ated to a sublethal endpoint and most species—9 out of 12—were diploid.

The search for a unifying concept of seed radiosensitivity can be carried one step further. The values in the last column of Table 1 may be estimates of a single number, representing the maximum energy (in Mev) which can be absorbed by a dormant nucleus in the apical meristem before growth of the ensuing seedling will be reduced by 50 percent; the average value is 610.4 ± 70.4 Mev. The only variables making up this "constant" value (k) are the nuclear volume and the 50 percent exposure; thus either could be used to estimate the other. Since the experimenter is usually interested in predicting radiation tolerance, it would be relatively simple to section a few embryos and measure apical nuclei. It follows that

$$\frac{k}{\text{nuclear volume}} = \frac{}{\text{the 50 percent exposure in roentgens}} \quad (1)$$

for dormant embryos in their most resistant state, and

$$k = \frac{610.4 \pm 70.4 \text{ Mev/nucleus}}{(1.77)\ (34)\ \text{ev}/\mu^3/\text{roentgen}} = (10.14 \pm 1.17) \times 10^6 \quad (2)$$

therefore

$$\frac{(10.14 \pm 1.17) \times 10^6}{\text{average nuclear volume } (\mu^3)} = \frac{}{\text{the 50 percent exposure in roentgens}} \quad (3)$$

for dormant embryos in their most resistant state.

This method was tested on the last two species of Table 1 prior to the performing of dose-response experiments. For the first 10 species, average energy per nucleus at the 50 percent exposure was 646.3 ± 80.1 Mev, hence k was calculated to be $(10.74 \pm 1.33) \times 10^6$. The 50 percent exposure for *Gossypium arboreum*, with an average nuclear volume of 435 μ^3, was thus predicted to be 24.7 kr with a 95 percent confidence interval of 17.5 to 32.1 kr and a 99 percent confidence interval of 14.8 to 34.6 kr. The experimental value (Table 1) was 16.8 ± 0.27 kr.

The data from *G. arboreum* were then added to the preceding 10 species and the average energy per nucleus at the 50 percent exposure became 627.5 ± 74.8 Mev, and k was thus $(10.43 \pm 1.24) \times 10^6$. *Daucus carota* (average nuclear volume 114 μ^3) was predicted to have a 50 percent exposure of 94.2 kr, with a 95 percent confidence interval of 65.2 to 119.4 kr and a 99 percent confidence interval of 57.0 to 125.9 kr. The experimental value (Table 1) was 61.8 ± 2.32 kr. Thus in both tests the observed values fell below the 95 percent but within the 99 percent confidence interval (*8*).

Since this report was first submitted, we have been permitted access to relevant unpublished data from two Spanish authors (*9*). Their study provides nuclear volume and LD_{50} (lethal dose to 50 percent of the population) values for 20 species. Pertinent technical features are: all species were from the family *Cruciferae* and 16 of the 20 species were diploid; dormant seeds were equilibrated at 70 percent relative humidity then x-irradiated at 1200 r/min; exposures reducing survival by 50 percent were determined after 2 months of growth; and nuclear volumes of apical meristems were measured in sprouted seedlings. Average nuclear volumes ranged from 25 to 270 μ^3; chromosome numbers, from 10 to 64; and LD_{50}'s, from 15 to 240 kr. Ranges of energy per chromosome and per nucleus at the LD_{50} were inconclusive, being 6.6-fold in the former case and 5.4-fold in the latter. Means and standard errors were $(24.97 \pm 2.99) \times 10^6$ ev per chromosome and $(553.4 \pm 65.5) \times 10^6$ ev per nucleus, values quite in agreement with ours of Table 1. From their data one obtains a k value of $(9.20 \pm 1.09) \times 10^6$, which compares favorably with our value of $(10.14 \pm 1.17) \times 10^6$.

The theoretical significance of such a constant is obscure, but the practical importance is clear. Heretofore a person embarking on a radiation study with dormant seeds of an untested species could not predict whether his material would be devastated by 1 kr or be unaffected by 100 kr. Now a few microscopic measurements and some easy arithmetic will reveal the approximate amount of radiation he can expect the seeds to tolerate before a significant reduction in growth will occur.

