THE UNIVERSITY OF CALGARY

SPECIFIC PROTEIN SYNTHESIS REQUIRED FOR THE INITIATION OF MITOSIS

by

J. Timothy Westwood

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGY

CALGARY, ALBERTA

SEPTEMBER, 1982

UNIVERSITY OF CALGARY

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Specific Protein Synthesis Required for the Initiation of Mitosis" submitted by J. Timothy Westwood in partial fulfillment of the requirements for the degree of Master of Science.

Supervisor, Dr. R.B. Church
Departments of Biology
and Medical Biochemistry

Dr. J.D. Duerksen
Department of Biology

Dr. C.C. Lin
Departments of Medical Biochemistry
and Pediatrics

September 30, 1982

ABSTRACT

The inhibition of protein synthesis in eukaryotic cells will prevent them from entering mitosis. Emetine inhibits peptide elongation and when it was added to asynchronous populations of Chinese hamster ovary (CHO) cells, the mitotic index decreased sharply 30 to 40 minutes later. It was found that the inhibitory effect of emetine could be reversed when it was removed and the reversibility was dependent on both the initial concentration of emetine and the pH of the media. Cell populations which were blocked by emetine for up to two hours showed a four- to five-fold increase in mitotic index approximately one hour after the emetine was removed. These results indicated that there may be a point or period in G_2 where there are critical 'mitotic proteins' being synthesized.

To determine whether or not there is a specific peptide or peptides synthesized just prior to mitosis in CHO cells the peptide patterns of several stages of the cell cycle were examined. Cells were first synchronized for the cell cycle phases by either mitotic shake-off or a combination of shake-off and hydroxyurea treatments. Early and late G_2, G_1/S arrested, early and late S, mid and late G_2 as well as mitotic cells were labelled for 30 minutes with ^{35}S-methionine. Protein samples were run on both

equilibrium (O'Farrell) and nonequilibrium pH gradient (NEPHGE) two-dimensional polyacrylamide gels and autoradiograms made. Over 600 peptides were clearly resolved on the autoradiograms. A few qualitative changes in protein synthesis which have never been reported previously were found. One protein of 22,000 MW and pI of 5.5 appears only to be synthesized during late G_2 and 2 to 4 very basic proteins having approximately the same molecular weight as histones were synthesized only during S phase. Several quantitative changes in peptide synthesis during the cell cycle were observed. Of these proteins the tubulins and histones were identified. The synthesis of alpha- and beta-tubulin appeared to be highest during late G_2 and mitosis and lowest during late G_1 and G_1/S arrest. Histone synthesis was restricted to the S phase of the cell cycle.

These results support the idea that synthesis of a specific protein(s) may be needed for the initiation of mitosis. Also, there appears to be a few proteins which are specific to the S phase of the cell cycle.

ACKNOWLEDGEMENTS

I would like to acknowledge the guidance, support, and useful criticisms provided by my supervisor, Dr. R.B. Church. I am also grateful for his financial support in the form of research materials and trips to scientific meetings.

I would like to thank Dr. Ron L. Brown and Dr. T.E. Stubblefield for all of their help and the use of their facilities at M.D. Anderson Hospital in Houston during the initial stages of this study.

I am also grateful for the technical assistance received from Joanne Moggert and Dr. Ted Zipf for the Ortho Cytofluorograf system 50 cell sorter.

I am indebted to Susie F. Carlson for her assistance in the typing of this manuscript.

Financial support was provided by the National Science Research and Engineering Council in the form of a scholarship and the Alberta Heritage Foundation for Medical Research in the form of a Research Allowance.

I would like to express my gratitude to all the friends I have found at the University for their support and encouragement and especially to Leo Dimnik who was always there when times got tough.

Finally, I would like to thank Dr. E.B. Wagenaar whose ideas, encouragement, enthusiasm, and friendship made this study possible.

This thesis is dedicated to
my family who have always given me
all the support anyone could ask for
and to
Dr. Emile B. Wagenaar who interested
me in science when I was
twelve and never let my interest die.

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LIST OF ABBREVIATIONS

CHO	Chinese Hamster Ovary
cpm	Counts per minute
DNA	Deoxyribonucleic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IEF	Isoelectric focusing
MW	Molecular weight
NEPHGE	Nonequilibrium pH gradient electrophoresis
PCC	Premature chromosome condensation
pI	Isoelectric point
RNA	Ribonucleic acid
SDS	Sodium dodecylsulfate
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
uCi	microcurie
ug	microgram
ul	microliter
uM	micromolar

INTRODUCTION

An essential property of any living organism is the ability of its cells to reproduce. Actively growing populations of cells usually divide on a regular basis. This regularity reflects that the events involved in the cell division process are probably cyclical. Early cell biologists divided this cycle into two phases based on what could be observed visually; the 'mitotic phase' and the 'resting phase' or interphase. However, as techniques in biochemistry improved it was found that the 'resting phase' was actually a time of great activity, namely cells were replicating their components in preparation for division. In 1953, Howard and Pelc observed that DNA synthesis in eukaryotic cells was limited to a discrete period within interphase. This period was later termed the S or synthetic period and was preceded by a presynthetic 'gap' (G_1 period) and followed by a premitotic 'gap' (G_2 period). These three phases plus mitosis and cytokinesis itself (M) make up what is termed the 'cell cycle'.

The M and S phases are defined by mitosis and chromosomal DNA replication respectively but no specific functions have been clearly defined for the G_1 and G_2

periods of the cell cycle. Knowledge of the events taking place during G_1 and G_2 is still rather limited but it is assumed that the G_1 period contains a sequence of events which culminates in the initiation of DNA synthesis. Similarly G_2 most likely contains critical events which leads to mitosis and cell division (For reviews see Mitchison 1971; Tobey et al., 1971 and Prescott, 1976).

A number of researchers have shown that both RNA and protein synthesis are needed within G_2 for the initiation of mitosis (see Tobey et al., 1971 for review). Because protein synthesis during G_2 is an absolute requirement for the successful initiation of mitosis, it is possible that proteins synthesized during this time are unique to that portion of the cell cycle and are directly involved with mitosis or cell divisional processes. Until recently, little attempt has been made to find, isolate or uncover the function of any G_2 specific proteins.

To examine the need for protein synthesis for the initiation of mitosis, studies to date have used inhibitors of protein synthesis. By scoring cells in a recognizable phase of the cell cycle (e.g. mitosis) behind a block produced by a specific inhibitor, the position in the cell cycle at which the inhibitor exerts its action can be determined. One of the earliest experiments attempting to localize such an inhibitor effect was conducted by Taylor

(1963). In this experiment, KB human carcinoma cells were exposed to various concentrations of the protein synthesis inhibitors chloramphenicol and puromycin and the rate of accumulation of mitotic cells (in the presence of colchicine) as well as the rate of increase in cell number was determined. The results of Taylor's study indicated that the rate of entry into prophase was related to the rate of protein synthesis, and the last protein-requiring step necessary for initiating mitosis occurred at a point approximately 30 minutes before the $G_{2/M}$ boundary.

In a study by Kishimoto and Leiberman (1964) the effect of actinomycin (a RNA synthesis inhibitor) and puromycin on the appearance of mitosis labelled with ^{3}H-thymidine in primary cultures of rabbit kidney cortex was examined. Their results indicated that cells which were one hour or more into G_2 could successfully divide in the presence of actinomycin (.33 ug/ml) indicating no essential RNA synthesis is occuring in mid and late G_2. Addition of puromycin (3.3 ug/ml) indicated that protein synthesis was required for most of G_2 if cells were to enter mitosis.

Buck and coworkers (1967) demonstrated that HeLa cells could enter and complete mitosis in the presence of puromycin and actinomycin indicating that all cellular constituents necessary for successful initiation and completion of mitosis are produced prior to the onset of

mitosis.

Perhaps the most critical study done on the temporal location of the 'essential' protein and RNA synthesis in G_2 was a study conducted by Tobey, Peterson, Anderson and Puck (1966). This study examined the effects of puromycin and actinomycin on the completion of mitosis in synchronized cultures of Chinese hamster ovary (CHO) cells. Their results indicated that the final division related process requiring RNA synthesis occurred 1.87 hours prior to cell division while the final process requiring protein synthesis happened 36 minutes before cell division. The mitotic index of cells treated with puromycin was always greater than zero for at least 12 hours even in the absence of cell division. They suggested that there were probably two different proteins being affected by puromycin; one which allowed cells to enter mitosis and a second, synthesized 8 minutes later, which was needed for the completion of mitosis. It was calculated that these proteins were synthesized 2 and 10 minutes before the $G_{2/M}$ boundary.

Using techniques involving time lapse microcinematography and autoradiography of Fernandes cells, Donnelly and Sisken (1967) demonstrated that actinomycin D (4 ug/ml) only affected those G_2 cells which were not within a 30 to 40 minute period prior to prophase. The addition of puromycin (25 ug/ml) resulted in a 30% decrease

in mitotic index by 30 minutes. They concluded that protein synthesis is essential for continued progression through G_2 until a point less than 30 minutes before prophase.

In a recent study by Wagenaar and Mazia (1978) it was shown that the addition of emetine (a protein synthesis inhibitor) to Strongylocentrotus purpuratus (sea urchin) zygotes approximately 50 minutes before prophase of the first cleavage division prevented chromosome condensation. Addition of emetine 45 to 50 minutes before prophase prevented nuclear membrane breakdown. Emetine added less than 40 minutes before the first cleavage division had no effects on mitosis. This study, like that of Tobey and coworkers (1966) indicates that there may be more than one protein being synthesized during the 'critical' G_2 period preceding mitosis.

There have been a few studies in recent years which have attempted to find qualitative and quantitative differences in peptide synthesis during the cell cycle of a number of cell types. One early study using only one-dimensional polyacrylamide gel electrophoresis of HeLa cells labelled with ^{14}C-leucine throughout the cell cycle and ^{3}H-leucine during G_2 revealed significant differences in the ratios of ^{3}H to ^{14}C on the bands in the gel (Kolodny and Gross, 1969). Therefore, either different proteins

were being synthesized at different rates during the cell cycle or that variations in synthetic rates for groups of proteins occur at various stages in the cycle. A single peptide or group of peptides could not be detected as being G_2 specific since one-dimensional electrophoresis could not resolve single peptides in whole cell preparations.

The advent of two-dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975) made high resolution peptide mapping during the cell cycle possible. A study by Milcarek and Zahn (1978) reported that of 90 proteins studied throughout the HeLa cell cycle, only six proteins were found to vary greater than fourfold during the cell cycle. Four of these had predominant synthesis near mitosis while the other two showed their lowest amount of synthesis in the G_2 period. Al-Bader, Orengo and Rao (1978) compared the peptide patterns of S, G_2-synchronized and G_2-arrested HeLa cells. S phase cells were found to have 25 fewer peptide spots than G_2-arrested and 35 fewer peptide spots than G_2-synchronized cells. There were at least nine proteins in the molecular weight range of 4 to 5 x10 ^{4} daltons in peptides of G_2-synchronized cells that were not seen in S or G_2-arrested cells. It was concluded that the missing proteins were probably involved in the transition of cells from G_2 phase to mitosis. Perhaps the most comprehensive study of differential peptide synthesis throughout the cell cycle was one conducted by Bravo and

Celis (1980). In this study synchronized HeLa cells were pulse-labelled with ^{35}S-methionine at various times during the cell cycle or for long periods starting at the beginning of each phase of the cycle. The polypeptides synthesized were analyzed by two-dimensional electrophoresis followed by fluorography. Over 700 peptides were visible in the fluorograms and the relative synthesis of the 99 most abundant peptides was determined. The results of this study indicated that none of the detected polypeptides synthesis was confined to only one of the cell cycle phases. However, some of the peptides were synthesized in significantly higher or lower amounts during M, and M and G_2. Other peptides were shown to be synthesized in variable amounts during S but no specific marker was found for the G_1 period.

Several studies have been conducted recently which examine the differential synthesis of histones during the cell cycle. Groppi and Coffino (1980) examined the synthesis of histones during the cell cycle of CHO and mouse S49 lymphoma cells and found no appreciable difference either qualitatively or quantitatively. Marashi and coworkers (1982) have presented evidence which indicates that histone synthesis occurs only during S phase and that histone messenger RNA sequence can only be detected in S phase and not in G_1 phase HeLa S3 cells.

The preceding information has hopefully established two facts: first, that protein synthesis is necessary during G_2 for eukaryotic cells to enter and/or complete mitosis and cell division; and second, that differential peptide synthesis occurs during the cell cycle.

A portion of this study further examines the need for protein synthesis in the initiation of mitosis. Like other studies which have examined the involvement of protein synthesis in specific cellular events, this study uses an inhibitor of protein synthesis. Protein biosynthesis can be inhibited at a number of places including; initiation (e.g. cycloheximide), elongation (e.g. emetine and cycloheximide), and peptide release (e.g. puromycin) (Ennis and Lubin, 1964; Grollman, 1966 and 1968; Obrig et al., 1970; and Oleinick, 1977). It is advantageous to use a protein synthesis inhibitor which is reversible since the time it takes to have an effect on a function can be measured from the addition of the inhibitor and the return of the function measured from the time of the removal of the inhibitor. Both cycloheximide and emetine have been shown to be reversible inhibitors of protein synthesis in mammalian cells. The reversibility of emetine induced protein synthesis inhibition in CHO cells has been fairly well characterized (Gupta and Siminovitch, 1976; Westwood et al., 1981) so emetine was chosen as the inhibitor for

this study.

The aim of this study is to:

(1) Determine the last point or 'span' in time during G_2 in which protein synthesis is absolutely necessary for Chinese hamster ovary (CHO) cells to enter mitosis and/or complete cell division.

(2) Examine the peptide synthesis pattern throughout the cell cycle of CHO cells and determine if there are any peptides synthesized specifically during the portion of G_2 found in (1).

An initial indication of the need for protein synthesis prior to mitosis and the temporal location of such synthesis was obtained by adding emetine to an asynchronous population of CHO cells and examining the effect this has on both mitotic index and cell division (as function of time from the addition of the inhibitor). A rough estimate of the duration of the above mentioned protein synthesis period was obtained by exposing asynchronous populations of CHO cells to emetine for various lengths of time after which the emetine was removed and the time it takes for the mitotic index to return to control levels determined.

Examination of differential peptide synthesis during the cell cycle was accomplished by exposing synchronous populations of CHO cells to high specific activity

^{35}S-methionine for short periods of time at various points throughout the cell cycle (i.e. early G_1, late G_1, G_1/S, early S, late S, mid G_2, late G_2 and Mitosis) then qualitatively analyzing the protein synthetic patterns at that time using both equilibrium (IEF) and nonequilibrium (NEPHGE) two-dimensional polyacrylamide gel electrophoresis and autoradiography.

MATERIALS AND METHODS

A. CULTURING AND MAINTENANCE OF CELLS

The Chinese hamster ovary (CHO) cells used in this study were a gift from Dr. T.E. Stubblefield (Department of Cell Biology, M.D. Anderson Hospital, Houston, Texas). This cell line was originally established in culture by Tjio and Puck (1958) and have a modal chromosome number of 22 with a deviation of 20 per cent (Puck et al., 1958). Under the growing conditions described below, CHO cells have a generation time of approximately 12.4 hours consisting of: G_1, 4.7 hours; S, 4.1 hours, G_2, 2.8 hours, and mitosis, 0.8 hours (Puck et al., 1964).

Cells were routinely cultured in sterile plastic flasks with modified McCoy's 5a media (Gibco) supplemented with 10% fetal calf serum (Flow) and 10mM Hepes buffer (pH 7.4). Penicillin (100 units/ml) and streptomycin (100 ug/ml) were also added to the media. Cells were usually subcultured daily with the cells being removed with a brief treatment of .02% trypsin in magnesium and calcium free Hank's balanced saline. Cells were incubated in a 5% CO_2 atmosphere at 37°C.

B. INHIBITION OF PROTEIN SYNTHESIS

1. Measurement of Protein and RNA Synthesis

The rate of protein or RNA synthesis was determined by assay of ^{3}H-leucine or ^{3}H-uridine incorporation into the TCA precipitable fraction of the cells.

Approximately 24 hours before each experiment identical monolayer cultures of CHO cells were grown in the bottoms of glass scintillation vials (Kimble) by seeding each vial with two mls of a cell suspension containing approximately $5x10^{5}$ cells/ml. Prior to each experiment, the media was poured from the vials and the vials were rinsed once with Hank's balanced saline solution. Then, two mls of leucine-free McCoys 5a media supplemented with 10% dialyzed fetal calf serum (dfcs) was added to each vial. At the specific sample times 50 ul of balanced saline solution containing 40 uCi/ml ^{3}H-leucine (New England Nuclear, Specific Activity 5.0 Ci/mMol) was added to the respective vials resulting in a final concentration of 1 uCi/ml. After 15 minutes, the media was poured from each vial and 5 ml of cold 5% TCA was added for 5 minutes at 4^{o} C. For the determination of RNA synthesis, cultures were continuously labelled in media containing 2.0 uCi/ml ^{3}H-uridine (New England Nuclear, Specific Activity 25.0 Ci/mMol) and fixed with cold TCA as just described. After

three five minute washes with cold TCA, cultures were rinsed twice with absolute ethanol (5-10 ml each rinse) followed by two rinses with toluene. Ten mls of Scintiverse (Fisher Scientific) was then added and the vials allowed to sit overnight in the dark before being counted on a Beckman Model LS200C liquid scintillation counter. All samples were done in either triplicate or quadruplicate.

