TEMPORAL ORDER IN MAMMALIAN CELLS

I. The Periodic Synthesis of Lactate Dehydrogenase in the Cell Cycle

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ABSTRACT

Chinese hamster cells were synchronized by the Colcemid-selection system. In cells with a division cycle time of 11.5–12 hr, the activity of the enzyme lactate dehydrogenase (LDH) underwent marked oscillations with a 3.5-hr period. Precipitation of labeled LDH enzyme with specific antibody indicated that the enzyme activity changes were the result of intermittent enzyme synthesis and relatively constant degradation. Inhibition of normal DNA replication with 4 mM of thymidine, while reducing the amount of new enzyme synthesized, did not prevent oscillations from occurring. Similarly, actinomycin D (AcD) added at the time of synchronization allowed some new enzyme synthesis to proceed in an oscillatory manner. LDH synthesis went on at nearly normal rates when AcD was added in the middle of S phase. However, addition of cycloheximide to cultures at any time in the cycle caused an immediate drop in levels of activity and in enzyme protein. The half-life of LDH, calculated either from loss of enzyme activity or precipitable radioactivity in cycloheximide-treated cultures, was between 2 and 2.5 hr.

INTRODUCTION

To understand cellular regulatory mechanisms, it may be useful to consider that metabolic processes occur sequentially and that the organization of these events in time is important to the control of cell growth and the timing of cell division. The arrangement of such events is difficult to question in randomly growing exponential cell cultures. This problem can best be approached with synchronized cell cultures in which the entire population can be taken to represent an individual cell.

Of obvious interest is the relationship between DNA replication and the transcriptional and translational activities of the cell. For example, does the doubling in gene dosage which occurs in S phase result immediately in a doubling in the rate of synthesis or accumulation of gene products either RNA or a particular protein? An affirmative answer came from the work of Masters, Kuempel, and Pardee (1) who demonstrated that in synchronous *E. coli* the gene product, in this case an enzyme, did accumulate at twice its initial rate following replication of the particular gene involved. Under conditions of induction or derepression, the rate of synthesis of a number of enzymes was shown to vary with the number of copies of the gene specifying their structure. Mapping studies showed that doubling times could be closely correlated with replication of the particular gene loci involved (1–3).

The situation in synchronous mammalian cell systems is not so clear. Several years ago, we were able to demonstrate that the rate of synthesis of rapidly labeling RNA increased sharply in the first part of S phase (4, 5). We also noted several

peaks in incorporation of the precursor and suggested that, although the potential for RNA synthesis doubled with DNA replication, the rate at any instant was not totally determined by gene dosage. Compatible results showing that the RNA synthesis rate increases slowly through S phase have since been reported for HeLa cells (6) and for another Chinese hamster cell line (7). RNA synthetic rate is a rather gross measure of gene activity and, with the possible exception of ribosomal RNA synthesis (7), such studies give little information about regulation of individual genes.

Klevecz and Ruddle (8) studied the behavior of several enzymes through the cell cycle and reported that the activities of the enzymes LDH and glucose-6-phosphate dehydrogenase (G6PD) oscillated through the cycle with maxima 3.5, 7, and 10 hr after division. LDH activity increased greatly after the initiation of S phase and, although no mechanism was provided, it was suggested that such oscillations in activity might be one manifestation of a cellular clock. Brent, Butler, and Crathorn (9), working with thymidine-synchronized HeLa cells, were able to observe a broad peak in thymidine kinase and thymidylate phosphokinase activities in late S phase, while Littlefield (10) observed a rather sharp maxima in thymidine kinase activity in the S phase of mouse L cells.

Recent work with enzyme regulation in mammals has shown that inducer-mediated increases in enzyme activity can be caused by an increase in synthesis (11, 12), a decrease in degradation (13), or possibly by conformational changes (14).

We have endeavored in this work to demonstrate that levels of a constitutive enzyme oscillate and that the oscillations are due to periodic bursts in enzyme synthesis.

MATERIALS AND METHODS

A Chinese hamster cell strain derived from the original Don C line (15) and designated CS133 was used in this study. Stock cultures were grown in Eagle's Minimum Essential Medium supplemented to a final concentration of 20% fetal calf serum 0.2 mM of nonessential amino acids, 1 mM of sodium pyruvate, and 2 mM of L-glutamine. This standard medium is referred to in the text as MEM. Synchronous populations were obtained by the Colcemid-selection method described originally by Stubblefield and Klevecz (16).

S Phase Synchronization

Synchronization at the beginning of S was accomplished by treating cultures for 12 hr with 4 mM of thymidine. Thymidine was washed out, and the cells were incubated for 6 hr in MEM. Amethopterin (5 μM) and adenosine (0.1 mM) (17) were then added for an additional 12 hr. This block was relieved by the addition of thymidine to a final concentration of 6 μg/ml.

Nucleic Acid Labeling

Determination of initiation and duration of S phase was done by labeling cells with tritiated thymidine (1 μc/ml; 5 c/mmole— New England Nuclear Corp., Boston) as described previously (5).

Protein Labeling and Estimation of Total Protein

Isotopic immunoprecipitation studies were carried out by incubating synchronized cultures with MEM lacking nonessential amino acids and containing tritiated reconstituted protein hydrolysate (RPH-^{3}H, 0.3–2 c/mmole—Schwartz Bioresearch Co., Orangeburg, N.Y.) and half the normal level of essential amino acids. The soluble pool was measured by washing and sonicating replicate cultures after allowing incorporation of amino acids-^{3}H for increasing periods of time. Trichloroacetic acid was added to the extract to a final concentration of 10%, and the precipitate was collected by centrifugation in a Beckman microfuge for 1 min. The supernatant was then counted directly. Estimation of total protein content in cultures and amino acid-^{3}H incorporation into total protein was performed as previously described (5).

