The Uptake of Topically Applied Tritiated Thymidine by Chick Embryos[1] (36427)

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(Introduced by J. W. Osborne)

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Tritiated thymidine (^{3}H-TdR) has been used to study cellular kinetics in normal and irradiated tissues (1–3). Thymidine is a specific precursor of DNA. When ^{3}H-TdR is made available to cells, those cells that are in the S (DNA synthesizing) phase of the cell cycle very quickly incorporate ^{3}H-TdR into their DNA. The tritium is usually detected by means of high resolution autoradiography or liquid scintillation counting techniques.

One of us (B.T.A.) had shown that cells of the rat lens epithelium could take up ^{3}H-TdR when it was topically applied to the cornea of the eye. The purpose of this present study was to determine whether or not chick embryo cells could utilize ^{3}H-TdR topically applied to the inner shell membrane. Assuming an affirmative result, secondary objectives were to determine (a) the feasibility of using liquid scintillation counting techniques to quantitate the uptake of ^{3}H-TdR and (b) some factors influencing the uptake.

Materials and Methods. Two experiments were done. In the first, either 0.1 μCi or 1.0 μCi of ^{3}H-TdR was topically applied onto the inner shell membrane of eggs that had been incubated for either 4 or 6 days. Sample embryos were obtained at 5, 15, 30, 60, 90, or 120 min after application of the ^{3}H-TdR. In this way, 24 different uptake values were derived, each being the mean of 10–14 different embryo samples values ($\pm$ SEM). In the second experiment, 0.5 μCi of ^{3}H-TdR was applied to embryos at 92, 103, and 118 hr of incubation. Embryos were killed 1 hr after the ^{3}H-TdR was applied. Each experimental value was the mean of 12 different embryo sample values.

A constant volume, 0.25 ml, of ^{3}H-TdR solution was applied to each embryo. Sterile aqueous solutions of methyl-^{3}H-thymidine (Schwarz/Mann, sp act 1.9 Ci/mmole) were diluted with 0.9% sterile saline to obtain suitable concentrations of the radionuclide. For the first experiment, stock solutions of ^{3}H-TdR were diluted to concentrations of 0.4 or 4.0 μCi/ml. A concentration of 2.0 μCi/ml was utilized in the second experiment.

White Leghorn chicken eggs of approximately the same size, 65 g/egg, were incubated at 37 $\pm$ 0.5° in an incubator with an automatic turner (Humidaire Incubator Co., New Madison, OH). Before the ^{3}H-TdR was applied, the eggs were candled. If the embryo was reasonably centrally located, a small hole was drilled through the egg shell into the air space above the embryo. Then a disposable 1-ml hypodermic syringe was used to carefully layer 0.25 ml of ^{3}H-TdR solution onto the inner shell membrane. The shell was wiped, the hole was covered with transparent tape, and the egg was returned to the incubator where it remained, blunt end uppermost, for the required ^{3}H-TdR exposure period.

At appropriate times, the embryos were dissected out of the shells and freed from all adhering membranes. Each embryo was placed in a linear polyethylene liquid scintil-

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lation counting vial (Isolab Inc., Akron, OH) containing 5 ml of Carnoy's fixative (3 parts absolute ethanol + 1 part glacial acetic acid). This fixed the tissues and also eluted any unincorporated ^{3}H-TdR and ^{3}H-TdR breakdown products (1). After about 4 hr, an aspirator was used to remove the fixative solution from the vial. The used fixative solution, which contained some tritium, was set aside for disposal by the Radiation Protection Officer. Immediately after removal of the fixative solution, 5 ml of chilled saline was added to the vial. This eluted essentially all of the Carnoy's fixative from the tissues. Otherwise, the fixative solution would have (a) tended to impede the solubilization of the tissues by neutralizing the strongly basic solubilizer and (b) added to the amount of quenching during the liquid scintillation counting. After about 2 hr, the saline solution was aspirated from the vial and the walls of the vial were wiped dry. Droplets of saline tended to adhere to the walls of the vial and these droplets, if they were not eliminated, formed a heavy precipitate when the liquid scintillation counting solution was added to the vial. Next, the embryo was gently dried by placing the embryo in its vial with the vial cap loosened on a warming plate. This was done to reduce the water content of the tissues and the likelihood that excess water would "overload" the solubilizer and cause precipitation of the solubilized protein. The development of a precipitate could, of course, introduce a counting error. It should be noted, however, that it was very difficult to solubilize the eye if too much drying occurred.

Soluene 100 (Packard Instrument Company, Inc., Downers Grove, IL) was added to each vial (0.75 ml–1.5 ml for 4-day embryos, up to 2.0 ml for 6-day embryos) and the tightly capped vials were placed on a warming plate at 40°. When solubilization was completed, 10 ml of the liquid scintillation counting solution was added to each vial. The mixture of primary and secondary fluors consisted of 5.0 g PPO and 0.3 g dimethyl POPOP/liter of toluene. Samples were counted in a Model 3380 Packard Tri-Carb Liquid Scintillation Counter equipped with a Model 544 Absolute Activity Analyzer.

Results and Discussion. In Figure 1 the uptake of topically applied ^{3}H-TdR by chick embryos *in situ* is plotted as a function of the dose of radioisotope used and the age of the embryos. From the graphs we may deduce that the uptake of ^{3}H-TdR is related to: (1) the length of time the isotope is made available to the embryo. The uptake of isotope is roughly proportional to the square of the exposure time: (2) the amount of isotope which is topically applied to the embryos. A tenfold increase in thymidine concentration produced an approximately proportional increase in the amount of radioactivity detected for embryos of either age: (3) the age of the embryo. The ratio of radioactivity detected (dpm) in the 6-day embryo to the 4-day embryo is approximately constant at 4.0. This compares with a value of 5.6 for the ratio of the wet wt of 6-day–4-day embryos (4).

