## DNA Synthetic Rates Among Cells of Populations in vitro

The DNA-synthetic period relative to total cell replication time can be measured by calculation of a time curve from a histogram of DNA-content distribution[1]. Calculation of such a curve requires several assumptions about the cell population: (a) it is homogeneous; (b) it exhibits logarithmic growth; (c) there are equal increments of DNA/unit time of synthesis for all cells; (d) a representative sampling is made for all cells in S, and (e) the cells are not synchronized in growth or division. Moreover, the concept that most cells in a population proceed through S, $G_2$ and M within relatively narrow limits has been the basis for interpreting data derived from emulsion grain counts following exposure to incorporated labeled thymidine.

The present studies were initiated to: (1) apply the method of photographic colorimetry[2] for DNA measurement to a biological problem, and (2) to test certain of the above assumptions about a cell population with regard to the S-period.

*Materials and methods.* Heart tissue explants from 8-day-old chick embryos were cultured on glass slides for periods of from 3–12 days. In the initial studies samples were fixed in methanol-formaldehyde (9:1) at 3, 5, 7, 9 and 12 days and stained according to the Feulgen technique. Feulgen-DNA measurements were performed with the method of photographic colorimetry, and synthesis curves constructed according to the method of WALKER[1]. Two additional schedules were followed using 3-day-old cultures. One entailed a 10-min-pulse label with $^3$H-thymidine (2 $\mu$C/ml; s.a. 12,600 mC/m$M$), and the other a 10-min-pulse with $^{14}$C-thymidine (0.25 $\mu$C/ml; s.a. 224 mC/m$M$). The cultures were washed in Hank's solution and fixed at the end of the pulse-labeling period. After a 20-min wash in 5% trichloracetic acid, liquid emulsion (Ilford, G5) was applied to the slides and exposed in the dark at 4 °C for 3 days ($^3$H-thymidine) or 3 weeks ($^{14}$C-thymidine). Following development of the emulsions, the slides were stained with azure B and grain counts made on individual nuclei. Photographic maps of these cells were prepared for their later identification. The Feulgen technique was carried out on the cells following grain removal, and Feulgen-DNA measurements made with the method of photographic colorimetry. In every instance, slides of chicken erythrocytes were stained alongside the cultured cells as a control against photographic and staining errors[2].

*Results.* No significant differences in the distribution of DNA contents (2C–4C values) were found in the samples taken at various intervals from 3–12 days. Consequently these data were pooled and are presented in the histogram of Figure 1. Construction of a synthesis curve (Figure 2) was based on these results. The fraction of interphase time occupied by S in these cells is 54%, by $G_2$ 16%, and by $G_1$ 30%. Furthermore, the shape of the curve, as found in other studies[3], suggests that DNA synthesis is continuous and the rate relatively constant. If this conclusion is valid and if there are equal increments of DNA/unit time for all cells (assumption c), a correlation should be found in labeled nuclei between grain-count values and Feulgen-DNA contents. However, when such a plot is made for the $^3$H-thymidine data (Figure 3) no significant correlation is observed. Since the path length of the $\beta$ particles emitted by tritium is rather short, differences in specimen thickness might affect the accuracy of grain counts as a measure of incorporation[4]. The use of $C^{14}$ therefore, should provide a more accurate determination of precursor incorporation. Nevertheless, when the $^{14}$C-thymidine grain counts are plotted against Feulgen-DNA contents, no significant correlation is found (Figure 4). It may be noteworthy that the range of scatter outside of the 2C and 4C values is greater with tritium than with $C^{14}$; some measurement error may be introduced with the use of tritium.

