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# Temporally Different Poly(Adenosine Diphosphate-Ribosylation) Signals Are Required for DNA Replication and Cell Division in Early Embryos of Sea Urchins

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**Abstract** To analyze the temporal relationship of poly(adenosine diphosphate [ADP]-ribosylation) signal with DNA replication and cell divisions, the effect of 3 aminobenzamide (3ABA), an inhibitor of the poly(ADP-ribose)synthetase, was determined in vivo during the first cleavage division of sea urchins. The incorporation of $^3$H-thymidine into DNA was monitored and cleavage division was examined by light microscopy. The poly(ADP-ribose) neosynthesized on CS histone variants was measured by labeling with $^3$H-adenosine during the two initial embryonic cell cycles and the inhibitory effect of 3ABA on this poly(ADP-ribosylation) was determined. The results obtained indicate that the CS histone variants are poly(ADP-ribosylated) de novo during the initial cell cycles of embryonic development. The synthesis of poly(ADP-ribose) is decreased but not abolished by 20 mM of 3ABA. The incubation of zygotes in 3ABA at the entrance into S1 phase decreased $^3$H-thymidine incorporation into DNA in phase S2, while S1 was unaltered. Alternatively, when the same treatment was applied to zygotes at the exit of S1 phase, a block of the first cleavage division and a retardation of S2 phase were observed. The inhibitory effect of 3ABA on both DNA replication and cell division was totally reversible by a release of the zygotes from this inhibition. Taking together these observations it may be concluded that the poly(ADP-ribosylation) signals related to embryonic DNA replication are not contemporaneous with S phase progression but are a requirement before its initiation. These results also indicate that a poly(ADP-ribosylation) signal is required for cell division; such signal is temporally different from that related to S phase initiation and occurs at the exit of S phase. 

**Key words:** cell cycle, chromatin, zygotes, sea urchin development, 3ABA, poly(ADP-ribose)synthetase

Poly(adenosine diphosphate [ADP]-ribosylation) of nuclear proteins is catalyzed by the poly(ADP-ribose)synthetase, which transfers the ADP moiety of NAD to chromosomal proteins to form either oligo- or poly(ADP-ribosyl) linear or branched homopolymers [reviewed in Althaus and Richter, 1987; Shall, 1989]. This enzyme contains two zinc-binding domains, participates in the recognition of DNA strand breaks, and its activity is strictly dependent on single or double stranded interruptions in DNA [Menissier-de-Murcia et al., 1989]. The removal of these polymers or oligomers from acceptor proteins is catalyzed by two enzymes, the poly(ADP-ribose) glycohydrolase and the poly(ADP-ribose)protein-lyase, yielding ADP-ribose that is further hydrolyzed to AMP and ribose phosphate [Miwa et al., 1974; Okayama et al., 1978]. The joint and coordinated action of these enzymes induces a transient modification of the chromosomal proteins that are involved in the regulation of DNA recombination or DNA replication/DNA repair [Farzaneh et al., 1988; Boulikas, 1990; Cesarone et al., 1990; Cleaver and Morgan, 1991].

Acceptors for poly(ADP-ribosylation) in the nucleus are somatic and embryonic histone variants [Riquelme et al., 1979; Huletsky et al., 1985; Imschenetzky et al., 1991a], as well as a great number of non-histone chromosomal proteins such as the high mobility group (HMG) proteins [Tanuma et al., 1985] and several nuclear enzymes that decrease their activities when they are poly(ADP-ribosylated) [Kawaichi et al., 1981; Creissen and Shall, 1982; Fero and Olivera, 1984; Tanaka et al., 1984, 1986; Darby et

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al., 1985; Taniguchi et al., 1985; Yoshihara et al., 1985].

Despite the conflicting results related to the phases of the cell cycle in which the major poly(ADP-ribosylation) of nuclear proteins occurs, it seems clear that this post-translational modification is relevant for the cell cycle progression in the majority of the biological systems thus far investigated, although it is still unclear in which step this post-translational modification is involved [Kidwell and Mage, 1976; Tanuma and Kanai, 1982; Adolph, 1985; Golderer et al., 1988; Boulikas et al., 1990; Imschenetzky et al., 1991a].

