SYNTHESIS AND SECRETION OF LIGHT-CHAIN IMMUNOGLOBULIN IN TWO SUCCESSIVE CYCLES OF SYNCHRONIZED PLASMACYTOMA CELLS

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ABSTRACT

Suspension-cultured mouse plasmacytoma cells (MPC-11) were accumulated in the late G1 phase by exposure to isoleucine-deficient medium for 20–24 h. The arrested culture was fed with complete medium enabling the cells to continue the cell cycle synchronously, undergo mitosis, and enter a second cycle of growth. This method of synchronization left the protein-synthesizing ability intact as judged by the polysome profile and the capacity of the cells to incorporate labeled amino acids into protein after the restoration of isoleucine. After incubation in isoleucine-deficient medium and the addition of isoleucine to the culture, cells entered the S phase after a short lag, as judged by [^{3}H]thymidine incorporation into nucleic acid and by spectrophotometric measurement of nuclear DNA. The cells were in mitosis between 12 and 18 h as judged by the increase in cell count and analysis of cell populations on albumin gradients.

Synthesis and secretion of light-chain immunoglobulin were maximal in the late G1/early S phase of the first cycle. During late S phase, G2 phase, and mitosis, both synthesis and secretion were observed to be at a low level; however, immediately after mitosis the cells which then entered the G1 phase apparently commenced synthesis of light chain immunoglobulin straight away, although secretion of labeled material remained at a low level.

Lymphocytes ordinarily are quiescent cells that produce little or no immunoglobulin. After stimulation by antigen, lymphocytes undergo a transition to active, immunoglobulin-producing cells. Several investigators, using immunoglobulin-secreting, tissue-cultured cells which had been synchronized by different techniques, have suggested that the bulk of immunoglobulin synthesis occurs in the late G1/early S phase of the cell cycle (4, 5, 16, 20), while other observations have not demonstrated any cyclical behavior with respect to immunoglobulin synthesis (6, 13).

As part of studies concerned with elucidating the details of immunoglobulin biosynthesis and of membrane biogenesis on a molecular level, it became important to isolate large quantities of

synchronized mouse plasmacytoma cells. Several methods have been recommended for synchronizing cells in culture with respect to stage of cell cycle: use of colcemid or thymidine-colcemid (7); use of thymidine or double thymidine blockade (11); use of amethopterin (18); transferring cells allowed to reach stationary phase to fresh medium (21); or use of isoleucine-deficient culture medium (10, 22). Enger and Tobey (10) found that cells incubated in isoleucine-deficient medium enriched with glutamine traversed the cell cycle and became arrested in late G1 phase, and although translation was diminished and DNA synthesis absent, the capacity to synthesize protein was preserved, as indicated by the amount of polysomes and the rate of protein synthesis 2 h after readding isoleucine to the medium. Of several methods tried in our laboratory for synchronizing cells, the use of isoleucine-deficient medium gave the largest fraction of cells in synchrony, the highest viability, and the best polysome profiles.

We have investigated the processes of synthesis and secretion of light-chain immunoglobulin (hereafter referred to as "light-chain") in plasmacytoma cells (MPC-11) which had been synchronized late in the G1 phase. To check whether or not the peaks of synthesis and secretion observed after the readdition of isoleucine to the culture were in fact real and not merely due to some artifact induced by the period of isoleucine starvation, we allowed the synchronized culture to pass through mitosis and then we investigated synthesis and secretion in the second cycle. If the peaks observed previously in the first cycle were indeed artifacts, then one would not expect to observe them in the next cycle in cultures now growing in complete medium.

MATERIALS AND METHODS

Cell Line and Culture Conditions

MPC-11 cells were grown in roller bottles in Dulbecco's modified Eagle's medium containing the following additions per liter: 5.7 g $NaHCO_3$, 0.1 g sodium pyruvate, and 1.16 g glutamine. The complete medium contained 15% heat-inactivated horse serum and 25 U penicillin-G and 25 μg/ml streptomycin. Cell counts were determined with a Celloscope counter, and viability was estimated by the exclusion of erythrosin B (0.03%).

