Effects of X-rays on nucleic acid metabolism during cleavage and gastrulation in *Pleurodeles waltl**ii** eggs

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**SUMMARY**

X-irradiation produces a rapid and substantial decrease in DNA synthesis in cleaving eggs of *Pleurodeles*, as shown by thymidine incorporation experiments; this decrease is presumably due to the arresting of the cell cycle in many blastomeres. DNA replication is partly resumed at the late blastula stage after irradiation at the 2- to 4-cell stage.

The present work demonstrates a high radiosensitivity of the metabolic pathway leading to the incorporation of uridine into DNA; this could be the result of the inhibition of the activity or the synthesis of such enzymes as ribonucleotide reductase, thymidylate synthetase and CTP synthetase. The radiosensitivity of this metabolic pathway becomes lower after gastrulation.

Finally, irradiation strongly inhibits the synthesis of new RNA species at gastrulation, but seems to have no effect on heterodisperse RNA (mRNA) synthesis during cleavage.

The results are discussed in relation to the biochemistry of early stages of development in Amphibians.

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**INTRODUCTION**

Although each stage of embryonic development is radiobiologically independent (Rugh, 1961), it is well established that the cleavage stages are the most sensitive in all animal species studied to date.

In the particular case of the *Pleurodeles* egg, it has previously been shown that, during the cleavage period, the undivided egg is the most radiosensitive. The threshold dose which blocks development at the end of cleavage has been defined: all gastrulation movements are inhibited after 500 R for undivided eggs and 1000 R for cleaving eggs (Alexandre, 1967). Moreover, the radiosensitivity of the undivided egg varies according to stage, amphimixis being most sensitive to gamma rays (Labrousse, 1967); during the first three cleavage cycles, sensitivity to X-rays increases from prophase to telophase and decreases throughout the DNA synthetic period (S), as found by measuring the percentage of embryos surviving during 3 days (Hamilton, 1969).

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Using autoradiography, a correlation has been established between the arrest of development at the late blastula stage and the inhibition (which is dose-proportional) of thymidine and uridine incorporation into the nucleus (Alexandre, 1970). Moreover, it was found that the metabolic pathways leading to the incorporation of $^3$H uridine into DNA seem to be particularly radiosensitive in young embryos (Alexandre, 1970, 1971).

The purpose of this paper is to present evidence obtained with a quantitative technique, confirming our previous autoradiographic experiments.

**METHODS**

All experiments were performed with early embryos of *Pleurodeles waltlii* (Urodela). The jelly surrounding the eggs was removed with a pair of forceps before exposure to the X-ray generator (Machlett; beryllium window, 50 kV, 30 mA, 1 mm aluminium filter). The rate used was 640 R/min.

The labelled precursors were micro-injected into the blastocoels of blastulae or young gastrulae, using a micropipette manufactured by means of a needle-pulling apparatus (Microforge de Fonbrune). This micropipette was joined to a glass hypodermic syringe by plastic tubing and the injection process was controlled by hand. The volume injected was approximately 0.5 $\mu$l/egg; the precursors used were 1 $\mu$Ci/ml [3H]thymidine (sp. act. 5 Ci/mM), 1 $\mu$Ci/ml [3H]uridine (sp. act. 2.5 Ci/mM) and 500 $\mu$Ci/ml [3H]deoxyuridine (sp. act. 27 Ci/mM). All labelled precursors were obtained from the Radiochemical Centre, Amersham, Bucks, England.

Extractions were carried out as follows: samples of 5 micro-injected embryos (Steinert, 1951) were fixed with 94% ethanol for 5 min; the extraction of the acid-soluble fraction was done twice with 0.4 ml of 2% perchloric acid (PCA) at 4 °C during 30 min; it was followed by washing in an equal volume of PCA (fraction I). Delipidation was carried out thrice in 94% ethanol and twice in ether during 30 min. The eggs were then vacuum-dried. The acid-insoluble fraction was hydrolysed with 0.8 ml 10% PCA for 24 h at 4 °C, followed by a wash with 0.4 ml of 10% PCA (fraction II: RNA). Thereafter, the eggs were extracted thrice with 0.4 ml 5% PCA at 70 °C during 20 min (fraction III: DNA).

Each fraction was added to 10 ml of Bray scintillation medium and counted in a Nuclear Chicago scintillator.

