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Acid-Soluble Nucleotides in the Unfertilized Eggs of the Sea-Urchin *Paracentrotus lividus*

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The acid-soluble nucleotides of the unfertilized eggs of the sea-urchin *Paracentrotus lividus* have been analysed by ion-exchange and paper chromatography. The following compounds have been identified: AMP*, ADP, ATP, GMP, GDP, GTP, UMP, UDP, UTP, CMP, CTP and inosine. An UDP-derivative and two compounds containing CMP have not been fully identified.

In their recent work on the RNA and DNA content of the sea-urchin embryo during early embryonic life Agrell and Persson [1] have also studied the variations in the acid-soluble nucleotide fraction. Only optical densities in the ultraviolet were measured and no attempt was made to identify the components of this fraction. Other authors, however, have identified some of the nucleotide compounds in the unfertilized echinoderm egg. Chambers and Mende [2] have shown by the use of barium-precipitation and differential spectrophotometrical methods that the eggs of *Asterias forbesii* and *Strongylocentrotus dröbachiensis* contain ATP, ADP, and AMP. Whithley [3] using the same methods found that *S. purpuratus* contains ATP and White and Chambers [4] have shown that purified actomyosin dephosphorylates the calcium salt derived from the trichloroacetic acid extract of *Arbacia punctulata*. The methods hitherto applied do not, however, separate similar components, especially those occuring in small amounts; these disadvantages can be overcame by the use of chromatographic methods. In a recent analysis by ion-exchange chromatography of embryos of *Psamechinus miliaris* the presence of uridine- and guanosine derivatives as well as adenosine compounds was

* AMP = 5′-adenosinemonophospate
ADP = 5′-adenosinediphosphate
ATP = 5′-adenosinetriphosphate
GMP = 5′-guanosinemonophospate
GDP = 5′-guanosinediphosphate
GTP = 5′-guanosinetriphosphate
UMP = 5′-uridinemonophosphate
UDP = 5′-uridinediphosphate
UTP = 5′-uridinetriphosphate
CMP = 5′-cytidinemonophosphate
CTP = 5′-cytidinetriphosphate
TMP = 5′-thymidinemonophosphate
RNA = ribonucleic acid
DNA = deoxyribonucleic acid

demonstrated [5]. The present paper is concerned with an analysis of the acid-soluble nucleotides from sea-urchin eggs by ion-exchange and paper chromatography.

MATERIAL AND METHODS

The sea-urchin species used was *Paracentrotus lividus* collected at *Station Biologique Roscoff*. Most of the animals were of a shallow water form taken at low water; some were a deep water form taken from outside the harbour of Roscoff. The ovaries were placed in beakers containing sea water where the eggs were shed. The egg suspension was filtered through gauze to remove the rests of the ovaries. The eggs were then washed several times (4—5) with sea-water and then twice with a solution of 3.5 % sodium chloride. Before the last washing samples were taken for the counting of the eggs. The last washing was ended by gentle centrifugation (200 g for 2 min) to concentrate the eggs.

Preparation of the acid extract. The washed egg suspension was homogenized in a glass homogenizer or a Waring blendor with an equal volume of 10 % cold trichloroacetic acid and filtered through Hyflo Supercel. The extraction was repeated three times with 5 % trichloroacetic acid. The filtrates were pooled and extracted 6 times with ether to remove the bulk of the trichloroacetic acid, neutralized with a few drops of ammonia and freed from ether by aeration. All operations were performed in the cold (1—2°C).

Ion-exchange chromatography. Dowex-1 (200—400 mesh) in the formate form was used. The pH of the solution to be chromatographed was brought to 9—11 by addition of ammonia. After adsorption the column was washed with water to neutrality and elution was performed by the formic acid — sodium formate system previously used [6]. The effluent was fractionated by a time-operated fraction collector. The optical density of every fraction was read at 250, 260 and 270 mμ. Appropriate fractions were pooled and the nucleotides were concentrated by adsorption on norite from acid solution followed by elution with 50 % ethanol containing 1 % ammonia.

For re-chromatography of some of the most complex fractions obtained in the formate system, a chloride system was used [7], it having been found that nucleotides with identical or very similar elution positions in the formate system were generally well separated in the chloride system.

