BIOLOGICAL ACTIVITY AND BIOCHEMICAL MECHANISMS OF ACTION STUDIES OF Ψ-OX AND THYMOX IN CULTURED L1210 CELLS

A Dissertation

Presented to

The Faculty of the Department of

Biophysical Sciences

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

By

Marian L. Moore Lewis

August 1979

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ABSTRACT

Thymox and Ψox induced time and concentration dependent cytotoxic and cytostatic effects in L1210 cells. Affected cells were irreversibly blocked in the G2 phase of the cell cycle. Minimum effective exposure time of cells to Ψox and thymox was 30 minutes and 2 hours respectively. In synchronized cells, both compounds were cytotoxic in all cell cycle phases, but exhibited greatest effect in late G1-early S and in G2.

Biochemical mechanisms of action included inhibition of DNA more than RNA synthesis. Activity of DNA and RNA polymerases in crude cellular extracts were reduced. Protein synthesis was inhibited, but some protein and RNA synthesis appeared to continue in G2 blocked cells. Microtubule protein levels, detected by the colchicine binding assay, were greatly reduced. Preliminary evidence suggests that Ψox and thymox inhibited the synthesis of microtubule protein. This may account in part for the arrest of cells in the G2 phase of the cell cycle.

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INTRODUCTION

The first recorded use of a cancer chemotherapeutic drug was in 1942 when nitrogen mustard was administered clinically to a patient with advanced lymphosarcoma (1). In 1948, Sidney Farber used the folate antagonist, aminopterine, in treatment of children with acute leukemia and obtained marked responses in 10 of 16 patients (2). Since 1942, chemotherapeutic drugs have been found which are effective in curing a number of malignancies among these are Hodgkin's disease, Burkitt's lymphoma (a virus associated malignancy), acute lymphocytic leukemia of children and others. The rational design, as opposed to serendipitous discoveries, of cancer chemotherapeutic drugs, though extremely advanced since 1942, is still complicated by such factors as diversity of types of cells involved in malignancies, the lack of, though dawning, understanding of gene expression in eukaryotic cells and the unpredictability of biological systems in interacting with drugs.

The classification of drugs to which pseudouridinedicarboxaldehyde (Ψox) and thymine ribosidedicarboxaldehyde (thymox) belong, as proposed by Guthrie (3) in 1961, is "periodate oxidate products". The reason for the broad terminology is because as many as six structures for such compounds may exist in equilibrium with each other.

Use of purine and pyrimidine oxidation products in cancer chemotherapy came about by a circuitous route. Related compounds, the arylglyoxals, were reported in 1953 to have antitumor activity (4). However, the interest in these compounds was mainly in their activity as antiviral agents (5, 6). Furst et al. in 1957 (7), reported the compounds to be carcinostatic in mouse tumors. In 1958 French and Freedlander reported antitumor activity against leukemic and ascites tumors (8).

Stock et al. reported in 1953 the results of testing the therapeutic activity of the periodate-oxidation products of carbohydrates and their derivatives (9). The first concerted study of the periodate-oxidation product compounds as chemotherapeutic drugs was conducted by Dvonch et al. in 1966 (10). In a screening program, Dvonch found that many periodate-oxidation product compounds "showed striking inhibition of Ehrlich ascites and inhibition of leukemia L1210 in mice". The compounds lacked activity against sarcoma 180 or adenocarcinoma 755.

Since 1966, several oxidation product drugs have been shown to be effective antitumor agents (11, 12, 13, 14, 15) in experimental systems. Based on inhibition of tumor cells in vivo and in vitro, inox, the dialdehyde derivative of inosine was started in clinical trials (16). Although no one class of drugs may be expected to possess totipotent antitumor capabilities, the feasibility of using the oxidation product drugs is well advised.

This dissertation describes studies with two such drugs, thymox, the oxidation product of thymine riboside, a ribonucleoside with a normal

pyrimidine carbon-nitrogen glycosidic linkage; and Ψox, the oxidation product of the naturally occurring carbon-carbon glycosidic linked ribonucleoside, pseudouridine. Both pseudouridine and thymine riboside are components of the tRNA. Pseudouridine and its ability to be oxidized by periodate were reported by Cohn in 1960 (17) but this oxidation product compound has not heretofore been tested for anti-tumor activity.

In order to design chemotherapeutic drugs it is advantageous to understand the molecular biology of the cell replication cycle. The biochemical control of cell replication still remains enigmatic. The discovery in 1953 by Howard and Pelc (18) that DNA is synthesized during a short period in eukaryotic cells led to the idea that interphase has three parts. Development of flow microfluorometric techniques by 1974 to follow progression of cells through the cycle has greatly facilitated understanding of cellular function mechanisms. The cell cycle consists of four phases. By cytological observation only two distinct conditions may be described, interphase and mitosis. During interphase replication of DNA, transcription and translation occur. Mitosis results in cytokinesis and division of the chromosomes into two daughter cells. Interphase, immediately following mitosis in diploid cells begins with G1 (gap 1). The G1 phase ends when cells begin to synthesize new DNA (S phase). During the S phase, DNA and the histone conponents of chromatin are synthesized as well as other division-related proteins. At the end of S, chromosomes have two complementary sets of chromatids and are in the G2 (gap 2) phase where they remain until mitosis (M)

begins. The synthesis of cytoplasmic proteins, ribosomes and other organells occurs continuously during interphase. During the M phase, snythesis of DNA and RNA are turned off.

Particular cell types differ in the length of time spent in these phases. For mouse L-strain cells with a 24 hour doubling time, G1 lasts for 12 hours, S for 6-8 hours, G2 for 3-4 hours and M usually lasts for only 1 hour (19). Using the high-speed flow cytometer system (flow microfluorometry or FMF) for automated single-cell analysis of DNA content to determine the cell cycle in logarithmically growing L1210 cells in ascites mice tumors, Crissman et al. have shown that the percentages of cells in G1, S, and G2 + M are 29.2, 65.5, and 8.3 respectively (20).

With this technology available, it is now possible to begin to understand the biochemical action of a drug and to attempt to correlate its cytotoxicity to biochemical effects in the phase of the cell cycle in which the drug is predominantly lethal. For instance, in treating solid tumors the cells which are not cycling would not be affected by phase-specific drugs and drugs such as cyclophosphamide, doxorubicin or dactinomycin which are cytotoxic in all phases of the cell cycle would be indicated (21). For small tumors with fast growing cells, phase-specific drugs such as hydroxyurea or Ara-C theoretically would be selected (22). Only by finding if there is cell cycle phase-specific activity can the effectiveness of a new drug by exploited. This type of study requires use of synchronized cells.

The purpose of the research reported herein was to define the effect of two heretofore untested drugs in a known malignant cell line (L1210), and to analyze the response of the cells at the molecular and biochemical level.

Section I describes the effect on cell proliferation and cytotoxicity of Ψox and thymox in cultured L1210 cells, the stability of biological activity, the perturbation of the cell cycle as shown by FMF and the cell cycle phase specificity of activity of the drugs.

Section I

MATERIALS AND METHODS

L1210 Cell Cultures

Cultured L1210 cells were a gift from Dr. Carol E. Cass of the University of Alberta, Edmonton, Alberta, Canada. The L1210 mouse leukemia was chosen for these studies because of its selection as the standard model tumor reference system for evaluation of candidate chemotherapeutic drugs by the World Conference on Drug Screening which met in Geneva, Switzerland in 1974 (23). The original tumor was induced in an 8-month-old DBA/2 mouse in 1948 by painting the skin with 0.2% methylcholanthrene in ethyl ether (24). Tumors of the spleen and lymph nodes developed initially and since that time, the tumor has been propagated in vivo in DBA/2 mice and tested for drug sensitivity in BDF_1 mice (C57B1/6 X DBA/2). *In vitro* cultured cells may be established in static suspension cultures by injection of mice with tissue culture medium and cells and subsequent passage *in vitro* (25), by addition of special growth factors to tissue culture medium (26), and by developing special culture media (27, 28).

Cells were cultured in loosely capped 75 cm (Falcon 3024) or 25 cm (Falcon 3013) flasks in a 37°C, 5% CO_2 95% air humidified incubator (Forma Scientific, model 3295). Proliferation was monitored with a Model ZB1 Coulter counter (Coulter Electronics) and cells were diluted to contain approximately 200,000 cells per ml on alternate

days three times a week. Population density was approximately 1 X 10^6 cells/ml at the time cells were diluted. Fisher's medium for leukemic cells of mice (10 X) was obtained from Grand Island Biological Company (GIBCO). Complete Fischer's growth medium (FGM) was prepared by addition of sterile stock solutions of 50 ml of 10X medium, 50 ml of horse serum (GIBCO), 5 ml of 7.5% $NaHCO_3$(GIBCO) and 5 ml of penicillin-streptomycin solution (10,000 units penicillin, 10,000 mcg streptomycin per ml) (GIBCO) to 390 ml of sterile double distilled deionized water.

Preparation of Ψox, Thymox and Inox

The periodate oxidation products, pseudouridinedicarboxaldehyde (Ψox), thymine ribosidedicarboxaldehyde (thymox) and inosinedicarboxaldehyde (inox) were synthesized by the method of Dvonch et al. (29). Synthesis of each drug was carried out in the dark for 30 minutes by reacting 1 millimole of pseudouridine (Sigma) or inosine (Sigma) or thymine riboside (Cal Biochem) with 1.1 millimole of periodate dissolved in 11 ml of deionized water. Solutions were subsequently passed through a 1 X 4 cm Dowex-1 formate column containing a sintered glass filter. The column was washed with two 15 ml volumes of double deionized water. The total eluate was collected, quick-frozen in a dry ice-acetone bath and lyophilized (Kimball et al. (30) and Bell et al. (31)).

Effect of Ψox, Thymox and Inox on Proliferation and Viability of L1210 Cells

Suspension cultures of L1210 cells were diluted in FGM to contain 100,000 to 150,000 cells per ml approximately 24 hours before drugs were added. The lyophilized drugs were dissolved in FGM and sterilized by filtration through a 0.45 um pore diameter HA membrane (Millex filter unit, Millipore Corp.) immediately prior to addition to cells. Addition of drugs contained in 0.03 volumes of FGM gave final concentrations of 400 to 10 uM when diluted and added to suspensions of 1-3 X 10^5 asynchronously growing cells per ml.

The viability of the cells at any given time was measured by the presumed ability of viable cells to exclude trypan blue (28, 32, 33). A 0.1 ml aliquot of cells was mixed with 0.1 ml of trypan blue (0.4% in normal saline, GIBCO), and allowed to stand 5, but not longer than 15 minutes. Viability was determined by counting total and unstained cells in a hemocytometer and was expressed as percent viable cells (34).

Soft Agar Cloning Technique

Cloning cells in soft agar was also used to determine viability of cells after drug treatment. The method was a modification of that described by Himmelfarb et al. (35). Aliquots of drug treated and control cells were diluted in FGM and subsequently in 2X cloning medium. Cloning medium was prepared as described by Cass and Au-Yeung (36) and consisted of FGM supplemented with 100 units/ml penicillin and 100ug/ml streptomycin, 2mM HEPES buffer (N-2-hydroxyethyl piperazine

-N'-2-ethanesulfonic acid) (pH 7.4), 15% horse serum, 0.75 %$NaHCO_3$, and 5% conditioned medium (consisting of medium from 24 hour cultures from which the cells were centrifuged). The final dilution of cells for cloning was made in 2X cloning medium and held not longer than 5 minutes at 37°C before mixing with an equal volume of Noble agar (Difco), (0.26% dissolved in water and sterilized by autoclaving at 15 lbs. pressure for 20 minutes in an AMSCO (American Sterilizer Co.) autoclave. Agar was cooled to 46° before mixing with cell suspension. Dilutions of cells were made so that replicate tubes contained 100-400 cells/ml and up to 3000 cells/ml in cases in which cells were treated with high concentrations of drugs. One ml volumes of these cell suspensions in 1X cloning medium containing 0.13% Noble agar were distributed into the wells of multiwell plates (Falcon 3008) (37, 38). Four wells per dilution were used for each sample. The multiwell plates were placed in the CO_2 humidified incubator and observed daily after the fifth day for development of clones. On day 10, clones were counted using a dissecting microscope at low magnification and the relative colony forming efficiency (RCE) of surviving cells, or the ratio of treated/control cells, was evaluated.

Stability of Biological Activity of Ψox and Thymox Incubated at 37°C in FGM

Drugs were dissolved in FGM and sterilized by filtration. The stock drugs in FGM were added in 0.03 volumes to cell suspensions to a final concentration of 200 uM. Thirty ml of FGM containing drugs were dispensed into each of 12 flasks for each drug and FGM with no drugs

was dispensed into 12 control flasks. All flasks were placed in the 37°C CO_2 incubator. At designated times after incubation, stock cultures of L1210 cells were centrifuged from their growth medium and resuspended in one flask of the incubated medium containing drug and one control flask with no drug. Cells were added to all flasks in the test at a concentration of 1 X 10^5 cells per ml. Cell proliferation was evaluated on days 1, 2 and 3 and the percent increase or decrease in cell number during each 24 hour period served as the criterion for drug stability.

Stability of Ψox: Lyophilized and in Solution

Ψox was dissolved in distilled water, sterilized by filtration as described and added at 100 uM final concentration to asynchronously growing L1210 cells at concentrations of 1.8 to 2.8 X 10^5 cells per ml. Cells were sampled and counts were expressed as percent of controls at 5, 24, 48 and 72 hours. The drug was handled in the following ways:

1. Synthesized, lyophilized, and dissolved and tested within 1 day.
2. Lyophilized, stored in a container packed in dry ice for 3 days, dissolved and tested.
3. Lyophilized and allowed to stand at room temperature for 3 days, dissolved and tested.
4. Lyophilized, dissolved and held at 4°C for 4 days.
5. Lyophilized, dissolved and tested immediately.

Minimum Effective Exposure Time of L1210 Cells to Wox, Thymox and Inox

L1210 cells at concentrations of 1 X 10^5 cells/ml were treated with drugs (100 uM final concentration) for 0.5, 1, 2, 4, 7, and 24 hours. At the stated times, cells were centrifuged from drug containing medium, resuspended in an equal volume of fresh medium and incubated at 37°C in the CO_2 incubator. Cells were counted at 24, 48, and 72 hours and proliferation over the 24 hour periods was stated as difference in increase or decrease between treated and control cells for each time point.

Flow Microfluorometry (FMF)

The instrument was constructed at the University of Houston by Dr. John C. Allred and Dr. Ed Hungerford, Department of Physics, and by Dr. Allen Bartel, Department of Biophysical Sciences and was patterned after the instrument constructed by the Biophysics and Instrumentation group at the Los Alamos Scientific Laboratory, Los Alamos, New Mexico (39).