When the means of the grain-count values for each Feulgen-DNA class (in increments of 10 units) are plotted, some information about the average rate variation may be noted. With the tritium-labeled cells the variation in rate during S seems impressive (Figure 5). The $^{14}$C-labeled cells, however, reflect a smoother, less-varying rate

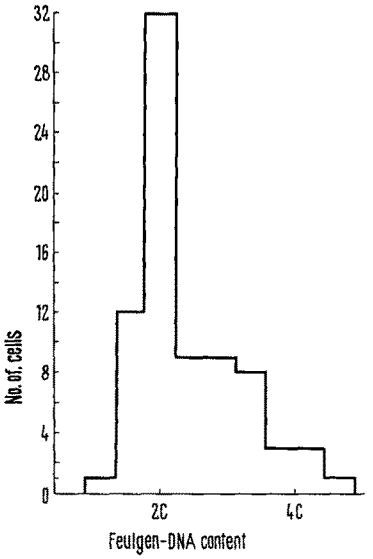


Fig. 1. Histogram of Feulgen-DNA contents of cultured cells of chick embryo heart. Measurement in arbitrary units by the method of photographic colorimetry.

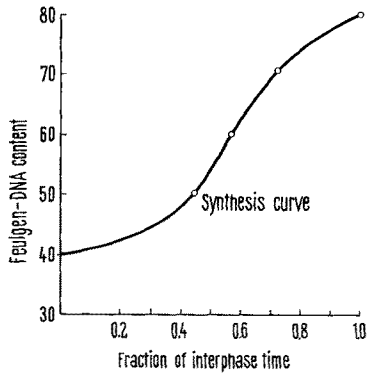


Fig. 2. Curve representing the proportions of interphase time occupied by $G_1$, S and $G_2$. Based on the histogram of Figure 1, and calculated by the method of P. M. B. WALKER[1].

[1] P. M. B. WALKER, J. exp. Biol. *31*, 9 (1954).
[2] E. M. DEN TONKELAAR and P. VAN DUIJN, Histochemie *4*, 1 (1964).
[3] A. ZETTERBERG and D. KILLANDER, Expl Cell Res. *39*, 22 (1965).
[4] A. ZETTERBERG, Expl Cell Res. *42*, 500 (1966).

(Figure 6). In neither graph is the wide grain-count distribution about each point shown; if it were these curves would not be meaningful. At the most, only a general indication of an average synthetic rate can be derived from this treatment of the data, but it appears that there may be higher rates near the beginning and/or end of S, with a depression somewhere near mid-S. However, these results do support the view that DNA synthesis is a continuous, albeit non-rate-constant, process.

*Discussion.* Examination of the population of cultured cells demonstrates that the population is homogeneous (assumption a), it exhibits logarithmic growth (assumption b), and it is not synchronized (assumption e). Although the sampling of cells may be nearly representative for all cells in S (assumption d), some selection is necessary to meet the requirements for measurements of grains and Feulgen-DNA contents, and selection has been followed by most investigators[3,5,6]. The assumption that there are equal increments of DNA/unit time of synthesis for all cells (assumption c) is not validated by the results reported here. Although several workers have accepted this assumption (see [5]), others have reported results indicating the contrary[5,7-9].

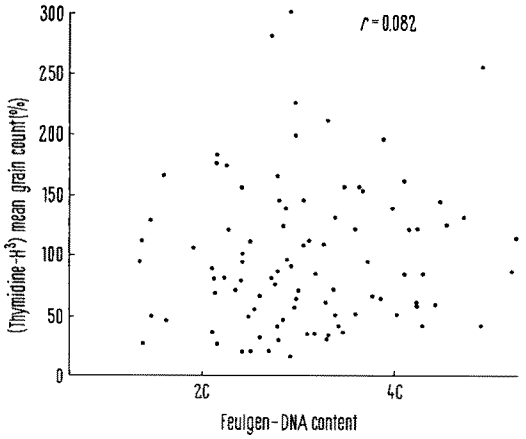


Fig. 3. Scattergram of percentages mean grain count relative to Feulgen-DNA content of cells labeled with $^3$H-thymidine. $r$ is the coefficient of correlation derived by least squares analysis.

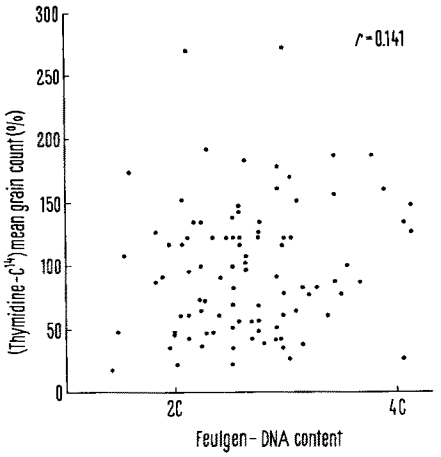


Fig. 4. Scattergram of percentages mean grain count relative to Feulgen-DNA content of cells labeled with $^{14}$C-thymidine. $r$ is the coefficient of correlation derived by least squares analysis.

