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NUCLEOTIDES IN EMBRYOS IN THE STAGES OF MORULA, GASTRULA, AND NEURULA*

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ABSTRACT

Levels of nucleotides and sugar nucleotides in embryos of *Bufo arenarum* at the stages of morula, gastrula, and neurula have been measured. The total amounts of purine nucleoside diphosphates decreased from morula to gastrula, but increased sharply from gastrula to neurula. The levels of ADP followed this pattern, but those of GDP did not change significantly through the three stages. Purine nucleoside triphosphate levels, which had increased immediately after fertilization, remained almost constant through morula, gastrula, and neurula. As with the purine nucleoside diphosphates, the adenine nucleotide decreased from morula to gastrula, and increased from gastrula to neurula. In contrast, the level of GTP showed a sharp maximum at gastrula. The total pyrimidine nucleoside triphosphate did not change significantly from morula through neurula. As in previous stages of development, only uridine sugar nucleotides were detected. A sharp increase of the galactosyl ester of nucleotides was found at gastrula.

INTRODUCTION

During early development of *Bufo arenarum*, neither ribosomal nor informational RNA is synthesized, and the process of synthesizing embryo proteins utilizes ribonucleic acids of maternal origin[2–5]. In mid-blastula, new m-RNA is detected, whereas synthesis of new r-RNA starts at early gastrula[6]. Both m-RNA and r-RNA are made from the same pool of nucleoside triphosphates, and a metabolite essential for regulation of their synthesis is supposedly of nucleotidic nature[7]. In addition,

*Nucleotides and Sugar Nucleotides in Early Development of *Bufo arenarum*, Part II. For Part I, see ref. 1.

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levels of sugar nucleotides* during early development can be related to the biosynthesis of membrane polysaccharides that are very active after fertilization. Therefore, a comparison is here made of the composition (in regard to nucleotides and sugar nucleotides) of embryos in morula, gastrula, and neurula, in order to detect possible variations during stages that differ noticeably in morphological and metabolic aspects.

As in the preceding paper[1], extreme care was taken to preserve labile structures, and 60,000 to 120,000 embryos were used (700 to 1,400 μmoles of nucleotides) to ensure the isolation of compounds present in very small proportions.

EXPERIMENTAL

Materials. — Unless otherwise stated, materials and methods were as described previously[1].

Extraction procedure. — Six to twelve females of *B. arenarum* were injected with 3 ml of an aqueous extract from two homologous hypophyses, and the animals were maintained at 25°. After ovulation, oocytes were extracted from the ovisacs. Insemination was achieved by pouring a suspension of sperm taken from the testes of two males of *B. arenarum* over the oocytes. After 15 min, fertilization was complete, and the eggs were dispersed in several Petri dishes with Holtfreter solution[8], and kept at 18–20°. Morula was taken at the stage of 16–32 blastomers (~6 h after fertilization), early gastrula at the appearance of the dorsal lip of blastopore (~22 h), and neurula at neural tube formation (~72 h). The jelly coat was removed by a brief treatment with sodium thioglycolate (2%, pH 9) and washed exhaustively with Holtfreter solution. Samples from each stage were homogenized in a blender with 2 volumes of cold, 10% trichloroacetic acid (TCA) for 1 min. The homogenate was centrifuged for 30 min at 3,000 *g*, and the precipitate was re-extracted with 10% TCA. The solutions were combined, and TCA was removed by three washings with cold ether. The pH of the aqueous phase was brought to 7 with concentrated ammonia. The extracts were concentrated *in vacuo* at room temperature, and the concentrate stored at −20° until processed. Acid-soluble extracts were chromatographed on a column (40 × 5 cm) of Dowex 1 X-4 (Cl^-) resin (200–400 mesh) at 5°. The bases and nucleotides adsorbed were eluted, first with water and then with a linear gradient of ammonium chloride (0–0.9M). Fractions (15 ml each) were collected, and their absorbance was measured at 260 nm. Each peak was pooled, and processed as already described[1].

