THE SYNTHESIS OF DNA, RNA, AND NUCLEAR PROTEIN IN NORMAL AND TUMOR STRAIN CELLS

II. Fresh Embryo Mouse Cells

JOHN SEED

From the Department of Radiotherapeutics, University of Cambridge, England

ABSTRACT

Interferometric and photometric measurements have been made on replicating embryo mouse cell cultures. From a study of the relations between successive physical measurements on individual cells, it was found that the net syntheses of DNA, nuclear RNA, nuclear protein, and cytoplasmic RNA are closely associated during interphase. In auxiliary experiments, an inhibition of the onset of DNA synthesis (produced by a dose of X-rays) was found to block the majority of the accumulation of nuclear protein and nuclear RNA. These results are consistent with others previously reported in dividing cell cultures freshly prepared from normal tissues.

INTRODUCTION

In earlier papers (Seed, 1961, 1962, 1963, 1966 *a*), the investigation of the syntheses of DNA, RNA, and nuclear protein in replicating animal cell cultures has been described. The present experiments with freshly prepared embryo mouse cell cultures had three aims:

1. To investigate the relations between net DNA, RNA, and protein syntheses during interphase in the nuclei of replicating cells, by making correlation plots of successive physical measurements on the same individual cells.
2. To investigate the relationship between DNA synthesis and the net synthesis of cytoplasmic RNA during interphase.
3. To prevent the initiation of DNA synthesis by administering a dose of X-irradiation to a replicating cell culture, and then to measure the amounts by which nuclear protein and nuclear RNA had increased after one interphase time. In this way it was planned to elucidate further the relations between DNA, RNA, and nuclear protein syntheses within the replicating cell.

EXPERIMENTAL PROCEDURE

Fresh embryo mouse cells were cultured on quartz cover slips as previously described (Seed, 1962, 1966 *a*): Eagle's medium (Eagle, 1959), supplemented with 0.25% lactalbumin hydrolysate, and 20% bovine serum were used for growing the cells. 14-day embryos were used for the cultures.

In preliminary experiments, where irradiated cell cultures were followed for extended periods by low-power time-lapse photography, it had been found that a single X-ray dose of 1250 R was sufficient to abolish mitosis for approximately one interphase time, and that there was no cell death during this period. Accordingly, at the start of the present experiments, six cultures were each irradiated with 1250 R (3½ min each); the cultures were then reincubated and were subsequently fixed at times up to 14 hr after the time of irradiation. (All cultures were rinsed briefly in warm saline before fixing in methanol.)

Two unirradiated (control) cultures were fixed at the beginning and two at the end of the experiment when the last irradiated culture was fixed. Over the time period of the experiment, an additional control culture was filmed by low-power time-lapse cinephotomicrography and, from subsequent examination of this film, the intermitotic time of the cells (690 min) was found.

Measurements are given below on a control culture fixed at the end of the experiment, and on two irradiated cultures fixed 540 and 730 min, respectively, after irradiation. Additional measurements, not shown, were made on a control culture fixed at the beginning of the experiment, in order to confirm that the nuclear dry mass per cell remained constant over the time period of the experiment.

Physical Measurements

Before making the physical measurements on the individual cells, low molecular weight compounds were first extracted from the cultures in 1% PCA (perchloric acid) at 4°C for 30 min. Four types of measurements were made: total UV absorption at 2536 A (total cell nucleic acid), nuclear UV absorption at 2536 A (nuclear nucleic acid, constituting roughly ⅔ RNA and the remainder DNA), nuclear dry mass ($\simeq$ ⅚ nuclear protein), and Feulgen stain (DNA) (see Seed, 1966 *a*). Cytoplasmic RNA values were obtained by subtracting nuclear UV absorption from total UV absorption. The excess nucleolar absorption was not included in the nuclear UV and dry mass measurements. (See Seed, 1966 *a*.)

The measurements shown in Figs. 1 to 6 are expressed in arbitrary units of nucleic acid and separately of dry mass: 1 nucleic acid unit $\simeq 6.3 \times 10^{-13}$ g, and 1 dry mass unit $\simeq 1.6 \times 10^{-12}$ g.

RESULTS

Control Cells

NUCLEAR METABOLISM: It was previously shown (Seed, 1962, 1963) that, the form of the time synthesis curve for DNA being known, it is possible to follow the progress of the interphase growth of nuclear dry mass by plotting the measurements for individual cells against the corresponding values of Feulgen DNA and nuclear nucleic acid (RNA + DNA) for the same cells.