Cleavage stages of sea urchin development offer a particularly attractive model to investigate the potential role of the poly(ADP-ribosylation) of nuclear proteins in DNA replication since they are characterized by very fast cell cycles composed by a predominant S phase, a practically inexistent G1, and very short G2/M intervals, except for the first embryonic cell cycle that exhibits a short period of pronuclear remodeling previous to the first DNA replication wave [Poccia, 1986]. Different from other eukaryotes the chromatin of sea urchin eggs and early cleavage embryos are organized in atypical nucleoparticles formed by CS histone variants that are bound to 126 base pairs of ADN [Imschenetzky et al., 1986, 1989]. The patterns of poly(ADP-ribosylation) of these atypical histone variants differ in S phase and at the G2/M transition and are a requirement for DNA replication and for the first cleavage division of early embryos [Imschenetzky et al., 1991a]. These finding were based on the immunobiochemical detection of polymers of ADP-ribose, but by such approach the short chains of ADP-ribose were undetectable [Kanai et al., 1978; Imschenetzky et al., 1991a].

In this report the zygotes were labeled with $^3$H-adenosine to determine the oligomers and polymers of ADP-ribose synthesized during the first cleavage cycle. We demonstrate herein that the poly(ADP-ribosylation) required for embryonic DNA replication does not coincide with S phase progression and is temporally different from that related to cell division. The results presented also indicate that both modifications—that linked to DNA replication and that linked to cell division—are partially and reversibly inhibited by 3 aminobenzamide (3ABA), an inhibitor of the poly(ADP-ribose)synthetase [Rankin et al., 1989].

## MATERIALS AND METHODS

### Gametes and Zygotes

Sea urchins *Tetrapygus niger* were collected from the bay of Concepción and maintained at room temperature in an aquarium containing natural sea water under constant aeration. Unfertilized eggs, sperm, and embryo cultures were obtained as described previously [Imschenetzky et al., 1986].

### Isolation of Chromosomal Proteins and Gel Electrophoresis

CS chromosomal proteins from zygotes harvested at different times after insemination were isolated as described previously [Imschenetzky et al., 1986, 1988]. Sample preparation and uni- and two-dimensional gel electrophoresis were performed essentially as described by Panyim and Chalkley [1969] for polyacrylamide gels containing urea and acetic acid and by Laemmli [1970] for polyacrylamide gels containing sodium dodecyl sulfate (SDS).

### DNA Synthesis

Zygotes were incubated continuously from 3 to 120 min after insemination in $^3$H-thymidine (NEN, Boston, MA; specific activity 108 Ci/mmol) at a concentration of 1 μCi/ml. At different times after insemination 1 ml samples of the zygote suspension were collected and processed as previously described [Imschenetzky et al., 1991a].

### De Novo Synthesis of Poly(ADP-Ribose) and Detection of Labeled ADP-Ribose Polymers

Zygotes were incubated continuously from 3 to 90 min after insemination in $^3$H-adenosine (NEN; specific activity 59 Ci/mmol) at a concentration of 350 μCi/ml. Two sets of experiments were performed in parallel, one in the presence and the other in the absence of 3ABA. At 90 min after insemination, 10 ml samples of the zygote suspension were collected, their chromatin was isolated, the CS histone variants were purified with 0.25 N HCl, and the radioactivity incorporated into these proteins was measured. Then the isolated CS histone variants were analyzed by electrophoresis in polyacrylamide gel (PAG)/SDS and PAG/urea/acetic acid and the radioactivity associated to each electrophoretic band was detected by the fluorography of these gels [Chamberlain, 1979].

