age. Lipid peroxidation aging processes may be a universal disease whose chemical deteriorative effects might be slowed by increased intake of antioxidants (*11*).

We have studied the fluorescent characteristics of products formed in lipid peroxidation reactions of subcellular organelles in vitro. Rat liver mitochondria, microsomes, and lysosomes, prepared according to the method of Ragab *et al.* (*12*), were suspended in 0.15*M* NaCl. Lipid peroxidation reactions proceeded when these suspended organelles were shaken in oxygen at 37°C (*5*). At intervals, the suspensions were centrifuged at 12,000*g* for 10 minutes, and the supernatants were analyzed. Fluorescence and excitation spectra and thiobarbituric acid reactions (*13*) were obtained for all samples. Fluorescence was measured (Fig. 1) with an Aminco-Bowman spectrophotofluorometer which was standardized with quinine sulfate.

In addition to the increases in the fluorescence and excitation spectra (Fig. 2), characteristic of the Schiff base product, an increase is found in flavin fluorescence in the 515-nm region as a result of enzymic hydrolysis of flavin-adenine dinucleotide (*14*). Lysosomes show an increase in fluorescence at 460 nm as a function of oxidation time, while the free thiobarbituric acid reactants remain constant. When oxidations were extended to 24 hours, the intensity of the fluorescence at 460 nm was greatly increased, with only a small increase in the flavin peak. Among the organelles, mitochondria gave fluorescence and excitation spectra most similar to those of age (lipofuscin) pigment and showed concurrent increases of fluorescence at 460 nm and thiobarbituric acid reactants as a function of oxidation. Microsomes showed an increase in fluorescence and thiobarbituric acid reactants beginning at 3 hours. Although there was a definite fluorescence peak for the Schiff base product, the fluorescence spectrum of microsomes was dominated by increased flavin fluorescence. For all three organelles the production of the chromophore fluorescent at 460 nm was inhibited approximately 75 percent by an antioxidant (0.02 percent propyl gallate).

K. S. Chio, U. Reiss
B. Fletcher, A. L. Tappel
Department of Food Science and Technology, University of California, Davis 95616

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15. Supported by PHS research grants AM-09933 and AM-05609.

20 August 1969; revised 2 October 1969

Temporal Coordination of DNA Replication with Enzyme Synthesis in Diploid and Heteroploid Cells

Abstract. *The rate of DNA synthesis in the S phase of growth of synchronized diploid Chinese hamster cells shows two maximums, while in heteroploid hamster cells the DNA replication rate is constant. In diploid cells a reciprocal relationship exists between maximum DNA synthetic rates and maximum lactate dyhydrogenase and thymidine kinase enzyme levels. Enzyme activity in heteroploid cells increases continuously through the cell cycle with no evidence of oscillations. It seems possible that these differences in molecular organization may accompany or precede the transition to heteroploidy.*

Oscillatory changes in the intracellular concentration of large and small molecules occur in both prokaryotic and eukaryotic cells. Some can be induced by altered nutrient levels, and involve transient perturbations in the concentration of metabolites (*1*), while others are revealed only by the establishment of a synchronous cell population and may involve fluctuations in levels of metabolites (*2*) or macromolecules (*3, 4*). Macromolecular periodicities show a great deal of regularity and occur at precise times in each cell cycle. Because of this stability they can be accorded an important function in the cellular timekeeping process.

DNA replication in metazoan cells is an intermittent process whose onset and termination are used to subdivide the cell cycle (*5*). Genetic and cytological studies have given additional significance to the temporal order of DNA replication, since certain chromosomes and chromosome segments, usually exemplified by the sex chromosomes in somatic cells of mammals, are distinctly and repeatedly the last to replicate their DNA in the S phase of growth (*6*). The correlation of late DNA replication with sex chromatin, and the many demonstrations that heteropyknosis and late-replicating DNA are associated with gene inactivation, suggest that time of replication might be a rather permanent and general method in a hierarchy of regulatory mechanisms, insuring that unwanted gene products will not be made (*7*). It seems evident that replication patterns are a manifestation of nuclear differentiation, since they can vary from tissue to tissue and have a distinct onset during embryogenesis (*8*).

