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NUCLEOTIDES AND NUCLEOTIDE SUGARS IN EARLY DEVELOPMENT OF *BUFO ARENARUM*

PART I. MATURE OOCYTES*

MARTA ELENA FERNÁNDEZ DE RECONDO, MARIA CRISTINA MAGGESSE, GRACIELA GUERRERO, ROSA C. SPAIZMAN, AND EDUARDO F. RECONDO†

Embriología Animal y Biología Molecular, Departamentos de Ciencias Biológicas y Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón 2, Ciudad Universitaria, Buenos Aires (Argentina)

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ABSTRACT

A study of nucleotides and nucleotide sugars in mature oocytes of *Bufo arenarum* has been performed. Two samples of oocytes from ovulations of ten females each were analyzed by ion-exchange chromatography and the following nucleotides and nucleotide sugars found**: NAD, UDPGNAc, UDPGalNAc, UDPG, UDP, NADP, CTP, UTP, UDPGA, ADP, GDP, GTP, and ATP. The nucleotides and sugar-nucleotides were identified on the basis of chemical, enzymic, and chromatographic analysis. To minimize the decomposition of unstable substances, the extraction was performed with trichloroacetic acid in the cold, the contact of the sample with the acid was minimal and ammonium chloride was used for the elution from the Dowex 1-resin. In order to detect compounds present in minute amounts, very large samples were used (60,000 to 120,000 oocytes). A nucleotide of complex structure, which binds weakly to the column, was detected in the two samples analyzed.

INTRODUCTION

The main objective of our research work is the study of the early development of an amphibian, *Bufo arenarum*, from a biochemical point of view and at a molecular level. Because of the external fertilization, all the steps of embryogenesis can be followed from the very beginning by *in vitro* fertilization. After fertilization and during cleavage, the embryo uses ribosomal ribonucleic acid of maternal origin, made during the oogenesis, for the protein-synthetizing machinery[1–4]. Synthesis of new

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†Career Investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina).

**The following abbreviations are used: ADP, adenosine 5′-pyrophosphate; ATP, adenosine 5′-triphosphate; CTP, cytidine 5′-triphosphate; GDP, guanosine 5′-pyrophosphate; GTP, guanosine 5′-triphosphate; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; UDP, uridine 5′-pyrophosphate; UDPG, uridine 5′-(D-glucosyl pyrophosphate; UDPGA, uridine 5′-(D-glucosyluronic acid pyrophosphate); UDPGalNAc, uridine 5′-(2-acetamido-2-deoxy-D-galactosyl pyrophosphate), UDPGNAc, uridine 5′-(2-acetamido-2-deoxy-D-glucosyl pyrophosphate), and UTP, uridine 5′-triphosphate.

rRNA starts only at the onset of gastrulation. On the other hand, informative RNA (mRNA), synthetized and stored in the oocytes at the lampbrush-chromosome stage of oogenesis, is still present in the embryo beyond the late blastulation[5–8]. All these observations emphasize the importance of ribonucleic acids in the early development of the amphibian embryo, particularly because they are synthetized during oogenesis, stored intact through ovulation and early cleavage, and consumed totally or partially at the beginning of gastrulation. Since both RNA (ribosomal and informational) are made from the same pool of nucleotide precursors, a systematic research was undertaken of possible changes in nucleotide composition during the complete process, from oogenesis through gastrulation and neuralization.

It was thought that a metabolite essential for regulation of both types of RNA synthesis could be of nucleotidic nature and could appear or disappear at some key points of development. It was also of interest to establish the modifications of the pattern of the pool of nucleotides and sugar nucleotides during embryogenesis. In addition, the appearance (or disappearance) of sugar nucleotides during the development could be related to the biosynthesis of membrane polysaccharides of the oocytes; these compounds might play important roles in oogenesis, ovulation, and fertilization.

Samples were taken from oocytes of the ovaries and the body cavity, from mature oocytes, and from eggs after fertilization and during the different stages of early development. Extreme precautions were taken to preserve labile structures. The extraction step was performed with trichloroacetic acid, in the cold, with a minimal contact of the samples with the acid. Ammonium chloride was employed for the elution from the Dowex-1-resin, since ammonium salts have been shown to be less harmful for the stability of nucleotides[9]. Special attention was also given to the detection of compounds present in very small amounts. Eggs from spawnings of 6 to 12 animals were used, which means between 60,000 and 120,000 eggs or 700 to 1,400 μmoles of nucleotides measured by u.v. absorption at 260 nm (ε 10^4, expressed as uracil). This first report describes the isolation and identification of nucleotides and sugar nucleotides from samples of mature oocytes.