Paper chromatography. For determination of the purine or pyrimidine components in the different nucleotide fractions one dimensional chromatography in *iso*propanol-hydrochloric acid-water (98:25:27) was used after hydrolysis of the fraction in N hydrochloric acid at 100°C for one hour. For identification of the unhydrolysed fractions they were chromatographed in at least three different solvent systems, in general the following: propanol-ammonia (*d* 0.880)-water (60:30:10), *iso*butyric acid-ammonia (*d* 0.880)-water (66:1:33) and ethanol- M ammonium acetate pH 7.5 (75:30). Descending or ascending chromatography on Whatman No 1 paper was used with developing times of 20—70 h. In some cases the chromatogram was developed twice to secure better separation. The spots were located by photography in ultraviolet light. For quantitative estimations, the spots were cut out and eluted with 0.1 N hydrochloric acid. Phosphorus was located by spraying with the molybdate reagent of Hanes and Isherwood [8]. The chromatograms were further sprayed with ninhydrine to reveal amino acids, and with anilinium hydrogen phthalate to demonstrate the sugar components.

Analytical procedures. Phosphorus was determined by the method of Allen [9]; acid-labile phosphorus was determined after hydrolysis for 10 min at 100°C in N hydrochloric acid.

Purine-bound pentose was estimated by the orcinol method according to Albaum and Umbreit [10] and pyrimidine-bound pentose by the same method after treatment of the nucleotide with bromine according to Massart and Hoste [11]. For the determination of phosphorus and pentose a Klett-Summerson photoelectric colorimeter was used with a red filter.

Optical densities were measured in a Beckman Universal Spectrophotometer, model DU or in a Hilger Uvispek Spectrophotometer. The following extinction coefficients were used: 14 500 for adenosinephosphates at 258 mμ, 10 500 for guanosine phosphates at 256 mμ, 10 080 for uridine phosphates at 262 mμ and 13 000 for cytidine phosphates at 280 mμ. The measurements were generally made in 0.1 M hydrochloric acid.

Oxidation with periodate was carried out according to Dixon and Lipkin [12].

Dephosphorylation with 5'-nucleotidase. For these experiments dehydrated venom of *Crotalus adamenteus* (Ross Allen Reptile Institute, Silver Spring, Florida) was used, following the procedure of Johnson *et al.*[13] The reaction products were identified by paper chromatography and phosphorus analysis.

Determination of the egg count. During constant stirring of the egg suspension a sample was taken with a spoon and quantitatively transferred to a graduated cylinder, where its volume was estimated. The sample was then diluted to appropriate volume from which a 2-ml sample was taken and counted under a microscope with low magnification. The values obtained from triplicate samples agreed within 5 %.

RESULTS

Most of the phosphorus in the trichloroacetic acid extract from unfertilized *P. lividus* eggs was determined as inorganic phosphate. This fraction amounts to about 60 % of the total phosphorus, with the remaining 40 % equally distributed between acid-labile and acid-stable phosphates. The analytical data are shown in Table 1.

Table 1. Determination of the phosphorus content of the trichloroacetic acid extract from unfertilized *Paracentrotus lividus* eggs.

$\mu g/10^6$ eggs				
P_0	P_{10}	P_T	$P_{10}-P_0$	P_T-P_{10}
515	735	915	220	180
537	671	791	134	120
529	707	869	178	162
612	769	982	157	213
765	1 050	1 325	285	275

The phosphorus contents of different batches of eggs are in reasonable agreement with exception of the last one, which shows a considerably higher concentration of phosphorus (Table 1). But the eggs giving this value came from sea-urchins taken from deep water while the other samples originated from the shallow water form.

The ultraviolet absorption spectrum of the neutralized trichloroacetic acid extract had a maximum around 265 mμ and a minimum around 240 mμ (Fig. 1) indicating that the extract might contain ultraviolet absorbing components other than adenosine derivatives. Paper chromatography of the hydrolysed extract in *iso*propanol-hydrochloric acid-water showed that the extract contained derivatives of adenine, guanine, 5'-uridylic acid and 5'-cytidylic acid. Spraying with ninhydrine revealed several amino acids, which impaired the quantitative evaluation of the ultraviolet absorbing spots. It was possible, however, to obtain a rough estimation of the components by two dimensional paper chromatography (Fig. 2). This figure shows strong spots corresponding to AMP, ADP, and ATP and weaker spots with R_F-values which indicate guanosine-, cytidine-, and uridine derivatives.

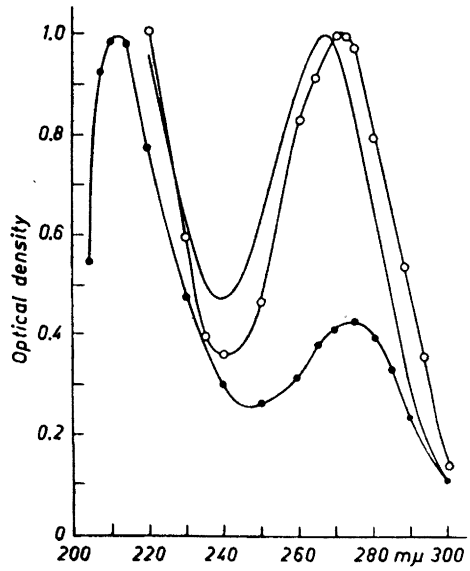

Fig. 1. Ultraviolet absorption curves 1) for the trichloroacetic acid extract from *P. lividus* eggs (—), 2) for the filtrate from the ion-exchange chromatogram of the extract (O—O) and 3) for the norite-eluate derived from the ion-exchange filtrate (●—●).