*Glycosyl esters of nucleoside 5′-diphosphates will be referred to as "sugar nucleotides". Abbreviations used: ADP, adenosine 5′-diphosphate; DNA, 2′-deoxyribonucleic acid, GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; NAD, nicotinamide adenine dinucleotide; RNA, ribonucleic acid; UDP, uridine 5′-diphosphate; UDP-Gal, uridine 5′-(D-galactosyl diphosphate); UDP-GalNAc, uridine 5′-(2-acetamido-2-deoxy-D-galactosyl diphosphate); UDP-Glc, uridine 5′-(D-glucosyl diphosphate); and UDP-GlcNAc, uridine 5′-(2-acetamido-2-deoxy-D-glucosyl diphosphate).

Analytical methods. — Paper chromatography was conducted on Whatman No. 1 paper with the following solvents (v/v): (I) ethanol–ammonium acetate (M) at pH 7.5, (II) ethanol–ammonium acetate[9] at pH 3.8, (III) 6:4:3 butanol–pyridine–water[10], and (IV) 41:170:39 hydrochloric acid–isopropyl alcohol–water.

In order to analyze a mixture of purine and pyrimidine nucleotides (peak VI), three methods were used. (*1*) Pentose determination by the orcinol method, with bromine[11] (for purine plus pyrimidine nucleotides), and without bromine[12] (for purine nucleotides). (*2*) Acid hydrolysis, and adsorption on a small column of an ion-exchange resin. Five μmoles of peak VI were hydrolyzed in 3M hydrochloric acid for 60 min at 100°, and the acid was removed by evaporation under diminished pressure. An aqueous solution of the residue was applied to a column (0.6 × 8 cm) of Dowex 1 X-4 (Cl^-) resin (200–400 mesh). The free base (corresponding to purine nucleotide before acid hydrolysis) was eluted with water, and the pyrimidine nucleoside monophosphates (corresponding to pyrimidine nucleoside triphosphates before acid hydrolysis) were eluted with M hydrochloric acid. Recoveries were in the order of 90%. The relative amounts of the pyrimidine nucleotides were measured by ultraviolet absorbance. (*3*) Chromatographic or electrophoretic separation of the nucleotides, followed by elution from the paper, and measurement by ultraviolet absorbance.

When two purine nucleotides or two pyrimidine nucleotides were eluted simultaneously, they were determined spectrophotometrically by making use of the differences in their spectra[13].

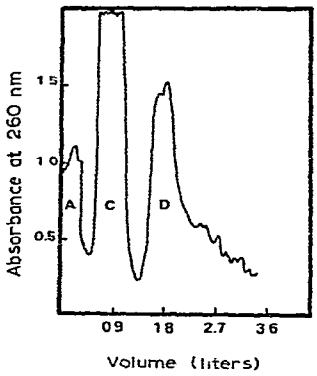
Acetamidodeoxyhexoses were determined as described by Reissig *et al.*[14].

UDP-GalNAc and UDP-GlcNAc were degraded to arabinose and lyxose by following the technique of Gardell *et al.*[15], and the sugars were separated chromatographically in solvent III.

Galactose was determined by the galactose oxidase technique[16]. Galacturonic acid was determined by a modification of the Ceriotti method[17] for DNA. The spectra of the colored compounds obtained with galacturonic acid and DNA are identical. The reaction is very sensitive, and specific (among uronic acids) for galacturonic acid. Glucuronic acid gave no reaction at all in concentrations 15 times greater than those satisfactory for galacturonic acid.

RESULTS

Figs. 1 and 2 show the profile of the ultraviolet-absorbing substances eluted directly with water and with a linear gradient of ammonium chloride (0–0.9M). The results were qualitatively similar in morula, gastrula, and neurula. Each peak was submitted to a series of analyses for identification of its constituents: drastic hydrolysis in 3M hydrochloric acid, and paper chromatography in solvent IV to identify the purine or pyrimidine base; chromatography in solvents I and II, and electrophoresis in phosphate buffer (pH 7.5) and acetate buffer (pH 3.8); chromatography in solvent III after acid hydrolysis, to identify the sugar moiety; and measurement of phosphate (total and labile), pentose, and sugar content.



