Characteristics of the Utilization of Nucleosides by Embryos of Paracentrotus lividus

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The sea urchin embryo develops normally in sea water without added nutrients. It draws on a reservoir of material for the formation of such cellular constituents as nucleic acids, but has the ability to use exogenously supplied precursors (1-3). Exogenous substrates can be used not only as a source of tracers for the study of metabolic pathways but also as a probe into the nature of the metabolic reservoir of the embryo, to relate this reservoir to its later metabolism. This study was a survey of the characteristics of utilization by the embryos of Paracentrotus lividus of several pyrimidine nucleosides and their analogues.

A part of this work takes advantage of the "natural" synchrony of division that occurs in large batches of these embryos during the early stage of their development. The temporal and quantitative aspects of the synthesis of deoxyribonucleic and ribonucleic acids have been studied in the course of the division cycle. Conclusions drawn from this biological system may avoid the artifacts of some artificially synchronized systems, such as those reviewed by Campbell (4).

EXPERIMENTAL PROCEDURE

Embryos—Gametes were obtained from the gonads of sea urchins (P. lividus) collected from the area of the bay of Naples from March to July. The eggs were suspended and washed in filtered sea water. Fertilization was performed by stirring with a small amount of sperm on a glass rod at 20-22°. Fertilized eggs were allowed to develop at 18° in filtered sea water in a constant temperature room, where their containers were tilted rhythmically, or in beakers in a constant temperature bath, in which they were slowly stirred. These conditions allowed development at least to the late pluteus stage. All experiments were performed with suspensions of 5000 embryos per ml. Concentrations of embryos and their stages of development were ascertained at 100 X magnification in a counting chamber. In the experiments on synchronously dividing embryos, two simultaneous samples were taken for biochemical assay and for determining the stage of development. To the former sample was added 5% trichloroacetic acid, to the latter 1% formalin in sea water. The average number of cells per embryo are plotted in Figs. 2, 3, and 4 to show the stage of development. These curves refer to the times at which given numbers of cell divisions are completed, i.e. when the cleavage furrows are completed. Incorporation caused by bacterial contamination may be regarded as negligible, since even in very long incubations of unfertilized eggs the amounts of incorporation in acid-precipitated material is very small (Table I).

Chemicals—Unlabeled uridine, cytidine, thymidine, deoxyuridine, deoxyxytidine, and thymidylic acid were California Corporation for Biochemical Research Cyp grade. Thymus DNA and 5-methyldeoxycytidine were gifts of Dr. E. Scarano of the Stazione Zoologica, Naples. 5-Fluorodeoxycytidine was a gift of Dr. D. Karnofsky of the Sloan-Kettering Institute, New York. 5-Idodeoxyuridine was supplied by Instituto Sieroterapico, Naples.

I prepared the C14-labeled compounds in the laboratory of Dr. S. S. Cohen in the University of Pennsylvania. They were prepared from uracil-2-C14 and thymine-2-C14, obtained from Isotopes Specialities, Inc. Conditions for enzymatic reactions with nucleoside phosphorylase and nucleoside phosphotransferases, used in these preparations, are described by Fizer and Cohen (5).

Deoxyuridine-2-C14 was obtained by an exchange reaction between deoxyuridine and labeled uracil, catalyzed by the enzyme of Escherichia coli W14, nucleoside phosphorylase, provided by Dr. L. Fizer of the University of Pennsylvania. The products were separated and purified by paper chromatography with ethyl acetate-formic acid water (6).

Cytidine-2-C14 and aridine-2-C14 were prepared from labeled RNA of E. coli B14, grown in the presence of uracil-2-C14. The methods for growing the organisms and extracting the 2',3'-ribonucleotides from RNA have been described (7). The pyrimidine ribonucleotides were dephosphorylated by calf intestinal alkaline phosphatase, prepared by M. Loeb of the University of Pennsylvania. The nucleoside products were purified by successive paper chromatography in isobutyric acid-ammonia (8) and ethyl acetate-formic acid-water.

