Biochemical Pharmacology, 1965, Vol. 14, pp. 1821–1829. Pergamon Press Ltd., Printed in Great Britain.

CELL VIABILITY AND NUCLEIC ACID METABOLISM AFTER EXPOSURE OF HeLa CELLS TO EXCESS THYMIDINE AND DEOXYADENOSINE

J. H. KIM, S. H. KIM and M. L. EIDINOFF

Biophysics Division, Sloan-Kettering Institute for Cancer Research, New York, N.Y., U.S.A.

(*Received* 25 *June* 1965; *accepted* 6 *August* 1965)

Abstract—Treatment of HeLa S-3 cells with excess thymidine (2 mM) caused blocking of DNA synthesis, which resulted in a synchronization of the cells after removal of thymidine. Continued synthesis of RNA and protein suggests an "unbalanced growth" syndrome with a deficiency in the deoxycytidylate moiety analogous to states induced by 1-β-D-arabinofuranosyl cytosine or 5-fluorodeoxyuridine. Exposure of cells to excess deoxyadenosine produces a loss of cell viability accompanied by an inhibition of DNA, RNA, and protein synthesis.

STUDIES of synchronous division of mammalian cells in culture induced by excess thymidine have been reported by several workers.[1–4] The use of such metabolic inhibitors as excess thymidine facilitates the production of large quantities of partially synchronized mammalian cells. The latter permit the study of metabolic events occurring during the division cycle, although the usefulness of these procedures for study of the normal relationship between intracellular events may be often diminished, owing to severe temporal distortions. During the course of our efforts to obtain large populations of synchronized cells induced by excess thymidine, substantial toxic effects were observed when the cells were exposed for longer than the period corresponding to one generation time of the cell. Consequently, more extensive studies were carried out dealing with aspects of cell viability and nucleic acid metabolism after exposure of HeLa cells to excess thymidine. Related studies were also carried out with excess deoxyadenosine in the culture medium. A principal effect of excess thymidine is a condition of "unbalanced growth' similar to that already observed with 5-fluorodeoxyuridine and 1-β-D-arabinofuranosyl cytosine.[5–7] Excess deoxyadenosine seems to affect both protein and nucleic acid synthesis and reduces the cell viability exponentially, with no apparent shoulder in the surviving fraction when it was plotted semilogarithmically as a function of time of exposure to this compound.

MATERIALS AND METHODS

Cell culture. HeLa S-3 cells (obtained from Dr. P. I. Marcus, Albert Einstein College of Medicine, New York, N.Y.) were grown on Eagle's minimum essential medium supplemented with 10% calf serum. Cells were subcultured weekly by trypsinizing fully-grown cultures (confluent monolayer) in 0·5% trypsin solution until most of the cells were freed from the surface. The trypsin was then inactivated by dilution with nutrient medium. In experiments involving exposure of cells to either excess thymidine or excess deoxyadenosine, cells from the stock cultures were trypsinized and plated in 60-mm plastic petri dishes containing 4 ml of the above

medium. After incubation for 24 hr the medium was removed by aspiration, and 4 ml of fresh prewarmed medium containing the appropriate compound was added to each plate. The plates were incubated in a humidified chamber at 38° in an atmosphere of carbon dioxide, to maintain the pH of the culture medium at 7·4.

Incorporation of labeled precursors into nucleic acid and protein. For studies involving the uptake of ^{3}H-labeled deoxycytidine, uridine, and histidine into DNA, RNA, and protein, respectively, the following procedure was used. Equal numbers of cells (approximately 4×10^{5} cells per plate) were labeled for 20 min with ^{3}H-deoxycytidine (1 μc/ml, 2·4 c/mmole) for DNA synthesis, ^{3}H-uridine (1 μc/ml, 2·0 c/mmole) for RNA synthesis, and ^{3}H-histidine (1 μc/ml, 1·2 c/mmole for protein synthesis). Immediately after the incubation, cells were washed three times with prewarmed saline and were harvested by trypsinization. The trypsinized cells were stored in 12-ml conical centrifuge tubes kept in ice. The trypsin solution was removed by centrifugation, and the cells were washed once with 4 ml of cold saline. Samples were acidified with cold 5% trichloroacetic acid, centrifuged, and the precipitates washed three times with cold 5% TCA. The residues were washed with 80% aqueous ethanol and then suspended in 0·2 ml of 0·2 M NaOH. Radioactivity was determined in samples of this suspension, in a liquid scintillation counter (Nuclear Chicago).