FMF analyses of cell cycle kinetics of drug treated and control populations of L1210 cells were done by modifications of the method of Tobey and Crissman (40). Four ml samples of suspension cells (not less than 200,000 cells/ml) were centrifuged and resuspended in one ml of 100 ug/ml mithramycin (a gift from the Pfizer Co.) in 15 mM $MgCl_2$. Cells were stained in the dark for 20-30 minutes and examined directly in the FMF with a laser wavelength setting of 457 nm. Mithramycin was chosen as the fluorescing stain for DNA because it selectively fluoresces when bound to DNA but does not interact with RNA (41).

Mitotic Index

Cells were prepared for mitotic index evaluations by modifications of the method of Tijo and Whang (42). One ml aliquots of uniformly dispersed cells were removed from suspension cultures, centrifuged at 1470 x g in a table top clinical centrifuge (Dynac, 0101, Clay Adams), the medium was decanted and the cells were washed twice with 5 ml of Dulbecco's phosphate buffered saline (PBS) without Mg^{++} and Cl^{++} (GIBCO). After the second wash, cells were resuspended in 10 ml of 0.075 M KCl and held at room temperature for 10 minutes. Cells were centrifuged as above and fixed for 5 minutes by dropwise addition of 4 ml of Carnoy's fixative (methanol, glacial acetic acid, 3:1). Cells were centrifuged, the fixative was decanted and 4 ml of fresh fixative was added to the pellet. Cells were resuspended by gentle pipetting with a Pasteur pipette and then centrifuged again. Several drops of fixative were added to the pellet, cells were dispersed and dropped onto an alcohol cleaned slide from a Pasteur pipette held approximately 2 feet above it. Slides were air dried and the cells were stained with giemsa. (See Appendix I for preparation of stain and solutions). Cells were stained by dipping slides into a staining jar containing phosphate buffered giemsa for 1.5 minutes, then placing them in a jar containing phosphate buffer for 7-10 minutes. Excess stain was rinsed away with distilled water, slides were air dried and coverslips were mounted with Permount (Fisher Scientific Co.). The mitotic index was evaluated by counting the number of discernable mitotic cells per 100 cells. A total of at least 200 cells was counted for each evaluation.

Cells stained in this manner were also examined microscopically for chromosome damage and aberrant cytological morphology.

Cell Cycle Phase Specific Activity of Ψox, Thymox, Inox and Vinblastine

L1210 suspension cells were synchronized by the method of Thilly et al. (43) as described for Hela suspension cultured cells. Suspensions of L1210 cells with a population doubling time of 20±4 hours were diluted to contain 2 X 10^5 cells/ml (200 ml total volume). Thymidine (TdR), (Swartz-Mann) made up and sterilized in FGM, was added at a final concentration of 0.25 mM. After incubation for 24 hours the cells were centrifuged from the TdR-containing medium and resuspended in fresh FGM without TdR. The cells were incubated 24 hours, counted, diluted to a concentration of 2 X 10^5 cells/ml and TdR (0.25 mM final concentration) was again added. Cells were incubated for 24 hours, centrifuged from the TdR-containing medium and resuspended in fresh FGM at 1 X 10^5 cells/ml. Forty-five flasks, each containing 15 ml of cell suspension of equally dispersed cells, were set up and divided into 5 groups of 9 flasks each. The cells, the majority of which were synchronized at the G1/S boundary, were then ready for treatment with the drugs. At 2-hour intervals, one flask from each of the 5 sets was subjected to Ψox, thymox, and inox at a final concentration of 200 uM; to vinblastine (Eli Lilly Co.) at 0.2 ug/ml; and to no drug. Cells were incubated with the drugs for 2 hours then centrifuged and resuspended in fresh FGM without drugs. These cells were incubated and proliferation was evaluated at selected times after drug treatment. Before addition of the drugs aliquots of cells were removed from

flasks for determination of the cell cycle phase at the time drugs were added. Aliquots consisted of 1 ml for Coulter counting, 4 ml for FMF profiles, 1 ml for mitotic index determinations, and 1 ml for tritiated thymidine uptake for determining the S phase of the cell cycle.

Determination of the S Phase of the Cell Cycle

To the 1 ml of cells removed from flasks as described for the synchronization study, [^{3}H]-thymidine (Swartz-Mann) (specific activitiy 6 Ci/m mole) was added in a 60 ul volume to give a final concentration of 1 uCi/ml. Cells and radioactive precursor were incubated in a Precision Scientific Dubnoff Metabolic Shaking water bath at 37°C for 20 minutes. At the end of the incubation time, 10 ml of ice-cold TdR, (10^{-4}M made up in 0.9% saline) was added to each incubation mixture. This was immediately poured onto 1.2 um pore size RAWP filters (Millipore Corp.) supported in a sampling manifold (Millipore 1225). The acid insoluble fraction was collected on the membranes after washing membranes 3 times with 10 ml volumes of ice-cold 5% trichloroacetic acid (TCA). Filters were air dried and placed in scintillation vials containing 10 ml of toluene cocktail. The toluene cocktail was made by adding 24g PPO (ICN) and 3 g POPOP (New England Nuclear) to 4 liters of Scintanalyzed toluene (Fisher Scientific Co.). Incorporated radioactivity was evaluated in a Beckman LS 9000 Scintillation Counter.

Section I

RESULTS

Growth Characteristics of L1210 Cell Cultures

The characteristic growth pattern of L1210 cells is shown in Figure 1. The broken lines indicate the number of cells/ml after diluting an aliquot of the cell suspension on alternate days. The slopes of the solid lines of cell counts plotted daily are generally parallel indicating that cells were in log growth phase and following a predictable growth pattern. The doubling time was established from these plots and was found to be 20 ± 4 hours.

Effect of Ψox, Thymox and Inox on Proliferation and Viability of L1210 Cells.

The time and concentration dependent effect of Ψox, thymox and inox on L1210 cell proliferation is shown in figures 2, 4 and 6 respectively. A concentration of 50 uM Ψox inhibited cell growth rapidly as shown by counts at 4 hours after exposure. No increase in cell number was observed at 24, 48 or 72 hours, however, no decrease was evident, indicating that a segment of the population was continuing to cycle. At drug levels of 50 uM or less at least 90% of the cells remained unstainable with trypan blue at 24 hours (Figure 3) but the proportion of stainable cells increased with higher drug dose and longer incubation time.

FIGURE 1

PROLIFERATION OF L1210 CELLS

Cells were cultured in FGM in the 37°C CO_2 incubator as described in Materials and Methods. Proliferation was monitored by counting daily using a Coulter counter and cells were diluted on alternate days to contain approximately 200,000 cells/ml.

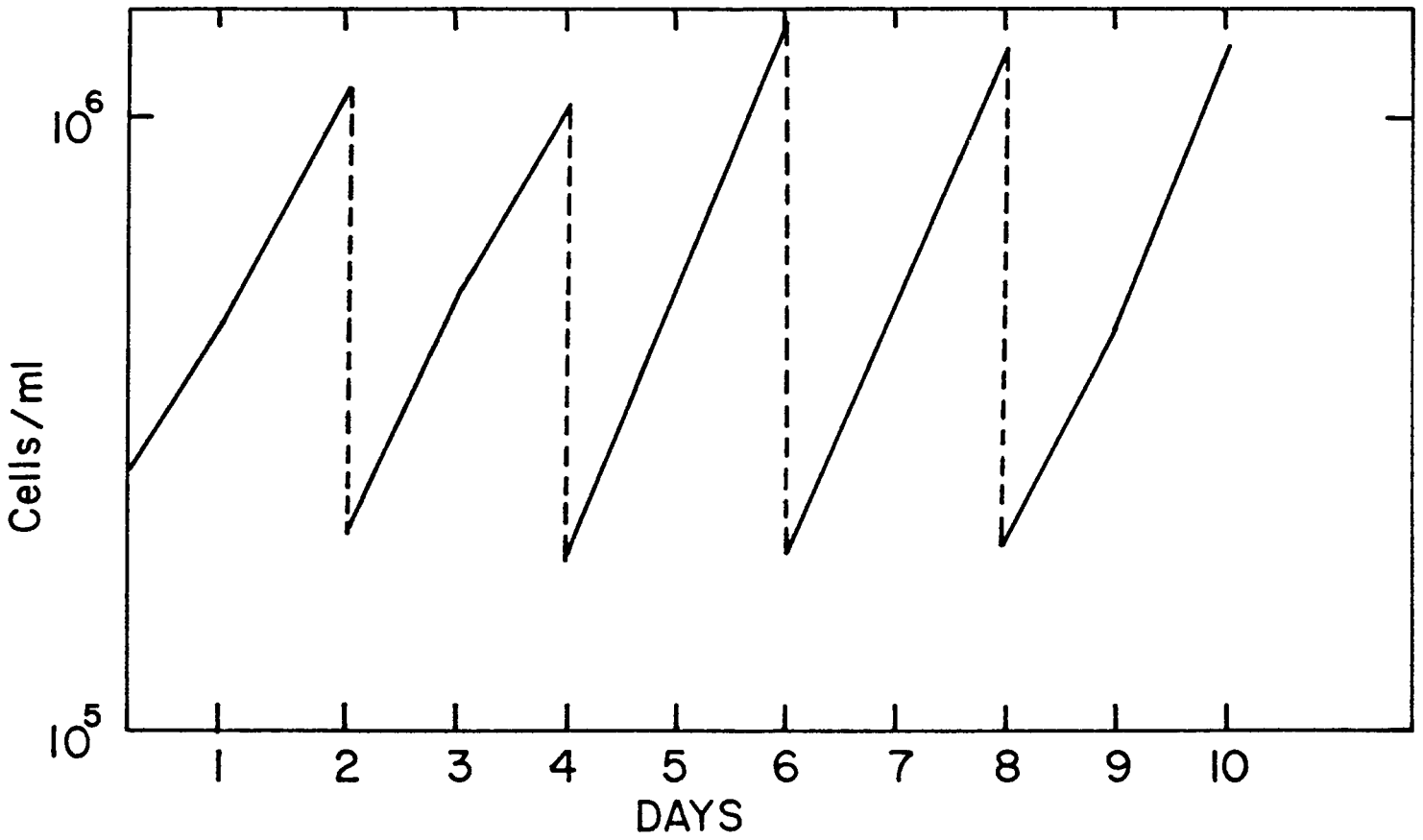

10^6
10^5
Cells/ml
1
2
3
4
5
6
7
8
9
10
DAYS

FIGURE 2

EFFECT OF Ψ-OX ON ASYNCHRONOUSLY GROWING CELLS

Cells were treated with various concentrations of Ψox made up in FGM and added to cell suspensions in 0.03 volumes. Cell counts were made with the Coulter counter at 5, 24, 48 and 72 hours of incubation.

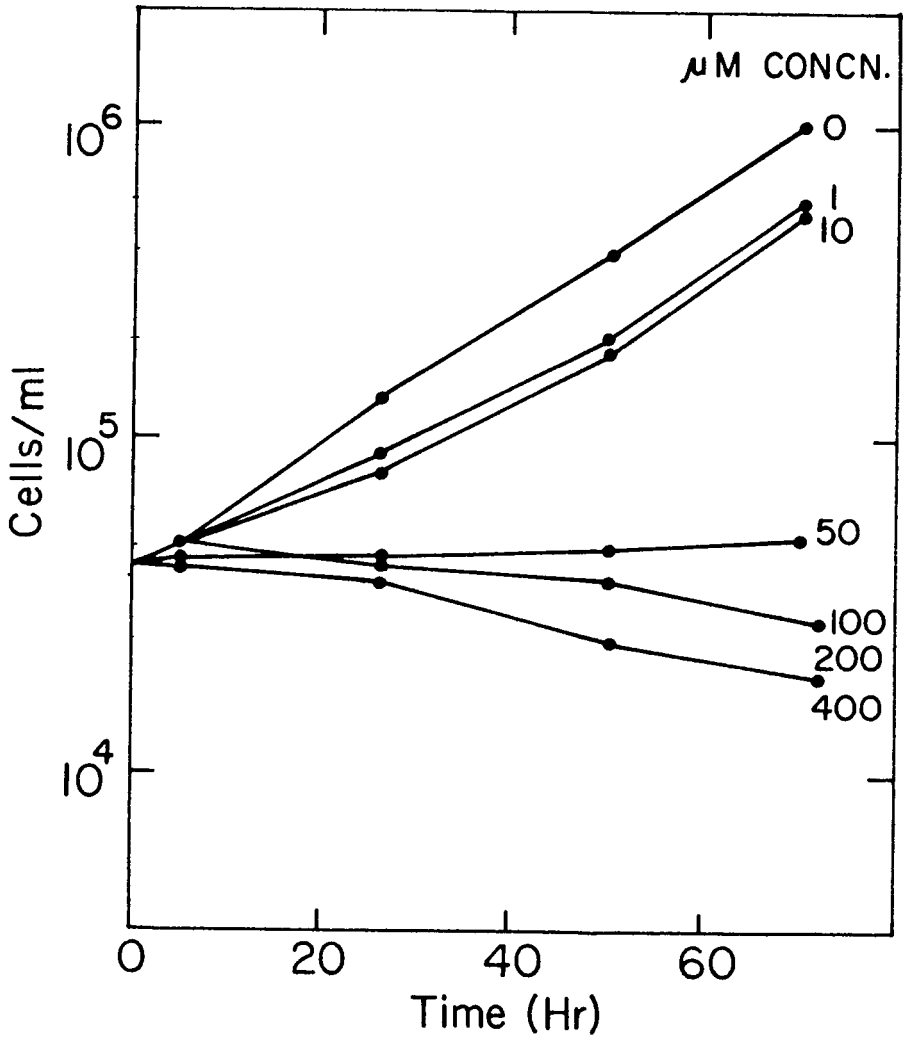
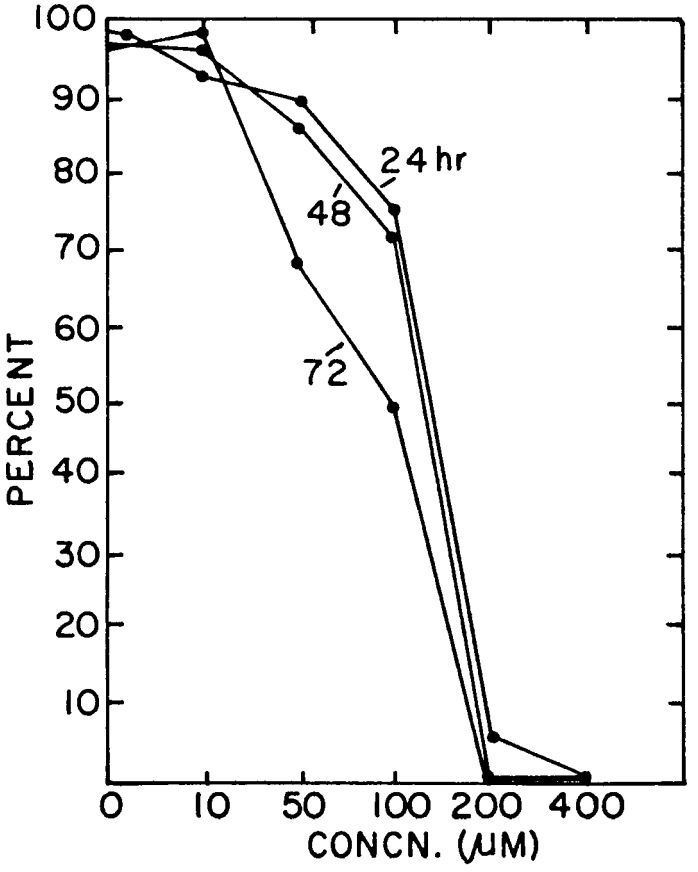

μM CONCN.
0
1
10
50
100
200
400
10^6
10^5
10^4
Cells/ml
0
20
40
60
Time (Hr)

FIGURE 3

PERCENT VIABILITY BY TRYPAN BLUE EXCLUSION
OF CELLS TREATED WITH Ψ-OX

Aliquots of 0.1 ml of cell suspension were mixed with 0.1 ml of 0.4% trypan blue and allowed to stand 5 but not longer than 15 minutes. Stained and unstained cells were counted in a hemacytometer and the percent viability of cells was determined.