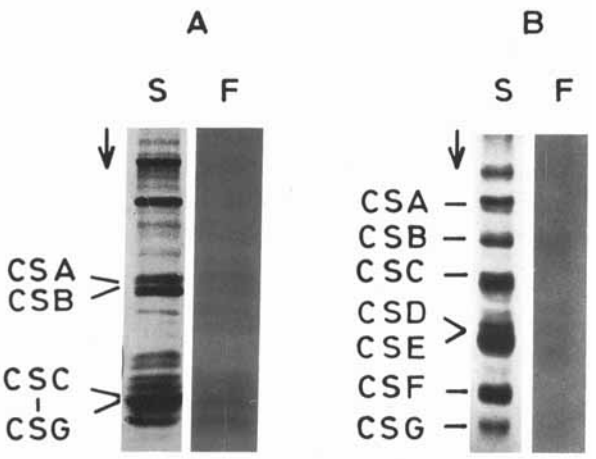


**Fig. 1.** Incorporation of $^{3}$H-adenosine into CS histone variants during the first cleavage cycle of sea urchin development. Fluorograph of whole CS histone variants isolated from sea urchin zygotes incubated continuously in $^{3}$H-adenosine from 3 min p.i. to 120 min p.i. and electrophoresed in uni-dimensional polyacrylamide gels. **(A)** 18% PAG/SDS gels; **(B)** 15% PAG/urea/acetic acid gels. **(Lane S)** Coomassie blue-stained gel slots containing the CS variants from zygotes; **(lane F)** fluorography of these gel slots. The direction of migration is indicated by arrows.

## RESULTS

### De Novo Poly(ADP-Ribosylation) of CS Histone Variants During the First Cleavage Division of Sea Urchin Development

In *T. niger,* as well as in other species of sea urchins, the first cleavage division is observed 90 min p.i. and the second 120 min p.i. As it has been reported previously, the first S phase proceeded from 20 min p.i. to 40 min p.i. and the second from 80 min p.i. to 120 min p.i. [Poccia, 1986; Imschenetzky et al., 1991a,b].

To determine the polymers or oligomers of ADP-ribose bound to the CS histone that are newly synthesized in vivo, the incorporation of $^{3}$H-adenosine into CS histone variants was determined from fertilization until the first cleavage division (90 min p.i.). The results shown in Figure 1A indicate that each of the CS variants resolved by uni-dimensional SDS gel electrophoresis were labeled with $^{3}$H-adenosine. An unequivocal identification of individual CS variants in one-dimensional SDS gels is difficult due to the poor resolution of CS C–E variants. To determine more accurately the $^{3}$H-adenosine-labeled CS variants, these proteins were analyzed in urea/acetic acid gels, which allows a better resolution of CS C–E variants (Fig. 1B). As shown in Figure 1B, CS B and D–F variants were preferentially labeled with $^{3}$H-adenosine while the label on CS A, C, and G variants was barely detectable. These results confirm previous observations indicating that all the CS histone variants (A–G) are ADP-ribosylated during the first cleavage division, although the intensity of $^{3}$H-labeled ADP-ribose polymers or oligomers bound to each CS variant is different.

### Temporal Relationship Between Poly(ADP-Ribosylation), DNA Replication, and Cell Division of Sea Urchin Zygotes

To define more precisely the temporal relationship between poly(ADP-ribosylation), the replication of the embryonic genome, and the first cell division, the zygotes were incubated continuously from the initiation of first S phase (30 min p.i.) until the second cleavage division (120 min p.i.) in 20 mM of 3ABA. The cleavage divisions were followed by light microscopy and the DNA replication was monitored by $^{3}$H-thymidine incorporation (Fig. 2). As shown in Figure 2, if the inhibition of poly(ADP-ribose)synthetase activity by 3ABA occurs at the time of the initiation of the first S phase (30 min p.i.), the first replication wave is unaffected, the second S phase is slightly decreased, but the first and the second cleavage divisions are inhibited.

To assess more clearly the timing of the requirement of poly(ADP-ribosylation) for the first cleavage division, the zygotes were incubated in 20 mM of 3ABA from the first G2/M interval (60 min p.i.) until completion of the second S phase (120 min p.i.). The results shown in Figure 3 indicate that the first and the second cell divisions were blocked while only a moderate inhibition of the second S phase was observed.

To determine the degree of inhibition by 20 mM of 3ABA on the poly(ADP-ribosylation) that occurs in vivo, the incorporation of $^{3}$H-adenosine into CS histone variants was compared for CS variants that were isolated from zygotes harvested 120 min p.i., cultured in normal sea water or in the presence of 20 mM of 3ABA from 3 min p.i. till 120 min p.i. (Table I), respectively. As shown, the poly(ADP-ribosylation) of CS variants was partially inhibited by 20 mM of 3ABA (47%), while embryonic DNA replication was almost abolished (more than 90% of inhibition) and the first cell divisions was blocked (Fig. 4).