Synchronization of Cell Cultures

Cells were synchronized by growth in Dulbecco's medium deficient in the essential amino acid isoleucine. After growth for at least 20 h, the majority of cells were arrested in the G1 phase of the cell cycle (1).

Labeling and Isolation of Intracellular and Secreted Light Chain

Approximately 2.5×10^7 cells were suspended in 5 ml of Dulbecco's medium diluted 20-fold with Earle's balanced salt solution containing 1.6 mg/ml HEPES, 0.6 mg/ml glutamine, 3.5 mg/ml glucose, and 6 μCi [U-^{14}C]amino acid mixture, and incubated for 60 min at 37°C. After incubation, the cells were harvested and the supernate was kept for immunoprecipitation of secreted, labeled light-chain. The cells were washed twice with medium and disrupted by suspension in 1 ml of phosphate-buffered saline (pH 7.4) containing 0.1% Kyro EOB (a nonionic detergent) and 0.1% deoxycholate (DOC) (3). The homogenate was centrifuged at 105,000 *g* for 60 min and the supernate was collected for the immediate assay of labeled light-chain.

In some experiments, immunoglobulins were concentrated by precipitation with 50% ammonium sulfate before immunoprecipitation. Light-chains were isolated by a sandwich technique using rabbit/anti-mouse and goat/anti-rabbit sera, reduced and alkylated by β-mercaptoethanol and iodoacetamide (8), and then subjected to acrylamide gel electrophoresis (17) using N,N'-diallyltartardiamide (2) as cross-linking agent. The gels were cut into thin slices after the run and the gel matrix was dissolved by suspending each slice in 0.5 ml of cold 3% periodic acid and 0.1 ml of glacial acetic acid. The samples were assayed for radioactivity after the addition of Unisolve 1 scintillation fluid.

Labeling of DNA with [^{3}H]Thymidine

Incorporation of ^{3}H-labeled thymidine was followed by incubating 1.6×10^6 cells in 2 ml of Dulbecco's medium containing 5 μCi [^{3}H]thymidine for 60 min at 37°C. After incubation, 8 ml of cold medium was added, the cells were pelleted and washed twice with 10-ml vol of media, and 3 ml of cold 5% trichloroacetic acid were then added. The pellet was washed with equal volumes of alcohol and ether then with ether alone. The final pellet was washed with ether before being dissolved in 0.5 ml of 0.5 N NaOH, and 50 μl aliquots were counted in Unisolve 1.

Spectrophotometric Determination of the DNA Content of Nuclei

Aliquots of cells were dried on microscope slides and fixed in alcohol-glacial acetic acid, 3:1. The nuclei were stained by Feulgen's hydrolyzed for 6 min at 60°C, and mounted in benzoyl benzoate (9, 14). Relative amounts of DNA in nuclei were calculated from measurements performed on single nuclei at 560 nm in a Zeiss Ultra-Micro Spectrophotometer 1 using aperture 1 (9).

Fractionation of Cells According to Stage of Cell Cycle using Albumin Gradients

Between 10^7 and 10^8 cells were sedimented at room temperature, washed with phosphate-buffered saline, and suspended in 0.5 ml of phosphate-buffered saline. The cell suspension was layered on a cold linear 12-ml 0–25% (wt/wt) gradient of purified human plasma albumin in phosphate-buffered saline and centrifuged in an IEC 488 rotor at 4,000 rpm for 24 min at 2°C (19). The gradient outflow was monitored at 660 nm with a 5-mm Gilford flow-through cell (Gilford Instruments, Oberlin, Ohio) and fractions were collected. The contents of the various fractions were determined by light microscopy.