100
90
80
70
60
50
40
30
20
10
PERCENT
24 hr
48
72
0
10
50
100
200
400
CONCN. (μM)

Inox was less effective than Uox and a 200 uM concentration was required to block cell proliferation (Figure 4). This is consistent with the report of Plagemann et al. (32) that concentrations of inox greater than 1 uM were required to inhibit cell proliferation completely. There was only a slight decrease in viability (Figure 5) with concentrations of up to 100 uM inox at 24 hours, but viability decreased rapidly with increasing doses after 24 hours.

Thymox, Figure 6, at concentrations greater than 100 uM was cytotoxic at 24 hours. A 100 uM concentration of the drug effectively blocked cell proliferation for 24 hours before producing cell death (Figure 7). As shown in Figure 6, different synthesis lots of these drugs gave slightly different results on proliferation rates of L1210 cells. Based on these titration curves, the concentrations of these drugs used for subsequent tests was 100 uM for Uox and thymox and 200 uM for inox unless otherwise stated.

Soft Agar Cloning Tests

Since the viability of drug treated cells, as measured by trypan blue exclusion at the time of sampling, may not reflect the number of cells which are moribund (but which may still exclude the dye or those cells which may have altered cellular membranes due to drug binding, and although dead, fail to take up trypan blue) cloning procedures were developed. The cloning efficiency (clones counted/input number of cells for untreated controls) ranged from 38 to 25% depending on the conditions of the different tests. This is slightly less than the efficiency of 50-60% reported by Himmelfarb (35) with L1210 cells, but is essentially the same as that obtained by Cass and Au-Yeung (36) of 25-38%.

FIGURE 4

EFFECT OF INOX ON ASYNCHRONOUSLY GROWING CELLS

Cells were treated with various concentrations of inox made up in FGM and added to cell suspensions in 0.03 volumes. Cell counts were made with the Coulter counter at 5, 24, 48 and 72 hours of incubation.

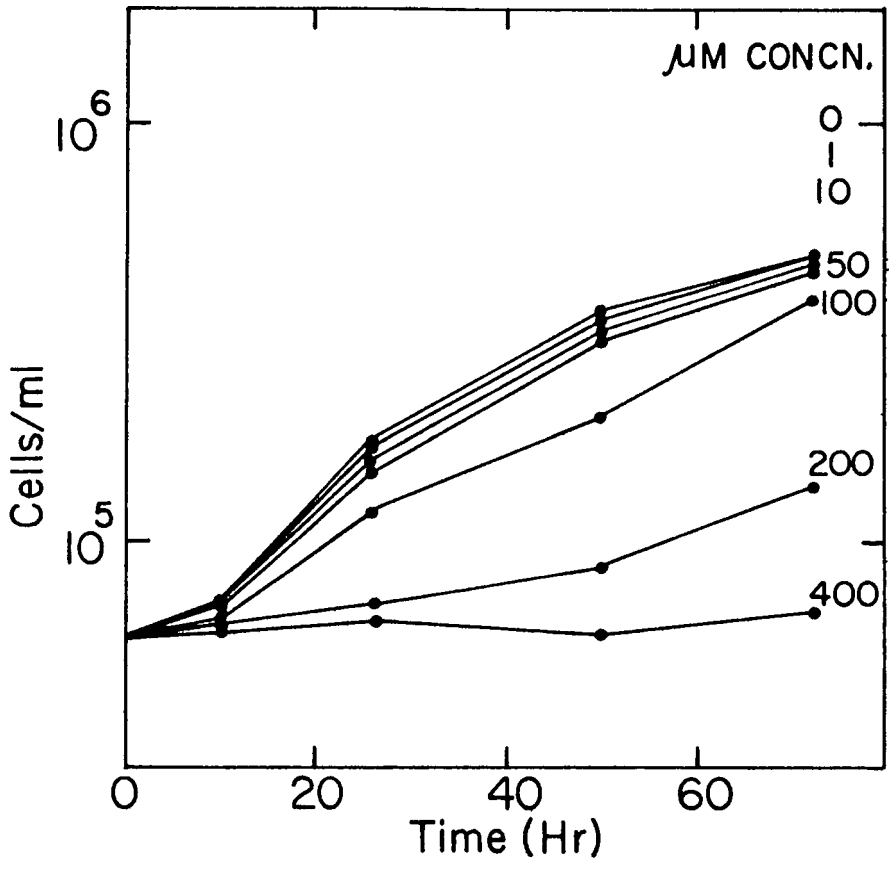

μM CONCN.
0
1
10
50
100
200
400
10^6
10^5
Cells/ml
0
20
40
60
Time (Hr)

FIGURE 5

PERCENT VIABILITY BY TRYPAN BLUE EXCLUSION OF CELLS TREATED WITH INOX

See legend for FIGURE 3.

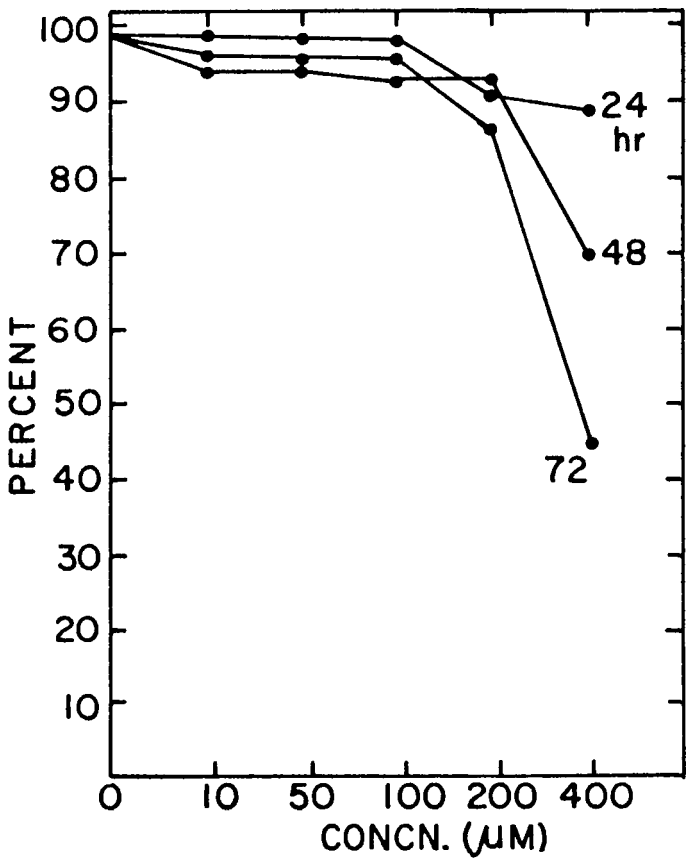

100
90
80
70
60
50
40
30
20
10
PERCENT
24
hr
48
72
0
10
50
100
200
400
CONCN. (μM)

FIGURE 6

EFFECT OF THYMOX ON ASYNCHRONOUSLY GROWING CELLS

Cells were treated with various concentrations of thymox made up in FGM and added to cell suspensions in 0.03 volumes. Cell counts were made with the Coulter counter at 5, 24, 48 and 72 hours of incubation.

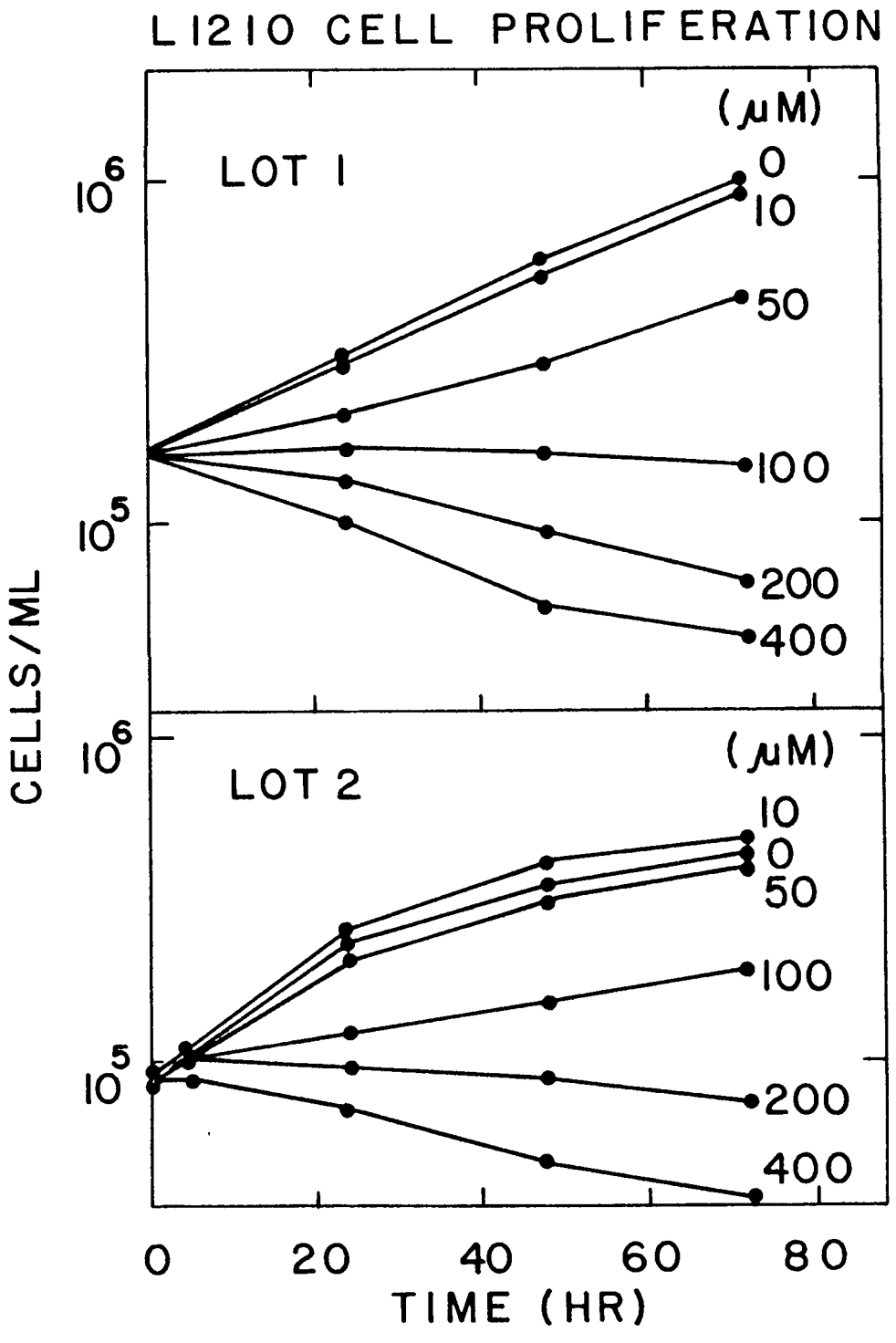

L1210 CELL PROLIFERATION
(μM)
LOT 1
0
10
50
100
200
400
LOT 2
10
0
50
100
200
400
10^6
10^5
CELLS/ML
0
20
40
60
80
TIME (HR)

FIGURE 7

PERCENT VIABILITY BY TRYPAN BLUE EXCLUSION OF CELLS TREATED WITH THYMOX

See legend for FIGURE 3.

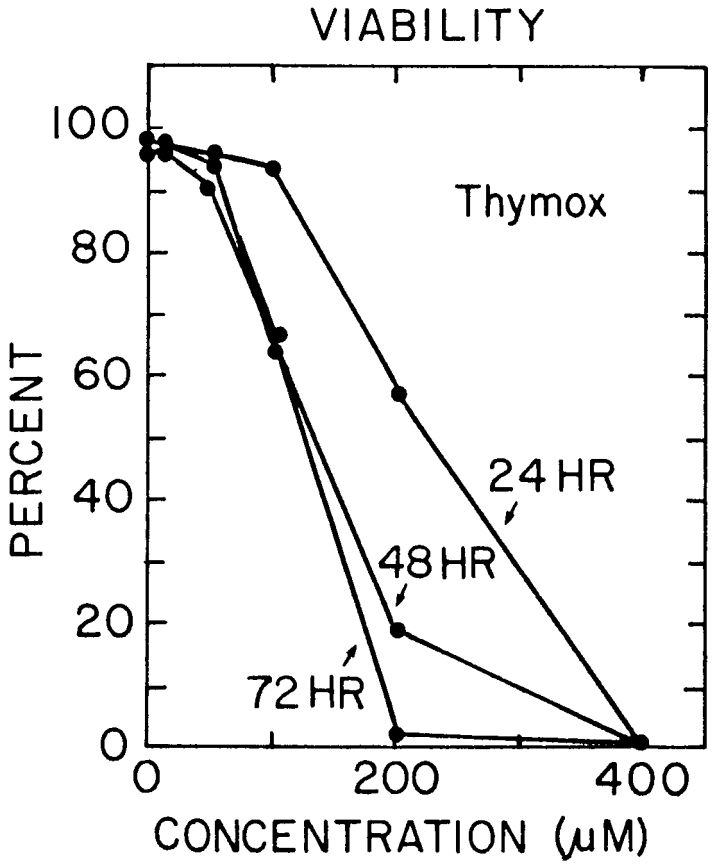

VIABILITY
100
80
60
40
20
0
PERCENT
Thymox
24 HR
48 HR
72 HR
0
200
400
CONCENTRATION (μM)

Table 1 shows the effect of inox and Uox on the relative cloning efficiency of L1210 cells. The relative cloning efficiency (RCE) is defined as the number of clones for treated cells/number of clones for untreated cells adjusted for the total input number of cells for both treated and control cells. After 5 hours of exposure of cells to inox, the number of cells surviving and forming clones was not significantly reduced at 1-100 uM levels of the drug. However, 29% of the cells did not survive 5 hours of treatment with 200 uM inox, and 90.4% failed to survive the 400 uM concentration. The higher RCE of cells treated with 1, 10 and 100 uM concentrations of inox relative to controls is unexplained, but the phenomenon was also observed with 2'dCF treated cells as reported by Cass and Au-Yeung (36). Cells sampled and cloned after 26 hour and 50 hour treatment with inox showed a marked decrease in the number of clones at inox concentrations greater than 10 uM.