To investigate the recovery of sea urchin zygotes from the inhibition by 3ABA, the zygotes were incubated continuously in 20 mM of 3ABA from 3 min p.i. until the first G2/M interval (60 min p.i.). The embryonic cells were subse-

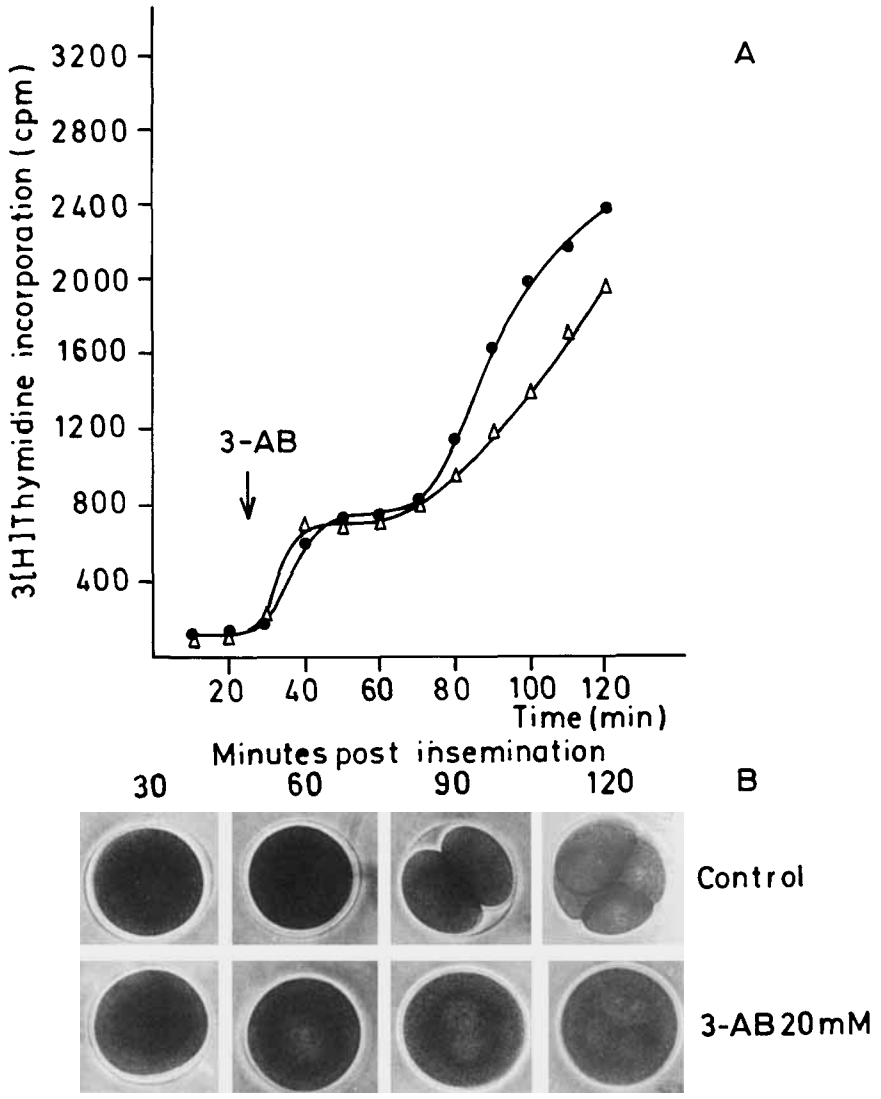


**Fig. 2.** Effect of the inhibition of poly(ADP-ribose)synthetase with 3ABA during S phase of the first cell cycle of sea urchin development. Zygotes were incubated in sea water containing 20 mM of 3ABA from 30 min p.i. until 120 min p.i. **(A)** The incorporation of $^{3}$H-thymidine into DNA was measured compared with sea urchin cultures performed in normal sea water (△) 3-ABA; (●) control. **(B)** The cell division of these cultures was observed by light microscopy.

quently washed in sea water to remove the 3ABA from the cultures and $^{3}$H-thymidine was added to measure the incorporation of this precursor into DNA. The cell divisions of these zygotes were followed by light microscopy. The results are shown in Figure 4 compared with zygotes that were not treated with the inhibitor or those that were inhibited during the time course of this experiment. As shown, the zygotes treated with 3ABA completely recovered from the inhibitory effect. The recovery of DNA replication was immediate and cell divisions were slightly deleted, but such delay did not induce any abnormal effect on further early embryonic development, which was followed until larval stages of development (Fig. 4).