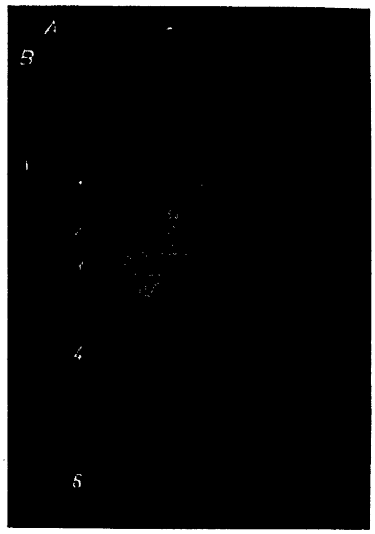

Fig. 2. Two-dimensional paper chromatogram of the extract from *P. lividus* eggs. Solvent: (A) *n*-propanol-ammonia-water (60:30:10) 72 h, (B) saturated ammonium sulphate-*iso*propanol-water (79:2:18) 12 h, Whatman No. 1 paper. (1) AMP, (2) ADP, (3) ATP, (4) guanosine derivatives, (5) uridine derivatives, and (6) cytidine derivatives.

The separation of the different components in the extract can be accomplished by ion-exchange chromatography on Dowex-1; Fig. 3 shows such a separation. While all the nucleotide material was adsorbed on the resin, most of the ultraviolet absorbing non-nucleotide components were removed in the filtrate. The present results indicate that about half of the ultraviolet absorption at 260 mμ in the acid extract is ascribable to non-nucleotide material; of an optical density at 260 mμ of 16 800 put on the column 8 600 were recovered in the filtrate and wash-water giving an ultraviolet absorption curve with a maximum around 273 mμ (Fig. 1). This fraction might contain purine and pyrimidine bases and ribosides which should be adsorbed on norite and then eluated with ammoniacal ethanol or with pyridine. Only about 40 % of the ultraviolet-absorbing material at 260 mμ from the filtrate and wash-water from the column was, however, adsorbed on norite in acid solution, and of the adsorbed material less than 10 % was eluted with 50 % ethanol (2 % ammonia) or with 25 % pyridine indicating the absence of any appreciable amounts of bases or nucleosides. Furthermore the eluates showed a strong ninhydrine reaction and an ultraviolet spectrum in 0.1 N hydrochloric

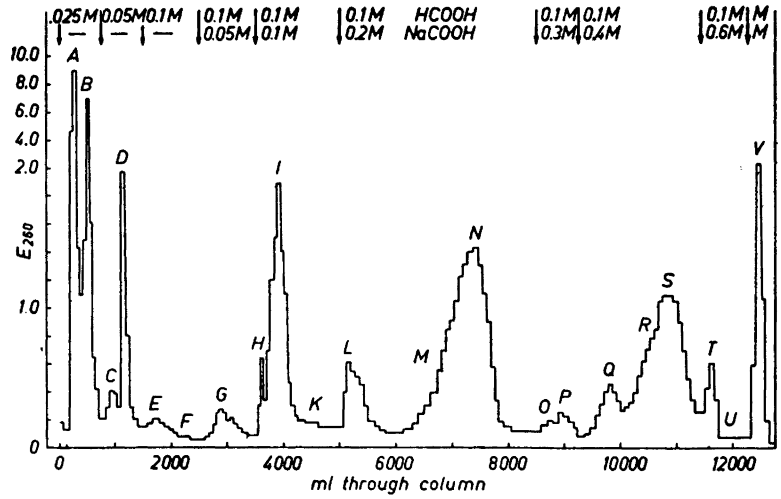

Fig. 3. Ion-exchange chromatogram of a trichloroacetic acid extract from *P. lividus* eggs on a Dowex-1 column (200—400 mesh, 2 cm² × 40 cm) in the formate system. Adsorbed material: extract with optical density E_{260} = 16 800. Recovery 103 %.

acid with maxima at 212 mμ and 275 mμ and a minimum at 250 mμ corresponding to the spectrum of tyrosine (Fig. 1).

Fractional elution of the nucleotides adsorbed on the column gave the separation shown in Fig. 3, resulting in the fractions indicated by the letters A—V. The fractions were concentrated by treatment with norite as described above. The concentrated eluates were then submitted to further analysis.

