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

I. Fresh Embryo Human Cells

JOHN SEED

From the Department of Radiotherapeutics, University of Cambridge, England

ABSTRACT

Interferometric and photometric measurements have been made on replicating embryo human 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. Additional measurements of DNA and cytoplasmic RNA on freshly prepared replicating monkey kidney cells gave similar results. In auxiliary experiments with embryo human cells, 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 RNA and about half the accumulation of cytoplasmic RNA. These results are consistent with others previously reported in dividing cell cultures freshly prepared from normal tissues.

INTRODUCTION

In recent years, much effort has been devoted to the study of the role of the nucleic acids in protein synthesis, and considerable speculation has occurred regarding the possible nature of processes that may control synthesis and reproduction, both in animal cells (Seed, 1963 b; Brachet, 1957; Caspersson, 1950; Swann, 1957, 1958) and in bacteria (Jacob and Monod, 1961).

It has been known for some time that, in animal and plant cells proceeding to division, the synthesis of DNA for a new cell takes place during only a part of interphase (see Fig. 1.), (Swift, 1950; Walker and Yates, 1952; Howard and Pelc, 1953; Firket, 1958; Richards et al., 1956). Until recently (Richards and Davies, 1958; McLeish, 1959, 1960; Seed, 1961, 1962, 1963 a, 1964), parallel investigations on the accumulation of protein during interphase had been neglected: however, if a tumor, characterized by an autonomy or by excessive and uncoordinated growth (e.g. Rusch, 1954; Willis, 1960), be associated with a deficiency in the control mechanisms leading to cell replication, then comparative studies of the relations between the nucleic acids and protein synthesized during the life cycle of cells of normal and tumor origin should be of particular interest.

In previous experiments (Seed, 1961, 1962, 1963 a), successive physical measurements of nucleic acids and protein were made on nuclei of individual cells grown in culture. The various ages of the cells in interphase (that is, the time since the last mitosis) were found by a time-lapse photographic technique (Walker and Yates, 1952; Seed, 1962, 1963 a), and, by plotting the measurements obtained for individual cells against the corresponding cell ages, average synthesis curves for nucleic acids and protein could be constructed.

With these techniques, it was shown that, during the interphase of rapidly dividing cells freshly prepared from normal tissues, the processes of DNA and the majority of net RNA and protein syn-
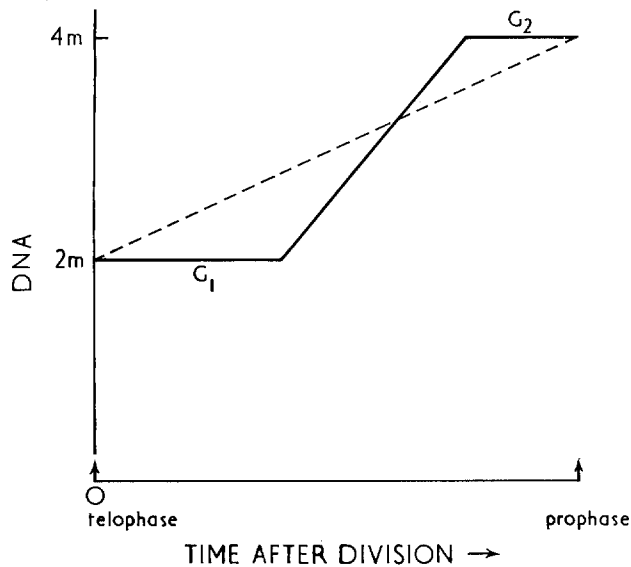
FIGURE 1 Model DNA synthesis curve for an animal cell (solid line).

theses in the nucleus began at approximately the same time and thereafter proceeded approximately together. However, in the rapidly dividing cell strains derived from or capable of producing tumors, a large part of nuclear RNA and protein accumulated continuously during interphase, in contrast with DNA synthesis which began only after a delay had elapsed after telophase, as in normal cells. In addition, for individual nuclei of replicating normal monkey kidney cells, similar high correlations were found between dry mass and DNA and between dry mass and (RNA + DNA): on the other hand, for the nuclei of HeLa strain cells, a significantly lower correlation was found between dry mass and DNA than between dry mass and (RNA + DNA) These correlation results were obtained from the same populations of cells as were used in the timing experiments, thus demonstrating the equivalence of the timing and correlation methods (Seed, 1963 a). Because of the genetic role attributed to DNA, it was suggested that this time disconnection of nuclear protein and RNA syntheses from the onset of DNA replication reflected a fundamental loss of control in tumor strain cells (Seed, 1961, 1962, 1963 a and b, 1964).

The aim of the present experiments was threefold:

1. To examine the relations between net DNA, RNA, and protein syntheses in the nuclei of replicating embryo human cells by making correlation plots of the successive

physical measurements on individual cells: as described above, the equivalence of this method to the timing experiments had already been demonstrated for monkey kidney and HeLa cells (Seed, 1963 a).

2. To investigate the relationship between DNA synthesis and the accumulation of cytoplasmic RNA during interphase in embryo human and also in freshly prepared replicating monkey kidney cells.

3. To produce a block in the initiation of DNA synthesis for one interphase time, and then to measure the amounts by which nuclear protein, nuclear RNA, and cytoplasmic RNA had increased during this time. In this way it was hoped to throw further light on the coupling between the syntheses of DNA, RNA, and protein in the replicating embryo human cell.

In these experiments it was planned to use radiation as a blocking agent for DNA synthesis (Seed, 1961). It had already been reported that the processes of RNA and protein syntheses themselves were relatively unaffected by radiation under conditions where DNA synthesis was inhibited in ascites tumor and other cells (see Klein and Forssberg, 1954; Caspersson et al., 1958; Hevesy, 1948; Mitchell, 1942). Similarly, it has been known for some time that radiation doses of the order of 1000 R produce little inhibition of DNA synthesis in progress, but act by delaying the initiation of a new cycle of synthesis, either in

the presynthetic period at the 2m-DNA level or in the premitotic period at the 4m-DNA level by preventing cells from entering mitosis (Howard and Pelc, 1953; Holmes and Mee, 1955; Pelc and Howard, 1955; Kelly et al., 1955, 1957; Lajtha et al., 1958; Caspersson et al., 1958; Dickson et al., 1958; Painter and Robertson, 1959; Whitfield and Rixon, 1959; Yamada and Puck, 1961; Seed, 1961). Thus, in experiments preliminary to the ones described in this paper, rapidly growing cultures of embryo human cells were irradiated with a single dose of X-rays and the cells were then followed by low power time-lapse photography for an extended period. In this way it was found that a radiation dose of 1250 R completely abolished mitosis in the embryo human cultures for longer than one interphase time, and that there was no cell death during this period. With this information, it was possible to set up the experiments.

METHODS

CELL CULTURES: Eagle's medium (Eagle 1959) supplemented with 10% human and 10% bovine sera with 0.25% lactalbumin hydrolysate was used for growing this cell type. Fresh cultures were prepared from 16 weeks-old embryo human skin in babies' feeding bottles as described previously (Seed, 1962). After three days' growth, cells were removed from the walls of the bottle by pipetting with growth medium or weak trypsin solution. The cells were then resuspended and equal volumes of suspension were used to prepare a series of similar cultures on quartz cover slips: these were then mounted in small culture slides, each of approximately 2 ml in volume. After allowing the cells to settle and incubate for 20 to 30 hr, the growth medium in the slides was changed (with prewarmed medium at pH 7.2), and the cultures were examined briefly under the microscope in readiness for the beginning of the experiments 5 to 6 hr later.

EXPERIMENTAL PROCEDURE: At the start of the experiments, two cultures were fixed for subsequent use as controls: these were rinsed briefly in warm saline and then fixed in methanol. Two more cultures were set aside in the incubator for use as controls, to be fixed at the end of the experiment. During the course of the experiment a low-power time-lapse cine film (on a microscope contained in an incubator) was made of an additional control culture, and from subsequent examination of this film the intermitotic time (850 min) of the cells was found. The remaining six cultures were each irradiated with an X-ray dose of 1250 R (3½ min each, 220 kv at 15 ma from a G. E. Maximar with 1 mm Al filter). The cultures

were then reincubated and were ultimately fixed at times up to 18½ hr after irradiation. After the last irradiated culture had been fixed, the two remaining control (unirradiated) cultures were fixed.