EXPERIMENTAL

Materials. — AMP-^{14}C (322 μC/mole), ATP-^{14}C (250 μC/mole), and UDP-D-glucose-^{14}C (204 μC/mole) were purchased from the Radiochemical Centre Amersham (England).

D-Glucose-oxidase (β-D-glucose:oxygen oxidoreductase, EC 1.1.3.4) and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) were obtained from Sigma Chemical Co. (St. Louis, Mo., U. S. A.). Purine and pyrimidine bases, nucleosides, nucleotides, sugar nucleotides, and sugars were commercial preparations. Animals were purchased from a local supplier.

Extraction procedure. — Six to twelve females of *Bufo arenarum* were injected each with 3 ml of an aqueous extract from two homologous hypophyses. After approximately 16 h at 25°, the oocytes were extracted from the ovisacs and poured

into Petri dishes with Holfretter solution. After they swelled, the jelly coat was removed by treatment with a 2% sodium thioglycolate (pH 9), and the oocytes were washed exhaustively with Holfretter solution. Then they were suspended in 10% trichloracetic acid (2 vol.) and homogeneized with a blendor for 1 min. The grey suspension was centrifuged at 5.000 r.p.m. for 30 min. The supernatant solution was extracted three times with cold ether to remove most of the acid and finally neutralized with ammonia to pH 7.0. The grey precipitate was treated again with 10% trichloracetic acid, centrifuged off, and the supernatant solution extracted with ether and neutralized as just described. The two extracts were pooled, the organic solvent was removed by evaporation at room temperature *in vacuo*, and the solution stored in a frozen state at −20° until used.

Ion-exchange chromatography. — Extracts together with markers of AMP-^{14}C, ATP-^{14}C, or UDP-D-glucose-^{14}C, were poured onto a column (40 × 5 cm) of Dowex 1 (X-4, Cl^-, 200–400 mesh) resin, and washed with 4 l of distilled water. Fractions of 15 ml were collected at a rate of 10 ml/min and their absorbancy was determined at 260 nm in a Zeiss spectrophotometer (Fig. 1). Under these conditions, the free bases were eluted in sequence. Then, the still adsorbed nucleotides were eluted with a linear gradient of ammonium chloride (0–0.9M). Fractions of 15 ml were collected and their absorbance measured at 260 nm (Fig. 2). The radioactivity of each fraction was determined in a Packard scintillation counter on aliquots (0.25 ml). The pooled fractions corresponding to each peak of u.v.-absorbing material were adsorbed on Norit A and eluted in sequence with 50% ethanol and 50:1:150 (v/v) ethanol–ammonia–water. Ammonia was eliminated immediately by evaporation under reduced pressure, which was continued until a concentration of about 1 μmole/ml of nucleotide, measured as uridine, was reached. The fractions were stored in a frozen state at −20° until the analyses were performed.

Analytical methods. — Inorganic orthophosphate was determined by the Fiske and SubbaRow method[10] and acid-labile phosphate by the same method after hydrolysis with 0.05M sulfuric acid at 100° for 15 min. Total organic phosphate was measured similarly after a previous treatment with perchloric acid–sulfuric acid. The solution was heated until a yellow color developed and boiling was maintained until it disappeared.

The pentose content in purine nucleotides was measured by the orcinol reaction[11]. Cohn's procedure[12], slightly modified by Yanagisawa and Isono was used[13] for pyrimidines nucleotides before the orcinol reaction was applied. 2-Acetamido-2-deoxyhexoses were determined as described by Reissig *et al.*[14], and D-glucose by the D-glucose oxidase technique, as described by Huggett and Nixon[15]. Bases were identified by paper chromatography with solvent systems V, VI, and VII, after acid hydrolysis with 3M hydrochloric acid at 100° for 1 h.

Paper chromatography was performed on Whatman paper No. 1 with the following solvents: (I) ethanol–M ammonium acetate, at pH 7.5; (II) ethanol–ammonium acetate[16] at pH 3.8; (III) 6:4:3 (v/v) butyl alcohol–pyridine–water[17]; (IV) 7:3 ethanol–ammonia; (V) 41:170:39 hydrochloric acid–2-propanol–water[18]; (VI)

100:1 butyl alcohol (saturated with water at about 23°)–ammonia[19]; (VII) 27:4:9 2-propanol–acetic acid–water[20]; (VIII) 0.1M buffer phosphate (pH 6.8)–ammonium sulfate–propyl alcohol[21].