Materials

Kyro EOB was a gift from Dr. D. H. Hughes, Miami Valley Laboratories, Procter and Gamble Co. N,N'-Diallyltartardiamide was synthesized according to Anker's directions (2). Sodium deoxycholate (DOC) and enzyme grade ammonium sulfate were purchased from Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N.Y. HEPES from Calbiochem; β-mercaptoethanol and iodoacetamide from Fluka; and Unisolve 1 from Koch-Light. Radioactive precursors were purchased from the Radiochemical Centre, Amersham, U.K. Tissue culture media, sera, and antibiotics were supplied by Bio-Cult/Gibco Laboratories, (Grand Island Biological Co., Grand Island, N.Y.). Anti-sera were purchased from Cappel Laboratories, Downingtown, Pa. All other reagents were standard analytical grade.

RESULTS AND DISCUSSION

Synchronization of Cell Cultures

When a nonsynchronized culture of MPC-11 cells was transferred to isoleucine-deficient medium, the cell count continued to increase for a time as cells in S phase, G2 phase, and mitosis completed the cell cycle. By 20–24 h no further increase in cell count was observed; the culture was apparently arrested. When isoleucine was restored to the medium most cells began to traverse the cell cycle in synchrony and an abrupt increase in the cell count occurred between 12 and 18 h (Fig. 1 A).

The incorporation of ^{3}H-labeled thymidine into nucleic acids began 1 h after the addition of isoleucine to arrested cultures, indicating the commencement of S phase (Fig. 1 A). That the cells indeed entered S phase synchronously was con-

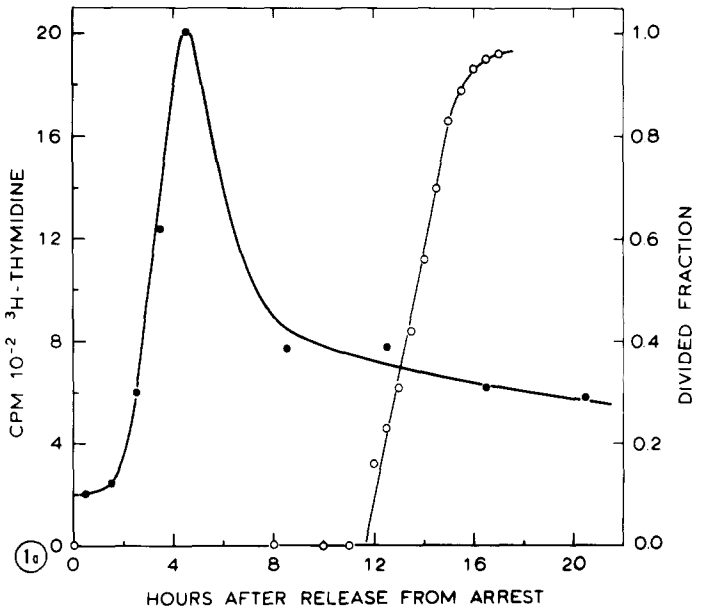

FIGURE 1 A Patterns of DNA synthesis and cell division obtained after the addition of isoleucine to a culture previously maintained for 20–24 h in Dulbecco's medium deficient in isoleucine. The culture had an initial cell count of approximately 4×10^5 cells/ml, and the fraction of dividing cells (O—O) was determined from the increase in cell count. Aliquots of 1.5×10^6 cells were removed at various times and incubated for 1 h with 5 μCi [^{3}H]thymidine, and radioactivity was determined in TCA-precipitable material (●—●).

