significant difference noted was the markedly lower viral titers obtained in the irradiated tissue cultures. Although direct evidence is lacking it is likely that this diminished ability of cells to support viral multiplication is due to cellular damage resulting from radiation exposure, as has been described in the case of a bacteriophage-X-ray inactivated bacteria system(11). That diminished viral multiplication may be a result of decreased cellular proliferation is suggested by the observation that normal tissue cultures derived from 10-14-day chick embryos yielded lower viral titers than did those derived from younger (6-day) embryos.

The diminished susceptibility of cells irradiated *in ovo* to support viral multiplication is in contrast to reports stating that the radiation of the intact animals results in an increase in susceptibility to viral infection. In another virus-host-cell system utilizing tissue culture technics, we have been unable to overcome the innate resistance of embryonic and adult cells to viral infection by means of exposure to ionizing radiations. Attempts to propagate the MEF1 strain of Type 2 poliomyelitis virus in tissue culture employing mouse embryonic skin and muscle fibroblast-like cells and adult mouse kidney epithelial-like cells previously exposed *in vitro* or *in vivo* to X-rays or beta rays have been unsuccessful.

Summary. The effect of X-radiation of chick embryonic tissue has been studied in regard to its subsequent ability to support the multiplication of vaccinia virus in tissue cultures. Under the stated experimental conditions radiation of the tissue *in vitro* had no significant effect; in contrast, exposure of the embryonic tissue to radiation *in ovo* appeared to diminish its ability to support viral growth in tissue culture. The differences noted were fairly uniform throughout the period studied. Evidence suggesting that the differences noted are due chiefly to secondary radiation effects is discussed.

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A New Count of Allantoic Cells of the 10-Day Chick Embryo. (21175)

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Numerous viruses and some other parasites are often propagated in the cells lining the allantoic cavity of the developing chick embryo. In the case of influenza and some other viruses, many quantitative and kinetic investigations on problems relating to virus reproduction have been carried out with allantoic cells, usually in eggs inoculated at the 10th day of incubation. In order to compute the ratio of adsorbed particles to available cells and the yield of virus particles per cell, it is necessary to know the number of cells lining the allantoic cavity.

This number has been estimated by several indirect methods and by direct count of the nuclei in a preparation, and the various estimates agree fairly well(1). However, recent data obtained in this laboratory indicated that

possibly these estimates were too high(2). The indirect methods employed are valid only if the assumptions on which they are based are also valid. Nuclear counts may have been in error because of the nuclei in other cells as well as the allantoic cells of the chorioallantoic membrane, and the difficulty of distinguishing one from another. In the present investigation the number of cells lining the allantoic cavity was estimated by direct measurement of the mean area of allantoic entodermal cells observed with the phase contrast microscope and by measurement of the area of the allantoic surface. The number of allantoic cells was found to be about 10-fold less than previous estimates.

Materials and methods. Microscopic observations and measurement of cells. The eggs came from a single flock of White Leghorn hens and were incubated for 10 days at 38°C in a commercial egg incubator. The contents of fertile eggs, weighing 54 to 57 g, were removed and the chorioallantoic membrane was rinsed with 0.85% NaCl buffered at pH 7.15 with 0.01 M phosphate buffer (B.S.). With a sharp cork borer (internal diameter 1.26 cm) a circular piece was punched out of the membrane. The piece of membrane with the allantoic surface uppermost was carefully floated from the shell onto a slide and was covered with a coverglass which was sealed to the slide with paraffin. The mean diameter of the piece of membrane was estimated after each count because it tended to shrink gradually to a variable extent. The preparation was examined with the oil immersion objective (97X) of a phase contrast microscope. All 3 layers of the membrane could be visualized, and the uppermost or allantoic layer apparently consisted of a single layer of epithelial cells. The nuclei and cell outlines were clearly seen, as indicated in Fig. 1. Cytoplasmic structures, *e.g.*, mitochondria, were also visualized. A scale drawing of the cell outlines in each field was made on graph paper with the aid of a grid (Howard disc) in the microscope ocular. The grid was calibrated with a stage micrometer employing the objective used in these studies. A grid of 16 squares was used representing a square area of membrane with sides 107 μ in length.

