Cytoplasmic DNA and basic protein synthesis in *Megalobatrachus davidianus* oocytes

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SUMMARY

Mature *Megalobatrachus* oocytes contain 43 μg DNA per oocyte, as compared with 250 pg DNA in a hepatocyte of the same animal. *Megalobatrachus* oocytes respond to CdR treatment by an increased incorporation of [3H]lysine into basic proteins associated with ooplasmic particles, with an optimal CdR concentration at 2 mM. The nucleolus is the most active site of [3H]lysine incorporation. It is suggested that CdR-stimulated basic protein synthesis is a common biochemical event during amphibian oogenesis. The dose response to CdR treatment may be a function of the c-DNA content or c-DNA synthesis potential in the ooplasm.

INTRODUCTION

We have recently reported that, in *Triturus sinensis*, the administration of exogenous deoxycytidine (CdR) stimulates an increase in [3H]lysine incorporation into a basic protein component in oocytes incubated in vitro. It was suggested that CdR may act by increasing the deoxyriboside-triphosphate pool, thus permitting an accelerated cytoplasmic DNA (c-DNA) synthesis (Lau & Kong, 1971). We report here similar experiments performed on *Megalobatrachus davidianus*, the largest living amphibian. We expected that larger oocytes would contain more c-DNA and thus manifest a more prominent response to CdR treatment. It is postulated that a greater c-DNA content per oocyte may be indicative of a higher rate of DNA synthesis in the cytoplasm and that this rate of synthesis can be further accelerated by the administration of exogenous CdR at a high concentration. The phosphorylated derivatives of CdR, in this case, would be readily consumed in DNA synthesis rather than accumulated in an abnormally large pyrimidine deoxyriboside-triphosphate pool which may be inhibitory to the reduction of purine ribotides. According to this hypothesis, the dose effect of exogenous CdR may be directly proportional to the content of c-DNA per oocyte. In other words, the response to CdR stimulation by an increase in basic protein synthesis may well be a function of

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the c-DNA content in the oocyte. To this end, the effect of exogenous CdR on [3H]lysine incorporation in *Megalobatrachus davidianus* oocytes *in vitro* was investigated, and the intracellular site of CdR-stimulated basic protein synthesis was examined by autoradiography. In order to substantiate the above hypothesis, DNA was extracted in a highly purified form from ooplasmic particles.

Finally, it would be interesting to know if CdR-stimulated basic protein synthesis is a common biochemical event during amphibian oogenesis.

**MATERIALS AND METHODS**

*Megalobatrachus davidianus*

*Megalobatrachus davidianus* of the Cryptobranchidae is the largest living urodele, in fact, the largest existing amphibian. There is no reference that this urodele has been used in experimental embryological studies. A mature female measures about 1 m in length and weighs 3-4 kg (Fig. 1). It has a pair of elongated sac-like ovaries, weighing 30-40 g, and they contain oocytes at all stages. Mature oocytes are yellow in colour with a light orange tinge, measuring 4-5 mm in diameter. Younger oocytes appear light yellowish at the onset of vitellogenesis and slightly pinkish when devoid of yolk, due to blood circulation in the surrounding follicle cell layer. We arbitrarily called these three categories of oocytes the third-year, second-year and first-year oocytes respectively, although oogenesis in *Megalobatrachus* may not last exactly three years.

The ovarian stroma, as well as the follicle cell layer, consist of tough fibrous tissues. This characteristic permits complete removal of the follicle cells with relative ease, either by manual manipulation or homogenization. Oocytes of all stages are non-pigmented, thus offering another advantage for biochemical processing and autoradiographic study.

Fig. 1. A mature *Megalobatrachus davidianus*. This individual measured 81 cm.
Materials

Tritiated L-Lysine-4-5-T, 100 mCi/mm, was supplied by Radiochemical Centre, Amersham, England. Deoxycytidine, pancreatic DNase-I (EP), pancreatic RNase A (5 x crystallized) and trypsin (2 x crystallized) were supplied by Sigma. Pronase B and alpha-amylase were the products of Calbiochem. In autoradiographic experiments, Ilford K2 nuclear emulsion was used.

