

Composition of the Nucleotides Pool in a Morphogenetic Compartment in Eggs of *Nassarius reticulatus* (Mollusca) Analysed by Capillary Isotachophoresis

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The spectrum of low molecular weight compounds, in particular of ribonucleotides, within first cleavage stage embryos of the polar lobe-forming mollusc *Nassarius reticulatus* and the distribution of the compounds within the embryo at the trefoil stage of first cleavage are analysed by means of capillary isotachophoresis after 0.5 M PCA extraction. The compounds which are found in the whole trefoil embryo (T), the lobeless part (LL), and the polar lobe (PL) respectively, and the mean quantities (nmol. μl^{-1} ; $n=6$) are: UTP (11.5, 4.8, 5.6), ITP (8.5, 3.6, 5.0), GTP (10.3, 3.0, 9.0), ATP (29.8, 13.4, 18.8), UDP (11.8, 3.4, 8.7), CTP (8.0, 3.1, 4.5), GDP (5.3, 2.6, 3.4), ADP (16.5, 6.1, 11.6), CDP (4.0, 1.4, 2.6), GMP (4.7, 2.7, 4.3), glucose-6-phosphate (G6P) (53.5, 38.8, 13.0). These compounds appear to be localized in the non-yolk cytoplasmic pool. As the volume ratio of PL/LL for total volume and for non-yolk cytoplasmic volume is about 0.74 and 0.60 respectively, the concentration of all nucleotides in PL as compared to LL is significantly higher (HO, $p<0.001$), both relative to the total volume and to the non-yolk cytoplasmic volume. The G6P concentration is considerably higher in the lobeless part. The morphogenetic role of the vegetal pole compartment of the egg apparently is correlated with a relatively high level of its nucleotides contents. © 1985 Academic Press, Inc.

In current concepts on the control of differentiation patterns during embryonic development it is generally assumed that patterns of gene expression are controlled by cell line-specific cytoplasmic determinants which are inherited from the egg [1–3]. In this context, species that exhibit determinate cleavage, e.g., ascidians, molluscs, and insects have been of special interest. In these systems, the egg itself is or becomes partitioned into developmental compartments, which are marked by the presence of so-called cytoplasmic localizations. Classical embryological studies have revealed a causal relationship between the segregation of these constituents to particular daughter cells and the determination of cell-specific developmental potentialities [3–7]. It is generally assumed that cytoplasmic localizations are endowed with morphogenetic determinants, which exert their influence at either transcriptional or at post-transcriptional levels. Polar

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lobe-forming molluscs, e.g. *Ilyanassa*, *Nassarius*, *Dentalium* and *Bithynia* are favourable material for experimental studies as they provide the possibility to isolate the cytoplasmic compartments of interest and to analyse their biochemical composition. In these species, a morphogenetic compartment of the egg is temporarily set apart during first cleavage in the form of a cytoplasmic protuberance at the vegetal pole, the so-called polar lobe, which at the height of its formation (at the so-called trefoil stage) is merely connected by a thin stalk to the dividing blastomeres [4–12]. The polar lobe can be easily isolated from the egg, either by surgical or chemical methods. After removal of the polar lobe the stem cells of adult mesodermal and ectodermal tissues and organs are formed as in the normal embryo, but lack the normal developmental potentialities. The restricted and highly reproducible spectrum of deficiencies of the lobeless embryo closely resembles the phenotypic expressions of a maternal effect mutant [cf 13]. Although the developmental significance of the vegetal pole constituents of the egg is well documented, little is known about their chemical composition.

We have studied the molecular composition of the isolated polar lobe in *Nassarius reticulatus* by means of capillary isotachopheresis, which is an elegant micromethod particularly well suited for analysing a high amount of material. Preliminary experiments have shown that the polar lobe and, *mutatis mutandis*, the vegetal pole region of the egg, is characterized by large quantities of nucleotides [14]. In the present paper we present a detailed account of the qualitative and quantitative differences in composition of the nucleotides pool of the polar lobe and the lobeless part of the embryo, isolated at first cleavage.

MATERIALS AND METHODS

Chemicals

Nucleotides were purchased from Boehringer, Mannheim (FRG). Agar Noble was from Difco Laboratories, Detroit Ill.; perchloric acid (PCA) (70%, SW 1.67); Imidazole, Na₂-EDTA (titriplex III); CTAB (cetyltrimethylammoniumbromide); capronate (sodium salt); and β -alanine were obtained from Merck, Darmstadt (FRG). Methanol was from Baker Chemicals BV, Deventer (Holland), and PVA (polyvinylalcohol) was obtained from Hoechst, Frankfurt (FRG). These and all other chemicals used in this study were of analytical reagent grade.

Maintenance of Animals and Handling of Embryonic Materials

The embryonic material which is used in these studies is from the species *Nassarius reticulatus*, a marine gastropod in which during first cleavage a large polar lobe is formed (fig. 1). Animals were collected at the coast of Brittany, France, and were kept in the laboratory in aquaria at 16°C, in conditioned natural sea water. They were fed alternately with meat from *Loligo vulgaris* (kept frozen in stock) and either freshly killed clam (*Mytilus vulgaris*) or *Patella vulgata*. Under these conditions, abundant amounts of egg masses are deposited spontaneously each day.