2. Protein Synthesis Inhibition and Reversibility of Inhibition

(a) Effect of drug concentration. Stock solutions of emetine-HCl ($1x10^{-3}M$) (Sigma Chemical, St. Louis, Missouri) were made in Hank's balanced saline solution. Leucine-free media containing the appropriate concentration of emetine was added to the respective vials at T = 0 minutes. At T = 30 minutes ^{3}H-leucine was added to the vials for a 15 minute pulse label. In the case of ^{3}H-uridine incorporation, cells were continuously labelled. Reversibility was checked by exposing the cells to emetine for 30 minutes, removing the media, rinsing the vials once with 2 mls of Hank's balanced saline, and then adding 2 mls of inhibitor and leucine-free media to each of the vials. ^{3}H-leucine was added thirty minutes later for a 15 minute pulse label.

(b) Rates of inhibition and reversibility. $5x10^{-7}M$ emetine was added to the cultures at zero minutes, followed by the

addition of ^{3}H-leucine (for a 10 minute pulse) at 0, 2, 5, and 10 minutes. For reversibility, the drug containing media was removed, the cells rinsed once with Hank's balanced saline, and inhibitor and leucine-free media added at time zero. ^{3}H-leucine was added for 10 minute pulses at 5, 10, 20 and 30 minutes.

(c) Effect of pH. Leucine-free 5a media containing 20 mM Hepes buffer (with 10% dfcs) was adjusted to pH 6.0, 6.5, 7.0 and 7.5 using HCl or NaOH. Approximately 40 minutes before the start of the experiment the media was poured off the cells and the pH adjusted media added. Approximately 30 minutes before the start of the experiment stock solution emetine was added to those vials which required it to a final concentration of $5x10^{-7}$M.

3. Effect of Emetine on Cells Entering Mitosis.

Asynchronous populations of CHO cells were grown to about 70-90% confluency in plastic T-25 flasks. Emetine in conditioned media (media taken from flasks in which cells had been growing) was added to the respective flasks at zero minutes at a final concentration of $5x10^{-7}$ or $1x10^{-6}$M. Samples were taken at 10 minute intervals after the addition of emetine. In a second experiment, cells were exposed to emetine ($5x10^{-7}$M) for either 60 or 120 minutes after which time the emetine media was poured off, the flasks rinsed once and conditioned media added. Samples

were taken at 15 minute intervals after the removal of emetine. Approximately 5 minutes before each sample time in both experiments, cells were scraped from the flasks using a rubber policeman and the cell suspension poured into a 15 ml conical centrifuge tube. The cells were pelleted in a clinical centrifuge and at the sample time fixed with 3 methanol:1 glacial acetic acid mixture. Air dried slides were prepared and stained with 2% aceto-orcein (Gibco). The mitotic indexes were determined by counting prophase, metaphase, anaphase, telophase, and interphase cells and dividing the total number of cells in first four groups (mitotic cells) by the total number of cells in all groups. A minimum of one thousand cells were counted to determine each mitotic index. Initial experiments involved blind controls.

C. SYNCHRONIZATION OF CELLS

1. Synchrony Techniques

A variety of synchronization techniques are available for mammalian cell lines such as CHO. These synchronization techniques are usually based on either; natural changes in shape and volume of cells which occur during the cell cycle (e.g. mitoic shake-off, centrifugal elutriation), cell starvation (e.g. serum or amino acid deficiences), or the use of metabolic inhibitors (e.g.

excess thymidine, hydroxyurea, fluorodeoxyuridine, etc.).

A number of synchronization techniques were tried in order to obtain a population of cells homogenous for a certain stage of the cell cycle including: mitotic shake-off and variations of that technique (Terasima and Tolmach, 1963; Peterson et al., 1968; Tobey et al., 1967; Stubblefield and Klevecz, 1965; Stubblefield et al., 1968); isoleucine deprivation of cells to arrest them in G_1 (Ley and Tobey, 1970; Tobey and Ley, 1971); addition of excess thymidine to arrest cells at the G_1/S border (Xeros, 1962; Bootsma et al., 1964; Peterson and Anderson, 1964); and the addition of hydroxyurea which also arrests cells at G_1/S (Sinclair 1965 and 1967; Tobey et al., 1974).

Only two of the techniques; shake-off of mitotic cells and the hydroxyurea block provided adequate and reproducible synchronous populations of cells.

(a) Shake-off of Mitotic Cells

It was first observed by Terasima and Tolmach (1963) that mitotic HeLa S3 cells in monolayer culture could be separated from interphase cells simply by shaking the vessel they were growing in since mitotic cells are 'rounded up' and are less firmly attached to their growing surface. This technique provided only a small number of synchronized cells relative to the number of cells growing in the vessel. To increase the yield of mitotic cells, the

mitotic spindle inhibitor colcemid was used at low concentrations for a short period of time (Stubblefield and Klevecz, 1965).

Depending on the experiment, anywhere from 8 to 20 T-175 plastic flasks (NUNC, Denmark) were seeded with approximately $7x10^6$ cells 24 hours before the experiment. The next day the flasks were struck once to remove any mitotic or dead cells and the media filtered through a .22 um membrane filter (Nalgene). Stock colcemid (Gibco) was added to the filtered media to a final concentration of .06 ug/ml. Equal amounts of this media was added to the flasks which were then placed in the incubator. After two and a half hours the flasks were removed from the incubator and each flask was shaken three times by striking it softly against the palm of one's hand. The cell suspension was then collected in sterile 50 ml centrifuge tubes and spun at 1000 rpm on a International clinical centrifuge. Usually a small portion of the suspension was collected in a 15 ml centrifuge tube, pelleted, fixed and scored for mitotic index. The colcemid media was then removed from the 50 ml centrifuge tubes and the cells resuspended in warm (37^{o} C) Hank's balanced saline. The cells were then repelleted, the saline removed, and resuspended again in warm conditioned media. This suspension was then distributed into culture flasks or dishes depending on the experiments. The time of reversal of mitotic arrest was

measured from the time the conditioned media was added.

(b) G1/S Block with Hydroxyurea

Hydroxyurea inhibits DNA synthesis by inhibiting nucleoside diphosphate reductase and therefore preventing the production of nucleoside diphosphate from deoxynucleoside diphosphate (Sinclair, 1968). If the hydroxyurea is removed, DNA synthesis will resume quite readily.

Before hydroxyurea treatment, CHO cells were first obtained by mitotic selection as described in the previous section. Two hours after the resuspension of mitotic cells in conditioned media, stock solutions of hydroxyurea (Calbiochem) were then added to the cultures to a final concentration of 2×10^{-4}M. After five hours in hydroxyurea, the media was removed and the cells rinsed twice with warm balanced saline solution. Conditioned media containing 11 ug/ml deoxycytidine was then added to the cells and the cells then placed in the incubator. This action signified time zero from hydroxyurea release.

2. Monitoring Synchrony

(a) Mitotic Index

The best method for examining the synchrony of a mitotic shake-off experiment is to sample the cells and determine what portion of the population are in mitosis. A portion of the mitotic shake-off was collected, fixed and stained as described in section B of Materials and Methods. Almost all of the mitotic figures should appear in metaphase since colcemid arrests cells at this stage. This method was also used to determine the degree of synchrony of cells entering mitosis after shake-off and hydroxyurea treatments had been applied. In this case cells were scraped from 35 mm petrie dishes at various times after release from hydroxyurea and pelleted, fixed and scored for mitotic index. A minimum of 500 cells were counted to determine each mitotic index.

(b) Incorporation of 3H-Thymidine

The incorporation of ^{3}H-thymidine into cells synchronized by a combination of mitotic shake-off and hydroxyurea treatments was monitored in order to determine how effective the hydroxyurea was holding cells at the G_1/S border and how synchronously they went through S. ^{3}H-thymidine incorporation can be monitored by autoradiography or quantitation through liquid scintillation counting. Liquid scintillation counting was

chosen for this study since it gives a better estimation how fast cells are entering S phase and where in S phase peak incorporation of ^{3}H-thymidine occurs. After mitotic shake-off the cell suspension is pelleted, rinsed once with warm rinsing solution, repelleted and resuspended in warm conditioned media to a concentration of approximately $5x10^{4}$ cells/ml. Two mls of this suspension was then added to a number of glass scintillation vials (Kimble) and then incubated. After two hours hydroxyurea was added to the vials to a final concentration of $2x10^{-4}$M. Thirty minutes before hydroxyurea removal (i.e. five and a half hours after the hydroxyurea was added) 50 ul of balanced saline containing 80 uCi/ml ^{3}H-thymidine (Amersham, 48 Ci/mMol) was added to the respective vials resulting in a final concentration of 2 uCi/ml. After a 15 minute labelling period incorporation was stopped and the TCA precipitable counts determined as described in section B1 of Materials and Methods. All samples were done in duplicate.

(c) Quantitation of Individual Cell DNA Content

An Ortho cytofluorograf system 50 fluorescent cell sorter (Ortho Scientific) was used to examine the DNA content of individual cells in a population. At various times after either mitotic shake-off or a combination of shake-off and hydroxyurea treatments cells were fixed with ice cold 70% ethanol. Before analysis on the

cytofluorograf cells were stained with propidium iodide (Calbiochem) by removing the fixative and resuspending the cell pellet in the staining solution (.01M Dulbecco's phosphate buffered saline; 0.6% NP-40; 50 ug/ml propidium iodide). After 10 minutes or more cells were run on the cytofluorograf for red fluorescence (488 or 514 nm). Cells were first sized then individual cell fluorescence was plotted on a forward scatter versus fluorescent intensity graph. This data was then taken and converted to a plot of cell frequency versus fluorescent intensity. All of these analytical funtions were performed on the cytofluorograf's microprocessor. The graphs shown in Figure 7 were traced from the original computor printouts.

D. SEPARATION OF PROTEINS BY TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

1. Equilibrium (O'Farrell) Two Dimensional Gels

The most widely used technique for high resolution separation of peptides is O'Farrell two-dimensional polyacrylamide gel electrophoresis. In this technique proteins are separated according to their isoelectric point by isoelectrofocusing in the first dimension then according to their molecular weight by sodium dodecylsulfate (SDS) electrophoresis in the second dimension. All two-dimensional gel electrophoresis was carried out

according to the methods described by O'Farrell (1975, 1978) except for a few modifications which are discussed below.

(a) Labelling of Cells in Culture with 35S-Methionine

CHO cells were grown under the conditions described in part A of Materials and Methods. To label an asynchronous population of cells, T-25 flasks containing monolayers of the synchronized cells were rinsed twice with Hank's balanced saline solution. One ml of low methionine (1 uM) media containing 10% dialyzed fetal calf sera and 100 uCi/ml ^{35}S-methionine (Amersham, 1300 Ci/mMol) was added to the cells for thirty minutes. Low methionine McCoy's 5a media is not available commercially and consequently was made up in the laboratory according to the formula (Iwakata and Grace, 1964) with the exception of 1 instead of 100 uM methionine added to the media. After the labelling period the media was removed and the cells rinsed twice with balanced saline. The cells were scraped from the flask using a rubber policeman, then 5 mls of balanced saline were added and the cell suspension pelleted. Cell pellets were prepared according to the procedure described in the next section -(b) Sample Preparation.

The labelling of cells at various stages of the cell cycle was done using the following protocol. Early and late G_1 cells were labelled for 30 minutes, one hour (for

early G_1) and three hours (for late G_1) after the cells from a mitotic shake-off were suspended in conditioned media. Cells at the G_1/S border were labelled for thirty minutes 6 hours after a mitotic shake-off experiment in which hydroxyurea was added at 2 hours after the shake-off. Early and late S and mid G_2 cells were labelled for 30 minutes at 1.5, 5 and 8 hours respectively after release from hydroxyurea in a shake-off/hydroxyurea synchrony experiment. Mitotic cells were labelled by adding 4 mls of low methionine media containing 25 uCi/ml ^{35}S-methionine to each of 8 T-175 flasks (NUNC, Denmark) which contained monolayers of asynchronous cells. After 30 minutes the media was carefully removed and the mitotic cells shaken-off in rinsing solution. Late G_2 cells were labelled in a similar manner except that after the labelling period, the media containing the label was carefully removed, the cells rinsed once and normal 5a media containing 10% fetal calf sera and twice the normal methionine concentration (200 uM) was added. After 30 minutes in this media the mitotic cells were shaken-off and collected.

(b) Sample Preparation

Protein preparations from whole cells were made using the technique devised by Garrels (1979). The O'Farrell method of sample preparation (1975) was tried but it was found that the sonication of the cells resulted in the

modification of a number of proteins. The major differences in the Garrels' preparation from other methods is the use of micrococcal nuclease to degrade the nucleic acids and the speed of preparation - the entire procedure taking less than sixty seconds.

The activity (cpm) in each sample was determined by taking duplicate 1 ul aliquots and dotting them on 13 mm glass fiber filters (Reeve Angel). The aliquots were then allowed to air dry and then washed with 5 mls of cold 25% trichloracetic acid (TCA) containing 1 mM methionine followed by three 5 ml portions of 8% TCA. The filters were allowed to dry again, placed in scintillation vials containing 10 mls Aquasol (New England Nuclear). Samples were counted on Beckman scintillation counter. The protein concentration of each sample was determined using the Bio-Rad protein assay procedure as described by Bradford (1976).

The specific activity of each sample was determined in relative terms by dividing the total activity (cpm) of each sample by the total amount of protein (ug) in each sample.

(c) Preparation, Loading and Running of IEF Gels (1st Dimension)

Isoelectric focusing gels were set up and run as described by O'Farrell (1975) except for the modifications described below. The gels were made in cylindrical glass

tubing (125 x 2.0 mm inside diameter) (Bio-Rad) that had been acid cleaned and siliconized with 'Siliclad' (Clay Adams). Gels were run in a commercial tube gel electrophoresis chamber (Bio-Rad, Model 150A). The acrylamide, ampholines, TEMED and ammonium persulfate were all obtained from Bio-Rad, the ultrapure urea from Swartz-Mann and the bis-acrylamide from Aldrich chemical. Either 100,000, 150,000, or 200,000 cpm of each sample were loaded onto the gels. After running the gels they were placed in scintillation vials containing O'Farrell sample buffer and stored at -70°C.

(d) Preparation Loading and Running of Discontinuous SDS Gels (2nd Dimension)

The discontinuous SDS gel system for separation of peptides by their molecular weight was first described by Laemmli (1970). Some minor modifications to the method described by O'Farrell (1975) of the second dimension gels were employed in this study. A commercially available gel apparatus (Hoeffer Model SE-600) specifically designed to run two-dimensional gels was used to run the second dimension. A linear gradient gel of 7.5 to 15% polyacrylamide having the approximate dimensions of 120(h) x 150(w) x 0.75(d) mm were used as it was found that those gels had improved resolution over straight concentration gels. A 4.5% acrylamide stacking gel (40 mm high) was also

used as described by O'Farrell (1975). Gels were run under constant current (20 mA/gel) until the tracking dye front ran off the gel (approximately 4 hours). During electrophoresis gels were cooled by running tap water through the cooling coils of the apparatus and the lower buffer constantly stirred using a magnetic stir bar and stir plate.

(e) Detection of Proteins

i. Silver Staining

After the SDS gels were run they were fixed overnight in 50% methanol and 12% v/v acetic acid. The gels were then photochemically silver stained according to the method of Merril and coworkers (1981 and 1982). The photochemical silver staining method offers the advantages of over 50 times more sensitivity than Coomassie brillant blue staining and less than an hour of time to stain the gel after fixation.

ii. Autoradiography

Gels containing ^{35}S-methionine labelled peptides were first fixed overnight in 50% methanol, 12% acetic acid. The gels were then soaked for one hour in 150 ml of 'Enhance' (New England Nuclear) followed by three rinses with deionized water. The gels were allowed to sit in the third rinse for 30 to 40 minutes and then soaked in a solution of 2% glycerol for 15 minutes. The gels were

dried under heat and vacuum onto filter paper using a Hoeffer Model SE540 gel dryer.

Kodak X-omat AR film (13x18 cm) (Picker Canada) was preflashed from a distance of approximately 50 cm using a Braun model 200 B electronic flash unit covered with approximately 10 layers of facial tissue and one piece of white 4 ply cardboard. The film was then taped onto a dried gel (flashed side towards gel). The gels were usually exposed 7 or 10 days at -70° C.

Some of the autoradiograms were scanned on a LKB Model 2002 Ultrascan Laser Densitometer. Each protein that was analyzed was scanned with several passes 480 um apart. The density of each scan was recorded (in terms of area) using a Hewlitt-Packard Model 3390 intergrator-plotter. Total density was calculated in relative terms by adding the area from all the scans of a particular protein.

(f) Determination of Molecular Weights and Isoelectric Points of Proteins.

The assignment of molecular weights and isoelectric points (pI's) to the peptides was accomplished primarily through the use of protein standards using a technique described by Peters and Comings (1980). In this technique a set of protein standards were run at the same time (but in different gels) as the samples being examined. After the standards were run in both dimensions they were silver stained and identified. Molecular weight values were

assigned using the log molecular weight versus Rf relationship described by Shapiro et al. (1967).

Protein standards were obtained in lyophilized form from LKB (Sweden) and contained phosphorylase b, albumin, ovalbumin, soybean trypsin inhibitor, and alpha-lactalbumin. To these standards beta-galactosidase (Sigma) was added. See Figure 8 for the molecular weights and pI's of these standard proteins.

The pI's of the protein were also estimated by comparing its position with the pH at the same position of an unloaded IEF tube gel which had been run at the same time as the sample. The pH along the gel was determined using a Bio-Rad pro-pHiler tube gel microelectrode.

2. Nonequilibrium (NEPHGE) Gels

Nonequilibrium pH gradient electrophoresis (NEPHGE) was first described by O'Farrell and coworkers (1977) and was designed to better resolve basic proteins. However, this method is not able to resolve all basic proteins so a modification of the technique by Sanders and coworkers (1980) was used so that the histones could be detected. The techniques used for running NEPHGE gels are virtually identical to those used for running IEF gels except for some modifications in sample preparation and running of the first dimension. These modifications are described below.