Deoxouridylic Acid-2-C14 was obtained by phosphorylation of labeled deoxyuridine with p-nitrophenyl phosphate, catalyzed by wheat germ nucleoside phosphotransferase, kindly supplied by H. Barner of the University of Pennsylvania. The products were separated and purified by the solvent systems, isobutyric acid-ammonia, ethyl acetate-formic acid-water, and n-butanol-water-ammonia atmosphere (9).

Thymidine-2-C14 was prepared by the exchange of thymine-2-C14 with thymidine, catalyzed by nucleoside phosphorylase, as above. Thymidylic acid-2-C14 was prepared by phosphorylation of labeled thymidine, as described for deoxyuridylic acid-2-C14 formation.

The identification and purity of all compounds were ascertained by comparison of their chromatographic and ultraviolet spectrophotometric characteristics with appropriate standards.

TABLE I
Incorporation of nucleosides and nucleotides in acid-insoluble material

Concentrations of the C-14-labeled compounds were all 200
mMoles per 10 embryos per 20 ml. Embryos were incubated for
5 hours. Incorporation into total trichloroacetic acid precipitate
was measured. Values are averages of duplicate determinations
of ±10% precision.

<table>
<thead>
<tr>
<th>Labeled compound</th>
<th>Amount incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unfertilized egg</td>
</tr>
<tr>
<td></td>
<td>mMoles × 105/embryo</td>
</tr>
<tr>
<td>Uridine</td>
<td>0.96</td>
</tr>
<tr>
<td>Cytidine</td>
<td>0.80</td>
</tr>
<tr>
<td>Deoxyuridine</td>
<td>0.76</td>
</tr>
<tr>
<td>Thymidine</td>
<td>0.16</td>
</tr>
<tr>
<td>Thymidylic acid</td>
<td>0.98</td>
</tr>
<tr>
<td>Deoxyuridylic acid</td>
<td>0.60</td>
</tr>
</tbody>
</table>

TABLE II
Incorporation of nucleosides and nucleotides in acid-soluble and
acid-insoluble components of early cleavage stage embryos

In incubations of 10 embryos per 20 ml were performed for 3
hours. Embryos developed from the 1- to approximately the
8-blastomere stage. Amounts of exogenous incorporation were
calculated with appropriate self-absorption corrections for acid-
soluble material and precipitates on Millipore filters. These
data are averages of duplicate determinations of ±10% precision.

<table>
<thead>
<tr>
<th>Labeled compound</th>
<th>Millimicromoles × 105/embryo</th>
<th>Amount incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acid-soluble</td>
</tr>
<tr>
<td>Cytidine</td>
<td>72</td>
<td>33</td>
</tr>
<tr>
<td>Uridine</td>
<td>118</td>
<td>27</td>
</tr>
<tr>
<td>Thymidine</td>
<td>101</td>
<td>6.5</td>
</tr>
<tr>
<td>Deoxyuridine</td>
<td>128</td>
<td>4.5</td>
</tr>
<tr>
<td>Thymidylic acid</td>
<td>134</td>
<td>2.5</td>
</tr>
<tr>
<td>Deoxyuridylic acid</td>
<td>38</td>
<td>0.5</td>
</tr>
</tbody>
</table>

5-Iododeoxyuridine-2-Cl4 was prepared by a modification of
the method of Prusoff (10). Deoxyuridine-2-Cl4 (3 to 4 μmoles)
was refluxed in 0.1 ml of chloroform and 2 ml of 1 N nitric acid
plus 20 mg of solid iodine for 3 hours at 75°. The excess iodine
was sublimed, and the mixture was applied to paper for chroma-
tography in the solvent system of ethyl acetate, saturated with
0.05 M phosphate at pH 6. Approximately 0.5 μmole of 5-iodo-
deoxyuridine-2-Cl4 was recovered. The ultraviolet spectrum
showed a maximum at 288 μm and a minimum at 278 in 0.01
N HCl, a maximum at 278 and a minimum at 253 in 0.01 N
NaOH.

Incorporation in Total Nucleic Acids—Reactions were stopped
by addition of cold 5% trichloroacetic acid. The total acid-
precipitated material was plated on membrane filters of 0.45 μ
porosity (Millipore Filter Corporation), affording essentially
complete recovery of nucleic acids. The filters were affixed to
aluminum planchets and dried. Samples were counted in a
Nuclear-Chicago automatic end window counter, to a counting
error of 2%.