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8. It is possible that the numbers in the last column of Table 1 are estimates, not of a single value, but of a distribution having a rather small variance. It may be that further work will reveal different k values for different classes of seeds—for example, different ploidy levels.
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10. We thank C. Gómez-Campo and L. Delgado for permission to use unpublished data, D. A. Gardiner and R. J. Monroe for statistical advice, and A. H. Sparrow for helpful discussions.

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Protein Synthesis During Development: Control through Messenger RNA

Abstract. *Utilization of long-lived messenger RNA appears to be the exception rather than the rule in cells which are differentiating and synthesizing large amounts of specialized product at the same time. The fact that polyribosomes synthesize protein after RNA synthesis is turned off by actinomycin D is used to demonstrate messenger RNA of long half-life. The data suggest that most tissues examined have short-lived messenger RNA's, but the ocular lens can synthesize protein after an incubation of 24 hours in 40 μg of actinomycin D per milliliter. A common basis for the presence of long-lived messenger RNA in the cells of the lens, the feather, and in reticulocytes is discussed.*

A mark of the differentiated cell is its capacity to synthesize structural or enzymatic cell specific proteins. Some cells, such as skin, liver, muscle, connective tissue, reticulocyte, pancreas, and thyroid, produce large amounts of one or a few kinds of protein. We have asked whether all or only some differentiating cells synthesize their specialized product on messenger RNA which has a long half-life. It has already been shown that hemoglobin (*1*) and feather

proteins (*2, 3*) are synthesized on messenger RNA which has a long life span. In bacteria the half-life of messenger RNA is about 2 minutes (*4*), while in HeLa cells the half-life of messenger RNA is about 3 hours (*5*). In some, if not all, cells of the down feather some messenger RNA's have a half-life which is longer than 24 hours (*3*).

Polyribosomal protein synthesis can be demonstrated as early as the first day of incubation (*6*) in the chick embryo, although, apart from blood cells, the earliest actual demonstration of messenger RNA of long half-life has been in the 9-day skin. The earliest chick blood cells in which hemoglobin can be detected have been shown to contain messenger RNA of long half-life (*7*). In sea urchin embryos it has been shown that immediately after fertilization proteins can be synthesized without production of new RNA (*8*). This condition appears to persist throughout cleavage and implies that stable messenger is present in the ovum and is put to use for a limited time after fertilization. Similar data on chick embryos of comparable age are not available.

There are at least two ways by which differentiating cells can make large amounts of one or a few characteristic proteins. The first is through the repeated use of limited amounts of long-lived specific messenger RNA's. The second is to make the specific protein through the continued synthesis of short-lived messenger RNA's. To determine which of these alternatives can best describe the use of messenger RNA in different cell types, a variety of embryonic and adult chicken tissues have been examined for long-lived polyribosomes. Long-lived polyribosomes are defined as those which will function to produce new protein for long periods after RNA synthesis has been reduced to less than 2 percent of controls by treating tissues (in vitro) with actinomycin D for varying periods. It is assumed that polyribosomes which function under the foregoing conditions provide a measure of the stability of messenger RNA.

Table 1. Results of 2-hour labeling period with uridine-2-C^{14} after 23 hours incubation of 14-day-old embryonic lenses (72 lenses in each sample) in 25 ml of Charity Waymouth medium. Experimental reaction mixtures contained 40 μg of actinomycin D per milliliter, and each contained penicillin and streptomycin. After dividing each group into two parts, RNA was extracted according to the method of Scherrer and Darnell (*13*).