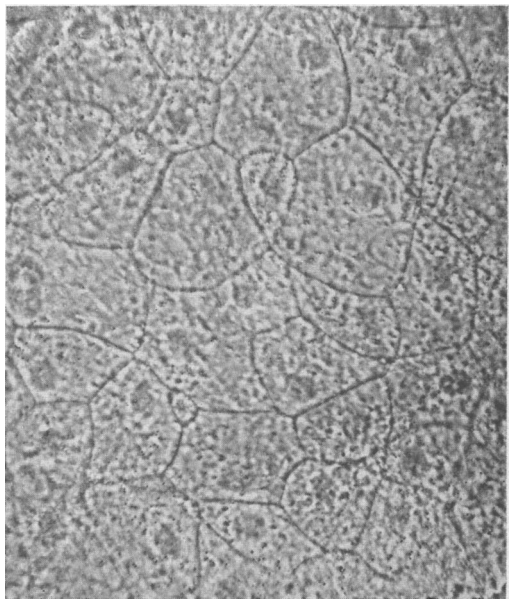

FIG. 1. Appearance of allantoic cells as seen with the phase contrast microscope. Observe clarity of cell outlines. Magnification 505×.

By cutting out the graph area representing the grid and from it the area representing complete cells, and by weighing the 2 parts of the paper, it was possible to calculate the mean area of the cells relative to the grid and hence the mean absolute area of the cells. To obtain the area of the cells in the original membrane, this value was corrected for the amount of shrinkage or stretching of the specimen using the ratio of the square of the internal diameter of the cork borer to the square of the mean diameter of the piece on the slide.

In counting allantoic cells from the amnion-yolk sac area it was not possible to punch out areas of the membrane so as to allow for shrinkage or stretching of the pieces. However, the preparations for microscopy and those for estimating the area of the membrane were prepared by the same operator in as similar a fashion as possible. Therefore, although the area of the cells measured may have been different from that existing before dissection of the embryo, the estimate of the total number of cells present should be correct.

Measurement of the area of the allantoic membrane. The allantoic membrane was removed from the embryonated egg by the following procedure: The pointed end of the egg

TABLE I. Illustrative Protocol of Allantoic Cell Counts on One Piece of Membrane (CA) from a 10-Day Chick Embryo.

Observer*	Diameters of membrane piece	(Mean diameter)^2	No. of complete cells	Wt of traced paper: Whole grid	Wt of traced paper: Complete cells	Mean No. of cells/cm^2†
	cm	cm^2		g	g	$\times 10^5$
1	.94 1.35	1.31	12	.6132	.3658	1.45
2	.9 1.31	1.21	11	.5955	.3094	1.41
3	.9 1.32	1.23	14	.5860	.2701	2.06
4	.92 1.32	1.25	7	.5956	.1950	1.48

* Each observer examined a separate field in the same piece of membrane.

† Calculated = Corrected No. of cells per grid $\times \frac{1}{\text{area of grid}}$ = No. of complete cells $\times \frac{\text{grid wt}}{\text{cell wt}} \times \frac{(\text{mean diameter})^2}{(1.26\text{ cm})^2} \times \frac{1}{1.14 \times 10^{-4}}$.

was cut off, the egg was immersed in warm buffered saline, and albumin and yolk were drained. The chorioallantoic membrane with the enclosed amnion, embryo, and yolk sac, as well as the remainder of the allantoic membrane, was floated free from the egg shell. The yolk sac then was removed by dissection, and the chorioallantoic membrane was dissected out. It should be emphasized that allantoic membrane in the region of the yolk sac is not fused with this structure. In the region of the amnion, however, the allantoic membrane is partly fused. The various membranes were floated onto a glass surface. The outlines were marked on the glass, from this traced to paper, and the area of the paper was measured by weighing. The area of the membrane attached to the egg shell, *i.e.*, the chorioallantoic membrane, was also measured by an independent method. The outline of the membrane was traced on the shell under the candler. The egg was emptied and the portion of the shell not covered by membrane was cut away. The remaining egg shell was weighed. Eight pieces were carefully punched from the shell with a cork borer of known internal diameter, and these pieces were weighed. From the 2 weights and from the diameter of the cork borer the total area of the shell covered by the chorioallantoic membrane was calculated.