Incorporation in DNA and RNA—The separation and isola-
tion of DNA and RNA were performed by the Schmidt-Thann-
hauser method (11). The lipid-extracted material was sub-
mitted to alkaline hydrolysis with 1 N KOH at 37° for 18 hours,
after addition of 1 mg of carrier thymus DNA, which was neces-
sary for complete precipitation of DNA in the next step (the
amount of DNA encountered in these analyses was generally 1
μg or less per sample). The use of either 1 or 5 mg of carrier
DNA gave the same results. After removal of K+ by precipita-
tion with perchloric acid, the undigested material, including
DNA, was precipitated with 5% trichloroacetic acid in the cold.
An aliquot of the acid-supernatant, digested material was
plated on polystyrene planchets for radioactive assay. The
DNA precipitate was extracted with 5% trichloroacetic acid at
90° for 20 minutes; aliquots of the acid-extracted material were
plated on polystyrene planchets. Samples were assayed for
radioactivity as above. Through these procedures, it was found
that 5% of labeled thymidine incorporated into total acid-pre-
cipitable material was in the RNA fraction. This value may
then represent the possible degree of contamination of RNA by
DNA.

Incorporation in Acid-soluble Material—This was assayed by
plating the supernatant material on polystyrene planchets after
precipitation with cold trichloroacetic acid.

Amounts of Incorporation—In all cases, the same number of
embryos per sample was used, and a constant volume of extract
was plated for assay. Consequently, for each fraction a charac-
teristic and essentially constant amount of material, hence self-
absorption was encountered. The counts per minute measured
in a given fraction referred to a given amount of compound as
compared to a standard measured under the same conditions.
Standards were plated with the volume of extract regularly em-
ployed for assay, to give the following specific activities (counts
per minute per mmole): thymidine and thymidylic acid in acid-
soluble material, 39; in DNA hydrolysate, 35; in RNA hydroly-
sate, 42; and in total acid-precipitable material, 43. The other
labeled compounds had the following respective specific activi-
ties: deoxyuridine, deoxyuridylic acid, and 5-iododeoxyuridine,
117, 105, 126, and 129; cytidine and uridine, 240, 215, 260, and
265.

RESULTS AND DISCUSSION

Uptake and Incorporation of Nucleosides and Nucleotides—In
preliminary experiments, the ability of 1-hour and 24-hour em-
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endogenous pool than deoxyribonucleoside. The concentration of cytidine in the medium was 3.6 \times 10^{-6} M initially and 1.8 \times 10^{-6} M at the end of the 3-hour incubation. The concentration of labeled material in the embryos, considering their volume as no more than \( \frac{1}{20} \) of the total volume, would be over \( 30 \times 10^{-6} \) M. There may be an active transport system at work here.

The embryos of the experiment described in Table II were resuspended in fresh sea water and incubated for an additional 3 hours with samples for analysis taken every hour. After this initial 3-hour "pulse," the decay curves (Fig. 1) for the amount of labeled acid-soluble material from the exogenous deoxyribonucleosides and deoxyribonucleotides describe half-lives of about 30 minutes. The half-lives of the pools supplied by exogenous ribonucleosides are 4 to 6 times longer than those of the deoxyribose compounds.

In the early cleavage stage embryo, measurements of incorporation in DNA and RNA show that the DNA:RNA ratio of incorporation from cytidine may be as high as 8, but only approximately 2 for uridine (Table III). When unlabeled cytidine or uridine are also included in the medium, the diminution of incorporation tends to be proportional to the ratio of unlabeled carrier to labeled precursor. This proportionality probably reflects the similar degree of uptake of these ribonucleosides (Table II). The proportionality holds for RNA incorporation, indicating a high degree of equilibration of RNA precursors from exogenous cytidine and uridine. However, the dilution of incorporation of uridine in DNA by carrier cytidine is twice that which would then be expected from their relative amounts of uptake alone. The presence of unlabeled uridine reduces the DNA:RNA incorporation ratio from cytidine by no more than 20%, but unlabeled cytidine reduces this ratio of uridine incorporation by 50%. The derivatives of exogenous cytidine may furnish metabolic intermediates between exogenous uridine and DNA. The same relations do not seem to pertain to the 24-hour embryo, in which the utilizations of cytidine and uridine are similar. In time course experiments up to 24 hours, incorporations of cytidine and uridine tend to reflect the following relations: cytidine in DNA > cytidine in RNA = uridine in RNA = uridine in DNA.