In contrast to inox, Uox treated cells at 5 hours of exposure showed only 66% survival at a Uox concentration of 100 uM.

In an attempt to correlate cell viability observed by the cloning technique with that of trypan blue exclusion, samples for both techniques were taken at the same time. Results are shown in Table 2. Inox, from 1-200 uM did not have a significant effect on viablity as shown by trypan blue exclusion (TBE) at 24, 48 or 72 hours. At 400 uM, viability by TBE was 89%, 70% and 40% respectively for these times indicating that cell mortality increased with the greater dose and longer exposure time. However, when cells were exposed to inox for 26 hours and cloned, the surviving fraction shown by RCE was markedly reduced at 50 uM (27%). At 400 uM essentially none of the cells was

TABLE 1

EFFECT OF INOX AND U-OX ON VIABILITY OF L1210 CELLS
AS SHOWN BY THE SOFT AGAR CLONING TECHNIQUE

One ml volumes of cell suspensions in cloning medium at 37°C were dispensed into 4 wells of Falcon multiwell plates for each duplicate sample. Plates were incubated at 37°C in the humidified CO_2 incubator. Clones were counted with a dissecting microscope after 10 days incubation. The RCE was calculated from the ratio of treated/control clones adjusted for the total input number of cells.

EFFECT OF INOX AND ψ-OX ON VIABILITY OF L1210 CELLS AS SHOWN BY THE SOFT AGAR CLONING TECHNIQUE

Sample Time (M)	Drug (uM)		Input Cells/Well	Viability Clones/Well 1	2	3	4	Avg. no Clones/Well	RCE[a]
5	0	Inox	260	112	102	90	110	103.5	100
	1		260	99	156	127	101	120.75	117
	10		270	107	125	111	112	113.75	106
	50		260	111	113	96	94	103.25	100
	100		240	112	95	ND	ND	103.5	108
	200		250	66	79	66	72	70.75	71
	400		230	6	4	13	12	8.75	9.6
26	0	Inox	350	87	104	72	ND	87.66	100
	1		350	79	105	72	62	79.5	90.7
	10		328	63	39	62	36	50.0	60.9
	50		302	24	19	19	ND	20.66	27.3
	100		488	37	29	36	32	33.5	27.4
	200		ND	ND	ND	ND	ND	ND	ND
	400		246	0	1	0	0	0.25	0.41
50	0	Inox	159	56	40	48	ND	48.0	100
	1		153	54	35	48	ND	45.7	99
	10		146	29	41	30	ND	33.3	75.6
	50		ND	ND	ND	ND	ND	ND	ND
	100		ND	ND	ND	ND	ND	ND	ND
	200		ND	ND	ND	ND	ND	ND	ND
	400		2300	3	2	2	1	2	0.28
5	0	ψox	161	-*	--	--	--	94	100
	1		161	--	--	--	--	86	91
	10		158	--	--	--	--	89	96
	50		152	--	--	--	--	72	81
	100		156	--	--	--	--	72	72
	200		143	--	--	--	--	44	53
	400		140	--	--	--	--	9	11

[a] Relative cloning efficiency
ND Not Done
* Individual values not recorded

TABLE 2

COMPARISON OF TRYPAN BLUE EXCLUSION AND RELATIVE CLONING EFFICIENCY FOR VIABILITY DETERMINATIONS

Cells were sampled and processed for TBE and the cloning technique as described in Materials and Methods.

COMPARISON OF TRYPAN BLUE EXCLUSION AND RELATIVE CLONING EFFICIENCY FOR VIABILITY DETERMINATIONS

Drug	Concn. uM	% Viability by TBE Sampling Time (Hr)			% Viability by RCE Sampling Time (Hr)		
		5	26	50	5	26	50
Inox	0	ND	99	98	ND	100	100
	1	ND	98	98	ND	91	99
	10	ND	98	98	ND	61	76
	50	ND	98	95	ND	27	ND
	100	ND	98	95	ND	27	ND
	200	ND	93	94	ND	ND	ND
	400	ND	89	70	ND	0.41	0.28
Wox	0	ND	97	97	100	ND	ND
	1	ND	96	97	91	ND	ND
	10	ND	93	97	96	ND	ND
	50	ND	90	87	81	ND	ND
	100	ND	75	71	72	ND	ND
	200	ND	5	1	53	ND	ND
	400	ND	0	0	11	ND	ND

ND Not Done

viable. These results show that the trypan blue exclusion technique may be valuable as a qualitative indicator of drug activity on cells for dose response studies since it shows the dead cells at the moment of sampling. TBE also reflects the recovery of the population since the percent of unaffected cells continues to increase thus it appears that viability does not decrease as rapidly as in the case of the cloning procedure. However, TBE does not account quantitatively for cells which are blocked by the drug, fail to cycle, and subsequently die after longer incubation periods. Table 2 also shows the comparison of TBE and RCE for Ψox treated cells. Comparison of the RCE and TBE after 5 hours of exposure at 50 and 100 uM Ψox concentrations, shows only 9% difference in the two techniques. Samples treated with 200 and 400 uM Ψox showed higher RCE than TBE probably due to the possibility that a small percent of cells initially affected by the drug recovered and began to grow. This was suggested because of the appearance of clones which were smaller in size than those of the control.

Although the cloning technique more precisely describes the true viability of the cells, it is laborious and subject to fluctuations in experimental conditions. The TBE method of determining viability is rapid, does not require extensive carefully controlled experimental conditions or reagents, and indicates viaibility effectively if data are viewed over a 2 or 3 day testing period as was the case in these experiments.

Effect of Storage on the Biological Activity of Ψox

Because shipping drugs to laboratories across the country for testing under different conditions is necessary, a container of lyophilized Ψox in a tightly sealed shipping container was stored in

dry ice for 3 days and subsequently tested for biological activity in L1210 cells. ψox stored in various other ways was also tested as shown in Figure 8. Cell counts at 72 hours of drug exposure show that there is an approximate 10% reduction between the drug dissolved in distilled water and stored in the refrigerator for 4 days and the drug freshly synthesized, lyophilized and tested immediately after dissolving. ψox appears to be stable after holding the lyophilized drug at room temperature for 3 days. Based on these experiments I was able to determine that shipping ψox in dry ice would not alter its biological activity in L1210 cells. Because of the slight increase in percent proliferation (10%) of ψox dissolved and tested immediately, we followed this procedure in subsequent testing.

Stability of Biological Activity of ψox and Thymox Incubated at 37°C in FGM

Cysyk and Adamson reported that the oxidation product of inosine, inox, reacts with amino acids which are components of the L1210 growth medium (FGM) (44). The cytotoxicity of inox on L1210 cells was reduced by these interactions. To detect whether the activity of ψox and thymox was significantly reduced by the conditions of testing (which included incubation of FMG at 37°C in the humdified CO_2 incubator), and to determine how long the drugs retained their biological activity under these conditions, experiments were done as described in Materials and Methods. Results are shown in Figure 9. The percent change in proliferation rates of treated and control cells over a 24 hour period (from 0-24, 24-48, and 48-72 hours) indicates that incubating ψox in FGM results in loss of biological activity after 1 hour of incubation.

FIGURE 8

STABILITY OF ψ-OX AFTER STORAGE

ψox was dissolved in H_2O, filtered and tested at a final concentration of 100 uM in suspensions of asynchronously growing cells containing 1.8-2.8 x 10^5 cells/ml. Cell counts were plotted as percent of controls. Treatment of the drug was as follows:

—·—·—Synthesized, lyophilized and dissolved and tested within 1 day.

. . . . . Lyophilized, stored in a container packed in dry ice for 3 days, dissolved and tested.

- - - - - Lyophilized and allowed to stand at room temperature for 3 days.

– – – – – Lyophilized, dissolved and held at 4°C for 4 days.

——— Lyophilized, dissolved and tested immediately.

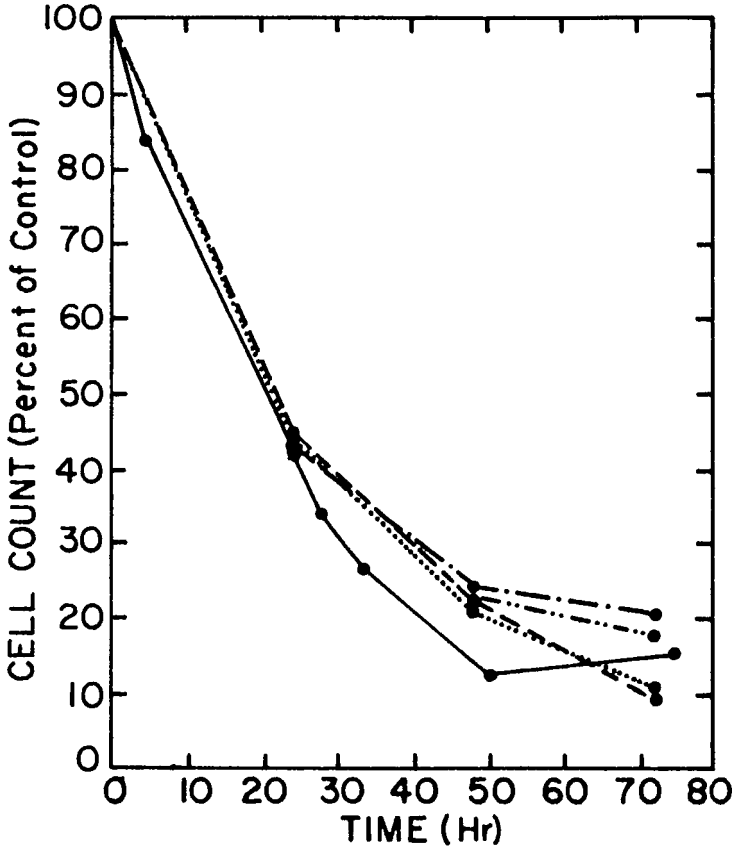
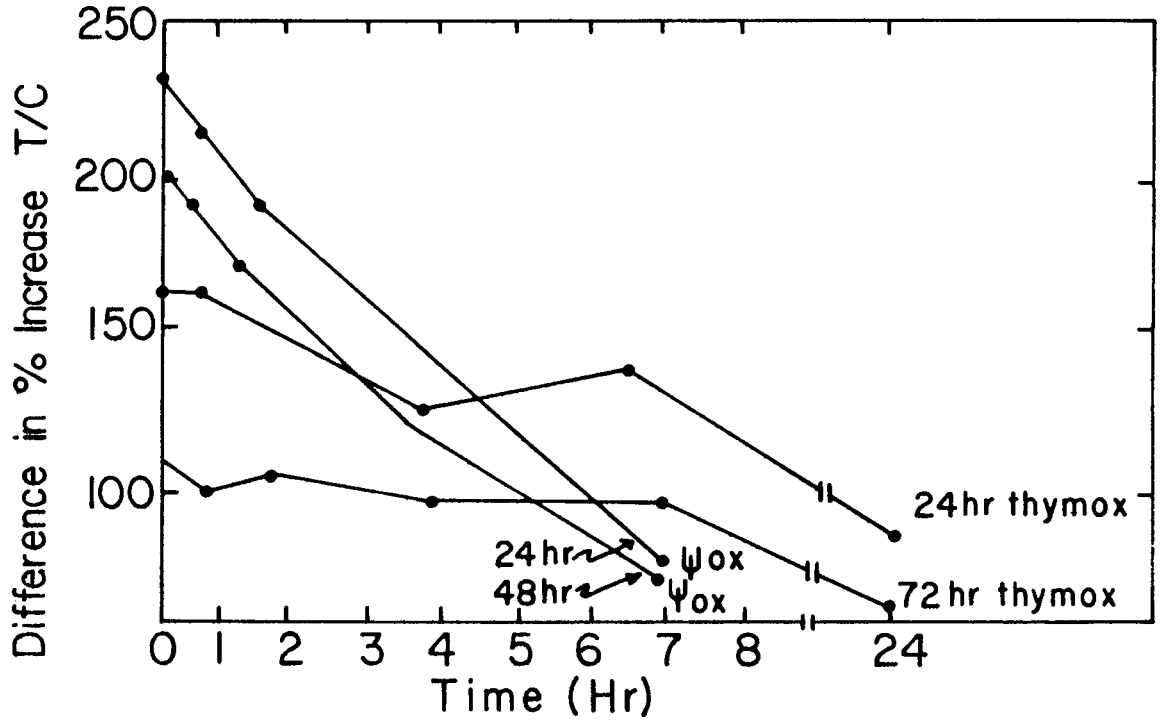

CELL COUNT (Percent of Control)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
TIME (Hr)

FIGURE 9

STABILITY OF BIOLOGICAL ACTIVITY AFTER INCUBATING DRUGS AT 37°C IN FGM

ψox and thymox at 200 uM were incubated at 37°C in FGM. At the times shown, L1210 cells were added to give a final cell concentration of 1×10^5 cells/ml. The cell proliferation rate was monitored at 24 hour intervals and the difference in percent increase between treated and control cells was plotted for the 24 hour period shown.


