Studies on Unbalanced Growth in Tissue Culture

I. Induction and Consequences of Thymidine Deficiency*

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SUMMARY

Thymidine-deficient growth of HeLa cells induced by either of two agents, amethopterin or 5-fluorouracil-2′-deoxyriboside, resulted in an unbalanced growth syndrome strikingly similar to that described for bacteria. Thus, mitosis and DNA synthesis were blocked while RNA and protein synthesis continued.

After an interval of thymidineless growth corresponding to about two-thirds of a generation, the cells abruptly began to lose their capacity to reproduce progeny at a rate of 90–99 per cent per generation. At about the same time the rate of RNA accumulation fell rapidly and after 24 hours ceased completely. Protein continued to accumulate only very slowly under such conditions. Such results are in accord with the hypothesis that thymidineless death represents a nuclear inactivation.

Restoration of thymidine after a suitable interval of deficient growth resulted in synchronous division of these cells. The growth kinetics of such cultures is described, and evidence suggesting a multiple role of thymidine or its derivative(s) in cellular reproduction is presented.

Thymine deficiency in a population of growing bacterial cells results in a condition called "unbalanced growth" (4, 5). This state is characterized by a cessation of DNA synthesis and cell division while RNA and protein continue to accumulate. As the unbalanced growth state develops there is also a rapid loss of viability and an associated high frequency of mutation (14). The present communication concerns the study of thymidine deficiency in HeLa cells as induced with two agents, 4-amino-N^{10}-methyl folic acid (amethopterin) and 5-fluorouracil-2′-deoxyriboside (FUdr), which interfere with the *de novo* synthesis of thymidine. The results of these experiments confirm and extend those of Eidinoff *et al.* (12, 23) and of Hakala and Taylor (13) and show clearly a striking parallel in the unbalanced growth syndrome exhibited by animal cells and bacteria. In the course of these studies it was also observed that reversal of the unbalanced growth state at the proper time by the addition of thymidine to the cultures had a synchronizing effect on DNA synthesis and cell division. Studies on the growth kinetics and compositional changes of such synchronized cultures are described, and preliminary evidence is presented which suggests a multiple role of thymidine or a derivative in the initiation of DNA synthesis.

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MATERIALS AND METHODS

The experiments described in this paper were performed with a human carcinoma cell[1] (HeLa) grown in our laboratory during the last 4 years on Eagle's HeLa medium (10) containing 10 per cent whole bovine serum[2] and 2×10^{-5}M inositol. Earle's balanced salt solution (11) was employed as the electrolyte base, and cultures were routinely gassed with 5 per cent CO_2 in air. This medium is hereafter referred to as BEHM.

[1] The HeLa cell strain used in these experiments was obtained originally from Dr. Gilbert Chang, State Laboratory of Hygiene, Madison, Wis.

[2] The serum was prepared in our laboratory from freshly defibrinated blood obtained from the Oscar Mayer Packing Plant, Madison, Wis. Sterilization was effected by passage through 03 Selas porcelain filters, and the serum was stored at −20° C.

In experiments with dialyzed bovine serum the medium was also supplemented with glycine and serine at a final concentration of 0.1 mM; this medium is referred to as DBEHM. Dialysis of the serum was carried out against two changes of 100 volumes of cold 0.9 per cent saline agitated with a magnetic stirring bar to facilitate diffusion.

Suspension cultures referred to in the text were grown in modified spinner flasks with the use of a rotating magnetic bar as described by McLimans *et al.* (18). In such cultures the cells were grown in BEHM from which the calcium and magnesium were omitted and which was supplemented with 0.1 mM glycine and serine and with 0.1 per cent carboxymethylcellulose.[3] This medium is referred to in the text as "spinner medium." Stock cultures in suspension were diluted at daily intervals to an initial concentration of 1.5×10^5 cells per ml.

"Amethopterin medium" consists of BEHM supplemented with amethopterin,[4] 10^{-6}M; thymidine, 10^{-5}M; adenosine, 5×10^{-5}M; and glycine and serine, 10^{-4}M, each. This medium supports rapid growth of the cells as shown in the text.