Flask	Treatment	RNA (OD 260 mμ units)	Count/min
Control 1	None	0.395	548
Control 2	None	.598	809
Expt. 1	Actinomycin	.627	8.6
Expt. 2	Actinomycin	.560	15.8

$$\frac{\text{Experimental average (count/min per OD)}}{\text{Control average (count/min per OD)}} \times 100 = 1.55 \text{ percent}$$

Protein synthesis on polyribosomes in ocular lenses from embryos 13, 14, 15, or 20 days old was not abolished after tissues were incubated for 21 to 24 hours in 40 μg of actinomycin D per milliliter (Fig. 1). Treatment of the labeled homogenate with ribonuclease at 4°C for 10 minutes moves all of the radioactivity measured in the polysome region to the 74*S* peak. The incorporation of uridine-2-C^{14} into 14-day lenses during a 2-hour period of labeling was 1.5 percent of controls after 22 hours in 40 μg of actinomycin D per milliliter as judged by radioactivity of RNA extracted by a hot-phenol procedure (Table 1). Incorporation of amino acid into protein on polyribosomes in lenses treated with actinomycin D ranged from 4 to 16 percent of controls. It is clear from these data that there are some messenger RNA's in the ocular lens which have a life span comparable to the long-lived messenger RNA in feather cells (*2, 3*).

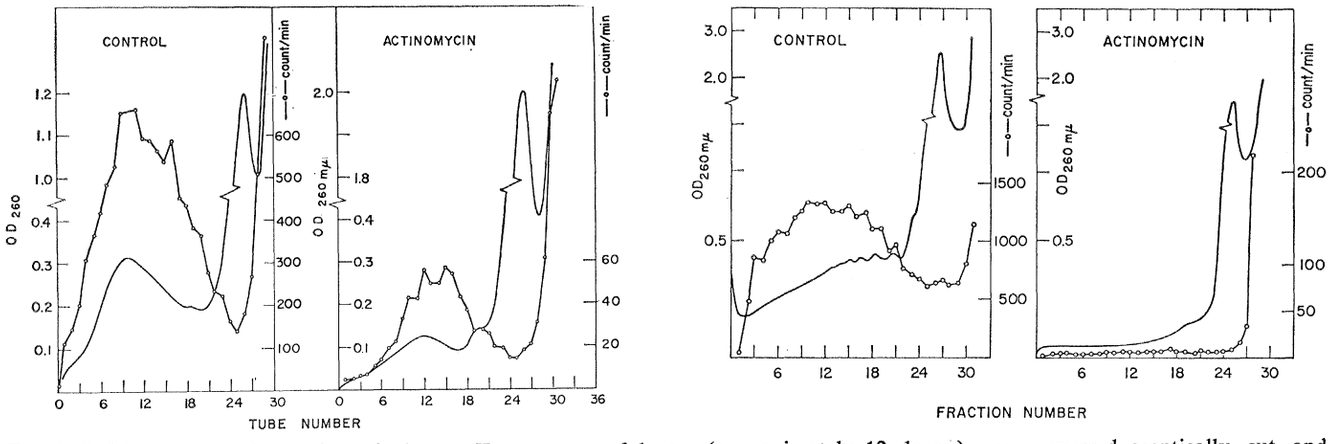

Fig. 1 (left). Fourteen-day embryonic lenses. Two groups of lenses (approximately 12 dozen) were removed aseptically, cut, and then incubated for 23 hours (with agitation) at 37°C in 25 ml Charity Waymouth medium (*14*) with 4 mg penicillin, 2.5 mg streptomycin, and phenol red indicator. The experimental flask also contained 40 μg of actinomycin D per milliliter (*15*). The medium was then removed, and tissues were labeled for 3 minutes at 37°C in buffered saline with 20 μc of C^{14}-labeled algal hydrolysate per milliliter but no actinomycin. The reaction was stopped with ice-cold saline, and the tissues were washed three times before suspending them in four volumes of cold hypotonic buffer (0.01*M* tris, *p*H 7.4; 0.0015*M* Mg^{++}; 0.01*M* KCl) for 30 minutes. Samples were homogenized gently with three to six strokes of a tightly fitting Dounce homogenizer. Nuclei were sedimented at 600*g* for 10 minutes, and sodium deoxycholate was added to the supernatant to give a concentration of 0.5 percent prior to layering on a linear 25-ml sucrose gradient (15 to 30 percent by weight) in the same buffer. Gradients were centrifuged at 24,000 rev/min in an SW-25 rotor of a Spinco model L centrifuge for 2 hours at the −9°C setting. Twenty (drop) fractions were collected and the optical density at 260 mμ was recorded continuously on a Gilford spectrophotometer. To each fraction 0.5 ml of 2*N* NaOH was added. Protein was precipitated with trichloracetic acid at a final concentration of 5 percent, and the precipitate was collected on Millipore filters. The radioactivity was counted on aluminum planchets in a low background gas flow counter. Fig. 2 (right). Optical density profile of polyribosomes of whole 3½-day chick embryo showing radioactivity of nascent protein in the control and the effect of actinomycin D (10 μg/ml). The peak at fraction 25 represents single ribosomes.