**Temporal and Quantitative Aspects of Nucleic Acid Synthesis in Synchronously Dividing Early Cleavage Stage Embryos**—Embryos were incubated in the presence of deoxyuridine-2-C\(^{14}\) and thymidine-2-C\(^{14}\), with samples taken at short intervals to measure incorporation into nucleic acids. Total trichloroacetic acid precipitates were plated on Millipore filters and assayed for radioactivity. In experiments in which DNA and RNA were separated (5-hour incubations of 1-hour embryos), it was found that less than 5% of the total incorporation of the thymidine and less than 10% of the deoxyuridine may have been incorporated into RNA (i.e., alkaline-digested trichloroacetic acid precipitate). We may assume then that the incorporations from these compounds, described below, are essentially into DNA. Both compounds are precursors of DNA thymine (12).

The incorporation of deoxyuridine in DNA from fertilization to the 8-cell stage is shown in Fig. 2. A small amount of incorporation is shown at the 1-blastomere stage long before the first cleavage. This early incorporation is in agreement with the autoradiographic demonstration by Simmel and Karnofsky (13) that the major portion of label appears in DNA during the first 20 to 30 minutes after fertilization, and is initiated before the pronuclei have fused in sand dollar zygotes. Since the incorporation of deoxyuridine is mostly in DNA, we may assume that at these 1- and 2-blastomere stages the embryo is capable of converting deoxyuridylate acid to thymidylate acid. The time course of the incorporation shows that there is a discontinuous synthesis of DNA. The incorporation into DNA occurs immediately following division; i.e., in early interphase.
In the previous experiment, the embryos were exposed continuously to an excess of substrate in the medium. To test whether these incorporations reflected permeability changes in the course of development or through the cell cycle, and to extend our study to another DNA precursor, the following experiment was performed. Embryos were incubated for 2 hours in the presence of exogenous, labeled thymidine, then resuspended in fresh sea water after washing, and allowed to develop. Samples were taken from the 4- to the 16-blastomere stage. Again the incorporation occurred immediately after division (Fig. 3). The beginning of incorporation was detected at about the time that 50% of the cells finished division. All of the incorporation in DNA came from a pool of labeled precursors taken up from the medium during the 2-hour preincubation period and stored as acid-soluble material.

The incorporation of labeled cytidine into DNA and RNA is described in Fig. 4. The synthesis of DNA from this source bears the same temporal relation to cell division as previously seen with thymidine and deoxyuridine; i.e. half of the DNA was synthesized approximately 5 minutes after the time at which half the cells had divided. The incorporation into RNA occurs only over a very short interval in the cell cycle at this early stage of development. It precedes or is concurrent with that into DNA.

In these experiments, a large amount of the DNA pyrimidine is derived from exogenously supplied nucleosides. An estimate of the amount of DNA pyrimidine or DNA thymine that must normally be synthesized in each transition from one interphase to the next (i.e. from one tetraploid level to the next) can be made from the measurements by Elson et al. (14) and Chargaff et al. (15) on the content and composition of the DNA of the sperm (haploid amount) of this same species. In the transition from the 4- to the 8-cell stage, $1.6 \times 10^{-4}$ mmoles of DNA thymine per 10^6 embryos per 20 ml, incubated at 18° for 2 hours. Embryos were washed twice and resuspended in fresh sea water, with samples then taken every 5 minutes. These data are single determinations from one of two similar experiments. O-O, incorporation in total tri-chloroacetic acid precipitate; X---X, cells per embryo.
mine is synthesized per embryo. According to Fig. 2, a large amount of this material could be derived from exogenous deoxyuridine. Similarly, in this transition, thymidine has been seen to supply 1.0 to 2.3 \times 10^{-4} \text{ mmole} of DNA thymine per embryo. In the transition from 8 to 16 cells (Fig. 4), exogenous deoxycytidine can supply 45\% of the 4.8 \times 10^{-7} \text{ mmole} of DNA pyrimidine synthesized per embryo, as calculated from the data of the above authors (14, 15). In these experiments, each embryo had taken up 3.2 \times 10^{-6} \text{ mmole} of cytidine (or ribonucleotide) per embryo. Such a reservoir of ribonucleotides could support DNA synthesis until the 128-cell stage, after which de novo pyrimidine synthesis would have to occur. The extensive use of exogenous substrates in this system, which is highly endowed with a supply of precursors, suggests that the added compounds exert a feedback regulation upon their own synthesis.