Studies involving the measurement of the rates of incorporation of cytidine-2-^{14}C (0·5 μc/ml; 16·8 mc/mmole) into DNA were carried out by methods previously described.[8, 9]

Nucleic acid and protein determination. These determinations were carried out by methods previously described.[5]

Cell and colony counts. Cell counts were performed with a Coulter counter, model B. Plating for colony counts was carried out in 60-mm plastic petri dishes. Control and treated plates prepared from trypsinized single-cell suspensions were incubated for 14 days at 38°. Colonies were fixed with methanol, stained, and counted after projection with a photographic enlarger. The average clone size exceeded 2 mm in diameter. All clones containing more than 50 cells were scored as reproductively intact.

RESULTS

Effect of excess thymidine or deoxyadenosine on cell division and rate of DNA synthesis

In order to obtain a partially synchronized population induced by excess thymidine, an experiment was carried out in which cells were exposed to 2 mM thymidine for 16 hr. After this time, thymidine was removed and normal medium was introduced. The results are shown in Fig. 1. The control cells showed a logarithmic growth with a doubling time of about 24 hr. After exposure to thymidine, the cell population increased for 8–10 hr and then remained stationary until the medium was changed after 16 hr. The number of cells then began to increase from 23 to 30 hr. It can be seen that HeLa cells exposed to the above concentration of thymidine exhibit a pronounced synchronization of cell division.

Cells exposed to 2–4 mM deoxyadenosine increase in number up to about 8 hr. The population then remains stationary up to about 40 hr. (Fig. 2). Some aspects of DNA synthesis in the partially synchronized system induced by the use of excess thymidine can be studied by the determination of the rate of DNA synthesis after the removal of the excess thymidine. Figure 3 shows the rate of incorporation of ^{14}C-cytidine into DNA immediately before and after the removal of the excess thymidine

from the culture medium. The rate of ^{14}C-cytidine uptake into DNA began to rise very sharply and decreased rapidly as cells passed out of the DNA synthesis phase, reaching minimal activity at 30 hr.

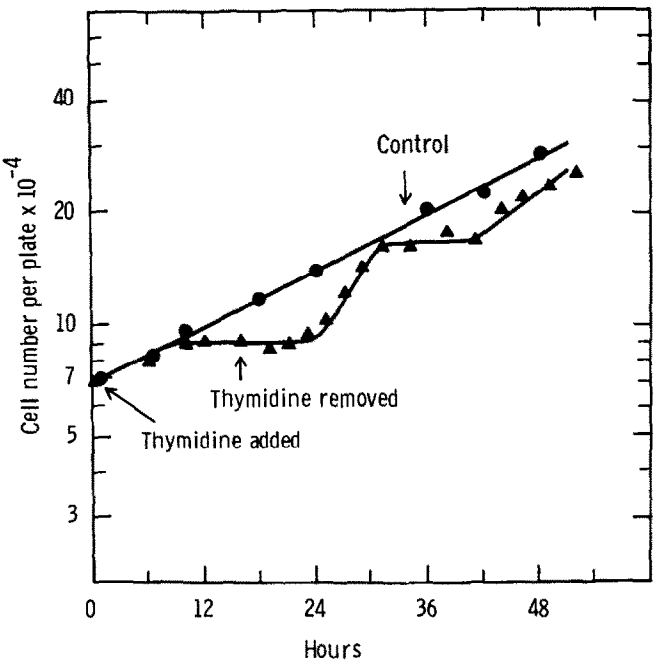

FIG. 1. Growth curves of HeLa S-3 cells after addition of 2 mM thymidine at 0 hr. Thymidine was removed at 16 hr. Thymidine was not present in the control cultures.