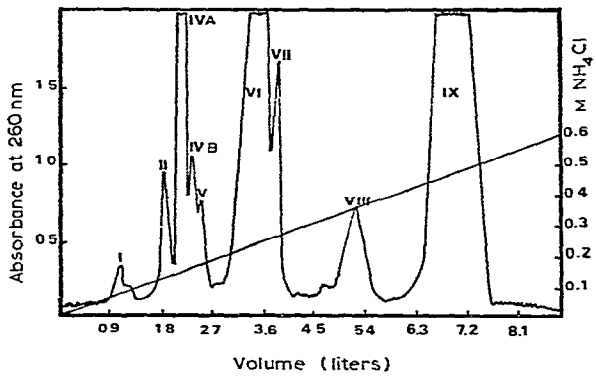


Fig. 1. Elution pattern of an extract from embryos in the gastrula stage, adsorbed on a column (40 × 5 cm) of Dowex 1 X-4 (Cl^-) resin (200–400 mesh) and eluted with water. (Fraction volume: 15 ml. The absorbance was measured at 260 nm.)

Fig. 2. Elution pattern of an extract from embryos in the gastrula stage, adsorbed on a column (40 × 5 cm) of Dowex 1 X-4 (Cl^-) resin (200–400 mesh) and eluted with 12 liters of a linear gradient of ammonium chloride from 0–0.9M. (Fraction volume: 15 ml. The absorbance was measured at 260 nm.)

The same bases, nucleotides, and sugar nucleotides were found at the three stages of development, but great quantitative differences were detected. As Fig. 3 shows, significant differences were found in the levels of guanine, with a maximum at the gastrula stage. A slow but significant increase in the amount of NAD was seen from morula to neurula, as is shown in Fig. 4.

Quantitative differences were also detected in the levels of purine nucleotides, pyrimidine nucleotides, and the nucleotide esters of glucose and galactose (see Tables I and II). The data are the averages for two (or three) samples.

The total amounts of purine nucleoside diphosphates decreased from morula to gastrula, but increased sharply from gastrula to neurula. The levels of ADP followed this pattern, but those of GDP did not change significantly through the three stages (see Fig. 5). The levels of purine nucleoside triphosphate, which had increased immediately after fertilization[1], remained almost constant through morula, gastrula, and neurula. Again, as was seen for the purine nucleoside diphosphates, the level of adenine nucleotide decreased from morula to gastrula, and increased from gastrula to neurula. In contrast, the level of GTP showed a sharp maximum at gastrula (see Fig. 6).

As in previous stages of development (ref. 1 and unpublished results), only uridine sugar nucleotides, UDP-*N*-acetylhexosamines, UDP-hexoses, and UDP-hexuronic acids were found in morula, gastrula, and neurula. The sugar moiety was always glucose or galactose, or their corresponding derivatives. As may be seen from Fig. 7, UDP-Gal increased from morula to gastrula, and UDP-Glc had a minimum at gastrula. The UDP-*N*-acetylhexosamines decreased significantly from morula to gastrula (see Fig. 8).

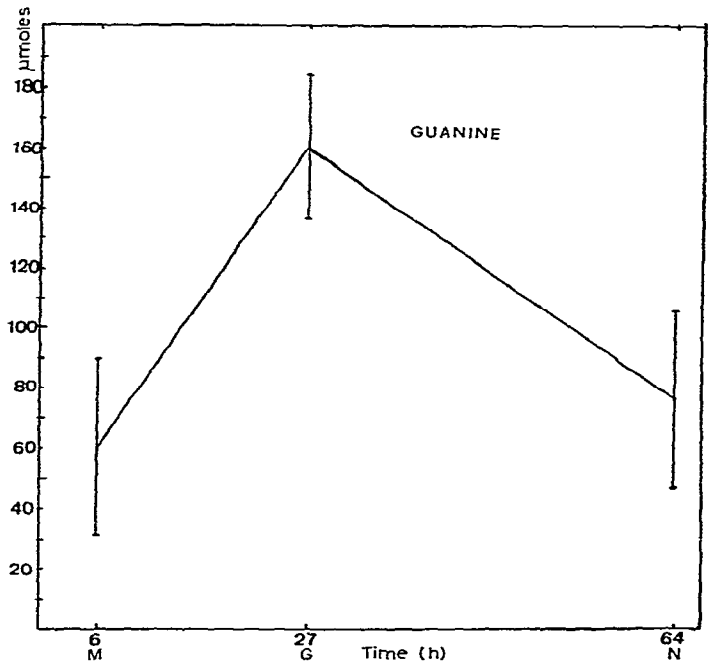


Fig. 3. Comparison between levels of guanine in extracts from embryos in the morula (M), gastrula (G), and neurula (N) stages.