250
200
150
100
Difference in % Increase T/C
0 1 2 3 4 5 6 7 8 24
Time (Hr)
24hr thymox
72 hr thymox
24hr
48hr
ψox
ψox

There was no significant reduction of activity for thymox until 2 hours of incubation in FGM as seen by the 24 hour plot. The half-life of Ψox in FGM under these conditions is 5 hours and only 13% of the activity remained after 7 hours of incubation. Thymox was more stable and retained 50% of its activity at 24 hours of preincubation in FGM.

Minimum Effective Exposure Time of L1210 Cells to Ψox, Thymox and Inox

In many instances, it is necessary to perform short-term tests such as chromosome damage or synchronization studies in which the cells are subjected to drug treatment for 1 to 2 hours and then the drug effect is evaluated. Minimum exposure times were determined and evaluated for effect on cell proliferation as described in Materials and Methods. Results shown in Table 3 are expressed as the difference in percent proliferation of treated and control cells for each 24 hour evaluation time (0-24, 24-48 and 48-72 hours).

Cells treated with Ψox for 0.5 hour and evaluated at 24 hours showed a 261% increase whereas the control population increased 394%, a difference of 133%. After 1 hour of exposure to Ψox, there was a 239% difference in proliferation rate between controls and treated cells. This represents about 60% greater effect than incubation for 0.5 hours. Since Ψox is 50% inactivated by incubation in FGM after 5 hours the greater reduction in proliferation rate is unexpected at 4 hours. It is possible that these drugs are concentrated within the cells as reported for vinblastine (45). The biological importance depends on the concentration of the drug in the cell. For vinblastine there was a 9-fold concentration intracellularly. Thus, the reduction in proliferation rates seen at 7 hours when the drug is only approximately

TABLE 3

MINIMUM EFFECTIVE EXPOSURE TIME OF CELLS TO DRUGS

Cells were treated with 100 uM concentrations of the drugs for the times shown, then centrifuged and resuspended in drug-free medium. Proliferation was monitored and results are shown as percentage increase in cell number over 24 hour periods.

MINIMUM EFFECTIVE EXPOSURE TIME OF CELLS TO DRUGS

Drug	Exposure (Hr)	Percent Proliferation Rate								
		24 Hours			48 Hours			72 Hours		
		T[a]	C[b]	Diff.	T	C	Diff.	T	C	Diff.
ψox	0.5	261	394	133	ND	ND	ND	204	154	50+
	1	160	399	239	ND	ND	ND	108	134	26
	2	78	301	223	ND	ND	ND	111	112	1
	4	34	297	263	ND	ND	ND	69	121	52
	7	36	252	216	ND	ND	ND	56	121	65
	24	82	455	373	ND	ND	ND	50	37	13+
Thymox	0.5	ND	ND	ND	153	163	10	115	110	5+
	1	78	112	34	151	131	20	120	123	3
	2	84	125	41	137	150	13	124	145	21
	4	56	103	47	132	161	29	115	104	11+
	7	ND	ND	ND	ND	ND	ND	ND	ND	ND
	24	62	134	72	104	150	46	178	133	45+
Inox	0.5	567	394	173+	ND	ND	ND	188	154	34+
	1	280	399	119	ND	ND	ND	125	134	9
	2	281	301	20	ND	ND	ND	119	112	7+
	4	195	297	102	ND	ND	ND	120	112	1
	7	195	252	57	ND	ND	ND	112	121	9
	24	255	455	200	ND	ND	ND	239	37	202+

[a] Treated
[b] Control
+ Increase of proliferation of treated was more than that of control.
ND Not done

13% active (Figure 9) may represent permeability and internal concentration of ψox within the cells early in the incubation period. The large percent difference in the rate at 24 hours (373%) is due to this and to a burst of proliferation of control cells.

After a 1 hour exposure the difference in the percent rate of proliferation between cells treated with 100 uM thymox and untreated controls was 35% (evaluated at 24 hours). From the previous section, we know that thymox is still 50% active after preincubation with FGM for 24 hours. It is, therefore, not surprising that the greatest effect on cell proliferation is seen at 24 hours when the difference in the proliferation rate of control and thymox treated cells is 72%. However, it can be stated that from these data thymox effectively reduces the proliferation rate of cells exposed for a minimum of 1 hour.

The proliferation rate of cells treated with inox for 0.5 hours actually increased during the 48 hour evaluation period. Treated cells increased 567% while controls increased only 394%. The first significant reduction in inox treated cell proliferation occurred after a 1 hour exposure and maximum effect was seen after 24 hours of exposure.

Conclusions from this data are that thymox and inox exert significant (though less than ψox) effect on cell proliferation after 1 hour of exposure. Subsequent short-term tests may be done using a 1 to 2 hour exposure time for these drugs. This data also shows that removing the drugs from the medium and adding fresh medium does not release cells from the effect of the drugs. From the results of these tests and the cloning data, it may be concluded that the effect of these drugs is irreversible.

Flow Microflurometry (FMF) Profiles of Ψox, Thymox and Inox Treated Cells

FMF has been used by Tobey and Crissman (46, 47) and Tobey et al. (48) to assess the effects of drug perturbations on cell cycle progression. This technique allows rapid precise measurements and has as an advantage the ability to monitor non-traversing or blocked cells. Figures 10, 11, and 12 show DNA profiles for Ψox, inox and thymox respectively. For cells treated with Ψox for 5 hours, the first observable effect is an increase in the number of cells in the S phase of the cell cycle at all drug concentrations. This effect does not appear to persist at the 26 hour sample time, however, with 50 and 100 uM concentrations of the drug, at 26 hours there is a drastic accumulation of cells in the G2 + M phase of the cell cycle. The G2 + M block persisted for the duration of the test period up to 72 hours but a part of the population was either unaffected by the drug or overcame the effect as shown by presence and variation in size of the G1 peak.

Inox has been reported by Plagemann et al. (32) to induce an accumulation of cells in G2 + M. My results (Figure 11) confirm this. The effect at 5 hours in slowing cell passage through S is dose dependent and the G2 + M block at 26 hours is similar to that of Ψox with the exception that higher concentrations of inox were required to produce comparable G2 + M arrest (200 uM for inox, 50 uM for Ψox).

FMF profiles for thymox (Figure 12) show the same patterns of early slow down in S and incomplete arrest in G2 + M as seen with Ψox and inox. The G2 + M block of these cells was maintained over the observation period of 72 hours. This suggests an irreversible block of affected cells as did the previous data.

FIGURE 10

THE EFFECT OF Ψ-OX ON THE DNA DISTRIBUTION OF CULTURED L1210 CELLS AS SHOWN BY FMF.

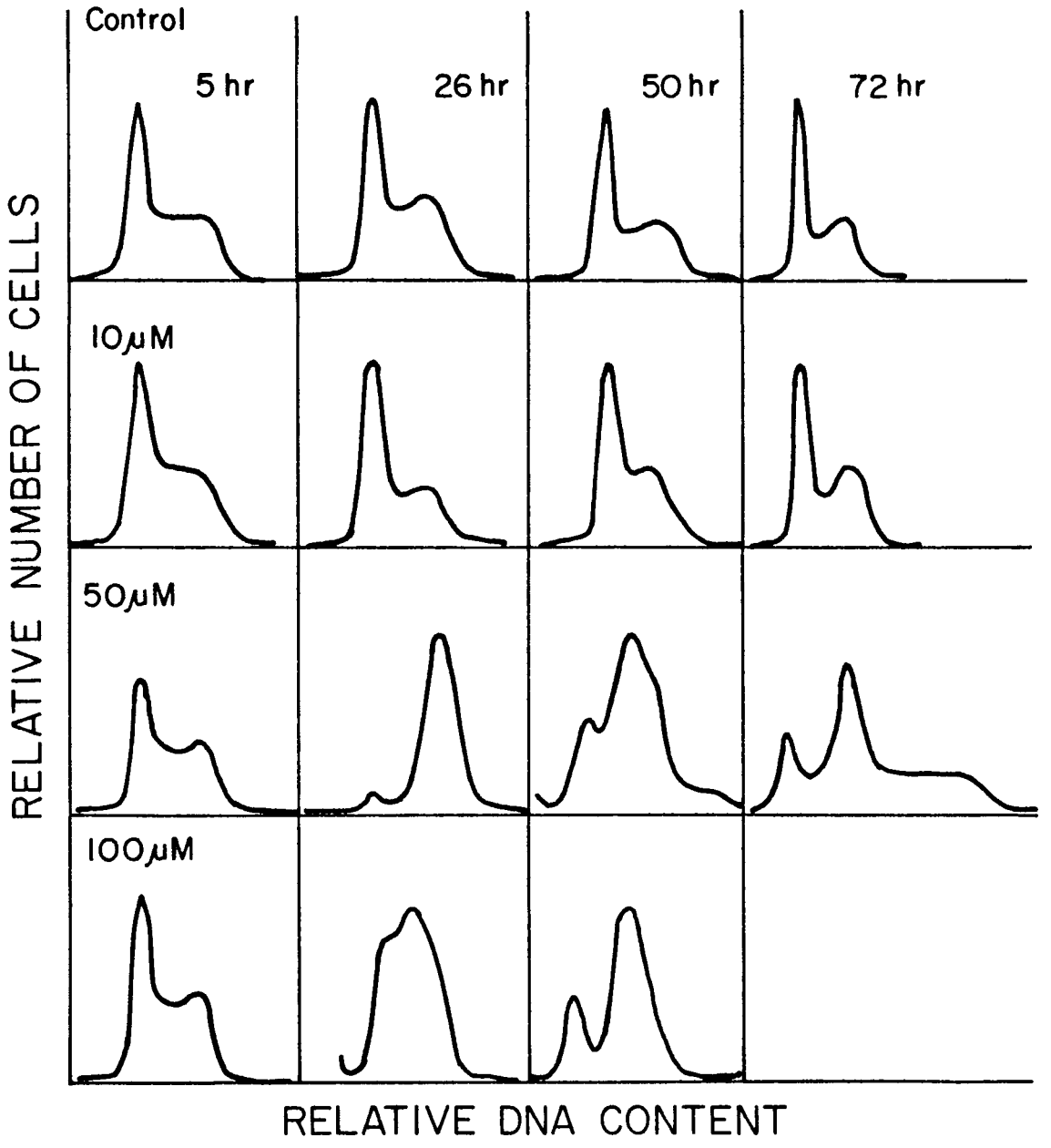

Control
5 hr
26 hr
50 hr
72 hr
10μM
50μM
100μM
RELATIVE NUMBER OF CELLS
RELATIVE DNA CONTENT

FIGURE 11

THE EFFECT OF INOX ON THE DNA DISTRIBUTION OF L1210 CELLS AS SHOWN BY FMF.

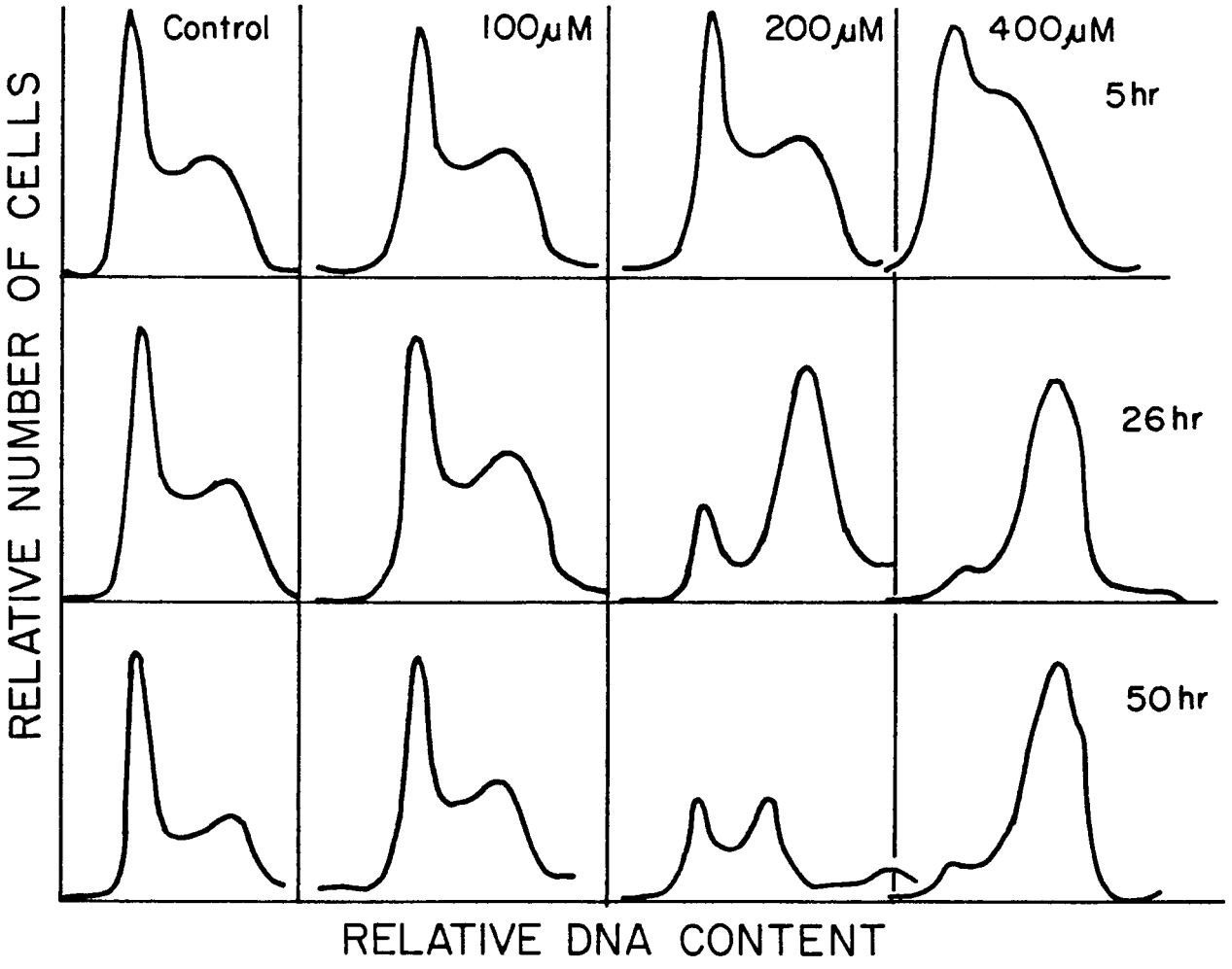
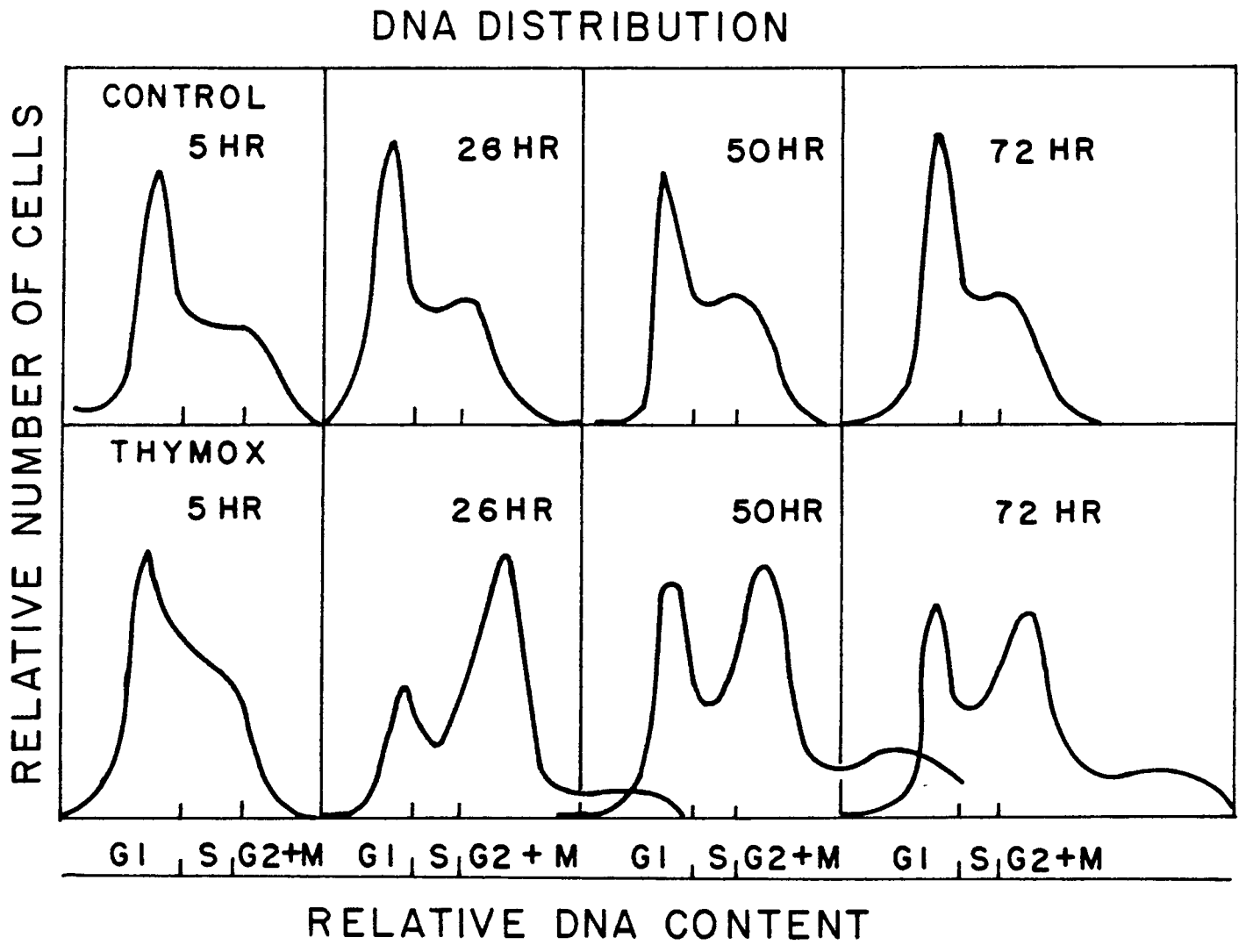