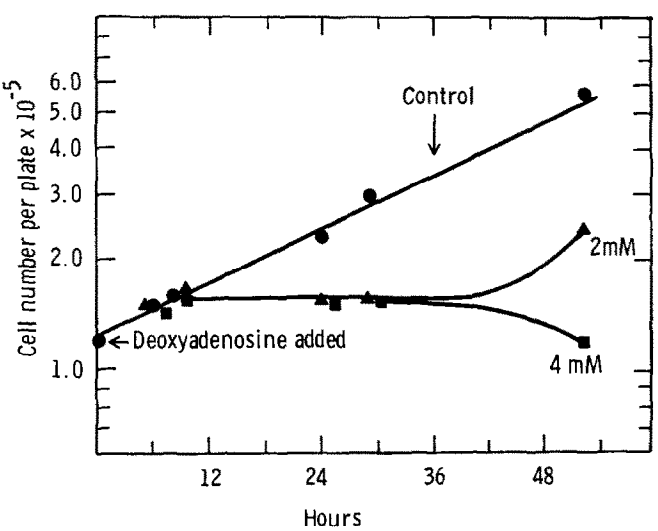

FIG. 2. Growth curves of HeLa S-3 cells after addition of 2 mM and 4 mM deoxyadenosine at 0 hr The control culture received no deoxyadenosine.

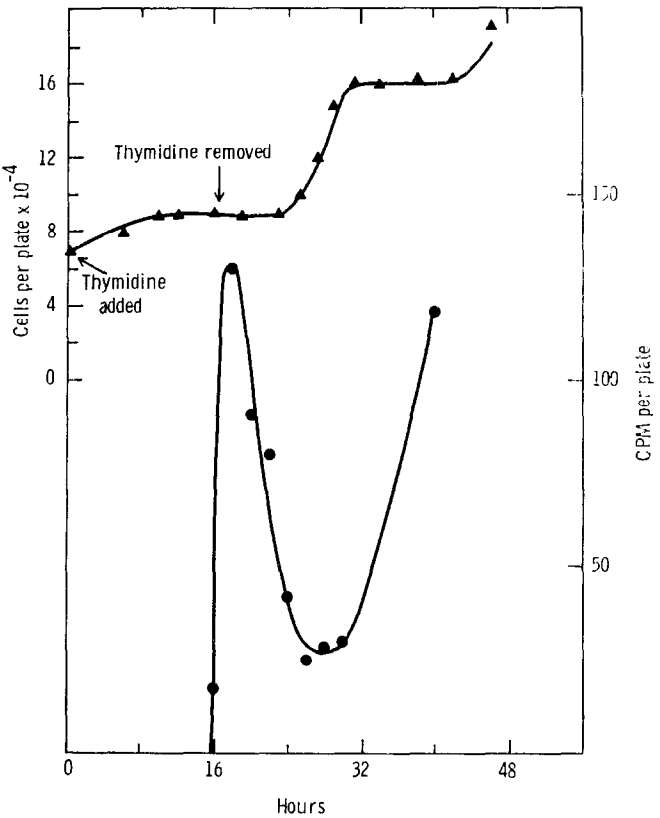

FIG. 3. The utilization of ^{14}C-cytidine for DNA synthesis during partially synchronized growth. Cells were incubated in media containing and lacking thymidine (2 mM) and supplemented with ^{14}C-cytidine (0·5 μc/ml) for 30 min. After incubation, the DNA was isolated from the cells and its radioactivity determined; ●——● counts per minute in DNA; ▲——▲ cells per plate.