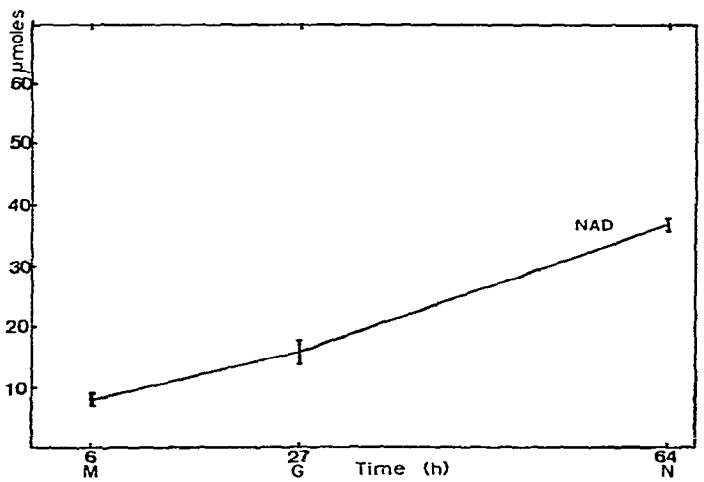


Fig. 4. Comparison between levels of NAD in extracts from embryos in the morula, gastrula, and neurula stages.

TABLE I

QUANTITATIVE DATA FOR THE PEAKS[a] OBTAINED BY ION-EXCHANGE CHROMATOGRAPHY OF EXTRACTS FROM EMBRYOS IN MORULA, GASTRULA, AND NEURULA

Peak	*Constituents*	*Morula*		*Gastrula*		*Neurula*	
		%	*μmol (mean ±s.d.)*	%	*μmol (mean ±s.d.)*	%	*μmol (mean ±s.d.)*
C	Hypoxanthine	—	295 ±14	—	273 ±12	—	273 ±20
D	Guanine	—	60 ±29	—	159 ±24	—	76 ±29
I	CDP-choline	—	16 ±4	—	19.5 ±1.1	—	26 ±12
II	NAD	30	8.4 ±0.8	—	15.9 ±2.4	—	36 ±1
	UMP	70	20 ±2	—	—		—
IVA	UDP-GlcNAc UDP-GalNAc	—	95 ±9	—	56 ±1	—	61.6 ±4.3
IVB	UDP-Glc	98.5	18.8 ±0.8	—	12.6 ±0.5	95.5	44 ±4
	UDP-Gal	1.5	0.3 ±0.0	30	5.4 ±0.2	4.5	2.1 ±0.2
V	TPN	—	1.8 ±0.1	—	9.6 ±1.1	—	2.6 ±0.2
VI	UTP	65	127 ±28	72.3	113 ±13	80	163 ±43
	CTP	35	68 ±15	25.5	40 ±5	20	41 ±11
	AMP	—	—	1.8	2.7 ±0.3	—	—
VII	UDP-GlcNAc UDP-GalNAc	—	44 ±11	—	29.9 ±1.6	—	29.6 ±1.6
VIII	ADP	54	17.1 ±1.3	40	10.6 ±2.2	73	32.6 ±4.5
	GDP	46	14.6 ±1.1	60	15.8 ±3.3	27	12.1 ±1.7
IX	ATP	73	150 ±7	58	110 ±3	75	162 ±3
	GTP	27	56 ±3	42	79 ±2	25	54 ±1

[a]Micromoles of each compound are referred to 1,000 μmoles of bases, nucleotides, and sugar nucleotides eluted from the column (average absorption coefficient used: $10 mM^{-1} \cdot cm^{-1}$). Data are the averages of three samples from different ovulations.