Egg masses consist of an urn-shaped capsule, containing about 300 already fertilized eggs. Egg masses were collected, freed from adhering debris, and were transferred to a glass culture dish containing Millipore-filtered artificial sea water (ASW) at 18°C, prepared according to the Woods Hole formula [15] with (g/l): 24.72 NaCl; 0.67 KCl; 1.36 CaCl₂·2H₂O; 4.66 MgCl₂·6H₂O; 6.29 MgSO₄·7H₂O; NaHCO₃, 0.18 g/l distilled water adjusted to pH 8.3 with Tris (hydroxy-methyl-aminomethane). The glass culture dishes, Boveri type, and all other glass-ware used for handling live

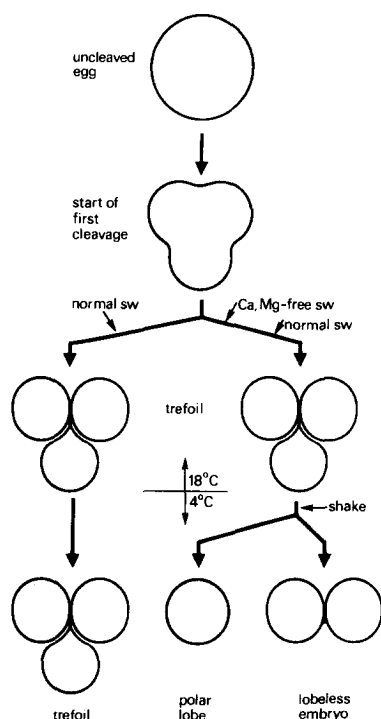


Fig. 1. Scheme representing successive steps in preparing PL and LL.

embryonic material, were coated with sterile agar noble (1% w/v in distilled water), and thoroughly dried in the stove at 37°C. The agar film thus produced is necessary in order to prevent the embryonic material from sticking to the glass surface. Eggs were freed from the surrounding capsule with iridectomy microscissors. They were washed in three changes of ASW to remove capsule fluid, and were kept at 18°C until the onset of first cleavage, which is indicated by the appearance of the polar lobe.

Preparation of Samples of Normal Embryos (Ts), Polar Lobes (PLs) and Lobeless Parts (LLs)

At the beginning of first cleavage, the eggs out of one egg mass were transferred to 18°C Millipore-filtered Ca^{2+} - and Mg^{2+} -free artificial sea water (Ca, Mg, FASW), prepared according to the Woods Hole formula [15] with (g/l): 27.0, NaCl; 0.8, KCl; 1.0, Na_2SO_4 ; NaHCO_3 , 0.18 g/l distilled water. They were washed in three changes of Ca, Mg, FASW and were left in this medium until the trefoil stage of first cleavage was reached (fig. 1). At this stage, the polar lobe is maximally constricted off and is connected to the dividing blastomeres by a very thin stalk only. The embryos were then transferred to ice-cold ASW in a glass Petri dish which was kept on ice. All egg masses were processed separately until this step. Polar lobes were detached from the cleavage-arrested embryos by gently rocking the Petri dish. This causes the embryos to roll over the lateral side. Thereby the lobes are detached, as they tend to roll over faster than the rest of the embryo. PLs and LLs were counted and were collected separately on ice within a microvessel, together with a minimal amount of sea water. Known numbers of normal trefoil stage embryos (T) were collected immediately after the initial washing steps with ASW. Sea water was removed from the embryos and isolates, which up to this step are still intact, by means of a sharply drawn out capillary under controlled suction. Next, the samples were frozen on dry ice and stored at -70°C. The sampling procedure up to this stage was always completed within 1 h. A series of isotachophoretic test runs, in which sampling times were varied deliberately from ½ to 2 h,

had shown previously [14] that changes in nucleotides contents in correlation with sampling time do not occur, at least not under the present conditions. So, the sampling procedure adopted in the present study was within safe time limits.

Perchloric Acid (PCA) Extraction

For preparing PCA extracts, one thousand of each T, PL and LL were used for each series of analyses. These numbers were obtained by combining separate smaller samples (2–4) which had been kept frozen at -70°C . The samples which were to be combined were thawed, briefly vortexed and 42 μl ice-cold 0.5 M PCA was added to the first of the samples. After resuspending by vortexing briefly, the sample contents were transferred quantitatively to the second microvessel, etc. The last reaction vessel, which contained the combined extracts of either T, PL or LL, was kept on ice for 15 min. The acid extracts were next resuspended and were centrifuged at 18000 rpm (Sorvall RC2-B, SW34 rotor) at 0°C for 5 min to remove precipitated macromolecular constituents. A fixed volume of supernatant (35 μl) was taken off with a Hamilton syringe and the pH was immediately brought near neutrality by adding in quick succession 5.0 μl 0.5 M imidazole and 5.0 μl 3.5 M KOH. The pH was routinely checked for each sample with pH-indicator-paper (Johnsons of Hendon Ltd., Hendon, England) using a small aliquot of supernatant. The pH appeared to be within the following ranges: 7.4–7.7 (T), 7.1–7.4 (PL), and 7.1–7.7 (LL). Neutralized supernatants were centrifuged as before to remove potassium precipitates. The supernatants of this centrifugation step were frozen on dry ice and stored at -70°C . Yolk material was isolated from 200 whole trefoil embryos (the same number as used in preparing PCA extracts) by centrifugation (Sorvall SW 34, 18000 rpm, 5 min), after lysis by one freezing/thawing cycle after removal of the residual sea water. Pellets were washed twice with cold (-20°C) MeOH-EDTA (50% v/v MeOH, 1.25 mM EDTA-Na), and were extracted with the same medium at -20°C . Extraction was facilitated by 5 min sonification (Bransonic model D-50) immediately after addition of the extraction medium. For analysis of the yolk contents, we had to use a different extraction procedure, i.e., MeOH-EDTA extraction, because yolk granules are difficult to homogenize, especially when they are first treated with acid, which hardens them by precipitation of the proteinaceous constituents. It is not possible to perform PCA extraction for longer periods of time, because the acid medium would entail the risk of nucleic acid degradation.