Stock cultures of growing cells were prepared by seeding 50,000 cells per ml. of BEHM into Roux bottles and permitting the culture to grow for 3 days. This procedure insured that the experimental inocula represented vigorously growing cells in the logarithmic phase of growth. Replicate monolayer cultures were prepared by pipetting an aliquot of a cell suspension containing 0.5×10^6 cells in 10 ml. of BEHM into 3-oz. prescription bottles. After 24 hours' incubation to permit attachment of cells to the glass the experiments were initiated either by replacing the medium or by adding an aliquot of complete medium containing the test substance. All operations in experiments on synchronous growth were carried out in a 37° C. room, and all media were pre-warmed to 37° C. in a thermostated water bath before use in cultures in an effort to avoid any possible synchronization which might result from thermal variation.

The viability of cells was determined by plating known numbers of trypsinized cells into BEHM by the methods described by Puck *et al.* (17) but omitting the use of agar. After removing the medium, the monolayer to be assayed for plating efficiency was washed briefly with 0.9 per cent saline and then incubated at 37° C. in 10 ml. of 0.05 per cent trypsin (obtained from Nutritional Biochemicals Corporation under the designation 1-300) in saline A (22). After incubation for 15 minutes at 37° C., an equal volume of warm medium was added, and the cells were dispersed by repeated aspiration and discharge from a pipette. With due precautions this method of preparation yields a monodisperse suspension of HeLa cells with a viability of 80–100 per cent when assayed for ability to form macroscopic colonies from single cells. Aliquots of such cell suspensions were plated into a final volume of 4.5 ml. of BEHM supplemented with 10^{-5}M thymidine in 60-mm. petri dishes and incubated under a water-saturated atmosphere of 5 per cent CO_2 in air for 7–10 days, at which time the colonies were fixed with Bouin's fixative and stained with Giemsa stain for counting.

Analyses of cell composition were carried out in duplicate on the monolayers after the cells were washed 3 times with cold saline to remove residual protein of the medium, and then washed successively with 10-ml. volumes of 4 per cent perchloric acid, 70 per cent ethanol, 95 per cent ethanol and absolute ethanol, followed by successive rinses with absolute ethanol and ether. The dry sheet of cell residue was dissolved in 2.0 ml. of 88 per cent formic acid, and suitable aliquots were desiccated in test tubes for analysis. RNA was determined by the method of Ceriotti (3), DNA by a modified fluorometric procedure of Kissane and Robins (15), and protein by Oyama and Eagle's (20) modification of the Lowry method. A modified anthrone procedure was used to determine glucose in the culture medium (16). Cell counts were usually performed with the Coulter Automatic Counter[5] (Model A) with a 100-μ orifice.

In the experiments to be reported all points are derived from duplicate cultures in which the respective analyses agreed within ± 5 per cent of the mean value. In all cases the individual experiments have been repeated at least once and in most cases 3 or 4 times with comparable results.

RESULTS

Induction of thymidine deficiency with amethopterin—effects on cell division and viability.—The recent observation of Hakala and Taylor (13) that amethopterin produces a nutritional requirement for thymidine in the HeLa cell offers a convenient method for studying the effects of thymidine-deficient growth in mammalian cells. Chart 1 illustrates the growth of HeLa cells in amethopterin medium (see "Methods") in the presence

[3] Carboxymethylcellulose, Type 12-HP, Hercules Powder Company, Wilmington, Delaware.

[4] We are indebted to Dr. H. Waisman for a generous gift of amethopterin.

[5] Coulter Automatic Blood Cell Counter and Cell Size Analyzer (Model A), Coulter Electronics, Chicago 40, Ill.

and absence of added thymidine. Whereas the thymidine-deficient cells rapidly ceased to divide, they continued to increase in size over a period of 72 hours or longer.

Continued growth of cultures in a thymidine-deficient medium beyond a period corresponding to approximately one generation time resulted in an irreversible loss of the capacity of individual cells to give rise to macroscopically visible colonies when plated into complete medium supplemented with thymidine (Chart 1). More detailed experiments on thymidine deficiency with cells grown in suspension culture have revealed that the reversible period lasts about 16–20 hours; thereafter the plating efficiency declines at a rate of 90–99 per cent per generation time. Whereas this criterion scores the ability of a single cell to give rise to sufficient progeny to form a macroscopically visible colony within 10 days, microscopic examination of the plates revealed that a significant percentage (1–10 per cent) of the "nonviable" cells survived the 10-day incubation without giving rise to visible colonies. The majority of these microcolonies were abortive, consisting of single or double giant cells or of a small number of cells which were degenerating. Such cells were highly reminiscent of the x-radiated cultures described by Puck *et al.* (21). The remaining cells which were unaccounted for as either macroscopic colonies or abortive microcolonies apparently died and disintegrated.