(a) Sample Preparation

The sample preparation of Garrels (1979) was used except that instead of the samples being dissolved in normal sample buffer after lyophilization they were dissolved in NEPHGE sample buffer (9.95 M urea, 4% NP-40, 2% pH 3-10 ampholines (Bio-Rad), .1% protamine sulfate, 5% B-mercaptoethanol, and 0.3 M NaCl).

The samples used for the NEPHGE gels were identical to the ones used in the IEF gels as the original sample was split into two portions after sample preparation but before lyophilization.

(b) Preparation, Loading and Running of Non-equilibrium Gels (1st Dimension)

The methods used for NEPHGE gels were identical to those used for the first dimension of IEF gels except for the formula for the gels in which 5% pH 3-10 ampholines were used instead of 4% pH 5-7 and 1% pH 3-10 ampholines and the amount of 10% ammonium persulfate and TEMED increased (20 and 14 ul/ 10 ml respectively) (Sanders et al. 1980).

In NEPHGE first dimension gels the upper and lower resevoirs are reversed from IEF gels; the upper buffer being .01 M phosphoric acid and the lower degassed .02 sodium hydroxide. Gels were electrophoresed for 2 hours at a constant voltage of 500 volts with the cathode on the

bottom of the apparatus (reverse of IEF gels).

The remaining procedures for running and detecting the NEPHGE gels were identical to those described for IEF gels.

RESULTS

Requirement of Protein Synthesis for the Initiation of Mitosis

The need for protein synthesis prior to mitosis in CHO cells was determined with the use of the protein synthesis inhibitor emetine. Before emetine's effects on mitosis were examined the drugs capacity to inhibit protein synthesis and the reversibility of this inhibition was thoroughly examined.

The effect of emetine concentration on inhibition of ^{3}H-leucine incorporation into CHO cells and the reversibility of this inhibition is shown in Figure 1. Increasing the concentration of emetine resulted in a decreased amount of ^{3}H-leucine incorporated into cells. Approximately 90% inhibition of ^{3}H-leucine incorporation occurs in cells treated with $5x10^{-7}$M emetine while $2x10^{-7}$M emetine resulted in 75% inhibition (Figure 1a). The ability of the cells to resume protein synthesis once the emetine was removed was also affected by the initial concentration of emetine used. Inhibition was virtually 100% reversible at $2x10^{-7}$M, 95% reversible at $5x10^{-7}$M, but less than 60% reversible at $1x10^{-6}$M emetine (Figure 1b).

These results indicate that the concentration range at which emetine provides adequate protein synthetic inhibition (90%) and retains reversibility properties (i.e.90% reversible) is quite narrow and lies between $5x10^{-7}M$ and $1x10^{-6}M$.

The rate at which emetine inhibits protein synthesis and the rate of recovery from emetine inhibition is illustrated in Figure 2. At a concentration of $5x10^{-7}M$ emetine, 75% inhibition of ^{3}H-leucine incorporation occurs by 7 minutes and 90% inhibition occurs at 15 minutes after the drug is added. When the emetine was removed, incorporation of ^{3}H-leucine reaches approximately 75% of control levels by 15 minutes and is greater then 90% by 25 minutes. Taking into account the degree of inhibition and recovery from inhibition that occurs at $5x10^{-7}M$ emetine it appears that maximum inhibition is achieved within 15 minutes after drug addition and maximum recovery from inhibition is attained 15 to 25 minutes after drug removal.

Altering the pH of the media affected both the inhibitory and reversibility properties of emetine. As the pH of the media was lowered the degree of protein synthesis inhibition in the presence of $5x10^{-7}M$ emetine decreases (Figure 3). At pH 7.5, $5x10^{-7}M$ emetine induces over 90% inhibition of protein synthesis while at pH 6.0, there was only 40% inhibition at the same concentration of emetine.

In respect to the reversibility of emetine inhibition, decreasing the pH increases the amount of reversibility. When emetine ($5x10^{-7}M$) was removed from cells at pH 7.5, there was virtually no recovery from the inhibitory effect while at pH 6.0, approximately 100% recovery occurs. Another interesting finding was the decrease in ^{3}H-leucine incorporation that occurs in control populations when the pH of the media was lowered below pH 7.0. ^{3}H-leucine incorporation a pH 6.0 was approximately 65% that of incorporation at pH 7.0 (results not shown). Based on these observations the experiments represented in Figures 1,2,4, and 5 and table 1 were carried out at pH 7.4 when cells were being inhibited by emetine and approximately pH 6.5 when reversal of inhibition was desired.

The effect of emetine concentration and length of emetine exposure on ^{3}H-uridine incorporation is demonstrated in Table 1. At lower concentrations ($5x10^{-7}$ and $2x10^{-6}M$) ^{3}H-uridine incorporation was 85% of control levels at 10 minutes after addition and 70% of controls at 150 minutes after the addition of emetine. Increasing the emetine concentration to $10^{-5}M$ only slightly decreased ^{3}H-uridine incorporation.

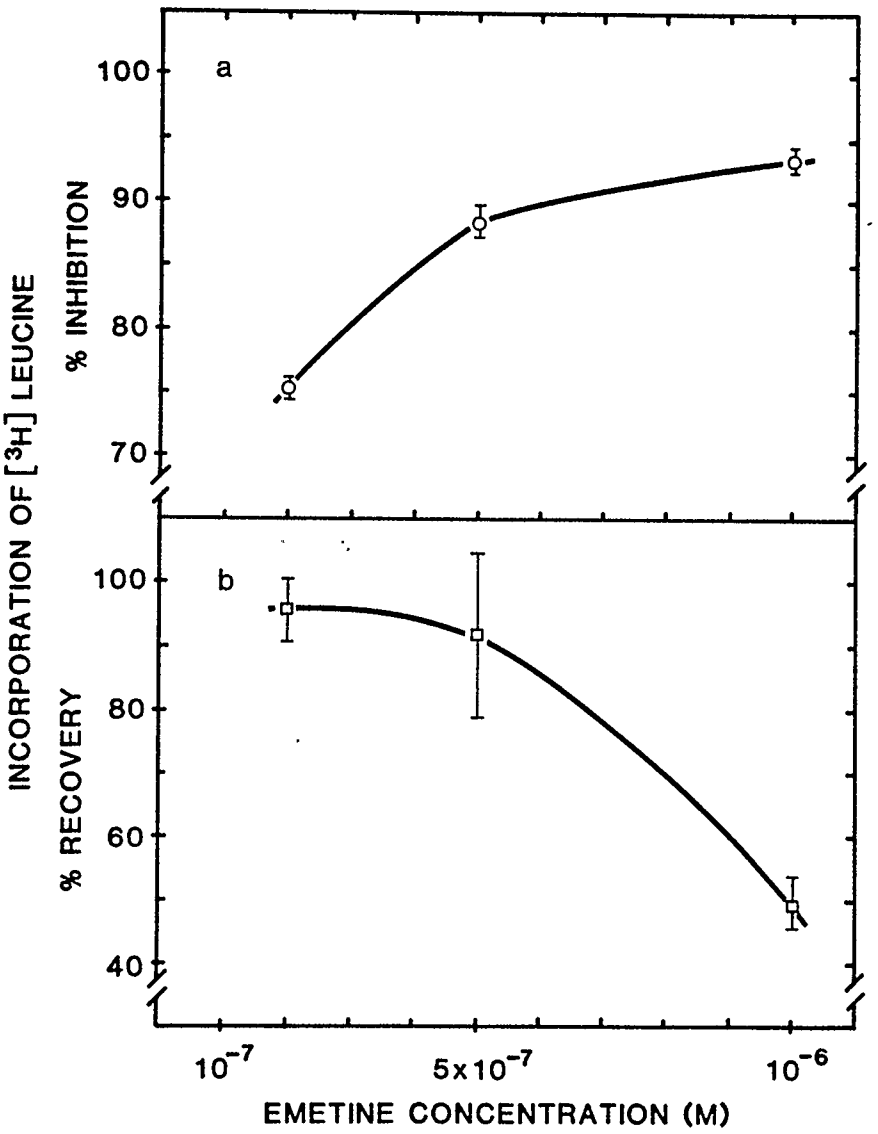

Figure 1. Effect of emetine concentration on protein synthesis. For % inhibition (a) emetine was added to a number of identical cultures and after 20 minutes ^{3}H-leucine was added for a 15 minute pulse label. For % recovery (b) cultures were exposed to emetine for 30 minutes and then placed in emetine-free media. After 30 minutes in this cells were pulse labelled with ^{3}H-leucine for 15 minutes. For this experiment and the experiments in Figures 2, 4, and 5 and Tables 1, 2, and 3 protein synthesis experiments were carried out in pH 7.4 media and recovery experiments in pH 6.5 media. % Inhibition = 100 - (CPM of sample/CPM of controls) x 100; % Recovery = (CPM of sample/CPM of controls) x 100. Control cultures had incorporated approximately 20,000 CPM. Cultures in the presence of emetine, O—O; cultures 30 minutes after emetine removal, □—□.

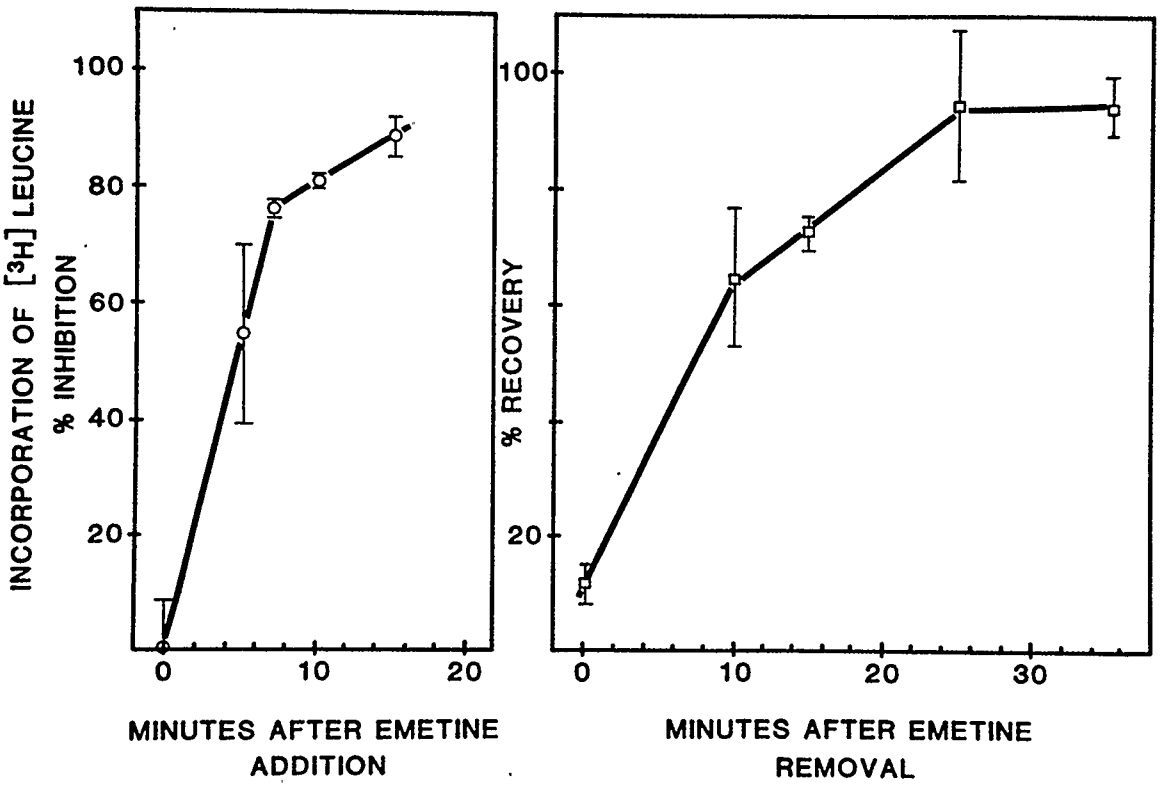

Figure 2. Rates of inhibition by emetine and recovery from inhibition. To determine the rate of inhibition (a), $5x10^{-7}M$ emetine was first added to identical cultures at zero minutes. At 0, 2, 5, and 10 minutes ^{3}H-leucine was added to cultures for a 10 minute pulse label. The result obtained for a particular 10 minute pulse period was plotted 5 minutes (midway) after the ^{3}H-leucine was added. For % recovery (b) cultures were exposed to $5x10^{-7}M$ emetine for 30 minutes after which the emetine was removed and replaced with normal media. ^{3}H-Leucine was added to the cultures at the times after emetine removal indicated in the figure. Percent inhibition and recovery was calculated as described in the legend to Figure 1. Cultures showed 11% recovery in the presence of emetine (0 minutes) because only 89% inhibiton was obtained by $5x10^{-7}M$ emetine. Control cultures (0% inhibition, 100% recovery) had incorporated approximately 10,000 CPM. Cultures in the presence of emetine, O——O ; cultures after emetine had been removed, □——□.

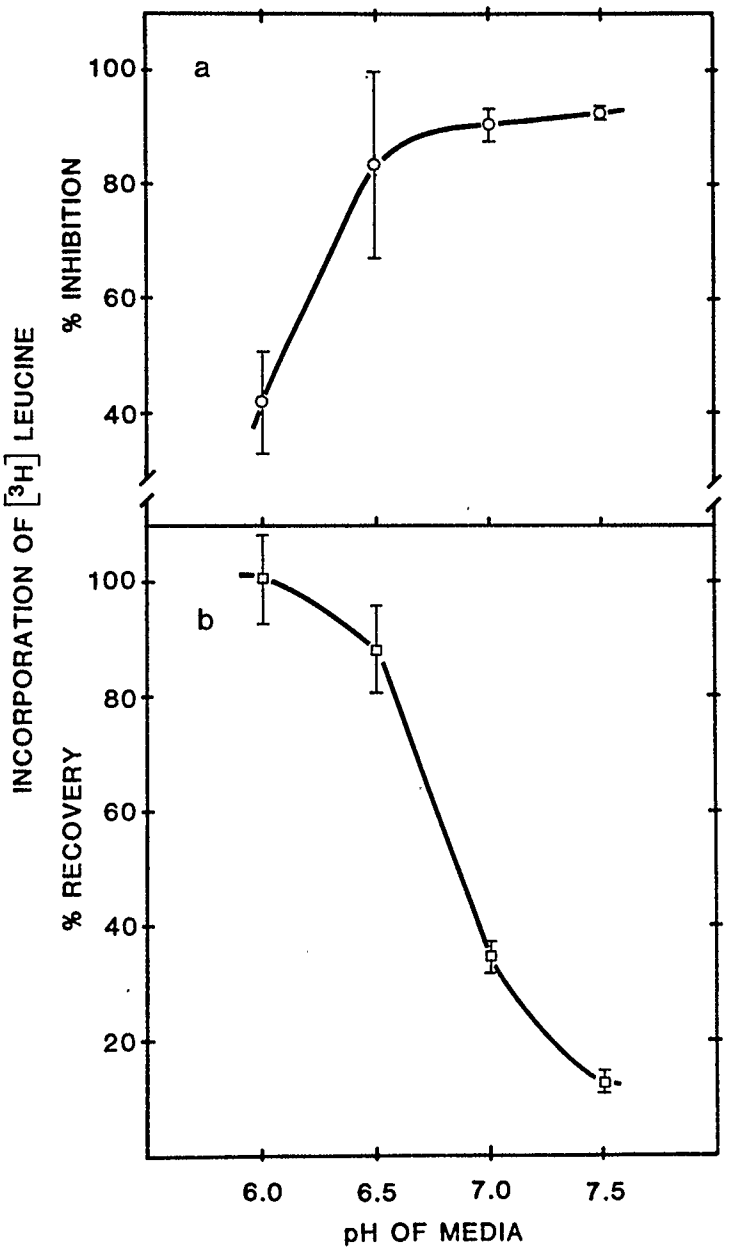

Figure 3. Effect of pH on emetine inhibition and recovery. Hepes buffered media was adjusted to a variety of pH's and added to cultures. ^{3}H-Leucine incorporation at these pH's was determined both in the presence of $5x10^{-7}$M emetine (a) and 30 minutes after its removal (b). Percent inhibition and recovery was calculated as described in the legend of Figure 1. Cultures in the presence of emetine, o——o; cultures 30 minutes after emetine removal, □——□.

Table 1. Effect of emetine on [^{3}H] uridine incorporation

Minutes After Emetine Addition	% Incorporation of [^{3}H] Uridine* Emetine Concentration		
	$5x10^{-7}$ M	$2x10^{-6}$ M	$1x10^{-5}$ M
10'	84.1 ± 9.3	82.6 ± 8.2	79.6 ± 4.2
30'	79.8 ± 13.0	N/A**	68.6 ± 3.8
90'	77.9 ± 2.3	80.8 ± 3.2	N/A**
120'	69.0 ± 3.2	69.2 ± 4.4	61.2 ± 5.2

* Emetine and 2 μCi/ml [^{3}H] uridine were added to the cultures at zero minutes. The cultures were continuously labelled with samples taken at the indicated times and processed as described in Materials and Methods. 100% incorporation is the amount of [^{3}H] uridine incorporated by control (non-emetine treated) cultures.

N/A** No samples taken at these time points

When emetine ($5x10^{-7}$ and $2x10^{-6}$M) was added to CHO cells in log phase growth, a slight depression in mitotic index occured about 30 minutes after the addition of the drug (Figure 4). An examination of the cells in each of the various stages of mitosis revealed that the number of metaphases in the population was reduced by one-half (when compared to control populations) between 20 and 30 minutes after the addition of emetine (see Table 2). By 40 minutes, the overall mitotic index was reduced to approximately one-fourth the control level and significant reductions are apparent in all the stages of mitosis. Beyond 40 minutes, the mitotic index remains depressed and by 105 minutes after the addition of emetine, the mitotic index was approximately 20% that of control values.