In Fig. 1, it is seen that the nuclear nucleic acid and nuclear dry mass measurements made on the same individual cells are approximately proportional during interphase, and that the Feulgen DNA and nuclear dry mass values are similarly proportional. A least squares analysis gives the high correlation of $r = 0.900$ between nuclear nucleic acid and dry mass, and a similar correlation of $r = 0.869$ between DNA and dry mass. The application of Fisher's z-transformation (Fisher, 1936; Fisher and Yates, 1948) shows that there is no significant difference between the correlations, giving $t = 1.05$, which does not reach the value to be attained at the $P = 0.05$ level ($t = 1.97$), nor at the $P = 0.1$ level ($t = 1.65$), but merely equals that at the $P = 0.3$ level ($t = 1.04$).

From the similarity of these high correlations between the physical measurements on individual cells at the various stages of interphase, it is concluded that the syntheses of all three components, DNA, RNA, and protein, are closely associated in interphase in the nuclei of freshly prepared replicating embryo mouse cells. This result is in accord with others previously reported, both from correlation methods and by time-lapse photography with freshly prepared normal cells (Seed, 1962, 1963).

CYTOPLASMIC RNA: It was extremely difficult to measure total cell nucleic acid (and thus cytoplasmic RNA) in this cell type because of the great propensity for cytoplasmic overlapping between neighboring cells. Of the 112 cells, in Fig. 1, on which nuclear measurements were made, it was possible to make measurements of total UV absorption on only 35 cells.

The synthesis of cytoplasmic RNA during interphase was followed by a method analogous to that used for nuclear dry mass: DNA and nuclear nucleic acid values for individual cells were each plotted separately against the corresponding total cell nucleic acid values and the correlations were compared. As before (Seed 1966 *a*), total nucleic acid rather than cytoplasmic RNA was plotted because the latter is obtained by subtraction and its use entails the combination of two errors (DNA and nuclear nucleic acid comprise $\simeq$ ⅔ and $\simeq$ ⅓, respectively, of total nucleic acid).

Now if, in embryo mouse cells, the synthesis of cytoplasmic RNA were independent from the synthesis of DNA, one would not expect to find a high correlation between total cell nucleic acid and DNA content, because DNA comprises only $\simeq$ ⅔ of the total nucleic acid. Nevertheless, in a cell engaged in RNA synthesis, it would be reasonable to expect a measure of correlation between the quantities of nuclear RNA and cytoplasmic RNA, because the synthesis of cytoplasmic RNA appears to occur in the nucleus (from pre-