Kinetics of cell division and macromolecular biosynthesis during thymidine-deficient growth.—Cell division ceased completely soon after initiation of thymidineless growth; typically, the cell number increased only 4–10 per cent after addition of amethopterin (Chart 2). Even in the presence of thymidine a brief anomalous suppression of mitosis was observed upon addition of amethopterin; however, in the presence of thymidine the cells rapidly resumed their usual proliferative rate.

In spite of the rapid cessation of mitotic activity the thymidine-deficient cells exhibited a limited increase (10–45 per cent in various experiments) of a DNA-like material (Chart 2). This material appeared to be physiologically unstable, since, on continued culture, an equivalent amount of DNA-positive material was lost from the cells. On the other hand, RNA and protein of thymidine-deficient cells continued to accumulate at normal rates for about 16 hours when a rapid decline in this rate was observed. While RNA accumulation ceased completely after 36 hours, protein continued to accumulate slowly.

Induction of thymidine-deficient growth with FUdr.—It has been previously demonstrated that FUdr effectively blocks DNA synthesis in animals (2) and microorganisms (6) by inhibiting the con-

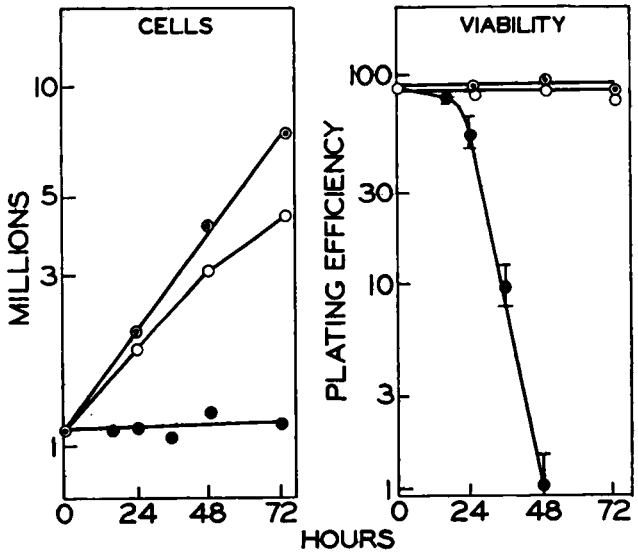

CHART 1.—Inhibition of cell division and loss of viability during thymidine-deficient growth induced with amethopterin. —Replicate cultures were prepared as described under "Materials and Methods." The experiment was initiated by replacing the medium with 10 ml. of experimental medium. ⊙ = BEHM; ○ = amethopterin medium (see under "Methods"); ● = amethopterin medium minus thymidine. "Viable" cells were titrated by plating them into 4.5 ml. "enriched" BEHM (containing glycine and serine, 0.1 mM each, and adenosine and thymidine at 0.01 mM each to reverse the action of any intracellular amethopterin). After 10 days' incubation plates were fixed, stained, and scored for macroscopically visible colonies. Each point represents the average variation obtained from quadruplicate plates.

version of deoxyuridylic acid to thymidylic acid. In growth inhibition studies with H.Ep. #1 cells, Eidinoff *et al.* (12) demonstrated that the toxic effects of this compound were specifically reversed by thymidine. These observations have been confirmed in our laboratory with the HeLa strain.

However, as shown in Chart 3, the capacity of 10^{-5}M thymidine to reverse the growth-inhibitory effects of FUdr was dependent upon the concentration at which this drug was employed. Thus, whereas thymidine (10^{-5}M) reversed the effect of 10^{-6}M FUdr (a concentration sufficient for complete inhibition of HeLa growth, as shown in Chart 4) it did not completely reverse the effect of 10^{-5}M FUdr. In view of the known suppressive effects of fluorouracil and its riboside