**Developmental and Biochemical Effects of 5-Fluorodeoxyuridine**

The deoxyuridine analogue, 5-fluorodeoxyuridine, was found to stop development of the embryos of *P. lividus* at the 8-blastomere stage in concentrations as low as 10^{-7} \text{ M}. This observation agrees with the conclusions drawn from the measurements of the thymidine content of the eggs of this species by Hoff-Jørgensen (16) and Kavanau (17). Kavanau found that they contained 4.6 \times 10^{-5} \text{ mmole per egg}, enough to support development to the 16-diploid blastomere stage. In Table IV, in which the concentration of 5-fluorodeoxyuridine is 8 \times 10^{-5} \text{ M}, thymidine at about one-tenth this concentration allowed the embryos to develop at least to the blastula stage (further observations were not made in this case), whereas deoxyuridine, at one-tenth, and deoxycytidine at one-half the 5-fluorodeoxyuridine concentration, had no effect. During the interval from the 1- to the 8-blastomere stage, the embryo is capable of incorporating exogenous deoxyuridine, but this incorporation is markedly inhibited by 5-fluorodeoxyuridine (Table V). In these embryos, which have enough thymidine to support DNA synthesis to the 8-blastomere stage, approximately one-third of the DNA thymine (0.8 \times 10^{-5} \text{ mmole per embryo}) could still be supplied by deoxyuridine up to that stage.

Reversal of 5-fluorodeoxyuridine inhibition, with development to the blastula stage, was achieved with 5-methyldeoxycytidine and with 5-iododeoxyuridine. The latter alone blocks development at the blastula stage (see below). It is likely that the 5-methyldeoxycytidine was phosphorylated and then converted to thymidylic acid by the enzyme 6-aminopyrimidine deoxyribonucleotide deaminase (18).

If increasing concentrations of thymidine are added to embryos exposed to 5-fluorodeoxyuridine, the embryos will develop only as long as they can be supported by the exogenous thymidine and thus their extent of development will be proportional to the amount of added thymidine. "Controlled" development to various stages, as far as gastrulation, has been achieved with a concentration of 5-fluorodeoxyuridine at 10^{-7} \text{ M} and concentrations of thymidine varied from 0.4 \times 10^{-7} to 5 \times 10^{-4} \text{ M}.

The response to inhibition of thymidine synthesis occurs much earlier in this species than in other embryos. Karnofsky (19) was able to block development of *Echinarchinus parma* no earlier than the blastula stage with 5-fluorodeoxyuridine, and Grant (20) stopped development of frogs at the blastula stage with folic acid analogues. The action of these analogues presumably

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### Table IV

*Blockage of embryological development by 5-fluorodeoxyuridine and its reversal*

<table>
<thead>
<tr>
<th>Supplement added*</th>
<th>Concentration of supplement</th>
<th>Comments on development</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>( \times 10^{-6} \text{ M} )</td>
<td>Death at 8-cell stage</td>
</tr>
<tr>
<td>Thymidine</td>
<td>5</td>
<td>Alive at blastula stage</td>
</tr>
<tr>
<td>Deoxycytidine</td>
<td>50</td>
<td>Death at 8-cell stage</td>
</tr>
<tr>
<td>Deoxyuridine</td>
<td>5</td>
<td>Death at 8-cell stage</td>
</tr>
<tr>
<td>5-Methyldeoxycytidine</td>
<td>50</td>
<td>Alive at blastula stage</td>
</tr>
<tr>
<td>5-Iododeoxyuridine</td>
<td>2.5</td>
<td>Alive at blastula stage</td>
</tr>
</tbody>
</table>

*At fertilization, 80 \times 10^{-6} \text{ M} 5-fluorodeoxyuridine was present together with the listed supplement.