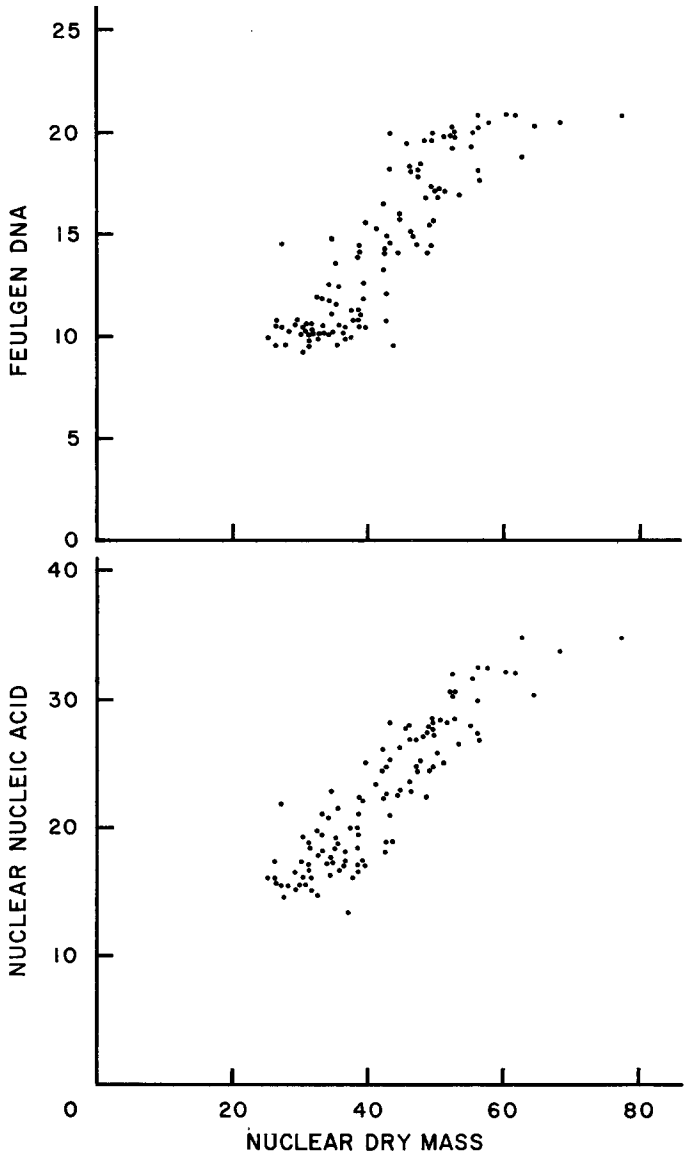

FIGURE 1 Feulgen stain (DNA) and nuclear ultraviolet absorption (at 2536 A, nuclear RNA + DNA) measurements plotted against the corresponding nuclear dry mass values for replicating embryo mouse cells. All quantities are expressed in arbitrary units.

cursor migration experiments and experiments with Actinomycin D (Seed, unpublished experiments). Therefore, if the growth of the cytoplasmic RNA is not associated with DNA replication, one would expect to find a higher correlation between nuclear nucleic acid and total cell nucleic acid than between DNA and total cell nucleic acid.

In reference to Fig. 2, the nuclear nucleic acid and DNA are each roughly proportional to the total cell nucleic acid, with a correlation of $r = 0.839$ between nuclear nucleic acid and total nucleic acid and a correlation of $r = 0.844$ between DNA and total nucleic acid; Fisher's z-transformation (Fisher, 1936; Fisher and Yates, 1948) gives $t = 0.071$, which does not reach the value to be attained at the $P = 0.05$ level ($t = 2.00$), nor that to be attained at the $P = 0.5$ level ($t = 0.68$): there is no significant difference between the two

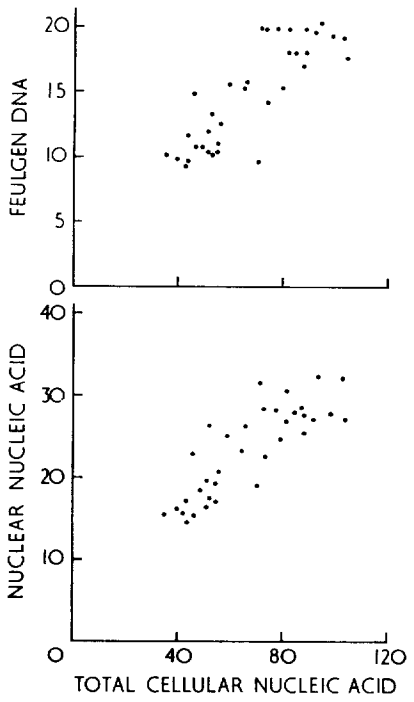

FIGURE 2 Feulgen stain (DNA) and nuclear ultraviolet absorption (at 2536 A, nuclear RNA + DNA) measurements plotted against the corresponding total cell ultraviolet absorptions (at 2536 A, total cell nucleic acid) for replicating embryo mouse cells. There are 35 cells on the plot, measurable out of the 112 cells in the population in Fig. 1.

correlations. Although there are fewer measurements in Fig. 2 than in previous experiments, this result is in agreement with that obtained for fresh embryo human and monkey kidney cells (Seed, 1966 *a*).

From the similarity of the high correlations between the physical measurements, it is concluded that the net syntheses of cytoplasmic RNA and of DNA are closely associated during the interphase of replicating embryo mouse cells. Supplementary evidence for this is afforded by the similarity of the ratios of cytoplasmic RNA/DNA at the 2m-DNA and 4m-DNA levels (3.06 and 3.11, respectively, from Fig. 6), a result that would not be obtained if a major component of cytoplasmic RNA accumulated independently from DNA replication (see Fig. 1 in the preceding paper).

After subtraction of the nuclear nucleic acid values from the total nucleic acid values, a least squares analysis of the plots of cytoplasmic RNA values against DNA values and of cytoplasmic RNA values against nuclear nucleic acid values gives correlations of $r = 0.741$ and $r = 0.715$, respectively: there is no significant difference between these correlations ($t = 0.228$). Because these cytoplasmic RNA values combine the errors of two measurements (see above), it is more accurate to test the total nucleic acid correlations as in Fig. 2.