Administration of deoxycytidine and [3H]lysine

Equal portions of oocytes (5–7 g) were incubated in 20 ml of Holtfreter solution containing 10 µCi/ml of [3H]lysine. CdR was added to a final concentration of 1–5 mM. In both control and CdR-treated samples the final volume and pH (7.6) of the incubation medium was adjusted before starting an experiment. Incubation was maintained at 18–20 °C for 16 h without shaking. At the end of the incubation period, oocytes were rinsed in two changes of cold Holtfreter solution; they were then ‘chased’ in 10 mM of non-radioactive lysine in Holtfreter solution for 1 h. Oocytes were broken up in saline-EDTA (0.15 M-NaCl, 1 mM-EDTA pH 7.2) in a loosely fitted ground-grass homogenizer and the total homogenate was filtered through glass-wool. Ooplasmic particles were collected at 170000 g in a Spinco L2–65B ultracentrifuge, operating at 4 °C. In an early experiment, total acid-precipitable protein was collected at 10000 g after direct homogenization in cold trichloroacetic acid (TCA) at 5 %. The particulate fraction was resuspended in saline-EDTA and collected under similar conditions. The final precipitate was directly taken up in 0.3 N-KOH or 1 N-NaOH with 0.05 M hyamine, and prepared for protein estimation and scintillation counting. Total acid-precipitable protein was purified by two cycles of TCA-KOH before it was eventually taken up in 0.1 N-KOH for protein estimation and scintillation counting. Further details for protein estimation, scintillation counting and tryptic digestion had been reported (Lau & Kong, 1971).

 Autoradiography

Oocytes were fixed in Bouin–Duboscq (alcoholic Bouin’s) after they were rinsed and ‘chased’ as mentioned above. Sections were cut at 10 µm and brought down to water. After the removal of picric acid and ample rinsing, slides were dipped into a 1:2 dilution of nuclear emulsion melted at 60 °C. All slides were dried over desiccating agents and exposed in total darkness for 1 month at 4 °C. Development with Kodak microdol-X (1:1 dilution) was carried out at 16 °C for 5 min. Slides were stained with methyl green–pyronine for microscopic examination.

 DNA extraction

Some 370 third-year oocytes were removed from the ovary of a mature female. The oocytes were homogeneous in colour and size. Smaller or atretic oocytes were eliminated. Fifty oocytes in a batch were stored in saline-EDTA at −20 °C.
Before processing, the oocytes were thawed and the follicle cell layer was carefully removed with fine forceps. The yolk platelets tend to stick together so that it was possible to obtain an intact oocyte denuded of its follicle cell layer. The follicle cell debris was rinsed once in saline-EDTA and collected at low-speed centrifugation; the supernatant fraction was pooled with the naked oocytes.

DNA extraction followed essentially the method of Berns & Thomas (1965). Details of this procedure are found in Fig. 2. Purified DNA was estimated according to Dische (1930), using highly polymerized calf thymus DNA (Sigma) as reference, assuming 1 mg of DNA to give 33 units of optical density at 260 nm.

In another experiment, instead of processing the ooplasmic particles as outlined in Fig. 2, the particulate fraction was directly digested by DNase-I (100 μg/ml, Mg$^{2+}$ 300, 37 °C, overnight). After an aliquot of the preparation was removed, deoxycholate was added to a final concentration of 0.2% and digestion continued for another 24 h. The DNase-I digest was pooled and acid-
Cytoplasmic DNA and basic protein synthesis

fied with perchloric acid. The ultraviolet absorption spectrum of the acid soluble fraction was recorded after prior neutralization.

DNA from *Megalobatrachus* liver was prepared and estimated as indicated above. For hepatocyte cell counts, 1–2 g of liver were weighed and macerated, then homogenized, in saline-EDTA plus trypsin (50 μg/ml, 15 min). Cell debris was removed by low-speed centrifugation and the hepatocyte nuclei were counted in a hemocytometer.

