Use of the Sea Urchin Egg for Quick Screen Testing of the Biological Activities of Substances
I. Influence of Fractions of a Tobacco Smoke Condensate on Early Development (36864)

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(Introduced by P. Shubik)

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Mammals are usually used in toxicity studies because their metabolism closely resembles the metabolism of man. Toxicity tests using mammals, however, are often very laborious. For example, animals must be genetically similar, of the same age, and should have been bred under the same circumstances. To obtain reliable data, large numbers of animals have to be used. A test is likely to last several days and may be costly. For these reasons, if, biologically, a substance has an equivalent effect on invertebrates, it is advantageous to use the invertebrate system which can be tested more simply than the mammalian system. Large numbers of genetically uniform invertebrates can be kept under constant conditions in a small space and their development proceeds quickly, thus lowering the cost of tests. Bacteria and some invertebrates have been used for testing; *e.g.*, the fruit fly *Drosophila* has been used to test mutagenic activity.

We will describe the use of the fertilized sea urchin egg as a new test system for routine analysis. Effects of different substances such as metabolic inhibitors on the sea urchin egg have occasionally been described. Most of these results have been reported by Harvey (1) and Mateyko (2). We have used different fractions of a condensate of tobacco smoke to demonstrate the significance of this system.

Materials and Methods. Sea urchins (*Psammechinus miliaris*) received from the Biologische Bundesanstalt Helgoland were used. Eggs and sperm were obtained after injecting 0.5 ml of a 0.5 *M* KCl solution. Sperm was kept dry and was diluted immediately before fertilization. Eggs were washed several times in sea water and filtered through 0.45 μ Schleicher & Schüll BA 85 Selectron filters. For fertilization, the cells were suspended in such a way that 1 ml contained 10^3 cells. After repeated washings, 10 ml of a suspension containing 10^4 cells/ml were added to a 25 ml Erlenmeyer beaker, incubated at 18° and shaken slightly. After certain intervals (see figures), samples were taken and observed under the microscope (Leitz Ortholux) at 100-fold magnification. Pictures were taken with the Orthomat camera (Leitz, Wetzlar) on Ilford FP 4 film 22 Din. One field contained an average of 50–60 embryos. At least three photographs were taken of each sample. The negatives were projected on normal Din A4 writing paper and the different stages of development were counted. One-, two-, four-, and eight-cell stages were differentiated.

Fractionation of cigarette-smoke condensate. The smoke condensate was prepared by smoking cigarettes of a tobacco mixture corresponding to mixtures of common cigarettes (3).

a) Nitromethane fraction (4). Five g smoke condensate from 130–190 g tobacco were dissolved in 80 ml methanol (90% v/v) and extracted twice with 80 ml of cyclohexane. The combined cyclohexane extracts were extracted twice with 160 ml of nitromethane (NM). The NM-phases were combined and NM was removed at 14 mm and 35°. The residue was then weighed. A yield of 250 mg was obtained, corresponding to about 0.2% which is referred to as the amount of burnt tobacco.

b) *Nitromethane fraction ovalbumin adduct* (*N*). Six hundred mg ovalbumin (Serva, Heidelberg) were dissolved in 10 ml of 0.06 *M* phosphate buffer pH 7.0 and heated at 37° for 30 min. Then a solution of 200 mg of the residue, obtained as described in (a) and dissolved in 2.0 ml of dimethylsulfoxide, was added slowly. The mixture was then heated to 37° for 2.5 hr. Finally, 40 ml phosphate buffer were added.

c) *Extraction of the ovalbumin adduct with benzene* (*NB*). Thirty-five ml of a solution of the adduct (containing 135 mg N) was shaken with 50 ml benzene. To remove residual benzene, the buffer phase was heated for 4 hr at 37° in an open beaker.