When emetine ($5x10^{-7}$M) was added to cultures for either 60 or 120 minutes the mitotic index was significantly lower than control values (Figure 5). When the emetine was removed from these cultures, the cells rinsed with saline and then replaced in emetine free media, the mitotic index remained low until approximately 60 minutes after emetine removal. In the cultures which had received a 60 minute emetine treatment, the mitotic index increased approximately three-fold between 45 and 60 minutes after emetine removal. Cultures which received a 120 minute emetine

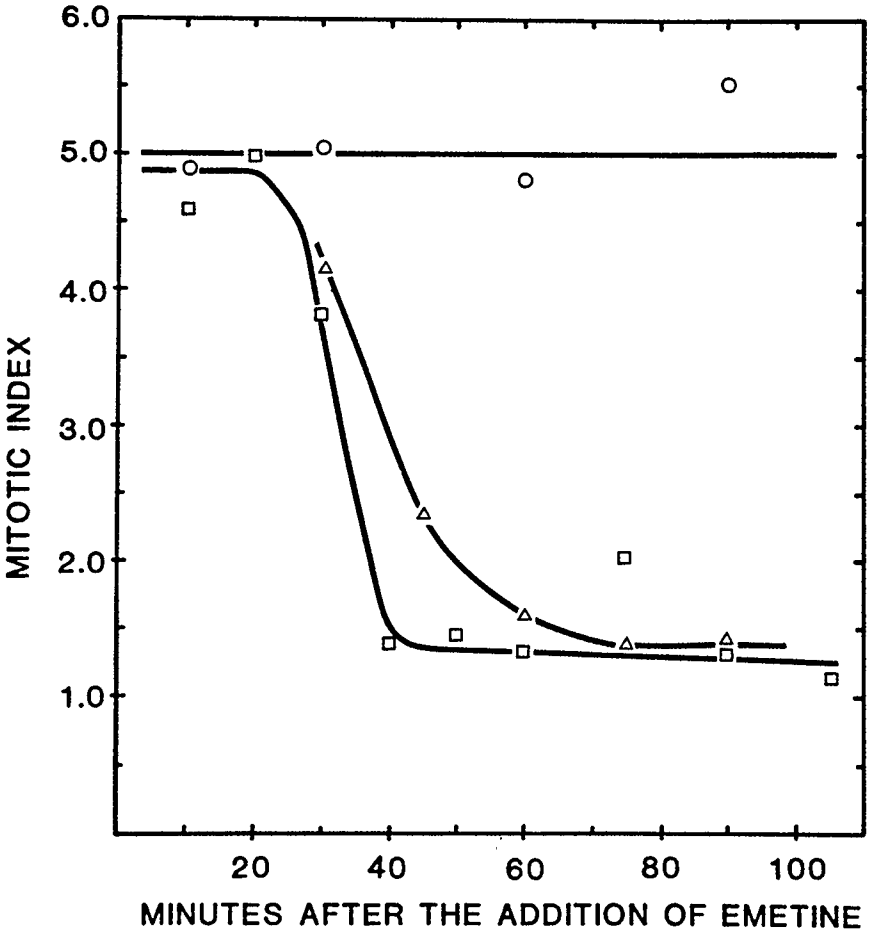

Figure 4. The relationship between protein synthesis inhibition and mitotic index. Emetine ($1x10^{-6}$M and $5x10^{-7}$M) was added to asynchronous cultures and the mitotic index scored at various times after emetine addition (see Materials and Methods). The number of cells in each of the various stages of mitosis for all of the samples plotted is given in Table 2. Control cultures,O; cultures in the presence of $1x10^{-6}$M emetine,□——□; cultures in the presence of $5x10^{-7}$M emetine,△——△.

Table 2. Effect of protein synthesis inhibition on mitotic index.*

	Time of Sampling	Mitotic** Index	Number of Cells			
			Prophase	Metaphase	Anaphase and Telophase	Interphase
control	10'	4.90	2	36	16	1010
	30'	5.05	2	30	22	1016
	60'	4.82	6	35	11	1027
	90'	5.54	6	35	20	1041
$5x10^{-7}$ M Emetine	30'	4.15	2	14	28	1022
	45'	2.32	5	13	6	1009
	60'	1.61	1	7	9	1042
	75'	1.40	1	8	6	1056
	90'	1.45	1	9	6	1087
$2x10^{-6}$M Emetine	10'	4.60	1	32	18	1059
	20'	4.97	1	37	15	1013
	30'	3.80	3	16	22	1037
	40'	1.39	0	6	9	1064
	50'	1.45	3	10	2	1022
	60'	1.35	1	8	5	1023
	75'	2.04	3	13	6	1058
	90'	1.32	1	7	6	1049
	105'	1.14	0	8	4	1042

* Two different emetine concentrations ($5x10^{-7}$ and $2x10^{-6}$ M) were added to asynchronous cultures at zero minutes and samples were taken at the indicated times. Cultures were fixed and scored for mitotic index as described in Materials and Methods.

** Mitotic Index = $\frac{\text{No. of Prophase + Metaphase + Anaphase and Telophase cells}}{\text{Total No. of Cells}} \times 100$

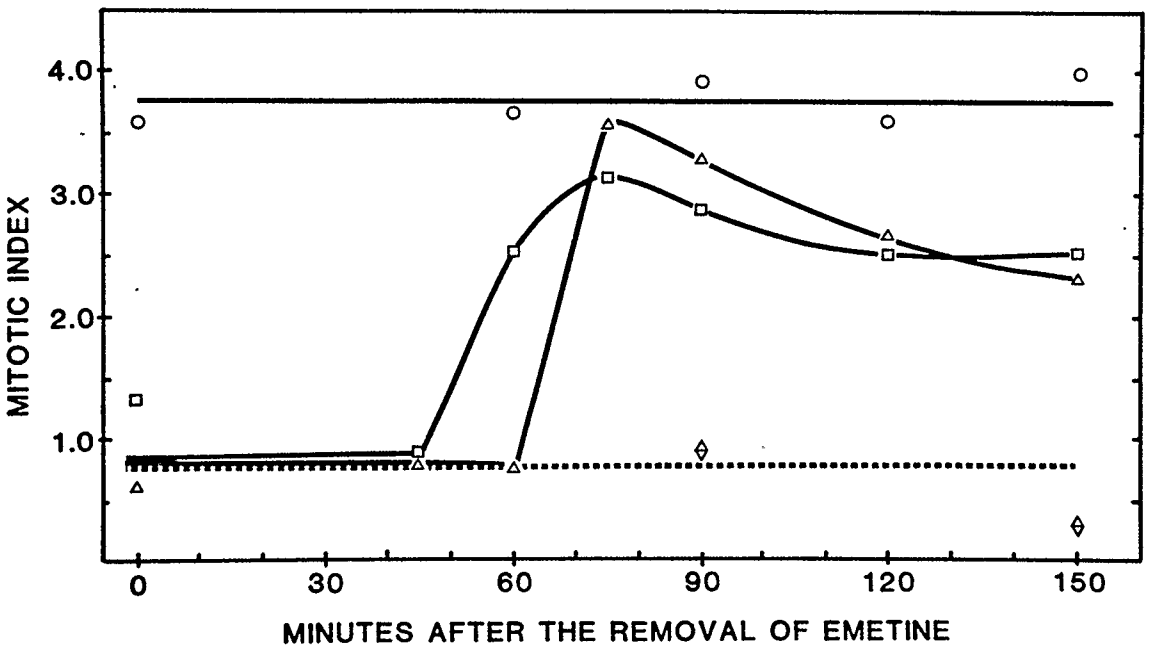

Figure 5. Changes in mitotic index after the removal of emetine. Emetine ($5x10^{-7}$M) was added to cells for either a 60 or 120 minute treatment. At zero minutes the emetine media was removed and replaced with normal media and the mitotic index scored at various times after removal. The number of cells in each of the stages of mitosis is shown in Table 3. Control cultures, ○——○ ; cultures which received a 60 minute emetine treatment, □——□ ; cultures which received an 120 minute emetine treatment, △——△ ;cultures in the continual presence of emetine, ◊---◊ .

Table 3. Changes in Mitotic Index after the removal of emetine.*

	Time of Sampling	Mitotic Index	Number of Cells			
			Prophase	Metaphase	Anaphase and Telophase	Interphase
control	-1' **	3.58	6	22	10	1024
	60'	3.65	7	22	12	1083
	90'	3.92	6	22	12	980
	120'	3.59	9	20	11	1073
	150'	4.00	7	25	12	1061
Emetine treated (60 minutes)	-1' **	1.32	1	5	8	1045
	45'	.88	1	4	4	1010
	60'	2.54	19	5	4	1071
	75'	3.13	9	17	7	1021
	90'	2.88	6	16	8	1010
	120'	2.50	4	13	9	1016
	150'	2.51	3	15	9	1047
Emetine treated (120 minutes)	-1' **	.59	0	3	3	1009
	45'	.77	3	3	2	1009
	60'	.76	5	2	1	1034
	75'	3.57	17	17	4	1025
	90'	3.29	5	22	10	1087
	120'	2.66	2	19	6	987
	150'	2.31	2	18	4	1052
Emetine continuous	90'	.79	0	6	1	1008
	150'	.30	0	1	2	1012

* Asynchronous cultures were treated with 5×10^{-7} M emetine for either 60 or 120 minutes. At zero minutes the emetine media was removed and replaced with ordinary media. In the emetine continuous samples emetine was added at -120 minutes and was not removed at zero minutes. Samples were taken at the indicated times and the mitotic scored as described in the legend of Table 2.

** -1 minute samples taken just before the emetine was removed.

treatment showed a five-fold increase in mitotic index between 60 and 75 minutes after emetine removal (see Table 3). In both cases, the sharp increase in mitotic index was due primarily to large increases in the number of prophase and metaphase cells (see Table 3). For example, in the cultures which received a 60 minute emetine treatment, the number of prophase configurations increased from 1 to 19 between 45 and 60 minutes after emetine removal. For both sets of cultures, the mitotic index only increased to control levels upon emetine removal and maintained this level for approximately one hour after which the mitotic index decreased slightly. Cells which were in the continual presence of emetine showed no fluctuation in mitotic index and maintained a mitotic index approximately one-sixth that of controls. Cytological observations of emetine arrested cells did not reveal any abnormalities in the mitotic configurations which were present.

Cell Cycle Specific Protein Synthesis

In order to determine if there was any cell cycle speific protein synthesis it was first necessary to obtain highly synchronous populations of cells. A variety of synchrony techniques were tried but not all were effective.

Some of the techniques that were found to be ineffective included; isoleucine deprivation, for synchrony in G_1 (Tobey and Ley, 1971), and excess thymidine treatment, for G_1/S synchrony (Bootsma et al., 1966). Both

of these techniques provided some degree of synchrony to the cells but not enough for the requirements of this study. For both of these techniques the duration of treatment is quite long; greater than 30 hours for the isoleucine deprivation, 16 hours or more for treatment with excess thymidine. The recovery of the cells from these synchrony techniques was never high (i.e. less than 80% of the cells) nor was it extremely synchronous with some cells taking longer to respond than others. It seems likely that the relative ineffectiveness of these techiques might be attributed to the long inhibitory periods used.

Selection of mitotic cells by shake-off obtained cells which were highly synchronous. When combined with a prior treatment of colcemid (Stubblefield and Klevecz, 1965) the shake-off technique usually provided greater than 95% mitotic cells. Recovery from the colcemid treatment was also good with less than 15% mitotic cells one hour after the removal of colcemid and less than 2% mitotic cells two hours after. This technique provided cells that were synchronous during mitosis, early and late G_1 and late G_2 (see Materials and Methods).

Cells synchronous for the early and late S and mid G_2 phases of the cell cycle were obtained by a combination of mitotic selection followed by treatment with hydroxyurea. Treatment of the cells with $2x10^{-4}M$ hydroxyurea seemed to

adequately prevent cells from entering S phase as measured by the incorporation of ^{3}H-thymidine (Figure 6). Incorporation of ^{3}H-thymidine in the presence of hydroxyurea was less than 3% the incorporation which occurred during the peak of S phase (270 minute time point) and 10% the incorporation of cells not treated with hydroxyurea (results not shown). Thirty minutes after the release from hydroxyurea there was already a significant increase in ^{3}H-thymidine incorporation. Half of the maximum ^{3}H-thymidine incorporation rate was reached approximately 120 minutes after the release from hydroxyurea and then decreased to half the maximum rate at 420 minutes. A peak in mitotic index of just under 20% occured at 520 minutes after release from hydroxyurea. These results indicate that S phase was approximately 300 minutes and G_2 100 minutes.

The effectiveness of the synchrony techniques was also examined by determining the DNA content of individual cells in a population by quantitating the fluorescence emitted by each cell after they had been stained with propidium iodide. This was accomplished with the use of an Ortho Cytofluorograf. As mentioned in the Materials and Methods, cells in G_1 phase of the cell cycle should have a 2n complement of DNA while cells in G_2 or mitosis should have a 4n complement. Figure 7 depicts the

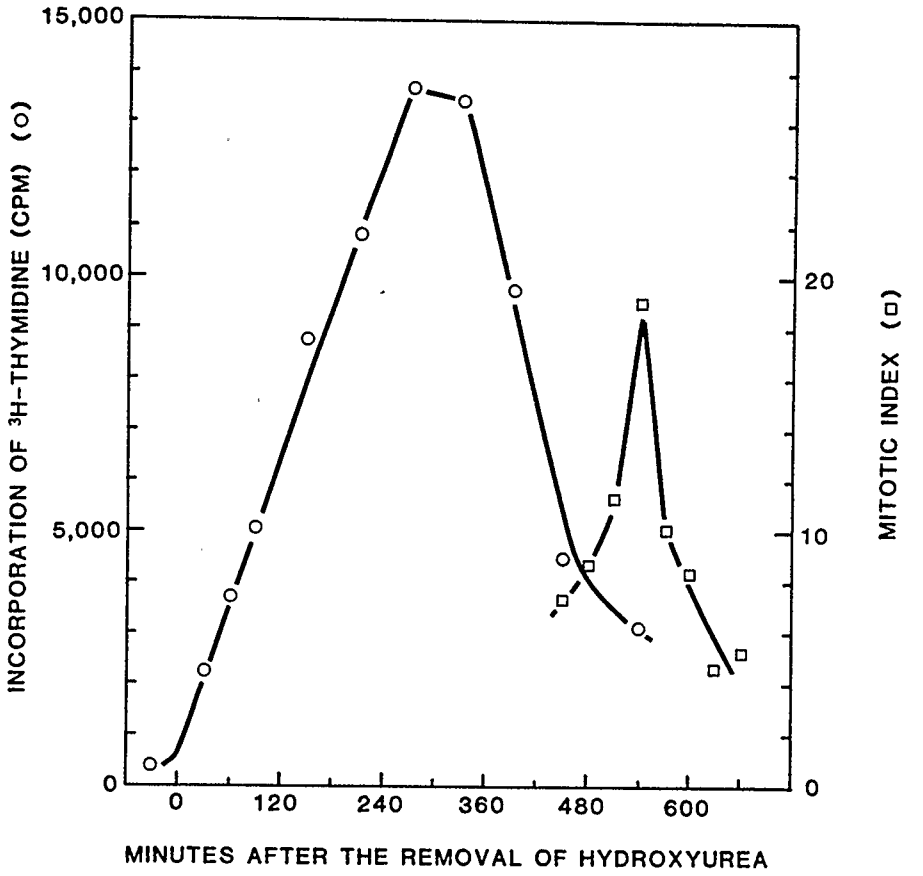

Figure 6. Monitoring of cell synchrony. Cells were synchronized by a combination of shake-off of mitotic cells and G_1/S arrest with hydroxyurea. Thirty minutes before the removal of hydroxyurea and various times after the removal of hydroxyurea identical cultures were pulse- labelled with ^{3}H-thymidine for 15 minutes. The mitotic index of the synchronized cells was also determined at various times after the removal of hydroxyurea.