Measurements were made on one control culture fixed at the end of the experiments, and on irradiated cultures fixed at 744 and 897 min after irradiation. In addition, a control culture fixed at the beginning of the experiment was measured in order to confirm that the nuclear dry mass per cell remained constant over the time of the experiment, i.e. that there was a steady state in the controls: these measurements are not given here as they were similar to those of the other control.

PHYSICAL MEASUREMENTS: The fixed cultures were examined under the microscope and areas of equivalent cell population density were retained for measurement in each culture: the remaining cells were scraped off the quartz cover slips with the aid of a cataract knife.

Measurements were made of (a) nuclear nucleic acid and total cell nucleic acid (ultraviolet microscope), (b) nuclear dry mass (interference microscope) and (c) DNA content (Feulgen stain). The excess nucleolar absorption was not included in the nuclear UV absorption and dry mass measurements. (See Notes on the measurements in the following section.)

ULTRAVIOLET PHOTOMETRIC MEASUREMENTS: The cultures were first extracted in 1% PCA (perchloric acid) at 4°C for 30 min to remove low molecular weight compounds. The quartz cover slips to which the cells were adhering were then mounted in glycerol for the ultraviolet absorption measurements: a series of overlapping photographs of the cells were made using a Cooke quartz monochromat (6 mm, NA 0.7) with the 2536 A mercury line as illuminant. Following King and Roe (1953), a condenser aperture of 0.42 was used, and alongside each photograph was superimposed the UV image of a rhodium step wedge (Walker and Davies, 1950; Walker, 1956) for contrast measurements.

The close proximity of the 2536 A line to the nucleic acid absorption peak at 2600 A enabled density measurements to be related directly to nucleic acid content: the total quantity of nucleic acid (N) in each cell nucleus was obtained by making spot density measurements (5 to 10 per nucleus), or density traces relative to background on the photographic image of each cell nucleus, from which:

$$N = \frac{DA \, 10^{-3}}{20} \, g$$

where D is the optical density, assuming an extinction coefficient of 20. The nuclear areas A were obtained from planimeter measurements on photographs of the Feulgen-stained nuclei.

From the UV plates, prints were made on which the cells were numbered to enable identification throughout the remainder of the experiments.

Neglecting the small contribution from protein, (at 2536 A, the ratio of nucleic acid/protein absorption in the nucleus is $\overset{\sim}{>} 10$), in rapidly dividing cells of this and similar types, RNA appears to contribute around $\frac{2}{5}$ of the nuclear UV absorption and DNA the remainder (Walker and Yates, 1952; Killander et al., 1962; Bonner et al., 1961, Seed, 1963 a; Lin and Chargaff, 1964). As will be seen later, in the present experiments the behavior of nuclear RNA during the cell cycle has been inferred from the comparison of plots or distributions of nuclear nucleic acid (RNA + DNA) and of DNA: in this way, the combination of errors that would result from subtraction of individual DNA from nuclear nucleic acid measurements has been eliminated. This procedure results in an appreciable gain in information that can be obtained from the results.

The integrated UV absorption of individual cells (total cell nucleic acid) was obtained from density measurements on the UV photographic plates using the Deeley (1955) scanning microdensitometer at low magnification. The instrument was used in conjunction with a leaf iris of adjustable shape, designed to enable individual cells to be isolated from others growing nearby.

When required, the amount of cytoplasmic RNA in a cell could be obtained by subtraction of the nuclear from the total cell UV measurements (Fig. 7).

DRY MASS MEASUREMENTS: The cells were remounted in water and were observed on the Cooke-Dyson interference microscope with even-field illumination. Using a photometer eyepiece, the optical retardation, relative to the mercury green line, was determined for each of the cell nuclei previously measured in the ultraviolet, from which

$$M = \frac{\Delta A}{0 \cdot 18} \, g$$

where A is the nuclear area and Δ is the optical retardation in centimeters (Davies and Wilkins, 1952; Barer, 1952). Usually between 4 and 8 readings were made in each nucleus. Because most of the nuclear dry mass consists of protein ($\overset{\sim}{=} \frac{5}{6}$), M is effectively the nuclear protein content. A correction has been made for the nucleic acid content where this is important (see p. 244).

DNA MEASUREMENTS: Next, the cells were stained with the Feulgen reaction: all the irradiated and control cultures were stained together during this procedure so that the DNA values could readily be compared. The cells were mounted in immersion oil of similar refractive index.

The amount of stain absorbing at 5600 A in each cell nucleus was then measured with the Deeley integrating microdensitometer (Deeley, 1955) to give the relative amount of DNA in each cell.

In Figs. 2 to 7 it was found convenient to work throughout in arbitrary units of nucleic acid and separately of dry mass. The reader can readily convert these to the absolute units of the equations above by noting:

1 nucleic acid unit $\simeq 5.8 \times 10^{-13}$ g

1 dry mass unit $\simeq 1.5 \times 10^{-12}$ g

NOTES ON THE MEASUREMENTS

1. The accuracy with which a single reading of optical path difference (OPD) can be measured is $\simeq \pm 1/100$ λ. Under the conditions of growth used here, the OPD of an embryo human control cell nucleus was usually $\simeq \frac{1}{7}$ λ, and that of a few nuclei was only $\simeq \frac{1}{10}$ λ. Although several (4 to 8) OPD readings were made for each nucleus, an accuracy of better than 10 to 12% for $\Delta \cdot A$ would not be expected for a single control nucleus of this cell type.

2. (a) The accuracy of the nuclear nucleic acid measurements for individual cells ($D \cdot A$) is expected to be around $\pm 10\%$, and the accuracy of the DNA measurements about $\pm 5\%$: the improved accuracy for DNA is shown by the closer grouping of the DNA values. (b) Any error in the measurement of area will affect nuclear nucleic acid and nuclear dry mass equally, leaving DNA unaffected as it is measured by a scanning method. The effect of (a) will be to improve slightly the DNA-dry mass correlation, and the effect of (b) will be to improve relatively the nuclear nucleic acid–dry mass correlation.

It must be remembered that the comparison of the correlations is made over the whole range of values which is much greater than the range of errors; in addition the comparison is made *within* the same cell population.

In the present experiments, areas of the Feulgen-stained nuclei were measured. These areas were some 5 to 10% lower than those found from phase-contrast micrographs before staining, at least partly because of the apparent thickness of the nuclear membrane in a phase-contrast micrograph. There was no difference in apparent per cent area reduction between cells of different interphase ages.

3. Nucleoli were omitted from the nuclear UV and dry mass measurements, that is, the nuclear values were calculated including the nucleolar areas and as if the nucleoli had the same mean UV absorption and dry mass as the remainder of the nucleus. However, the nucleoli are included in the measurements of the integrated UV absorption of the whole cell; thus the cytoplasmic RNA values in Figs. 3 and 7, obtained by subtraction of nuclear UV absorption from total

UV absorption, also contain the excess nucleolar UV absorption and omit the absorptions of the thin cytoplasmic layers overlapping the nucleus in the flat cells grown in culture. Each of these effects is ∼5%: they act in opposite directions and can be neglected. Radioautography and dry mass measurements (Seed 1963 a, 1965, and unpublished) showed that the nucleolar syntheses followed a time course approximately similar to the remainder of the nucleus.

4. Cells in telophase or early prophase were not measured.

5. Nuclei that appeared to be overlapped by cytoplasm of neighboring cells were not measured. These were few in the present cultures: however, measurement of total cell UV absorption could be made on only one-third to two-thirds of the numbers of cells on which nuclear measurements had been made, on account of cytoplasmic overlapping. Because of the greater difficulties in measuring the total cell UV absorption (e.g. associated with overlapping, uniformity of illumination), errors of up to ±15% might occur in certain cases.