Preparation and Purification of LDH

Four female Chinese hamsters (*Cricetulus griseus*) were sacrificed, and a homogenate of the muscle was prepared by blending in 10 volumes of 5×10^{-3} M phosphate buffer pH 7.0. The disrupted tissue was then sonicated, stirred in the cold for 2 hr, strained through gauze, and centrifuged. The initial steps in the purification of this crude extract were performed according to the method of Kornberg (18) and involved repeated ammonium sulfate fractionations (Table I). The partially purified enzyme was then drawn onto a DEAE[1]-cellulose column equilibrated in 0.005 M phosphate buffer and eluted by exponentially increasing the phosphate buffer concentration from 0.005 to 0.25 M. A spot test for LDH was performed, and the fractions showing activity were pooled and concentrated to 20 ml by using Aquacide 1 (Calbiochem, Los Angeles). This material was then eluted from a Sephadex G-200 column. The fractions containing LDH were again pooled, concentrated, and the enzyme was precipitated by addition

[1] Diethyl amino ethyl.

TABLE I

Summary of LDH Purification Procedure

Fraction	Total vol.		Total units	Protein	Specific activity	Recovery
	ml	*Units/ml*		*mg/ml*	*Units/mg*	%
1 Crude homogenate	700	19.8	13,860	9.3	2.1	100
2 Supernatant	660	20.9	13,794	5.2	4.1	99.5
3 Ammonium sulfate 0.52–0.72 paste	200	58.0	11,611	7.4	7.8	83.8
refractionation	*a.* 125	64.2	8,025	6.7	9.6	57.9
	b. 40	194.8	7,792	8.1	24.0	56.2
	c. 15	356.9	5,354	2.8	127.5	38.6
4 DEAE cellulose followed by Sephadex G200-aquacide	21	88.5	1,858	0.129	686.0	13.4

A unit is defined as amount of LDH necessary to reduce 1 μmole of pyruvate/min at 37°C.

of saturated ammonium sulfate to 72%. The precipitate was then suspended in a small volume of phosphate buffer and dialyzed against 0.01 M phosphate pH 7.0. The total yield of protein by the Biuret determination was 1.2 mg.

Production of Antibody to LDH

To prepare the antibody, 0.6 mg of LDH in 0.14 M NaCl was mixed with a slightly smaller volume of complete Freund's adjuvant and injected into a rabbit subscapularly. 2 wk after the 1st injection, a 2nd injection of 0.1 mg of LDH was given. Beginning 9 days after the 2nd injection, weekly bleedings were made. The rabbit was fasted for 24 hr prior to bleedings, and 50 cc of blood were taken. Sera from the first four bleedings were pooled and used in these experiments. The specificity of the antiserum was tested by double diffusion in agar against the original antigen and supernatant extracts of cell line CS133 (Fig. 1, insert). The single precipitin band which appeared was shown to have LDH activity by specific staining, using lactate and DPN with nitro blue tetrazolium as the final hydrogen acceptor. Anti-LDH serum was titrated by reacting with two-fold serial dilutions of the original antigen. Quantitative precipitin curves were constructed for three dilutions of antiserum, and the point of equivalence of the serum was determined in this manner (Fig. 1, graph).

Determination of LDH Synthesis during the Cell Cycle

Cells were inoculated into culture vessels in the usual manner following synchronization ($1–5 \times 10^6$ cells for continuous labeling, 10×10^6 cells for pulse labeling). At hourly intervals, culture flasks were rinsed three times in cold BSS, and 0.5 ml of 0.01 M phosphate buffer (pH 7.4) containing five units of unlabeled LDH (approximately 25-fold excess) was added at this time to assure that all subsequent precipitations were carried out at the equivalence point. Sonication was performed in a sealed container for 1 min at 4°C in an external sonifier. Extracts were then centrifuged at 27kg for 60 min, and the supernatants were collected. Rabbit anti-LDH slightly in excess of equivalence was then added to the supernatant, and the mixture was incubated at 37°C for 2 hr and then refrigerated overnight. Reacted mixtures were centrifuged at 10,000 rpm in a Sorvall RC-2B, and the precipitate was washed twice in phosphate-buffered saline, dissolved in 0.2 N NaOH, and counted to 2% error in Bray's solution. A second precipitation of the supernatant indicated that essentially all LDH-specific radioactivity had been removed. Precipitation assays with preimmune rabbit serum gave less than 10% as much radioactive material as did the specific antisera.

RESULTS

Assessment of the Synchrony System

Since all interpretations in synchrony studies depend on the assumption that a population of synchronized cells can be considered to magnify accurately the activities of a single cell growing without perturbation, it was essential to establish that the observed changes were not an artifact of the methods used.

The Colcemid-selection system represents a compromise between the forced synchrony methods involving prolonged exposure to inhibitors which results in unbalanced growth, and the selection