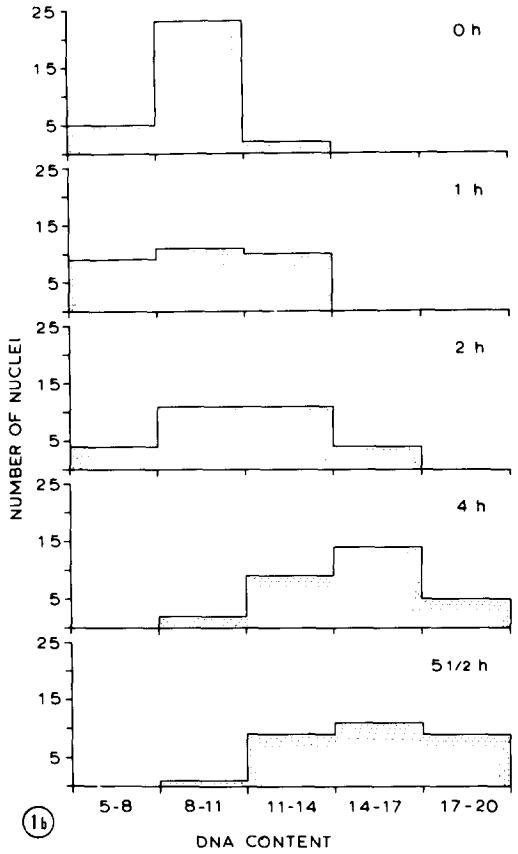

FIGURE 1B Spectrophotometrical determination of the DNA content of nuclei from synchronized cells. Aliquots of cells were removed at the times indicated from a culture synchronized by 20–24 h of isoleucine starvation, fixed, and stained by Feulgen's. The relative amounts of DNA in the nuclei were calculated from spectrophotometrical measurements made at 560 nm. For convenience, the observations have been classified into groups.

firmed by Feulgen spectrophotometry (Fig. 1 B) which showed an approximate doubling of the DNA content within 5.5 h after the addition of isoleucine. These results were in good agreement with earlier findings (1) showing that more than 90% of thymidine incorporation (followed for a period of 14 h) occurred within the space of 3–5 h after the addition of isoleucine. The observations were consistent with the interpretation that plasmacytoma cells become arrested in G1 phase in isoleucine-deficient medium, and upon restoration of isoleucine, enter S phase synchronously.

A comparison of cell viability, polysome content, and the fraction of cells commencing a traverse of the cell cycle in synchrony obtained by using several methods for synchronization is set out in Table I. From many experiments of this type we were able to conclude that of the various methods for synchronizing the cell cycle in suspension-cultured plasmacytoma cells, growth in isoleucine-deficient medium resulted in arrest of cells in late G1 phase, and that this method of synchronization gave the greatest cell viability, the greatest polysome content, and the greatest capacity to synthesize light-chain (see below).

Thus, our experience with mouse plasmacytoma cells corresponds generally to the findings of Tobey and co-workers (10, 22) for Chinese hamster ovary cells. Arrest by growth in isoleucine-deficient medium resembles contact inhibition in monolayer cultures; in both circumstances the protein-synthesizing machinery of the cell is preserved during arrest. Synchronization obtained by permitting cells to grow well into stationary phase results in cells with particularly diminished polysome content and diminished capacity for protein synthesis, as previous investigators have found for Chinese hamster cells (10, 22) and Ehrlich ascites cells (23).

In the present experiments, an "enriched" Dulbecco's medium was used in contrast to F-10 medium which was used by Tobey and co-workers (10, 22). Using this medium, we found that effective arrest occurred without the need for dialyzing the serum.

TABLE I

Characteristics of Several Methods of Synchronization

Method of synchronization	Cell viability*	Cells traversing cell cycle‡	r-RNA in polysomes§
Isoleucine deprivation	96%	95%	84%
Medium depleted of serum	83%	73%	73%
Stationary phase cells (reference 21)	70%	55%	60%
Cold treatment (24 h at 0°C)	50%	33%	44%

* Cell viability determined before release from arrest.

‡ Cells having divided 20 h after release from arrest calculated from cell counts and mitotic index.

§ Total area under the 260-nm absorption curve of polysomes (including dimers) expressed as a percent of total absorption (polysomes + monosomes).