6. In the comparison of the control and irradiation measurements, the chief potential source of error in the calculation of the dry mass (and nucleic acid) increases in the irradiated cultures arises through a possible inconsistency of Feulgen-staining between control and irradiated cultures. The 2m-DNA and 4m-DNA values for control cultures stained together were always found to be within ±5%, and were within ±3% in the present series of experiments. However, a shift of 5% in DNA values between a control culture and an irradiated culture would lead to an error of ≃0.06 to 0.07 in the dry mass increase observed: this source of error is, of course, reduced by using more than one culture. The extent of nonuniformity of growth between cultures can be estimated by noting that, throughout the present series of papers (Seed, 1966 a, b, c), successive irradiated cultures always showed a shift to higher mean dry mass values with increasing time after irradiation. In these experiments, the time intervals between successive irradiated cultures were 120 to 190 min and so a nonuniformity of ±60 to 95 min would probably not be exceeded: this would lead to an error of ≃ ±0.03 in the dry mass increase observed in one culture. Because two or more irradiated cultures were used to determine the dry mass increase for a cell type, a final error of < ±0.09 is not unreasonable. The UV measurements for the control and the irradiated cells were each made on 6 to 10 plates, and deviations between plates would probably even out: the error in the comparison of a UV increase with a dry mass increase within the same culture would probably not exceed ±0.07, but comparisons between cell types would be less accurate. Greater accuracy could have been achieved by using a direct recording UV micro-

photometer, but such an instrument was not available.

7. A slight shift to higher values of DNA in cells blocked at the 2m-DNA and 4m-DNA levels appeared to take place in the irradiated cultures in all the experiments (Seed, 1966 a, b, c).

8. In the control cultures of all the cell types studied (Seed, 1966 a, b, c), the ratios of nuclear dry mass/DNA are different at the 2m-DNA and 4m-DNA levels: there is also a difference in the nuclear nucleic acid/DNA ratios: There are two reasons for this: (a) The synthesis of a fraction f of nuclear RNA and protein dissociated from DNA synthesis: this would reduce the 4m-DNA ratios and increase the 2m-DNA ratios (see Fig. 1). (b) The nuclear UV absorption and dry mass measurements include small contributions from the thin cytoplasmic layers overlapping the nucleus above and below. In the flat cells grown in culture, cytoplasmic granules are rarely seen in these layers because of their thinness (Walker and Yates, 1952) and hence their contribution has been neglected in the measurements. However, unless these cytoplasmic layers increase in OPD in proportion to the nuclear OPD during interphase, the older cells will have a measured "nuclear" dry mass too small in comparison with cells at the beginning of interphase. Similar considerations apply to nuclear UV absorption.

In principle it is possible to distinguish between effects (a) and (b). This is because the ratio of OPD/UV absorption at a point in the cytoplasm near the nucleus is similar to the corresponding ratio within the nuclear area: thus a small contribution from a cytoplasmic overlap will affect the nuclear dry mass/DNA and nuclear UV absorption/DNA ratios equally. On the other hand, a fraction f of synthesis dissociated from DNA synthesis will affect the UV absorption/DNA ratio by only ≃⅖ of its effect on the nuclear dry mass/DNA ratio, because RNA contributes only ⅖ of the nuclear UV absorption.

In practice, the measurements are not sufficiently accurate to permit calculations of f from the ratio differences. However, it is of interest that in each cell type the ratio differences indicate a contribution from both sources, (a) and (b):

For the embryo human cells we have, from Figs. 4 and 5,

fractional difference between 2m-DNA and 4m-DNA levels in the ratio

$$\text{Nuclear dry mass/DNA} = \frac{3.30 - 2.95}{3.12} = 0.11$$

and that in the ratio

Nuclear UV absorption/DNA =
$$\frac{1.65 - 1.52}{1.58} = 0.082.$$
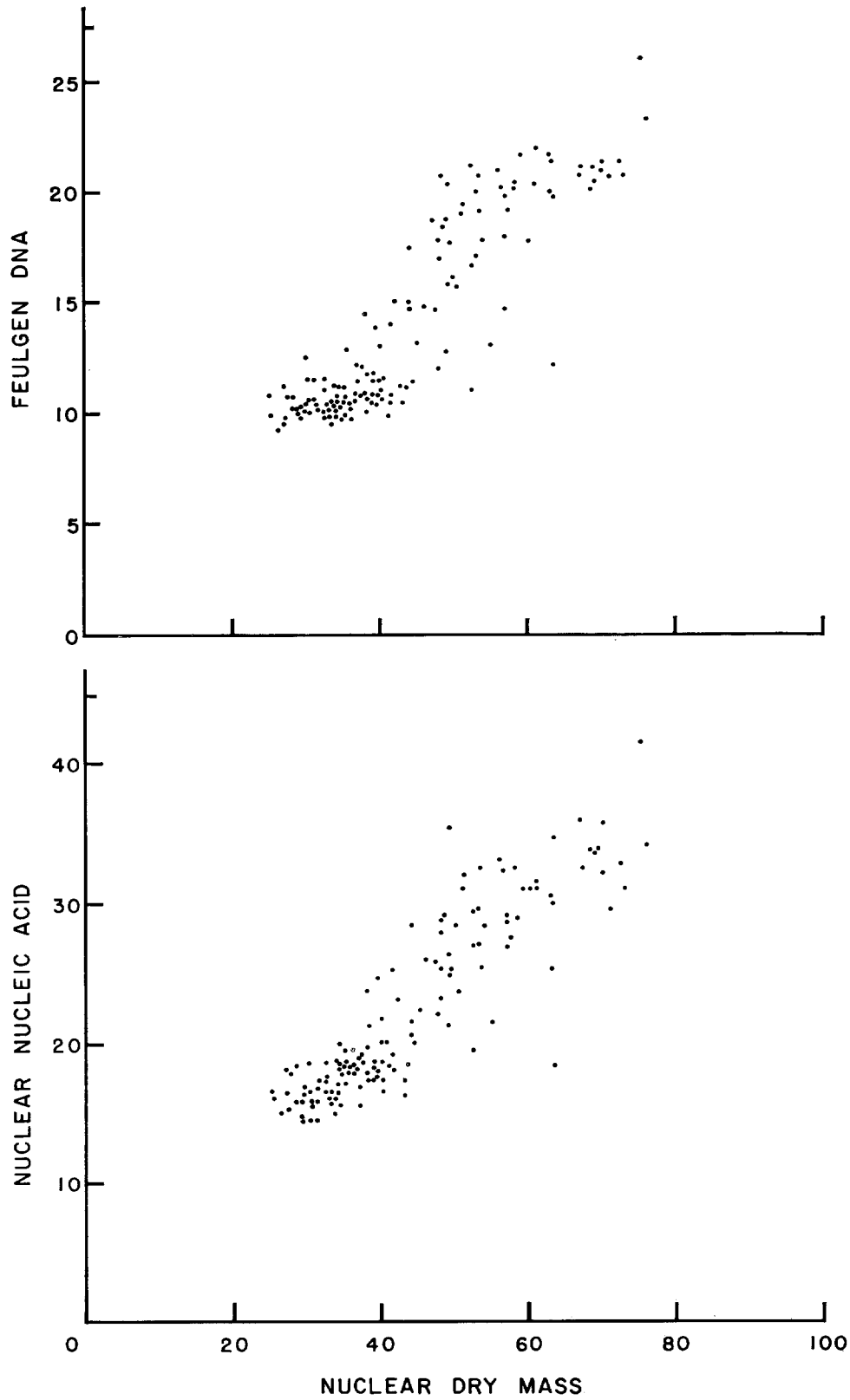
We have, then, from the ratio differences,

