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SHORT COMMUNICATION

EFFECT OF 4-NITROQUINOLINE-1-OXIDE ON THE EARLY STAGE OF THE DEVELOPMENT OF SEA URCHIN EGGS

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In the previous papers we reported on carcinogenesis induced in vitro in cultured hamster cells by 4-nitroquinoline-1-oxide (4NQO) (KAMAHORA and KAKUNAGA, 1966a, 1966b, 1967). While investigating the mechanisms of carcinogenesis by 4NQO attempts were made to find particular abnormalities of chromosomes in the transformed cells. We observed, however, that the chromosomes remained more or less intact in form and size, except for a certain increase in aneuploidy with frequent occurrence of trisomy and monosomy, suggesting that 4NQO might affect the process of cell mitosis (KAMAHORA and KAKUNAGA, 1968).

Evidence has been obtained that the biological action of 4NQO is closely associated with its chemical affinity for sulfhydryl (SH-) groups (OKABAYASHI, 1953; ENDO, 1958; HAYASHI, 1959; SEARLE and WOODHOUSE, 1963; NAKAHARA and FUKUOKA 1964; HOZUMI *et al.*, 1967). The mitotic apparatus of fertilized sea urchin eggs apparently contains SH-groups in metaphase (BRACHET, 1940; MAZIA and DAN, 1952; KAWAMURA and DAN, 1958). It seems most probable, therefore, that 4NQO interacts with cellular SH-groups at metaphase causing abnormal mitosis.

Sea urchin eggs are valuable in these kinds of studies because their cell division is synchronized and accordingly we studied the effect of 4NQO on their mitosis.

The sea urchins, *Psudocentrotus depressus* and *Hemicentrotus pulcherrimus* were used in these experiments. Spawing into sea water was induced by 0.05 M KCl, and eggs were inseminated with suspensions of sperm, placed in watch glasses, and kept at about 20°C throughout the experiments. Since the experiments were to test the effect of 4NQO on the eggs and mainly on the process of cell division, experimental observations were limited to the early stage of development, i.e, elevation of the feritilization membrane, hyaline layer formation, appearance of the streaks, and the pattern and rate of cleavage from the first to the third cleavage. At short intervals fertilized eggs were examined under the microscope at 100-fold magnification. A stock solution of 4NQO was prepared in sea water, and used in the experiments at various stages of development. In each set of experiments eggs and sperms from identical parents were used, and observations were repeated several times to insure valid comparisons.

Fertilized eggs were transferred to sea water containing various concentrations of 4NQO or 4-nitroquinoline (4NQ), ranging from 10^{-7} to 10^{-4} M at intervals of 10 to 50 min. To measure the rate of development, the number of eggs with streaks or those undergoing cleav-

age were counted under the microscope at short intervals, and the fifty percent streak and cleavage times were calculated. Table 1 shows results of one experiment. A concentration of 4NQO of as low as 10^{-6} M caused noticeable retardation of the early stages of development, whereas the same concentration of 4NQ was ineffective. The inhibitory action of the non-carcinogenic 4NQ was about one tenth of that of 4NQO. Thus the minimum concentrations causing complete inhibition when given 10 min after fertilization were 3×10^{-6} M and 10^{-4} M, respectively, for 4NQO and 4NQ. On addition of these agents at the stage when the streak appeared, however, their effects in retarding the time of the first cleavage were less. Whether the agent was added just before or just after fertilization, the results were the same.

It was of particular interest that sperms which had been treated with 4NQO at concentrations of as high as 10^{-3} M for a full three hours inseminated without any noticeable changes in the process of fertilization, e.g. elevation of the fertilization membrane, and the series of phenomena thereafter.

However, after the same treatment unfertilized eggs showed retardation or inhibition of development depending on the concentration of 4NQO employed, although the fertilization membrane was elevated normally. It should be noted that concentrations of 1% or less of acetone, which is a solvent of 4NQ, appeared to have no visible effect on the development of eggs or the retarding effect of 4NQO.

For staining of SH groups the following procedure was employed: eggs in various stages were fixed in 5% trichloroacetic acid for 12 hr.