Paper electrophoresis was performed on Whatman paper No. 4, as described by Markham and Smith[22], with 0.05M potassium tetraborate buffer, pH 9.2; disodium phosphate buffer, pH 7.5; sodium carbonate–sodium hydrogen carbonate buffer[23], pH 9.2; sodium acetate buffer, pH 3.8; or pyridine acetate buffer, pH 6.5.

Sugar spots were revealed with the reagent of Trevelyan *et al.*[24] and phosphate compounds by dipping the papers into the molybdate reagent described by Burrows *et al.*[25]. U.v.-absorbing substances were located with a Mineralight lamp.

RESULTS

Elution with water of the extract from mature oocytes adsorbed on Dowex 1 (X-4) gave three main peaks of u.v.-absorbing substances (Fig. 1). The properties of Peak A were characteristic for a protein and were not further analyzed. Peak B was present in a very small proportion and was not identified. Peak C was identified as hypoxanthine by the u.v. absorption spectrum and by its behavior in paper chromatography with solvents V and VII and paper electrophoresis with a borate buffer at pH 9.2. Peak D was identified as guanine by the u.v. absorption spectrum and its

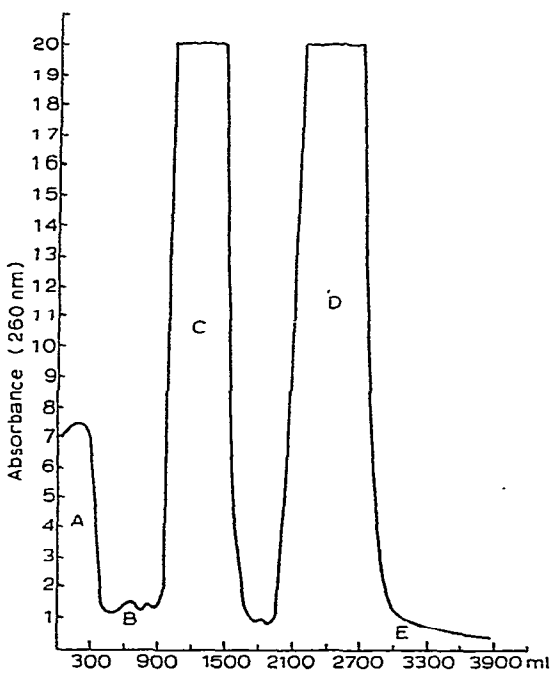

Fig. 1. Elution pattern of an extract of mature oocytes adsorbed on a 40 × 5 cm column of Dowex 1 (X-4, Cl^-, 200–400 mesh) and eluted with distilled water at a rate of 10 ml/min. Fractions of 15 ml.

behavior in paper chromatography with solvents V, VI, and VII and paper electrophoresis with a borate buffer at pH 9.2. Area E had a yellow color and a u.v. absorption spectrum and chromatographic behavior similar to those of riboflavine or some related compound.

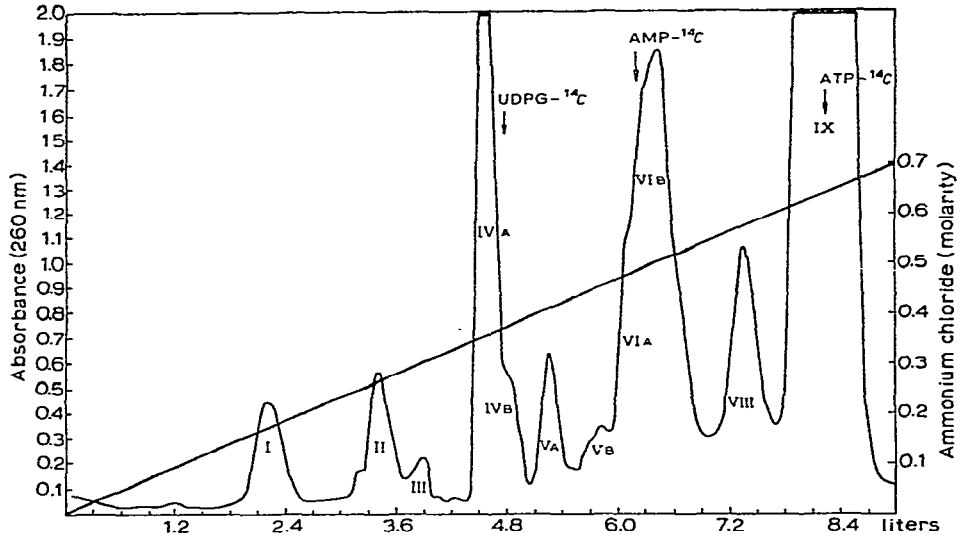

Fig. 2. Elution pattern of an extract from mature oocytes on the same column as described in Fig. 1 and eluted with a linear gradient of 0–0.7M ammonium chloride at a rate of 10 ml/min. Fractions of 15 ml.