The different purine and pyrimidine components in the ion-exchange fractions were determined by paper chromatography of the hydrolysed fractions. R_F-values and ultraviolet absorption curves of the eluted spots were used for identification (Table 2).

It is clear from the table, that some of the fractions contained more than one component showing that the ion-exchange separation of the complex mixture of nucleotides in the acid extract of sea-urchin eggs had not been complete. In the first half of the chromatogram the separation was good but some overlapping had occurred between consecutive fractions, *e.g.* between B and C, C and D *etc.* In these cases complete separation could be achieved by preparative paper chromatography in the propanol-ammonia-water system. The mixture was applied to the paper as a line and after development, the formed zones were eluted with 0.01 N hydrochloric acid and concentrated by the norite-method. In those cases where it was obvious that the same components were present in more than one fraction, the eluates were pooled; in other cases the components were analysed separately. In the later part of the ion-exchange chromatogram the separation was not good, and some of the fractions were composed of a mixture of two compounds (fractions R and V)

Table 2. Paper chromatography of the hydrolysed fractions from the ion-exchange chromatogram shown in Fig. 3. R_F-values and ultraviolet-maxima of the eluted spots, together with those of authentic substances. Solvent: *iso*propanol-hydrochloric acid-water (98:25:27). Time: 24 h.

Fraction	R_F-value	UV max mμ	Fraction	R_F-value	UV max mμ
A	0.07	<220	M	0.40	262
	0.35	250		0.67	262
	0.50	280	N	0.40	262
B	0.38	262	O	0.39	262
	0.67	262		0.50	280
C	0.40	262	P	0.39	262
	0.67	262		0.50	280
D	0.50	280	Q	0.22	250
	0.67	263		0.68	262
E	0.38	—	R	0.38	262
F	0.38	—		0.68	262
G	0.22	249	S	0.38	262
H	0.22	249	T	0.38	262
	0.67	262	U	0.38	262
I	0.67	262	V	0.21	250
K	0.67	262		0.38	262
L	0.40	262	Guanine	0.22	249
Adenine	0.38	262	2′-CMP	0.55	280
2′-UMP			3′-CMP		
3′-UMP	0.75	262	5′-CMP	0.50	280
5′-UMP	0.67	262			

which could not be separated by paper chromatography in the propanol-ammonia system. In these cases the separation was achieved by re-chromatography of the fraction on Dowex-1 in the chloride system previously used (Fig. 4).

By these further partitions most of the fractions obtained in the original ion-exchange chromatogram could be divided into subfractions which were homogeneous in the following respects: On paper chromatography in propanol-ammonia-water they gave only one spot, their ultraviolet absorption spectra corresponded to one of the four ribosides adenosine, guanosine, uridine, and cytidine and after hydrolysis they gave only one ultraviolet absorbing spot on paper chromatography in *iso*propanol-hydrochloric acid-water (Table 3). Most of the original ion-exchange fractions were thus divided into components which were well separated and characterized at least with regard to the purine and pyrimidine part of the nucleotides. In some of the smaller fractions, however, the amounts available were not large enough to permit a final identification of the base-component. After hydrolysis fraction F gave a spot with an R_F-value identical with that of adenine; the spot, however, was too weak for determination of the ultraviolet absorption curve. The unpurified fraction did not give any definite nucleotide absorption spectrum; no maximum was obtained, only a shoulder between 250 and 265 mμ indicating the presence of a nucleotide heavily contaminated with non-nucleotide impurities. On

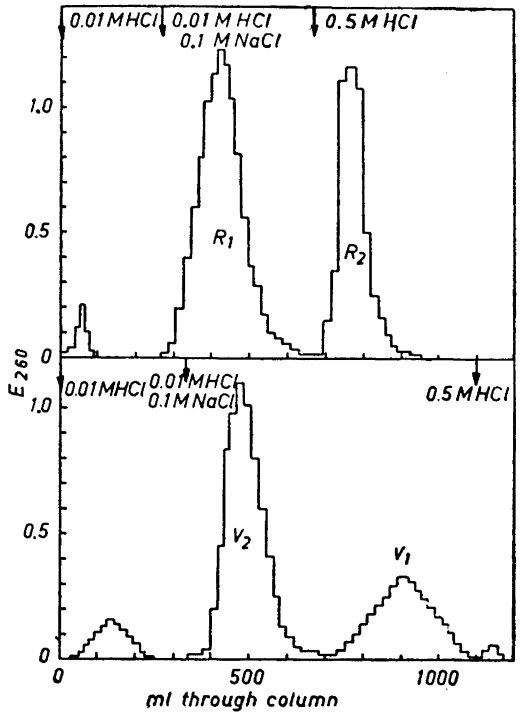

Fig. 4. Chromatogram on a Dowex-1 column (200—400 mesh, 0.3 cm² × 10 cm) in the chloride system of two complex fractions from the ion-exchange chromatogram shown in Fig. 3. Lower diagram fraction V. Upper diagram fraction R.