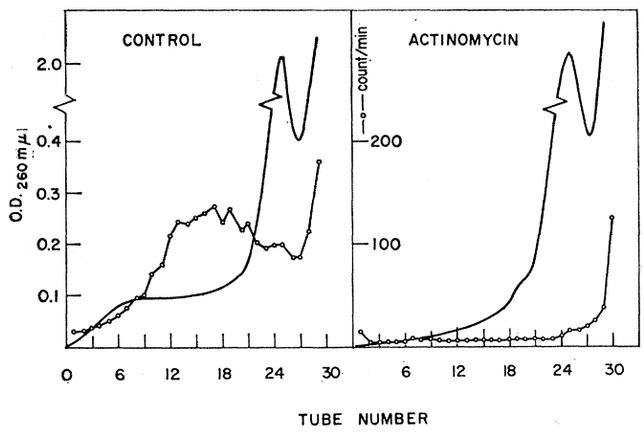

Fig. 3 (left). Polyribosomal protein synthesis in the smooth muscle of the proventriculus of 15-day chick embryos and the effect of actinomycin (20 μg/ml).

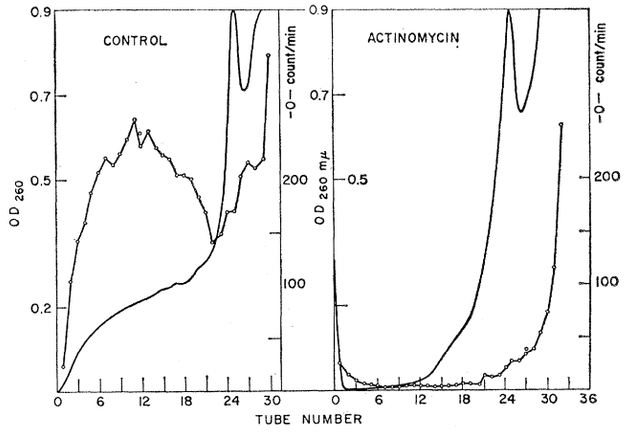

Fig. 4 (right). Polyribosomal protein synthesis in the brain of 9-day chick embryos showing inhibition by actinomycin (20 μg/ml).

In contrast to the foregoing results, in studying the sedimentation in a sucrose density gradient of the polyribosomes from 3½-day-old chick embryos it is seen that no labeled amino acids were incorporated into proteins on polyribosomes after tissues were incubated for 21 hours with actinomycin D (10 μg/ml). In fact, by 6 hours in 6-day-old whole embryos treated with actinomycin D, protein synthesis on polyribosomes is already less than 10 percent of that on polyribosomes of untreated controls. Examination of 3-, 4, 5-, or 6-day-old embryos shows the same absence of long-lived messenger RNA after actinomycin treatment. After 5 days of incubation many organs of the embryo are morphologically well differentiated; these include liver, brain, and skeletal and smooth muscle. However, the skin is poorly developed, and feather primordia have not yet appeared. It should be pointed out that there is essentially no contribution to the polyribosome profile from circulating blood cells because the tissues are washed free of blood during preparation.

Some older embryonic tissues were similarly treated with actinomycin, and homogenates were studied for persistence of polyribosomes (Figs. 3 and 4). Protein synthesis on polyribosomes in 15-day liver and smooth muscle (proventriculus), 14- and 16-day skeletal muscle, and 9-day brain was completely inhibited by treatment with 20 μg of actinomycin per milliliter.