FIGURE 2   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 human cells. All quantities are expressed in arbitrary units.
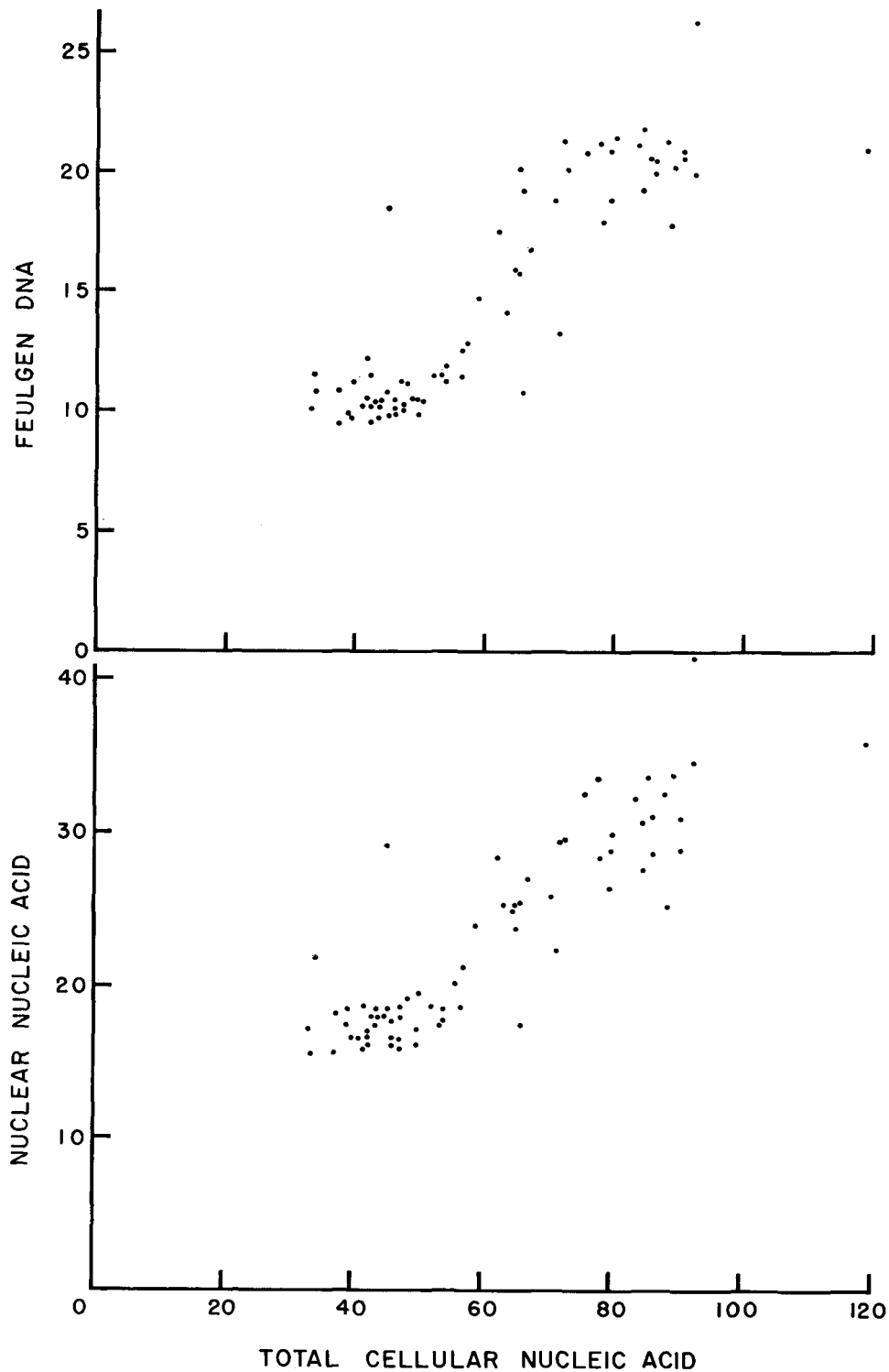
FIGURE 3   Feulgen stain (DNA) and nuclear ultraviolet absorption (at 2536 A, nuclear RNA + DNA) measurements plotted against the corresponding total cell ultraviolet absorptions (at 2536 A, total cell nucleic acid) for replicating embryo human cells.

dissociated synthesis + cytoplasmic overlap = 0.11
2/3 dissociated synthesis + cytoplasmic
overlap = 0.082

Solving the equations, we have:

Ratio difference attributed to cytoplasmic
overlap $\simeq$ 0.06

Ratio difference attributed to dissociated
synthesis $\simeq$ 0.05

The errors in these quantities are large owing to their sensitivity to the exact values of the ratios. However, it is interesting to note that if we work on the basis that the nuclear area and OPD each increase by $\simeq \sqrt{2}$ during interphase, then a constant cytoplasmic overlap of the order of $\frac{1}{10}$ the thickness of the early interphase nucleus would account for the ratio differences attributed to it in all the experiments (Seed, 1966 a, b, c). Relative thicknesses of this order are consistent with the interferometric and refractometric work of Barer and Dick (1957) and with the thicknesses observed by Perry et al. (1961) in transverse sections of cultured cells.

RESULTS

Control Cells

NUCLEAR METABOLISM: The progress of the three syntheses (DNA, RNA, and protein) in the nucleus during interphase is followed in Fig. 2 where the amounts of nuclear UV absorption (nuclear nucleic acid) and Feulgen DNA for individual cells are plotted separately against the corresponding dry mass values for the same cell nuclei. From the figure, it is seen that, in general, the nuclear nucleic acid (RNA + DNA) and dry mass values are approximately proportional during interphase, and that the DNA and dry mass are similarly proportional. A least squares analysis of the plots confirms this, giving the high correlation coefficient $r = 0.891$ between nucleic acid and dry mass and a similar correlation of $r = 0.889$ between DNA and dry mass. Applying Fisher's z-transformation (Fisher, 1936; Fisher and Yates, 1948) to test the significance of the difference between the two correlations, $t = 0.07$, which does not reach the value to be obtained at the $P = 0.05$ level ($t = 1.97$) nor does it reach that to be attained at the $P = 0.5$ level ($t = 0.675$). The difference is therefore not significant.

It is reasonable to conclude, therefore, from the similarity of these high correlations between the nuclear measurements, that the net syntheses of all three components, DNA, RNA, and protein, occur closely together in interphase in the nuclear

chromatin of rapidly growing embryo human cells.

This result is similar to that obtained previously for freshly prepared monkey kidney cells in culture, where time-lapse photography was used to show that the DNA, RNA, and nuclear protein syntheses curves were similar: in this case it was shown that the two methods, using timing and correlation plots, gave equivalent results (Seed, 1962, 1963 a).

CYTOPLASMIC RNA: Of the 140 cells on which nuclear measurements were made (in Fig. 2), it was possible to make measurements of total UV absorption on 72 cells without cytoplasmic overlapping.

In order to compare the increase of cytoplasmic RNA during interphase with the progress of DNA replication, an approach was adopted similar to that employed above for nuclear dry mass: the measurements for integrated cell UV absorption (total cell nucleic acid) for individual cells were plotted separately against the corresponding DNA and nuclear nucleic acid values for the same cells (Fig. 3). Total cell nucleic acid was plotted, in preference to cytoplasmic RNA, in the comparison of correlations, because this procedure eliminates the combination of errors resulting from the subtraction of nuclear nucleic acid from total cell nucleic acid. (DNA comprises $\simeq \frac{2}{3}$ of total nucleic acid and nuclear nucleic acid (RNA + DNA) comprises $\simeq \frac{1}{3}$ of total nucleic acid).

Now, from experiments with labeled precursors in the embryo human cell, the synthesis of cytoplasmic RNA appears to take place in the nucleus (Seed, unpublished), as found by other authors in other replicating cells (Woods, 1960; Goldstein and Micou, 1959; Feinendegen et al., 1960; Woods and Taylor, 1959; Marshak, 1948; Jeener and Szafarz, 1950; Perry, 1960; Goldstein and Plaut, 1955; Zalokar, 1960): therefore, in a cell synthesizing RNA, it is reasonable to expect some kind of correlation between the amounts of nuclear RNA and cytoplasmic RNA. On the other hand, one would not expect to find a correlation between the DNA and cytoplasmic RNA contents if the two syntheses are largely dissociated in time: in this case, one would expect a higher correlation between nuclear (RNA + DNA) and total cell nucleic acid than between DNA and total cell nucleic acid.

Returning to Fig. 3, where the nucleic acid measurements are plotted, the DNA and the

nuclear nucleic acid are each approximately proportional to the increase of total cell nucleic acid during interphase. A least squares analysis gives the high correlation coefficient of $r = 0.873$ between DNA and total nucleic acid, and a similar correlation of $r = 0.885$ between nuclear nucleic acid and total nucleic acid. Applying Fisher's z-transformation (Fisher, 1936; Fisher and Yates, 1948), to test the significance of the difference between the two correlations, $t = 0.311$, which does not reach the value to be attained at the $P = 0.05$ level ($t = 1.98$), nor at the $P = 0.5$ level ($t = 0.676$).