Young blastulae injected with deoxyuridine were fixed by freeze-substitution 3 h after micro-injection and embedded in paraffin. Sections 10 $\mu$m thick were autoradiographed using Ilford emulsion K2 and the usual procedure (Ficq, 1959) and stained with Unna (methyl green–pyronin). Grain counts were made over the nuclei of the animal pole of the blastulae. The autoradiographs were developed after 10 days exposure.
Table 1. *Total uptake and distribution of[^3H]thymidine and[^3H]uridine at late blastula stage after irradiation at the 2–4 cell stage*

<table>
<thead>
<tr>
<th></th>
<th>[^3H]Thymidine (c.p.m.)</th>
<th>[^3H]Uridine (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>5000 R</td>
</tr>
<tr>
<td>Acid-soluble fraction</td>
<td>13167</td>
<td>23170</td>
</tr>
<tr>
<td>Fraction II RNA</td>
<td>394 (1.3%)</td>
<td>1072 (5.3%)</td>
</tr>
<tr>
<td>Fraction III DNA</td>
<td>29208 (98.7%)</td>
<td>19039 (94.7%)</td>
</tr>
<tr>
<td>Total incorporated</td>
<td>29602</td>
<td>20111 (68% of control)</td>
</tr>
<tr>
<td>Total uptake</td>
<td>42769</td>
<td>43281</td>
</tr>
</tbody>
</table>

**RESULTS**

*Precursor incorporation in control and 5000 R irradiated embryos*

**DNA and RNA synthesis at the advanced blastula stage (stage 7–8a, Gallien & Durocher, 1957) after irradiation at the 2–4 cell stage**

The injection of 0.5 µl per embryo of the precursor-containing solution was performed 20 h after irradiation. Fixation of the embryos was carried out 3 h later.

The results (Table 1) are given in counts per minute (c.p.m.). The distribution between fractions II and III is given as the percentage of incorporated radioactivity.

The results show that the method used discriminates well between the radioactivities incorporated into RNA and into DNA, since hydrolysis at 4 °C for 24 h removes only 1.3% of the radioactivity which had been incorporated into DNA after incubation with labelled thymidine.

The data show that DNA replicates at a high rate (68% of the control value) 20 h after a high dose of irradiation (5000 R) received at the beginning of cleavage. However, more radioactivity is extracted with cold PCA from irradiated eggs than from the controls.

After incubations of equal time, much less uridine than thymidine (only 5%) is incorporated into the control embryos. It seems clear, moreover, that approximately twice as much uridine is incorporated into RNA as into DNA at the beginning of gastrulation.

Irradiation inhibits total uridine incorporation; however, this inhibition is stronger in the case of DNA synthesis than in that of RNA synthesis. This stands in good agreement with previous autoradiographic evidence.
Table 2. Total uptake and distribution of $[^3H]$thymidine and $[^3H]$uridine soon after irradiation of young blastulae

<table>
<thead>
<tr>
<th></th>
<th>$[^3H]$Thymidine (c.p.m.)</th>
<th>$[^3H]$Uridine (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>5000 R</td>
</tr>
<tr>
<td>Acid-soluble fraction</td>
<td>19040</td>
<td>38373</td>
</tr>
<tr>
<td>Fraction II RNA</td>
<td>969 (5%)</td>
<td>1364 (44%)</td>
</tr>
<tr>
<td>Fraction III DNA</td>
<td>18270 (95%)</td>
<td>1751 (56%)</td>
</tr>
<tr>
<td>Total incorporated</td>
<td>19239</td>
<td>3115 (16% of control)</td>
</tr>
<tr>
<td>Total uptake</td>
<td>38279</td>
<td>41488</td>
</tr>
</tbody>
</table>

DNA and RNA synthesis at the young blastula stage (stage 6, Gallien & Durocher, 1957) immediately after irradiation

Young blastulae were irradiated with 5000 R, micro-injected 1.5 h later and fixed 3 h after injection.

The results for thymidine and uridine uptake and incorporation, in c.p.m. and in percentages (distribution between fractions II and III) are given in Table 2. It is clear that irradiation considerably inhibits DNA synthesis during the first 4 h; moreover, the small amount of newly synthesized DNA seems to have become more acidolabile, since 44% is extracted with cold PCA.

A small uridine incorporation takes place at this stage; it corresponds to a synthesis of heterodisperse RNA (mRNA) (Bachvarova, Davidson, Allfrey & Mirsky, 1966; Davidson, Crippa & Mirsky, 1968). Its incorporation into DNA represents only 12% of the total incorporation at this early stage. This result is not in agreement with those previously obtained by autoradiography (Alexandre, 1970), although the latter took only nuclear uridine incorporation into account. However, the high radiosensitivity of this metabolic pathway is confirmed by the present experiments.