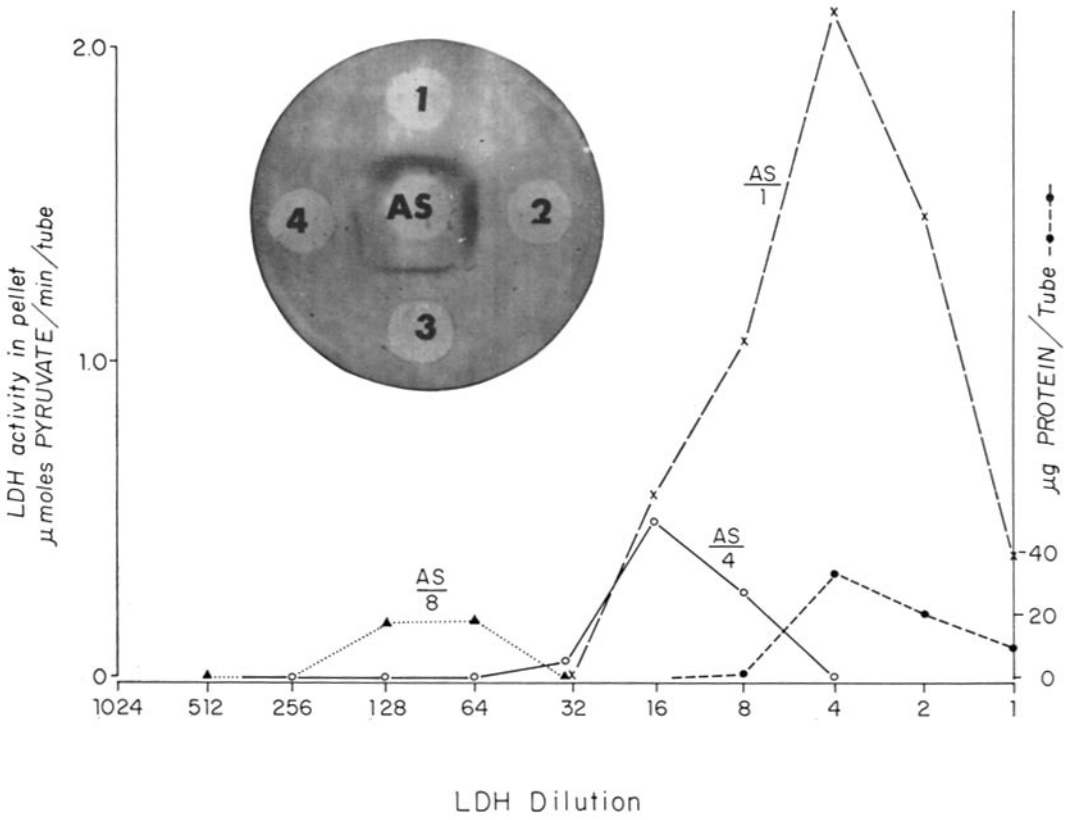

FIGURE 1 Double diffusion in Agar and quantitative precipitin curves of rabbit anti-hamster LDH. Hamster LDH activity precipitated by rabbit anti-hamster LDH (*AS*) used at dilutions of *1* (X---------X), *4* (—O—O—), and *8* (▲. . . . .▲). Protein in precipitate of 1:1 dilution is correlated with LDH activity (●---------●). LDH activity was determined as described previously (8) and recorded as μmoles of pyruvate reduced. *Insert,* Double diffusion plate of rabbit anti-hamster LDH (*AS*) reacted against: *1*, supernatant of distilled water extract from 1×10^8 CS133 cells/ml. *2*, supernatant of 0.01 M phosphate buffer pH 7.0 extract from 1×10^8 CS133 cells/ml. *3*, original LDH antigen in distilled water. *4*, original LDH antigen in 0.01 M phosphate buffer pH 7.0. Specific staining of precipitin bands was accomplished by incubating the plates at 37°C with a solution containing 2 g of sodium lactate, 30 mg of nitro blue tetrazolium chloride, 4 mg of phenazine methosulfate, and 60 mg NAD^+ in 100 ml 0.05 M Tris-HCl buffer pH 7.0.

approach in which large yields of cells are difficult to obtain. If cells must be blocked to achieve a synchronous state, mitosis is probably the best time to block them. No DNA synthesis occurs, RNA synthesis does not occur at detectable levels (19), and protein synthesis is greatly reduced (20). In addition, the time of blockage in this system is brief (less than 3 hr).

The evidence indicates that the cells suffer no lasting effects from the brief Colcemid arrest. Even following prolonged treatment with colchicine, a more toxic alkaloid, it has been shown that no changes in DNA, RNA, or protein synthesis are detectable for the equivalent of a generation (21). The kinetics of DNA synthesis in Colcemid-synchronized cultures agree with the results obtained using cultures synchronized with FUdR block and thymidine reversal (22).

We have previously reported that unselected populations treated for a brief period with Colcemid, and subcultured, show none of the characteristic enzyme oscillations of a synchronized culture. To further substantiate the idea that the ability to detect oscillations is limited by the extent of synchrony, we treated cultures for the usual 2.5-hr period with Colcemid, selected mitotic cells,

and then subcultured the remaining interphase cells after adding varying numbers of mitotic cells (Fig. 2 *b*). Oscillations in LDH activity are clearly proportional to the extent of synchrony.

While we can eliminate subculturing and the effects of Colcemid on exponential cultures as causing the oscillations in enzyme activity, it is entirely possible that mitotic cells, since they are specifically affected by Colcemid, may give rise to aberrantly growing G_1 cells. One can best answer this objection by measuring enzyme activity changes in cells synchronized by an entirely different method (Fig. 2 *a*). Cell cultures were synchronized by using 4 mM thymidine as the initial