## DISCUSSION

The data presented in this report demonstrate that in sea urchin zygotes each one of the CS histone variants is a target for poly(ADP-ribosylation) during the first embryonic cleavage cycle. The results reported also demonstrate that the inhibition of the poly(ADP-ribose)synthetase by 3ABA can selectively and temporally impair the first round of replication and the first cleavage division. These results strongly suggest that an ADP-ribosylation signal determines com-

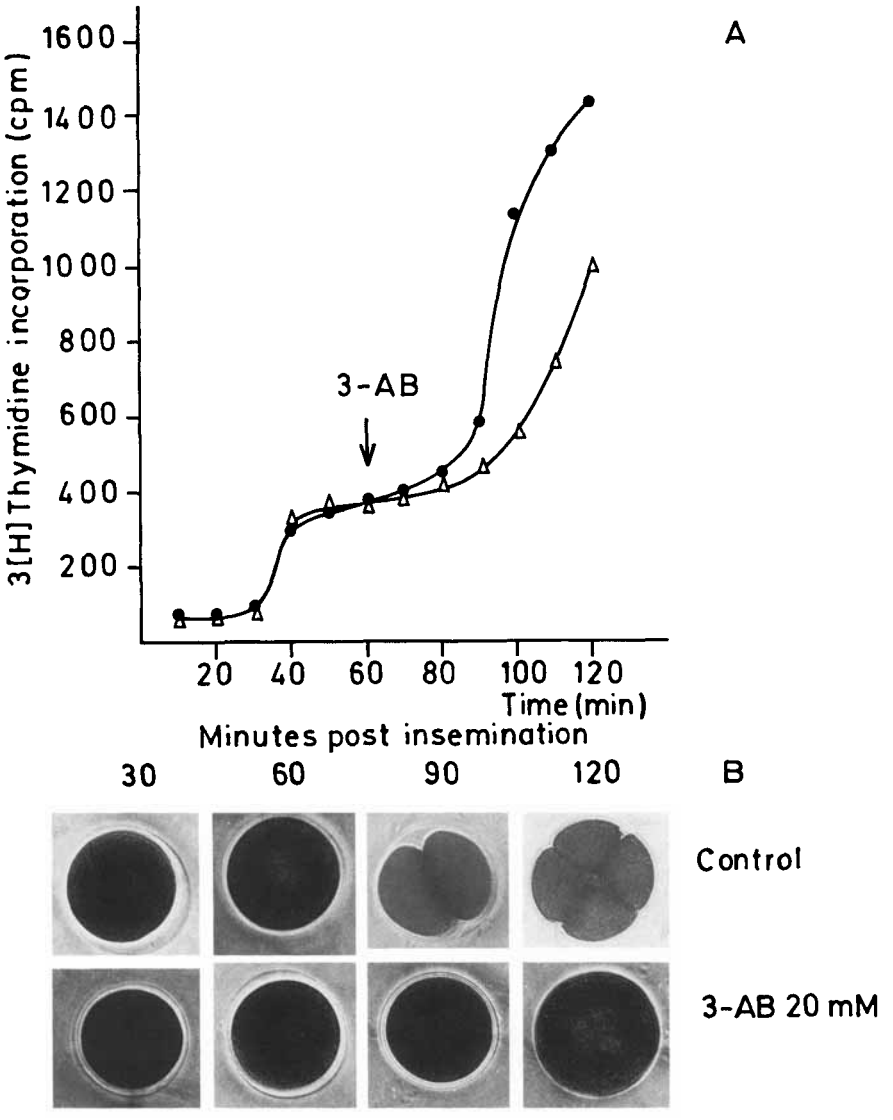


Fig. 3. Inhibitory effect of 3ABA added to the cultures of sea urchin embryos during the G2/M interval of the first cell cycle of embryonic development Zygotes were incubated in sea water containing 20 mM of 3ABA from 60 min p i until 120 min p i (A) The incorporation of $^3$H-thymidine into DNA was determined compared with cultures of sea urchin zygotes performed in normal sea water (△) 3-ABA, (●) control (B) The cell division of these cultures was observed by light microscopy