Analytical System

Samples were analysed by capillary isotachopheresis (ITP), which is a micromethod permitting separation, identification and quantitation at the picomole level. In ITP, charged sample constituents are separated under the influence of an electrical field, and migrate as a train of contiguous zones in the order of decreasing effective mobilities, enclosed between a leading electrolyte (with highest mobility) and a terminating electrolyte (with lowest mobility). Sample components that under the chosen operational conditions (pH, concentration, etc.) are uncharged or have effective mobilities outside the range specified by the leading and terminating ions are not separated, and consequently could not be analysed. When separation is completed, an equilibrium is reached at which all zones move with the same velocity (effective mobility \times local field strength). This steady-state is maintained by the voltage drop (sh_T) between leading and terminating zone. Conductivity typically shows stepwise increments at each zone boundary (zonal step height, sh), as is described by the Kohlrausch regulating function [16]. The relative zonal step height ($rsh = sh/sh_T$) is dependent on the effective mobility of the ionic species and, given the operational conditions, is a qualitative measure. Zonal length at steady state is dependent on the concentration of the compound in the sample and on its effective mobility, and again under the operational conditions is a direct quantitative measure. Quantitative estimates can be calculated from response factors (nmol/mm). The sequence of zones is traced continuously by measuring UV absorption (A_{254} , A_{280}) and conductivity (measure for the local field strength) as the zones pass the respective detection units (fig. 2). Traces of UV absorption (A_{254} , A_{280}) and of conductivity (R) are plotted with constant chart speed. In the present study, a column-coupling system as described previously was used [17].

Operational Procedure

Operational conditions used in the present study are given in table 1. The conditions are such, that anionic species with effective mobilities between those of chloride (leader) and capronate (termina-

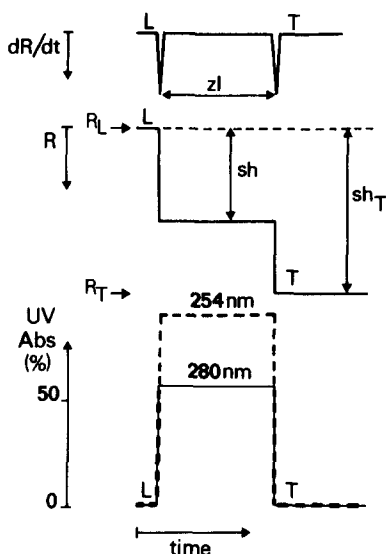


Fig. 2. Scheme representing the UV-absorption traces (A_{254} , A_{280}), the conductometric trace (R), and the differential recording of the conductometric trace (dR/dt). z_l , Zone length, R , resistance; R_L , R_T , conductivity of R_L leading; R_T terminating ion; sh , zonal step height; sh_T , total voltage drop.

tor), like uncomplexed nucleotides, are separated. Immediately before ITP, the samples were thawed. The time during which the samples were at room temperature was always kept at a minimum. Volumes to be injected into the column were taken off with a Hamilton syringe, which was adjusted with the aid of a binocular microscope. Injection volumes, which varied from 2.3 to 5.0 μ l, were chosen such that in all cases the extract of equal volumes of biological starting material was analysed. Consequently, comparison of zone lengths in the traces of T, PL and LL respectively gives a direct impression of the concentrations of the respective nucleotides pools. In all cases undiluted extracts were analysed.

Zone lengths (mm) were measured from a differential recording of the conductometric traces, which gives the exact positions of the zone boundaries. Lengths were determined with a micrometer device (accurate to 0.1 mm). Mean values of zone lengths were calculated for each component from each series of samples (T, PL and LL respectively). Nucleotides quantities were calculated by multiplication with the appropriate response factor ($\text{nmol} \times \text{mm}^{-1}$). From the values thus obtained, quantities and concentrations of nucleotides were calculated by making appropriate corrections for differences in number of T and of both isolates in the initial homogenate and of the cytoplasmic volumes of each of them, taking losses of sample volume in the successive procedural steps into account. Estimates of the cytoplasmic volumes of T, PL and LL respectively were obtained from diameter measurements of isolated polar lobes and dissociated blastomeres of lobeless embryos, which were performed by means of a microscope equipped with a calibrated ocular micrometer.