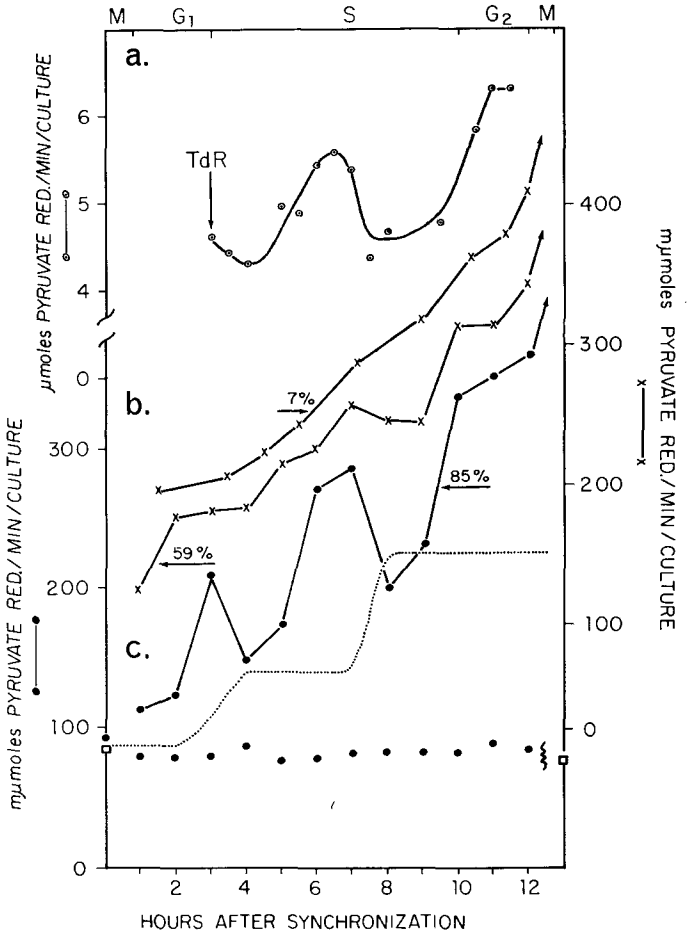

FIGURE 2 Changes in LDH activity with degree and type of synchrony. *a*, CS133 Chinese hamster cells (2.5×10^6 cells/culture) were synchronized by a double S phase block as described in the Methods section. Enzyme activity was assayed as described previously (8) using a Gilford 2400 multiple sample recording spectrophotometer to measure the change in absorbance at 340 nm. Data are plotted as hours after beginning of S phase. *b*, Semisynchronous cultures (1×10^6 cells/culture) treated for 2.5 hr with Colcemid, and subcultured after removal of mitotic cells. In cultures with 7% initial synchrony, no mitotic cells were added. In cultures with 59% initial synchrony, equal numbers of mitotic cells were added to the residual population. *c*, LDH activity in a synchronous population (1×10^6 cells/culture) and in the media removed from these cultures at the time of harvest. Dotted line (. . . . .) indicates predicted amount of LDH in culture medium if all LDH lost from cells was being secreted into the medium. Points (● ● ●) indicate actual LDH in medium. Squares (□) indicate LDH activity in fresh medium.

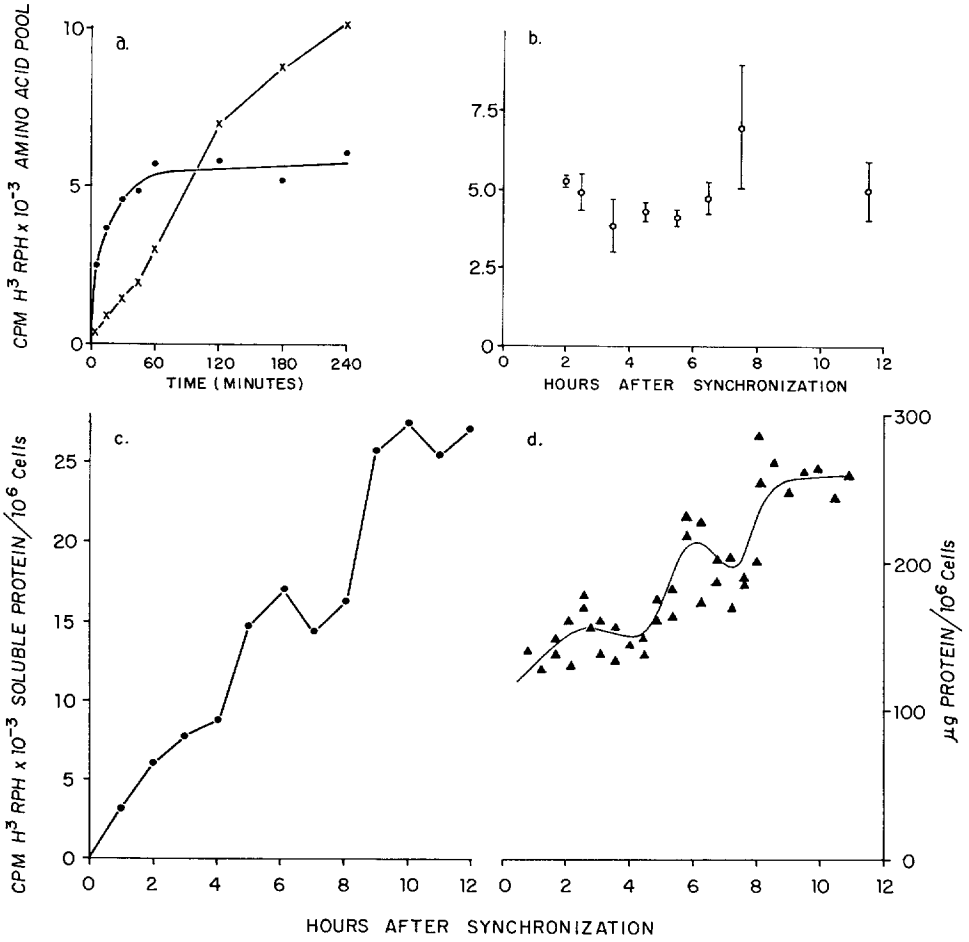

FIGURE 3 Incorporation of tritiated amino acids into protein and their equilibration with the pool. *a*, cpm in 10% trichloroacetic acid-soluble supernatants (—●—●—) and precipitates (—×—×—) from replicate exponential cultures labeled with 5 μc/ml RPH-^{3}H in MEM. *b*, cpm in 10% trichloroacetic acid-soluble supernatants from replicate synchronized cultures pulsed for 1 hr with 5 μc/ml RPH-^{3}H in MEM. Brackets indicate standard error of the mean for each point. *c*, Total incorporation into soluble protein in synchronized cells labeled continuously from mitosis (Data in Figs. 3 *c* and 4 are from the same experiment). *d*, Total Lowry protein content of synchronized cells.

blocking agent, followed by reversal and a second block with amethopterin. LDH activity was assayed, and the oscillations were shown to occur at the predicted times when the data from the amethopterin-reversed cells were plotted from the beginning of S phase. It is interesting to note that LDH levels are approximately ten times normal. This aspect of S phase blockage has been noted before (23), as have other manifestations of unbalanced growth following this type of synchronization (17). Oscillations are not due to passive leakage or active transport of the enzyme out of the cell since LDH activity of media is nearly constant through the cycle and is equal to the activity in media not used to grow cells (Fig. 2 *c*). Furthermore, the phenomenon is not due to the method of preparation of samples for analysis. Sonication and freeze-thawing give similar results, and activity of LDH is the same whether the sonicate is centrifuged at 27,000 *g* for 1 hr and the supernatant analyzed or whether the analysis is done directly after sonication. Preliminary experiments revealed that trypsin treatment of attached cells lowered the activity of soluble enzymes. Consequently, this method of collection was rigorously avoided.