Table 3. Nucleotide fractions from the ion-exchange chromatogram shown in Fig. 3 after further purification by chromatography on paper or on Dowex-1 chloride. The products of hydrolysis were determined by paper chromatography in *iso*propanol-hydrochloric acid after hydrolysis for one hour at 100°C in N hydrochloric acid.

Fraction	UV max. mμ	Product of hydrolysis	Fraction	UV max. mμ	Product of hydrolysis
B	257	adenine	M_2	258	adenine
C	263	5′-UMP	N	258	adenine
D	265	5′-UMP	O	258	adenine
E	—	adenine	P	280	5′-CMP
F	—	—	Q	256	guanine
G	256	guanine	R_1	258	adenine
H_1	256	guanine	R_2	263	5′-UMP
H_2	263	5′-UMP	S	258	adenine
I	263	5′-UMP	T	258	adenine
K	263	5′-UMP	U	258	adenine
L	258	adenine	V_1	256	guanine
M_1	263	5′-UMP	V_2	258	adenine

hydrolysis fraction C gave not only 5'-UMP but also a faint spot corresponding to TMP, and the same spot was also found when the unhydrolysed fraction was chromatographed in *iso*propanol-hydrochloric acid-water. The ultraviolet absorption curve of the eluted spot had the same shape as that of thymidine with a maximum between 265 and 270 mμ. The amount obtained was, however, too small for further analysis. Fraction A was not included in the table, as it consisted of a mixture of at least four components which could not be resolved by the propanol-ammonia-water solvent. This fraction will be dealt with separately below.

Further identification of the purified fractions was then carried out by determination of acid-labile and total phosphorus and of pentose. The results of the analyses are shown in Table 4.

Some of the fractions from Table 3 are not mentioned in Table 4. Fractions E and F could not be analysed on account of the small amounts available. Furthermore, as mentioned above, some of the consecutive ion-exchange fractions contained the same component; on paper chromatography before and after hydrolysis they thus gave one and the same ultraviolet absorbing spot. Such cases are H_1 which is identical with G, H_2, identical with I, M_2 and O identical with N and fraction R_1, identical with S. T is probably the same substance as S; it appeared as a well defined fraction as the elution solution was changed before the base absorption in fraction S was low enough. Fraction V_2 gave the same analysis and R_F-value as fraction S.

The analysis of the purified ion-exchange fractions together with the establishment of the products of hydrolysis resulted in the identification of the different nucleotide compounds with reference to the purine or pyrimidine

Table 4. Analysis of the purified fractions from the ion-exchange chromatogram shown in Fig. 3.

Fraction	labile P μmole	total P μmole	base μmole	pentose μmole	base:pentose labile P: total P
B	0	0.117	0.118	0.117	1:0.00:—:0.99
C	0	0.122	0.122	—	1:—:—:1.00
D	0	0.170	0.170	0.156	1:0.92:—:1.00
G	0	0.155	0.154	0.151	1:0.98:—:1.01
I	0.109	0.216	0.110	—	1:—:0.99:1.96
L	0.076	0.227	0.181	0.174	1:0.96:0.41:1.25
M_1	0.066	0.134	0.066	0.070	1:1.06:1.00:2.03
N	0.128	0.252	0.126	0.126	1:1.00:1.02:2.00
O	0.094	0.198	0.093	—	1:—:1.01:2.12
P	0.180	0.270	0.091	0.086	1:0.94:1.98:2.96
Q	0.114	0.228	0.114	0.123	1:1.08:1.00:2.00
R_1	0.181	0.272	0.095	0.095	1:1.00:1.91:2.86
R_2	0.306	0.460	0.151	0.141	1:0.93:2.04:3.06
S	0.206	0.312	0.103	0.102	1:0.98:2.00:3.02
T	0.160	0.248	0.082	0.081	1:0.99:1.95:3.02
U	0.140	0.216	0.071	0.072	1:0.99:1.95:3.00
V_1	0.185	0.551	0.185	0.184	1:1.00:1.00:3.00
V_2	0.211	0.309	0.104	0.112	1:1.07:2.03:2.97

Table 5. R_F-values of the nucleotides obtained by ion-exchange chromatography of the trichloroacetic acid extract from unfertilized *P. lividus* eggs. Solvents: (1) *n*-propanol-ammonia (*d* 0.880)-water (60:30:10) 70 h, (2) *iso*butyric acid-ammonia (*d* 0.880)-water (66:1:33) 60 h, (3) ethanol-M ammonium acetate pH 7.5 (75:30) 48 h. R_F-values of authentic specimens are included.