RESULTS

Volumes of Trefoil Embryos (T), Lobeless Embryos (LL), and Polar Lobes (PL)

The mean diameter of the polar lobe, calculated from measurements of 75 isolated polar lobes out of three egg masses (3×25) is 0.137 ± 0.0036 mm. As the isolated polar lobe is spherical in shape, the mean total volume of PL is 1.36 nl. For estimating the volume of the lobeless part of the trefoil stage embryo, separated LL blastomeres from the same three egg masses (again 3×25) were measured. We preferred not to perform direct measurements on the lobeless

Table 1. Operational conditions for nucleotide analysis with capillary isotachopheresis

Parameter	Specification
Leading electrolyte	
Leading ion	Chloride
Concentration	0.01 M
Counter ion	β -Alanine
pH	3.90
Additives	0.05 M PVA ^a 0.02 mM CTAB ^b
Terminating electrolyte	
Terminating ion	Capronate
Concentration	ca 5 mM
Counter ion	Sodium
pH	ca 6
Preseparation capillary	
Inner diameter	0.8 mm
Current	350 μ A
Detection capillary	
Inner diameter	0.2 mm
Current	25 μ A
Detection	AC conductivity UV absorption at 254 and 280 nm
Recording velocity	1 mm sec ⁻¹

^a PVA, polyvinylalcohol; ^b CTAB, cetyltrimethylammoniumbromide.

embryo, because the blastomeres tend to flatten somewhat against each other, giving them a non-spherical appearance. Upon separation of the blastomeres, they round off. The mean diameter of LL blastomeres is 0.121 ± 0.0040 mm. Consequently, the mean volume of LL can be estimated as 1.84 nl (two times the volume of an isolated LL blastomere). The mean volume of T, estimated as the sum of vol_{PL} and vol_{LL} , is 3.20 nl.

ITP Analysis of Standard Mixtures

Reference values for the identification and quantitation of sample components were obtained from ITP analyses of standard mixtures containing compounds which can be separated under the chosen operational conditions. An example of ITP traces obtained by analysis of a standard mixture is given in fig. 3. From such traces reference values for identification parameters, i.e., A_{254} , A_{280} , A_{254}/A_{280} , rsh, position in the zone sequence and also response factors were deduced.

As explained in Material and Methods, the position of a zone is an important parameter for zone identification, as it allows discrimination between compounds which are not significantly different with respect to UV-absorption and conductometric step height. The relative position of all zones of interest can only be deduced from ITP traces of either extensive or overlapping standard mixtures.

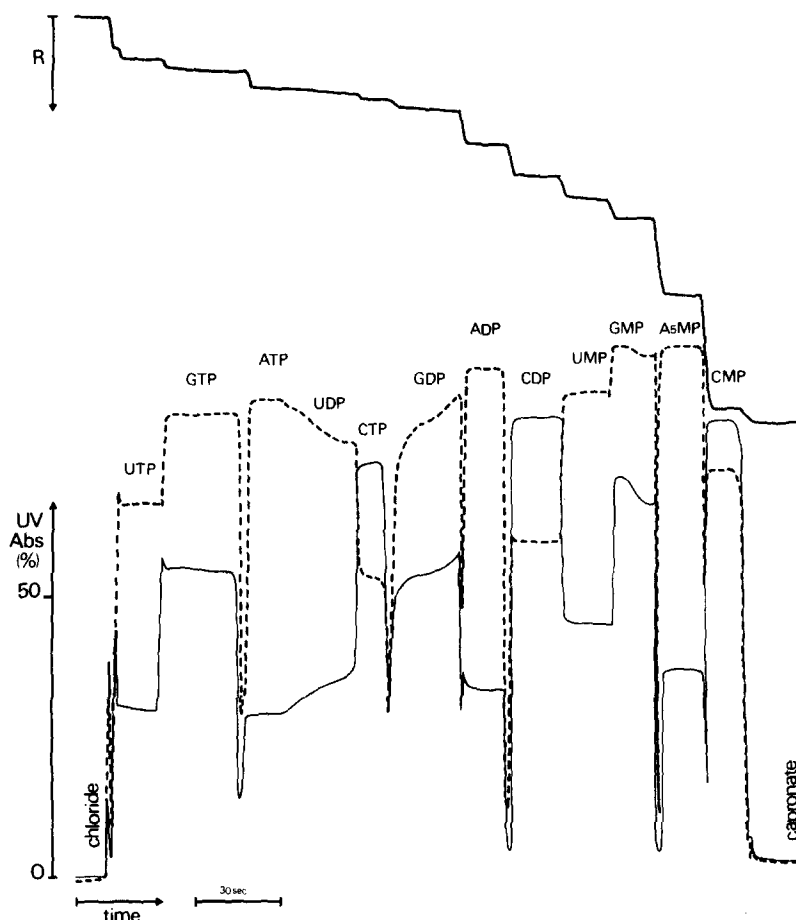


Fig. 3. An example of UV-absorption traces (A_{254} , A_{280}), and conductometric trace (R) of a standard mixture of nucleotides. All traces correspond exactly relative to the time base.

