either 0.25 mg or 0.50 mg crystalline cyproterone acetate (CYP A) placed directly on the blastodisc. Another 2 groups received either 0.25 mg or 0.50 mg CYP A suspended in sesame oil. A control group received only the sesame oil. Preparations in solution were evenly administered onto the entire blastodisc with the aid of a tuberculin syringe.

In the remaining 3 groups a small hole was placed in the blunt end of the egg with an egg punch. A 21 gauge needle attached to a syringe containing the substance to be injected was placed into this opening and passed through the air sac into the yolk sac where the solution was deposited. 1 group received 0.50 mg CYP A suspended in sesame oil and another received half this dosage or 0.25 mg. A control group received only the sesame oil vehicle. Following administration of the test substances, the openings in the eggs were sealed with scotch tape. The eggs were returned to the incubator until the end of the 5th day of incubation (Stages 24–27[11]) at which time the embryos were removed and examined.

Results. The results of this investigation are listed in the accompanying table. Chick embryos exposed to crystalline CYP A had a high incidence of mortality, especially those exposed to 0.50 mg (87.5%). Exposure to CYP A suspended in sesame oil and placed directly above the embryo resulted in 80% and 100% mortality in embryos receiving 0.25 mg and 0.50 mg, respectively. Even sesame oil controls did not escape the high mortality rate. The injection of CYP A into the yolk sac resulted in a significant decrease in the number of embryo deaths when compared to embryos treated directly. Only 3 of the 24 embryos exposed to 0.25 mg CYP A failed to reach 5 days of embryonic life. 50% of those receiving 0.50 mg managed to survive. No deaths were observed in the 19 embryos receiving injections of sesame oil into their yolk sac.

Discussion. Cyproterone acetate exerts its antiandrogenic effects by competitively inhibiting the action of androgens at target sites[12]. The data presented here shows a significant increase in chick embryo mortality when CYP A is placed directly on the embryo in either a crystalline form or suspended in solution when compared to that of embryos exposed to the same concentrations of the chemical injected into the yolk sac. These results differ from those of Caplan[4] who observed that the injection of nicotinamide analogs into the yolk sac of chick embryos resulted in a much higher mortality rate than in those in which the chemicals were placed through a window in the egg directly on the embryo. Another report appeared in which specific sugars placed directly above the chick embryo, exhibited a lesser degree of mortality than did control embryos[13].

These conflicting reports could be due to the fact that these different chemical agents have different absorptive rates in the chick embryo. The fact that even sesame oil, when placed directly over the embryo in the present study, resulted in such a high incidence of mortality suggests that the mere weight of a substance resting on the developing blastodisc may be responsible for restricting the growth of the embryo and resultant death. Applying the test substance directly to the embryo might allow for more rapid absorption than via the yolk sac and might be the method of choice where only short exposure to a drug is required. Studies using different chemical agents are difficult to equate. It is necessary, therefore, that a standard technique of evaluating the toxicity or teratogenicity of chemical agents on the chick embryo be employed.

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Fibrin membrane endowed with biological function. III. Fixing living cells in fibrin gel without impairing their functions

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Summary. Chlorella cells, sea urchin eggs and Paramecium were embedded in fibrin gel which was formed by fibrinogen-fibrin conversion in the presence of thrombin. The embedded Chlorella cells retain the ability of photosynthesis by illumination. The embedded sea urchin eggs develop to normal blastulae and gastrulae. Samples of Paramecium survive for more than several h beating their cilia. It is suggested that this technique of fixing living cells is useful for handling free cells as a mass like a tissue, and for holding free cells in micrurgical experiments.

In previous reports, immobilized asparaginase and immobilized adenosine triphosphatase were prepared by embedding them in fibrin polymer by fibrinogen-fibrin conversion in the presence of thrombin[1,2]. The principal advantages of fibrin polymer as a matrix are as follows; the polymerization of fibrin from fibrinogen proceeds by adding thrombin under physiological conditions (neutral pH and room temperature) and blood coagulation factor XIII serves as a cross-linking reagent between fibrin molecules. In the present paper, the authors succeeded in fixing living cells such as Chlorella, sea urchin eggs and Paramecium in fibrin membrane or block of fibrin gel without impairing their functions.

Materials and methods. Human fibrinogen was obtained from plasma by the method of Blombäck and Blombäck[3], and human thrombin was gifted from the Green Cross Cooperation. Chlorella ellipsoidea cells were grown photoautotrophically at 25 °C by the method of Tamiya et al.[4]. Egg and sperm were obtained from the sea urchin, Hemicentrotus pulcherrimus. Specimens of Paramecium candatum reared in hay infusion at 20 °C were used. Fixing sea urchin eggs in fibrin gel was carried out as

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follows: in a plastic Petri dish, a drop of suspension of fertilized eggs was mixed with 0.2 ml of sea water containing 0.5, 1 or 2% fibrinogen. Then 0.01 ml of thrombin solution (25 IU/ml) was added to the mixture, which was throughly mixed with a glass rod. Fertilized eggs with fertilization membrane, as well as those without membrane, were used. For removing the membrane, eggs were treated with 1 M urea solution for 4 min shortly after insemination. Specimens of Paramecium were embedded in a similar way. Observation was made with a Nikon inverted microscope through the flat bottom of Petri dish. Fixing Chlorella cells in fibrin membrane was performed as described previously[2]. To a mixture of 2.5% fibrinogen (1.5 ml) and cell suspension (0.1 ml) was added 0.04 ml of thrombin (80 IU/ml) and kept standing for 30 min at room temperature and pressed to 0.4 mm thickness. Measurement of oxygen production from the fibrin membrane containing Chlorella cells by illumination was carried out with a Clark oxygen electrode in a closed plastic chamber at 37 °C with constant stirring.