Results. Determination of the area of allantoic cells. The cells enclosed in the grid were drawn independently by 4 different observers. The values for the cell areas were calculated by weight from these drawings; the range of values was about 10% of the mean in one set of 4 drawings and 5% in another set. It was found in later experiments that even when counting cells in different areas individual observers did not count systematically higher or lower than the others. Of approximately 90 intercellular boundaries in one area drawn by 4 observers there was disagreement on the presence of only one such boundary.

Because only about 10 cells were included in each grid drawing on the average and because there was a wide range in cell size, it is possible that the results were biased in that there was more chance of a small cell than a large cell being completely enclosed in the grid. The effect would be to raise slightly the estimate of the total number of allantoic cells present. In one experiment it was found that the effect of increasing the area of the grid 4-fold was to lower the estimate of the mean cell area by 2.5%. This source of error could, therefore, be disregarded.

The allantoic membrane can be described in 2 portions: 1) that fused with the chorion and opposed with it to the shell membrane,

TABLE II. Mean Allantoic Cell Counts on Various Areas of Membrane of 10-Day Eggs.

Area of membrane	Estimated No. of cells per cm² × 10^{-6}			
	Egg 1	Egg 2	Egg 3	Mean
CA near air sac	1.21 (3)*	2.38 (2)	1.58 (4)	1.63 (9)
" " middle	1.90 (3)	1.76 (4)	1.49 (4)	1.71(11)
" " albumen	1.43 (3)	1.57 (3)	2.06 (4)	1.72(10)
Mean	1.51 (9)	1.84 (9)	1.70(12)	1.68(30)

	Egg 4	Egg 5	Egg 6	Egg 7	Egg 8
AYA†	2.60 (3)	2.39 (3)	3.97 (3)	1.60 (3)	4.46 (3)

* Figures in parentheses show No. of counts from which mean is taken.
† Not corrected for shrinkage or stretching of this portion of membrane (*cf.* text).

and 2) that reflected over the yolk sac and amnion and partly fused with the latter. The first portion will be referred to as CA and the second as AYA allantoic membrane. The 2 portions were studied separately.

The CA portion of the membrane was studied first. Table I shows the protocol of counts of allantoic cells made on separate fields of one piece of the CA membrane obtained near the air sac of a 10-day-old egg and illustrates the calculations used. It also indicates that variations occur from field to field which are much greater than variations in drawing cells.

Table II shows an analysis of the results of 30 counts made by 4 observers on 3 areas of CA membrane from each of 3 eggs. It appears that there is no systematic variation in mean allantoic cell area from area to area of the CA allantoic membrane or from egg to egg. Analyses of variance were made which showed that there was no significant difference ($p > .05$) when comparing the variance "within areas" with that "between areas" or that "within eggs" with that "between eggs." On the basis of 27 consecutive counts on 3 eggs it is estimated that the mean number of allantoic cells per cm² is 1.66×10^5 ($\pm$SD 0.082×10^5).

Membranes from twelve 10-day eggs in 4 groups of similar egg weights (one group each: 51-51.5 g and 63-64.5 g; 2 groups: 54-56 g eggs) were counted. Analysis of variance showed no significant relation of egg weight to mean cell area ($p > 0.05$). The mean number of allantoic cells per cm² in this experiment was 1.735×10^5.

The number of allantoic cells per cm² of the AYA portion of the allantoic membrane as shown in Table II was estimated as 3.00×10^5 ($\pm$SD 0.315×10^5) on the basis of 15 counts made on 5 eggs. Analysis of variance showed as with the CA portion of the membrane that the variance "between eggs" was not significantly greater than that "within eggs" ($p > .05$).