### Table V

*Inhibition of incorporation of deoxyuridine-8-C\(^{14}\) by 5-fluorodeoxyuridine*

5-Fluorodeoxyuridine was present at 4 \times 10^{-5} \text{ M}; uridine-2-C\(^{14}\) at 28 \times 10^{-8} \text{ M}. Incubations were started 30 minutes after fertilization at the 1-blastomere stage, and terminated 200 minutes later at the 8-blastomere stage. The values are derived from duplicate determinations of 10\% agreement.

<table>
<thead>
<tr>
<th>Time of addition of 5-fluorodeoxyuridine (after addition of uridine-C(^{14}))</th>
<th>Incorporation of uridine-C(^{14}) in acid precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>( \text{mmole} \times 10^{1}/\text{embryo}/200 \text{ minutes} )</td>
</tr>
<tr>
<td>0</td>
<td>0.13</td>
</tr>
<tr>
<td>30</td>
<td>0.45</td>
</tr>
<tr>
<td>90</td>
<td>0.64</td>
</tr>
<tr>
<td>180</td>
<td>0.75</td>
</tr>
<tr>
<td>200</td>
<td>0.80</td>
</tr>
</tbody>
</table>

### Table VI

*Incorporation of 5-iododeoxyuridine-2-C\(^{14}\) in nucleic acid*

Concentration of 5-iododeoxyuridine-2-C\(^{14}\) was 8.6 \times 10^{-8} \text{ M} (10,000 total c.p.m.). Embryos developed from the 1- to approximately the 8-blastomere stage.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Concentration of supplement</th>
<th>5-Iododeoxyuridine-C(^{14}) incorporation in acid precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>( \times 10^{-6} \text{ M} )</td>
<td>376, 390</td>
</tr>
<tr>
<td>Thymidine</td>
<td>11.4</td>
<td>122</td>
</tr>
<tr>
<td>5-Fluorodeoxyuridine</td>
<td>4.0</td>
<td>412, 420</td>
</tr>
</tbody>
</table>

reflects the extent to which the embryo depends upon thymidine synthesis.

**Developmental and Biochemical Effects of 5-Iododeoxyuridine**

The incorporation of 5-iododeoxyuridine-2-C\(^{14}\) in total acid-precipitated material was measured (Table VI). This incorporation was large and was reduced to about one-third by the presence of an essentially equimolar amount of thymidine, indicating that in a competition for incorporation thymidine is favored.
Effect of 5-fluorodeoxyuridine on cytidine incorporation into DNA of early cleavage stage embryos

Incubations were performed at initial concentration of 34 mmoles of cytidine-2-C\textsubscript{14} per 10\textsuperscript{5} embryos per 20 ml. 5-Fluorodeoxyuridine was added at 195 minutes after fertilization (between the 4- and 8-cell stage) at 200 mmoles per 10\textsuperscript{5} embryos per 20 ml. The 5-fluorodeoxyuridine-treated embryos were blocked in development at the 16-cell stage, whereas the controls continued developing. Values are averages from duplicate incubations of \pm 10\% precision.

<table>
<thead>
<tr>
<th>Duration of preincubation with 5-fluorodeoxyuridine</th>
<th>Incorporation of thymidine-C\textsubscript{14} c.p.m./10\textsuperscript{5} embryos/2 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>070</td>
</tr>
<tr>
<td>30</td>
<td>704</td>
</tr>
<tr>
<td>60</td>
<td>324</td>
</tr>
</tbody>
</table>

Effect of 5-fluorodeoxyuridine on incorporation of cytidine into DNA and RNA of early cleavage stage embryos

Embryos at a later stage (11-hour blastulae) were preincubated with 5-fluorodeoxyuridine. The preincubation period of 30 or 60 minutes was followed by exposure to thymidine-2-C\textsubscript{14} for 2 hours. The 60-minute pretreatment with 5-fluorodeoxyuridine resulted in a 50\% diminution of incorporation (Table VII). The deprivation of an endogenous supply of thymidine began to have a deleterious effect at some point during this interval. In spite of this action of 5-fluorodeoxyuridine, the thymidine incorporation of the 30-minute 5-fluorodeoxyuridine-treated embryos was just slightly greater than that of the controls.