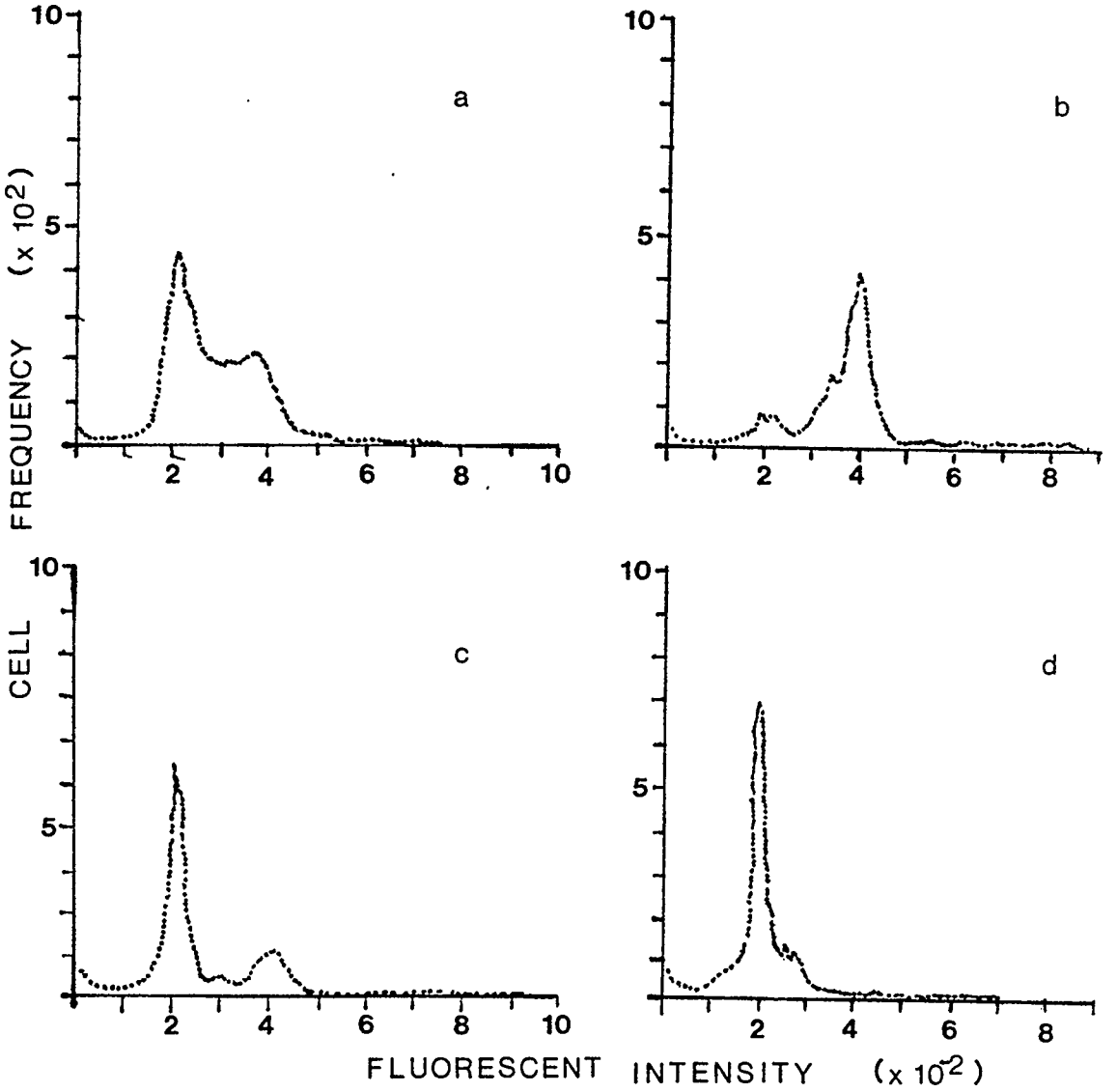

Figure 7. Cytofluorograf analysis of cell synchrony. Cells were synchronized by shake-off of mitotic cells or a combination of shake-off and G_1/S arrest with hydroxyurea. Samples from various stages of the cell cycle were fixed and stained with propidium iodide as described in Materials and Methods. Individual cell fluorescence was measured at 488 nm and plotted as cell frequency versus relative fluorescent intensity. Cells containing a diploid (2n) complement of DNA fluoresce at an intensity of approximately 200 while cells with a tetraploid (4n) complement fluoresce at just below 400. a, Asynchronous CHO cells; b, Mitotic cells; c, Early G_1 cells (1 hour after shake-off); d, Late G_1 cells (3 hours after shake-off).

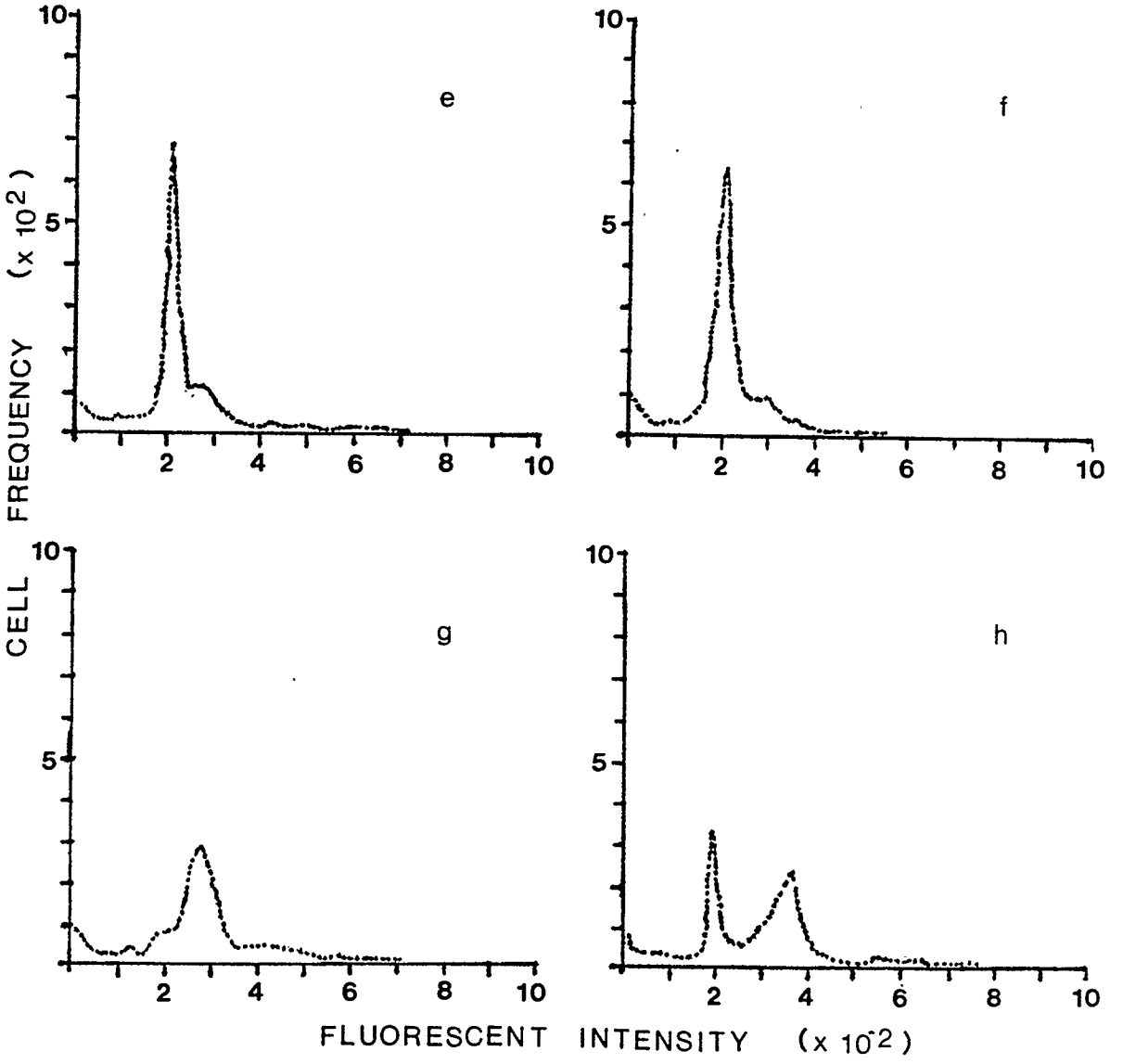

Figure 7 (continued). Cytofluorograf analysis of cell synchrony. e, G_1/S arrested cells (hydroxyurea present); f, Early S cells (1.5 hours after hydroxyurea removal); g, Late S cells (5 hours after hydroxyurea removal); h, Mid G_2 cells (8 hours after hydroxyurea removal).

type of data obtained from the Ortho Cytofluorograf. The abscissa represents the relative intensity (quantity) of fluorescence emitted at 488 nm and the ordinate the frequency (number of cells) at that intensity. Ten thousand cells were analyzed in each sample to attain the configurations shown in Figure 7.

Figure 7(a) represents an asynchronous population of CHO cells. A 2n complement of DNA appears to occur at an intensity of approximately 200 while 4n occurs at just under 400. One can see from this Figure that the highest frequency occurs at 200 or G_1 phase. There is also a very minor peak at the G_2/M position (400). The fluorescence between these two areas represent cells in the S phase of the cell cycle. Mitotic cells obtained from a shake-off experiment are shown in Figure 7(b). As one would expect the great majority of cells fall under the G_2/M peak. The cells which appear in the peak at 200 represent cells which have just divided and were not firmly attached when the shake-off was done. Cells obtained one and three hours after release from colcemid are shown in Figure 7 (c) and (d) and represent early and late G_1 cells respectively. In (c), a portion of the population is still in the G_2/M peak signifying that some of the cells have not yet completed mitosis whereas in (d) this peak is virtually gone and only the G_1 peak exists. Figure 7(e) shows cells arrested at the G_1/S border with hydroxyurea. Cells one and one-half

and five hours after the release from hydroxyurea represent early and late S cells respectively and are shown in Figure 7 (f) and (g). Figure (f) is virtually identical to (e) with only a small shoulder of cells moved into S while in (g) the population is clearly all in S. Cells primarily in mid G_2 are shown in Figure 7(h) and are cells 8 hours after the release from hydroxyurea. Although the majority of cells are in the G_2/M peak some cells have appeared to have already divided and are in the G_1 peak.

Once highly synchronous cells were readily available, cells were labelled with ^{35}S-methionine for brief periods at a variety of times during the cell cycle. Whole cell preparations were made and examined using two dimensional polyacrylamide gel electrophoresis.

Before samples were loaded onto gels, their specific activities (cpm/ug protein) were determined. Interestingly, the specific activities varied even though the early G_1 through mid G_2 samples were labelled for the same amount of time (30 minutes) with 100 uCi/ml ^{35}S-methionine. Early and late G_1 cell samples had specific activities of 263 and 523 cpm/ug respectively. Early S cells had the highest specific activity - 635 cpm/ug but both late S (511 cpm/ug) and mid G_2 (541 cpm/ug) also had high specific activities. Late G_2 and mitotic cells which were both labelled with 25 uCi/ml ^{35}S-methionine had dramatically different specific

activities of 452 and 122 cpm/ug respectively.

As mentioned earlier, protein samples were separated by both equilibrium (IEF) and nonequilibrium (NEPHGE) gel electrophoresis. In order to accurately and clearly follow the distribution of the proteins, protein standards were run under identical conditions as the equilibrium two-dimensional gels. The peptide pattern of the standards is shown in Figure 8. The standards are; (a) carbonic anhydrase, (b) phosphorylase b, (c) bovine serum albumin, (d) beta-galactosidase, (e) ovalbumin, (f) soybean trypsin inhibitor, and (g) alpha-lactalbumin. For the gels that were examined in detail (Figure 11, 13 and 16) reference grids were made on the photographs to mark the molecular weight and pH reference lines originally indicated on Figure 8.

Figure 9 illustrates a two-dimensional separation of proteins obained from a whole cell preparation of asynchronous CHO cells. This gel was silver stained as described in Materials and Methods. Over 300 peptides were easily discerned on the original gel.

Figures 10 through 17 are autoradiograms of two-dimensional separations using ^{35}S labelled samples from various times during the cell cycle. Over 600 spots were clearly identifiable on the original autoradiograms and over 500 can be seen on the photographs. Only three of these autoradiograms were examined in great detail - late

G_1 (Figure 11), early S (Figure 13), and late G_2 (Figure 16). The remaining autoradiograms -early G_1 (Figure 10), G_1/S arrest (Figure 12), late S (Figure 14), mid G_2 (Figure 15), and mitosis (Figure 17) were included also and used to support statements describing increasing or decreasing synthesis of certain peptides during the cell cycle.

Figure 11 is an autoradiogram of peptides synthesized during late G_1. Proteins r1, r2 and r3 are designated 'reference' proteins as these three peptides were scanned with the laser densitometer and their total density determined (see Materials and Methods). Protein A is actin and was identified by its molecular weight, isolectric point and comparison of its position with actin in other researchers two-dimensional gels (Bravo and Celis, 1980). Proteins aT and bT are alpha- and beta-tubulin were also identified using the criteria described above. Alpha-tubulin does not even appear to be present (although it does comigrate with another protein) in this autoradiogram and beta-tubulin is synthesized in much lower amounts when compared to late G_2 (Figure 16). The open arrows indicate proteins which are synthesized at a considerably lower rate during this phase of the cell cycle when compared to other phases of the cycle.

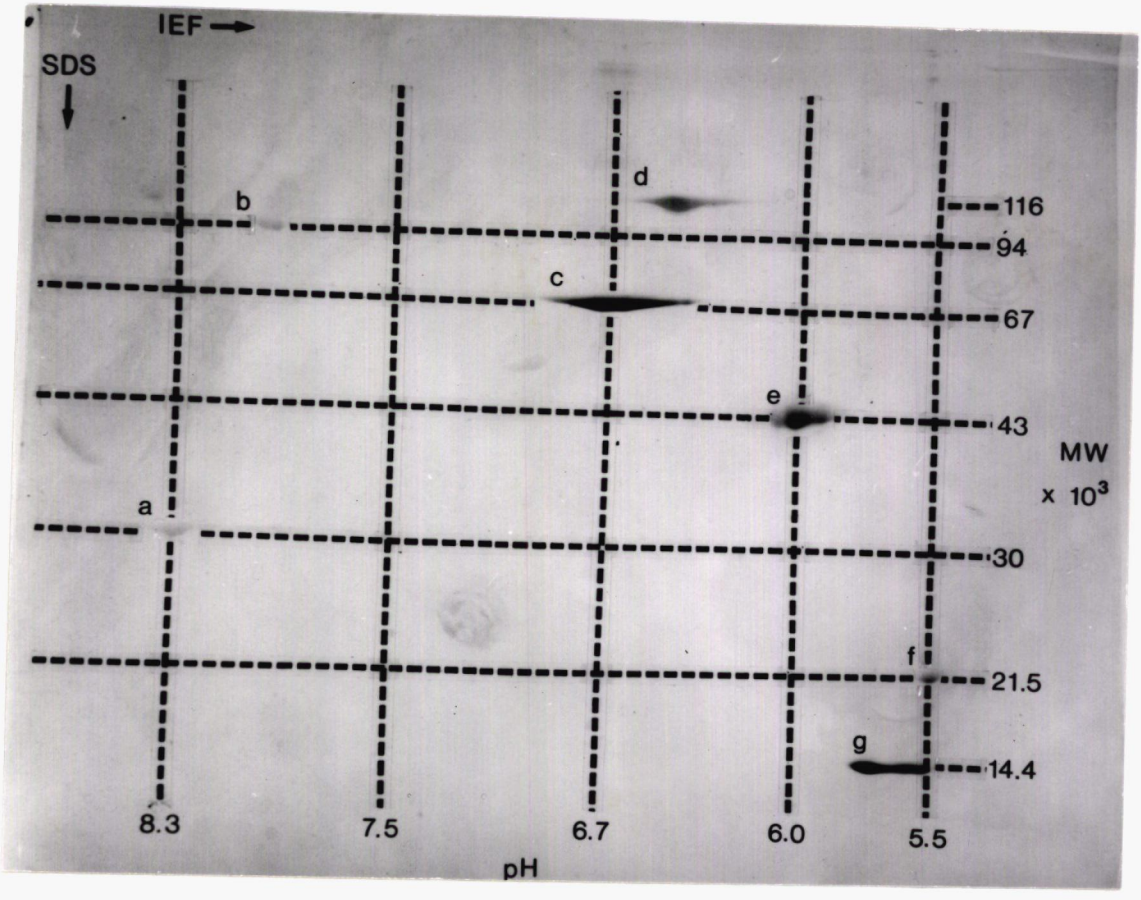

Figure 8. Two-dimensional separation of protein standards. Standards were separated by equilibrium two-dimensional gel electrophoresis, and silver stained as described in Materials and Methods. For this figure and Figures 9 through 17, the isoelectric focusing separation is from left to right while the SDS separation is from top to bottom. a, carbonic anhydrase; b, phosphorylase b; c, bovine serum albumin; d, beta-galactosidase, e, ovalbumin; f, soybean trypsin inhibitor; g, alpha-lactalbumin.

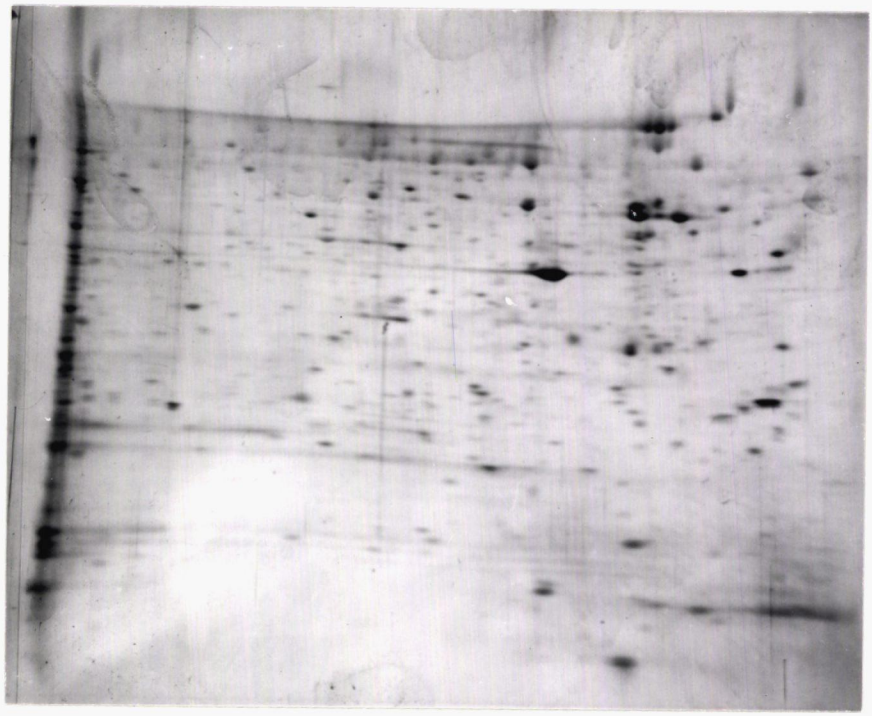

Figure 9. Two-dimensional separation of proteins from asynchronous CHO cells. Gel was run and silver stained as described in Materials and Methods.