Analysis of Cell Distribution using Albumin Gradients

The distribution of cells in various phases of the cycle was assessed by isopycnic sedimentation of a cell suspension in a linear 0–25% (wt/wt) albumin gradient. When an aliquot of cells from an actively growing culture was investigated, several bands were found (Fig. 2). Microscope examination of the various fractions showed that fraction 1 contained mainly cells which did not exclude erythrosin B and were judged to be nonviable. Fractions 3, 4, and 5 contained viable interphase cells but very few mitotic figures. On the basis of changes in the distribution of cells grown in isoleucine-deficient medium and correlation with light-chain synthesis, fraction 5 was judged to contain cells in the G1 phase and fraction 4 to contain mainly cells in the S phase, while fraction 3 was judged to contain cells in the G2 phase. Cells in fractions 7 and 8 were largely in mitosis.

Sedimentation of an aliquot of cells grown for 20–24 h in isoleucine-deficient medium gave a very large band in fraction 5—that judged to contain cells in the G1 phase—while only small numbers of cells were observed in the other fractions. When cells were incubated with radioactive amino acids before sedimentation and light-chain was then isolated from washed cells in the various fractions, the most predominant incorporation of radioactivity into light chain was found in cells in fraction 5. These results confirmed that fraction 5 indeed contained the bulk of cells in the G1 phase.

The technique of isopycnic banding of plasmacytoma cells in albumin gradients may be useful not only for analysis of cell population distribution according to stage of the cell cycle, but also as a preparative technique.

Light-Chain Synthesis during Various Phases of the Cell Cycle

After incubation in isoleucine-deficient medium for 20–24 h, the greatest incorporation of labeled amino acids into light-chain occurred 2 h after transfer to complete medium (Fig. 3), i.e. at the end of G1 phase/early S phase. These results are in agreement with those of earlier investigators (4, 12, 20).