Thus, we can conclude that oscillations are not

an artifact of the method; the amplitude of the oscillation decreases with decreasing synchrony. Results from other methods of synchronization show the same oscillations in enzyme activity.

Incorporation of Tritiated Amino Acids into Protein

Studies which base their interpretations on radioactive precursor incorporation are always open to objections regarding changes in pool size. If equilibration time of an exogenous precursor with the internal pool is relatively long, it can be argued that results from continuous labeling studies will be distorted by the changing specific activity of the internal pool. In the present case (Fig. 3 *a*), it is apparent that equilibration of the labeled precursor is very rapid. Radioactivity in the pool has reached half its plateau value after a 5-min exposure, and equilibration is completed within the 1st hr. Thus, radioactivity measurements will underestimate actual LDH levels by about 30% in the 1st hr, but not at all thereafter. Changes may also occur in the pool size during a cell cycle. This would have little effect on continuous labeling studies but might distort somewhat the results of pulse labeling. Indeed, small changes in pool size (Fig. 3 *b*) do occur in synchronous cultures. Midway through S phase a greater amount of label is incorporated into the TCA-soluble pool than at other periods. Since this is the time when noticeable increases in total protein are evident (Fig. 3 *c*, *d*), it would be anticipated that a transient decrease in the amino acid pool might occur concomitantly. A reciprocal experiment was performed in which continuously labeled synchronous cultures were chased with unlabeled amino acids at hourly intervals. Again, the data were consistent with the view that a transient decrease in pool size occurs in middle S phase. These observations have pertinence in pulse labeling studies and will be discussed in greater detail later.

Synthesis of LDH in the Cell Cycle

Several workers have demonstrated that changes in enzyme levels may represent changes in activity rather than an increase or decrease in the amount of actual enzyme protein (14). More recently, however, it has been (12) demonstrated that induction of tyrosine aminotransferase in a rat hepatoma cell line is the result of new enzyme synthesis.

After obtaining antiserum to a suitably pure enzyme and testing it to assure that only LDH would be precipitated, we thought it was necessary to make certain that rather large changes in the amount of LDH present in synchronized cells would not change the equivalence point of the precipitin reaction. To accomplish this, a 25-fold excess of unlabeled enzyme was added at the time of extraction, as described in the Methods section. Labeling experiments were begun immediately after synchronization, and ^{3}H-amino acid-labeled LDH was precipitated at short intervals thereafter. The results from these continuous labeling experiments, which are shown in Fig. 4, support the idea that the concentration of LDH enzyme molecules oscillates through the cycle and that the enzyme activity data do repre-

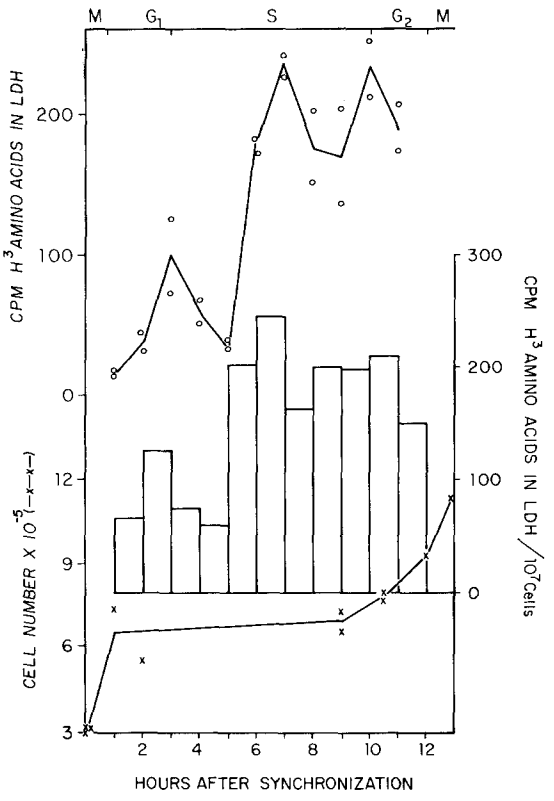

FIGURE 4 LDH levels and rate of synthesis in the cell cycle. Continuous labeling of 1×10^6 cells/culture with 10 μc/ml RPH-^{3}H for one generation. Samples were harvested at the indicated times (—O—O—) and prepared as described in the Methods section. Histogram indicates 1-hr duration pulse labeling of 1×10^7 cells with 20 μc/ml RPH-^{3}H.

sent at least a constant proportion of the actual concentration of enzyme molecules.

If synthesis of the enzyme is an intermittent phneomenon and destruction is relatively constant through the cell cycle, then one might predict from the cumulative labeling data that the rate of enzyme synthesis should be high at some times and very low at others. Pulse labeling the cultures with tritiated amino acids gave a partial answer to the question. Periodic increases in rate of amino-^{3}H acid incorporation did seem to occur at the expected times. The histogram in Fig. 4 presents the results of one such experiment. Increased rates of incorporation are closely associated with increases in the level of enzyme, with maximum rates occurring slightly before the achievement of maximum enzyme levels. Theoretically, the curve of pulse incorporation into LDH should be the first derivative of the cumulative labeling curve. In fact, the differences in rate of synthesis at various points in the cycle were not so great as one might predict from consideration of the cumulative labeling data.