![Specific radioactivity graph](image)

**RESULTS**

**CdR effect on [³H]lysine incorporation**

[³H]lysine incorporation into the ooplasmic particles increased with increasing CdR concentration. It was immediately noted that *Megalobatrachus* oocytes responded to a high CdR concentration (Table 1). In experiments where only first- and second-year oocytes were used, a linear relationship up to 5 mM-CdR was observed, leading to a 150 % increase in [³H]lysine incorporation into total acid precipitable protein. However, a peak value at 2 mM-CdR was obtained when particulate protein was rendered acid-soluble by tryptic digestion. This optimal concentration of CdR can be easily appreciated when the profiles from four experiments are superimposed (Fig. 3).
Table 1. Effect of CdR on the incorporation of $[^3H]$lysine into different protein fractions of Megalobatrachus davidianus oocytes

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Specific radioactivity of <em>Megalobatrachus</em> oocytes treated with deoxycytidine (cpm/mg protein $\times 10^3$)</th>
<th>Deoxycytidine concentrations (mm)</th>
<th>State of oocytes</th>
<th>Methods of extraction*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>I. Acid precipitable protein</td>
<td>75·3</td>
<td>94·5†</td>
<td>124·7‡</td>
<td>—</td>
</tr>
<tr>
<td>II. Tryptic digest of particulate protein</td>
<td>10·5</td>
<td>11·6</td>
<td>12·7</td>
<td>—</td>
</tr>
<tr>
<td>III. Particulate protein</td>
<td>17·7</td>
<td>24·5</td>
<td>34·2</td>
<td>—</td>
</tr>
<tr>
<td>IV. Particulate protein</td>
<td>22·1</td>
<td>31·2</td>
<td>12·6 (?)</td>
<td>—</td>
</tr>
</tbody>
</table>

* Details in Lau & Kong (1971). † 0·8 mM-CdR. ‡ 2·4 mM-CdR.
Examination of the autoradiograms revealed that silver grains were mainly distributed in the germinal vesicle, particularly over the nucleoli. Silver grains in the ooplasm were scarce and distributed homogeneously. With the addition of CdR, an abrupt increase in nuclear silver grains was observed (Fig. 4a, b). Although the density of silver grains cannot be correlated strictly with the increase in CdR concentration, incorporation of [³H]lysine appeared to be
maximum at 2 mM-CdR. The number of silver grains in the ooplasm increased only slightly as compared to that observed in the germinal vesicle.

**DNA extraction**

DNA extracted by the pronase-SDS-phenol method was measured by reaction with the Dische reagent. It was found that there are $43 \pm 1 \mu g$ per oocyte. A slightly higher value was obtained by calculation from the u.v. absorption spectrum. The amount of DNA, or its equivalent, in the high-speed supernatant fraction was $8 \mu g$ per oocyte, while the total number of follicle cells from an oocyte contained only $0-4 \mu g$. This value could be 2–3 times higher considering the fact that 50 $\mu g$ DNA/ml constitute the lower sensitivity limit of the Dische reaction; follicle cells from 50 oocytes in a sample contained at best 20 $\mu g$ DNA (Table 2).

**Table 2. c-DNA content in mature oocytes of Megalobatrachus davidianus estimated by Dische reaction ($\mu g$ DNA/oocyte)**

<table>
<thead>
<tr>
<th>Material</th>
<th>DNA content in ooplasm</th>
<th>DNA content in follicle cells</th>
<th>DNA content in high-speed supernate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Teased oocytes with follicle cells and ooplasm separated</td>
<td>44</td>
<td>50-9*</td>
<td>0-21</td>
</tr>
<tr>
<td>As above, ooplasmic particles collected after high-speed centrifugation</td>
<td>42</td>
<td>37*</td>
<td>0-58</td>
</tr>
<tr>
<td>As above, ooplasmic particles digested by DNase-DOC</td>
<td>—</td>
<td>17-4*</td>
<td>—</td>
</tr>
</tbody>
</table>

* Calculation based on u.v. absorption at 260 nm. (DOC = deoxycholate.)

Hepatocytes processed by the same procedure yielded only 250 pg DNA per cell. Both liver and ooplasmic DNA showed a typical u.v. absorption spectrum, with a maximum at 257 nm. The absorption ratio of 260/280 nm for liver DNA was 1-71 and for ooplasmic DNA 1-8 (Fig. 5).

**DISCUSSION**

Deoxycytidine plays a unique role in the synthesis of deoxyriboside-triphosphates. It provides dCTP by direct phosphorylation as well as dTMP by deamination to dUMP and subsequent methylation under the action of thymidylate synthetase. dTMP is rapidly accumulated as dTTP through the successive action of TMP kinase and TDP kinase. The appearance of dTTP in turn facilitates the reduction of purine ribotide (Larsson & Reichard, 1967). Thus, CdR is not only the source of pyrimidine deoxyriboside-triphosphates, it also initiates
the reduction of purine ribotides which together with the former, are prerequisites for DNA synthesis.