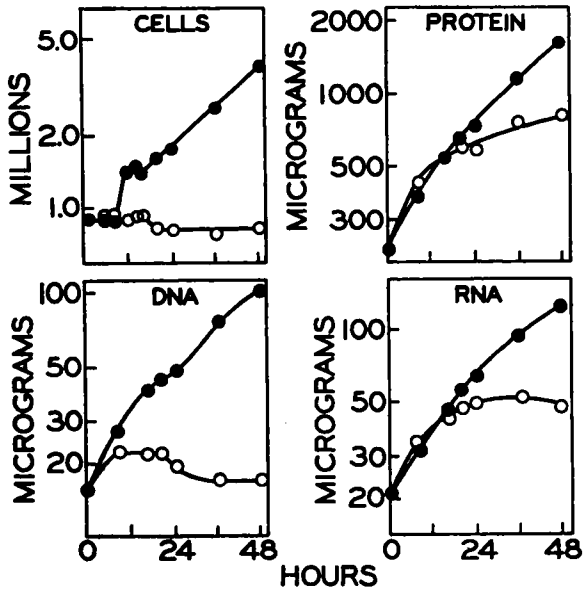

CHART 2.—Cell division and macromolecular biosynthesis during unbalanced growth in the HeLa cell.—Replicate monolayer cultures were prepared as described under "Materials and Methods." The experiment was initiated by adding 1 ml. warm complete growth medium containing sufficient amethopterin, serine, glycine, adenosine, and thymidine to bring each of the components to the respective final concentrations routinely employed in amethopterin medium (see "Materials and Methods"). The thymidineless cultures were identical, except that thymidine was omitted and replaced by water. ● = control; ○ = thymidine omitted.

derivatives on RNA biosynthesis (2), it is possible that some FUdr was converted to other derivatives (e.g., by transdeoxyribosylation-ribosylation reaction) and that such side reactions contribute to the toxicity of FUdr at elevated levels. However, this aspect of FUdr toxicity was not investigated further, since it was not of great importance to the experimental design.

Alternatively it is necessary to use a concentration of FUdr sufficiently high to maintain a sustained inhibition. Thus with cell populations greater than 10^5 cells per ml. only transient inhibition of cell division was observed, which was followed by continued growth. Such "breakthrough" was not observed when low cell populations or sufficiently high concentrations were employed, suggesting that the cells were capable of metabolizing the drug to less inhibitory products. Consequently, 10^{-6}M FUdr (a concentration permitting complete, yet relatively specific, inhibition of DNA synthesis while providing a sufficient reservoir of drug to prevent "breakthrough" with population densities of 10^5 cells per ml.) was chosen as the best compromise.

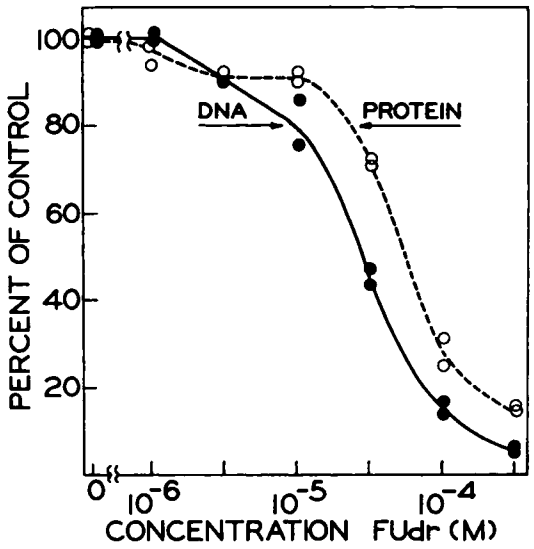

CHART 3.—Influence of FUdr concentration on the growth of thymidine-supplemented HeLa cells.—Replicate cultures containing 0.20×10^6 cells in 10 ml. BEHM supplemented with 10^{-5} M thymidine and the indicated initial concentration of 5-fluoro-2'-deoxyriboside were grown 98 hours at 37° C. and the monolayers analyzed for DNA and protein as described under "Materials and Methods."

Chart 4 illustrates the rapid cessation of cell division and blockade of DNA synthesis produced by FUdr. In contrast, RNA and protein initially accumulated almost as rapidly as in the control cultures containing thymidine; subsequently, however, RNA accumulation ceased, and protein accumulated only very slowly in a manner quite analogous to that observed in amethopterin-treated cultures. A similar decline in viability was also observed after 24 hours of unbalanced growth. Thymidineless death was almost entirely avoided however, in FUdr-treated cells which were simultaneously starved of arginine, a treatment which,

as shown in Chart 4, virtually abolished RNA or protein accumulation. DeMars and Hooper (7) have recently reported similar observations in which cells were protected against death by unbalanced growth by superimposing various amino acid deficiencies.