Table 3. Total uptake and distribution of $[^3H]$thymidine and $[^3H]$uridine soon after irradiation of gastrulae

<table>
<thead>
<tr>
<th></th>
<th>$[^3H]$Thymidine (c.p.m.)</th>
<th>$[^3H]$Uridine (c.p.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>5000 R</td>
</tr>
<tr>
<td>Acid-soluble fraction</td>
<td>16412</td>
<td>29528</td>
</tr>
<tr>
<td>Fraction II RNA</td>
<td>345 (1-1%)</td>
<td>598 (4-1%)</td>
</tr>
<tr>
<td>Fraction III DNA</td>
<td>31547 (98-9%)</td>
<td>13959 (95-9%)</td>
</tr>
<tr>
<td>Total incorporated</td>
<td>31892</td>
<td>14557 (45-6% of control)</td>
</tr>
<tr>
<td>Total uptake</td>
<td>48304</td>
<td>44085</td>
</tr>
</tbody>
</table>
Table 4. **Total uptake and distribution of labelling after irradiation of \([3H]\)thymidine pretreated blastulae (c.p.m.)**

<table>
<thead>
<tr>
<th></th>
<th>Acid soluble fraction</th>
<th>Total incorporated</th>
<th>Fraction II RNA</th>
<th>Fraction III DNA</th>
<th>Total uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7417</td>
<td>28439</td>
<td>278 (1%)</td>
<td>28161 (99%)</td>
<td>35856</td>
</tr>
<tr>
<td>5000 R</td>
<td>11318</td>
<td>24728</td>
<td>528 (2%)</td>
<td>24200 (98%)</td>
<td>36046</td>
</tr>
</tbody>
</table>

**DNA and RNA synthesis at the young gastrula stage (stage 9, Gallien & Durocher, 1957) immediately after irradiation**

The same experimental procedure was used, i.e. injection 1½ h after irradiation (in this case, at the young gastrula stage) and incubation for 3 h before fixation and extraction.

The results (expressed as in Table 2) are given in Table 3.

Synthesis of DNA at this stage is still very important in the controls; it is reduced by more than 50% in embryos treated with 5000 R. This corresponds to a lower radiosensitivity at the gastrula stage and is correlated with the mitotic index.

Again, an increase in the acidolability of the newly synthesized DNA is observed after irradiation.

In the case of uridine incorporation, a difference between the total uptake by the control and treated eggs is probably attributable to a loss of precursor in the medium: consequently, the apparent decrease in total incorporation probably has no significance.

It can be concluded that, in the gastrula, the metabolic pathway most affected by radiation is that of incorporation of uridine into DNA. As a result of this inhibition, there is an increase in the intracellular pool of uridine; the fact that more uridine becomes available might explain the apparent increase of nuclear uridine incorporation into RNA which has been observed by autoradiography (Alexandre, 1970).

**Irradiation of \([3H]\)thymidine preincubated blastulae**

In order to establish whether the previously mentioned higher acidolability of DNA in irradiated embryos reflects a modification of total DNA or only of the DNA which had been synthesized after irradiation, blastulae were irradiated with 5000 R, 4 h after they had been injected with 0.5 µl \([3H]\)thymidine. Fixation and extraction were carried out 2 h after irradiation.

The results are given in Table 4.

At the time of fixation, 6 h after the beginning of the incubation, a small part of the labelled thymidine remains unincorporated. It seems that this incorporation has been slowed down after irradiation and that under these
Table 5. Nuclear incorporation of [\( ^3H \)] deoxyuridine in control and irradiated young blastulae

<table>
<thead>
<tr>
<th>Doses</th>
<th>Labelled nuclei (%)</th>
<th>No. of grains/labelled nucleus</th>
<th>Mean no. of grains/nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 R</td>
<td>72</td>
<td>61</td>
<td>44.0</td>
</tr>
<tr>
<td>500 R</td>
<td>10</td>
<td>21</td>
<td>2.1</td>
</tr>
<tr>
<td>1000 R</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5000 R</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

experimental conditions, there is only a small increase in acidolability; it can thus be concluded that only the DNA which has been synthesized after irradiation has become more acidolabile.