Results. Chlorella cells embedded in fibrin membrane produced oxygen when it was illuminated, and the oxygen production stopped when the light was put off (curve B in figure 1). The full activity of oxygen production by illumination was observed even after the membrane was kept in the medium several h. The Chlorella cell suspension of the same concentration produced oxygen by light as shown by curve A in the same figure. The rate of oxygen production of cells embedded in fibrin membrane was smaller by only 30% than that of Chlorella suspension. This may be due to the physical effects, such as effectiveness in the absorption of light by cells and diffusion of oxygen into the medium. The absorption spectrum of Chlorella cells embedded in fibrin membrane was in good agreement in peak position with that of free suspension of cells. The fact mentioned above may indicate that fibrin membrane acts as a matrix suitable for holding the Chlorella cells without impairing their ability for photosynthesis.

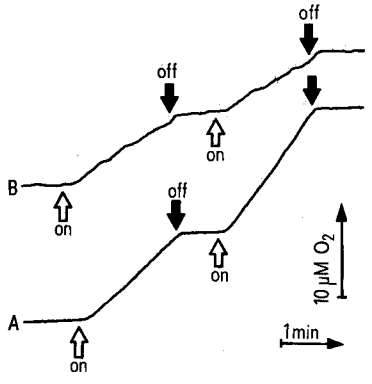

Fig. 1. Oxygen production by illumination of Chlorella cells (8 µg chlorophyll/ml) suspended in 40 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl(curve A) and embedded in fibrin membrane (curve B) 22°C.

Sea urchin eggs with fertilization membrane embedded in fibrin gel normally developed to blastulae and gastrulae (figure 2a), irrespective of the concentrations of fibrin used in the present experiment. The larvae were swimming in narrow spaces enclosed by fibrin gel. Cleavage of the egg without membrane embedded in fibrin gel was modified: elongation of the cell during cleavage was more or less impeded by the presence of the fibrin gel as shown in figure 2b (compare with eggs in normal sea water shown in figure 2c). Some eggs embedded in 1.6% fibrin gel did not divide, while eggs embedded in 0.4 or 0.8% fibrin gel developed to blastulae though the balstocoels were smaller than those in normal blastulae in sea water. Control eggs without membrane in sea water did not develop to blastula because blastomeres were separated with one another owing to the absence of hyline layer. Some samples of Paramecium embedded in fibrin gel were swimming in narrow spaces around them, while some others were completely embedded in the gel with no free space around them. Most samples with a free space around them continued swimming for more than 5 h and a few for more than 20 h. The microscopic appearance of cells was scarcely impaired by the presence of fibrin gel around them, because fibrin gels were almost transparent and homogeneous in the concentrations used in the present experiment. Nevertheless, fibrin gels were sufficiently stiff to hold the cell. This fact may provide an excellent technique for holding single cells during micromanipulation, such as insertion of microneedles, micropipettes and microelectrodes.

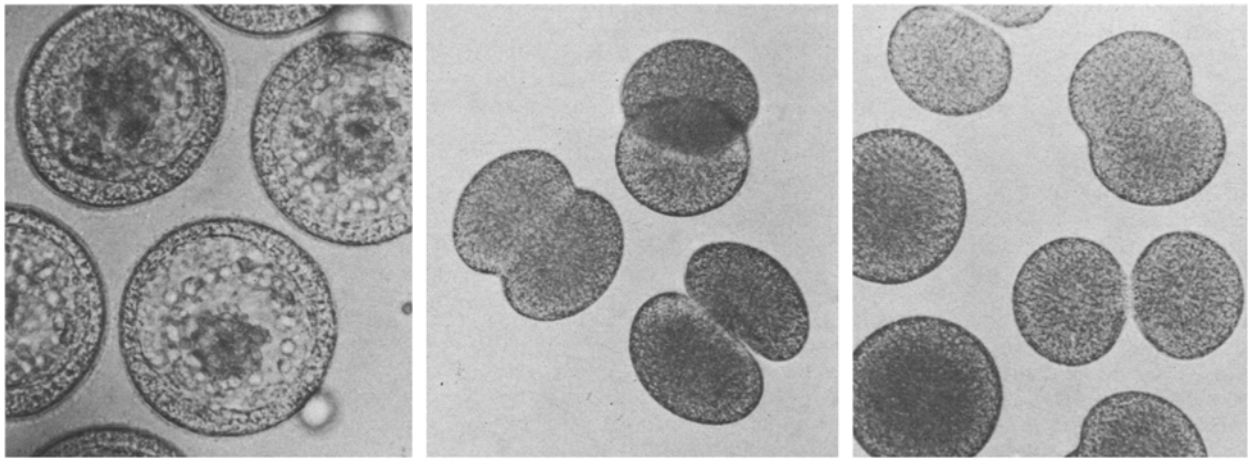

Fig. 2. Fertilized eggs of the sea urchin, Hemicentrotus pulcherrimus embedded in fibrin gel. a) Larvae developing in 0.8% fibrin gel (22.3 h after insemination at 18 °C); b) Eggs without fertilization membrane cleaving in 0.8% fibrin gel (1.3 h after insemination at 20 °C); c) Eggs without fertilization membrane cleaving in sea water (1.3 h after insemination at 20 °C). ×300.