The results of such analyses have been presented previously [14]. A relevant list of nucleotides which were analysed and the values obtained for respectively UV absorption A_{254} , A_{280} , UV-absorption ratio (A_{254}/A_{280}), and relative conductometric step height (rsh) is presented in table 2. The system reproducibility of the values for each parameter is within 2% [16]. In table 2 the components are arranged according to their effective mobilities, i.e., according to their position in the sequence of zones (top: first component).

ITP Analysis of PCA Extracts of T, PL and LL

ITP traces of T, PL and LL are represented in figs 4, 5 and 6 respectively. Components which are identified are indicated. Components were accepted as identified, if the mean values for UV absorption, UV-absorption ratio and conductometric step height all differed by no more than 5% from the value of the

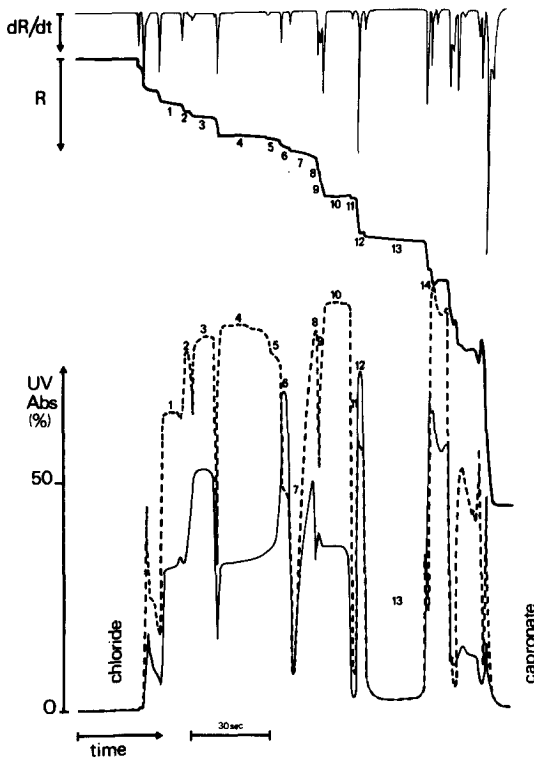


Fig. 4. An example of UV-absorption traces (A_{254} , A_{280}), conductometric trace (R) and its differential recording (dR/dt), obtained from whole first cleavage stage embryos (T). The PCA extract of 43 embryos was analysed, which corresponds to approx. 0.137 μ l of total cytoplasmic volume. Identified components: 1, UTP; 2, ITP; 3, GTP; 4, ATP; 5, UDP; 6, CTP; 7, phosphate; 8, GDP; 9, citrate; 10, ADP; 11, lactate; 12, CDP; 13, G6P; 14, GMP.

Table 2. Reference values for UV absorption (A_{254} , A_{280}), UV-absorption ratio (A_{254}/A_{280}), relative step height (rsh), and response factor (rf) for identified nucleotides obtained from analyses of standard mixtures

Comp.	A_{254} (%)	A_{280} (%)	A_{254}/A_{280}	rsh	rf (nmol/mm)
UTP	67	30	2.2	0.10	0.064
ITP	73	16	4.5	0.11	0.123
GTP	83	54	1.5	0.13	0.056
ATP	86	28	2.9	0.18	0.067
UDP	78	35	2.2	0.19	0.081
CTP	54	73	0.7	0.20	0.093
GDP	86	57	1.5	0.23	0.038
ADP	91	32	2.8	0.31	0.067
CDP	60	81	0.7	0.39	0.055
GMP	95	70	1.4	0.50	0.080

Investigators who wish to use these values as a reference for their own studies, should realize that they depend on (1) the operational conditions chosen; (2) the physical characteristics of the detection devices (UV-absorption detectors, conductometer) used. They should not be regarded as absolute.

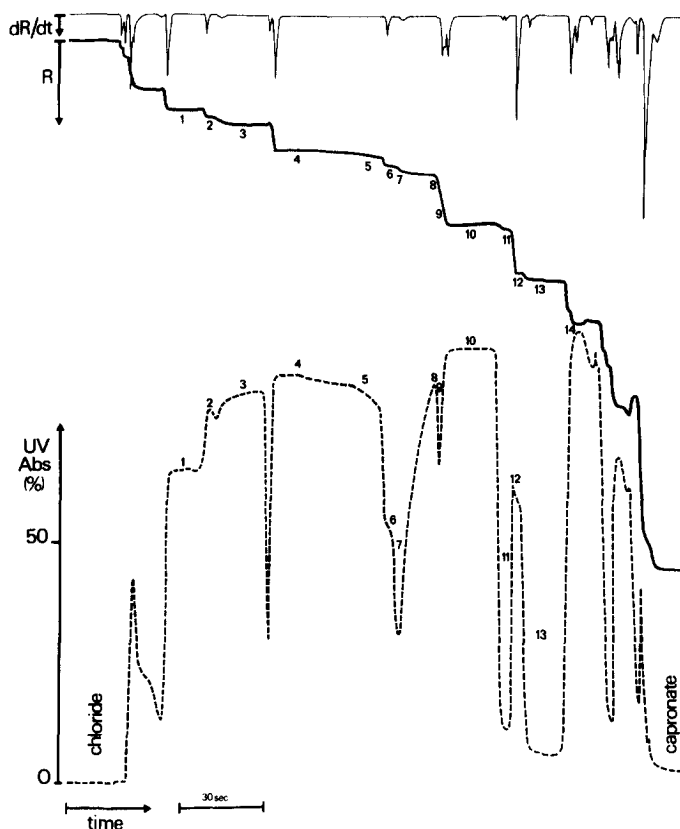


Fig. 5. An example of UV-absorption trace (A_{254}), conductometric trace (R) and its differential recording (dR/dt) obtained from polar lobes (PL). The PCA extract of 102 PLs was analysed, which corresponds to approx. $0.139 \mu\text{l}$ of total cytoplasmic volume. For numbering of identified compounds see caption to fig. 4.