In neither of the above experiments did we find a pronounced stimulation of the incorporation of exogenous thymidine in the presence of 5-fluorodeoxyuridine. Such a stimulation might have been expected, since 5-fluorodeoxyuridine does block the supply of endogenous thymidine (31), hence the source of dilution of the labeled exogenous thymidine. In each case, we compared the experimental embryos with controls, which were similarly "exposed" to exogenous thymidine. It might be concluded that if 5-fluorodeoxyuridine is inhibiting endogenous thymidine synthesis in the experimental embryos, then thymidine may be blocking its own synthesis in the controls. The preferential utilization of exogenous thymidine demonstrated previously also indicated a feedback control by thymidine.

Effect of 5-Fluorodeoxyuridine on Incorporation of Cytidine-2-C\textsubscript{14} into DNA and RNA—Embryos that had been incubated with an irregular pattern of embryonic death. In both species, the embryo develops with what may be a very abnormal DNA. The incorporation of 5-iododeoxyuridine-2-C\textsubscript{14} in our experiments indicates that the entire cleavage stage can be traversed with abnormally halogenated DNA.

The nucleic acid synthesis of embryos developing in the presence of 5-iododeoxyuridine was compared with that of normal embryos. They were exposed to uridine-2-C\textsubscript{14} or cytidine-2-C\textsubscript{14} for successive 2-hour intervals in the course of their development. The high concentration of 5-iododeoxyuridine (2 \times 10\textsuperscript{-4} M) blocked development at the blastula stage. At 19 hours, the controls were gastrulae, whereas 80\% of the 5-iododeoxyuridine-treated embryos were hatched blastulas, the rest early blastulae. Incorporation of both nucleosides in DNA was reduced 70 to 90\% by 5-iododeoxyuridine. This reduction may be attributed to competition with and replacement of endogenous thymine, and possibly to toxic and inhibitory effects of the large 5-iododeoxyuridine concentration. During the period before hatching, which normally occurs about 12 hours after fertilization, the incorporation in RNA of 5-iododeoxyuridine-treated embryos was somewhat reduced, but still increasing. After about 13 hours, the ability of the 5-iododeoxyuridine-treated embryos to incorporate cytidine or uridine into RNA remained at a constant level, whereas control values continued to rise.

Characteristics of Incorporation of Thymidine-2-C\textsubscript{14} in Presence of 5-Fluorodeoxyuridine—Fertilized eggs, which were preincubated with thymidine-C\textsubscript{14} until the 4-blastomere stage, were resuspended in fresh sea water, and their incorporation of labeled substrate in DNA was followed subsequently in the presence and absence of 5-fluorodeoxyuridine. Equal incorporations were observed up to the 16-blastomere stage, indicating that the same amounts of endogenous thymidine were produced by the treated and control batches. The development of the 5-fluorodeoxyuridine-treated embryos was blocked between the 16- and 32-blastomere stages.

Embryos at a later stage (11-hour blastulae) were preincubated with 5-fluorodeoxyuridine. The preincubation period of 30 or 60 minutes was followed by exposure to thymidine-2-C\textsubscript{14} for 2 hours. The 60-minute pretreatment with 5-fluorodeoxyuridine resulted in a 50\% diminution of incorporation (Table VII). The deprivation of an endogenous supply of thymidine began to have a deleterious effect at some point during this interval. In spite of this action of 5-fluorodeoxyuridine, the thymidine incorporation of the 30-minute 5-fluorodeoxyuridine-treated embryos was just slightly greater than that of the controls.