Control
100μM
200μM
400μM
5hr
26hr
50hr
RELATIVE NUMBER OF CELLS
RELATIVE DNA CONTENT

FIGURE 12

THE EFFECT OF THYMOX ON THE DNA DISTRIBUTION OF L1210 CELLS AS SHOWN BY FMF.


DNA DISTRIBUTION
CONTROL
5 HR
26 HR
50 HR
72 HR
THYMOX
5 HR
26 HR
50 HR
72 HR
RELATIVE NUMBER OF CELLS
G1 S G2+M G1 S G2+M G1 S G2+M G1 S G2+M
RELATIVE DNA CONTENT

Effect of Ψox, Thymox, Inox and Vinblastine (VBL) on the Mitotic Index of L1210 Cells

Plagemann et al. (32) have presented indirect evidence that cells treated with inox are blocked irreversibly in G2 by stating that mitotic spindle inhibitors which block cells in mitosis may be washed away and cells will continue progression through the cycle. Their data shows, and this research confirms, that the effect of inox cannot be washed away. To determine whether the cells accumulated in G2 + M were blocked in mitosis as with VBL or colchicine, the mitotic indices of cells treated with the dialdehyde derivative drugs and VBL were examined. Results are shown in Table 4. Each value represents three to six separate experiments and 100-200 cells were counted for each value. The ranges of mitotic indices for different experiments for the control cells was 4-8% with a mean of 6 and 5% as shown in Table 4 for 24 and 48 hours respectively. For thymox treated cells the mean of four experiments was 5, for both the 24 and 48 hour sample times, and the range was 2-8%. The mean of three experiments for Ψox was 3 and 2 respectively and the range was 1-4%. For three inox experiments the mean mitotic index was 5% and 4% and the range was 3-6% at the times tested. Cells treated with 0.1 ug/ml VBL, which gives FMF profiles similar to the oxidation product drugs, accumulated 62% of the cells in mitosis at 24 hours (See Figure 13).

Table 5 shows the accumulation of cells in mitosis over a 5 hour period. Cells were treated with 100 uM Ψox inox and thymox for 2 hours and VBL was added at a final concentration of 0.2 ug/ml. Control cells which received no drugs accumulated metaphase mitotic figures as shown. The cells treated for 2 hours with drugs and subsequently

TABLE 4

MITOTIC INDEX OF DRUG TREATED CELLS

Cells were prepared for mitotic index determinations as described in Materials and Methods at 24 and 48 hours after drug exposure. The dialdehyde derivative drugs were tested at final concentrations of 100 uM for Nox and thymox and 200 uM for inox. VBL was tested at 0.1 mg/ml.

MITOTIC INDEX OF DRUG TREATED CELLS

EXPOSURE TIME (HR)	% MITOSES CONTROL	THYMOX	U-OX	INOX	VBL
24	6	5	3	5	62
48	5	5	2	4	ND

FIGURE 13

ACCUMULATION OF CELLS IN MITOSIS BY VINBLASTINE (VBL)

VBL was tested at concentrations ranging from 0.1 mg/ml to 1.0 mg/ml. Cells were treated for 24 hours with the drug, then sampled and the mitotic index determined as described in Materials and Methods.

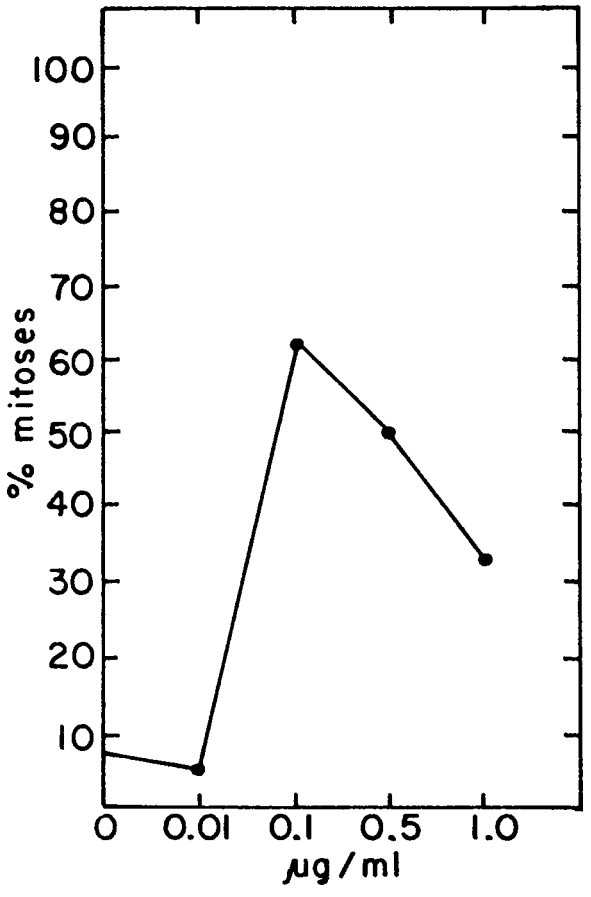

100
90
80
70
60
50
40
30
20
10
% mitoses
0
0.01
0.1
0.5
1.0
µg / ml

TABLE 5

ACCUMULATION OF DRUG TREATED CELLS IN METAPHASE BY ADDITION OF VBL

Cells were treated with 100 uM ψox, thymox and inox for two hours and VBL was added at a final concentration of 0.2 ug/ml. Cells to which no VBL was added served as controls. The mitotic indices were determined as described in Materials and Methods.

ACCUMULATION OF DRUG TREATED CELLS IN METAPHASE BY ADDITION OF VBL

TIME AFTER ADDITION OF VBL (Hr)	MITOTIC INDEX			
	Thymox	Inox	Ψox	Control
2	1	5	3	9
4	4.6	8	2	19
6	4	12	4	31
no VBL added				
2	ND	ND	ND	ND
4	5	4	1	6
6	4	7	1.3	7

with VBL showed slight increases in numbers of mitotic figures (when compared to those with no VBL) probably due to some cells being unaffected by Ψox and thymox, but the numbers were significantly less than controls. Inox treated cells had a mitotic index at 6 hours after addition of VBL of 12% which is higher than Ψox or thymox. Since inox at 100 uM is less effective than Ψox or thymox at equimolar concentrations this result is apparently due to drug concentration and probably does not represent a different mechanism of action.

These data show conclusively that Ψox, thymox, and inox block cell cycle progression as shown by FMF and the cells are accumulated in G2 and not in mitosis as with VBL.

Synchronization of L1210 cells

For purposes of designing cancer chemotherapeutic drugs and with analytical technology available such as FMF, the practicality of understanding the biochemical action of drugs and correlating biological activity to the cell cycle phase in which the drug exerts its greatest effect is obvious. The oxidation product drugs block cells in G2 as shown by FMF and mitotic index studies, however, very few drugs exert maximum cytotoxicity on the cells in G2. Among drugs most toxic to cells in G2 are bleomycin and BCNU (1, 3-bis(2-chloroethyl)-1-nitrosourea) (49). To determine if the G2 block of cells treated with oxidation product drugs was due to maximum effect in G2 per se, the L1210 cells were synchronized with low dose TdR as described in Materials and Methods. Excess thymidine blocks DNA synthesis by a feedback mechanism in preventing formation of deoxycytidine in the pathway to DNA (50).

Cells synchronized by this method are blocked at the G1/S border and when excess thymidine is removed, cells proceed through the cycle in a synchronous wave.

The phases of the L1210 cell cycle after release from the TdR block were monitored by FMF (indicative not absolute) and by sampling at 2 hour intervals for the 16 hour test period to determine a cell count, DNA synthesis and mitotic index. Results of FMF analyses are shown in Figure 14. The single peak of the synchronized cells indicates that most of the cells in the population contained only one relative amount of DNA or that they are relatively well synchronized at 0 hour after release (Figure 14A). With time the peaks move to the right (Figure 14B) indicating an increase in the amount of DNA as the population progressed through S into G2 and the beginning of M. Figure 14C indicates relative amounts of DNA in G2 and mitotic cells. The shoulders are assumed to be due to cells in various stages of G2 and mitosis. As cells proceed thorugh G2 and M back to G1 and S (Figure 14D) one major peak is again seen. Figure 14E shows cells in early S and peak S at 14 and 16 hours respectively. This FMF data in conjunction with data in Figure 15, shows visually the position of the majority of cells during the cycle. Also, the narrowness of these peaks gives some indication of the degree of synchrony.

The method for measuring the phases of the L1210 cell cycle was that Volpe and Volpe (51). Figure 15 shows that cells synchronized by Thilly's method (43) have a cycle length of at least 15 hours. After release, the cell number remained constant for 6 hours then abruptly increased 1.6 times over a 6 hour period to a second plateau beginning at 12 hours. There was a burst of mitosis peaking at 8 hours (Figure 15B)