Irradiation Experiments Blocking DNA Synthesis

In the preceding section, a study has been presented of the relations between successive physical measurements on the one population of control cells; in the following section we shall compare irradiated with control cell populations.

The DNA and nuclear nucleic acid values are

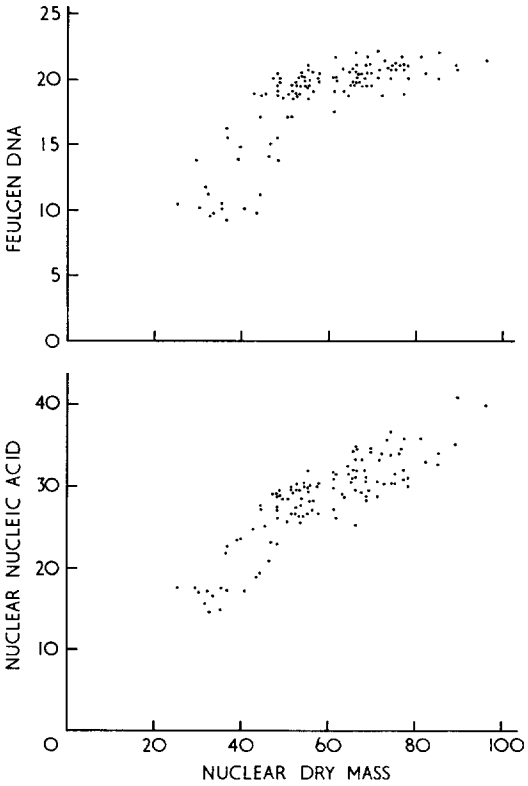

FIGURE 3 Feulgen stain (DNA) and nuclear ultraviolet absorption (at 2536 A, nuclear RNA + DNA) measurements plotted against the corresponding nuclear dry mass values for embryo mouse cells fixed 540 min after X-irradiation with 1250 R (I_1).

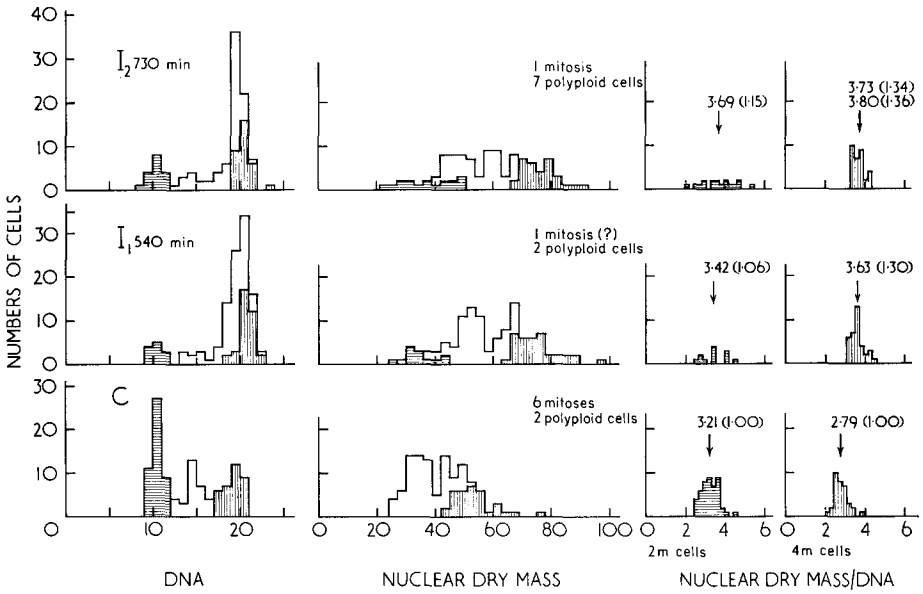

FIGURE 4 Distributions of Feulgen stain (DNA) and nuclear dry mass measurements on individual embryo mouse cells, intermitotic time 690 min. At the right of the figure are plotted the nuclear dry mass/DNA ratios for controls and for cells having DNA synthesis blocked since the time of irradiation: ≣ (horizontal shading), cells with 2m-DNA values; ||| (vertical shading), cells with 4m-DNA values. Lower section, C control cells (from Fig. 2); center section, I_1 cells fixed 540 min after X-irradiation with 1250 R; upper section, I_2 cells fixed 730 min after X-irradiation with 1250 R (from Fig. 3) All quantities are expressed in arbitrary units. Cells in mitosis and polyploid cells are not included in the measurements, but the numbers of such cells found among those measured are given above the dry mass distribution for each culture.

plotted in Fig. 3, against the corresponding nuclear dry mass values for cells in an irradiated culture (I_1) fixed 540 min after irradiation. The distributions of values of DNA, nuclear dry mass, and nuclear nucleic acid for control and irradiated cultures are plotted in Figs. 4 and 5.