It is concluded, therefore, from the similarity of these high correlations, that the syntheses of DNA and of cytoplasmic RNA are closely associated during the interphase of replicating embryo human cells. Additional evidence that the majority of the synthesis of cytoplasmic RNA is associated with DNA synthesis arises from the similarity of the mean ratios of cytoplasmic RNA/DNA at the 2m-DNA[1] and 4m-DNA levels (2.63 and 2.49, respectively, from Fig. 7): this result would not occur if a major RNA component accumulated independently of DNA replication (e.g. broken line in Fig. 1). Similarity of the ratios would also not occur if the time delay between initial RNA synthesis in the nucleus and appearance of RNA in the cytoplasm were long, as this would result in a staggering in time of two stepwise accumulation curves. The kinetics of RNA transfer from nucleus to cytoplasm in these cells appear to be similar to those reported by Woods (1960) in *Vicia faba* where, after a short period in tritiated cytidine followed by incubation in unlabeled cytidine, the labeled RNA in the cytoplasm had reached 80% of its final value at 2 hr later.

It is apparent from inspection of Fig. 3 that the DNA and nuclear nucleic acid are each correlated with the total cell nucleic acid to a much greater extent than is warranted from their contributions to the total nucleic acid ($\simeq$ ⅔ and $\simeq$ ⅓, respectively). By subtracting the values of nuclear nucleic acid from total nucleic acid we can obtain correlations between DNA and cytoplasmic RNA of $r = 0.772$, and between nuclear nucleic acid and cytoplasmic RNA of $r = 0.764$: there is no significant difference between these values ($t = 0.12$). Because these correlations combine two errors (see above), it is more informative to com-

pare the total nucleic acid correlations; nevertheless they are given here for the sake of completeness.

Corresponding nucleic acid measurements on individual cells in a replicating fresh monkey kidney culture (grown as described previously, Seed, 1962, 1963 a) are shown in Fig. 8. From the figure, the DNA and total cell nucleic acid values are seen to be roughly proportional to one another, as are also the nuclear nucleic acid and total cell nucleic acid values. A least squares analysis confirms a high correlation of $r = 0.861$ between DNA and total cell nucleic acid, and a similar correlation of $r = 0.828$ between nuclear nucleic acid and total cell nucleic acid. Applying Fisher's z-transformation (Fisher, 1936; Fisher and Yates, 1948) to test the significance of the difference between the two correlations, $t = 0.684$, which does not reach the value to be attained at the $P = 0.05$ level ($t = 1.98$), and in fact merely attains the value at the $P = 0.5$ level, ($t = 0.677$). The difference is therefore not significant.

From the similarity of these high correlations between the nucleic acid components, it is concluded, therefore, that the syntheses of DNA and of cytoplasmic RNA are closely associated during interphase in replicating monkey kidney cells. Supplementary evidence is afforded by the similarity of the ratios of cytoplasmic RNA/DNA at the 2m-DNA and 4m-DNA levels (3.5 and 3.25, respectively): again, this result could not be obtained if there were present a major component of RNA synthesis dissociated from DNA replication.

It is clear from inspection of the plots in Fig. 8 that these relations exist independently of the contributions ($\simeq$ ⅔ and $\simeq$ ⅓, respectively, in monkey kidney cells) that DNA and nuclear nucleic acid make to total cell nucleic acid. By subtracting the values of nuclear nucleic acid from total nucleic acid, we can obtain correlations between DNA and cytoplasmic RNA of $r = 0.761$, and between nuclear nucleic acid and cytoplasmic RNA of $r = 0.702$: there is no significant difference between these values ($t = 0.769$). As stated above, these correlations combine two errors, and it is, therefore, more informative to compare the total nucleic acid correlations.

It must be emphasized that these observations refer to *net* synthesis, as they measure total amount of substance present at a given time in the cell or nucleus. The experiments throw no light on any
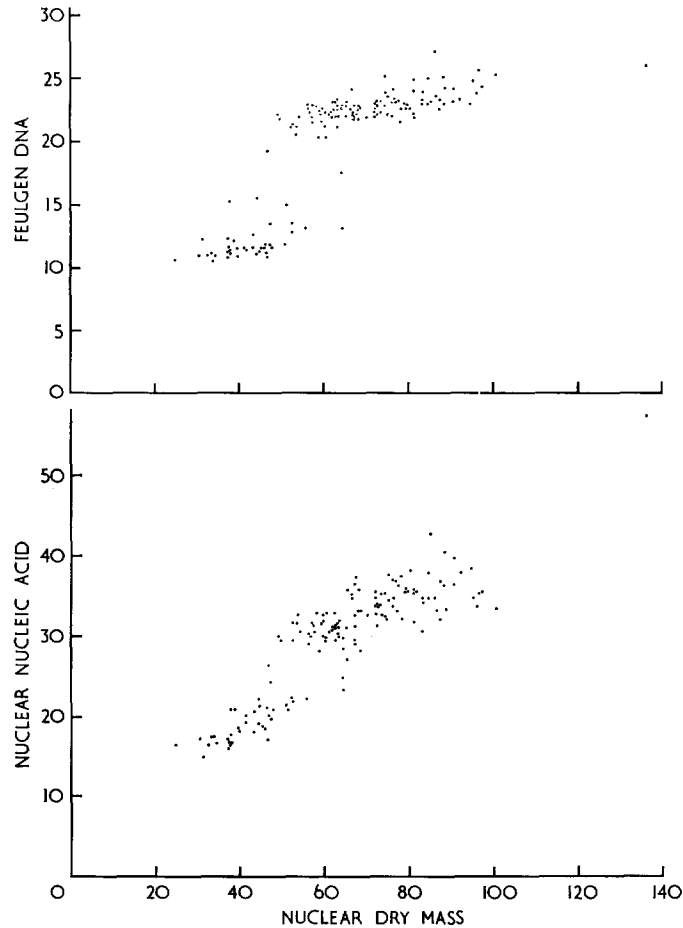
---

[1] The DNA values corresponding to telophase and prophase are 2m and 4m, respectively.

FIGURE 4 Feulgen stain (DNA) and nuclear ultraviolet absorption (at 2536 A, nuclear RNA + DNA) measurements plotted against the corresponding nuclear dry mass values for embryo human cells fixed 897 min after X-irradiation with 1250 R (I_2).

RNA turnover that may exist, although previous radioautographic observations (Seed, 1963 *a*) showed that a large fraction of the uptake of cytidine into nuclear RNA was in fact associated with DNA synthesis in monkey kidney cells. The present experiments show that the accumulation of RNA in the replicating cell is derived mainly from the RNA synthesis associated with DNA *replication*.

Irradiation Experiments Blocking DNA Synthesis

In the last section, the relations between successive physical measurements were studied in a population of replicating cells. In the present section, by comparison of measurements on ir-

radiated and control cell populations of embryo human cells, we shall study the coupling between the syntheses when the initiation of DNA synthesis is blocked.

In Fig. 4 the nuclear nucleic acid and DNA measurements for individual cells are plotted separately against the corresponding nuclear dry mass values for an irradiated culture fixed 897 min after irradiation (I_2). The distributions of values of DNA, nuclear dry mass, and nuclear nucleic acid measurements for individual control and irradiated cells are shown in Figs. 5 and 6: the measurements for the control culture are shown in the lower and those for the irradiated cultures are shown in the upper parts of the figure.

DNA SYNTHESIS: In the control culture,

the Feulgen DNA distribution shows a large group corresponding to the posttelophase value (2m) and a smaller group corresponding to the pre-prophase value (4m), together with a number of intermediate values appropriate to synthesizing cells (Fig. 5). This occurs because, in animal cells preparing for division, DNA synthesis occupies only a part of interphase (Swift, 1950; Walker and Yates, 1952; Howard and Pelc, 1953; Richards et al., 1956; Firket, 1958) and, therefore, in a randomly growing population it is more probable to find DNA values corresponding to either the presynthetic or premitotic delay periods (cf. Fig. 1).