Fraction	R_F-value in system			
	1	2	3	Identified as
B	0.30	0.62	0.25	AMP
C	0.21	0.44	0.32	UMP
D	0.21	0.44	0.31	UMP
E	0.30	0.62	0.25	?
F	0.30	0.62	0.25	?
G	0.15	0.41	0.16	GMP
H_1	0.15	0.40	0.16	GMP
H_2	0.35	0.36	0.50	UDPX
I	0.35	0.36	0.50	UDPX
K	0.35	0.37	0.50	UDPX
L	0.29	0.62	0.25	?
M_1	0.18	0.30	0.17	UDP
M_2	0.22	0.50	0.12	ADP
N	0.21	0.50	0.12	ADP
O	0.21	—	—	ADP ?
P	0.12	0.32	—	CTP
Q	0.12	0.30	0.06	GDP
R_1	0.17	0.45	0.08	ATP
R_2	0.13	0.24	0.10	UTP
S	0.17	0.45	0.08	ATP
T	0.17	0.42	0.09	ATP
U	0.17	0.43	0.08	ATP
V_1	0.11	0.23	0.06	GTP
V_2	0.17	0.44	0.08	ATP
AMP	0.30	0.62	0.25	
ADP	0.21	0.50	0.12	
ATP	0.17	0.42	0.08	
GMP	0.15	0.40	0.16	
GDP	0.13	0.30	0.10	
GTP	0.11	0.23	0.05	
UMP	0.22	0.44	0.32	
UDP	0.17	0.30	0.19	
UTP	0.13	0.25	0.10	
CMP	0.23	0.50	—	
CDP	0.17	0.44	—	
CTP	0.12	0.33	—	

component and to the degree of phosphorylation. The identification was finally checked by paper chromatography in three different solvent systems together with authentic compounds. As developing systems an alkaline, an acid and a neutral solvent were used. The R_F-values in the different systems are shown in Table 5, which also gives the identification of the fractions.

Most of the fractions have been definitely identified as the nucleotides indicated in Table 5 by the analyses shown in Table 4 and by chromatography. Some of the fractions may, however, need some further comments.

Fraction C was eluted from the ion-exchange column by the same eluent as D, which it preceded as a small peak. The main component of C showed the same analytical results and the same R_p-values in four solvent systems as fraction D, a fraction in all respects identical with authentic 5′-UMP. Fraction C also contained AMP but the occurrence of thymidylic acid or some other tymidine derivative as indicated by paper chromatography after hydrolysis in *iso*propanol-hydrochloric acid-water, mentioned above, could not be confirmed by chromatography before hydrolysis in the systems used in Table 5.

On paper chromatography fractions E and F behaved like 5′-AMP but their elution positions on ion-exchange were different from that of 5′-AMP. They may possibly be labile derivatives of 5′-AMP, transformed to 5′-AMP during the working up of the original ion-exchange fractions. They cannot be 2′- or 3′- derivatives as by treatment with 5′-nucleotidase they were completely dephosphorylated to adenosine.

The peak represented by fraction H was possibly a consequence of a premature change of eluting solution resulting in a nearly equimolar mixture of H_1 identical with GMP (G) and H_2 identical with I. Fraction I appeared as a well defined peak entirely free from interfering ultraviolet absorbing compounds. It is a derivative of UDP containing ninhydrine positive components; it has not yet been fully identified. L is also a complex fraction with an analysis suggesting a mixture of several components. The fraction gave, however, only one spot on paper chromatography in the three systems mentioned above. The R_p-values corresponded to those of 5′-AMP, indicating in fraction L the presence of a compound containing the 5′-AMP moiety which probably was released by the norite treatment. ATP was found to occur in several fractions, R_1, S, T, U, and V_2. The ATP samples recovered from all these fractions were, however, in all tested respects identical with ATP isolated from muscle. It was probably the unsatisfactory resolution in the formate system of a mixture of higher phosphorylated nucleotides which was the cause of the broad elution of ATP. The separation was further impaired by the high background absorption given by non-nucleotide components present in the crude extract. If, however, an extract purified by norite treatment was used an ion-exchange chromatogram was obtained in which ATP was eluted only in fractions R and S, T and U were absent and V consisted entirely of GTP.

To complement the identification described above, the fractions were also tested to verify the position of the phosphate groups in the 5′-position. Two analytical methods were used; periodate oxidation and the action of a specific 5′-nucleotidase from snake venom. The results of the analysis are shown in Table 6.