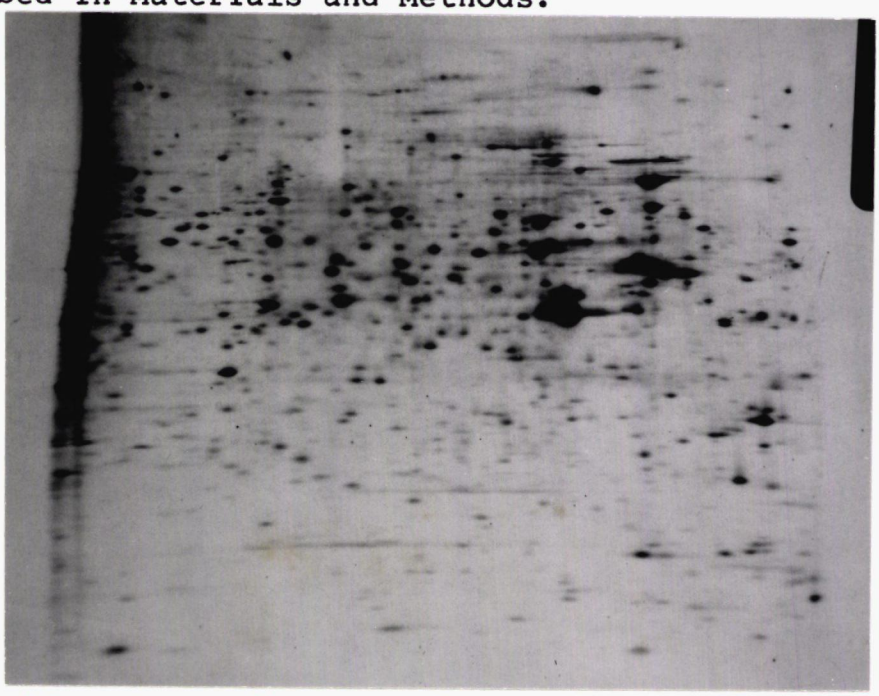

Figure 10. Autoradiogram of peptides synthesized during early G_1 phase. For this figure and Figures 11-17 cells were labelled with ^{35}S-methionine for 30 minutes and the proteins samples prepared, separated, and autoradiogrammed as described in Materials and Methods. All gels were loaded with 150,000 cpm and exposed for 7 days except where otherwise noted.

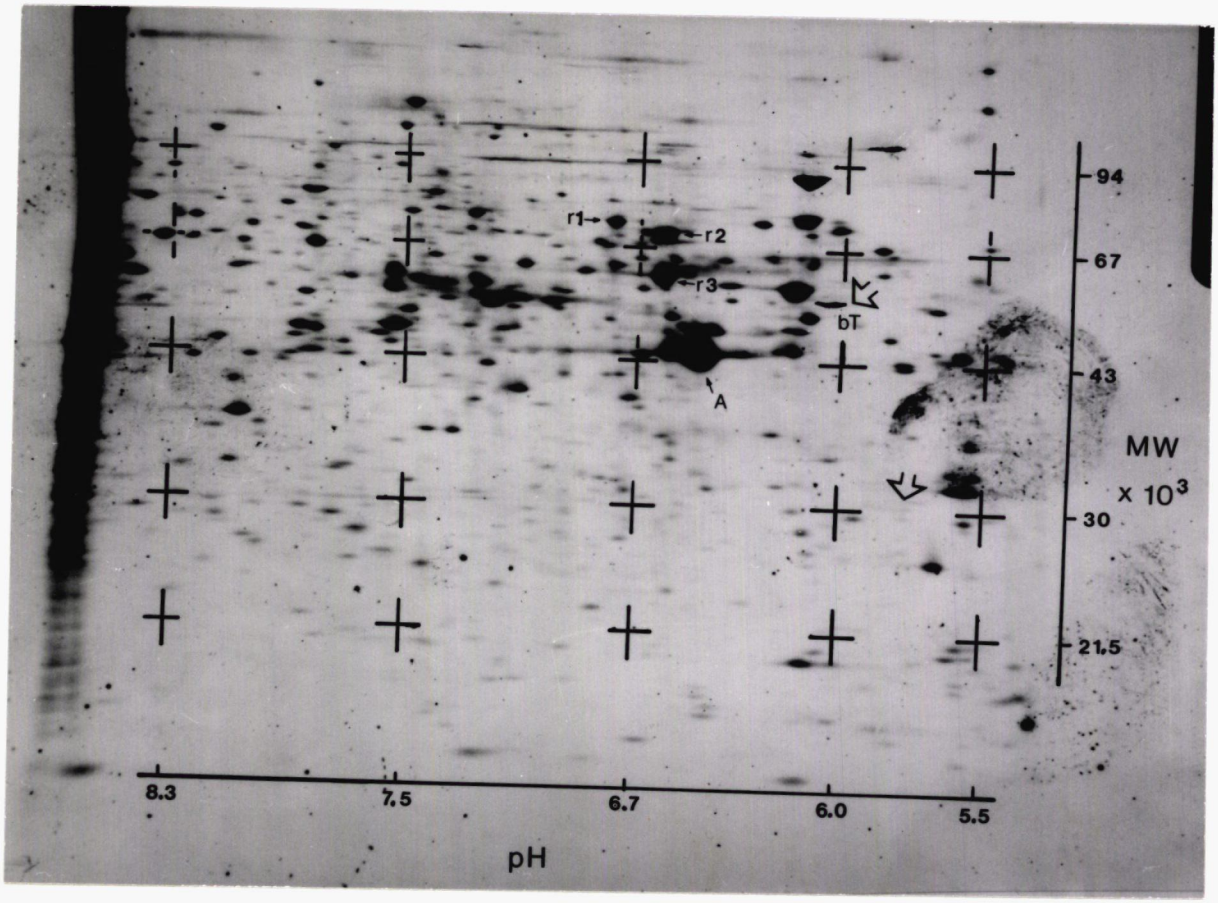

Figure 11. Autoradiogram of peptides synthesized during late G_1 phase. The grid marks in this figure and in Figure 13 and 16 correspond to the molecular weight and pI standards seen in Figure 8. Protein A is actin and aT and bT are alpha- and beta-tubulin respectively. Proteins r1, r2, and r3 are reference proteins (see Results). Open arrows designate proteins which have decreased synthesis (intensity) when compared to the mitotic autoradiogram (Figure 17).

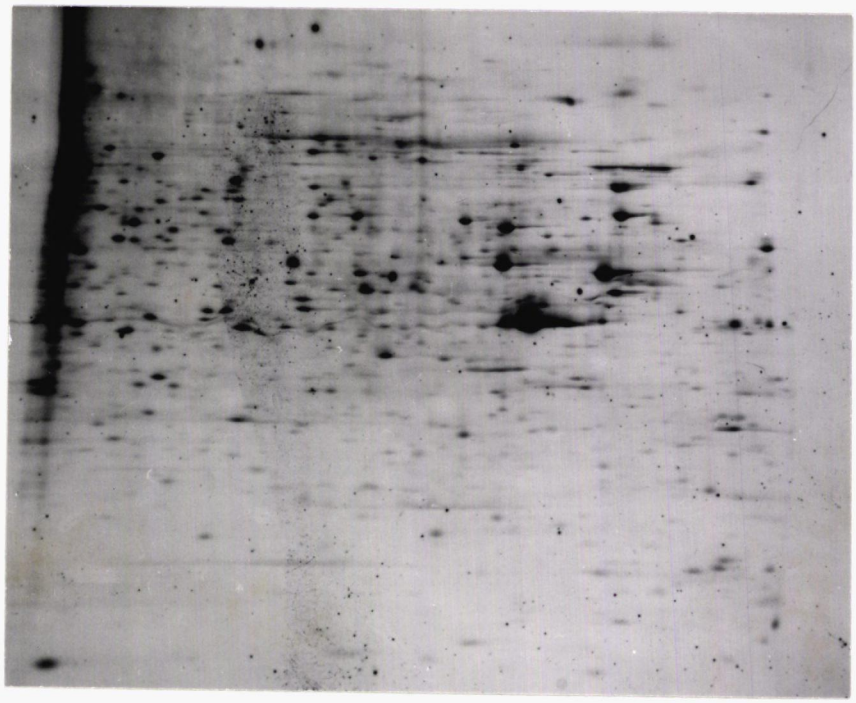

Figure 12. Autoradiogram of peptides synthesized during G_1/S arrest. The open arrow designates two proteins which have decreased synthesis when compared to late G_2 or mitotic cells. These proteins have been identified as alpha- and beta-tubulin.

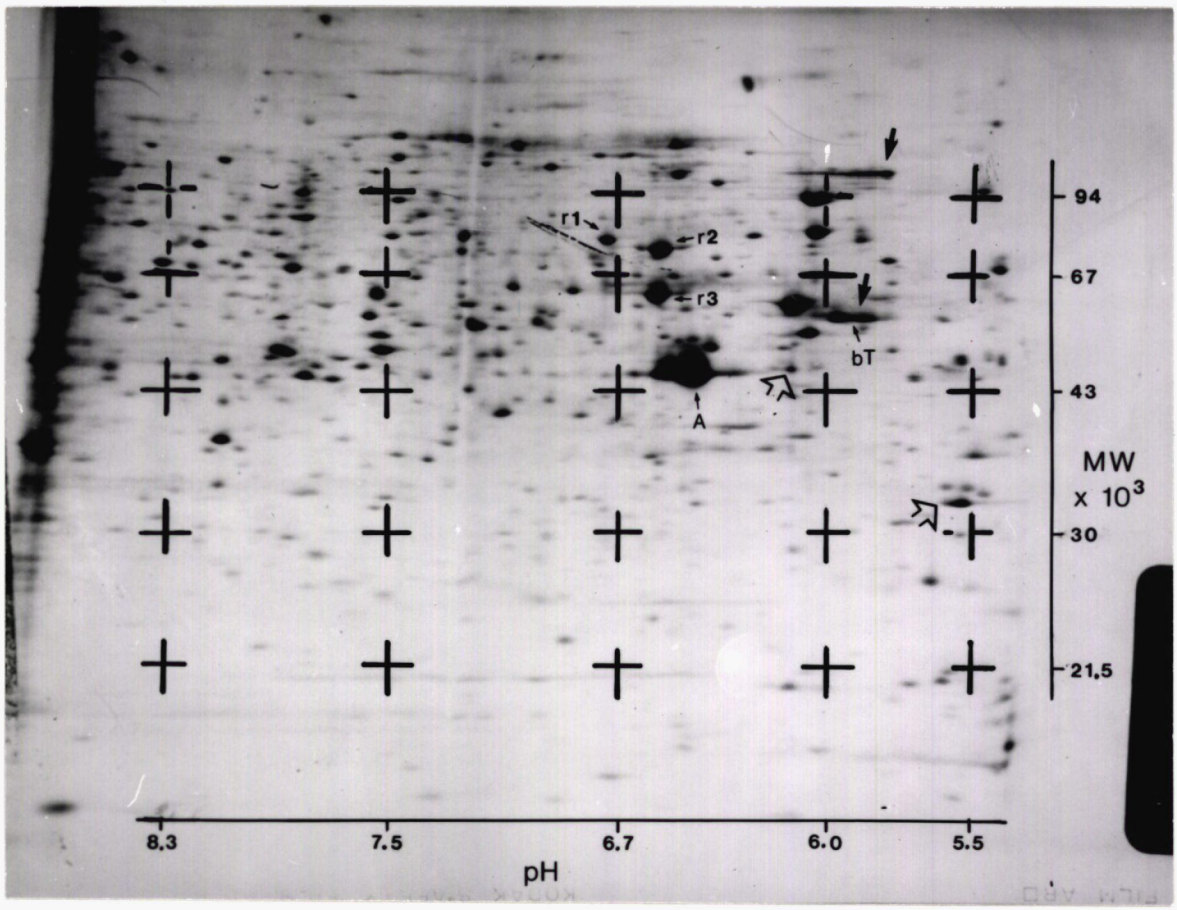

Figure 13. Autoradiogram of peptides synthesized during early S phase. The proteins that have been marked with letters are identified in the legend to Figure 11. Closed arrows show proteins that have increased synthesis and open arrows decreased synthesis when compared to the same peptides on the late G_1 autoradiogram (Figure 11).

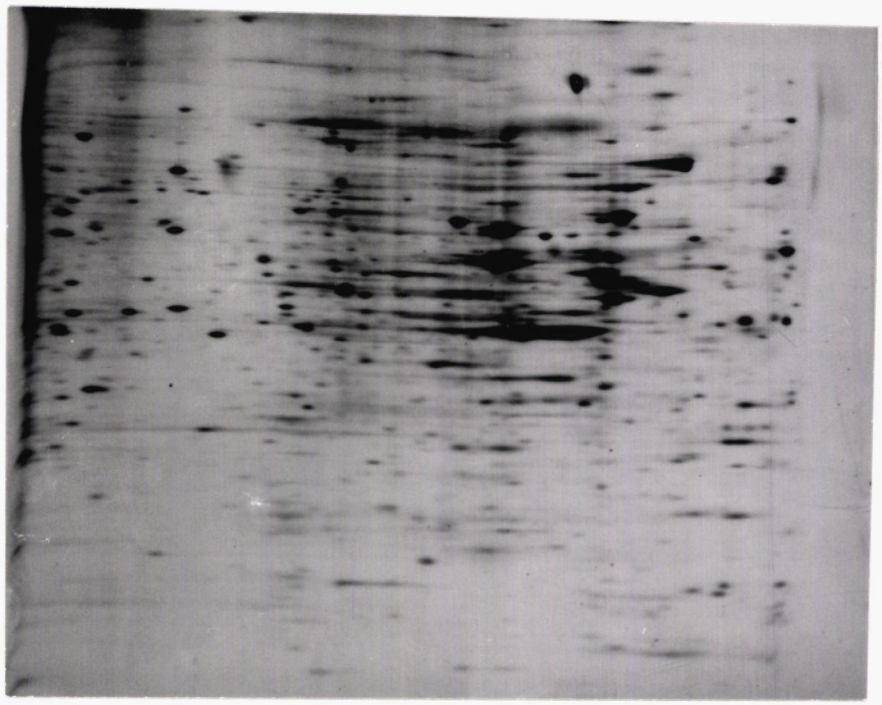

Figure 14. Autoradiogram of peptides synthesized during late S phase. Closed arrows indicate peptides which have increased synthesis when compared to the same peptides in the early S autoradiogram (Figure 13).

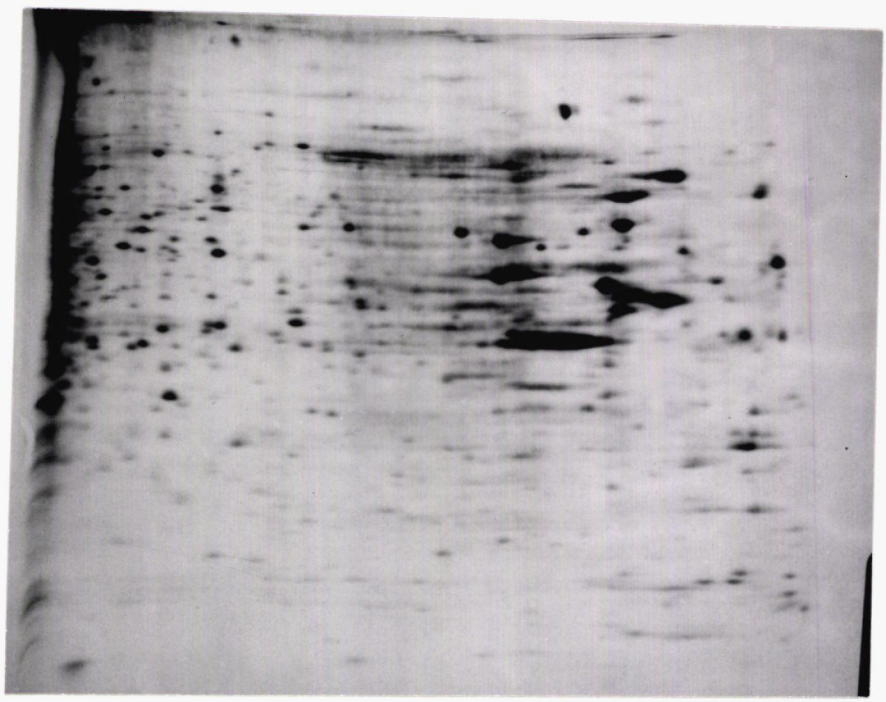

Figure 15. Autoradiogram of peptides synthesized during mid G_2 phase. Closed arrows indicate peptides which have increased synthesis when compared to early S phase cells (Figure 13).