In the DNA distribution for the irradiated cultures (Fig. 5), there is a large accumulation of cells at the 4m-DNA level (preprophase), with relatively few cells in synthesis. In comparison with the DNA distributions for the control cells just described, these distributions are consistent with results of other authors already quoted, in that

radiation doses of the order of 1000 R have little effect on DNA synthesis already in progress: such cells go on to complete the cycle but are later blocked on reaching the 4m-DNA level because of the inhibition of mitosis. In addition, some cells remain blocked at the 2m-DNA level in the irradiated cultures, whereas others have obviously moved into DNA synthesis (perhaps after a temporary block at 2m) up to the 4m-DNA value (compare with control DNA distribution in Fig. 5) (see Howard and Pelc, 1953; Holmes and Mee, 1955; Pelc and Howard, 1955; Kelly et al., 1955, 1957; Dickson et al., 1958; Caspersson et al., 1958; Lajtha et al., 1958; Painter and Robertson, 1959; Whitfield and Rixon, 1959; Yamada and Puck, 1961; Seed, 1961).

NUCLEAR DRY MASS: It is clear from Fig. 5 that in the irradiated cultures there has been a general shift to higher dry mass values as there was to higher DNA values: that is, cells already in protein synthesis have gone on to completion of the
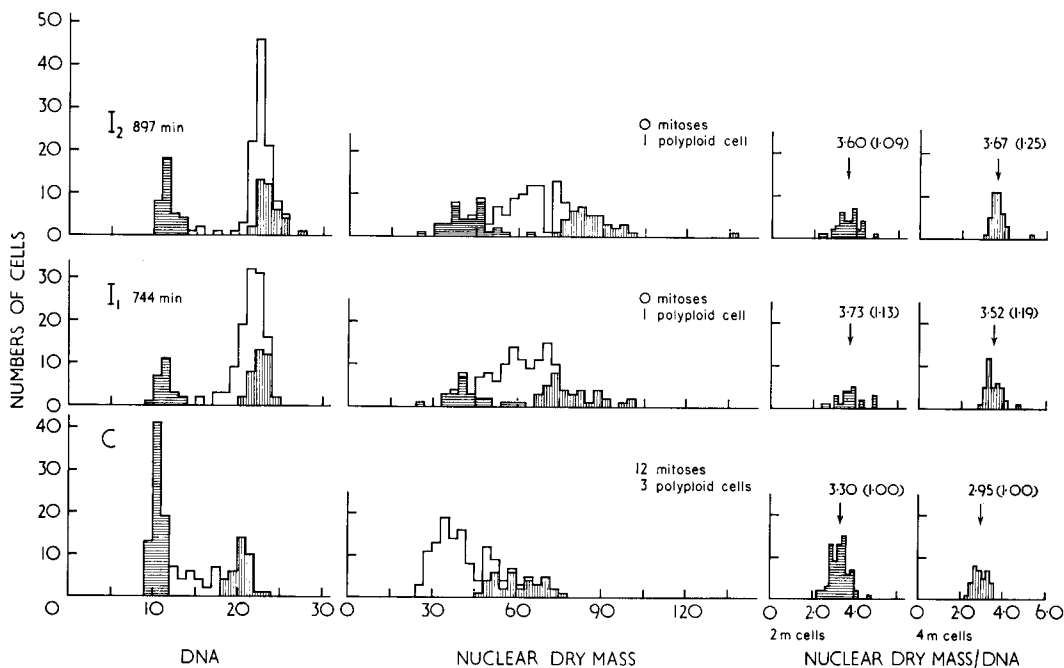

FIGURE 5   Distributions of Feulgen stain (DNA) and nuclear dry mass measurements on individual embryo human cells, intermitotic time 850 min. Lower section, C control cells; center section, I_1 cells fixed 744 min after X-irradiation with 1250 R; upper section, I_2 cells fixed 897 min after X-irradiation with 1250 R. 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. At the right of the figure the nuclear dry mass/DNA ratios are plotted 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.

cycle just as they did for DNA. The question in which we are interested is: By what amount has the nuclear dry mass increased in those cells where the initiation of DNA synthesis has been blocked at at the 2m or 4m levels? It is obviously difficult to draw quantitative conclusions about cells that were in DNA synthesis at the time of irradiation, or were at the 2m-DNA level and later moved into synthesis, because the extent of blocking of DNA synthesis in these cells is unknown. However, there are two classes of cells from which deductions can be made: (a) those that have been blocked at the 2m-DNA level since the time of irradiation (shaded horizontally in Fig. 5); and (b) those blocked at the 4m-DNA level since the time of irradiation (shaded vertically in Fig. 5).

(a) Let us consider first the irradiated culture fixed 744 min after irradiation (I_1). In the 24 cells with DNA values at the 2m level, the initiation of DNA synthesis has been blocked since the time of irradiation, mitosis having been abolished. In order to determine what fraction of the nuclear mass has been synthesized in these cells in the absence of DNA synthesis, the nuclear dry mass/DNA ratios are plotted for the individual cells and are compared with the corresponding ratios for cells at the 2m-DNA level in the control culture (Fig. 5). The arrows in the figure indicate the mean nuclear dry mass/DNA ratios in arbitrary units, and the numbers in parentheses show the increase over the control value, the controls being taken as 1.00 for this purpose. Similar plots are made for the 35 cells blocked at the 2m-DNA level in the culture fixed 897 min after irradiation (I_2).

It is seen that for the 2m-DNA cells the nuclear dry mass has increased by 0.13 in 744 min after irradiation (I_1), and by 0.09 in 897 min after irradiation (I_2). Assuming a constant rate of increase, this gives a mean increase of 0.11 in one interphase time (850 min). Allowing for the presence of nucleic acid, this result for I_1 and I_2 gives a mean increase of 0.13 in protein in one interphase time in the absence of DNA synthesis at the 2m level.

(b) In the control culture there are 36 cells (shaded vertically in Fig. 5) at around the 4m-DNA level out of a total of 139 cells. Because mitosis was blocked in I_1 and I_2 by the radiation, there will therefore be a corresponding number $36/139 \times 141 \simeq 37$ out of a total of 141 cells in I_1 and a number $36/139 \times 146 \simeq 38$ out of a

total of 146 cells in I_2, that have been blocked at the 4m-DNA level since the time of irradiation.

In order to identify the 37 and 38 cells in the dry mass distributions of I_1 and I_2, respectively, the reasonable assumption has been made that these cells have increased most in value: thus, distributions of values, containing 37 and 38 cells, respectively, have been drawn in the higher ends of the dry mass distributions of the irradiated cultures I_1 and I_2. In constructing these distributions, the attempt was made to include as many high values as possible, consistent with maintaining a general similarity in shape to the dry mass distribution of the corresponding 4m-DNA cells in the control culture: because of this method, the dry mass increase observed at the 4m-DNA level will tend to represent a *maximum* value for the syntheses dissociated from DNA synthesis at the 4m-DNA level. These cells are shaded vertically in Fig. 5, along with the corresponding Feulgen DNA values. The ratios of nuclear dry mass/DNA are also plotted for these 4m cells, along with the corresponding ratios for the 4m-DNA cells in the control culture. Again, the amounts of increase are shown in brackets, referred to the control as 1.00.

It is seen that, at the 4m-DNA level, the 37 cells (I_1) have increased in dry mass by 0.19 over the control in 744 min, and the 38 cells (I_2) have increased by 0.25 in 897 min after irradiation. Assuming a constant rate of accumulation, this gives a mean increase of 0.23 in 850 min, one interphase time, and, allowing for the presence of nucleic acid in the dry mass, we have an increase of 0.27 in nuclear protein in the absence of DNA synthesis.

With reference to Fig. 5, there were no mitoses found among the irradiated cells, and the numbers of polyploid cells were less than those in the controls: no correction is therefore necessary on this account.