**Autoradiographic study of the effect of irradiation on deoxyuridine incorporation in young blastulae (stage 6, Gallien & Durocher, 1957)**

Young blastulae were irradiated with 500, 1000 and 5000 R, micro-injected 1½ h later and fixed by freeze-substitution 3 h after injection. In each case, grains were counted in five embryos and counts were made over 100 nuclei. DNAase treatment on slides removes all the label; in contrast, RNAase has no effect.

The results are given in Table 5. Incorporation of deoxyuridine into DNA is drastically inhibited by X-rays. This inhibition seems to be of the same order of magnitude as that of uridine into DNA (Alexandre, 1970).

**DISCUSSION**

*Regulation of nucleic acid synthesis during cleavage and gastrulation*

In agreement with previous autoradiographic studies (Alexandre, 1970), our results clearly indicate that the early stages of development are characterized by a high level of thymidine incorporation; uridine incorporation is much less important (the relative percentages are respectively 60–70 % and 5 %, for similar concentrations and specific radioactivity). The low level of uridine incorporation corresponds to two different metabolic pathways, which are summarized below (from Marré et al. 1968):
Uridine incorporation into DNA involves the presence of two types of enzymes: ribonucleotide reductase (RNRase) and either thymidylate synthetase (Th synth.) or CTP synthetase (CTP synth.).

Previous autoradiographic studies indicating the existence of an enzymic reduction of ribonucleotides (Bielavsky & Tencer, 1960; Alexandre, 1967, 1970) led to the conclusion that most of the nuclear uridine incorporation during early cleavage is resistant to RNAase. It has been shown more recently that labelled DNA can be isolated in a CsCl gradient after $[^3H]$uridine incorporation in amphibian eggs (Tencer, 1970). On the other hand, it is well known that during cleavage, uridine incorporation is predominantly linked to a synthesis of mRNA (Brown & Littna, 1964, 1966; Bachvarova et al. 1966) and tRNA (Gurdon, 1967).

The present results indicate that the incorporation of uridine into DNA, which is typical of the early stages of development in amphibians, represents only 20% of its total incorporation. Nevertheless, it has been established that this biochemical pathway is essential for further development since specific inhibitors of ribonucleotide reductase, such as hydroxyurea (Brachet, 1967) and 2'-d-adenosine (Brachet, 1968) block amphibian development at the late blastula stage.

Radiosensitivity and DNA synthesis

Irradiation of young blastulae with 5000 R reduces the incorporation of thymidine into DNA to 16% of the control value 4½ h after irradiation. This result agrees very well with the autoradiographs showing a 60% reduction in the number of the nuclei synthesizing DNA during the first hours after irradiation (Alexandre, 1970). This might be linked to the fact that the radiosensitivity of mammalian cells varies greatly during the cell cycle; the cells have been found to be most resistant to X-rays during the last part of the DNA synthetic period (S) and to be about equally sensitive before (G1) and after (G2) this period, M being the most sensitive (Sinclair & Morton, 1966). In the case of cleaving amphibian eggs, the situation is simplified because of the almost complete absence of G1 and G2; the sensitivity to X-rays increases during mitosis, reaches its maximum at telophase/early interphase and decreases during the S period (Hamilton, 1969); the high mitotic rate during cleavage would explain the strong inhibition of DNA synthesis after irradiation. This is specially relevant, since it is known that irradiation of Ilyanassa eggs during telophase and early interphase causes the greatest delay in the next cleavage and inhibits most DNA synthesis (Cather, 1959).

The fact that the rate of thymidine incorporation reaches a high value (68%) at the late blastula stage after irradiation of 2- to 4-cell stages leads to the conclusion that the early effect of radiation on DNA synthesis is linked to the delay in mitotic activity; the fact that the mitotic rate normally decreases at the
gastrula stage might explain the lower sensitivity of thymidine incorporation at this stage.

The higher acidolability of the DNA synthesized after irradiation remains to be explained. It can be suggested that the acidolable DNA corresponds to small fragments of newly synthesized DNA which are set free in the nucleus and perhaps even in the cytoplasm. In fact, Ide (1969) has shown that prelabelled DNA of embryonic chicken cells leaves the nucleus after irradiation. In addition, fragmentation of chromosomes during abnormal anaphase has been observed in irradiated *Pleurodeles* embryos (Alexandre, 1967).