Area of the allantoic membrane. The results of a number of measurements appear in Table III. The area of the CA portion of the membrane obtained by weighing of the shell covered by the membrane was consistently higher than that obtained by direct measurement of the membrane. Since the membrane was observed to shrink somewhat while floating in saline, the higher estimate was used for the final calculations. The area of the AYA portion of the membrane was determined by direct measurement only, but the measurements were consistent.

It was found that in eggs incubated from the 4th day without being turned the area of the CA membrane was 31% less than in eggs incubated in the usual fashion. It appears that a previous estimate of the area of the allantoic membrane(3), which agreed with

TABLE III. Area of the Allantoic Membrane.

Egg	Chorionic portion (CA)		Amnion-yolk sac portion (AYA)
	By direct measurement	By wt of egg shell covered	By direct measurement
	cm²	cm²	cm²
A	49.5	58.2	26.4
B	44.2	57.8	30.5
C	49.7	57.8	30.4
Mean*	47.8	57.9	29.1

* Estimated mean total area = 57.9 + 29.1 = 87.0 cm².

TABLE IV. Mean Number of Cells Lining Allantoic Cavity.

Membrane	Mean area of membrane/egg	Mean No. of cells/cm²	Mean No. of cells/egg
Chorionic portion (CA)	57.9 cm² (SD .135)	1.66×10^5 (SD $.082 \times 10^5$)	9.60×10^6 (SD 0.15×10^6)*
Amnion and yolk sac portion (AYA)	29.1 cm² (SD 1.35)	3.00×10^5 (SD $.315 \times 10^5$)	8.73×10^6 (SD 0.97×10^6)*
CA + AYA	87.0 cm²	—	1.83×10^7 (SD 0.1×10^7)

* Approximate estimate ignoring possible covariance.

that reported earlier(1), was in error because in this measurement the area of the yolk sac itself was included. The area of the yolk sac is 35.2 cm² . Subtracting this area from the earlier estimate of 121 cm² gives 85.8 cm² which agrees well with the present estimate which is 87.0 cm².

The number of cells lining the allantoic cavity. From the above data it is possible to estimate the number of cells lining the entire allantoic cavity, and the details of the calculation are set out in Table IV. In the case of the 10-day chick embryo, the total number of cells lining the allantoic cavity is 1.8×10^7. If nearly all the allantoic cells of the CA portion of the membrane were included, the number of allantoic cells in a deembryonated egg would be about 8×10^6.

Discussion. The present value obtained for the number of cells in the allantoic cavity of 10-day chick embryos is about 10-fold lower than that of earlier estimates which have been reviewed recently(1). It is probable that the new value is more nearly correct because the living cells were directly observed in a way that avoided confusion with other membrane layers, and because the present estimate does not rest on unproved assumptions as did some earlier computations. Since much quantitative work, summarized in recent papers(1,2), on the multiplication of influenza virus has been based on the assumption that there are at least 10^8 susceptible cells lining the allantoic cavity, certain interpretations may require reexamination in the light of the present estimate of 1.8×10^7 cells. Recent data from this laboratory(2) indicated that no more than 10^7 virus particles were needed in the inoculum to cause marked alterations in the reproductive process in the allantoic cavity.

Summary. 1. The mean number of allantoic cells per square centimeter of the allantoic membrane in 10-day embryonated chicken eggs has been estimated by direct counting with the phase contrast microscope. 2. In the chorionic (CA) portion of the allantoic membrane there were 1.66×10^5, and in the amnion-yolk sac (AYA) portion 3.00×10^5 allantoic cells per cm². 3. The total area of the allantoic membrane was measured directly. The area of the CA portion was 57.9 cm², that of the AYA portion was 29.1 cm², and that of the entire membrane was 87.0 cm². 4. The number of cells lining the allantoic cavity of the 10-day chick embryo was estimated to be 1.8×10^7.

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