From the measurements, the dry mass increase after blocking DNA synthesis is apparently bigger at the 4m-DNA than at the 2m-DNA level: this was also observed in embryo mouse cells (Seed, 1966a). As already stated, the method of construction of the dry mass distribution for cells blocked at the 4m-DNA level in the irradiated cultures is expected to give a maximum value for the accumulation there. The presence of a few cells not participating in the replication cycle at the 2m-DNA level might influence the results there
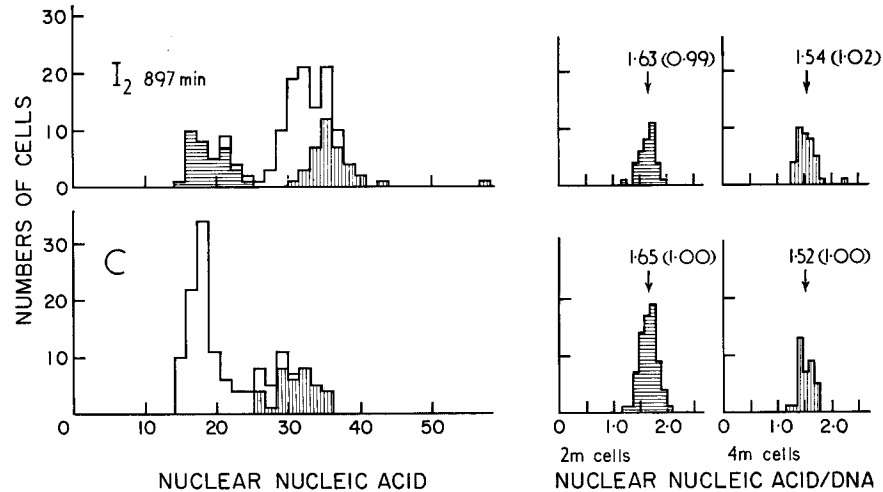
FIGURE 6   Distributions of nuclear ultraviolet absorption (at 2536 A, nuclear RNA + DNA) measurements on embryo human cells. At the right of the figure are plotted the nuclear nucleic acid/DNA ratios for controls and for cells having DNA synthesis blocked since the time of irradiation: ☰, cells with 2m-DNA values; ‖‖, cells with 4m-DNA values. Lower section, C control cells; upper section, I₂ cells fixed 897 min after X-irradiation with 1250 R.

slightly: however, such cells would not form more than ≃5% of the total in these cultures (Seed, 1962).

NUCLEAR NUCLEIC ACID: The distributions of nuclear UV absorption are shown in Fig. 6 for the control and for one of the irradiated cultures (I₂). Again we can make deductions about the same two classes of cells used above.

1. In the 35 cells with 2m-DNA values in the irradiated culture, the nuclear nucleic acid/DNA ratio shows no increase over the corresponding ratio for the control 2m-DNA cells, after a block in DNA synthesis for 897 min.

2. Cells blocked at the 4m-DNA level since the time of irradiation. When we refer to the plots for the irradiated culture I₂ in Fig. 4, we see that there is an approximate relation between cells increasing in nuclear dry mass and in nuclear nucleic acid at the 4m-DNA level: it is appropriate therefore to take the same nuclear nucleic acid values for the same 38 cells as were used in Fig. 5 in the dry mass distributions (shaded vertically in Figs. 5 and 6). Again in Fig. 6 the nuclear nucleic acid/DNA ratios are plotted and it is seen that the 38 cells blocked at the 4m value of DNA show an increase of 0.02 in nuclear nucleic acid at 897 min after the time of irradiation.

Now, if the nuclear RNA and nuclear protein have increased proportionately in the irradiated cells, we should expect an increase in nuclear nucleic acid of $0.11 \times \frac{2}{5} \simeq 0.04$ at the 2m-DNA level and of $0.3 \times \frac{2}{5} \simeq 0.12$ at the 4m-DNA level (since RNA contributes $\simeq \frac{2}{5}$ of the nuclear UV absorption and if we neglect the small contribution from protein). These figures are not significantly different ($\pm$ 0.07) from the increase observed (0 and 0.02, respectively).

CYTOPLASMIC RNA: Out of the 146 cells in the irradiated culture I₂ on which nuclear measurements were made in Figs. 5 and 6, it was possible to make cytoplasmic measurements on 98 cells not suffering from cytoplasmic overlapping. The distributions of values of DNA and of cytoplasmic RNA for these cells are shown in Fig. 7 along with the corresponding distributions for the control cells (from Fig. 3). There are again two classes of cells which are of interest:

1. The 29 cells (measureable out of the 35 cells in the nuclear measurements of Figs. 5 and 6) where DNA synthesis has been blocked at the 2m level since the time of irradiation are shaded horizontally in Fig. 7. The cytoplasmic RNA/DNA ratios for these cells are also plotted, along with the corresponding ratios for the 2m-DNA cells in the control culture: the amounts of increase are shown in brackets. It is seen

that the cytoplasmic RNA/DNA ratio has increased by 0.71 in 897 min, giving an increase of 0.67 in cytoplasmic RNA in 850 min, one interphase time.

2. Cells blocked at the 4m-DNA level since the time of irradiation. Again the increase in cytoplasmic RNA in the irradiated cells is correlated with increase in nuclear dry mass (Seed, unpublished): however, of the 38 cells shaded in the dry mass distribution of Fig. 5, where DNA synthesis was blocked at the 4m level (corresponding to the 4m-DNA cells in the control culture), the cytoplasm of only 19 cells could be measured because of cytoplasmic overlapping. These 19 measured cells are indicated in Fig. 7 in order to give an estimate of the cytoplasmic RNA increase at the 4m-DNA level: they give a mean increase of 0.4 over the 897 min since the time of irradiation.

It must be emphasized that, in the irradiated culture, the increases observed for the cytoplasm are inherently less reliable than those for the nuclear measurements. This is because the nuclear measurements were made on cells comprising almost the entire population in a field, whereas the cytoplasmic measurements could be made on only about ⅓–⅔ of the same population, because of cytoplasmic overlapping. Although this is not expected to influence the conclusions made from correlations between cytoplasmic measurements *within* the same group of cells (e.g. Fig. 3), it might affect those made from a comparison of cytoplasmic values in two different populations (e.g. an irradiated culture and a control): overlapping might occur with one size-group more than another to give a nonrandom distribution.

DISCUSSION

The evidence here presented from the measurements on the control cells shows that, during the interphase of embryo human cells that are proceeding to a further division, the net syntheses of DNA, nuclear protein, nuclear RNA (in the chromatin), and cytoplasmic RNA are closely associated within the individual cell. The additional measurements on fresh monkey kidney cells show that the net syntheses of DNA and cytoplasmic RNA are also closely associated in this cell type. These results are in agreement with others previously reported for fresh embryo human and monkey kidney cells, where a time-lapse photographic technique showed that the onset of the syntheses of DNA and the majority of nuclear RNA and protein were coupled in interphase (Seed, 1962, 1963 a).

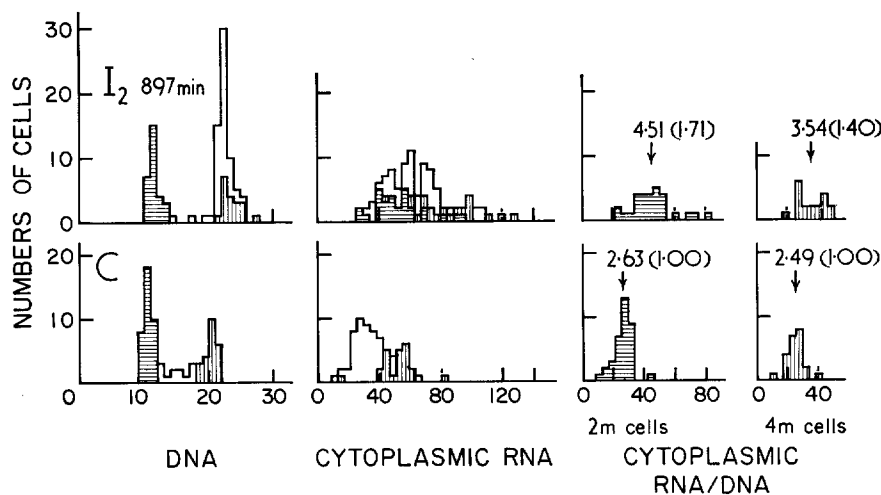The experiments with radiation utilize an entirely different approach which generally con-

FIGURE 7 Distributions of Feulgen stain (DNA) and cytoplasmic UV absorption (cytoplasmic RNA) in individual embryo human cells. At the right of the figure are plotted the cytoplasmic RNA/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, I₂ cells fixed 897 min after X-irradiation with 1250 R.