Not only does the rate of DNA synthesis vary from cell to cell, but it may vary from the beginning to the end of S in an individual cell. Intracellular variation has been

[5] A. J. Hale, E. H. Cooper and J. D. Milton, Br. J. Haemat. *11*, 144 (1965).
[6] J. Seed, Proc. R. Soc. B. *156*, 41 (1962).
[7] E. L. Alpen and M. E. Johnston, Expl Cell Res. *47*, 177 (1967).
[8] E. H. Cooper, G. L. Frank and D. H. Wright, Europ. J. Cancer *2*, 377 (1966).
[9] P. P. Dendy and J. E. Cleaver, Int. J. Radiat. Biol. *8*, 301 (1964).

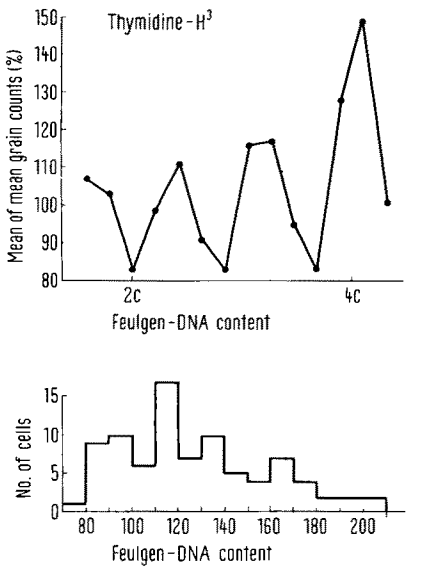


Fig. 5. Estimated average rate of DNA synthesis in $^3$H-thymidine-labeled cells. Each point is the mean of percentages mean grain count for each class interval of DNA content shown in lower figure.

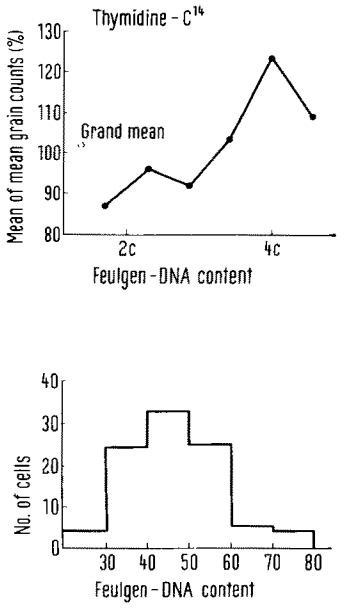


Fig. 6. Estimated average rate of DNA synthesis in $^{14}$C-thymidine-labeled cells. Each point is the mean of percentages mean grain count for each class interval of DNA content shown in lower figure.

reported in *Vicia faba* primary roots[10], and in synchronized cells of cultured human carcinoma[11]. In both studies[10,11] grain counts were measured against time rather than against DNA contents.

It is unlikely that grain-count variations of the type reported can be accounted for by errors in methodology or statistical variation due to radioactive decay[7]. An alternative explanation is necessary. Dependence of cell-to-cell variation in rates of S on chromosome replication patterns in individual nuclei has been discussed in detail[7]. It may not be necessary, however, to invoke an explanation based on random varying rates. Although DENDY and CLEAVER[9] have concluded that in experiments of this type metabolic pool size is of minor significance, it merits some attention. Variation in precursor pool size, resulting in variable degrees of dilution of the incorporated label, would result in a non-linear relation between grain counts and rates of synthesis during the pulse-labeling period. CRADDOCK and NAKAI[12] have suggested that the rate of incorporation of thymidine should not be considered as a measure of DNA synthesis, but as a reflection of the extent of intracellular pools of DNA precursors, and the activities of phosphorylating kinases and DNA polymerase Grain count variation may reflect the rate of filling the pool and the concentration of radio-active thymidine finally reached in it.