TABLE II

STUDENT TEST OF THE DATA IN TABLE I

Peak	*Constituents*	*Morula–Gastrula*	*Morula–Neurula*	*Gastrula–Neurula*
D	Guanine	t 2.6 p <0.05	n. s.[a]	t 2.2 p <0.05
II	NAD	t 2.9 p <0.025	t 2.9 p <0.025	t 7.9 p <0.0025
IVA	UDP-GlcNAc UDP-GalNAc	t 4.3 p <0.01	t 3.3 p <0.025	n. s.
IVB	UDP-Glc	t 6.7 p <0.0025	t 6.2 p <0.005	t 7.9 p <0.0025
	UDP-Gal	t 23.1 p <0.0005	t 9.5 p <0.0025	t 11.8 p <0.0025
VIII	ADP	t 2.5 p <0.05	t 3.3 p <0.025	t 4.4 p <0.0125
	GDP	n. s.	n. s.	n. s.
IX	ATP	t 5.1 p <0.0025	n. s.	t 11.5 p <0.0025
	GTP	t 6.5 p <0.0025	n. s.	t 9.4 p <0.0025

[a]n.s., not significant.

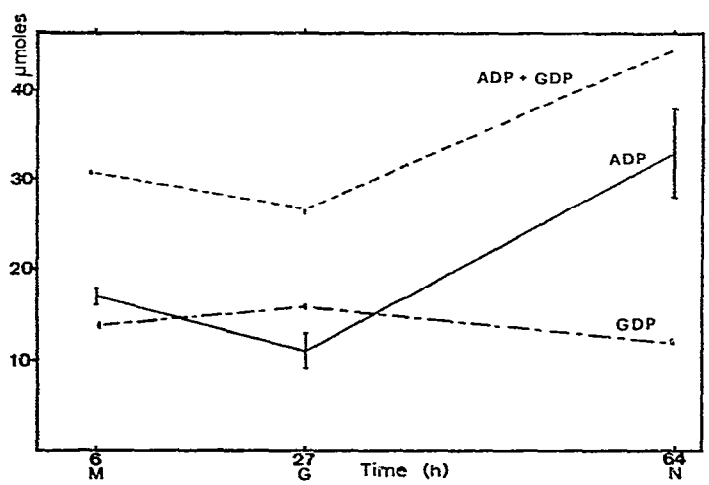


Fig. 5. Comparison between the levels of purine nucleoside diphosphates in extracts from embryos in the morula, gastrula, and neurula stages.

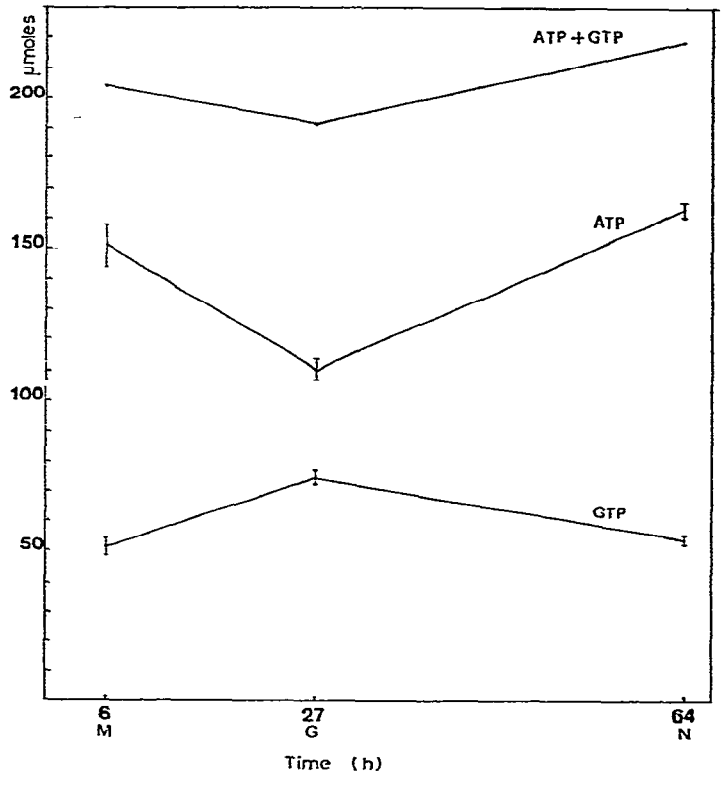


Fig. 6. Comparison between the levels of pyrimidine nucleoside triphosphates in extracts from embryos in the morula, gastrula, and neurula stages.