In the middle of the S phase, light-chain synthe-

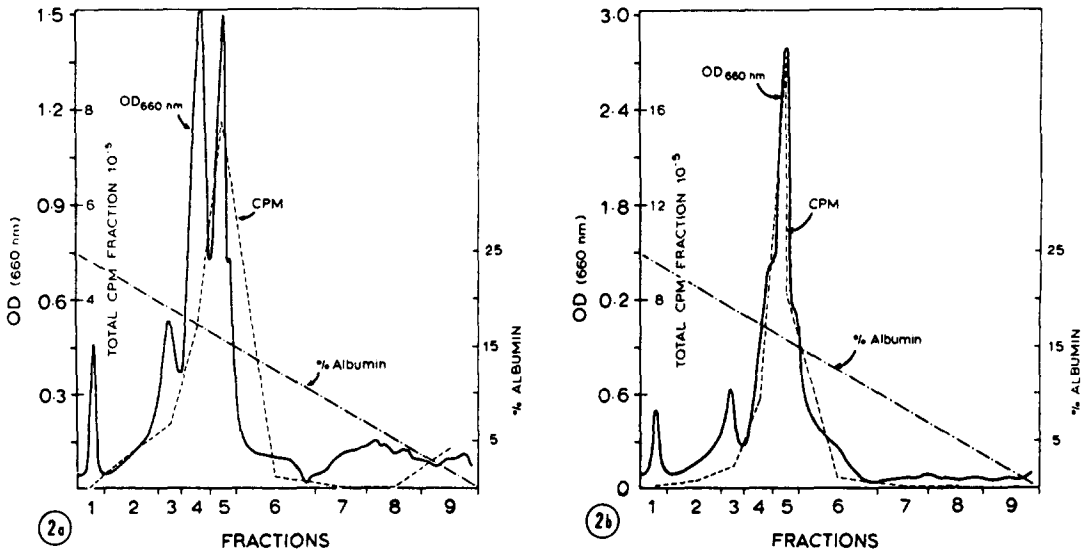

FIGURE 2 A About 10^8 MPC-11 cells, actively growing in complete Dulbecco's medium, were incubated for 60 min in medium containing 2 μCi [U-^{14}C]amino acids. The cells were washed with phosphate-buffered saline, layered on a 12-ml linear 0–25% (wt/wt) albumin gradient in phosphate-buffered saline, and sedimented at 4,000 rpm for 24 min (IEC 488 rotor). The gradient outflow was monitored at 660 nm (—), and fractions were collected for the assay of radioactivity in light-chain (-----). B, MPC-11 cells were grown in isoleucine-deficient medium for 20–24 h. 2 h after isoleucine had been restored to the medium, 10^8 cells were harvested and were incubated for 60 min in medium containing 24 μCi [U-^{14}C]amino acids. The cells were washed and sedimented in an albumin gradient, the outflow was monitored at 660 nm (—), and fractions were collected for the assay of radioactivity in light chain (-----).

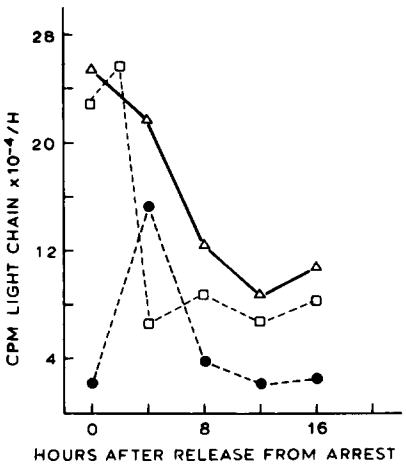

FIGURE 3 MPC-11 cells were grown for 20–24 h in isoleucine-deficient medium and isoleucine was then restored. Aliquots were removed periodically and incubated for 60 min in medium containing 24 μCi [U-^{14}C]amino acids. The cells were sedimented, washed, and disrupted, and a ribosome-free supernate was prepared for immunoprecipitation. Light-chain was also immunoprecipitated from the incubation medium (secreted light-chain). After reduction and alkylation, light-chain immunoglobulin was isolated by acrylamide gel electrophoresis. Calculated counts per hour in intracellular light-chain (□—□), secreted light-chain (●—●), and total light-chain (△—△) are shown.

sis was decreased to about one-third of the maximal amount observed earlier in the cell cycle and remained at a lower level through mitosis. The amount of labeled light chain secreted into the medium reached a peak 2–3 h after isoleucine was restored. Data for a typical experiment are depicted in Fig. 3.

Light-Chain Synthesis in the Cell Cycle after Mitosis

Since the experiments described above were performed with cultures shortly after the restoration of isoleucine which had been omitted from the culture medium for 24 h, one explanation for the results might be that they were artifacts caused directly by the conditions of isoleucine starvation. In order to overcome this we decided to allow the synchronized cells to traverse the first cell cycle and undergo mitosis, and then we examined the processes of synthesis and secretion in the G1 and S phases in the second growth cycle.

Synthesis and secretion of light-chain in the G2 phase in the first cycle were shown to be at a low level (Fig. 4); however, immediately after mitosis the rate of synthesis increased rapidly as the cells entered the G1 phase of the second cell cycle. Although the rate of synthesis accelerated during the early G1 phase, secretion remained at a low level during this time (Fig. 4). Late in the G1 phase, however, the amount of secreted material became almost identical to that synthesized during a 1-h incubation with radioactive amino acids. This rapid increase in the rate of secretion matched the rise previously observed 2 h after the restoration of isoleucine to a culture which had been starved for 24 h. Since the observations concerning synthesis and secretion in the second cycle matched those in the first, it was apparent that isoleucine starvation had not introduced artifacts.

These experiments indicate that synthesis and secretion of light chain do not occur at the same rate throughout the cell cycle. Both processes appear to reach a maximum at the end of G1 phase/early S phase and a minimum during G2 phase/mitosis. In vitro studies lend support to the above observations. The respective abilities of G1 and G2 membrane-bound polysomes to synthesize light-chain in vitro were studied (15). Membrane-bound polysomes isolated from cells in the late G1/early S phase were approximately seven times more effective in the synthesis of light chain in vitro than those isolated in the G2 phase.