Data of the kind reported here and by other workers can be made to fit a general concept of DNA synthesis. However, such manipulation conceals information regarding individual cells, and clouds the interpretation of mechanisms controlling synthesis. When DNA content alone is used for determination of DNA synthetic activity, the rate appears to be relatively constant, but with labeled cells the rate is not constant, and it varies from cell to cell. The assumption that there are equal increments of DNA/unit time of synthesis for all cells in a population is unfounded[13].

*Zusammenfassung.* In vitro gezüchtete Hühnerembryozellen werden mit $^{3}$H-Thymidin oder $^{14}$C-Thymidin «pulse-labeled», wobei «grain counts» und DNS-Gehalt in individuellen Zellen gemessen werden. Für keine der beiden Isotopen wurde eine Beziehung zwischen DNS-Gehalt und «grain count» gefunden, woraus folgt, dass die Geschwindigkeit der DNS-Synthese von Zelle zu Zelle variiert.

N. S. COHN

*Department of Botany, Ohio University, Athens (Ohio 45701, USA),*
*26 February 1968.*

[10] A. HOWARD and D. L. DEWEY, Expl Cell Res. *24*, 623 (1961).
[11] F. H. KASTEN and F. F. STRASSER, Nature *211*, 135 (1966).
[12] C. G. CRADDOCK and G. S. NAKAI, J. clin. Invest. *41*, 360 (1962).
[13] The author expresses his appreciation to Prof. P. VAN DUIJN for valuable discussions and for the use of laboratory facilities at the University of Leiden, The Netherlands, where these studies were initiated.

## Beitrag zur Frage der Entstehung mehrkerniger Zellen

Vor einiger Zeit haben wir die Bildung zweier mehrkerniger Zellen aus einer Mutterzelle ohne experimentellen Eingriff beschrieben[1]. Bei der hier beschriebenen Beobachtung haben wir eine unbeeinflusste HeLa-Zellkultur mit dem Zeitrafferfilm festgehalten und dabei festgestellt, dass aus einer durch normale Mitose sich teilenden einkernigen Zelle eine einkernige und eine zweikernige Tochterzelle entstanden sind. Der Entstehungsablauf dieser unterschiedlichen Tochterzellen ist auf einer Serie von Mikrophotographien mit dem 35 mm-Kinofilm festgehalten (Figur). Es sind nur die zur Demonstration des erwähnten Prozesses notwendigen Stadien angeführt. Die Zeiten sind auf die mit Null bezeichnete Aufnahme bezogen.

Auf der ersten Teil-Mikrophotographie (O h) ist die verfolgte Zelle durch einen Pfeil bezeichnet. Auf der zweiten Photographie befindet sich dieselbe Zelle in Mitose. Nach 2 h 3 min ist die Teilung in 2 Tochterzellen festzustellen. Auf einer weiteren Aufnahme ist die Rekonstruktionsphase (2 h 44 min) festgehalten, die jedoch kurze Zeit später zu Ende sein wird. Das Ergebnis der Rekonstruktion ist auf einem weitern Bild festgehalten (3 h 20 min). Man kann beobachten, dass die linke Tochterzelle 2 dicht aneinanderliegende Kerne enthält, deren Gesamtfläche nur wenig grösser ist als die Fläche des Kerns der rechten Tochterzelle. Die zweikernige Zelle lebt längere Zeit, ohne sich zu teilen oder zu degenerieren. Nur die Kerne der zweikernigen Zelle rücken etwas auseinander, wie aus unserer letzten Photographie zu ersehen ist, und zwar nach 5 h 23 min vom Anfang der Beobachtung gerechnet.

Obwohl die Ursachen und Folgen dieses Prozesses verhältnismässig schwierig zu erklären sind, zeigt unsere Beobachtung eine der Bildungsarten mehrkerniger Zellen. Ein ähnlicher Befund wurde von SCHLEICH und MAYER[2]

[1] V. PŮŽA, Experientia *19*, 529 (1963).
[2] A. SCHLEICH und A. MAYER, Z. Krebsforsch. *60*, 47 (1954).