In order to obtain adequate samples of adrenal, pancreas, and spleen, pullet tissues were used, and the ovary was studied in adult hens. Only that portion of the ovary containing the relatively immature ova was selected for incubation. Satisfactory polysome profiles were difficult to obtain in some of these older tissues, but, in homogenates not treated with deoxycholate, radioactivity incorporated into the membrane-bound ribosomes (*9*) could be compared with and without actinomycin treatment. Long-lived messenger RNA was found in none of these tissues. It is possible that some tissues are more adversely affected by actinomycin D than others and that our failure to demonstrate long-lived messenger RNA's in some reflects this. However, in tissues such as the pancreas or adrenal, protein synthesis (greater than 10 percent of control levels) can still be measured after incubation of 17 or 15 hours, respectively, in actinomycin D. By 24 hours, protein synthesis in both is brought to a halt.

From our data and previous data, it is now evident that large fractions of relatively stabilized protein-forming complexes (messenger RNA and ribosomes) can be demonstrated in some but not all highly specialized cells which produce large amounts of a limited variety of specific protein. It has been reported that lamb thyroid (*10*) and adult rat liver (*11*) continue to synthesize protein after treatment with actinomycin D.

Cells which clearly have long-lived messenger RNA, namely lens, down feather, and reticulocyte, share a number of features. Each type makes large quantities of a limited number of proteins (crystallins, keratins, and hemoglobin, respectively), and in each case long-lived messenger RNA is found during periods of intense protein synthesis and cell differentiation. Also common to these cell types, which convert virtually their entire substance into a single product, is early cell death or loss of the nucleus shortly after a spurt of intense protein synthesis which occurs, at least in part, through the repeated use of long-lived messenger molecules. In the differentiation of mammalian erythrocytes, the nucleus is lost and the cytoplasm then functions independently for a limited period. The fate of nuclei in the feather cells and in cells of the lens is not clear, although it has been shown that in the central, more differentiated, portions of the lens no DNA can be detected (*12*). In feather cells, however, the nucleus along with the cytoplasm seems to "keratinize." We have no information on the fate of the DNA. In all three cell types the nucleus is effectively "turned off" as differentiation of the cell progresses towards its terminal state. By the time this occurs, differentiation of these cell types is a one way street paved with messenger RNA molecules which have a long half-life.

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Staphylococcal Alpha-Hemolysin: Detection on the Erythrocyte Membrane by Immunofluorescence

Abstract: *Purified staphylococcal α-hemolysin (but not the toxoid) was demonstrated on the surface of rabbit and human erythrocytes by immunofluorescence. This occurred during the period of maximal hemolysis and was a transient event. These findings have been analyzed in relation to previous data on the kinetics of leakage of both small and complex molecular constituents of the erythrocyte.*

While the kinetics of the lytic action of staphylococcal α-hemolysin on cells with rapid leakage of large molecules implicated the cell membrane rather than intermediary metabolism as the primary site of action (*1*), this point has not hitherto been demonstrated by direct evidence.

Analysis of the time course of hemolysis by highly purified toxin (*2*) revealed a sigmoid curve with a lag phase before the onset of hemolysis (prelytic lag phase), followed by a period of rapid, linear release of hemoglobin. Release of cell potassium takes place early in the prelytic period, with 50 to 70 percent of this loss occurring before leakage of hemoglobin is detectable (*3*). Specific antitoxin prevents hemolysis only when added before the end of the prelytic phase (*4*).

There is disagreement as to whether adsorption of α-hemolysin to the red cell is reversible. Forssman (*5*) has suggested that crude hemolysin is fixed for a brief time and then released to act on other red cells. On the other hand, Levine (*6*) has contended that this adsorption is not reversible. Evidence was presented recently by Lominski and Arbuthnott (*7*) that α-lysin is completely recoverable. The observations of Cooper *et al.* (*4*) indicate that, when purified hemolysin has reacted with erythrocytes, the products of hemolysis inhibit its attachment to other cells.