Further elution from the column of Dowex 1 (X-4) was obtained with a linear gradient of ammonium chloride between 0 and 0.7M (Fig. 2). The u.v.-absorbing substances were identified as follows:

Peak I. — This peak generally contained cytosine and guanine, but in some samples only cytosine was found. The results of many analyses showed the presence of a complex mixture of compounds rather than a single component, as reported earlier[26].

Peak II. — This component contains adenine and has properties similar to those of NAD in paper chromatography with solvents I and II, and in paper electrophoresis at pH 7.5 and 3.8 with phosphate and acetate buffers, respectively. The proportions of P–pentose–adenine was found to be 1.95:2.18:1. The identity with NAD was confirmed by the increase in absorbance at 327 nm when potassium cyanide was added[27].

Peak III. — This peak was present in very small proportion and was not identified further.

Peak IV. — This peak was shown to consist of a mixture of UDPGNAc and UDPGalNAc. Before hydrolysis, it migrated like UDP-2-acetamido-2-deoxyaldoses in paper chromatography with solvents I and II and in paper electrophoresis at pH 7.5

and 3.8. After acid hydrolysis at pH 2, paper chromatography in solvents I and VII and paper electrophoresis in borate buffer pH 9.2 showed a mixture of 2-acetamido-2-deoxyglucose and 2-acetamido-2-deoxygalactose. The latter chromatographic system distinguishes clearly between the two sugars. The substance that migrated like 2-acetamido-2 deoxy-D-glucose gave a much stronger reducing spot than the one corresponding to 2-acetamido-2-deoxy-D-galactose. In addition, pentose determination after treatment with bromine indicated 1 mole of pentose per mole of base, and analysis of the total and labile phosphorus showed 2 and 1 phosphate groups, respectively, per mole of base. Degradation with ninhydrin of the sugar moiety according to Gardell *et al.*[28] and paper chromatography in solvent III gave a mixture of arabinose

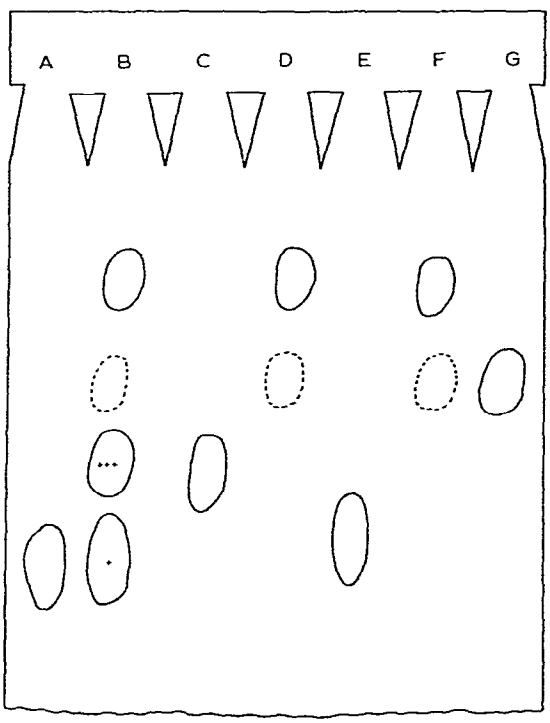

Fig. 3. Paper chromatography with ethanol–ammonium acetate[16] (pH 3.8) of peak IVa (Fig. 2): (D) Hydrolyzed with 2M hydrochloric acid for 1 h at 100°; (B) Hydrolyzed and treated with ninhydrin. Standards: (A) D-Lyxose; (C) D-arabinose; (E) 2-acetamido-2-deoxy-D-glucose; (F) 2-acetamido-2-deoxy-D-glucose, hydrolyzed with 2M hydrochloric acid for 1 h at 100°; (G) D-glucose.

and lyxose, as expected (Fig. 3). The relative intensities of the reducing spots of the pentoses, showed again that UDPGNAc was predominant over UDPGalNAc. Finally, drastic hydrolysis with hydrochloric acid and paper chromatography in solvent V showed that uracil was the only base present.