In rapidly dividing mammalian cells the temporal coordination of DNA synthesis with RNA and protein synthesis is clearly expressed. Both RNA and protein synthesis show gene dosage effects or stimulated synthesis following DNA replication (*9–11*). In pursuing this effect Klevecz and Ruddle observed that lactate dehydrogenase and glucose-6-phosphate dehydrogenase levels oscillated through the cell cycle of a Chinese hamster diploid strain (*4*), and subsequently I observed that these oscillations represented changing amounts of enzyme protein (*11*). In this report I show that the rate of DNA synthesis is constant through the S phase of growth of heteroploid (*12*) hamster cells, but intermittent in diploid cells. A reciprocal relationship exists between maximum rates of DNA synthesis and maximum enzyme levels in diploid but not in heteroploid cells, and I suggest that this is a general distinction between diploid and heteroploid cells.

The following Chinese hamster cells were used: the diploid line Don (CCL-16); a pseudodiploid strain (CS134) derived from diploid Don; a cell line

(G3, CCL14) with a modal chromosome number of 22, but with altered chromosome morphology and chromosome replication pattern; and a true heteroploid line (CHO) with a modal chromosome number of 21. Both Don and CS134 are severely contact-inhibited and contain all late-replicating chromosome segments originally described for Chinese hamster cells. Chromosome analysis and growth characteristics of these four cell types have been reported previously (*13*). Variations in chromosome number of the two diploid cell types were minimized by always performing synchrony studies on cells which had been thawed from liquid nitrogen for not more than 2 months. Consequently, the chromosome constitution of these two cell types remains the same as described in the original reports (*13*). Following prolonged culturing, both diploid lines tended to gain small metacentric chromosomes. Methods of cell culture, synchronization, assay of lactate dehydrogenase activity (*4, 9*), and determination of isotopic precursor incorporation (*9*) have all been described previously. Thymidine kinase activity was assayed as described by Bresnick and Karjala (*14*). Cytological preparations and autoradiographic procedures were the same as those reported by Hsu (*13*).

The changes which occur in rates of RNA synthesis and enzyme activity in the mammalian cell cycle have been studied almost exclusively in established heteroploid cell lines such as HeLa. The rate of DNA synthesis in HeLa cells is constant through the S phase of growth, the same DNA molecules are replicated late in each cell generation, and some of the cytologically distinct blocks of late-replicating DNA are evident (*15*). RNA synthesis in synchronized HeLa cells increases through the cell cycle, and in some cases rather abruptly following DNA replication. Enzyme studies show continuously increasing activities through the G_1 and early S growth phases, which reach a maximum in the late S phase and then decrease slightly in the G_2. This pattern has held true for thymidine kinase and thymidylate kinase in HeLa cells, for ornithine transaminase in Chang's liver cells, and for thymidine kinase in mouse L cells (*16*). It seems likely that heteroploid cells increase their transcriptional and translational activities in a rather continuous fashion through the cell cycle.

Chinese hamster diploid cells have a very distinct chromosomal late-replication pattern (*13*). The rate of replication was measured in the diploid CS134 cell strain by pulse-labeling with tritiated thymidine (Fig. 1). DNA synthesis does not go on at a constant rate through the S phase, but rather shows a bimodal distribution. One maximum occurs 5 hours after mitosis (M) or about 2 hours into the S phase. A second maximum occurs 8 hours after mitosis, or about 5 hours into the S phase. Autoradiographs prepared of cells pulsed 5 and 8 hours after mitosis indicate that these maximums correspond to the early and late replicating regions of the chromosome. Rather than simply accelerating in rate as the sex chromosomes replicate, a pause in DNA synthesis seems to occur in the mid S phase. The cessation or slowing in replication rate was particularly interesting in view of our earlier work (*4*)

