THE EFFECT OF 5-AZACYTIDINE ON DEVELOPMENT, NUCLEIC ACID AND PROTEIN METABOLISM IN SEA URCHIN EMBRYOS

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Summary

5-azacytidine, an analog of cytidine, is a strong inhibitor of both ribonucleic and deoxyribonucleic acid synthesis during the earliest stages of sea urchin development. Protein metabolism is not affected by this inhibition. Morphological development is not hampered by the presence of the drug up to the 8-cell stage as well, while pronounced retardation of development at later stages takes place.

5-azacytidine, a synthetic analog of cytidine is an inhibitor of nucleic acid synthesis in eucaryotic cells due to its interference with the activities of two enzymes participating in nucleic acid anabolism: uridine kinase and orotidilic acid decarboxylase(1,2).

We have found that during the earliest stages of sea urchin development 5-azacytidine is a powerful inhibitor of nucleic acid synthesis also. However, contrasting other eucaryotic cells(3) such an inhibition of nucleic acid synthesis had no effect on protein metabolism and only differential effect on morphological development.

Materials and methods

Ripe eggs of Arbacia lixula were obtained by electrical stimulation. 40 ml of freely sedimented eggs were placed in 1600 ml of filtered sea water supplemented with 50 µg/ml of streptomycin and 100 IU/ml of penicillin. The eggs were fertilized with 3-4 drops of concentrated sperm. Only those cultures were used where more than 95% of eggs were fertilized. This stock culture was subdivided into 4 subcultures to which, except for

the control, 5-azacytidine was added to the final concentracion of 0.1 ,1 and 10 µg/ml respectivly. At the beginning ofevery cell cycle of control from each of the four subcultures two aliquots were withdrawn and separatly incubated in the presence of H^3-uridine, H^3-thymidine or H^3-amino acid mixture(New England Nuclear) at the final concentracion of the label of 0.6 µC/ml. At the same time small aliquots of cultures were also taken to be fixed with formaldehyde, photographed under an optical microscope and examined for their morphological development.

The pulse labelling usually lasted about 45 minutes except for the first cell division(from fertilization to the two-cell stage)where incubation lasted longer, about 80 minutes. After incubation with the label the eggs were washed twice in sea water and resuspended in cold 5 % trichloracetic acid(TCA) and centrifuged. The treatment with TCA was repeated twice. The TCA washed pellet was extracted with a mixture of ethanol:chloroform:ether(2:1:1) and centrifuged. Finally the pellet was dissolved in 0.3 M KOH(for RNA hydrolysis the pellet was incubated for 18 hours at 37°C)and heated for a short time at 60°C. The alkaline solutions were neutralized with 3 N perchloric acid. After removing the $KClO_4$, the concentration of protein in the supernatant was measured by the method of Lowry et al.(4), while aliquots of the same solutions were placed on filter papers, air dried and counted in toluene scintillation liquid in a LS-100 Beckman scintillation counter.

In the case of RNA hydrolysis after neutralization with 3 N perchloric acid, the mixture of ribonucleotides was placed on a Dowex column and nucleotides fractionated by different concentrations of formic acid and ammonium formate solutions(5). The nucleotides were identified by their absorbance patterns.

Results and discussion

Table I presents the effect of 10 µg/ml of 5-azacytidine on RNA metabolism. The specific activities of both uridilic acid and cytidilic

TABLE I

The effect of 10 µg/ml of 5-azacytidine on incorporation of H^3-uridine into high molecular weight RNA;expressed as specific radioactivities of uridilic and cytidilic acid.

Stage of development	UMP cpm/µM C*	T*	% of inhibition	CMP cpm/µM C*	T*	% of inhibition
4 cells	844	299	65	355	143	60
16 cells	1064	146	87	683	35	95
blastula	4611	261	95	1385	78	95

C*=controls; T*=5-azacytidine treated embryos

acid are drastically decreased.In both cases an inhibition of 65-95% was observed.

In figure 1A the effect of different concentrations of 5-azacytidine on the rate of cell division of sea urchin embryos is summarized. The pyrimidine analog was added immediately following fertilization and was present continuously.5-azacytidine at any concentrations used had no effect on the rate of cell division up to the 8-cell stage.Following this stage the effect of the drug became visible.A retardation of development occured,extent of it depended on the dose of the analog used.It is difficult to establish with high precision the degree of delay of development in the presence of this pyrimidine analog because of its variable effect in different experiments.The numbers given in figure 1A therefore represent an average delay observed in several experiments.