respective parameters obtained from measurements of standard mixtures. This close correspondence for three parameters, together with the correct position in the zone sequence, allows identification with a high degree of reliability.

Identification of sample components was primarily done on a series of ITP traces from normal embryos (T), in which different extraction procedures and different operational conditions were tried out. PCA extraction according to the procedure described in Material and Methods gave the best results (both in quantitative and qualitative respects). A few remarks are appropriate with respect to the separations which are obtained. First, some adjacent zones are not completely resolved. This produced mixed zones and unsharp zone boundaries. This was the case with the ATP/UDP and phosphate/GDP zone boundaries. In these two cases the correct zone lengths had to be estimated by applying approximations for actual zone lengths based on theoretical considerations [16]. Separation can be improved by modifying the operational conditions, but this

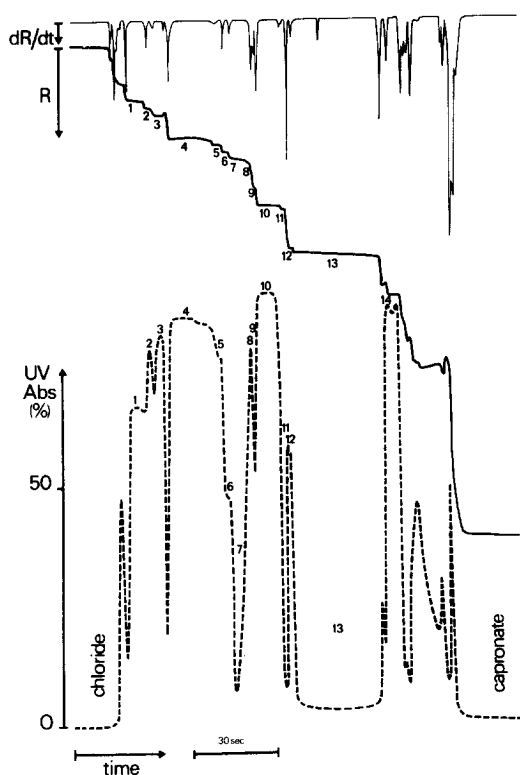


Fig. 6. An example of UV-absorption trace (A_{254}), conductometric trace (R) and its differential recording (dR/dt) of first cleavage stage lobeless embryos (LL). The PCA extract of 75 LL s was analysed, which corresponds to approx. 0.138 μ l of total cytoplasmic volume. For numbering of the identified compounds see caption to fig. 4.

appeared to be detrimental for the overall resolution of the nucleotide spectrum. Application of the approximation strategy to the standard mixtures, in which known quantities of components were analysed, exhibited close correspondence between estimated and actual quantities. For these zones, the response factors were determined by analysing the components concerned in separate runs.

The second point we wish to make, is that under the present conditions the monophosphate nucleotides, with the exception of GMP, are not well resolved. That is why we are not able to present qualitative and quantitative results with respect to these components.

Comparison of the ITP traces of PL and LL (figs 5, 6) immediately reveals that on a per volume basis the PL is highly enriched in most nucleotides. On the other hand, the LL embryo appears to contain much more glucose-6-phosphate (G6P), a non-UV-absorbing compound. A survey of components present in the normal embryo and in both isolates, with the calculated mean concentrations is given in table 3. Mean quantities for each nucleotide are given for whole trefoil embryos (T) only. For the two isolates (PL and LL), these values can easily be obtained by multiplying the concentrations with the appropriate volume. The last column shows estimates for quantities per T, which for each nucleotide were obtained as the sum of the quantities per PL and LL. The actual and estimated values for

Table 3. *Pool concentrations of low molecular weight components in trefoil (T) embryos; lobeless embryos; (LL) polar lobes (PL)*

Comp.	Concentrations						Quantities ^b	
	T ^a		LL ^a		PL ^a		T	PL+LL
	(n=6)		(n=6)		(n=6)			
	C	s(n-1)	C	s(n-1)	C	s(n-1)	T	PI+LL
	(nmol/μl)		(nmol/μl)		(nmol/μl)		(pmol)	(pmol)
UTP	3.6	0.24	2.6	0.18	4.2	0.41	11.6	10.4
ITP	2.7	0.37	1.9	0.25	3.7	0.84	8.5	8.5
GTP	3.2	0.20	1.6	0.11	6.6	0.96	10.3	11.9
ATP	9.3	0.93	7.3	0.72	13.9	0.72	29.8	32.3
UDP	3.7	0.54	1.8	0.45	6.4	0.63	11.8	12.1
CTP	2.5	0.22	1.7	0.15	3.3	0.31	8.0	7.7
GDP	1.7	0.38	1.4	0.17	2.5	0.33	5.3	6.0
ADP	5.2	0.60	3.3	0.29	8.6	0.82	16.5	17.7
CDP	1.3	0.15	0.8	0.10	1.9	0.22	4.0	3.9
G6P ^c	16.7	1.20	21.1	1.23	9.6	0.79	53.5	51.8
GMP	1.5	0.30	1.5	0.21	3.2	0.84	4.7	7.0

Student's *t*-test for statistical significance of differences between sample means of nucleotides concentrations in PL and LL revealed that for all nucleotides the polar lobe contains higher levels than the lobeless part of the embryo ($p < 0.001$).