Peak IV$_B$. — The component of this peak migrated similarly to UDPG in paper chromatography with solvents I and II and in paper electrophoresis at pH 7.5

and 3.8. After acid hydrolysis in 3M hydrochloric acid for 1 h at 100° and paper chromatography in solvent V, a single u.v.-absorbing spot, which had the mobility of uracil derivatives, was detected. After milder acid hydrolysis and paper chromatography with solvent III or paper electrophoresis with borate buffer, a reducing spot having the mobility of D-glucose was found with both techniques. Identification of the sugar moiety was confirmed by the D-glucose oxidase test. Pentose determination, after treatment with bromine, gave 1 mole of pentose per mole of base and analysis of the total and labile phosphorus showed the presence of 2 and 1 phosphate groups per mole of base, respectively. Consequently, the component of Peak IV_B is UDPG.

Peak V_A. — After drastic acid hydrolysis and paper chromatography with solvent V, uracil and adenine were detected. Paper chromatography in solvents I and II and paper electrophoresis at pH 3.8 and 7.5 showed that this peak consists of a mixture of UDP and NADP. It was purified further by preparative paper chromatography with solvent I. Identification of the band having NADP mobility was confirmed by a complex formation with potassium cyanide.

Peak V_B. — This peak is very complex; after drastic acid hydrolysis and paper chromatography in solvent V, four u.v.-absorbing spots consisting of adenine, cytosine, uracil, and guanine, respectively, were detected. Paucity and complexity of the material prevent further identification at the present time.

Peak VI_A. — Drastic acid hydrolysis and paper chromatography in solvent V showed the presence of cytosine as base. Before hydrolysis, the component behaved as CTP in paper chromatography with solvents I and II and in paper electrophoresis at pH 3.8 and 7.5. Analysis of phosphorus confirmed the identification: it gave 3 moles of total phosphorus per mole of base and 2 moles of labile phosphate per mole of base.

Peak VI_B. — It was shown to be a mixture of UTP and UDPGA. After adsorption on Norit A, most of the UTP was eluted with water [Peak VI_B (water)]. UDPGA was adsorbed tightly to the carbon and elution with ethanol–ammonia was necessary [Peak VI_B (ethanol–ammonia)].

Peak VI_B (*water*). Identity with UTP was confirmed by paper chromatography in solvents I and II, paper electrophoresis at pH 3.8 and 7.5, and analysis of total and labile phosphorus.

Peak VI_B (*ethanol–ammonia*). The component behaved as UDPGA in paper chromatography in solvents I and II, and paper electrophoresis at pH 3.8 and 7.5. After mild acid hydrolysis and paper electrophoresis at pH 7.5 or 3.8, a reducing spot having D-glucuronic acid mobility was detected. The unhydrolyzed compound was not attacked by alkaline phosphatase. Analysis of total and labile phosphorus indicated 2 and 1 moles per mole of base, respectively. In paper chromatography with an alkaline solvent (solvent IV), the compound decomposed to give UMP and glucuronic acid 1,2-cyclic monophosphate as UDPGA does. The presence of galacturonic acid as the sugar moiety was discarded, because of a negative Dische reaction which is specific for galacturonic acid[29].

Peak VIII. — The component had the same mobility as ADP in paper chro-

matography with solvents I and II, and in paper electrophoresis at pH 3.8 and 7.5. The main base present was adenine, but a small amount of guanine was also detected. Paper chromatography with solvent VIII, which distinguishes ADP from GDP, confirmed the presence of both nucleoside diphosphates.

Peak IX. — The component behaved as ATP in paper chromatography with solvents I and II, and in paper electrophoresis at pH 3.8 and 7.5. After drastic acid hydrolysis, guanine and adenine were observed in paper chromatography with solvent V. U.v.-absorbing spots having ATP and GTP mobilities were detected after paper chromatography with solvent VIII. Analysis of total phosphorus indicated 3 and 2 moles per mole of base, respectively. All the evidence shows that this peak consists of a mixture of ATP and GTP.

A summary of the data just described is reported in Tables I and II.