Only small changes in pool size were observed (Fig. 3 *b*) during the first 6.5 hr of the cycle. Consequently, we must believe that the pulse-incorporation data up to this point accurately reflect rates of synthesis. However, the pool appears to shrink somewhat at 7.5 hr, possibly as a result of the vigorous protein synthesis which has gone on in the previous 2 hr. A pulse administered at 7.5 hr

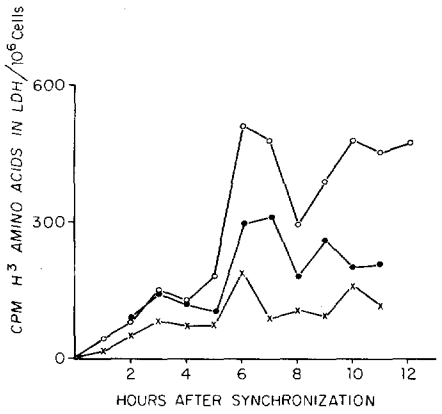

FIGURE 5 Effect of high thymidine and AcD on new enzyme synthesis. CS133 cultures (5×10^6 cells/culture) were treated at time of selection with 4 mM of thymidine (—●—●—) and 5 μg/ml of AcD (-X-X-X-). Counts per minute of incorporated RPH-^{3}H (10 μc/ml) compared with controls (—O—O—).

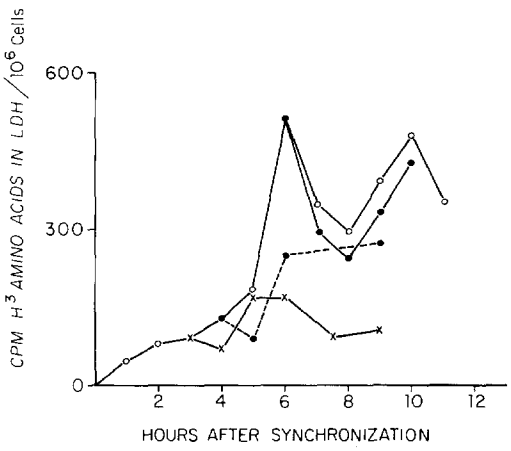

FIGURE 6 Effect on LDH synthesis of AcD addition at points in the cell cycle. AcD 5 μg/ml was added to cultures (5×10^6 cells/culture) 3 (-X-X-X-), 4 (---●---●---), and 6 (—●—●—) hr after synchronization. Control (—O—O—). Labeling as described in Fig. 5.

would result in a relatively greater number of labeled amino acids being available as precursors, and consequently a true reduction in the rate of protein biosynthesis would be difficult to detect. Taken together, the changes in enzyme activity and the pattern of LDH immunoprecipitation can most comfortably be reconciled by considering that the synthesis of LDH is an intermittent process.

Inhibitor Effects

Inhibition of DNA synthesis in the present study was accomplished by addition of 4 mM of thymidine to cultures immediately after selection. The amount of amino acid-^{3}H accumulated in LDH was then measured as before (Fig. 5). No effect on LDH synthesis was observable until 5 hr after mitosis, or early S phase, when thymidine-inhibited cells showed a decreased capacity to make new LDH as compared with controls. We noted previously the apparent relationship between the initiation of DNA replication and the sudden increase in enzyme activity and isotopic incorporation into RNA (5, 8). It is tempting to consider that replication of a particular template may stimulate its transcription, so that RNA and protein syntheses are entrained to DNA synthesis. The results also add strength to the idea that one

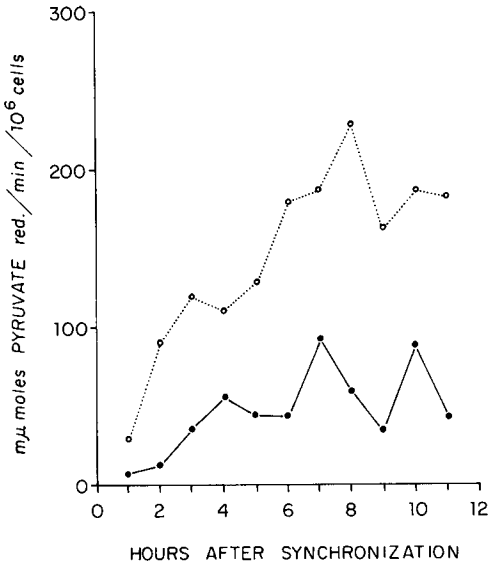

FIGURE 7 Enzyme activity after AcD treatment. AcD (5 μg/ml) was added at the time of synchronization. LDH activity was determined at intervals thereafter in treated cells (—●—●—) and controls (· · ○ · · ○ · · ○ · ·).

parameter of cellular synthetic capacity is gene dosage.

An attempt was made to determine whether RNA from one division cycle could function in protein synthesis in the next. AcD, when added at the time of Colcemid reversal, did not prevent synthesis of new LDH (Fig. 5). Presumably, sufficient RNA survives from the previous G_2 to permit some new protein synthesis to occur. This is in agreement with the findings of Steward, Shaeffer, and Humphrey (24) who studied the assembly of new polyribosomes in cells treated at mitosis with AcD. The requirements for new RNA synthesis were further tested by adding AcD at various intervals after the first synchronous division (Fig. 6). AcD does not seem to affect the intermittent synthesis and destruction of the enzyme, but it does reduce the amplitude of the oscillations if added prior to mid-S phase. AcD added at later times is decreasingly effective in preventing normal levels of enzyme from being made. The most noticeable difference in inhibitory effect occurs between 4 and 6 hr. Addition of AcD at 6 hr results in nearly normal LDH synthesis. The data in Fig. 7 indicate that enzyme activity behaved in the same manner as enzyme synthesis following AcD treatment. Addition of the antibiotic between 0 and 6 hr