TABLE 1 *Effects of 4NQO[a] and 4NQ on the early development of fertilized sea urchin eggs*

Agent	Final conc. of agent (M)	Time of addition of agent (min after fertilization)	Time of various stages after fertilization[b] (min)			
			Streak stage[c]	First cleavage stage	Second cleavage stage	Third cleavage stage
Control			51	68	115	155
4NQO	3×10^{-6}	10	(—)[d]	(—)	(—)	(—)
		55	51	73	150	(—)
	1×10^{-5}	10	(—)	(—)	(—)	(—)
		55	51	76	155	(—)
	3×10^{-5}	10	(—)	(—)	(—)	(—)
		55	51	85	(—)	(—)
	1×10^{-4}	10	(—)	(—)	(—)	(—)
		55	55	87	(—)	(—)
4NQ	3×10^{-6}	10	54	71	120	165
		55	51	68	115	155
	1×10^{-5}	10	78	95	150	(—)
		55	51	68	120	175
	3×10^{-5}	10	93	(—)	(—)	(—)
		55	51	70	150	(—)
	1×10^{-4}	10	(—)	(—)	(—)	(—)
		55	55	70	(—)	(—)

a Abbreviations: 4NQO, 4-nitroquinoline-1-oxide; 4NQ, 4-nitroquinoline.
b Time at which 50% of eggs entered the stage.
c The streak stage means that which appears prior to first cleavage.
d Not observed within 210 min after fertilization.

Then they were washed with several changes of distilled water, dehydrated by serial transfer through graded concentrations of ethanol, embedded in paraffin wax, and sectioned at 12–15 μ. Sections were stained by a specific SH reagent, Bennett's Reagent, according to the procedure of KAWAMURA and DAN (1958). Observations on normal eggs stained with Bennett's Reagent were as follows: Unfertilized eggs did not stain (Fig. 1). In the streak stage, part of the streak stained with Bennett's Reagent (Fig. 2), and in metaphase the color was strongest at the astral centers, spindle and chromosomes (Fig. 3). As the chromosomes separated and spindle fibres disappeared, the stainability of eggs decreased. Pretreatment of sections with p-chloromercuribenzoic acid caused complete loss of stain uptake by mitotic eggs (Fig. 4). These observations were consistent with those described by KAWAMURA and DAN (1958).

In eggs exposed to 10^{-4}–10^{-5} M 4NQO 10–40 min after fertilization, neither streaks nor spindle fibres were formed, and there was no uptake of stain (Fig. 5). On the other hand, eggs treated with 4NQO at 55 min after fertilization, when they were in the streak stage, showed almost the same stainability as normal fertilized eggs, though the mitotic stage was somewhat delayed (Fig. 6).

Many substances have been reported to inhibit or retard the cleavage of fertilized sea urchin eggs, such as anaesthetics (LILLIE, 1914; BLUMENTHAL, 1928; WATERMAN, 1936), detergents (GUSTAFSON and SÄVHAGEN, 1949), pyrimidine derivatives (STEARNS *et al.*, 1962), mitomycin C (MARKMAN and RUNNSTRÖM, 1963), actinomycin D (MIURA, 1953; WOLSKY and WOLSKY, 1961; BRACHET *et al.*, 1963; LALLIER, 1963; MARKMAN and RUNNSTRÖM, 1963; GROSS and COUSINEAU, 1963, 1964; MARKMAN, 1964; GUIDICE and HÖRSTADIUS, 1965; MATEYKO, 1965) and lipoic acid (WOLFSON and FRY, 1965). However, 4NQO seems to be a more powerful inhibitor than any of these and to affect the development of eggs through some particular process. It seems that 4NQO inhibits the early, but not the later stages of formation of spindle fibres, and does not affect the function of spindle fibres which have already been formed, although since the agent may take some time to reach the site of its action it is still uncertain exactly when it acts.

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FIGURE 1 to 6. Eggs of *Hemicentrotus pulcherrimus*, fixed in 5% trichloroacetic acid and stained with Bennett's Reagent.

FIGURE 1 Unfertilized egg.