Byars and Kidson (5) synchronized C1 myeloma cells at the beginning of S phase by double thymidine block and shortly after release from the block were able to demonstrate a peak of immunoglobulin synthesis. Liberti and Baglioni (13), using the same cell line and same synchronization conditions, were unable to confirm these observations. After labeling and preparation of a cell-free extract, Liberti and Baglioni froze their extracts at −20°C and stored them at this temperature before immunoprecipitation. In our experience the freezing of such extracts before immunoprecipitation has a profound effect on the amount of light-chain which can be finally isolated (Table II). We found that a good deal of light-chain was lost, presumably in the form of nonimmunoprecipitable aggregates, and that the recovery of light-chain was only about 30% of that from nonfrozen extracts. Since Liberti and Baglioni centrifuged for 10 min at 12,000 *g* after thawing to remove insoluble material, which most probably had

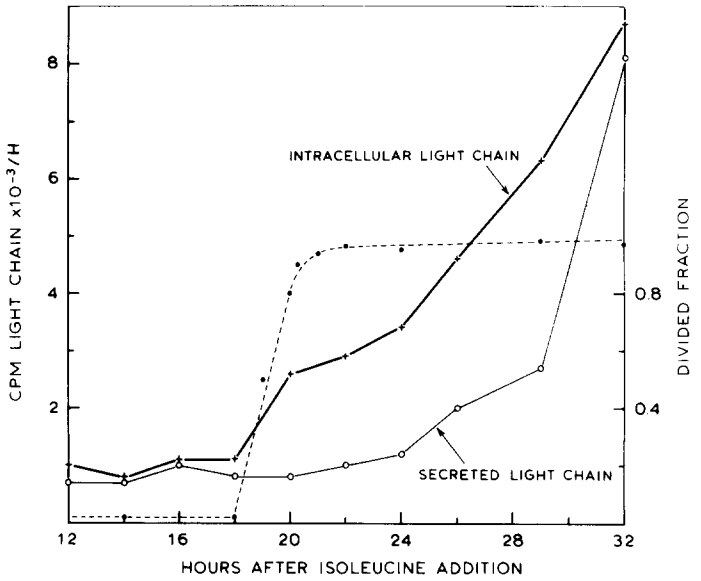

FIGURE 4 MPC-11 cells were grown in isoleucine-deficient medium for 20–24 h and isoleucine was then restored. Aliquots of 10^8 cells were removed periodically and incubated for 60 min in medium containing 24 μCi [U-^{14}C]amino acids. The cells were sedimented, washed, and disrupted, and a ribosome-free supernate was prepared. Labeled light-chain immunoglobulin was immunoprecipitated from both the ribosome-free supernate and the incubation medium, reduced and alkylated, and isolated by acrylamide gel electrophoresis. Calculated counts per hour in intracellular light-chain (+—+) and secreted light-chain (O—O). The cell count of the culture after the addition of isoleucine was 4×10^5 cells/ml. Aliquots of cells were counted at various times and the divided fraction was calculated (●- - -●).

TABLE II

The Amount of Light-Chain in Ribosome-Free Supernates before and after Freezing

Treatment	Exp. no.	Cpm in light chain	Cpm remaining in light chain
			%
Immunoprecipitation of supernate before freezing	1	4,850	100
	2	4,750	100
Immunoprecipitation of supernate after freezing (48 h)*	1	1,780	37
	2	1,500	32

* The ribosome-free supernate was frozen at −20°C and stored at this temperature for 48 h before thawing.

formed as a result of the freezing treatment, it is quite possible that they lost a large percent of their labeled material before immunoprecipitation was performed. This perhaps could explain the discrepancy between their results and those of Byars and Kidson who did not freeze their extracts.

In conclusion, the present results support the previous findings showing that human lymphoma and mouse myeloma cells grown in culture synthesize immunoglobulin maximally toward the end of G1 phase and the beginning of S phase.

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