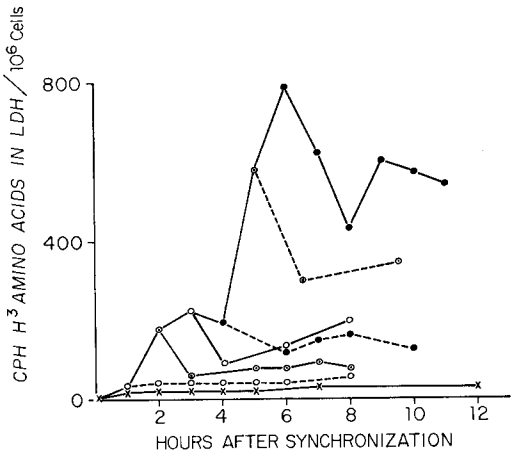

FIGURE 8 Synthesis and decay of LDH in cycloheximide-treated cultures. Cell cultures (5×10^6 cells/culture) were labeled with 10 μc/ml RPH-^{3}H and treated with 100 μg/ml cycloheximide at 0 (-X-X-), 1 (---○---○---) 2 (—⊙—⊙—⊙—), 3 (—○—○—), 4 (--●--●--), and 5 (--⊙---⊙--) hr after synchronization. Untreated controls (—●—●—).

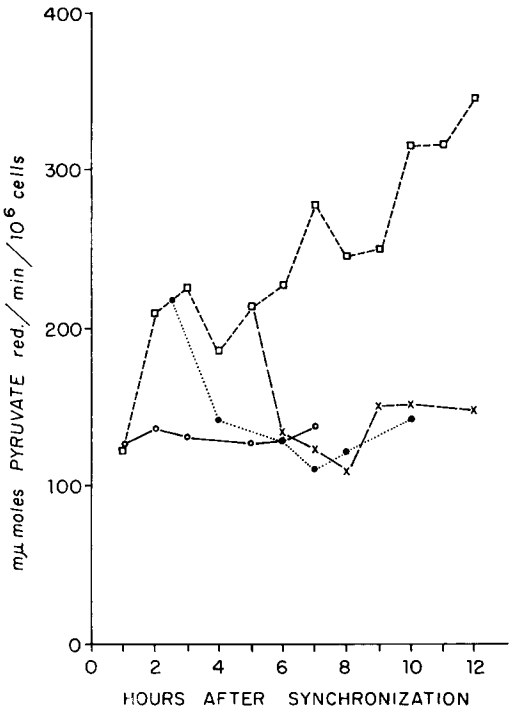

FIGURE 9 Enzyme activity in cycloheximide-treated cultures. CS133 cells (2.5×10^5) were treated with cycloheximide (100 μg/ml) at 0 (—○—○—), 2.5 (. . ● . . . . ● . .), and 5 (--x--x--) hr after synchronization. Control cultures (-□---□-).

permits increasing amounts of LDH to be made. The impression given is that most of the essential RNA for LDH synthesis is made 4.5–6 hr after mitosis. The fact that neither 4 mM of thymidine nor AcD prevents the characteristic oscillations from occurring suggests that the oscillations are not directly dependent upon replication or transcription.

Cycloheximide, an inhibitor of peptide elongation, was added to synchronized cells at various intervals after reversal (Fig. 8). In contrast with the results of either AcD or 4 mM of thymidine treatment, this drug completely suppressed oscillations. When added at mitosis, no new LDH was synthesized. However, enzyme activity (Fig. 9) remained essentially constant throughout the ensuing cycle. Activity curves from experiments in which cycloheximide was added at points after mitosis demonstrate that there is a tendency for enzyme activity to fall to the early G_1 level.

Enzyme Half-Life

The half-life for LDH was determined in several ways: cultures were labeled for the first 6 hr of the cell cycle and then treated with cycloheximide or chased with unlabeled amino acids in MEM. Since there is a tendency for activity to drop rapidly following cycloheximide addition, calculations of half-life from enzyme activity curves were necessarily determined from repeated measurements made in the first 2 hr of treatment. Enzyme activity was also assayed directly following cycloheximide addition in both synchronized and exponential cultures. Results from the two methods are in

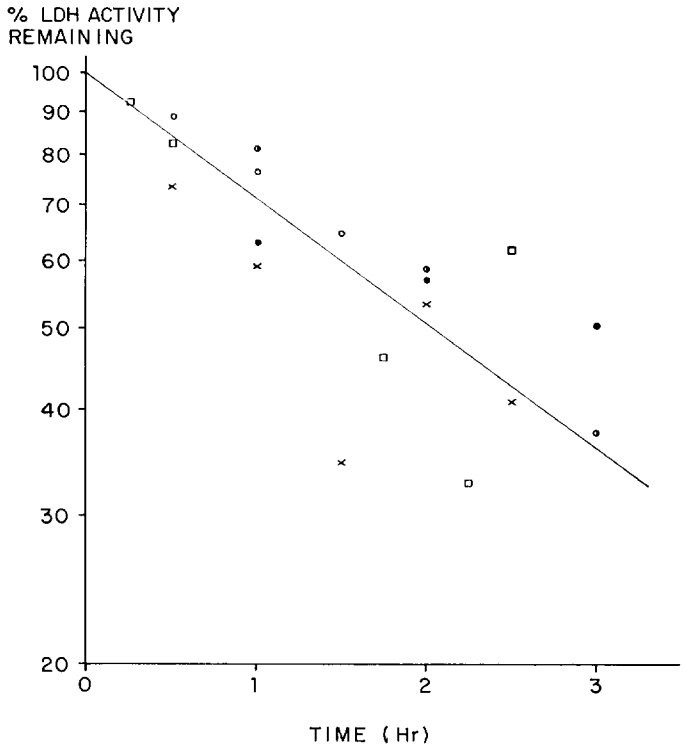

FIGURE 10 Estimate of enzyme turnover in synchronized and exponential cultures. Calculations of per cent of LDH activity remaining were made either directly from the spectrophotometric assay of activity (SA) described previously (8) or from immunoprecipitation (IP) of labeled LDH.*X*, (SA) synchronized culture, 100 μg/ml cycloheximide added 7 hr after mitosis. ◑, (IP) synchronized culture labeled with RPH-^{3}H from 0–6 hr and then chased with normal MEM. ○, (SA) synchronized culture treated with cycloheximide 3 hr after mitosis. □, (IP) exponential culture, cycloheximide (100 μg/ml) added 6 hr after addition of labeled amino acids. ●, (SA) synchronized culture, cycloheximide added 5 hr after mitosis. Time is scored from the addition of cycloheximide or unlabeled amino acids. Labeling was done as described in Fig. 5.