DNA SYNTHESIS: The distribution of Feulgen-stain values for the control culture (Fig. 4) shows a well defined group at the 2m-DNA (postelophase) level and a less well defined group at the 4m-DNA (preprophase) level, together with a number of intermediate synthesizing cells. The cells in the 2m and 4m groups are in the presynthetic and postsynthetic delay periods.

In the irradiated cultures (Fig. 4) there is a large accumulation of DNA values at the 4m level, with relatively few cells in synthesis. This is in accordance with work previously quoted on the lack of effect of radiation (1250 R) on DNA synthesis already in progress (Kelly et al., 1957; Caspersson et al., 1958; Dickson et al., 1958; Painter and Robertson, 1959; Yamada and Puck, 1961; Seed, 1961); cells irradiated in DNA synthesis go on to complete the cycle but are prevented from progressing further by the radiation block in mitosis. In the present case, some cells also remain at the 2m-DNA level (Holmes and Mee, 1955; Kelly et al., 1955; Lajtha et al., 1958; Howard and Pelc, 1953; Pelc and Howard, 1955).

NUCLEAR DRY MASS: A general shift to higher dry mass values was apparent in the irradiated culture (Fig. 4), in the same way as was described previously (Seed, 1966 *a*). There are two classes of cells from which useful information can be gained: those in which DNA synthesis has been blocked at the 4m-DNA and at the 2m-DNA levels, respectively, since the time of irradiation.

In the control culture, out of a total of 112 cells, there are 34 cells with DNA contents at about the 4m value. Therefore, in the irradiated culture I_1, out of a total of 118 cells, there will be a number $34/112 \times 118 \simeq 36$ of cells in which the initiation of DNA synthesis has been prevented since the time of irradiation (540 min), mitosis having been abolished. Similar considerations apply to the $34/112 \times 105 \simeq 32$ cells out of a

total of 105 cells in the irradiated culture I_2 (730 min).

In order to identify these cells in the dry mass and DNA distributions of the irradiated cultures, it has been assumed that they will have increased most in nuclear dry mass: therefore, distributions of values, containing 36 and 32 cells, respectively, have been drawn in the right ends of the dry mass distributions of the irradiated cultures I_1 and I_2 shown in Fig. 4. As before, (Seed, 1966 *a*), in constructing these distributions, the attempt has been made to include as many high values as possible, consistent with maintaining a general resemblance in shape to the dry mass distribution for the 4m-DNA cells in the control culture (shaded by vertical lines in Fig. 4). For this reason the dry mass increases observed will tend to represent a *maximum* value for the fraction of nuclear dry mass synthesis dissociated from DNA replication: this is clear from inspection of Fig. 4 where the distributions are shaded vertically, along with the corresponding DNA values for the cells.

The ratios of nuclear dry mass/DNA for these 4m-DNA cells are plotted at the right of Fig. 4, where they are compared with the corresponding ratios for the 4m-DNA cells in the control culture: the mean ratios, in arbitrary units, and the amounts of increase (in parentheses) relative to the controls as 1.00 are indicated by arrows.

It is seen that, relative to the controls, the irradiated groups of cells under consideration have increased in nuclear dry mass by 0.30 in 540 min (I_1) and by 0.34 to 0.36 in 730 min (I_2). The two ratios for I_2 arise because it is necessary to make a correction for the presence in the I_2 culture of 1 mitosis and 5 extra polyploid cells (over the control culture). If these 6 cells escaping the DNA block have come from the group of 36 cells blocked at the 4m-DNA level since the time of irradiation, then we must take a dry mass distribution of only 30 cells to determine the mean nuclear dry mass increase (0.36): on the other hand, if the 6 cells have not come from this group, then no correction is necessary (0.34). Although we do not know which is the case, the consequent ambiguity in the result is a small one. In the time-lapse films of irradiated embryo mouse cultures, it was found that mitosis began sporadically at about one interphase time after irradiation: divided cells could be recognized as such for some time after mitosis, and it is not possible that numbers of cells could have "leaked" past the mitotic block in I_2 without being noticed. In the irradiated culture I_1 there was a rounded cell not positively identified as a mitosis: this cell has been ignored.