d) *Chromatography on sephadex G 150* (*S*). Two ml of the NB extract were chromatographed in phosphate buffer on 8 g sephadex G 150 (Pharmacia, Sweden). Fractions containing 100 drops each were collected. Fractions 8–10 were concentrated in collodium bags versus H_2O at 500 torr. To obtain enough material, chromatography was repeated several times. The solutions used for the tests were twice as concentrated as the initial solutions within fractions 8–10.

e) *Benzo(a)pyrene ovalbumin adduct.* Three hundred and sixty mg benzo(a)pyrene, dissolved in 0.2 ml dimethylsulfoxide, were added to 1 ml of a 6% ovalbumin solution and heated to 37° for 2.5 hours. Finally, 4.2 ml phosphate buffer were added.

f) *Nicotine solution.* One hundred thirty-four mg nicotine (highest purity, distilled under N_2) were diluted with 100 ml phosphate buffer.

g) *Test of fractions.* For each test 10 ml of an egg suspension containing 10^4 cells/ml in artificial sea water were incubated in a 25 ml Erlenmeyer beaker as controls. The substances were added 30 minutes after fertilization.

N: 0.2, 0.1, 0.05 ml were added corresponding to 0.8, 0.4 and 0.2 mg residue bound to ovalbumin.

NB: 0.4 and 0.2 ml were added. The beakers contained the benzene extracted residue of 1.5 and 0.8 mg N.

S: 0.4 ml were added containing about 0.06 μg benzo(a)pyrene which was four times the amount of this polycyclic hydrocarbon present in 0.2 ml N.

The benzo(a)pyrene ovalbumin adduct and nicotine were also added 30 minutes after fertilization. (For concentrations, see "Results").

Results. Tobacco smoke is an unstable aerosol containing at least 10,000 different substances. For our test we distinguished between the "gas–vapor phase" and the "particle phase." The fractions mentioned above were obtained from the particle phase.

The aim of the fractionation procedure was to separate polycylic aromatic hydrocarbons from other substances and to enrich these hydrocarbons to a high degree in one fraction. For this purpose, polar substances were removed in the first step with methanol. The separation of aliphatic compounds from unsaturated compounds, heterocyclic components and aromatic substances was performed by extraction of the cyclohexane phase with nitromethane. The nitromethane phase contained almost all the benzo(a)pyrene. Because of certain effects, the nitromethane fraction still contained, among other substances, traces of different phenols and nicotine with most of the nicotine left in the methanol fraction.

The majority of the components enriched in the nitromethane fraction were only slightly soluble in water and therefore could not be added as such to a suspension of sea urchin eggs in sea water. To render them soluble in water, they were bound to ovalbumin. (The details of this procedure are described later). This fraction was marked as N and contained nearly all components of the nitromethane fraction.

Substances which were only weakly bound to ovalbumin were removed by extraction with benzene. More than 90% of the residue bound to ovalbumin was removed with benzene but benzo(a)pyrene and other aromatic hydrocarbons were still bound to ovalbumin. The residual buffer phase was marked NB. Out of this fraction the ovalbumin adducts of polycyclic aromatic hydrocarbons obtained after chomatography on Sephadex were designated S. For comparison, a pure benzo(a)pyrene ovalbumin adduct was prepared.

The influence of nicotine on development was observed after adding a 0.13% solution of