Effect of excess thymidine or deoxyadenosine on nucleic acid and protein synthesis

Measurements of the total DNA, RNA, and protein content of HeLa S-3 cells in replicate cultures were carried out at different times after the addition of either thymidine (2 mM) or deoxyadenosine (2 mM and 4 mM). Figure 4 shows the unbalanced growth induced by continuous exposure to thymidine (2 mM). It may be seen that the total DNA in the culture increased by only about 25% in the presence of thymidine. During this period the RNA and protein per plate increased by a factor of approximately 2. These results demonstrate that RNA and protein continued to be synthesized during the period that DNA synthesis was sharply inhibited by thymidine. In contrast to the above findings, the content of DNA, RNA, and protein after the addition of deoxyadenosine was reduced at all the times studied (Figs. 5 and 6).

Incorporation of some labeled precursors into DNA, RNA, and protein in the presence of excess thymidine

As shown above, exposure to excess thymidine resulted in an unbalanced growth of HeLa cells. The RNA and protein content of a plate approximately doubled,

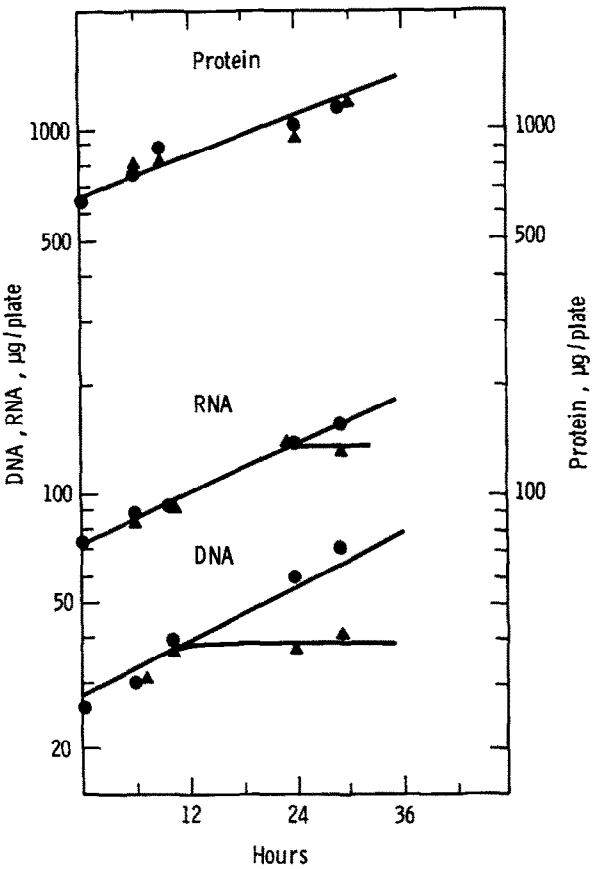

FIG. 4. The increase in DNA, RNA, and protein as a function of time in a culture given 2 mM thymidine at 0 hr; ●——● control; ▲——▲ treated.

TABLE 1. INCORPORATION OF SOME LABELED PRECURSORS INTO DNA, RNA, AND PROTEIN IN THE PRESENCE OF EXCESS THYMIDINE*

Labeled precursors	Thymidine concentration (mM)	Utilization of precursor (cpm/plate)
^{3}H-Deoxycytidine	0	365
	1·2	306
	2·4	277
	4·8	332
^{3}H-Uridine	0	3,200
	1·2	855
	2·4	442
	4·8	373
^{3}H-Histidine	0	187
	1·2	176
	2·4	190
	4·8	182

* Cells were exposed to varying concentrations of thymidine for 1 hr and incorporation of the various labeled precursors was carried out for 20 min.

whereas the DNA content increased slightly. It was of interest to determine whether the utilization of some selected ^{3}H-containing precursors would lead to a similar conclusion about this unbalanced growth. Cells were exposed to varying concentrations of thymidine for 1 hr, and incorporation of the various labeled precursors was carried out for 20 min. Table 1 shows a representative set of data for an experiment carried out many times. The extent of the incorporation of ^{3}H-deoxycytidine and ^{3}H-histidine into DNA and protein respectively was not appreciably different from that of the control value. However, the rate of utilization of ^{3}H-uridine for RNA synthesis was sharply reduced at all the thymidine concentrations. It is thus evident that the use of labeled precursors is not adequate for cellular imbalance studies. This is further discussed below.