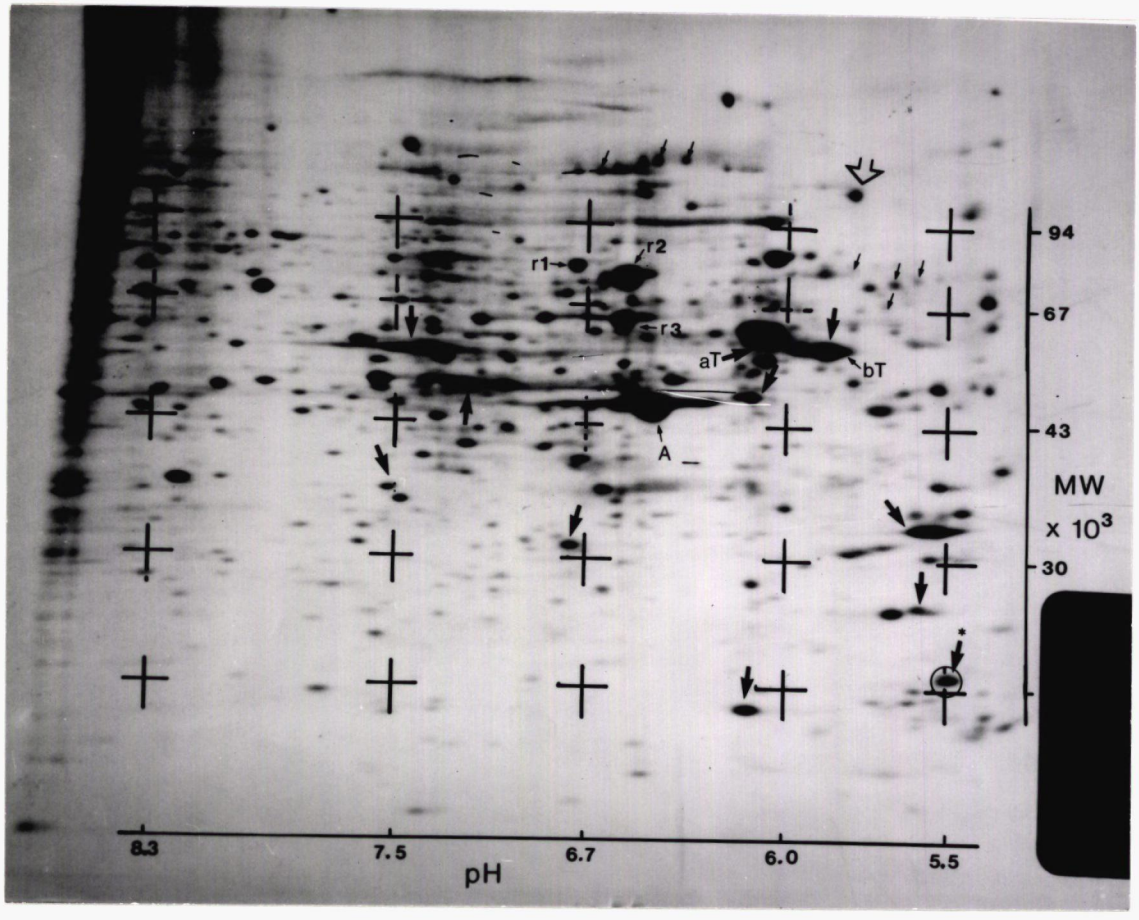

Figure 16. Autoradiogram of peptides synthesized during late G_2 phase. The proteins that have been marked with a letter are identified in the legend to Figure 11. Closed arrows mark peptides which appear to have increased synthesis when compared to S phase cells (Figures 13 and 14) while open arrows indicate proteins with decreased synthesis. The protein marked with an asterik and circled appears to be synthesized during late G_2 only as it is not found in any other autoradiogram.

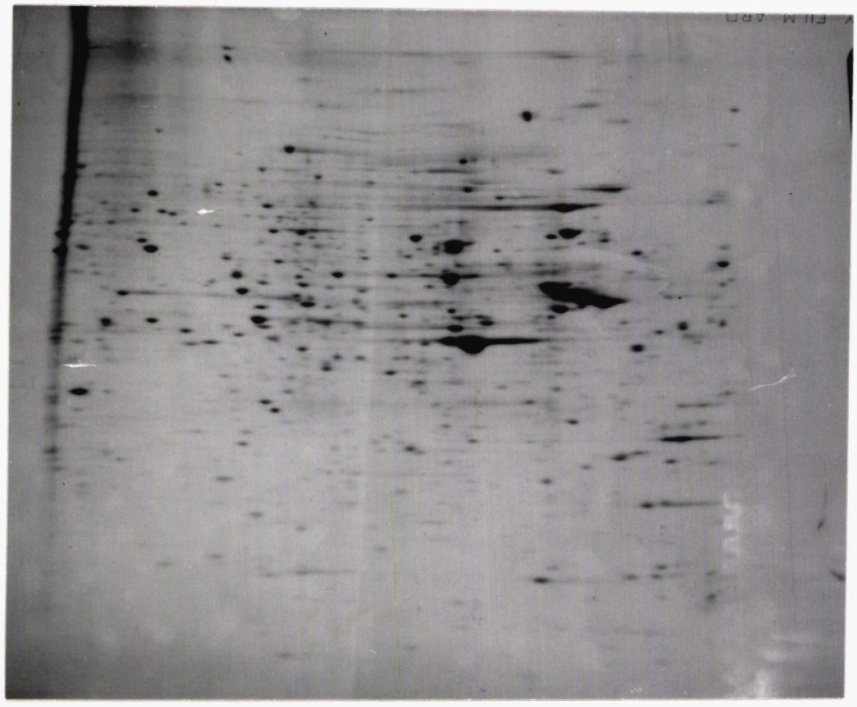

Figure 17. Autoradiogram of peptides synthesized during mitosis. The gel from which this autoradiogram was made was loaded with 40,000 cpm and exposed for 20 days.

The peptide synthetic patern of cells arrested at the G_1/S border is shown in Figure 12. Some of the most notable differences in this autoradiogram is a further decrease in alpha- and beta-tubulin synthesis. There doesn't appear to be a dramatic appearance or disappearance of any peptide in this autoradiogram.

Peptides synthesized in early S are shown in Figure 13. Beta-tubulin appears to be synthesized at a higher rate during this time but there is still not much synthesis of alpha-tubulin. The open arrows signify proteins which appear to have less synthesis than during late G_1. The closed arrows indicate proteins whose synthesis appears to have increased.

In Figures 14 and 15 which represent peptide synthesis during late S and mid G_2 respectively, both alpha- and beta-tubulin show increased synthesis when compared to synthesis during early S. One other protein which is marked by the closed arrow, also appears to be synthesized at a much higher rate. The resolution of these two autoradiograms is not as good as the rest of the cell cycle stages primarily due to a poorer preparation of the samples. Because of this, it is difficult to compare these autoradiograms to the early S and late G_2 autoradiograms.

Figure 16 illustrates peptides synthesized during late G_2. There appears to be 9 or 10 peptides synthesized at a higher rate during late G_2 than in S or mid G_2 phase and

are marked by large closed arrows. Amoung these proteins, alpha- and beta-tubulin can be identified. The protein marked with an asterik appears to be the only protein present during late G_2 that is not synthesized in any other phase during the cell cycle. The open arrow marks a protein that has decreased synthesis when compared to late S and mid G_2 autoradiograms. The small arrows mark out peptides which are actually modifications of the protein found to the very left of it. These modifications are possibly artifacts such as carbamylations of the peptide (hence their migration to a more acidic pI) and arise occasionally because of excessive heating during sample preparation (O'Farrell, 1975). Alternatively, they could also represent actual modifications of the peptides such as phosphorylations which are occurring specifically during late G_2.

Proteins synthesized during mitosis are shown in Figure 17. Both alpha- and beta-tubulin appear to be synthesized at approximately the same rate as in late G_2. Some of the proteins which appeared to have increased synthesis during late G_2 do not have the same increased synthesis during mitosis. The protein that is uniquely synthesized during G_2 can not be seen on the mitotic autoradiogram. It is also difficult to compare this autoradiogram to other ones because of its lower density. Because of the low specific activty of peptides obtained

from mitotic cells only 40,000 cpm was applied to the original gel, followed by a 20 instead of 7 day exposure to the film.

Although it was planned that the gels in Figures 11, 13, and 16 all have equal counts loaded (150,000 cpm) and a 7 day exposure it is apparent that the densities of identical spots are not equal from gel to gel. For this reason, three peptides (r1, r2, and r3) were scanned on a densitometer and the relative densities between gels compared. Protein r1 appears to be the same as one identified by Bravo and Celis (1980) as protein 12. They determined that this protein was synthesized at a very constant rate during the entire cell cycle. Protein r1 had a density of 43.4, 38.9 and 57.7 units (relative comparison) for late G_1, early S, and late G_2 autoradiograms respectively (Table 4). This means that late G_1 had approximately 10% more radioactivity label on the gel than early S and late G_2 had 32% more label assuming that r1 is synthesized at an equal rate throughout the cell cycle. This being the case, only those proteins which appeared to have more than a 30% increase in synthesis (when compared to other proteins in the vicinity) were marked with large arrows in the late G_2 autoradiogram (Figure 16).

The density of protein r1 was also compared to densities of r2 and r3 at each of various cell cycle stages

(Table 4). The ratio of densities of r1 to r3 was .29, .36 and .31 for late G_1, early S and late G_2 cells respectively. These ratios are more or less constant and if r1 is assumed to be synthesized at a constant rate during the cell cycle then r3 is also probably synthesized at a constant rate. The ratio of densities of r1 to r2, however, are not constant being .37, .41, and .21 for late G_1, early S, and late G_2 autoradiograms respectively. This result indicates that the rate of synthesis of r2 during late G_2 is roughly twice that what occurs during the late G_1 and early S phases of the cell cycle.

Protein samples from the various stages of the cell cycle were also examined with nonequilibrium (NEPHGE) two-dimensional gel electrophoresis. Figure 18 shows a silver stained peptide pattern of asynchronous cells. The histones were identified in this Figure by comparison with NEPHGE gels run by other researchers (Sanders et al., 1980; Groppi and Coffino, 1980). Figure 19 (a) through (f) illustrates autoradiograms of ^{35}S-methionine labelled proteins taken from various stages of the cell cycle. Only the section of the gel which contains or should contain the histones is shown. It should be noted that histone H1 or H2A are not visible in any of the autoradiograms since it does not contain methionine.

Table 4. Comparison of reference protein densities during the cell cycle.

Protein	Late G1	Early S	Late G2
r1	43.4	38.9	57.7
r2	117.1	93.8	268.7
r3	151.0	108.4	186.0
r1/r2	0.37	0.41	0.21
r1/r3	0.29	0.36	0.31

Reference proteins r1, r2, and r3 densities were determined by scanning the late G1, early S, and late G2 autoradiograms using an LKB Model 2002 Ultrascan Laser Densitometer as described in Materials and Methods. Total density for each protein was calculated by summing all of the area integrations obtained for that protein and is expressed in relative terms.

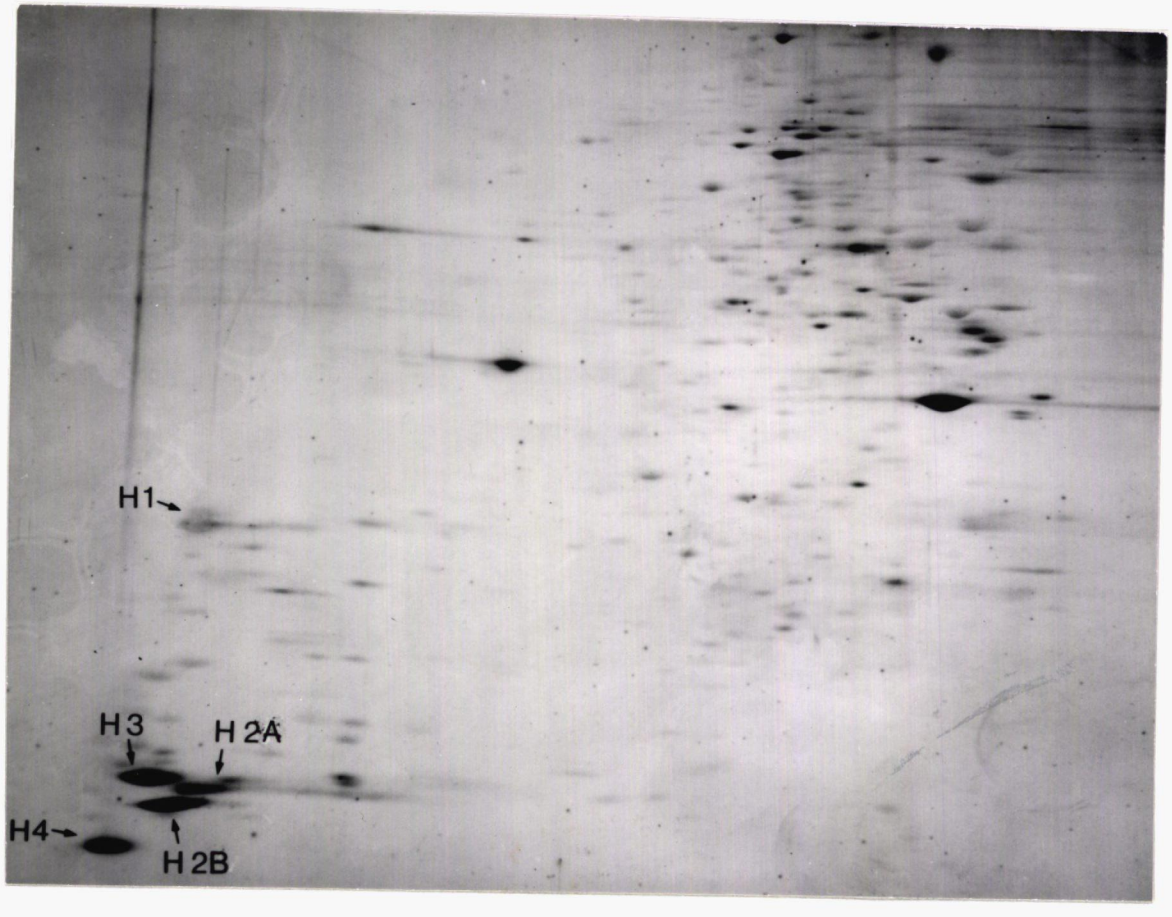

Figure 18. Nonequilibrium (NEPHGE) two-dimensional separation of proteins from asynchronous CHO cells (silver stained). Protein samples shown in this figure and Figure 19 were prepared and separated by NEPHGE gel electrophoresis as described in Materials and Methods. Histones H1, H2A, H2B, H3 and H4 are identified in this figure.

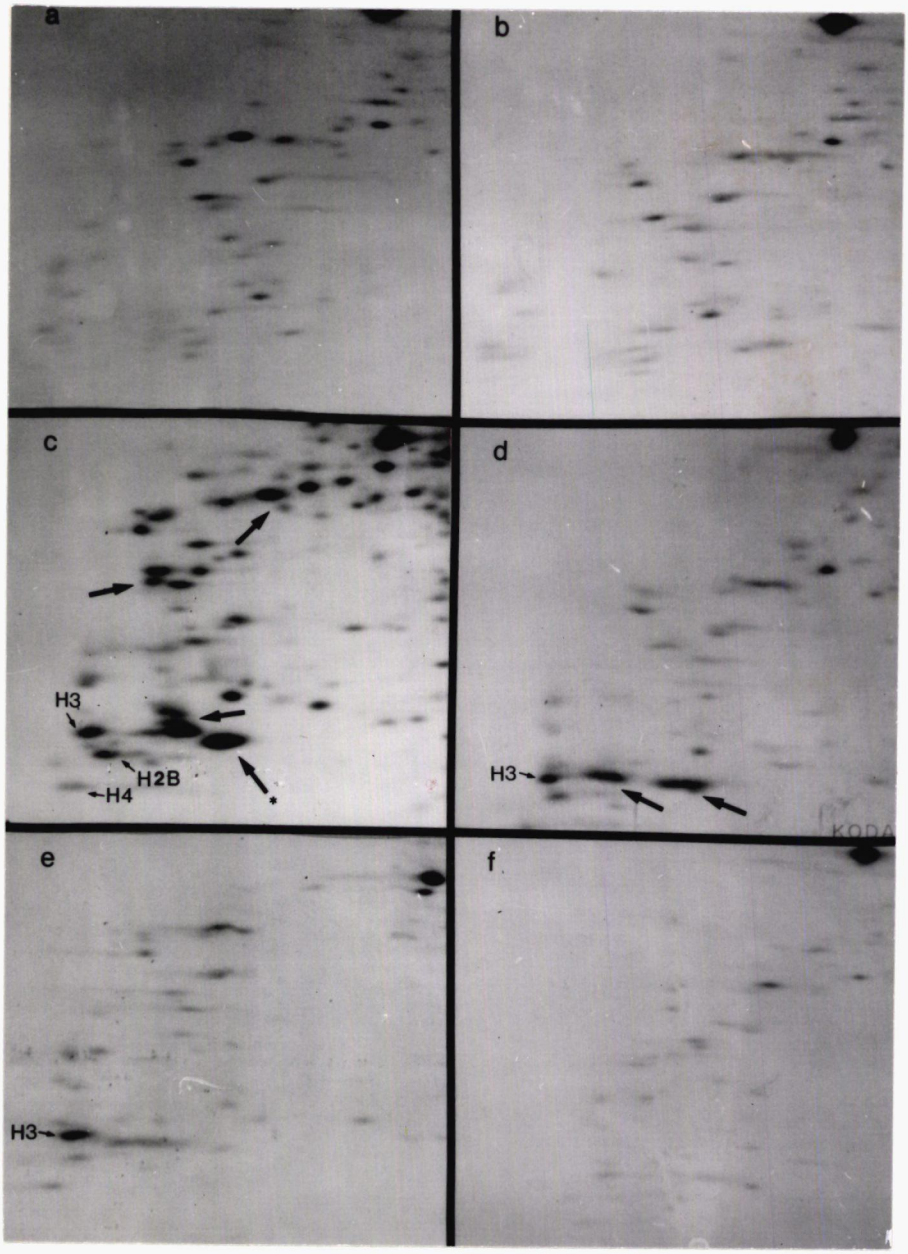

Figure 19. Histone synthesis during the cell cycle. ^{35}S-Labelled protein samples from a variety of stages of the cell cycle were separated by NEPHGE two-dimensional gel electrophoresis. Only the section of the gel which contains the histone proteins is shown. a, Early G_1; b, Late G_1; c, Early S; d, late S; e, Mid G_2; f, Late G_2.

Figure 19 (a) and (b) represent proteins synthesized during early and late G_2. Although the histone synthesis is not entirely absent it is barely visible. However, in (c) and (d) which represent early and late S cells, it is clear that histones are being synthesized at these times. The fact that a number of basic proteins are prominant in this area (especially in (c) and (d)) might indicate that more than one type of each histone (eg. modified histones). Alternatively, these might be some other type of chromosomal protein since either their synthesis or methionine content is considerably greater than any of the core histones. In 19 (e) which represents mid G_2 protein synthesis, histone synthesis is appreciably lower and continues to decrease so that by late G_2 (Figure 19 (f)) histone synthesis is practically non-existent again. The entire autoradiograms of the NEPHGE gels from the various cell cycle stages were also compared but no qualitative differences in protein synthesis were found (results not shown).