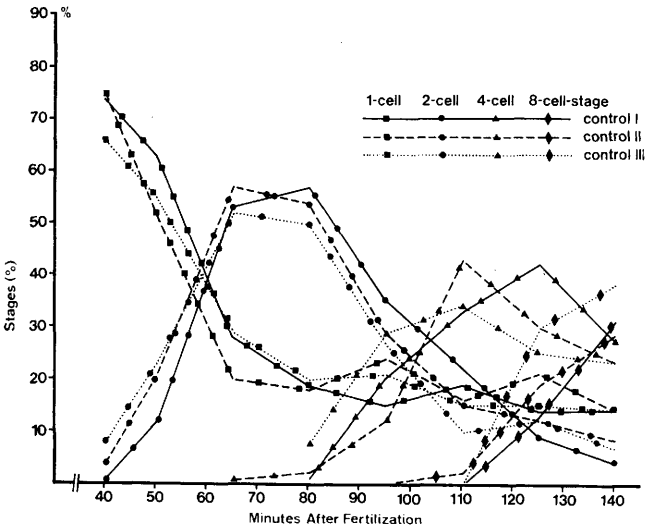

FIG. 1. Untreated fertilized sea urchin eggs (*Psammechinus miliaris*) shown from the one-cell stage to the eight-cell stage of development. All eggs described in the figures were of the same origin and were incubated at 18°. The first photograph was taken 40 min after fertilization; the following ones were made at 15 min intervals.

pure substances dissolved in phosphate buffer.

The sea urchin eggs proved to be very suitable for the test. They have a diameter of about 100 μ and are only weakly pigmented, which facilitates evaluation of different stages on the negatives. After fertilization they develop almost synchronously, if ripe eggs of only one animal are collected in the spawning season. Figure 1 shows the development of three control groups observed every 15 min. The eggs were collected when the spawning season was almost completed. The synchronization was not as complete as could be expected if eggs from the spawning season had been used. Seventy percent of the eggs had been fertilized and had started to develop. Most of the eggs showed a cleavage 80 min after fertilization. The four-cell stage was reached 20 min later. The maxima in the four-cell stage were lower than those of the two-cell stage because abnormal development had not been considered. Because exact classification and differentiation of normal and abnormal embryos after the eight-cell stage was difficult, samples were systematically registered until the eight-cell stage.

Test fractions were added 30 min after fertilization. Concentrations of the fraction N were chosen so that the highest concentration completely inhibited development and the lowest did not disturb development. Control groups and test groups contained eggs of the same origin and control eggs and test eggs were incubated in the same incubator and shaken at the same frequency.

Figure 2a shows the influence of 0.2 ml N-fraction on the development of the fertilized egg which contained 0.8 mg of the N-fraction. The development of the fertilized egg was inhibited almost completely. No four-cell stage embryos could be detected. Also, when eggs were incubated for 24 hr after adding the fraction, development did not proceed. The eggs did not appear to be destroyed by the fraction after a few hours, but after one day they began to lyse. If the concentration was lowered by one half, the inhibition of development was decreased significantly, as shown in Fig. 2b. Compared with the controls, the development of the two-cell stage was inhibited by about 70% in the test group; nevertheless, some embryos developed at least up to the four-cell stage. The first two-cell stage embryos appeared at the same time in the control and test groups, while the development of the four-cell stage was obviously delayed in the test group.