The most interesting result is the high radiosensitivity of the metabolic pathway leading to the incorporation of uridine into DNA. It is tempting to suggest that there is a selective inhibition of the synthesis of the enzymes involved in this metabolic pathway. In fact, there is indirect evidence that the key enzymes for the regulation of DNA synthesis are synthesized during cleavage, since the inhibitors of protein synthesis very quickly block the incorporation of labelled uridine into DNA during cleavage in amphibian eggs (Legros, 1970). The fact that the use of [³H]deoxyuridine as a precursor of DNA has led to the same results as those obtained with uridine after treatment of the autoradiographs with RNAase (Alexandre, 1970) could be a good indication that thymydylate synthetase is the main target of irradiation. The radiosensitivity of enzymes involved in the regulation of DNA synthesis has been verified in other systems; Sugino & Potter (1960) have demonstrated, in rat thymus, the great radiosensitivity of deoxycytidine-5'-monophosphate deaminase activity, with total inhibition after 400 R.

It must, however, be said that all these metabolic pathways are directed by many enzymes; feedback inhibitions due to the accumulation of one product like thymidine-5'-triphosphate, which is known to inhibit the activity of the dCMP deaminase in young embryos of *Rana pipiens* (Atkinson & Roth, 1971) could well play a role. The possibility of an inhibition due to the accumulation of a nucleotide should be considered in the light of the work of Adelstein & Manasek (1967a, b) and of Little (1970), who have clearly demonstrated such a ‘pool-size effect’ in several strains of mammalian cells after irradiation.

**Radiosensitivity and RNA synthesis**

It has been previously concluded that RNA synthesis, as judged by nuclear uridine incorporation (autoradiography, after treatment of the slides with DNAase), is more radiosensitive than DNA synthesis, in terms of nuclear incorporation of thymidine (Alexandre, 1970).

The present work shows that RNA synthesis remains almost normal even after irradiation with 5000 R. It is to be remembered that the autoradiographs demonstrate only a very low level of RNA synthesis; 21 days exposure to the slides was needed, whereas 5 days was sufficient for studying thymidine incor-
Radiosensitivity of amphibian development

poration. Moreover, Gurdon & Woodland (1969) have shown that the mRNA which is synthesized during the cleavage period migrates very quickly into the cytoplasm; consequently, DNAase-resistant nuclear incorporation of uridine, as observed by autoradiography, represents only a small fraction of the newly synthesized RNA. It could correspond to heterogeneous RNA present in the nuclei of *Xenopus* embryos at stage 8 (Gurdon & Woodland, 1969) or to a small fraction of tRNA.

The comparison of results obtained by the two methods suggests that the synthesis of the mRNA which is rapidly found in the cytoplasm is much more radioresistant than that of nuclear heterogeneous RNA or rRNA.

The high inhibition of total RNA synthesis at the late blastula stage, after irradiation at the beginning of cleavage, is in good agreement with this conclusion, since it is well known that nuclear accumulation of RNA has become more important at this stage. Moreover, quantitative RNA determinations (Alexandre, 1971) have already shown that the most important effect of X-rays is inhibition of the increase in RNA content at gastrulation; thus, irradiation could act on the induction (or depression) of several genes, notably by inhibiting the induction of rRNA synthesis.

This particular effect of X-rays can be correlated with the inhibition, by X-rays, of formation of the outer granular constituent in the nucleoli during the last part of cleavage, which has been observed by electron microscopy (Alexandre & Gérin, 1971).

**RÉSUMÉ**

L’irradiation X d’œufs en segmentation de Pleurodèle ralentit considérablement la réplication du DNA pendant les quelques heures qui suivent son application. Cette inhibition est probablement due à l’arrêt du cycle cellulaire dans une proportion importante de blastomères. La réplication du DNA est rétablie partiellement au stade de la blastula avancée, après irradiation de stades 2-4 blastomères.

La très haute radiosensibilité de la voie métabolique qui conduit à l’incorporation de l’uridine dans le DNA a pu être vérifiée; elle pourrait être la conséquence de l’inhibition de l’activité, ou de la synthèse d’enzymes spécifiques telles que la ribonucléotide réductase, la thymidylate synthétase et la CTP synthétase. La radiosensibilité de cette voie métabolique diminue après le début de la gastrulation.

Enfin, l’irradiation inhibe quasi totalement la synthèse des nouvelles espèces de RNA qui président à la gastrulation; elle semble, par contre, n’avoir que peu d’effet sur la synthèse des mRNA durant la segmentation.

Ces résultats sont discutés en fonction des connaissances biochimiques relatives aux jeunes stades du développement.

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