Effects on glucose metabolism.—Table 1 shows that the thymidine deficiency state did not significantly influence the amount of glucose utilization by HeLa cells during the first 48 hours of culturing; this occurred despite the fact that cell reproduction had been blocked by the thymidineless state. In fact, the observation that the amount of glucose used in the second 24-hour period was increased over the amount used in the first 24-hour period is in accord with the synthesis of new enzymes concerned with glucose metabolism even in the absence of DNA synthesis. In contrast, the arginine-deficient cells maintained the same rate of glucose utilization in the presence or absence of FUdr; this observation is consistent with a failure to accumulate more enzymes involved in glucose metabolism. These results are in accord with other experiments in which the activity of a number of amino acid-activating enzymes has been observed to increase in proportion with the protein accumulated during thymidine-deficient growth.[6]

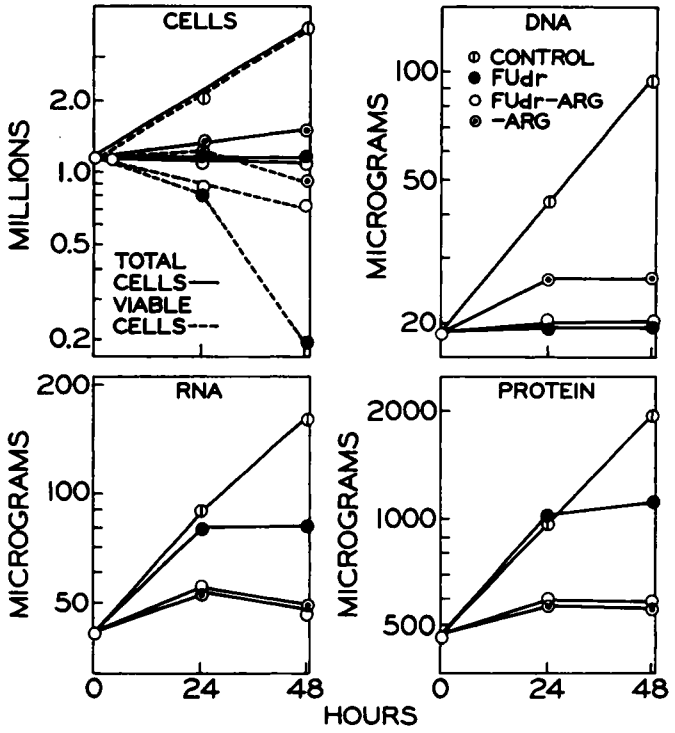

CHART 4.—Unbalanced growth induced by FUdr and the protective effect of arginine starvation.—Replicate cultures containing 0.5×10^6 cells (previously grown through four generations on DBEHM) in 10 ml. DBEHM were incubated 1 day to permit the cells to attach to the glass. The experiment was initiated by replacing the medium with 10 ml. of the respective experimental media supplemented with 10 per cent dialyzed bovine serum. FUdr was employed at a final concentration of 10^{-6} M and thymidine at 10^{-5} M. Arginine was omitted from the amino acid stock of the medium where indicated. Control cultures contained FUdr plus thymidine. "Viable" cells were titrated by plating them into 4.5 ml. BEHM supplemented with 10^{-6} M thymidine to reverse the action of any intracellular antagonist.

TABLE 1

GLUCOSE UTILIZATION BY HELA CULTURES IN VARIOUS GROWTH STATES

	Mg. glucose used	
	24 hours	48 hours
Control	2.60	5.58
FUdr	2.38	5.04
Minus arginine	1.16	2.32
FUdr minus arginine	1.20	2.26

Cultures were prepared and grown as described under Chart 1. Glucose used represents the difference between glucose present at zero time and that present at the time intervals indicated in a standard culture.

[6] Unpublished data of Mr. Judson Spalding in this laboratory.