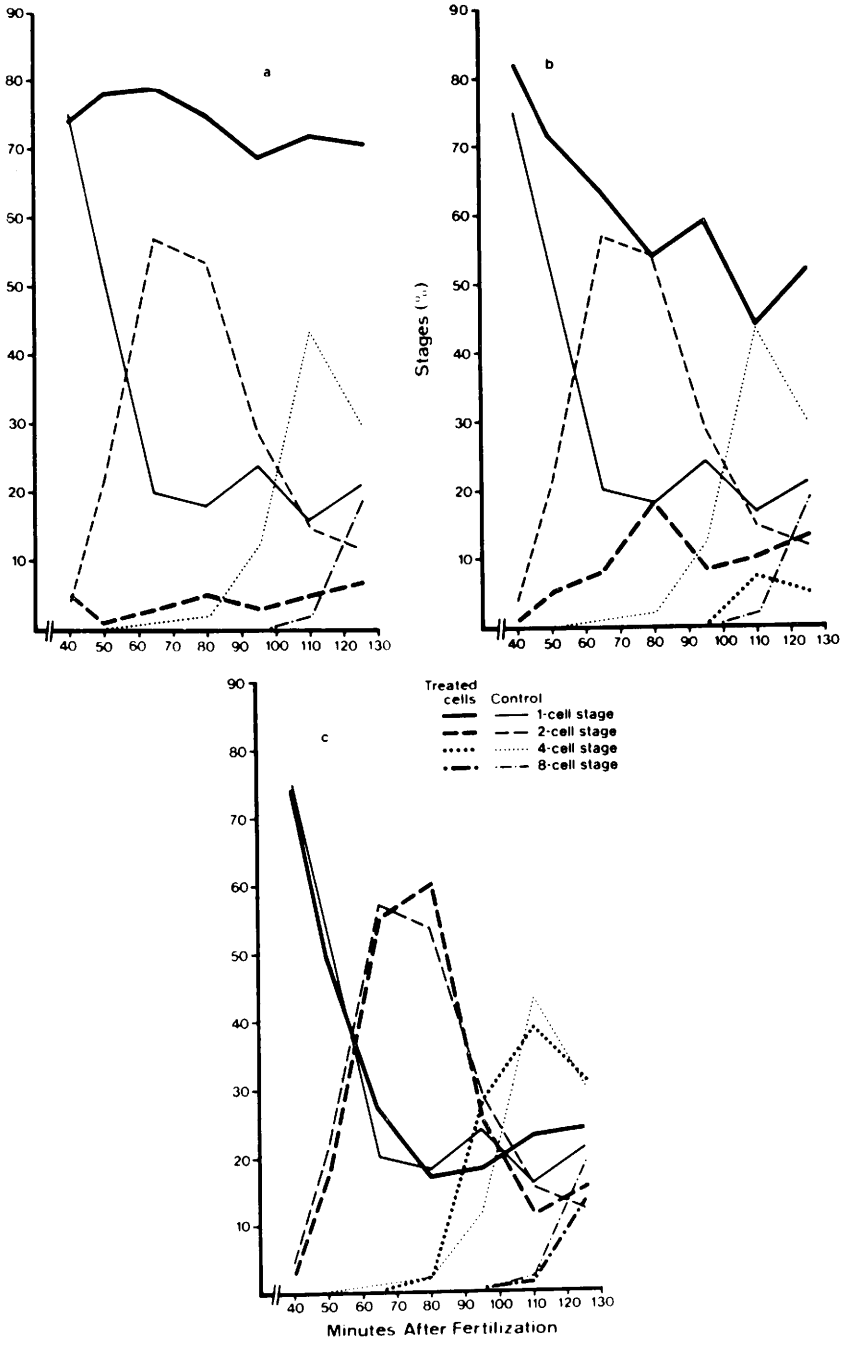

FIG. 2a. Fertilized eggs treated with 0.2 ml of fraction N. Fraction N was added to a 10 ml egg suspension 30 min after fertilization. The first registration was made 10 min after adding N.

FIG. 2b. Fertilized eggs treated with 0.1 ml of fraction N.

FIG. 2c. Fertilized eggs treated with 0.05 ml of fraction N.

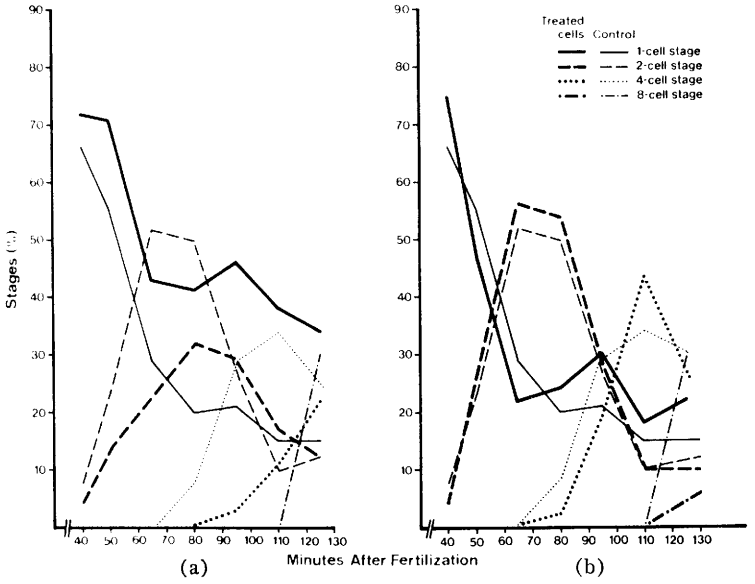

FIG. 3a. Fertilized eggs treated with 0.4 ml of fraction NB.
FIG. 3b. Fertilized eggs treated with 0.2 ml of fraction NB.