The discrepancy in these ratio values may be due to a number of factors of which at least two may be particularly significant. The 6-day embryo may be too large to permit the diffusion of thymidine to the cells at the centre of the embryo thus preventing them from becoming labeled. Secondly, the organs of the 6-day embryo may have differentiated to a level at which the proportion of cells synthesizing DNA is no longer directly proportional to total embryonic weight. Whichever factor is involved it begins to predominate during the sixth day of incubation. When 0.5 μCi of ^{3}H-TdR was topically applied to three groups of 12 embryos after 92, 103, and 118 hr of incubation, the amount of isotope subsequently detected increased with age, as previously shown, and the ratio of detectable activity for 118–92-hr embryos was approximately 3.0. This is in good agreement with the factor of 2.7 which represents the wet weight ratio of 5–4-day embryos (4). The fact that the 6-day embryo was more difficult to solubilize than the 4-day one may indicate that embryo size is the limiting factor in this experimental technique.

Apart from technical limitations there are a few potential sources of error against which an experimenter must guard. In some eggs the embryo is not centrally located. Conse-

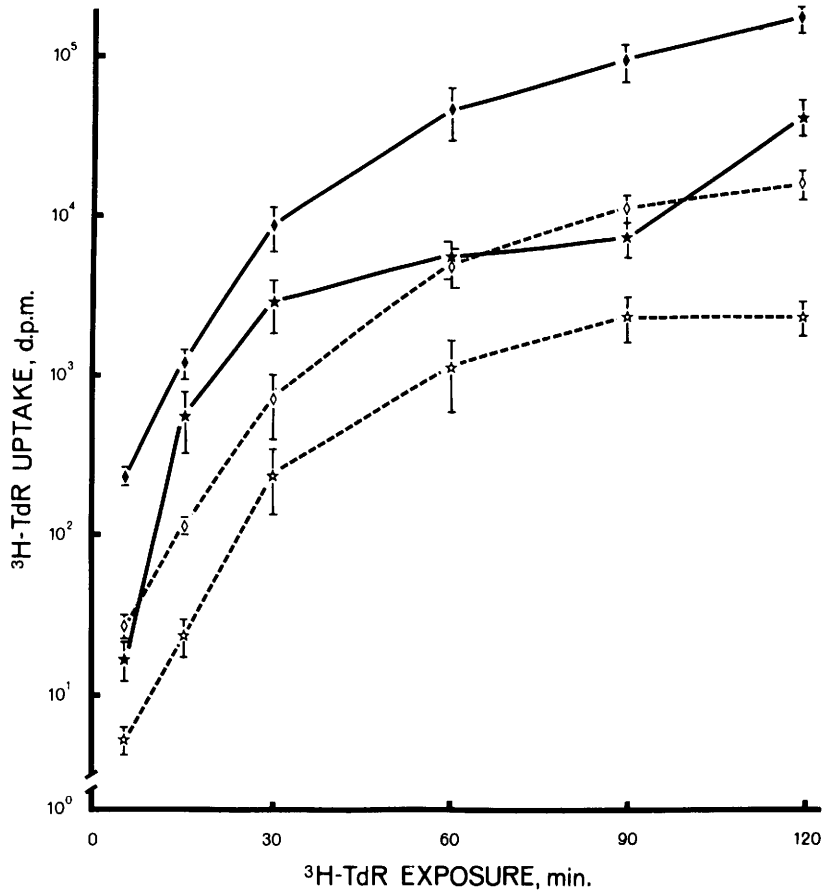

FIG. 1. Uptake (dpm) of topically applied ^{3}H-TdR by chick embryos plotted as a function of exposure (min) to the isotope. Stars indicate data obtained from 4-day embryos, diamonds = 6-day embryos, open symbols and interrupted lines = 0.1 μCi dose, filled symbols and continuous lines = 1.0 μCi dose. Each experimental point is an average of 10–14 embryo sample values ± SEM.

quently the isotope solution, when injected through the outer shell membrane, will not be layered upon the embryo. In these instances the uptake of the radioactive thymidine over a fixed period of time will decrease as a function of the time necessary for the isotope to penetrate the intervening medium in order to reach the embryo. This problem can only adequately be overcome by rejecting those eggs which, on prior candling, reveal an eccentric embryonic location.

Any volume of liquid injected into an otherwise "balanced" system may have a physical effect on that system which, in turn, may perturb the growth rate of the embryo. On the other hand we were unable to detect any effect on embryonic growth due to the injection of 1.0 ml of saline into a number of eggs. Nevertheless, the volume of injected solution should be kept to a minimum commensurate with other experimental requirements.

Chick embryos which have been incubated for a fixed length of time may show a considerable variation in their stage of development. It is, therefore, important to include as many subjects as possible within each treatment group in order to improve the statistical significance of the results. For the same reason it is important to incorporate a control

group in each part of the experiment.

Conclusion. The results of our experiments indicate that tritiated thymidine, after penetrating the inner shell membrane of the chick egg, is incorporated into those embryonic cells which are synthesizing DNA. The amount of radioactive tracer absorbed is a function of the age of the embryo, the concentration of the isotope dose and the length of the embryos' exposure to the isotope. The uptake of isotope by the embryo may be reproducibly determined and thus the process may be used to determine the effect of various agents on the DNA synthesis of embryonic chick cells. Using this system we are presently studying the effect of ionizing radiation on developing chick embryos.

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