Synchronization of cell division by thymidine rescue following unbalanced growth.—As shown above the thymidine-deficient growth of HeLa cells, whether induced with amethopterin or with FUdr, is characterized by a cessation of cell division and blockade of DNA synthesis. The cells retain their viability for a period of 16–20 hours, when they undergo an irreversible change resulting in a rapid "killing" of the cells. Addition of thymidine to a culture which has undergone unbalanced growth for a period of 16 hours (the brink of imminent death) rescues them and results in a burst of mitosis 6–8 hours later. Chart 5 illustrates a typical synchronization experiment in which a growth suspension culture was subjected to 16 hours of unbalanced growth with amethopterin, then rescued with thymidine. Division began about 6 hours after thymidine addition and ceased after a population increase of about

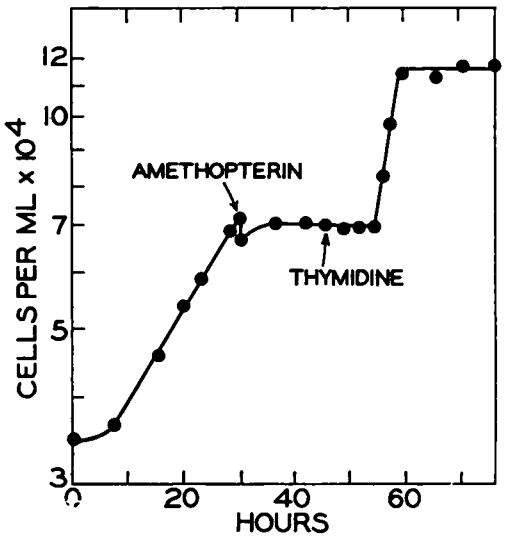

CHART 5.—Synchronization of HeLa in suspension culture by thymidine rescue following unbalanced growth.—After cell growth was well under way one-tenth of a culture volume of warm "spinner" medium (see under "Materials and Methods") containing amethopterin, 10^{-5} M, and adenosine, 5×10^{-4} M, was added to the culture at the point labeled "amethopterin." Sixteen hours later at the point labeled "thymidine" a hundredth culture volume of 10^{-3} M thymidine was added.

70 per cent. A similar synchronization has been achieved with 10^{-6}M FUdr used in place of amethopterin. Population increases of 70–95 per cent have been routinely obtained with either amethopterin or FUdr used as the blocking agent with both glass-attached monolayers and suspension cultures. Accordingly, the reversal of the thymidine deficiency state appears to be a useful tool for production of synchronized cultures. Extended time studies on such synchronized cultures are under current investigation.

Growth kinetics of synchronized cultures.—Chart 6 summarizes the kinetics of cell division, DNA synthesis, and protein accumulation during the course of a thymidine rescue experiment. In this experiment, upon addition of amethopterin cell division ceased promptly; however, the synthesis of a DNA-like material continued until an amount equivalent to 45 per cent of the initial DNA had accumulated. Similarly, failure to rescue the cells with thymidine resulted in the loss of a like quantity of "DNA" 16–20 hours after unbalanced growth was initiated. This corresponds to the time when cell death usually begins and the rate of RNA and protein synthesis begins to fall.

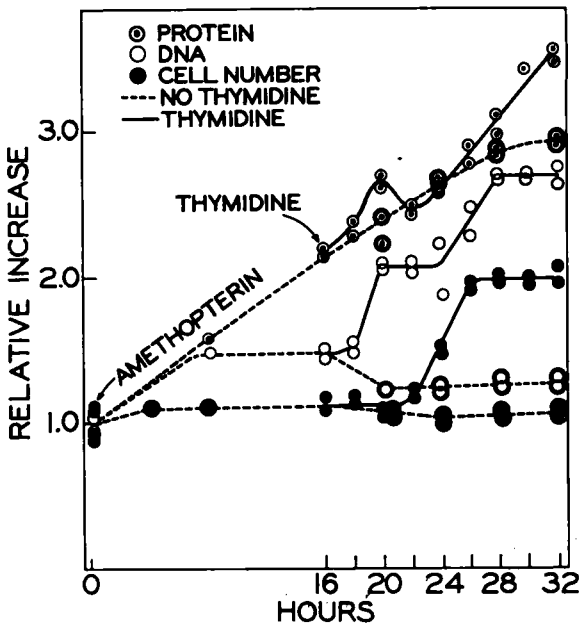

CHART 6.—Growth kinetics in a synchronized HeLa monolayer culture.—Replicate cultures were prepared as described under "Materials and Methods." The experiment was initiated by adding 1.1 ml. warm BEHM containing sufficient amethopterin, serine, glycine, and adenosine (but not thymidine) to bring each of these components to the respective final concentrations routinely employed in amethopterin medium (see "Methods"). After 16 hours' thymidineless growth, 0.11 ml. 10^{-3} M thymidine was added to one set of cultures and an equal volume of warm sterile water added to the others as indicated. Zero time cultures contained 1.03×10^6 cells. The composition of these cells at zero time was DNA, 11.6, and protein, 290, μg. per 10^6 cells, respectively. The solid line indicates growth in presence of thymidine; the dotted line, growth in absence of thymidine.