The initial reaction between staphylococcal α-hemolysin and the erythrocyte is probably a surface phenomenon. We have used the fluorescent antibody technique (*8, 9*) to study this and to attempt to relate the findings to the kinetics of hemolysis of rabbit and human erythrocytes. Purified α-hemolysin [40,000 hemolytic units (HU) per milliliter] was prepared by the method of Madoff and Weinstein (*10*). This material, when treated by incubation with 0.2 percent formalin (final concentration) at 37°C for 20 hours, was no longer hemolytic. It formed a precipitate with staphylococcal antitoxin (*11*) and induced active immunity in mice. A γ-globulin fraction of rabbit antibody to horse γ-globulin, conjugated with fluorescein isothiocyanate (*9, 12, 13*), was mixed in a ratio of 7 parts to 3 parts of normal human serum containing 500 mg per 100 ml of Evans blue dye for counter staining. Fresh erythrocytes were collected daily into Alsever's solution, washed, and prepared as a 2 percent suspension in phosphate-buffered saline, *p*H 6.9. Hemoglobin and potassium release were determined by methods described previously (*2, 3*).

The erythrocytes were mixed with purified hemolysin and the suspension was incubated in a water bath at 37°C, the test tubes being subjected to constant agitation. At intervals, excess antitoxin was added directly to samples of the cell-hemolysin suspension and incubated at 37°C for 15 minutes. The cells were centrifuged and washed twice with phosphate-buffered saline. The fluorescein-labeled antibody to horse γ-globulin was added and the mixture was incubated at 37°C for 30 minutes and centrifuged; the cells were washed three times with the phosphate buffer. The cell buttons were then smeared on glass slides, air dried, mounted with a solution containing 9 parts glycerol to 1 part veronal buffer (0.01*M*, *p*H 8.6), and examined by fluorescent microscopy.

In a simultaneous study, red cells exposed to hemolysin and antitoxin, as described above, were immediately smeared, air dried, fixed in acetone at −70°C for 2 hours, washed with phosphate-buffered saline, and covered with the fluorescein conjugate in moist

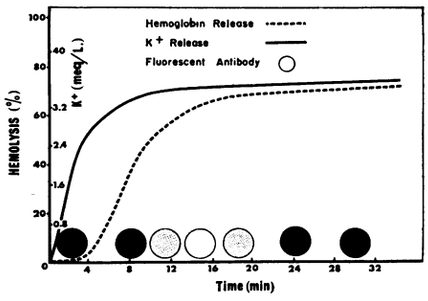

Fig. 1. Time course of hemolysis and release of potassium ions correlated with immunofluorscence when rabbit erythrocytes are exposed to 25 hemolytic units per milliliter of purified staphylococcal α-hemolysin. The gradient of brilliance of fluorescence is designated by the shading of the red cells (indicated by circles along the abscissa): black indicates no fluorescence; stippled, moderate fluorescence; white, bright fluorescence.

petri dishes at 37°C for 1 hour. The preparations were then washed twice with the phosphate buffer, once with veronal buffer, and mounted. Standard controls to document specific fluorescence (*9*) were included in each study.

More cells became fluorescent when conjugate was added directly to erythrocytes in a test tube and the mixture agitated for 30 minutes than when a red cell smear was flooded with conjugate. This was not unexpected, since there is a greater opportunity for contact between conjugate and erythrocytes with the former method. In each case, however, many cells remained unstained; this reflects the natural

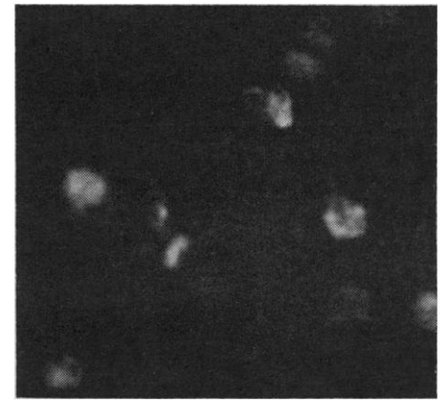

Fig. 2. Rabbit erythrocytes treated with 45 hemolytic units of purified staphylococcal α-hemolysin (45 HU per milliliter of cell suspension) for 15 minutes and then exposed to anti-hemolysin, washed, and stained with fluorescent antibody on slide (× 120). The large cells with the granular cytoplasm and nonfluorescent nuclei are leukocytes.