The percantage of inhibition of incorporation of labeled H^3-thymidine into high molecular weight DNA varied depending both on concentration of 5-azacytidine and the stage of development(figure 1B). At the lowest dose used the inhibition of DNA synthesis ranged from 25% at the 2-cell stage up to 75% at the hatching-blastula stage.For higher doses,even at the beginning of development,the inhibition was much more

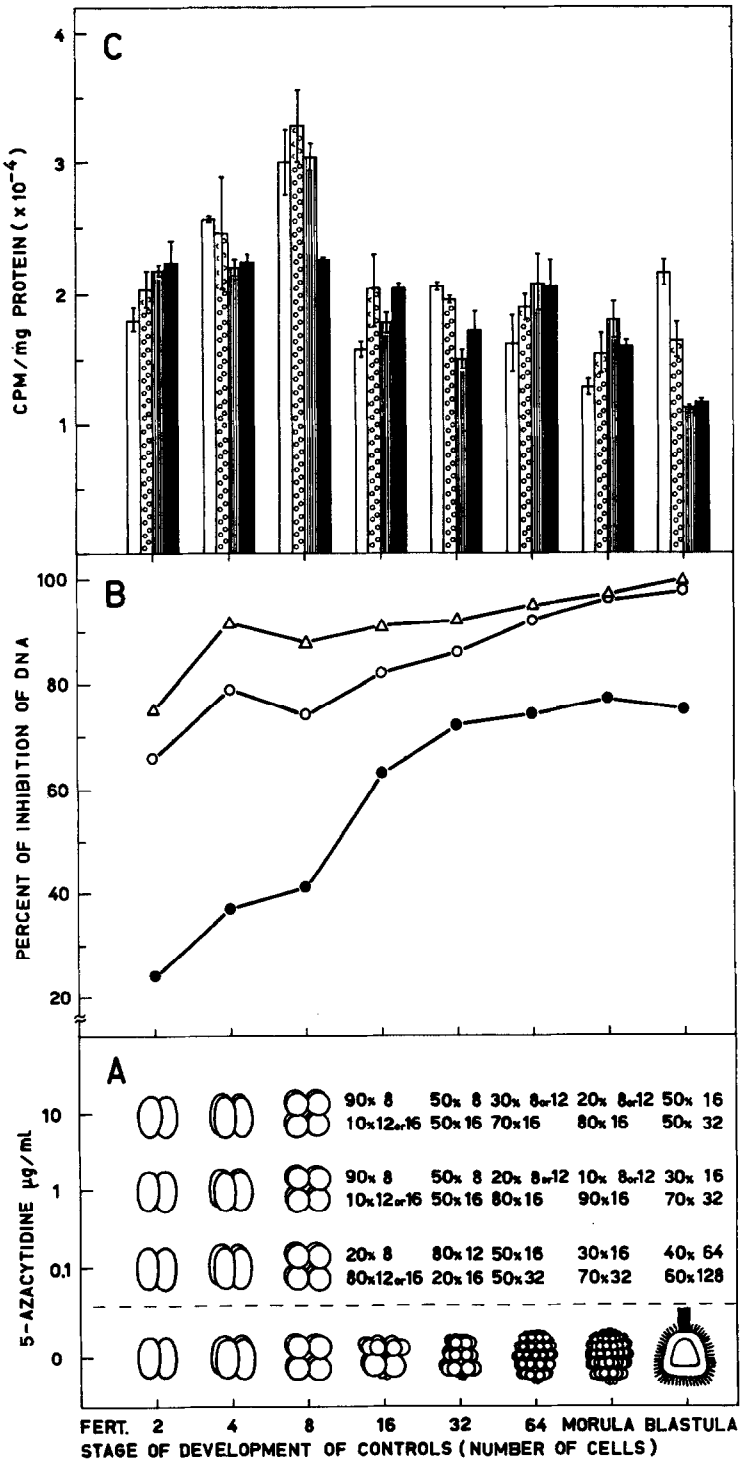

Figure 1 The effect of different doses of 5-azacytidine on:
A morphological development of sea urchin embryos,

B inhibition of uptake of H^3-thymidine into high molecular weight DNA.(•——•) o,1 µg/ml;(○——○) 1 µg/ml;(△——△) lo µg/ml.The percentage of inhibition was calculated by comparing specific radioactivities of controls and analog treated samples.Specific radioactivities were expressed as cpm/mg of proteins because the amount of protein does not change until the feeding stage(ref.8).

C total protein synthesis.[——] control;[○○○○] o,1 µg/ml; [▤] 1 µg/ml;[■] lo µg/ml.The duplicates are separatly expressed at each point.

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pronounced,starting in the range of 65-75% reaching 90% already at the stage of 8-16 cells.

We are faced with a fact here not encountered in regular mitotic processes.5-azacytidine when present in concentrations which inhibit almost 90% of de novo synthesis of DNA,had no effect on cell division (the first three cleveages).This situation can be explained by assuming that cell divisions can take place without completion of synthesis of the genetic material;if this is so caryotype should be different i.e. deffective(under current investigation).

Protein synthesis of the control embryos does not change considerably between the very early postfertilization period up to the hatching-blastula stage(figure 1C).Similar observations were reported by Westin et al.(6) recently.Nearly the same patterns of protein synthesis were obtained with cultures treated by different concentrations of the analog. This then suggests that the rate of protein synthesis is independent of the rate of cell division.It seems that the time following fertilization is the governing factor.

To measure the rate of total protein synthesis has a limitation since minor changes in types and rates,though being important,can not be detected.However,such a parameter gives an indication of the overall metabolic activity of the majority of proteins.

Our data indicate that the majority of proteins synthesized during the early embryogenesis of sea urchins is not connected with the metabolic

activities of nucleic acids. Most of the proteins synthesized during this period are made on stable, maternal messenger RNA's(7). We have described a phenomenon where cell divisions can occur without de novo synthesis of deoxyribonucleic acid.

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