Effect of excess thymidine or deoxyadenosine on cell viability

The cell viability (defined in terms of the capacity of a cell to grow out into a macroscopic colony) is shown in Fig. 7 as a function of time of exposure to excess thymidine. Cell-plating efficiencies (defined here as the ratio of total colonies formed to the number of single cells initially inoculated per plate) were generally 0·7 for HeLa cells. The general pattern of the survival curves was similar to those obtained by treatment with 1-β-D-arabinofuranosyl cytosine or 5-fluorodeoxyuridine.[5–7]

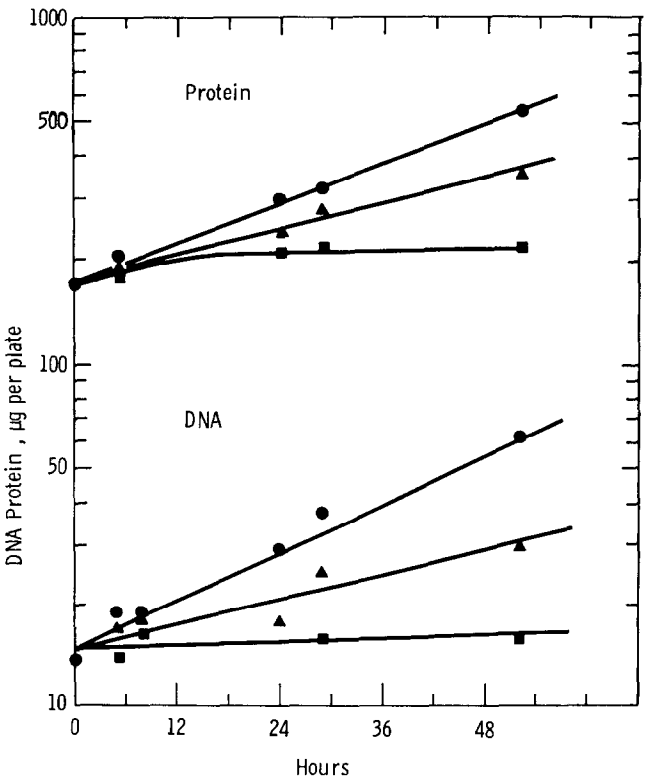

FIG. 5. The increase in DNA and protein as a function of time in a culture given 2 mM and 4 mM deoxyadenosine at 0 hr; ●——● control; ▲——▲ 2 mM; ■——■ 4 mM.