Assuming a constant rate of increase of nuclear dry mass, the increases observed for the I_1 and I_2 cultures, weighted for the numbers of cells in each group, give a mean increase of 0.36 in nuclear dry mass in 690 min, one interphase time. This corresponds to an increase of 0.40 in nuclear protein, allowing for the contribution from nucleic acid.

There are relatively few cells (12 and 17, respectively) remaining at the 2m-DNA level in the I_1 and I_2 cultures since the time of irradiation, although it is clear from Fig. 4 that some cells have recently left this block and are now in DNA synthesis. The nuclear dry mass/DNA ratios for these 2m-DNA cells, with the comparison values for the 2m-DNA control cells, are plotted at the right of Fig. 4 along with the mean ratios and amounts of increase indicated by arrows. It is seen that, relative to the controls, these cells have increased in nuclear dry mass by 0.06 and 0.15

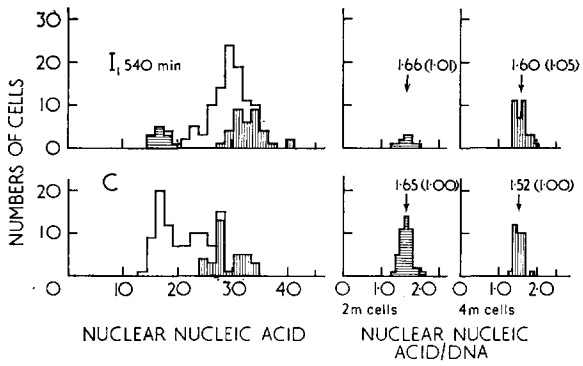

FIGURE 5 Distributions of nuclear ultraviolet absorption (at 2536 A, nuclear RNA + DNA) measurements on embryo mouse cells. At the right of the figure are plotted the nuclear nucleic acid/DNA ratios for controls and for cells having DNA synthesis blocked since the time of irradiation: ≣, cells with 2m-DNA values; |||, cells with 4m-DNA values. Lower section, C control cells; upper section, I_1 cells fixed 540 min after X-irradiation with 1250 R.

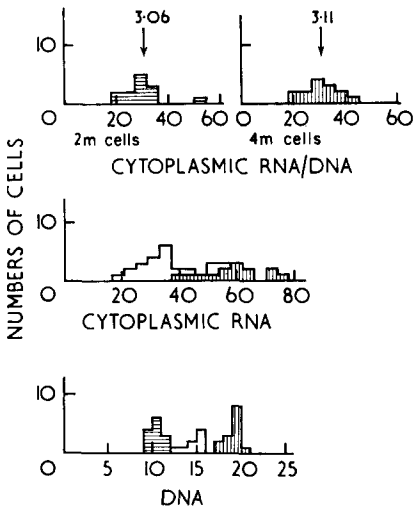

FIGURE 6 Distributions of Feulgen stain (DNA) and cytoplasmic ultraviolet absorption (cytoplasmic RNA) on individual replicating embryo mouse cells. The cytoplasmic RNA/DNA ratios for cells with 2m-DNA values (≣) and for cells with 4m-DNA values (|||) are plotted at the top of the figure.

since the time of irradiation and in the absence of DNA synthesis. Correcting for the presence of nuclear nucleic acid, this gives an increase of 0.13 in nuclear protein in one interphase time. Because of the relatively small number of cells remaining at the 2m-DNA level in this experiment, these values are not so accurate as the corresponding ones at the 4m-DNA level.

NUCLEAR NUCLEIC ACID: We can make useful conclusions from the same two classes of cells used in the dry mass distributions above.