25 ⎯
20 ⎯
FEULGEN DNA 15 ⎯
10 ⎯
5 ⎯
0 ⎯

40 ⎯
NUCLEAR NUCLEIC ACID 30 ⎯
20 ⎯
10 ⎯

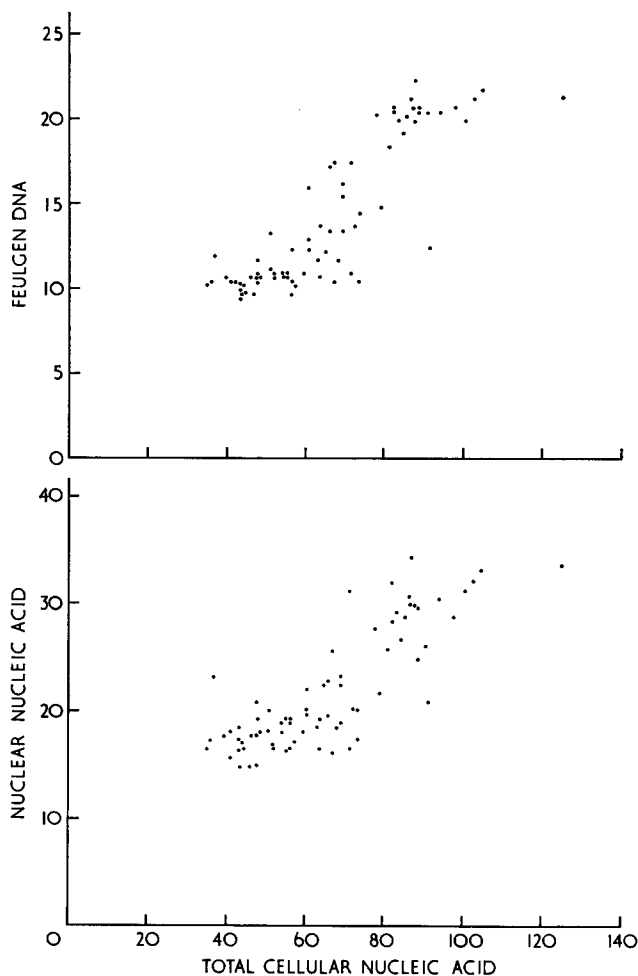0   20   40   60   80   100   120   140
TOTAL CELLULAR NUCLEIC ACID

FIGURE 8 Feulgen stain (DNA) and nuclear ultraviolet absorption (at 2536 A nuclear RNA + DNA) measurements plotted against the corresponding total cell ultraviolet absorption (at 2536 A, total cell nucleic acid) for a population of fresh replicating monkey kidney cells.

firms these conclusions on nuclear metabolism: by preventing the *initiation* of DNA synthesis in replicating embryo human cell cultures, it is found that the majority of the accumulation of nuclear protein and RNA is also blocked and is thus associated with the *event initiating* DNA synthesis. Thus a block in DNA synthesis for one interphase time resulted in increases in nuclear protein of 0.13 at the 2m-DNA level, and 0.27 at the 4m-DNA level, with corresponding increases of 0 and 0.02, respectively, in nuclear nucleic acid; an increase of about one-half was observed in cytoplasmic RNA. Because the arrest of the synthetic processes leading to mitosis may result in diversion of metabolites into other channels, it is not possible to derive, from the increases observed, the fractions of protein synthesis and RNA synthesis normally independent from DNA synthesis in rep-

licating cultures. Nevertheless it is reasonable to regard the increases observed as representing upper limits for the dissociated syntheses.

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

REFERENCES

BARER, R., 1952, *Nature,* **169,** 108, 366.

BARER, R., and DICK, D. A. T., 1957, *Exp. Cell Research Suppl.,* **4,** 103.

BONNER, J., HUANG, R. C., and MAHESHWARI, N., 1961, *Proc. Nat. Acad. Sc.,* **47,** 1548.

BRACHET, J., 1957, Biochemical Cytology, New York, Academic Press Inc., 1957.

CASPERSSON, T., 1950, Cell Growth and Cell Function, New York, W. W. Norton and Company, Inc., 1950.

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

DAVIES, H. G., and WILKINS, M. H. F., 1952, *Nature,* **169,** 541.

DEELEY, E. M., 1955, *J. Sc. Instr.,* **32,** 263.

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

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

FEINENDEGEN, L. E., BOND, V. P., SHREEVE, W. W., and PAINTER, R. B., 1960, *Exp. Cell Research,* **19,** 443.

FIRKET, H., 1958, *Arch. biol.,* **69,** 1.

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.

GOLDSTEIN, L., and MICOU, J., 1959, *J. Biochem. and Biophysic. Cytol.,* **6,** 1, 301.

GOLDSTEIN, L., and PLAUT, W., 1955, *Proc. Nat. Acad. Sc.,* **41,** 874.

HEVESY, G., 1948, Radioactive Indicators, New York, Interscience Publishers, Inc., 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.

JACOB, F., and MONOD, J., 1961, *J. Mol. Biol.,* **3,** 318.

JEENER, P., and SZAFARZ, D., 1950, *Arch. Biochem.,* **26,** 54.

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.

KILLANDER, D., RICHARDS, B. M., and RINGERTZ, N. R., 1962, *Exp. Cell Research,* **27,** 321.

KING, R. J., and ROE, E. M. F., 1953, *J. Roy. Micr. Soc.,* **73,** 82.

KLEIN, G., and FORSSBERG, A., 1954, *Exp. Cell Research,* **6,** 211.

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

LIN, H. J., and CHARGAFF, E., 1964, *Biochem. et Biophysica Acta,* **91,** 691.

MARSHAK, A., 1948, *J. Cellular and Comp. Physiol.,* **32,** 481.

McLEISH, J., 1959, *Chromosoma,* **10,** 686.

McLEISH, J., 1960, *in* The Cell Nucleus (J. S. Mitchell, editor), London, Butterworth & Co. (Publishers) Limited, 1960, 91.

MITCHELL, J. S., 1942, *Brit. J. Exp. Path.,* **23,** 285.

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

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

PERRY, R. P., 1960, *Exp. Cell. Research,* **20,** 216.

PERRY, R. P., ERRERA, M., HELL, E., and DURWALD, H., 1961, *J. Biophysic. and Biochem. Cytol.,* **11,** 1.

RICHARDS, B. M., and DAVIES, H. G., 1958, *in* General Cytochemical Methods. I, (J. F. Danielli, editor), New York, Academic Press Inc., 1958, 130.

RICHARDS, B. M., WALKER, P. M. B., and DEELEY, E. M., 1956, *Ann. New York Acad. Sc.,* **63,** 831.

RUSCH, H. P., 1954, *Cancer Research,* **14,** 407.

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

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

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

SEED, J., 1963 b, *Nature,* **198,** 153.

SEED, J., 1964, *J. Cell Biol.,* **20,** 17.

SEED, J., 1965, *in* Cells and Tissues in Culture, (E. N. Willmer, editor), New York, Academic Press Inc., 1965, **1,** 325.

SEED, J., 1966 a, *J. Cell Biol.,* **28,** 249.

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

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

SWANN, M. M., 1957, *Cancer Research,* **17,** 727.

SWANN, M. M., 1958, *Cancer Research,* **18,** 1118.

SWIFT, H. H., 1950, *Physiol. Zool.,* **23,** 169.

WALKER, P. M. B., 1956, *in* Physical Techniques in Biological Research, (G. Oster and A. W. Pollister, editors), New York, Academic Press Inc., 1956, **3,** 402.

WALKER, P. M. B., and DAVIES, H. G., 1950, *Discussions Faraday Soc.,* **9,** 461.

WALKER, P. M. B., and YATES, H. B., 1952, *Proc. Roy. Soc., London, Series B,* **140,** 274.

WHITFIELD, J. F., and RIXON, R. H., 1959, *Exp. Cell Research,* **18,** 126.

WILLIS, R. A., 1960, The Pathology of Tumours, London, Butterworth & Co. (Publishers) Limited, 1960.

WOODS, P. S., 1960, *in* The Cell Nucleus, (J. S. Mitchell, editor), London, Butterworth & Co. (Publishers) Limited, 1960, 127.

WOODS, P. S., and TAYLOR, J. H., 1959, *Lab Inv.,* **8,** 309.

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

ZALOKAR, M., 1960, *Exp. Cell. Research,* **19,** 559.