However, the stimulatory effect of CdR is entirely dose-dependent. The accumulation of dTTP and, particularly, that of dATP, will rapidly inhibit further reduction of all ribonucleotides through an allosteric regulation of the riboside-diphosphate reductase system (Larsson & Reichard, 1967). It is apparent that the permissible dose of exogenous CdR administered is strictly correlated to the rate that deoxyriboside-triphosphates are drained from their intracellular pool to form polymerized DNA. In more explicit terms, a cell can benefit from a high concentration of CdR in as much as the deoxyriboside-triphosphates are effectively consumed; beyond that critical concentration, further addition of exogenous CdR can only lead to inhibition of DNA synthesis due to the reduction of deoxyriboside-triphosphates pool size. This logic evidently holds true in *Megalobatrachus* oocytes, in which CdR begins to show its inhibitory effect at a concentration greater than 2–3 mM. In *Triturus* oocytes the optimal CdR concentration falls at 1 mM. This discrepancy of optimal CdR
concentration is correlated with the higher c-DNA content in *Megalobatrachus* oocytes.

With 43 \( \mu \text{g} \) DNA per oocyte, *Megalobatrachus* will undoubtedly rank first in the amount of c-DNA per egg in any species of amphibian. This makes its DNA content \( 1.6 \times 10^5 \) times in excess of that of a single hepatocyte, which contains only 250 pg per cell. In *Amphiuma*, 168 pg DNA per diploid cell was reported (Vendrely, 1955). The value found in *Megalobatrachus* is probably exaggerated by polyploidy and diminished cell count due to over-trypsinization and mechanical breakage during homogenization.

Since 8 \( \mu \text{g} \) DNA or its equivalent per oocyte were found in the high-speed supernatant fraction, it does not seem exaggerated to think that 43 \( \mu \text{g} \) of highly polymerized DNA in the ooplasm can withstand the drastic method of extraction adopted in our experiments. *Triturus alpestris* eggs and those from related species contain 1–2 \( \mu \text{g} \) DNA/egg (Chen, 1960) and c-DNA in *Pleurodeles waltl* is 5000 times in excess of that of a diploid cell (Baltus & Brachet, 1962). Simple arithmetic shows that a *Megalobatrachus* oocyte 3–4 times larger in diameter should contain 27–64 times more c-DNA; that is, \( 1.35 \times 3.2 \times 10^5 \) times in excess of a somatic cell. A still higher value could be reached in mature ovulated eggs if further increase of the c-DNA content occurs during maturation (see discussion by Brachet, 1969).

It is interesting to note that, out of the 43 \( \mu \text{g} \) DNA present in a mature oocyte, only 40\% (17-4 \( \mu \text{g} \)) was made acid-soluble by the combined action of DNase-I and deoxycholate. It has been suggested that c-DNA may exist in two forms differing in stability and sensitivity towards DNase action (Brachet, 1965).

Although the increase in total protein synthesis was linear with the increasing dose of CdR up to 5 mm, \([^3H]\)lysine incorporation was maximum at 2 mm-CdR when particulate protein was characterized by trypsin digestion. This confirms very well the observations in *Triturus* oocytes, where 0.5 mm-CdR was optimal for specific basic protein synthesis (as characterized by acid extraction and trypsin digestion), while increase in total particulate protein synthesis responded to a range of 0.5–1.5 mm-CdR. There is no reason to assume that all fractions of histone-type proteins respond to CdR treatment. Furthermore, the increase in basic protein synthesis may possibly entail the increased synthesis of other protein components associated with ooplasmic particles. Preliminary experiments with *Xenopus* oocytes treated under identical conditions showed a 150\% increase of basic protein synthesis at an optimal CdR concentration of 0.5 mm; the specificity of the protein fraction was controlled by extraction with 2 m-NaCl and dissolving in dilute mineral acid. We may tentatively conclude that CdR-stimulated basic protein synthesis may well be a common biochemical event during amphibian oogenesis.