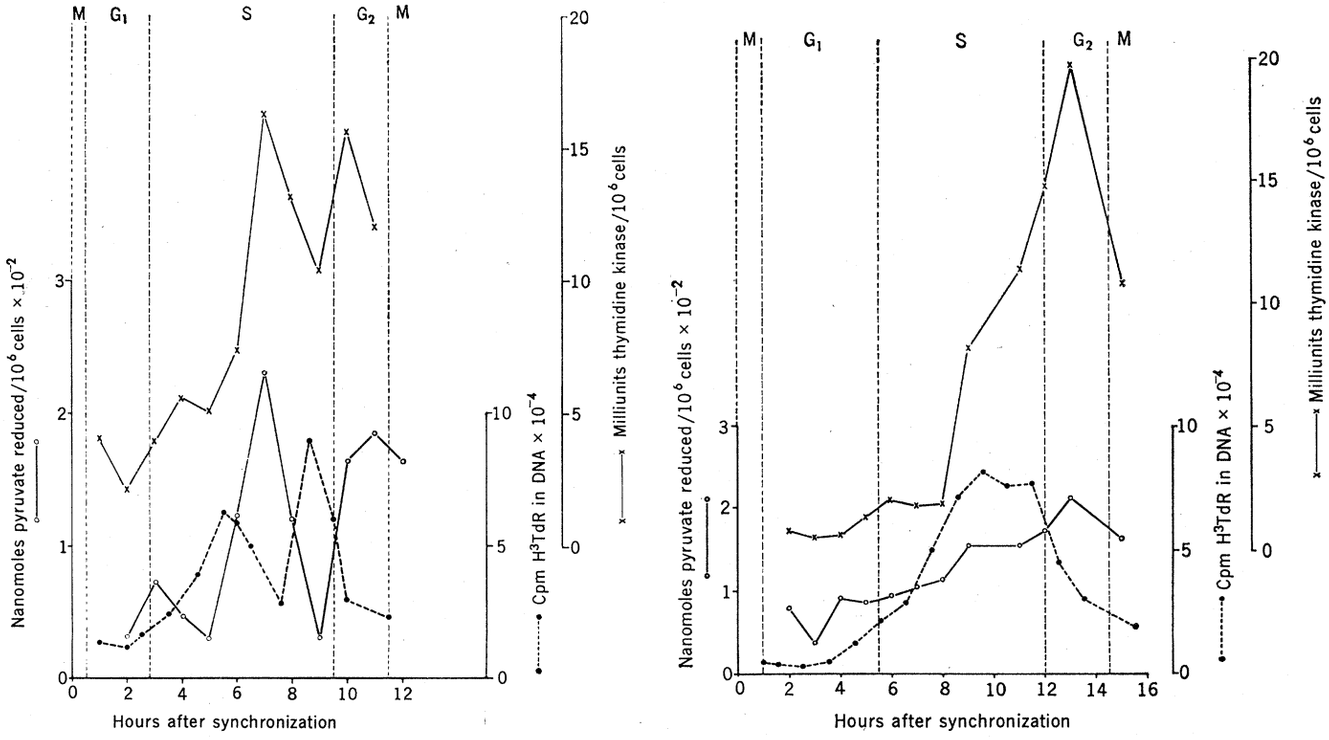

Fig. 1 (left). DNA synthesis and lactate dehydrogenase and thymidine kinase activity in the CS134 pseudodiploid cell strain. Cultures synchronized by the Colcemid-selection method and subcultured directly into scintillation vials were exposed at hourly intervals to a 1-hour pulse of H^3-thymidine (5 μc/ml, 5 c/mmole; Amersham-Searle). Extraction of the soluble pool, precipitation of DNA-specific tritium counts, and assay of lactate dehydrogenase were performed as previously described (*4*). Thymidine kinase assay was performed as described by Bresnick and Karjala (*14*). Activity is expressed as milliunits of thymidine kinase per 10^6 cells, with one unit being the amount of enzyme required to phosphorylate 10^{-7} mole of TdR per hour. Fig. 2 (right). DNA replication and enzyme activity in the heteroploid hamster cell line G3. Replicate cultures of G3 cells were synchronized and analyzed for DNA replication rate and enzyme activity as described in Fig. 1.

showing a dramatic increase in enzyme activity and in total protein 3 hours after the initiation of DNA synthesis.