Out of a total of 118 cells in the irradiated culture I_1, there will be $34/112 \times 118 \simeq 36$ cells in which DNA synthesis has been blocked at the 4m-DNA level since the time of irradiation: these cells correspond to the 34 cells at the 4m-DNA level in the control culture. Now, in the plots of the measurements on the irradiated culture I_1 (Fig. 3), there is an approximate relation at the 4m-DNA level between the increase in nuclear dry mass and that in nuclear nucleic acid within a cell; it is therefore valid to draw, in Fig. 5, a distribution of the same 36 cells as were used in the dry mass distribution in Fig. 4. In Fig. 5 the nuclear nucleic acid/DNA ratios are also plotted, showing a mean increase (in parentheses) of 0.05 over the control ratios in 540 min: this corresponds to an increase of 0.06 in 690 min, one interphase time, when DNA synthesis is blocked at the 4m-DNA level.

In the 12 cells in which DNA synthesis has remained blocked at the 2m-DNA level in the irradiated culture I_1, the mean nuclear nucleic acid/DNA ratio has increased by 0.01 over the control in 540 min.

If, in the irradiated culture, the nuclear RNA has increased in proportion to the nuclear protein, one would expect the increase of 0.4 in nuclear protein at the 4m-DNA level to be accompanied by a corresponding increase of $\simeq 0.4 \times \frac{2}{5} = 0.16$ in nuclear nucleic acid. The observed increase (0.06) is not significantly different (± 0.07) from that predicted.

CYTOPLASMIC RNA: No measurements of total cell RNA were made on the irradiated embryo mouse cultures because of the difficulties caused by frequent overlapping of cells. The distributions of cytoplasmic RNA and DNA values of measurable cells in the control culture are shown in Fig. 6.

DISCUSSION

The evidence here presented from the measurements on a population of replicating embryo mouse cells shows that the net syntheses of DNA, nuclear protein, nuclear RNA (in the chromatin), and cytoplasmic RNA are closely associated during interphase. The results agree with other experiments previously reported (Seed, 1961, 1962, 1963) on cells freshly derived from normal tissues.

The experiments with radiation constitute a different method which achieves the same end: by preventing the *initiation* of DNA synthesis it is shown that the majority of the accumulation of protein and RNA in the nucleus is associated with that event. A block in DNA synthesis at the 2m level for one interphase time resulted in an increase of 0.13 in nuclear protein and no increase in nuclear nucleic acid: a similar block at the 4m-DNA level resulted in increases of 0.40 in nuclear protein and 0.06 in nuclear nucleic acid.

These experiments are discussed along with others (Seed, 1966 *a*, *b*) in a later paper (1966 *c*).

Received for publication 15 July 1965.

REFERENCES

CASPERSSON, T., KLEIN, E., and RINGERTZ, N. R., 1958, *Cancer Research*, **18**, 857.

DICKSON, M., PAUL, J., and DAVIDSON, J. M., 1958, *Biochem. J.*, **70**, 18P.

EAGLE, H., 1959, *Science*, **130**, 433.

FISHER, R. A., 1936, Statistical Methods for Research Workers, Edinburgh, Oliver & Boyd Ltd., 1936.

FISHER, R. A., and YATES, F., 1948, Statistical Tables, Edinburgh, Oliver & Boyd Ltd., 1948.

HOLMES, B. E., and MEE, L. K., 1955, *Radiobiol. Symp. Proc., Liege, 1954*, 220.

HOWARD, A., and PELC, S. R., 1953, *Heredity*, **6**, (suppl.), 261.

KELLY, L. S., HIRSCH, J. D., BEACH, G., and PAGE, A. H., 1955, *Radiation Research* **2**, 490.

KELLY, L. S., HIRSCH, J. D., BEACH, G., and PETRAKIS, N. L., 1957, *Proc. Soc. Exp. Biol. and Med.*, **94**, 83.

LAJTHA, L. G., OLIVER, R., KUMATORI, T., and ELLIS, F., 1958, *Radiation Research*, **8**, 1.

PAINTER, R. B., and ROBERTSON, J. S., 1959, *Radiation Research*, **11**, 206.

PELC, S. R., and HOWARD, A., 1955, *Radiation Research*, **3**, 135.

SEED, J., 1961, *Nature*, **192**, 944.

SEED, J., 1962, *Proc. Roy. Soc., London, Series B*, **156**, 41.

SEED, J., 1963, *Nature*, **198**, 147.

SEED, J., 1966 *a*, *J. Cell Biol.*, **28**, 233.

SEED, J., 1966 *b*, *J. Cell Biol.*, **28**, 257.

SEED, J., 1966 *c*, *J. Cell Biol.*, **28**, 263.

YAMADA, M., and PUCK, T. T., 1961, *Proc. Nat. Acad. Sc.*, **47**, 1181.