Eight-cell stages were not observed. If the concentration was lowered again by one half, no more inhibition was observed as is shown in Fig. 2c.

The applied concentration of fraction NB was twice as high as fraction N. The eggs treated with fraction NB were inhibited in their development but to a significantly lower extent than in fraction N (Fig. 3a). A part of the toxic activity was therefore removed with benzene. By reducing the concentration by one half, only the formation of the eight-cell stage was slightly inhibited (Fig. 3b).

When fraction S, which contained the polycyclic aromatic hydrocarbons adducted to ovalbumin, was tested at a concentration having four times the amount of benzo(a)pyrene as fraction N tested at the highest concentration, no inhibition was observed (Fig. 4). The polycyclic aromatic hydrocarbons did not seem to influence development significantly, at least when adducted to ovalbumin. In a previous test we observed that a pure benzo(a)pyrene adduct in concentrations of 14 and 28 μg/10 ml egg suspension (50 eggs/ml) did not affect development up to the pluteus stage. These concentrations were several hundred-fold higher than the tested concentration in fraction S.

The effect of nicotine on sea urchin egg development has been studied by the Hert-

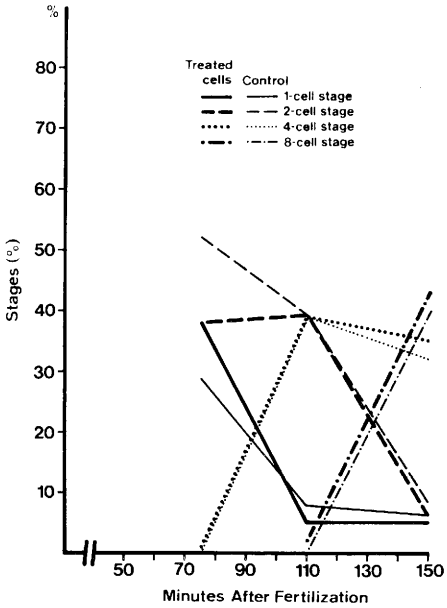

FIG. 4. Fertilized eggs treated wtih 0.4 ml of fraction S. (This is four times the amount of hydrocarbon of fraction N [Fig. 2a]). Only three registrations were made.

wigs. In contrast to the effect on unfertilized eggs, they observed that nicotine had no effect when added after fertilization in a concentration of 1:100 (5). The addition of 500 μg nicotine to 10 ml egg suspension, which corresponded to 100 times the amount in our tested concentration of fraction N, also did not affect development up to the pluteus stage.

Discussion and Summary. We have tested the influence of cigarette-smoke condensates on the fertilized sea urchin egg. Our results show that neither nicotine nor benzo(a)pyrene adducted to ovalbumin showed any effect on early egg development when they were added after fertilization, even though the applied concentrations were many times higher than the substances which occurred in the tested fractions. The egg, however, is very sensitive to fractions containing unsaturated hydrocarbons and other substances. For further subfractionation of these fractions, the sea urchin egg offers a certain advantage, compared with the mammalian system, because it is not sensitive to nicotine or polycyclic hydrocarbons still present in these fractions. If the mammalian system is used, nicotine has to be removed in order to test the toxicity of other substances. Technically this problem cannot easily be solved.

Examination of substances to determine their toxic effect on human beings is done mostly with mammals. For certain investigations, however, the sea urchin embryo seems to be equally useful and may even supply further information in certain cases.

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