In replicate cultures, the levels of thymidine kinase and lactate dehydrogenase showed nearly identical patterns. Maximum thymidine kinase activity occurred 7 and 10 hours after mitosis, while lactate dehydrogenase showed an additional maximum 3 hours after division. When plotted together, a reciprocal relationship results between maximum enzyme levels and maximum rate of DNA synthesis (Fig. 1). Don cells were also studied in this work and showed the same pattern of DNA synthesis and enzyme activity changes as CS134, except that maximum rates of DNA synthesis occur 7 and 10 hours after mitosis and maximum enzyme activity occurs 4, 8.5, and 12 hours after mitosis.

Since most studies of enzyme behavior in the cell cycle have been performed with heteroploid human lines (*16*) it was essential to compare DNA replication and enzyme synthesis in heteroploid and diploid Chinese hamster lines. In the absence of such comparisons, contradictory results can always be ascribed to differing methodologies or to some unique characteristic of Chinese hamster cells, rather than to loss of normal replication and translational order accompanying the transition to heteropoloidy.

This possibility was tested by synchronizing the hamster lines G3 and CHO in the same manner as the CS134 and Don cells. The kinetics of DNA synthesis in the G3 line (Fig. 2) are very different from the kinetics in the CS134 cells and much the same as in HeLa cells. Similarly, the chromosomal pattern of DNA synthesis is different from diploid cells, although some distinct blocks of late-replicating DNA are still identifiable. The rate of DNA synthesis reaches a maximum soon after the initiation of the S phase and remains constant until cells begin to enter the G_2 phase. Again, in contrast to the results in CS134 cells, lactate dehydrogenase and thymidine kinase activities in the heteroploid hamster line increase slowly and uniformly through the G_1 and S phases, reach a maximum in the late S, and decrease slightly in the G_2, in the manner of other heteroploid lines. Klevecz and Stubblefield commented before (*9*) on the differences between heteroploid and diploid lines and suggested that the extreme heterogeneity characteristic of many heteroploid cells, and in particular the variations in duration of the G_1, S, or G_2 phase within an apparently synchronous population, might make synchronization of all molecular events very difficult or impossible. The G3 line, which was cloned from the original B14FAF and which has a modal chromosome number of 22, was purposely chosen to eliminate this possibility. The time of entry into and exit from the S phase shows no more spread than for the diploid line. The generation time for this cell (15 to 16 hours) is slightly longer than that of the CS134 cell (11.5 to 12 hours), but is very close to the generation time of the diploid strain Don (14.5 to 15 hours). Studies of DNA synthetic rate and enzyme levels in the CHO line gave results essentially identical to those obtained with G3.

The discontinuous synthesis of DNA in the S phase demonstrated here for diploid hamster cells has been indirectly deduced by other workers (*17*), and is not surprising in view of the rapid replication of the inactive X chromosome (*18*) and portions of other chromosomes in the last half hour of the S phase. The molecular mechanism remains to be elucidated. A direct causal relationship between the two events, reflecting the mutually exclusive nature of DNA replication and its transcriptional-translational activities, is refuted by our earlier work in which inhibition of DNA synthesis was shown to reduce the amount of enzyme made, but not to alter the time at which it was made. Repression of enzyme synthesis and initiation of DNA replication may be coordinately controlled, and, if so, it seems likely that the enzyme regulatory components must have a broad specificity.

This study suggests that the differences in the pattern of DNA and enzyme synthesis depend on whether cells are established heteroploid lines with unlimited capacity for further cell division, or diploid or near diploid cell strains with possibly a finite capacity for propagation. It follows that all true diploid cell strains might be expected to synthesize DNA in two discrete portions with a slowing in synthetic rate in the mid S phase, and to synthesize many enzymes in an oscillatory manner. Heteroploid cells should show a unimodal rate curve for DNA synthesis and a uniformly increasing rate of protein synthesis, with possible maximums prior to and following DNA synthesis. Whether the observed changes accompany or precede the shift to heteroploidy or are the result of subsequent selection in culture cannot be determined. But comparative studies of freshly explanted cells of normal and neoplastic tissues may provide some insight into this phenomenon.

ROBERT R. KLEVECZ
Department of Biology,
City of Hope Medical Center,
Duarte, California 91010

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19. Supported by NIH grant CA 10619 and in part by the Isaac Ratner Research Fellowship Fund.

27 June 1969; revised 18 September 1969