reasonable agreement (Fig. 10). The half-life values of 2 hr obtained for Chinese hamster cells in culture were anticipated in other mammalian systems by Bresnick et al. (25), who demonstrated that thymidine kinase in regenerating rat liver had a half-life of 3.7 hr, and by Kenney (26) who showed convincingly that the half-life of steroid-induced tryptophan pyrolase in rat liver is 1.5 hr. However, at the same time Kenney observed that inhibitors of protein synthesis also prevented normal degradation.

DISCUSSION

As Pittendrigh (27) has pointed out, negative feedback is a common feature of many regulatory processes operating at different levels of organization in biological systems. An inherent property of such systems is a tendency for the components to oscillate. Perhaps the best known examples of such periodic phenomena are the circadian or diurnal rhythms manifested in the bioluminescence of *Gonyaulax* (28), in the spike frequency of *Aplysia* ganglia (29), and in the eclosion rhythms of *Drosophila* (30). On a much shorter time scale, Chance et al. (31) observed oscillations in the level of reduced pyridine nucleotide in intact yeast cell suspensions.

The ability of cells to recognize time is expressed most obviously in the constancy of generation times, in the constancy of the subdivisions of the cell cycle, and in the fact that generation times differ in different species. Bruce (32) has pointed out that many cells in culture have a generation time close to 24 hr. However, there are few other similarities between the circadian clock and the cellular clock. It is probably misleading to speak of *the* cellular clock. Even when subtly phrased, it implies a more mechanistic situation than actually exists. Cells can respond to the information stored in a sequence of nucleotides in DNA through its transcription into RNA and translation into functional proteins. These macromolecules can, in turn, respond to the information inherent in a population of small molecules whose concentration changes with time (33). It is the second type of response which is pertinent to cellular time-keeping. In all likelihood, timing of division and the occurrence of distinct cellular events is the end-product of a large series of coupled oscillations in populations of large and small molecules.

In synchronous bacterial and yeast cultures, a single peak in enzyme activity for a number of enzymes occurs in each cycle of replication and division (1, 34). Intermittent synthesis of enzymes has also been reported previously in synchronous microspores of *Lillium*. Hotta and Stern (35) demonstrated that thymidine kinase activity increases in a brief burst just prior to DNA synthesis. These authors consider that thymidine kinase acts as a "trigger" initiating DNA replication. Again, the trigger concept carries with it an unfortunately static impression of cellular processes. It is probably more accurate to consider oscillating enzyme synthesis as a continuous process coupled to metabolism and energy conversion and perhaps entrained to DNA replication by a burst in messenger synthesis.

Apart from any possible function in the cellular time-keeping process, oscillations in enzyme activity may offer insight into the kinetics of enzyme synthesis and degradation.

The changes which occur in enzyme activity and synthesis in the mammalian cell cycle have been studied almost exclusively in heteroploid, established cell lines. To this time, no studies have been performed using euploid or near euploid cell strains besides our own and those of Hooper-Conrad and Ruddle (36). The differences in potential for continued cell division between heteroploid and diploid cell types are well known (37). Heteroploid lines are either of neoplastic origin or have survived phase III crises (37), and, as a consequence, hold common properties with cancer cells. The results from studies of enzyme activity in such cell types are in remarkable agreement. All enzymes thus far studied show continuously increasing activity through G_1 and early S, they reach a maximum in late S phase and then decrease slightly in G_2. This pattern has held true for thymidine kinase, thymidylate kinase in HeLa cells (9), ornithine transaminase in Chang's liver cells (38), and for thymidine kinase in mouse L cells (10). Recently, Martin et al. (39) have studied the synthesis of tyrosine aminotransferase in synchronized HTC cells. Their data also indicate that the levels of LDH, G6PD, and alcohol dehydrogenase increase slowly to a maximum activity per cell in G_2. Similarly, we have found[2] that LDH and thymidine kinase activities in the heteroploid Chinese hamster line B14-G3 increase slowly through G_1 and S, reach a maximum in late S, and decrease slightly in G_2, in the manner of other heteroploid lines.

There can be little doubt that heteroploid cells

[2] R. R. Klevecz. Manuscript in preparation.

in culture have escaped from the stringent controls exercised by normal somatic cells in vivo. Certainly, the differences in mode of growth and regulation through the cell cycle which exist between diploid and heterodiploid lines should be investigated more thoroughly.

To summarize, the synthesis of LDH is an intermittent process. Synthesis of DNA and RNA was required for maximal LDH levels, but considerable new enzyme synthesis will go on in the absence of either. We were not able to observe the paradoxical effect of AcD reported by Garren et al. (40), possibly owing to the difficulty in treating synchronized cultures at precisely the right time. Degradation of LDH could be measured following cycloheximide treatment in agreement with the results of Peterkofsky and Tomkins (41) and in contrast to the findings of Kenney (26). The oscillations were halted by inhibitors of protein synthesis, but not by inhibitors of RNA or DNA synthesis.

This study was supported by National Institutes of Health grant CA 10619MB and a City of Hope Medical Center fund established in the name of Harry Fox.

The author wishes to thank Dr. T. J. Kindt of the immunochemistry section for his helpful suggestions and Dr. F. H. Ruddle of Yale University in whose laboratory this work was begun. The author also wishes to acknowledge the excellent technical assistance of Mrs. Norma Lee, Mrs. Sonia Ivey, and Mrs. Mercedes Lemos.

Received for publication 10 March 1969, and in revised form 14 May 1969.

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