FIGURE 14

PROGRESSION OF SYNCHRONIZED CELLS THROUGH THE CELL CYCLE AS SHOWN BY FMF

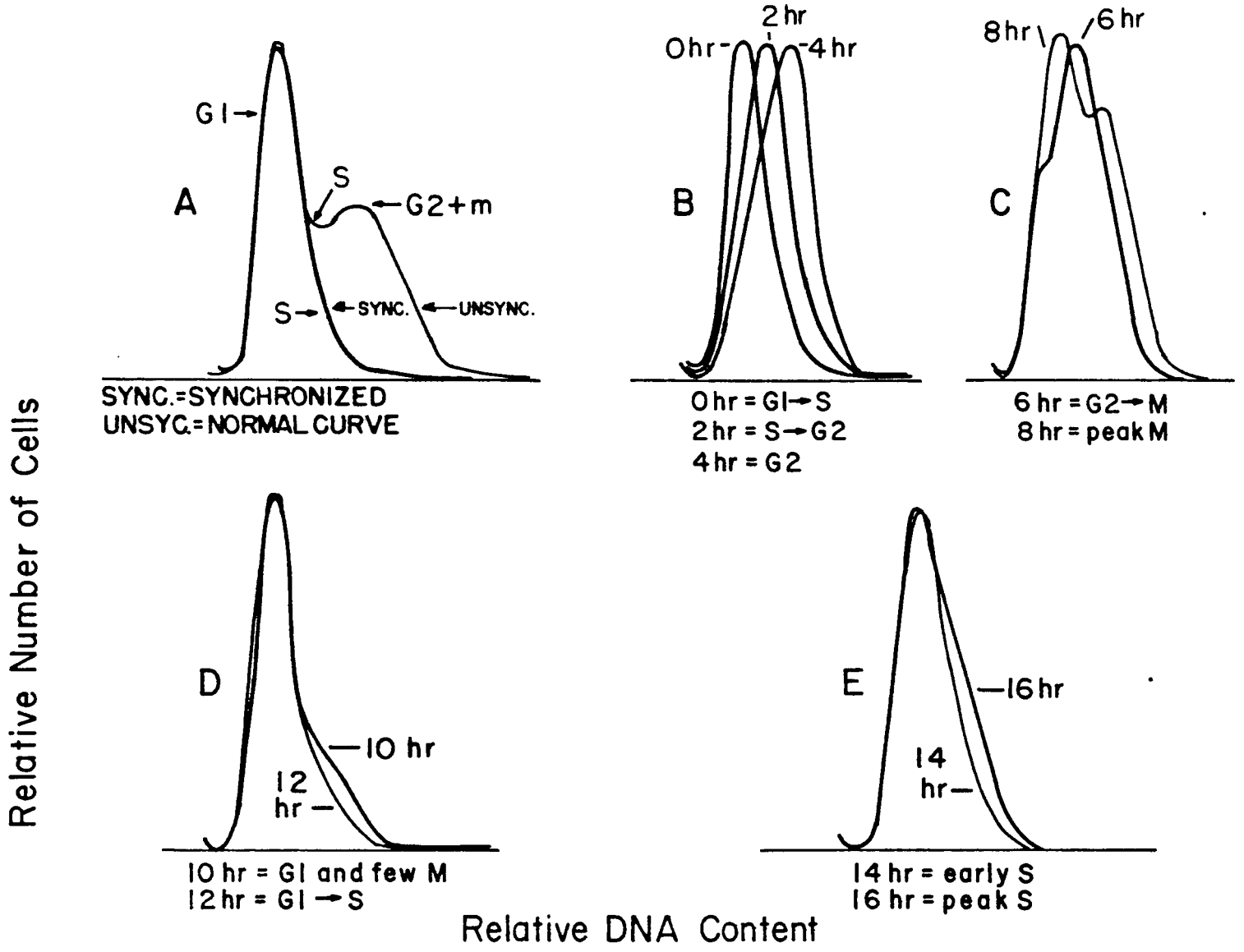
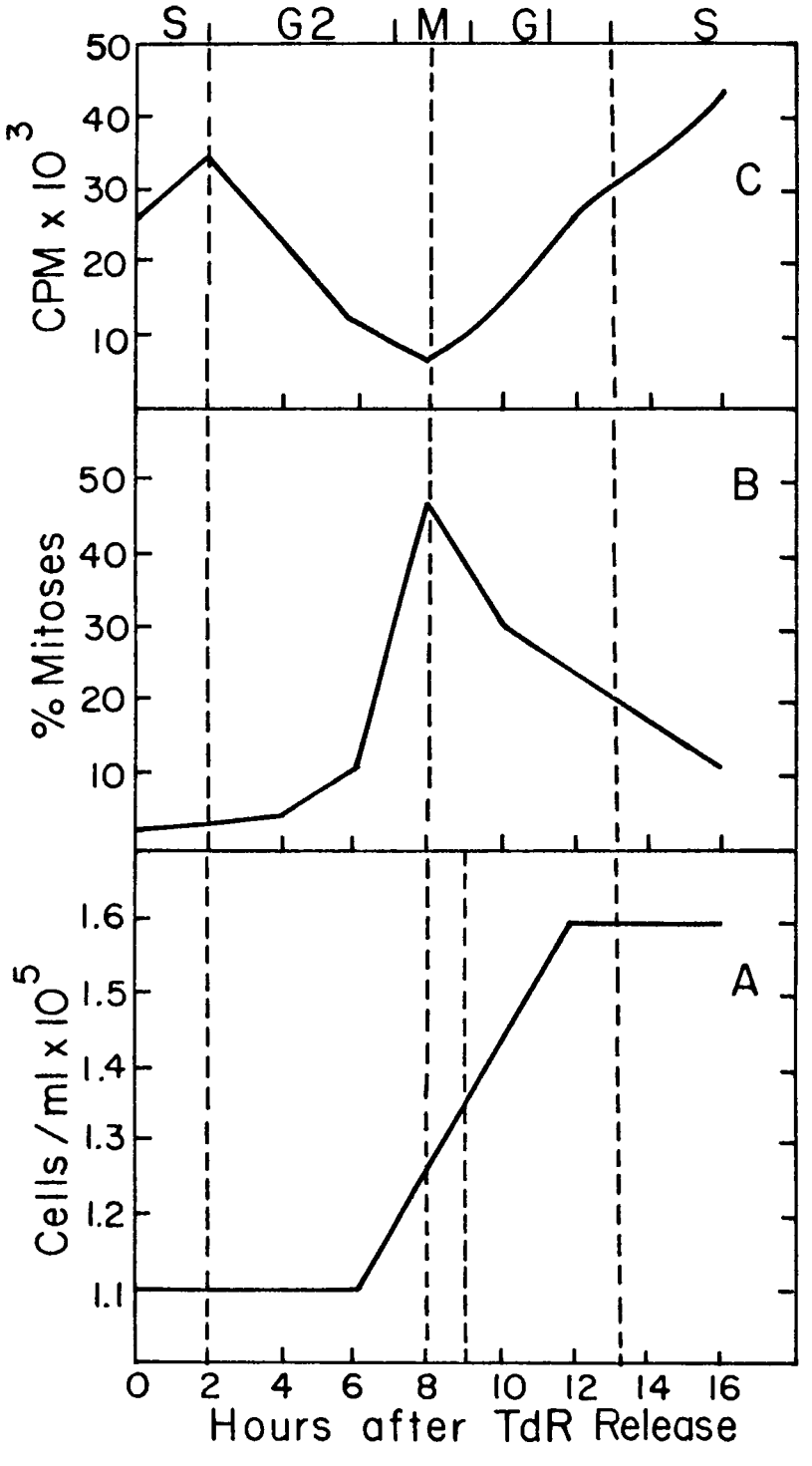

Relative Number of Cells
A
G1
S
G2+m
S
SYNC.
UNSYNC.
SYNC.=SYNCHRONIZED
UNSYC.=NORMAL CURVE
B
2hr
0hr
4hr
0hr = G1→S
2hr = S→G2
4hr = G2
C
8hr
6hr
6 hr = G2→M
8 hr = peak M
D
10 hr
12 hr
10 hr = G1 and few M
12 hr = G1→S
E
16hr
14 hr
14 hr = early S
16 hr = peak S
Relative DNA Content

FIGURE 15

LENGTH OF THE PHASES OF THE L1210 CELL CYCLE
OF CELLS SYNCHRONIZED BY TdR

Cells were synchronized as described in Materials and Methods. Samples were removed at 2 hour intervals and tested to determine the cell cycle phase. Curve A represents cell growth, curve B shows cell division as percent mitoses, and curve C represents DNA synthesis.


S
G2
M
G1
S
50
40
30
20
10
CPM x 10^3
C
50
40
30
20
10
% Mitoses
B
1.6
1.5
1.4
1.3
1.2
1.1
Cells / ml x 10^5
A
0
2
4
6
8
10
12
14
16
Hours after TdR Release

after the release of the TdR block. At this point, at least 47% of the cells in the culture were dividing. Maximum DNA labeling occurred at 2 and 16 hours which was the last time point evaluated (Figure 16C). The midpoint of the logarithmic increase cell growth curve (Figure 15A) is defined as the end of M and the beginning of G1 (51). If M is defined as twice the time between the peak of curve B and the midpoint of curve A (51), then in this case M is 2 hours long. G1 lasts from the midpoint of curve A to the beginnning of the new S phase, curve C and is approximately 3 hours. The S phase lasts from the beginning of S to the peak of the S curve (C) or at least 4 hours. G2, defined as beginning from the peak of S (curve C) at 2 hours to the peak of M (curve B) at 8 hours (51), is approximatley 6 hours long.

From the shape of these curves, it can be seen that some mitosis is occurring in the G2 and G1 phases and that some DNA synthesis is occurring in the G1 phase. However, the degree of synchrony of these cells was acceptable since the majority of the cells were within defined phases when drugs were added. It is difficult to determine the exact length of the cycle because no analyses were done after 16 hours. However, the cells had a cycle time of at least 15 hours which included 2 hours for mitosis, approximatley 4 for S, 3 for G1 and 6 for G2.

Effect of Ψox, Thymox, Inox and VBL on Synchronized L1210 Cells

The criterion for effectiveness of drugs added to cells at any given time after release from the TdR block was the cell count 48 and 72 hours after addition for 2 hours and removal of the test drug. Cell counts were expressed at percent of untreated control cell counts for each time point. FMF profiles are presented as visual supportive data.

Table 6 shows the evaluation times and effect of 0.1 ug/ml VBL on the proliferation synchronized L1210 cells. VBL had a major effect on cells when added at 0, 10, 12 and 14 hours after TdR release. A graph of the plots of treated/control cells at 56-72 hours after addition and removal of VBL is shown in Figure 16. As shown, the major effect of VBL was in late G1-early S, during S, and mitosis. This is consistent with results of Madoc-Jones and Mauro (52) in experiments with HeLa cells synchronized by mitotic selection. Using 0.3 ug/ml VBL and exposing cells for 3 hours (this research, 0.2 ug/ml VBL for 2 hours), they reported that cells were more sensitive in S and in late G1 than in G2. Cells exposed during peak S periods proceed at normal rates to mitosis and were arrested irreversibly. This accounts for the reduction of cells in my experiments during the period 2-4 hours representing peak S and early G2. There is less effect on cells in mid-G2, but during mitosis (7-9 hours), there is some reduction and again in G1 and early S at 10-14 hours cell counts were reduced.

FMF profiles confirm these conclusions. Figure 17 shows profiles of cells sampled at the same times that plots were made for Figure 16. Cells treated from 0-2 hours gave profiles representative of mostly dead cells (personal communication Don Schomer, Univ. Houston). Control cells were still progressing at 72 hours after TdR release in pseudo-synchronous waves (see dashed-line profiles). At 2 hours there is a slow-down of progression of VBL treated cells in S and at 4 and 6 hours there is no difference between treated and control cells. At 8 hours, VBL treated cells show a reduction in the G2 + M peak as compared to the control consistent with the effect during mitosis. Patterns for controls were not done for 10, 12 and 14 hour time points, however,

TABLE 6

THE EFFECT OF VBL ON SYNCHRONIZED L1210 CELL PROLIFERATION

Cells were synchronized and the TdR block removed. At two hour intervals thereafter VBL, 0.1 ug/ml, was added to cells. After incubation with the drug for two hours, cells were centrifuged and resuspended in drug-free medium. Cells were counted at the times shown in brackets and the percent of the control cell count for each time point over 24 hour periods was calculated.

THE EFFECT OF VBL ON SYNCHRONIZED L1210 CELL PROLIFERATION

Time After TdR release (Hr)	Cell Proliferation					
	Cells/ml	% of Control	Cells/ml	% of Control	Cells/ml	% of Control
0	109,700 (0)	100	64,420 (48)	14	65,860 (72)	8
2	106,720 (0)	98	278,000 (46)	64	536,000 (70)	57
4	103,880 (0)	100	135,100 (44)	29	180,860 (68)	19
6	103,500 (0)	97	173,080 (42)	41	298,000 (66)	36
8	117,820 (0)	99	167,280 (40)	33	280,000 (64)	30
10	152,700 (0)	105	114,280 (38)	24	125,960 (62)	15
12	158,800 (0)	99	112,880 (36)	27	115,320 (60)	15
14	164,260 (0)	101	124,700 (34)	21	124,240 (58)	11
16	163,060 (0)	100	125,440 (32)	27	126,280 (56)	14

FIGURE 16

CELL CYCLE SPECIFIC ACTIVITY OF VBL

Plots were made of the percent of control values of counts of cells made at 72-56 hours after treating synchronized cells for 2 hours at 2 hour intervals with VBL.

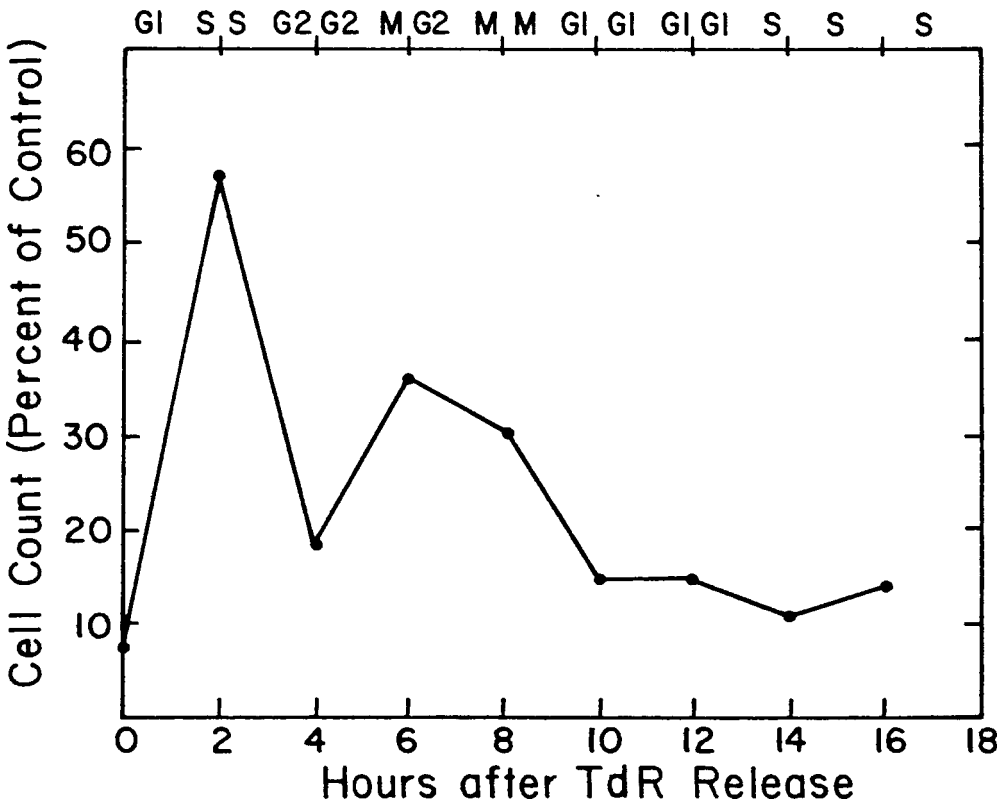

G1 S S G2 G2 M G2 M M G1 G1 G1 G1 S S S
Cell Count (Percent of Control)
10
20
30
40
50
60
0 2 4 6 8 10 12 14 16 18
Hours after TdR Release