In neither of the above experiments did we find a pronounced stimulation of the incorporation of exogenous thymidine in the presence of 5-fluorodeoxyuridine. Such a stimulation might have been expected, since 5-fluorodeoxyuridine does block the supply of endogenous thymidine (31), hence the source of dilution of the labeled exogenous thymidine. In each case, we compared the experimental embryos with controls, which were similarly "exposed" to exogenous thymidine. It might be concluded that if 5-fluorodeoxyuridine is inhibiting endogenous thymidine synthesis in the experimental embryos, then thymidine may be blocking its own synthesis in the controls. The preferential utilization of exogenous thymidine demonstrated previously also indicated a feedback control by thymidine.

Effect of 5-Fluorodeoxyuridine on Incorporation of Cytidine-2-C\textsubscript{14} into DNA and RNA—Embryos that had been incubated with...
cystidine-2-C\textsuperscript{14}, so that about $3.2 \times 10^{-4}$ m\textsuperscript{mole} per embryo was taken up into the acid-soluble material, were blocked at the 16-blastomere stage by 5-fluorodeoxyuridine, when the analogue was added at the 4- to 8-blastomere stage. Apparently, the large accumulation of ribonucleoside did not offset the action of 5-fluorodeoxyuridine by an augmentation of the deoxyribonucleoside concentration. The incorporations in DNA and RNA were compared with that of untreated embryos (Table VIII). Values for interphase embryos are given. The 5-fluorodeoxyuridine-treated embryos, which were blocked at the 16-blastomere stage, attained DNA incorporation intermediately between those of 8 and 16 blastomeres. This suggests that their amount of DNA was between diploid and tetraploid. However, the treated embryos were able to synthesize the same 16-blastomere complement of RNA as the controls.

**Summary**

1. Embryos of *Paracentrotus lividus* can utilize exogenously supplied cystidine, uridine, deoxyuridine, thymidine, thymidylic acid, and deoxythymidylic acid. Their uptake of ribonucleosides (mainly into acid-soluble components) was several times greater than that of deoxyribonucleosides. In the early embryo, exogenous cystidine furnishes a greater share of deoxyribonucleic acid pyrimidine than uridine, but at 24 hours of development, the utilizations of these ribonucleosides become fairly similar.

2. The extent of incorporation of exogenous cystidine in deoxyribonucleic acid indicates that an endogenous reservoir of related ribonucleotides of $4 \times 10^{-4}$ m\textsuperscript{mole} may be present in each unfertilized egg. Similarly, the large amounts of thymidine and deoxythymidine incorporated in deoxyribonucleic acid from exogenous sources would be possible only with a small endogenous reservoir of thymidine. It seems that the supply of available thymidine in the egg may not be enough to support deoxyribonucleic acid synthesis beyond the 8-blastomere stage, since 5-fluorodeoxyuridine blocks development at that stage. Deoxyuridine is incorporated into deoxyribonucleic acid as early as the 1-blastomere stage, indicating that its conversion to thymidylate is possible quite early in development. A feedback control by thymidine on its biosynthesis is indicated by the preferential exogenous utilization and by the failure of 5-fluorodeoxyuridine to stimulate greatly the incorporation of exogenous thymidine.

3. The block in development caused by 5-fluorodeoxyuridine is reversed by thymidine, 5-methyldeoxyctydine, or 5 iododeoxy uridine, with development at least to the blastula stage. The embryos incorporate 5-iododeoxyuridine-2-C\textsuperscript{14} into their nucleic acids. This incorporation is greatly diminished by the presence of thymidine; it is slightly stimulated by 5-fluorodeoxyuridine.

4. It was observed in batches of synchronously dividing embryos that cell division is followed immediately by incorporation of cystidine into ribonucleic acid and by synthesis of deoxyribonucleic acid, measured by incorporation of either thymidine, deoxyuridine, or cystidine. Both ribonucleic and deoxyribonucleic acid incorporations occur only during early interphase.

The addition of 5-fluorodeoxyuridine between the 4- and 8-blastomere stages in the presence of exogenous cystidine caused a block in development at the 16-blastomere stage. The incorporation of labeled cystidine in ribonucleic acid was equal to that of the 16-blastomere control, whereas that in the deoxyribonucleic acid was halted between the 8- and 16-blastomere levels.

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