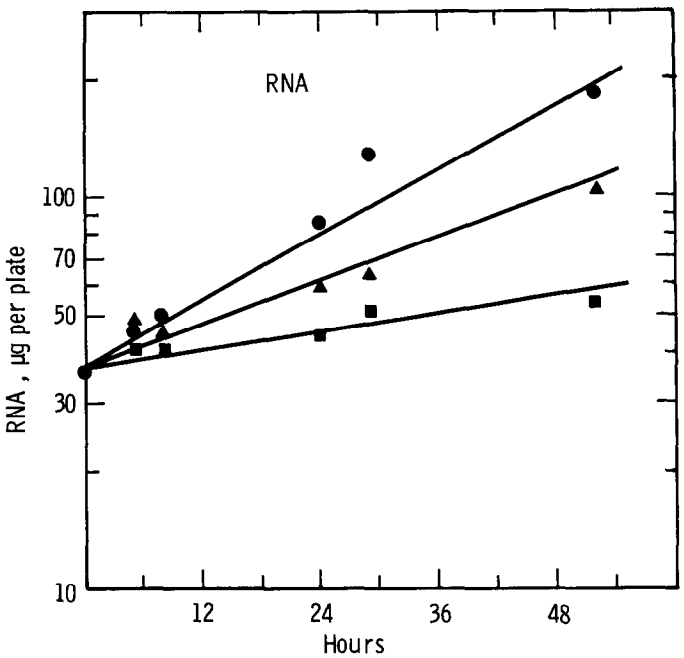

FIG. 6. The increase in RNA as a function of time in a culture given 2 mM and 4 mM deoxyadenosine at 0 hr; ●——● control; ▲——▲ 2 mM; ■——■ 4 mM.

Exposure for a period less than one generation time results in a relatively small loss in viability. Exposure for a period greater than one generation time yields a sharply lowered viability. Figure 8 shows the cell viability as a function of time of exposure to varying concentrations of deoxyadenosine. The survival fraction was

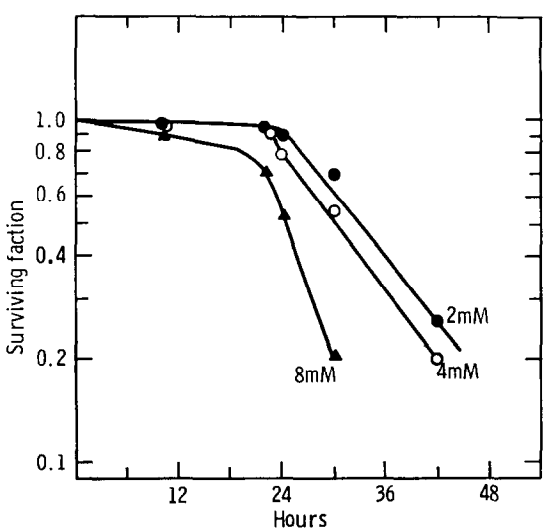

FIG. 7. The surviving fraction of HeLa S-3 cells as a function of time of exposure to various concentrations of thymidine. The control cells (not exposed to thymidine) had a plating efficiency of 70%.

reduced exponentially without having any shoulder (that could be observed in the case of thymidine treatment).

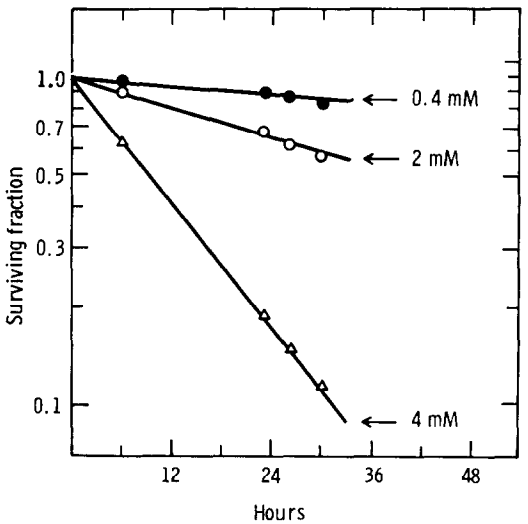

FIG. 8. The surviving fraction of HeLa S-3 cells as a function of time of exposure to various concentrations of deoxyadenosine. The control cells (not exposed to deoxyadenosine) had a plating efficiency of 65%.

DISCUSSION

The present study shows that thymidine (2 mM) is an effective inhibitor of DNA synthesis in HeLa S-3 cells. The accumulation of the cell population in the DNA synthetic (S) phase or at the start of the S phase readily accounts for the partial synchrony present following removal of excess thymidine after a 16-hr exposure to this compound.[2] The above results clearly demonstrate that the continued synthesis of RNA and protein in the presence of excess thymidine leads to a state of unbalanced growth analogous to that induced by 1-β-D-arabinofuranosyl cytosine, 5-fluorodeoxyuridine, or amethopterin.[5-7] Cell viability (as measured in terms of capacity to grow out into macroscopic colonies) was sharply depressed after exposure longer than one generation time but was only slightly reduced when exposures were less than one generation time. Thus, the viability curve (Fig. 7) exhibits a shoulder at low exposure times.