Addition of thymidine at 16 hours induced a single wave of cell division 6 hours later corresponding to a population increase of 85 per cent over the next 4-hour period. During this period

of observation two waves of DNA accumulation were observed repeatedly, the first of which occurred between 2 and 4 hours and was clearly premitotic; the second, between 8 and 12 hours, occurred after thymidine addition. Whereas the meaning of this second wave is more difficult to interpret, its initiation and cessation lagged by 2 hours the initiation and cessation of cell division and strongly suggest that this wave of DNA synthesis followed cell division.

The possibility that these twin steps of DNA accumulation represented the activities of a population of cells containing two genetic variants, one synthesizing DNA soon after thymidine addition, the other responding late, was tested by following the growth kinetics of a synchronized culture of HK-1,[7] a freshly isolated clonal strain of HeLa. This experiment yielded a result similar to that described above. It now remains to be established whether these twin bursts of synthesis correspond to (*a*) a single population of cells with bimodal DNA synthesis or (*b*) a dual cell population segregated by thymidine-deficient growth into two groups, each of which synthesized DNA at different times. These alternatives should be readily distinguishable by suitable autoradiographic labeling studies.

Of additional interest was a small but definite burst in protein accumulation which invariably occurred 2–4 hours after thymidine addition and which disappeared prior to a second acceleration in the rate of protein accumulation. Since preliminary experiments with leucine-1-C^{14} revealed little stimulation of protein synthesis immediately following thymidine addition and since small amounts of protein are extracted from the cells during the acid and alcohol washes used in preparing the cells for analysis, it is suggested that the apparent increase in protein following thymidine addition may reflect primarily an alteration in the physical state of the cell proteins rendering them less extractable. The nature of these changes which are brought about only on thymidine addition bears further investigation.

DISCUSSION

The acute interruption of cell division in spite of continued accumulation of a DNA-like material following the initiation of thymidine-deficient growth deserves further study, since one might reasonably expect that cell division would continue with DNA synthesis. It seems not unlikely that this material may represent an altered DNA which is unable to initiate division. With respect to the latter possibility it is of interest that Dunn and Smith (8, 9) have isolated a 6-methylaminopurine-containing DNA from thymine-starved bacteria. It will be of interest to see whether a similar phenomenon occurs in thymidine-starved HeLa cells.

The synchronization of cell division by thymidine rescue after a period of unbalanced growth was first described in bacteria by Barner and Cohen (1) and represents another point of similarity in the effects of thymidine deficiency in animal and bacterial cells. However, the growth kinetics of HeLa monolayer cultures following thymidine addition show some interesting new features including (*a*) apparent changes in the physical properties of the protein shortly following thymidine addition and (*b*) a biphasic burst of DNA synthesis, accompanied by a single wave of cell division. The lags which precede DNA accumulation suggest that the cellular machinery is not poised for DNA synthesis at the time of thymidine addition but becomes competent only after a period of exposure to this material. Accordingly this interval is presently being investigated in an effort to clarify a possible auxiliary role of thymidine or its derivatives in poising DNA synthesis over and above its presently known role as a fundamental building block for synthesis of the DNA molecule. Preliminary experiments with the blockade of protein synthesis with puromycin suggest that protein synthesis may play a role in preparing at least a portion of the cell population for DNA synthesis. Thymidine diphosphate rhamnose (19) and several other similar nucleotide forms (24) distinct from those known to be involved in DNA synthesis, have been isolated from bacterial cultures. The latter substances are thymidine nucleotides containing additional unidentified deoxy-sugars. It will be of interest to ascertain whether or not such derivatives play auxiliary roles in mammalian cellular reproduction.

[7] Isolated from our stock strain by Dr. K. Kajiwara.