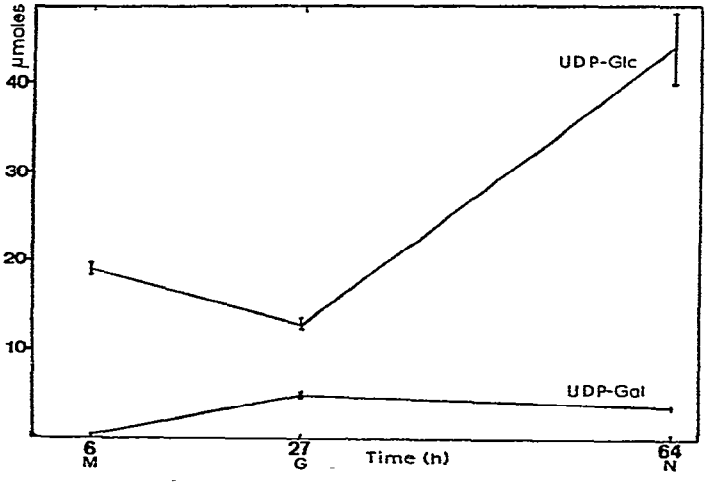


Fig. 7. Comparison between the levels of UDP-hexoses in extracts from embryos in the morula, gastrula, and neurula stages.

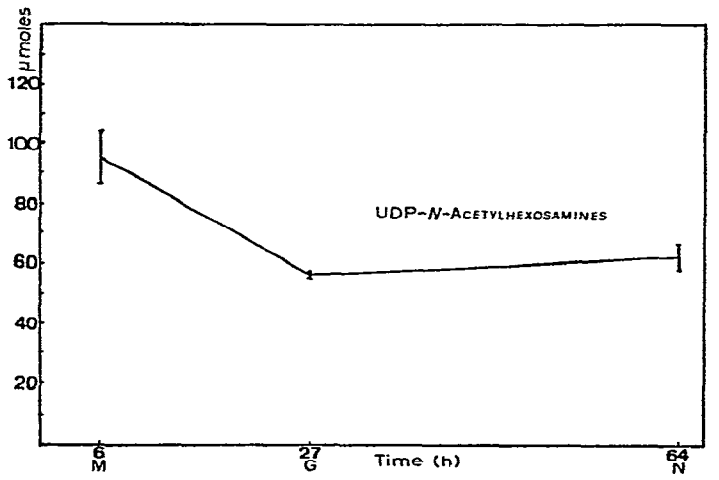


Fig. 8. Comparison between the levels of UDP-*N*-acetylhexosamines in extracts from embryos in the morula, gastrula, and neurula stages.

DISCUSSION

In most vertebrates, cellular differentiation is visible at the gastrula stage, because of the presence of arquenteron cells having very specialized functions; but, at the molecular level, differentiation starts before, at blastula, when the synthesis of new, informational[6] RNA induces synthesis of new proteins which seem to act as regulatory signals[18] for the utilization of the old m-RNA built at late oogenesis and during ovulation[6].

Morula, gastrula, and neurula are, then, very important stages of development as regards changes at the molecular level. We could not find qualitative differences in nucleotides or sugar nucleotides during the three stages. On the contrary, significant quantitative differences were detected (see Tables I and II). High levels of guanine found at gastrula could be related with the increased proportion of GTP detected at the same stage. Guanine and hypoxanthine could be precursors of purine nucleotides.

Sugar nucleotides change significantly at gastrula. A sharp increase in the level of galactosyl ester of nucleotides was found. One possible explanation for this could be changes in the proportions or activities of specific galactosyltransferases. In this respect, it is interesting that a galactosyltransferase has been found on the surface of chick-embryo, retinal cells[19], and it was postulated that this enzyme might be involved in the process of intercellular adhesiveness. Similarly, movements during gastrulation are the consequence of changes in cell adhesiveness. It is not yet known whether, in this key stage of embryogenesis, a galactosyl polymer is also built up.

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