DISCUSSION

The aim of this study was to examine whether specific protein synthesis is required for the initiation of mitosis in CHO cells.

The need for protein synthesis prior to mitosis was demonstrated by using the protein synthesis inhibitor emetine. Emetine was found to be a very potent and fast acting reversible inhibitor of protein synthesis in mammalian cells. Early studies on the use of emetine as an inhibitor of protein synthesis in mammalian cells had indicated that emetine was an effective inhibitor but irreversible even at low concentrations (10^{-7} to 10^{-6}M) (Grollman, 1966 and 1968). More recent studies have shown that the inhibitory effect of emetine could be reversed at low concentrations ($2x10^{-7}$M) (Gupta and Siminovitch, 1976 and 1977; Brinckerhoff and Lubin, 1977). This study clearly indicates that the conditions under which emetine can be used as a reversible inhibitor are quite stringent. First, the concentration range at which emetine exhibits both sufficient inhibitory and reversibility properties lies between $5x10^{-7}$ M and $1x10^{-6}$M for CHO cells. Second, the pH of the media has a very dramatic effect on both

inhibition and reversibility capacities of the drug. Inhibition of protein synthesis by emetine is significantly reduced when the pH of the media is lowered below 7.0. Also, the reversibility characteristics of emetine is affected by pH with reversibility decreasing as the pH is raised above 6.5. At pH 7.5 or greater, emetine appears to act as an irreversible inhibitor of protein synthesis. The reason pH has such a profound effect on both the inhibition and reversibility properties of emetine may be related to the drug's mechanism of action, more specifically, its ability to bind to ribosomes. It has previously been demonstrated that emetine's inhibitory effect is presumably a result of its binding to the 40S ribosomal subunit in eukaryotic cells (Gupta and Siminovitch, 1977; Boersma et al., 1979). Therefore, as the pH of the media is changed the conformation of the ribosome or the microenvironment of the emetine binding site might also be changed such that emetine binding is significantly reduced at lower pH's. The fact that relatively small changes in the pH of the media have such a profound effect on the reversibility of emetine's inhibitory action may explain why earlier investigators (Grollman, 1966 and 1968) described this drug as being irreversible. The effect of emetine concentration and pH of the media on inhibition of protein synthesis and recovery from inhibition has not been reported before.

The effect emetine exhibits on other macromolecular synthesis including DNA and RNA synthesis has been well documented by previous investigators. DNA synthesis, for example, is reduced by about 80% within 15 minutes after addition of emetine while inhibition of RNA synthesis varies depending on the concentration of emetine used. At high concentrations (approximately 10^{-3}M) all types of RNA synthesis (i.e. 4S, 5S and 18S) are inhibited whereas at low concentrations (10^{-7} to 10^{-6}M), emetine reduces the synthesis of only 18S RNA (pre rRNA) with practically no effect on 4S and 5S RNA synthesis (Gilead and Becker, 1971). Because only low concentrations of emetine were used in this study, it is assumed that the effects that emetine is exhibiting are probably a result of emetine's primary affect on protein synthesis rather than RNA synthesis since others have shown that messenger and transfer RNA synthesis appear not to be inhibited at low concentrations of emetine (Gilead and Becker, 1971).

When emetine was added to asynchronous growing populations of CHO cells the mitotic index began to decrease approximately 30 to 40 minutes after the drug was added. This result suggests that protein synthesis is necessary for cells to initiate mitosis and that there maybe a point roughly 30 minutes or so before mitosis where one or a group of hypothetical 'mitotic proteins' are being synthesized. This finding is not completely in agreement

with the findings of previous investigators who reported a similar essential protein synthetic period occurring approximately ten minutes before mitosis in CHO cells (Tobey et al.,1966). It should be noted, however, that the study conducted by Tobey and co-workers (Tobey et al.,1966) used the rate of cell division rather than mitotic index to determine the effects of the protein synthesis inhibitor puromycin.

In the experiment in which emetine was added to growing cells for either 60 or 120 minutes and then removed, the mitotic index increased sharply approximately 60 minutes after the drug was removed. If one includes a 20 to 25 minute lag time for protein synthesis to resume after drug removal, the period at which the mitotic proteins are synthesized occurs roughly 40 minutes before mitosis. In addition, there was very little difference in the amount of time it took for the mitotic index to recover from either a 60 or 120 minute emetine pulse. The fact that the mitotic index resumed to almost control levels 60 to 75 minutes after a two hour inhibition of protein synthesis indicates that the period in which the 'mitotic proteins' are being synthesized is probably quite short since a long synthetic period would most likely result in a longer delay in the recovery of mitotic index (i.e. equal to the amount of time protein synthesis was inhibited).

Once it had been established that protein synthesis was necessary during a critical time before mitosis, experiments were conducted to determine if a specific protein (or proteins) is synthesized during that time. If a specific protein was synthesized only during late G_2 it may be involved in the control of cells entering mitosis.

Cells were synchronized by either shake-off of mitotic cells or shake-off plus G_1/S arrest with hydroxyurea. The efficiency of synchrony was monitored by incorporation ^{3}H-thymidine, quantitation of individual cell DNA content, and scoring of mitotic index. These methods revealed that the cells were synchronized quite effectively and the degree of synchrony was comparable with that obtained by other researchers (for reviews see Tobey et al., 1974 and Prescott, 1976).

Synchronized cells were labelled for a short time with ^{35}S-methionine at a variety of stages of the cell cycle. A comparison of the specific activities of labelled protein samples revealed that they differed with highest specific activity occurring during S phase. The lowest specific activity was recorded in mitosis and was approximately one-fourth that of late G_2 (see Results). This observation compares well with cell cycle specific activities reported by other researchers (Kolodny and Gross, 1969 and Bravo and Celis, 1980).

The analysis of the peptides synthesized during the

cell cycle was accomplished by comparing autoradiograms of ^{35}S-methionine labelled peptides separated on two-dimensional gels. Qualitative changes were determined by the appearance or disappearance of spots on the autoradiogram while quantitative changes were determined by changes in spot intensity. When doing comparisons of the autoradiograms , a number of conditions regarding the incorporation of the radionucleotide were assumed. That is, it was assumed that all peptides were incorporating the ^{35}S-methionine in a random fashion and that the available pool size of the ^{35}S-methionine was equal for all peptides during every phase of the cell cycle.

The equilibrium two-dimensional autoradiograms revealed that there may indeed be at least one protein that is synthesized during late G_2 that is not synthesized at any other time during the cell cycle. There is also a few peptides which show quantitative differences in synthesis during the cell cycle. When the autoradiograms of late G_1 (Figure 11) and early S (Figure 13) were compared no qualitative differences in peptide synthesis could be found. However, quantitative differences were observed and included increased synthesis of three peptides; alpha- and beta-tubulin, and a peptide of 110,000 MW and pI of 5.8. Two peptides, approximately 44,000 MW, pI 6.1, and 32,000 MW, pI 5.5 showed decreased synthesis in early S. No qualitative changes in peptide synthesis were observed in

the late S (Figure 14) or mid G_2 (Figure 15) autoradiograms were compared with the early S autoradiogram. The quantitative changes noticed in the early S autoradiogram when compared to the late G_1 autoradiogram became more pronounced at the late S and mid G_2 stages. In particular the 110,000 MW protein had maximum synthesis during the late S and mid G_2 stages.

The protein which appears to be uniquely synthesized during late G_2 has an approximate molecular weight of 22,000 and isoelectric point of 5.5 (Figure 16). In addition to this protein there appears to be 9 or 10 other proteins that have increased synthesis during late G_2. Alpha- and beta-tubulin were found to have maximum synthesis during late G_2 and mitosis. Only one peptide, the 110,000 MW protein described above, showed a dramatic decrease in synthesis in late G_2. Proteins synthesized during mitosis (Figure 17) had most of the quantitative differences in synthesis seen in the late G_2 autoradiograms. Actin, which was easily identified in all autoradiograms, appeared to be synthesized at a constant rate during the cell cycle.

The results from the peptide gel analysis during the cell cycle generally agrees with the majority of findings of Milcarek and Zahn (1978) and Bravo and Celis (1980) who have conducted similar studies. Both of these studies conclude that there was not sufficient evidence to indicate

that the synthesis of any of the detected polypeptides was confined to only one of the cell cycle phases. However, the rate or quantity of synthesis of some peptides did vary during the cell cycle. Milcarek and Zahn (1978) found that four peptides showed increased synthesis near mitosis and two other proteins showed decreased synthesis during G_2. Bravo and Celis (1980) found that the synthesis of only about 12 peptides varied throughout the cell cycle to any significant degree. Of these they identified alpha- and beta- tubulin which showed increased synthesis during mitosis. Both of these studies also concluded that actin synthesis was more or less constant during the cell cycle. The results of this study differ from the two studies mentioned above in that there appears to be at least one peptide that is specific to a cell cycle stage (i.e. late G_2) and that most of the proteins identified as to having variable synthesis throughout the cycle have different molecular weight and isoelectric point assignments (with the exception of tubulin). Some of these differences perhaps can be attributed to the use of different cell lines in the experiments - both Milcarek and Zahn, and Bravo and Celis used HeLa cells in their studies. None of these studies agree with results of Al-Bader and co-workers (1978) who reported the appearance of nine new peptides when cells traverse from S phase into G_2.

A number of other recent studies have provided evidence that the synthesis of certain peptides does vary quantitatively throughout the cell cycle. Tubulin synthesis, for example, is also found to be synthesized at a much higher rate just before mitosis in Physarum (Laffler et al., 1981) and in Tetrahymena (Bird and Zimmerman, 1981). Also Chafoulease and co-workers (1982) reported the levels of calmodulin approximately double when cells progress from G_1 into S phase.

Examination of the protein synthesis from various parts of the cell cycle on NEPHGE two-dimensional electrophoresis revealed that the synthesis of histones and some other very basic proteins were more or less restricted to the S phase of the cell cycle. There appears to be a trace amount of histone synthesis during G_1 and late G_2 and slightly more synthesis during mid G_2. These results are in agreement with recent studies by Marashi and co-workers (1982) and Rickles and co-workers (1982) who found that both histone synthesis and histone gene transcription were limited to the S phase of the HeLa cell cycle. These results contrast those of Groppi and Coffino (1980) who found that histones were synthesized at equivalent rates during the cell cycle of mouse lymphoma and CHO cells.

The results of this study have hopefully demonstrated two facts; that there is a critical protein synthetic

period occurring approximately thirty minutes before mitosis in CHO cells and secondly, that there are a few proteins whose synthesis, or quantity of synthesis, appears to be coordinated or regulated during the cell cycle. Although this study does not provide absolute proof that there is a specific protein(s) synthesized during G_2 which is responsible for the initiation of mitosis the evidence presented does support the idea that such a protein(s) may exist.

To date, this protein or proteins have not been clearly identified or isolated. Consequently, the function of this protein(s) has not been characterized directly. However, there is a considerable amount of evidence which imply that a protein(s) is involved in the events which lead up to mitosis.

As mentioned in the Introduction, the G_2 period can be viewed as a time in the cycle where elements necessary for chromosome condensation and the construction and operation of the mitotic apparatus are synthesized or activated (Prescott, 1976). Mazia's theory on the chromosome cycle (1974) brings to our attention that perhaps the G_2 period in most cell types is concerned with the initial stages of chromosome condensation which are not visible with the light microscope. It has been assumed that the visible mitotic condensation of chromosomes is the result of the synthesis or activation of a single or several factors at

the point of transition of the cell into prophase (Prescott, 1976). This concept has been strengthened by the results of studies such as those conducted by Johnson and Rao (1970) which showed that a mitotic HeLa cell has the capacity to induce the condensation of chromatin in any interphase nucleus which has been physically fused to it. This phenomenon termed premature chromosome condensation (PCC) can occur even when mitotic cells are fused to cells which have not replicated their DNA (i.e. G_1 cells) (Matsui et al., 1972). The condensation factor(s) responsible for the PCC effect is affective over a wide variety of phylogenetic lines as the PCC effect was observed when HeLa metaphase cells were fused to bull sperm, chicken erythrocytes, Xenopus cells and mosquito cells (Johnson et al., 1970). The PCC effect has even been observed in higher plants. In a study by Van der Haar and co-workers (1981), nuclei of carrot protoplasts were injected into maturing Xenopus oocytes causing the nuclear membrane of the plant cells to disrupt and the chromatin to condense prematurely within 30 minutes after injection.

In recent years there have been a number of studies which indicate that the factor(s) responsible for premature chromosome condensation is a protein and can be found in mammalian cells during G_2 and mitosis or in mature Xenopus oocytes which are about to undergo meiosis (Sunkara et al., 1979b). In one study cytoplasmic extracts of HeLa cells

from various stages of the cell cycle were injected into Xenopus oocytes to detect the presence of factors which might induce meiotic maturation (i.e. germinal vesicle breakdown and chromosome condensation). No maturing activity was found in the extracts from G_1 or S phase cells and only a small amount of activity from early and mid G_2 extracts. Maturation activity increased greatly in extracts from late G_2 cells (Sunkara et al., 1979a). Initial characterization of the factor revealed that it was a protein whose activity was completely inhibited when calcium (but not magnesium) was added to it (Sunkara et al., 1979b). The mitotic factor or meiotic maturation activity was three times higher in fractions isolated from chromosomes then from the cytoplasm of metaphase arrested cells (Adlakha et al., 1982). Mitotic factor activity was found in low levels in early and mid G_2 cells but only in nuclear and not in cytoplasmic extracts. Late G_2 cells exhibited mitotic factor activity in both nuclear and cytoplasmic extracts. These results led the authors to conclude that the mitotic factors become preferentially bound to chromatin, perhaps as soon as they are synthesized, and as the cells synthesize more of these factors in preparation for mitosis increasing amounts of them are retained in the cytoplasm. The mitotic factor(s) found were also partially purified and found to be quite

stable (active after 2 months when stored at 0 or -70° C) as long as protease inhibitors and a carrier protein (bovine serum albumin) are added to the preparation (Adlakha et al., 1982).

It is still not absolutely clear whether this mitotic factor or some other protein(s) is in fact synthesized during late G_2 and mitosis or whether it is always present and/or synthesized but merely 'activated' during late G_2 and mitosis. The results of this and other studies have shown that protein synthesis is required during late G_2 and that there might be one peptide specifically synthesized during this time.

Whether a protein(s) is synthesized or activated it seems that there must exist certain critical events which control the initiation of mitosis. It may be that once one or two crucial steps are accomplished a 'cascade' of events follows which ultimately results in the cell going through mitosis and dividing. Obviously, inducing premature chromosome condensation is not enough to initiate mitosis in cells which are not prepared to divide. Therefore, other events or conditions within the cell are likely to be involved in the initiation of mitosis. One of these may be the completion of DNA synthesis such that two complete genomes are contained within the cell. Another might be that sufficient components involved in mitosis (eg.tubulin) be present in large enough amounts so that the division may

be successful.

If the factors responsible for the initiation of mitosis are indeed 'activated' rather than synthesized, what might be the nature of this activation? Some studies have indicated that the phosphorylation of certain proteins might be involved. For example, the phosphorylation of some of the histones appears to be cell cycle dependent (for review see Gurley et al., 1981). Histone H1 phosphorylation has been shown to be absent during G_1 arrest in CHO cells (Gurley et al., 1973) whereas phosphorylation of H1 during S and G_2 does occur (Gurley et al., 1975). More intriguing is the very high phosphorylation or 'superphosphorylation' of H1 during mitosis. All of the H1 molecules are superphosphorylated during this time suggesting that it may serve a specific function, eg. it may be involved in the formation of a condensed chromatin structure. In addition, all histone H3 molecules in CHO cells have also been reported to be phosphorylated during mitosis and this is the only time in which they are found phosphorylated (Gurley, et al., 1978). An intermediate filament protein of 58,000 daltons has also been found to be phosphorylated to a much higher degree during mitosis than other stages of the cell cycle of CHO cells (Robinson et al., 1981).

Besides protein phosphorylation, there are a number of other possible 'events' or conditions which could activate

(or deactivate) specific proteins involved in mitosis. One such possibility is changes in cellular (or nuclear) ion concentration. Although several ions may be involved there have been some evidence to suggest that both pH (Gerson, 1978) and calcium ions (Wang and Waisman, 1979; Cheung, 1980; Means and Dedman, 1980) might be involved in the control of mitosis and cell division. The role of calcium might be multifaceted as it plays a role in a number of other cell functions including protein phosphorylation and microtubule assembly (see reviews cited above). As mentioned previously, the mitotic factor found by Sunkara and co-workers (1979b) is totally inactive in the presence of calcium ions.

The results of this study have shown that protein synthesis is needed during late G_2 in order for CHO cells to enter mitosis and that there may be a specific protein being sythesized at that time. Examination of protein synthesis patterns during the cell cycle revealed that a few qualitative and quantitative changes in synthesis occur. This is the first study to demonstrate qualitative differences in peptide synthesis other than histones during the cell cycle. More specifically, there is a protein uniquely synthesized during G_2 that has a molecular weight of approximately 22,000 and a pI of 5.5. There also appears to be some very basic proteins other than the core

histones that are only synthesized during S phase. Of the proteins showing quantitative changes during the cell cycle, two groups of proteins have been identified- tubulin and histones. The synthesis of alpha- and beta- tubulin appear to be highest during late G_2 and mitosis and lowest during late G_1 and G_1/S arrest. Histone synthesis appears to be restricted to the S phase of the cell cycle with practically no synthesis during G_1, G_2, or M. Assuming that the techniques used resolved the majority of proteins being synthesized these results imply that there may be a specific protein(s) involved in the initiation of mitosis and perhaps of S phase as well.

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