mitment of the cell to enter into the initial embryonic replication rounds. Such a signal occurs before the beginning of the first S phase. The results reported also demonstrate that an ADP-ribosylation signal that occurs between the G2/M transition and the first cleavage division (60–90 min p.i.) is related to the initial cleavage divisions. These observations are further supported by the high specificity of the inhibitory effect of 3ABA, both in vivo and in vitro [Rankin et al., 1989], and the low toxicity demonstrated for this compound by complete recovery of the inhibitory effect on sea urchin embryos (Fig. 4). A poly(ADP-ribosylation) signal that precedes S phase described herein is consistent with previous findings reported by Cesarone et al. [1990] indicating that a marked increase of endogenous poly(ADP-ribose)synthetase activity is found pre-

**TABLE I. Effect of 3ABA on the Poly(ADP-Ribosylation) of CS Variants***

| | cpm/100 μg protein |
|---|---|
| Control | 15,648 ± 320 |
| + 3ABA | 8,316 ± 290 |

*Average data of four experiments

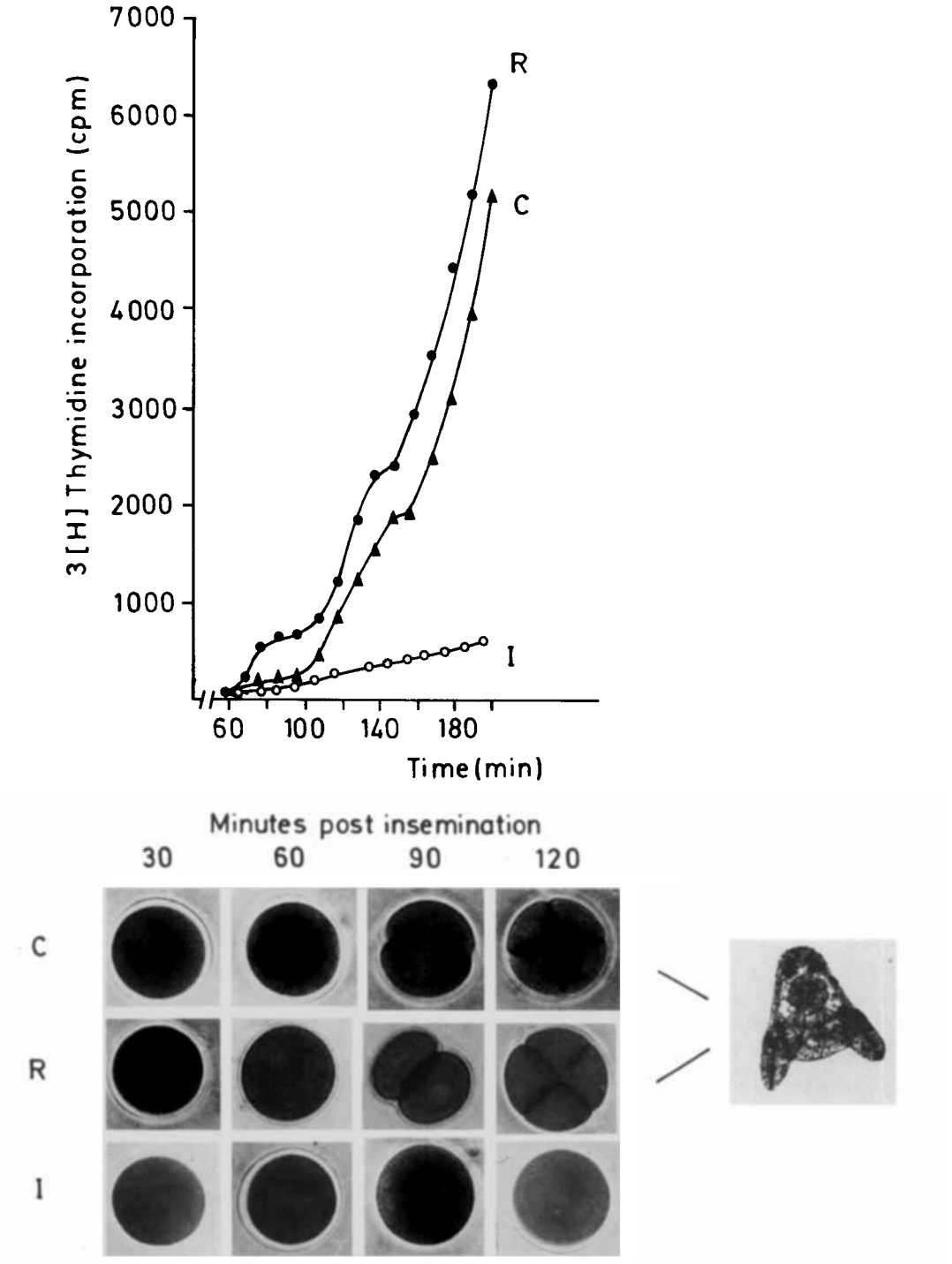


**Fig. 4.** Recovery of sea urchin zygotes from the inhibitory effect of 3ABA Zygotes were incubated continuously in 20 mM of 3ABA from 3 min p f until 60 min p f, then the cells were gently washed with sea water and $^{3}$H-thymidine was added to measure the incorporation of this precursor into DNA The $^{3}$H-thymidine incorporation into DNA was determined and the cleavage divisions and early developmental stages were observed by light microscopy (R) Cultures that were released from the inhibitor 60 min p f, (C) zygotes that were incubated in normal sea water, (I) zygotes that were incubated continuously in 3ABA during the two initial cell cycles

ceding DNA synthesis in rat hepatocytes stimulated by epidermal growth factor. Such results also agree with the increased levels of the mRNA encoding poly(ADP-ribose)synthetase found in mitogen-stimulated rat liver before a detectable DNA synthesis could be observed [Menegazzi et al., 1990]. According to such increased poly(ADP-ribosylation) preceding S phase it has been reported recently that the activity of the nuclear (ADP-ribose)glycohydrolase that degrades these polymers is twofold lower in early G1 than in late G1 [Tanuma and Fuminori, 1991].