TABLE I

NUCLEOTIDES ISOLATED FROM MATURE OOCYTES

Peak	*Total amount* (‰)	*Base*[a]	*Nucleotides*[b]	*Cyanide complex*	*Pentoses*[c]	*Phosphorus*[c]	
						Total	*Labile*
I	10.25	Cytosine Guanine		—	—	—	—
II	33.77	Adenine	NAD	(+)	2.18	1.95	1.10
III	—	Adenine Thymine		—	—	—	—
V_A	13.55	Adenine Uracil	NADP UDP	(+)	—	—	—
V_B	9.36	Adenine Cytosine Guanine Uracil		—	—	—	—
VI_A	16.63	Cytosine	CTP	—	.90 1.00	3.00	2.00
VI_B (water)	21.31	Uracil	UTP	—	.804	3.00	2.20
VIII	27.98	Adenine	ADP GDP	—	.93	—	—
IX	194.01	Adenine Guanine	ATP GTP	—	.80	2.90	1.8

[a]Identification by paper chromatography in solvent V after hydrolysis with 3M hydrochloric acid for 1 h at 100°. [b]Identification by paper chromatography in solvents I and II and electrophoresis at pH 7.5 and 3.8. [c]Moles per mole of base.

DISCUSSION

In echinoderms and amphibians, the process of ovulation is accompanied by a decline in the ability of the oocytes to incorporate labelled amino acids into proteins[30,31]. Mature, unfertilized eggs show little, if any, incorporation of labelled

TABLE II

SUGAR NUCLEOTIDES ISOLATED FROM MATURE OOCYTES

Peak	*Total amount* (‰)	*Base*[a]	*Nucleotides*[b]	*Pentoses*[c]	*Phosphorus*[c]			*Sugar component after acid hydrolysis*		
					Total	*Labile*	D-*Glucose*[c]	*Chromatography*[d]	*Degradation with ninhydrin*[e]	*Electrophoresis*[f]
IV_A	51.59	Uracil	UDPGNAc	0.816	1.85	1.10	0.00	2-Acetamido-2-deoxyglucose	Arabinose and lyxose	2-Acetamido-2-deoxyglucose and 2-acetamido-2-deoxygalactose
IV_B	10.39	Uracil	UDPG	0.800	1.85	1.00	0.91	D-Glucose		Glucose
VI_B (ethanol–ammonia)	12.93	Uracil	UDPGA	—	2.10	1.00	0.00			Glucuronic acid

[a,b,c]See footnotes in Table I. [d]Paper chromatography in solvent III. [e]Identification by paper chromatography in solvent III. [f]Paper electrophoresis at pH 3.8 and 7.5.

precursors into proteins[32]. In the mature oocyte, the chain of reactions leading from amino acids to proteins is blocked until fertilization. Several hypotheses have been put forward to explain this phenomenon[33], but it is still unknown how the inhibition takes place in the maturing egg. The results obtained in this paper show that this inhibition could not result from the lack of a source of energy or from an insuficient supply of the pool of ribonucleotides for RNA synthesis. The four common ribonucleotide triphosphates were found in mature oocytes, although in different proportions. ATP showed the highest level, although this was significantively lower than in fertilized eggs[34]. NAD and NADP were also present in small proportions.

In regard to the pool of deoxyribonucleotides, we could not detect any of these compounds, in spite of the great amount of starting material used. According to Woodland and Pestell[35] pyrimidine deoxyribonucleoside triphosphates are present in the mature oocytes of *Xenopus laevis*, but ribonucleoside triphosphates are 20–100 times more abundant. These authors at first destroyed the ribonucleotides by treatment with sodium periodate and methylamine, and then passed the products through a Dowex-1 column which retained only the deoxyribonucleotides. In our case, the pyrimidine deoxyribonucleotides were probably masked by the great excess of the ribonucleotides present in the oocytes of *Bufo arenarum*.

Four sugar-nucleotides were found in the sample of mature oocytes and all of them have uridine as nucleoside moiety. UDPG, UDPGNAc, and UDPGalNAc have been found previously in unfertilized eggs of the sea urchin, *Hemicentrotus pulcherrimus*[13]. As far as we know, the presence of UDPGA in unfertilized eggs is here reported for the first time since this compound was not found in unfertilized sea urchin eggs. In our system, UDPGA was eluted together with UTP, which was present in much larger amount, but its presence was detected after adsorption and selective elution from Norit A.

A considerable amount of the u.v. absorbing material was not adsorbed on the column and was separated into two main peaks, which were identified as hypoxanthine and guanine, respectively, in agreement with the results of Woodland and Pestell[35]. The levels of hypoxanthine and guanine decreased notably in eggs after fertilization, with a corresponding increase in the level of ATP and GTP[34].

A nucleotide, which binds weakly to the column, was detected in all samples analyzed (peak I). It showed to be a mixture of more than one compound and complete identification is in progress.

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