All fractions analysed by periodate oxidation and with 5′-nucleotidase were established as 5′-phosphates; no evidence was obtained for the occurrence of 2′- or 3′-derivatives. Dephosphorylation by snake venom 5′-nucleotidase resulted — under the conditions used — in the liberation of one mole of orthophosphate per mole of mono- or triphosphate, while two moles were liberated from one mole of nucleoside diphosphate. These findings are in agreement with the results of Johnson *et al.*[13]

Table 6. Action of periodate and 5′-nucleotidase on the purified nucleotides from unfertilized *P. lividus* eggs. Oxidation with periodate: the reaction mixture contained in 0.4 ml 0.050 μmole of nucleotide and 0.100 μmole of periodate. The change in extinction at 227 mμ was read against a periodate blank and a water blank. Reaction time 45 min. Action of 5′-nucleotidase: 0.5—1.0 μmole of nucleotide, 0.5 mg of snake venom, 10 μl of 0.5 M $MgCl_2$ and 0.05 M Tris-hydroxymethylaminomethane buffer (pH 8.5) to 2 ml. After 2 h at 38—39°C the reaction mixture was cooled to 0°C and deproteinized by the addition of 0.2 ml of 50 % trichloroacetic acid. After centrifugation the supernatant was analysed for total and inorganic phosphorus. The reaction products were determined by paper chromatography in butanol-formic acid-water (77:10:13).

Fraction	Oxidation with periodate μmole		Action of 5′-nucleotidase μmole		
	nucleotide used	periodate consumed	nucleotide used	P_0 formed	reaction product
B	0.050	0.047	0.52	0.52	adenosine
D			1.00	0.98	uridine
E + F			0.36	0.31	adenosine
G	0.050	0.051	0.55	0.50	guanosine
I	0.050	0.047	0.63	1.39	uridine
L			0.66	1.32	adenosine
M_1	0.050	0.053	0.61	1.18	uridine
M_2	0.050	0.046	0.61	1.18	adenosine
N	0.050	0.047	0.97	1.94	adenosine
P			0.50	0.50	cytidine
Q	0.050	0.048	1.07	2.05	guanosine
R_2	0.050	0.047	0.88	0.90	uridine
S	0.050	0.047	0.60	0.63	adenosine
T	0.050	0.047	1.03	1.07	adenosine
U	0.050	0.055	0.72	0.72	adenosine
V_1	0.050	0.048	0.86	0.89	guanosine
V_2	0.050	0.053	0.88	1.04	adenosine
5′-AMP	0.100	0.102	0.80	0.78	adenosine
2′-,3′-AMP	0.100	0.009	0.74	0.02	2′-,3′-AMP
ADP			0.64	1.26	adenosine
ATP			0.72	0.74	adenosine

Fraction A has hitherto only been mentioned in Table 2 where it was shown that on paper chromatography in *iso*propanol-hydrochloric acid-water the hydrolysed fraction gave three ultraviolet absorbing spots. The spot with the lowest R_F-value was not of purine or pyrimidine type since it did not show any maximum in the region between 220 and 300 mμ. The second spot corresponded to hypoxanthine possibly contaminated with small amounts of adenine, according to the R_F-values and ultraviolet spectra in acid and alkali. The third spot was 5′-CMP according to R_F-values and ultraviolet maxima at 280 mμ and 270 mμ at pH 1 respectively 11.

The components of the unhydrolysed fraction A were then separated by paper chromatography in butanol-formic acid-water (77:10:13). Four spots were obtained; spot I with $R_F = 0.03$, containing cytidylic acid and ninhydrine positive compounds, spot II ($R_F = 0.15$) with hypoxanthine spectrum, spot III ($R_F = 0.28$), a faint spot with an absorption maximum around 260 mμ

and spot IV ($R_F = 0.35$), also a faint spot which on account of the spectrum — maxima at 275 mμ and 213 mμ — and its positive reaction with ninhydrine was identified as tyrosine.

From spot I 5′-CMP was isolated free from ninhydrine reactive components by paper chromatography in *iso*propanol-hydrochloric acid-water and was further characterized by its R_F-values in propanol-ammonia-water (0.22) and *iso*butyric acid-ammonia-water (0.50). Analysis of the pure fraction gave the ration of 1:1.05:0:1.07 for cytosine:pentose:labile phosphate:total phosphate. Furthermore, a consumption of 0.98 moles of periodate per mole base was obtained and on treatment with 5′-nucleotidase 1.00 mole orthophosphate was released with cytidine as the other reaction product. As mentioned above, small amounts of cytidine compounds were also found in fractions B, C, and D and in two other ion-exchange chromatograms larger amounts of three cytidine derivatives were eluted before AMP. One of these was 5′-CMP; on hydrolysis the other two gave 5′-CMP but before hydrolysis they moved differently from 5′-CMP on paper chromatography in ethanol-ammonium acetate (pH 7.5). Both these substances contained phosphorus and pentose and are possibly cytidine derivatives of the type reported by Baddiley *et al.*[14] and by Kennedy and Weiss.[15] Further identification of these compounds had to be postponed.