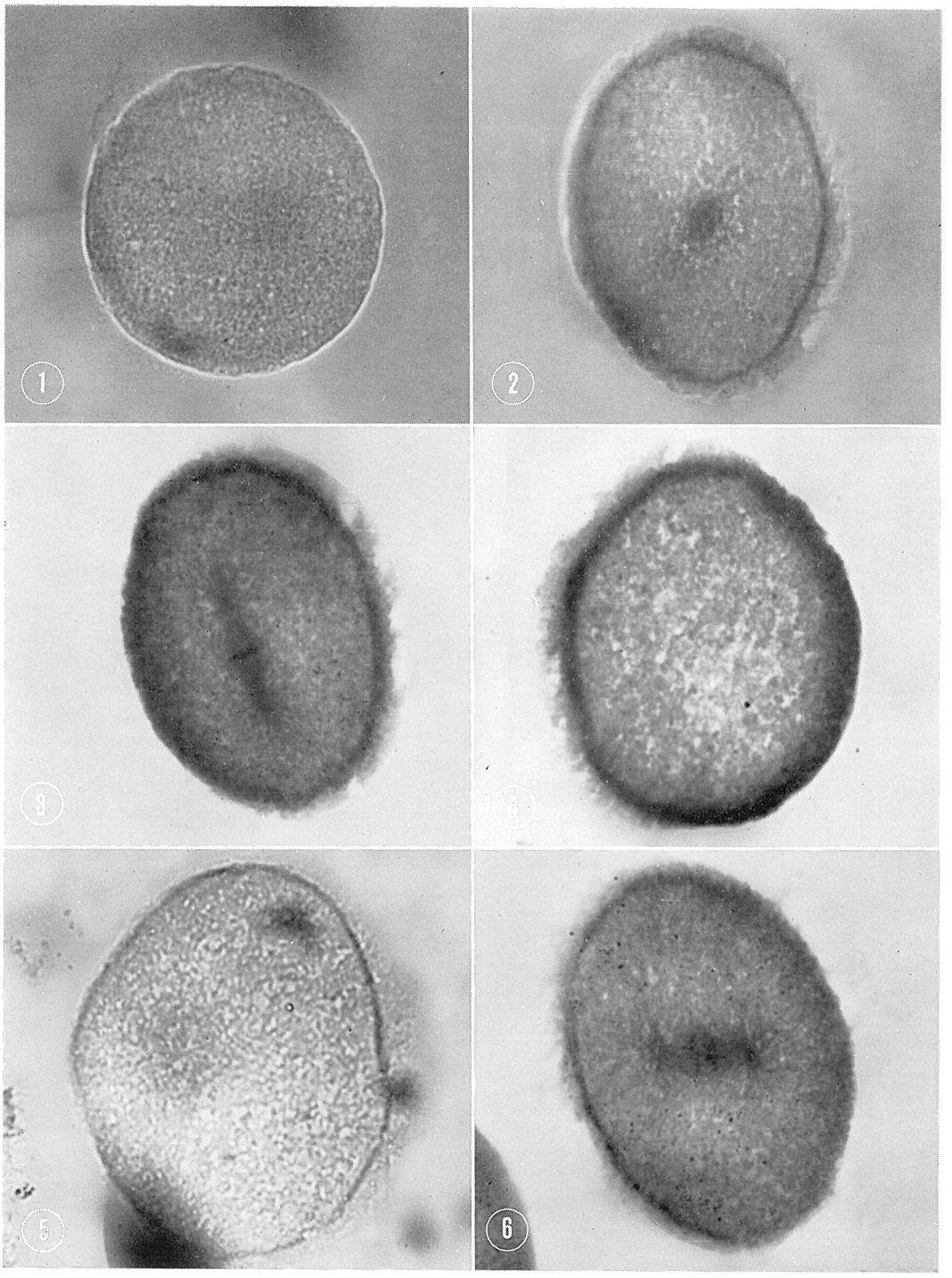
FIGURE 2 Fifty min after fertilization, streak stage.

FIGURE 3 Sixty-four min after fertilization, metaphase.

FIGURE 4 The same as Fig. 3, but pretreated with p-chloromercuribenzoic acid before staining.

FIGURE 5 Sixty-four min after fertilization, treated with 4NQO 10^{-5} M at 10 min after fertilization.

FIGURE 6 Seventy-four min after fertilization, treated with 4NQO 10^{-5} M 55 min after fertilization.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