The involvement of an ADP-ribosylation signal in chromatin replication/repair is supported by several lines of evidence that include the correlation of the poly(ADP-ribosylation) of histones or other chromosomal proteins with DNA replication/repair mechanisms [Farzaneh et al., 1988; Boulikas, 1990; Cesarone et al., 1990; Cleaver and Morgan, 1991; Imschenetzky et al., 1991a,b]; the reports indicating a prolonged doubling time of mutant cells that are clearly deficient in poly(ADP-ribose) synthesis [Chatterjee et al., 1989]; the finding that expression of the poly(ADP-ribose)synthetase is subjected to several post-transcriptional control mechanisms that are cell cycle dependent [Bhatia et al., 1990]; the studies with inhibitors of the poly(ADP-ribose)synthetase, such as 3ABA, indicating that DNA replication can be altered in a dose-dependent manner by this compound in embryonic cells in vivo [Imschenetzky et al., 1991a]; and the accumulation of 10 kb DNA, putative replication intermediates, in cells treated with this inhibitor [Lönn and Lonn, 1985].

The molecular mechanism by which poly(ADP-ribosylation) signals are related to DNA replication is not completely understood, but the requirement of poly(ADP-ribosylated)histones in the DNA-histone replication intermediates has been suggested previously by Boulikas [1990]. Alternatively, poly(ADP-ribosylated) proteins might have a role in the molecular arrangement of the nuclear matrix that may be required for DNA replication [Getzenberg et al., 1991]. The association of the poly(ADP-ribose)synthetase with the nuclear matrix was recently reported; the functional meaning of such an association remains to be determined in the future [Kaufman et al., 1991].

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