No definite conclusion can be drawn concerning the intracellular site of the CdR-stimulated basic protein synthesis. The nucleolus is frequently the most active site of amino acid incorporation (Ficq, 1961). In pea seedlings and
Novikoff ascitic hepatoma it has been suggested that the nucleolus is the site of nuclear histone synthesis (Birnstiel & Flamm, 1964; Hnilica, Liau & Hurlbert, 1966) and that in *Xenopus* it is responsible for the synthesis of certain fractions of this complex group of basic proteins (Berlowitz & Birnstiel, 1967). A kinetic study of the changes in specific radioactivity of isolated nucleoli could bring a straightforward answer to this question. It is also highly desirable to define the physico-chemical parameters of ooplasmic or nucleolar basic proteins newly synthesized in response to CdR.

**RéSUMÉ**

*DNA cytoplasmique et synthèse des protéines basiques dans les oocytes de Megalobatrachus davidianus*

Les oocytes mûrs de *Megalobatrachus* contiennent 43 μg de DNA, tandis que l'hépatocyte du même animal ne contient que 250 pg de DNA par cellule. Les oocytes de *Megalobatrachus* montrent une augmentation de l'incorporation de la [3H]lysine dans les protéines basiques associées aux particules ooplasmiques sous l'effet de désoxyctidine exogène, avec une concentration optimale de 2 mM. Le nucleolé constitue le site le plus actif de l'incorporation de la [3H]lysine. Il est suggéré que l'augmentation de la synthèse des protéines basiques serait un phénomène général pendant l'oogenèse des batraciens. Il semble que le degré de la réaction provoquée par le désoxyctidine soit lié à la quantité du DNA cytoplasmique ou au potentiel de sa synthèse dans l'oooplasme.

**References**


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NOTE ADDED IN PROOF

In an attempt to prove that 43 μg DNA per egg found in *Megalobatrachus davidianus* is neither exaggerated nor artificial, we have compiled data on DNA content and egg volume from four amphibian species (Table 1). When the DNA value is plotted against the cube of egg radius, a perfect linear relation is

![Graph showing linear relationship between DNA content per egg and egg volume of four amphibian species.](image)

Fig. 1. Linear relationship between DNA content per egg and egg volume of four amphibian species presented in Table 1.

Table 1. *Cytoplasmic DNA and egg size in four amphibian species*

Egg radius in *Pleurodeles waltillii* is measured through the courtesy of Dr R. Tencer (U.L.B., Belgium); other values are obtained in this laboratory.

<table>
<thead>
<tr>
<th>Species</th>
<th>Egg radius (mm)</th>
<th>DNA (ng/egg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Xenopus laevis</em></td>
<td>0.55</td>
<td>6.6</td>
<td>Dawid, 1965</td>
</tr>
<tr>
<td>2. <em>Pleurodeles waltillii</em></td>
<td>0.85</td>
<td>80.0</td>
<td>Baltus &amp; Brachet, 1962</td>
</tr>
<tr>
<td>3. <em>Triturus sinensis</em></td>
<td>1.25</td>
<td>1180.0</td>
<td>Kong <em>et al.</em> 1971a</td>
</tr>
<tr>
<td>4. <em>Megalobatrachus davidianus</em></td>
<td>2.50</td>
<td>43000.0</td>
<td>Kong <em>et al.</em> 1971b</td>
</tr>
</tbody>
</table>
observed (Fig. 1). At present we cannot define the upper and lower limit of this relationship; we have also no idea whether this relationship applies to telolecithal (avian) and oligolecithal (echinoderm) eggs. The total DNA content in mature eggs must be a summation of factors involved in DNA neo-synthesis; for example, nucleoside permeability, nucleoside kinase and/or de novo synthesis, ribonucleotide reductase and DNA polymerase (ligase). It would be interesting to determine which of these factors is more important to the increase in DNA content as the egg volume increases. In rat liver hepatomas, ribonucleotide reductase activity is directly proportional to the tumour growth rate (Elford, Freese, Passamani & Morris, 1970).

It would not be surprising to find that increase in DNA content is directly triggered by an increase in cytoplasm volume. However, in the nucleo-cytoplasmic relationship, the upper limit is set at 4C (C = haploid) value and is brought back to 2C value by mitotic division. So the problem still remains unresolved for cytoplasmic DNA, i.e. a particular regulatory mechanism, or the lack of it, permits the accumulation of an enormous amount of DNA many times above the diploid value.

REFERENCES