The fact that the excess deoxyadenosine results in the reduction of the biosynthesis of DNA, RNA, and protein (Figs. 5 and 6) is of interest. Deoxyadenosine has been reported to inhibit synthesis of DNA in Ehrlich ascites tumor cells,[10, 11] and in chick embryo mince[12-14] by interference with the endogenous synthesis of guanine and cytosine deoxyribosides. However, the reduction of RNA synthesis in our system could not be easily explained by the above mechanisms and should be sought in some other metabolic steps in a future study. The viability curve (Fig. 8) does not exhibit a shoulder as is the case with excess thymidine (Fig. 7).

Elimination of the block to DNA synthesis following the removal of excess thymidine (Fig. 3) permits resumption of DNA synthesis for the duration of the S

period. This properly accounts for the sharp maximum at 18 hr. The results of tracer studies in Table 1 require independent information concerning mechanisms of action in order to be correlated with the data in Figs. 5 and 6. Addition of ^{3}H-deoxycytidine supplies the moiety that is considered to be blocked by excess thymidine. Under these conditions uptake of label is close to that in control plates. The pronounced reduction of ^{3}H-uridine incorporation into RNA (Table 1) in the presence of excess thymidine may reflect a postulated increase in the acid-soluble pool of uracil-containing nucleotides. This may arise from an increase in the pool of cytosine-containing nucleosides after exposure to excess thymidine. Changes in permeability in the presence of excess thymidine may also be a factor.

The experiments reported here confirm previous reports[1-4] that excess thymidine may be used to obtain a certain degree of synchronization. The use of a metabolic inhibitor may distort the normal time-interval parameters of the mitotic cycle. The increasing toxicity of the excess thymidine for exposures greater than one division time (Fig. 7) makes it desirable to choose an exposure time between G2 + M + G1 and one division time. However, abnormal biochemical changes are associated with this method for producing synchronized populations. Imbalance in cellular content of DNA, RNA, and protein after exposure to excess thymidine was described above. Furthermore, changes in the enzyme composition of the emerging synchronized population may occur.[15]

Acknowledgement—We wish to acknowledge the able technical assistance of A. G. Perez and R Weingart.

REFERENCES

1. N. Xeros, *Nature (Lond.)* **194**, 682 (1962).
2. D. Bootsma, L. Budke and O. Vos, *Exp. Cell Res.* **33**, 301 (1964).
3. D. F. Petersen and E. C. Anderson, *Nature (Lond.)* **203**, 642 (1964).
4. T. T. Puck, *Science* **144**, 565 (1964).
5. J. H. Kim and M. L. Eidinoff, *Cancer Res.* **25**, 698 (1965).
6. M. L. Eidinoff and M. A. Rich, *Cancer Res.* **19**, 521 (1959).
7. R. R. Ruckert and G. C. Mueller, *Cancer Res.* **20**, 1584 (1960).
8. F. C. McIntire and M. F. Sproull, *Proc. Soc. exp. Biol. (N.Y.)* **95**, 458 (1957).
9. J. H. Kim, M. L. Eidinoff and J. S. Laughlin, *Nature (Lond.)* **204**, 598 (1964).
10. H. Klenow, *Biochim. biophys. Acta* **35**, 412 (1959).
11. L. Langer and H. Klenow, *Biochim. biophys. Acta* **37**, 33 (1960).
12. G. F. Maley and F. Maley, *J. biol. Chem.* **235**, 2964 (1960).
13. P. Reichard, Z. N. Canellakis and E. Canellakis, *Biochim. biophys. Acta* **41**, 588 (1960).
14. P. Reichard, Z. N. Canellakis and E. Canellakis, *J. biol. Chem.* **236**, 2514 (1961).
15. P. A. Morse and V. R. Potter, *Cancer Res.* **25**, 499 (1965).