^a Mean values (*C*) and SE *s*(*n*-1).

^b Mean quantities (*Q*) of components per T and estimates of *Q* (*Q'*) calculated as the sum of quantities per PL and LL respectively.

^c G6P, Glucose-6-phosphate.

quantities per T are in close agreement with each other. This demonstrates reproducibility of the extraction procedure and of the analytical approach.

In order to define the differences in nucleotides concentrations between PL and LL more precisely, we have analysed low molecular weight compounds in yolk material. PLs contain a relatively large amount of yolk, and localization of nucleotides within the yolk might be the reason for the observed high concentrations in the PL. Extraction of isolated yolk material with MeOH-EDTA even for periods as long as 17.5 h did not release any significant amounts of nucleotides. MeOH-EDTA extracts of total Ts, however, contain considerable amounts of nucleotides, although less than found in PCA extracts. These results indicate that virtually all of the low molecular weight components are present in the non-yolk cytoplasmic pool.

DISCUSSION

Our studies reveal that capillary ITP is an appropriate technique for studying the spectrum of low molecular weight compounds of early developmental stage embryos, and of differences in distribution of individual compounds. As prelimi-

nary investigations pointed out that nucleotides are among the most predominant low molecular weight compounds [14], the extraction procedure and operational conditions were optimized for analysis of these substances in particular. Several extraction procedures were tested, all of which are variations of either PCA or MeOH extraction, two procedures commonly met in the literature. These extraction procedures are directed towards rapid precipitation and inactivation of enzymes that might either cause transformation of metabolites (e.g., nucleotide triphosphates) or decomposition of macromolecular compounds (e.g., nucleic acids). For *Nassarius*, PCA extraction appears to be superior to MeOH-EDTA extraction [32]. The procedure for sample preparation in combination with PCA precipitation, which was finally adopted, appears to give good results, both quantitatively and qualitatively, although not satisfactory in all respects. The prolonged sampling times which were necessary do not introduce changes in the total spectrum, nor do they cause additional variance in quantities at least if sampling is terminated within 2 h [14].

It is of interest to compare the results of these studies with data described for other systems. Measurements of nucleotides pool sizes of the egg have been performed in several species, e.g., *Ilyanassa* [18, 19], mouse [20–22], star fish [24] and sea urchin [25–27], and were directed towards analysis of particular ribonucleotides [18–20, 23] or deoxyribonucleotides [23, 25, 26]. Analyses of nucleotide spectra by means of ITP have been described for adult material [28], but to our knowledge not for embryonic systems. The results of nucleotides measurements on embryonic systems, which have been reported in the literature, have in all cases been obtained with a different methodological approach. The luciferine/luciferase enzymatic assay has been used for measuring ATP levels in among others sea urchin [25, 27], and *Ilyanassa* [18]. A more sophisticated approach has been followed for analysing UTP and ATP pool sizes in the mouse egg using a synthetic polynucleotide (poly(dA-dT)) in combination with *E. coli* RNA polymerase and either radiolabeled UTP or ATP as exogenous second precursor for in vitro RNA synthesis [20, 21]. The ATP levels found for sea urchin [25], *Ilyanassa* [18] and mouse [20, 21] are 1.4, 25.39 and 1.1 pmol per egg respectively. On a per volume basis, these values are about 0.3, 11.6 and 0.29 nmol/ μ l. The quantity and concentration of ATP in the *Nassarius* egg are 29.8 pmol per egg and 9.26 nmol/ μ l respectively, values which are close to those described for *Ilyanassa* [18]. These two species, both molluscs, are characterized by relatively high ATP levels. Quantitative data on other nucleotides are not available for *Ilyanassa*. In the mouse the UTP level measures about 0.2 nmol/ μ l as compared with 3.4 nmol/ μ l for *Nassarius*. A high concentration of nucleotides in the egg might be a characteristic feature of the molluscan egg. Recovery experiments using internal standards, in which the same analytical approach was followed as in the present studies, have shown that recovery of nucleotides with the present analytical set-up is about 95% [16].