REFERENCES

1. Barner, H. D., and Cohen, S. S. Synchronization of Division of a Thymineless Mutant of *E. coli*. J. Bacteriol., 72:115–23, 1956.
2. Bosch, L.; Harbers, E.; and Heidelberger, C. Studies on Fluorinated Pyrimidines. Cancer Research, 18:335–43, 1958.
3. Ceriotti, G. Determination of Nucleic Acids in Animal Tissues. J. Biol. Chem., 214:59–70, 1958.
4. Cohen, S. S., and Barner, H. D. Studies on Unbalanced Growth in *Escherichia coli*. Proc. Nat. Acad. Sc., 40: 885–93, 1954.
5. ———. Studies on the Induction of Thymine Deficiency and on the Effects of Thymine and Thymidine Analogues in *Escherichia coli*. J. Bacteriol., 71:588–97, 1956.
6. Cohen, S. S.; Flaks, J. G.; Barner, H. D.; Loeb, M. R.; and Lichtenstein, J. The Mode of Action of

5-Fluorouracil and Its Derivatives. Proc. Nat. Acad. Sc., **44**:1004–12, 1958.

7. DEMARS, R. L., and HOOPER, J. L. A Method for Selecting for Auxotrophic Mutants of HeLa Cells. J. Exper. Med., **111**:559–71, 1960.
8. DUNN, D. B., and SMITH, J. D. Occurrence of a New Base in the Deoxyribonucleic Acid of a Strain of *Bacterium coli*. Nature, **175**:336–37, 1955.
9. ———. The Occurrence of 6-Methylaminopurine in DNA. Biochem. J., **68**:627–36, 1958.
10. EAGLE, H. Nutritional Needs of Mammalian Cells in Tissue Culture. Science, **122**:501–4, 1955.
11. EARLE, W. R. Production of Malignancy *in vitro*. IV. The Mouse Fibroblast Cultures and Changes Seen in the Living Cells. J. Nat. Cancer Inst., **4**:165–212, 1943.
12. EIDINOFF, M. L., and RICH, M. A. Growth Inhibition of a Human Tumor Cell Strain by 5-Fluoro-2′-deoxyuridine. Cancer Research, **19**:521–24, 1959.
13. HAKALA, M., and TAYLOR, E. The Ability of Purine and Thymine Derivatives and of Glycine To Support the Growth of Mammalian Cells in Culture. J. Biol. Chem., **234**:126–28, 1959.
14. KANAZIR, D. The Apparent Mutagenicity of Thymine Deficiency. Biochim. et Biophys. acta, **30**:20–23, 1958.
15. KISSANE, J. M., and ROBINS, E. The Fluorometric Measurement of Deoxyribonucleic Acid in Animal Tissues with Special Reference to the Central Nervous System. J. Biol. Chem., **233**:184–88, 1959.
16. KOEHLER, L. H. Differentiation of Carbohydrates by Anthrone Reaction Rate and Color Intensity. Anal. Chem., **24**:1576–79, 1952.
17. MARCUS, P. I.; CIECIURA, S. J.; and PUCK, T. T. Clonal Growth *in vitro* of Epithelial Cells from Normal Human Tissues. J. Exper. Med., **104**:615–27, 1956.
18. MCLIMANS, W. R.; DAVIS, E. V.; GLOVER, F. L.; and RAKE, G. W. The Submerged Culture of Mammalian Cells. J. Immunol., **79**:428–33, 1957.
19. OKAZAKI, R. Isolation of a New Deoxyriboside Compound, Thymidine Diphosphate Rhamnose. Biochem. & Biophys. Research Commun., **1**:34–39, 1959.
20. OYAMA, V. E., and EAGLE, H. Measurement of Cell Growth in Tissue Culture with a Phenol Reagent (Folin-Ciocalteau). Proc. Soc. Exper. Biol. & Med., **91**:305–7, 1956.
21. PUCK, T. T., and MARCUS, P. I. Action of X-rays on Single Mammalian Cells. J. Exper. Med., **103**:653–60, 1956.
22. PUCK, T. T.; MARCUS, P. I.; and CIECIURA, S. J. Clonal Growth of Mammalian Cells *in vitro*. J. Exper. Med., **103**:273–84, 1956.
23. RICH, M. A.; BOLAFFI, J. L.; KNOLL, J. E.; CHEONG, L.; and EIDINOFF, M. L. Growth Inhibition of a Human Tumor Cell Strain by 5-Fluorouracil, 5-Fluorouracilriboside and 5-Fluorouracil-2′-deoxyriboside-Reversal Studies. Cancer Research, **18**:730–35, 1958.
24. STROMINGER, J. L., and SCOTT, S. S. Isolation of Thymidine Diphosphosugars from *Escherichia coli*. Biochim. et Biophys. acta, **35**:552–53, 1959.