Spot II obtained from fraction A was identified as inosine. The R_F-values obtained by paper chromatography in four different solvent systems, — propanol-ammonia-water $R_F = 0.35$ (0.36), butanol-formic acid-water (77:10:13) $R_F = 0.15$ (0.15), butanol saturated with 10 % urea $R_F = 0.18$ (0.19) and ethanol-M ammonium-acetate pH 7.5 $R_F = 0.31$ (0.30) — were the same as those of authentic inosine, shown within the brackets. The ultraviolet spectrum of the eluted spot in acid and alkali was identical with that of inosine. The ratio 1:0.95 was obtained for hypoxanthine and pentose; no phosphorus was found. In the same way, spot III was identified as adenosine.

Extracts from four different batches of unfertilized sea urchin eggs were analysed by ion-exchange either as the crude extract (3) or after treatment

Table 7. The nucleotide content of unfertilized *P. lividus* eggs by ion-exchange chromatography of trichloroacetic acid extracts.

Fraction	μmole/10^7 eggs	Fraction	μmole/10^7 eggs
AMP	3.0	UMP	1.0
ADP	3.1	UDP	1.1
ATP	6.0	UDPX	3.3
Unidentified		UTP	1.0
Ad-compounds	1.3	ΣUr-compound	6.4
ΣAd-compounds	13.4	CMP	2.1
GMP	0.3	CTP	0.3
GDP	1.6	Unidentified	2.8
GTP	1.1	Cy-compounds	
ΣGu-compounds	3.0	ΣCy-compounds	5.2
Inosine	0.7		

with norite and with essentially the same results. The amounts of the nucleotide derivatives obtained are shown in Table 7.

The nucleotide content had the following general pattern: nearly one half of the nucleotides consists of adenosine derivatives (49 %) and the other half of equal amounts of uracil- and cytosine compounds (22 % respectively 17 %) and a smaller quantity of guanine nucleotides (10 %).

Even if adenine nucleotides dominate, the distribution of the nucleotides from the different purine- and pyrimidine base is fairly even. The pattern of acid-soluble nucleotides is quite different from that of *e.g.*, skeletal muscle [16] and the body wall musculature of *Annelida* [6] where the nucleotide fraction consists of adenine compounds to 90 %, or from that of the oviduct of the hen which has a high content of uridine nucleotides [17]. Muscle and the oviduct may be taken as examples of organs with a higly specialized function, a function which is reflected in the nucleotide composition; in muscle, which for its work needs easily accessible energy, adenine nucleotides, especially ATP, predominate, while the oviduct — whose main function is thought to be the synthesis of glycoproteins containing hexosaminederivatives — has a high content of uridine compounds, particularly in the form of different UDP-derivatives, which are thought to participate in the biosynthesis of mucopolysacharides.

As an example of reversed conditiones, *i.e.* an organ with a variety of functions, the liver can be taken. The more even composition of the nucleotide fraction of the liver [16] with regard to the different purines and pyrimidines, compared with that of the muscle and the oviduct, may be taken as a consequence of the multiplicity of functions.

Even the unfertilized egg which shall develop into a complex organism, has to have a manifold equipment of different nucleotides as a consequence of the multiplicity of processes essential for the differentiation and development of the embryo. Interesting is the occurrence of rather large amounts of cytosine derivatives, a type of nucleotides until recently not found to be present in biological material in higher concentrations. Kennedy and Weiss isolated different cytidine nucleotides from the liver of the hen and the rat, and have also shown that these compounds take part in the biosynthesis of phospholipids [15]. Furthermore, in a preliminary note Sugino *et al.*[18,19] have reported the occurrence of deoxycytidine derivatives in several organisms, among others in sea-urchin eggs. The unidentified cytosine compounds in the present investigation may consist of similar substances.

The only nucleosides found in the ion-exchange fractions were inosine and traces of adenosine. Part of the ultraviolet absorbing compounds from the extract was, however, not retained by the ion-exchange column; bases and nucleosides could be included in this fraction. But, as mentioned above, the major part of the ultraviolet absorption in the filtrate and wash water from the column consisted probably of amino acids and the only purine or pyrimidine compounds found were small amounts of substances — detected by paper chromatography — with spectra resembling those of uracil and cytosine. Therefore it seems unlikely that the unfertilized sea urchin egg contained larger amounts of free bases and nucleosides.

The further identification of the unknown fractions and the variations in nucleotide content during the mitotic cycle and the early development of the sea-urchin embryo is receiving attention.

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