The levels of ATP reported for *Ilyanassa* [18] and found by us for *Nassarius*,

are in the same order, although they are obtained with different analytical methods (luciferin/luciferase and ITP respectively). A difference between these two methods is, however, met in the variance of the analytical data. In studies on *Ilyanassa* and sea urchin, in which the luciferin/luciferase method was used to measure ATP quantities in the egg, a coefficient of variation between 2.5 and 5% was reported [18, 26]. In our study, the value for this variance parameter for the respective nucleotides is about 10%. This value is comparable to that reported for ATP and UTP quantities in the mouse ovum [20, 22] obtained with isotope incorporation measurements. As the system reproducibility of ITP is known to be approx. 2% [16], the higher variance values in our studies, as compared with those conducted on *Ilyanassa*, may be caused by a larger component of biological variation. The additional variance caused by differences in nucleotides levels within an egg mass, and among egg masses in *Nassarius* is not known. The blurred zone boundaries between ATP/UDP and phosphate/GDP necessitated an interpolation strategy. As explained in Results, this is a valid approach, although some of the variance within, e.g., ATP quantities might herewith be explained.

Measurements of deoxyribonucleotides cannot be performed with the present analytical set up, because the levels are much too low. The amounts of deoxyribonucleotides in embryonic material are about two orders of magnitude lower than those of ribonucleotides. In sea urchin, for example, the ratio of ATP/dATP is about 400 [26]. In *Ilyanassa*, like in *Nassarius*, a significantly higher level of ATP is found in the polar lobe. In *Ilyanassa*, the polar lobe contains about 37.8% of the ATP store of the egg, and the concentration of ATP is about 1.27 times as high as in the lobeless part of the egg. In *Nassarius*, the ATP concentration in the lobe is about 1.9 times as high. For the other nucleotides this ratio varies from 1.6 (UTP) to 4.1 (GTP). In *Nassarius*, like in *Ilyanassa*, the polar lobe apparently is highly enriched in its ribonucleotide contents. This is still more obvious, if the concentrations of nucleotides are calculated relative to the free cytoplasmic (non-yolk) volume. This is legitimate, as the yolk in *Nassarius* apparently does not contain appreciable amounts of nucleotides. Free cytoplasmic volumes have not been estimated in this study, but are reported for *Ilyanassa* [29]. Assuming roughly the same yolk content and yolk distribution in the two species, the concentrations of acid-extractable low molecular weight compounds in the polar lobe must be multiplied by a factor of 1.66. The ratio of ATP concentration between the polar lobe and lobeless part then becomes approx. 3.2. This corroborates the statement above, that the polar lobe is highly enriched in its nucleotides contents. The differences between the two isolates, when comparing these corrected values, for all nucleotides are highly significant (Student's *t*-test, Ho, $p < 0.001$).

Our analyses have revealed some other characteristic differences between the two isolates (PL and LL). The lobeless part is characterized by a significantly higher level of glucose-6-phosphate (G6P). This suggests, that the energy metabolism in the lobeless part is considerably higher than in the polar lobe.

In view of the morphogenetic role of the polar lobe, it is reasonable to postulate that the high concentration of ribonucleotides in the lobe has some bearing on polar lobe-dependent developmental control. It is difficult, however, to envisage how elevated levels of nucleotides, as such common metabolites, might be connected to the highly specific determinative and differentiative steps in early development. We are reticent in assigning a status of "morphogenetic determinant" to the enriched nucleotides contents of the polar lobe. The concept of "determinant" is generally used to designate a specific molecule or set of molecules (e.g., RNA or protein), which (irreversibly) sets the program of gene expression in the cells to which they are segregated (in *Nassarius* the D-quadrant cells). Our analyses have not provided evidence for the presence of polar lobe-specific compounds. These results have prompted us to reconsider the concept of "morphogenetic determinant". The question is, whether specific prelocalized morphogenetic determinants do exist at all. It seems feasible that the high concentration of nucleotides in the polar lobe brings about differential expression of maternal genome products like mRNA or protein (which not necessarily have to be polar lobe-specific) in D-quadrant cell lines. This in turn might give rise to reaction products, which determine programming of the embryo genome. The high concentration of nucleotides in the polar lobe might thus provide a permissive scaffold for these processes to occur. Such a view stresses the importance of post-transcriptional control mechanisms, which is in accordance with molecular studies on early molluscan development [30, 31]. Determination might thus be the result of the concerted interplay of different molecular systems, that encompass common molecular species, but whose distribution is inhomogenous with respect to the polar axis. According to this view, the determined state of D-quadrant cells is the consequence of a sequence of subcellular molecular processes, rather than of one specific decisive step. The idea that the nucleotides pool in the polar lobe provides permissive conditions for D-quadrant-specific processes is feasible in view of the many roles that nucleotides (especially the energy-rich triphosphates) play in cellular processes at physiological, cell biological and molecular levels. First, the energy-rich nucleotides (in particular triphosphates) are generally involved in energy-requiring processes, e.g., pathways in the expression of the animal genome (RNA and protein synthesis, post-translational modifications of proteins), synthesis of polysaccharides (as carrier of metabolic intermediates), assembly of macromolecules into polymeric structural configurations (e.g., assembly of tubulins into microtubules requires GTP). Second, the ribonucleotide pool may be utilized as a source for *de novo* RNA synthesis within cell lines that receive polar lobe materials. Concentrations and relative amounts of ribonucleotides may be of significance for regulation of transcription of the embryo genome, both quantitatively and qualitatively. To envisage the polar lobe as a morphogenetic store which is utilized predominantly during later development seems to be corroborated by the significantly lower G6P level in the lobe.

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