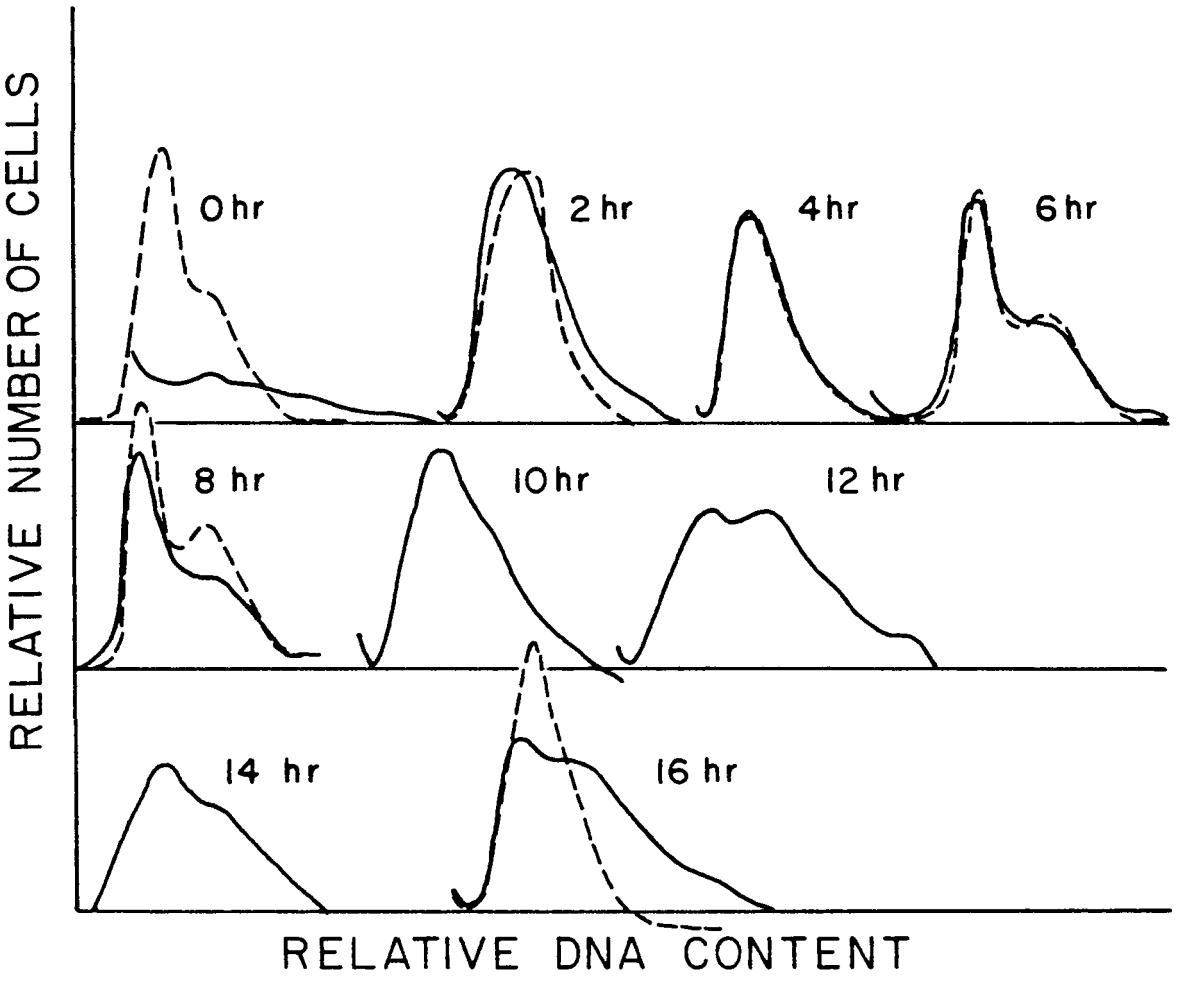
FIGURE 17

FMF PROFILES OF SYNCHRONIZED L1210
CELLS TREATED WITH VBL

Cells were synchronized, released and exposed to VBL for 2 hours at the 2 hour intervals shown. The FMF profiles are of cells sampled at 72-56 hours after treatment of the synchronized cells with VBL. Profiles show treated cells normalized to control cell numbers.

—— VBL

---- Control


RELATIVE NUMBER OF CELLS
0 hr
2 hr
4 hr
6 hr
8 hr
10 hr
12 hr
14 hr
16 hr
RELATIVE DNA CONTENT

at 16 hours, (during S), VBL treated and control cells show gross differences indicating blocking in G2 + M and apparent cell clumping in VBL treated cells.

Cell counts and sampling times for cells treated with 200uM Ψox are shown in Table 7. Ψox, when evaluated at the times shown in brackets, was toxic to cells in all phases of the cell cycle. Figure 18 shows, however, that there is apparently less toxicity during peak mitosis and early G1. This effect during mitosis is to be expected since cells in mitosis do not synthesize proteins, RNA or DNA and assuming that Ψox may affect the synthesis of proteins as well as the synthesis of RNA and DNA. Figure 18 also shows a slight depression at 4 hours (G2) and at 12 hours (G1/S). But, since the curve for Ψox shows general cytotoxicity and significant differences in effects at any phase are difficult to detect, in order to find if Ψox had a significant effect during any specific phase, FMF profiles showing the characteristic G2 block were more conclusive. Figure 19 shows that at 0 hour, the peaks of cells for FMF analyses were sampled at 72-56 hours as for Figure 17. Ψox treated and control cells are almost the same shape indicating that cells are in the same phase of the cell cycle (mostly S). However, there is a slight shift of the peak to the left indicating that fewer cells are progressing through S (consistent with slow down in S observed in asynchronous populations of cells). When cells were exposed to Ψox between 2 and 4 hours after TdR release, during peak S and early G2, cells which were slowed in S began to advance into G2, at exposure 4-6 hours after TdR release (during mid-G2 and early M) Ψox treated cells showed a greater accumulation in the G2 peak. When cells were exposed to Ψox during late G2 and early M (6-8 hours) there was a drastic

TABLE 7

THE EFFECT OF Ψ-OX ON SYNCHRONIZED L1210 CELL PROLIFERATION

Synchronized cells were treated with 200 uM Ψox for two hours at two hour intervals after release of the TdR block. Cells were counted at the times shown in brackets and expressed as percent of control counts.

THE EFFECT OF ψ-OX ON SYNCHRONIZED L1210 CELL PROLIFERATION

Time After TdR release (Hr)	Cell Proliferation					
	Cells/ml	% of Control	Cells/ml	% of Control	Cells/ml	% of Control
0	109,700 (0)	100	59,540 (48)	13	68,820 (72)	8
2	106,720 (0)	100	59,460 (46)	14	71,380 (70)	8
4	104,480 (0)	100	60,540 (44)	13	62,420 (68)	7
6	103,940 (0)	97	64,740 (42)	15	65,120 (66)	8
8	120,640 (0)	101	86,800 (40)	17	85,140 (64)	9
10	156,720 (0)	107	104,000 (38)	22	106,860 (62)	12
12	160,980 (0)	100	92,780 (36)	23	80,960 (60)	10
14	160,560 (0)	99	131,680 (34)	21	127,460 (58)	12
16	165,740 (0)	101	120,080 (32)	26	109,740 (56)	12

FIGURE 18

CELL CYCLE SPECIFIC ACTIVITY OF Ψ-OX, THYMOX AND INOX

Plots were made of the percent of control values of counts of cells made at 72-56 hours after treating synchronized cells for 2 hours at 2 hour intervals with the oxidation product drugs.

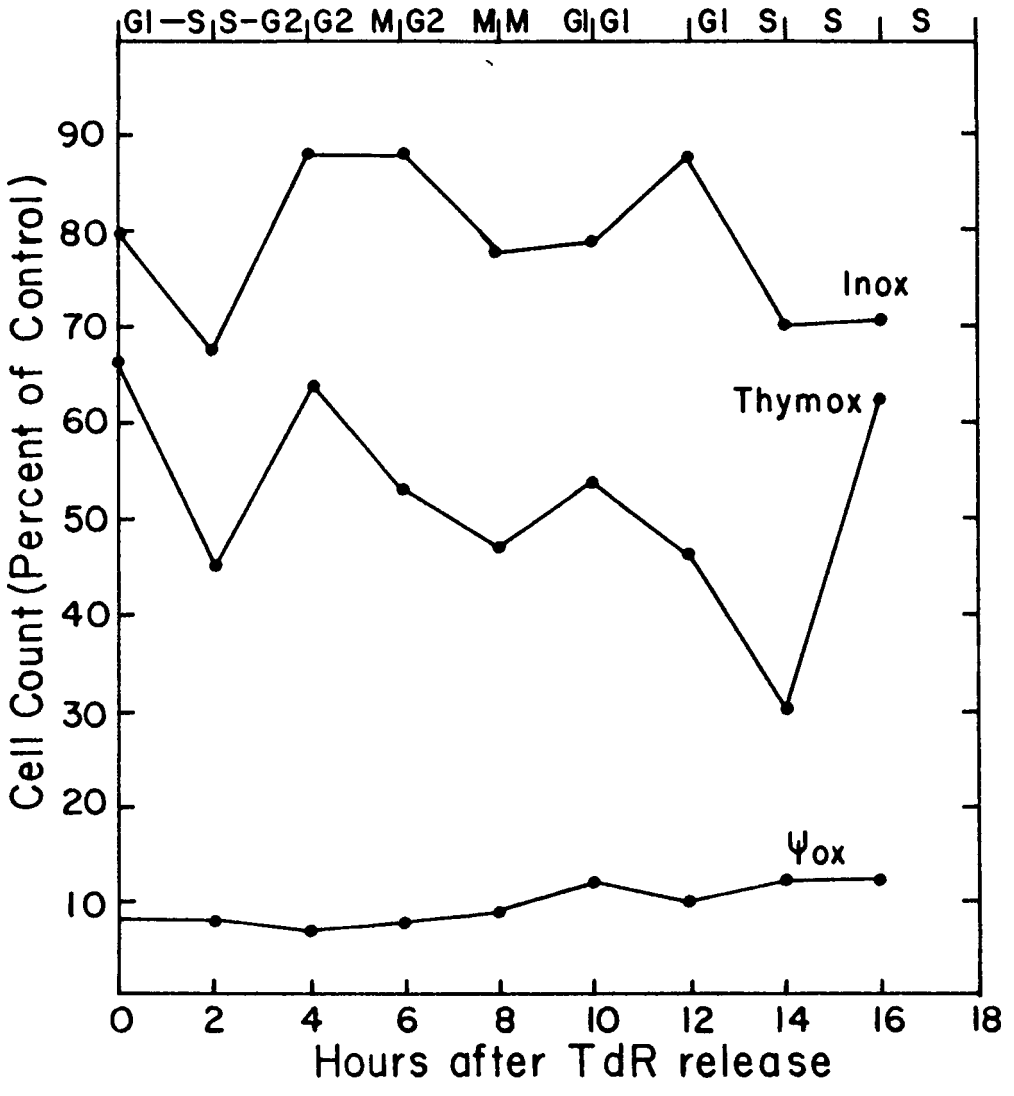

G1—S S-G2 G2 M G2 M M G1 G1 G1 S S S
90
80
70
60
50
40
30
20
10
Cell Count (Percent of Control)
Inox
Thymox
Ψox
0
2
4
6
8
10
12
14
16
18
Hours after TdR release

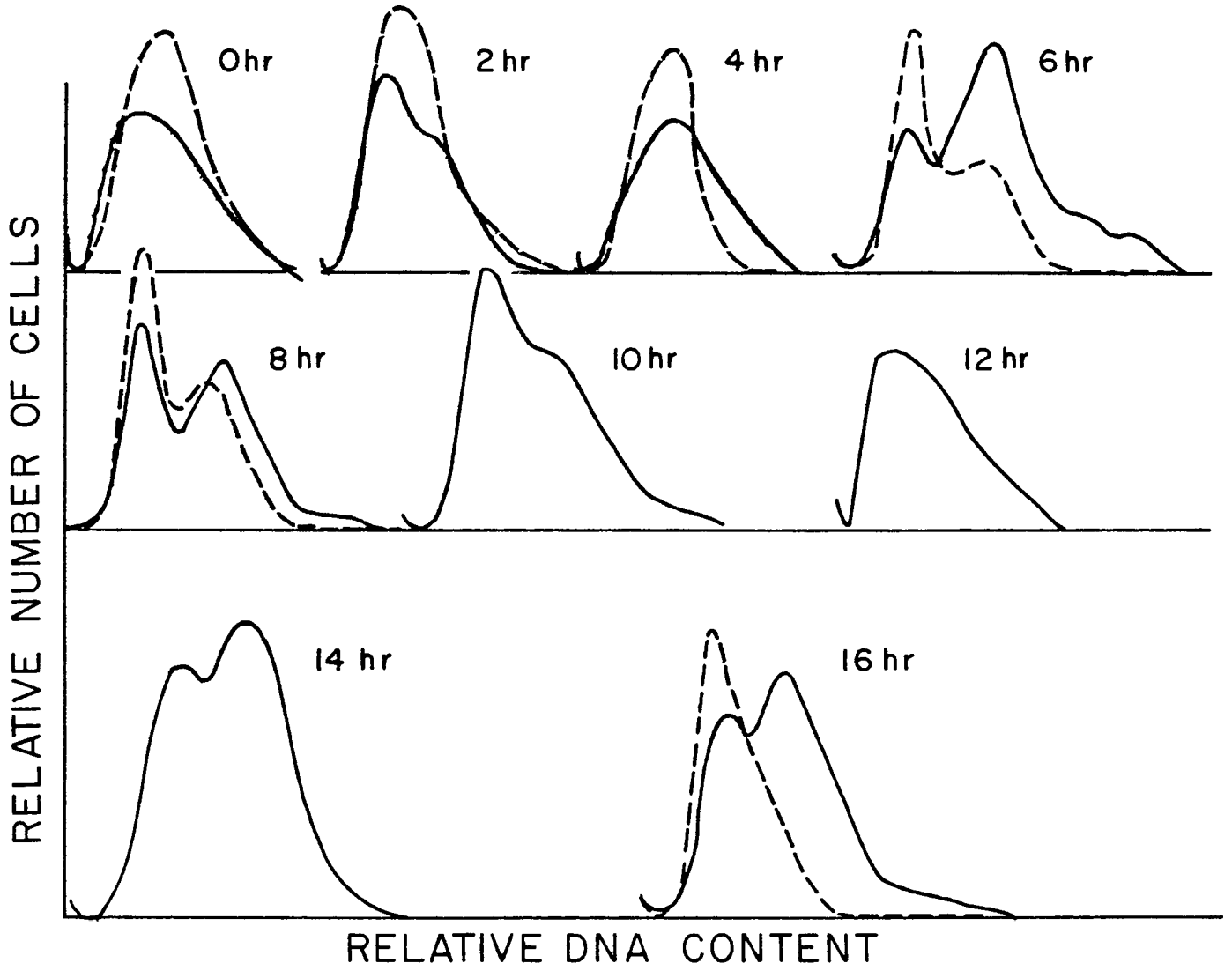
FIGURE 19

FMF PROFILES OF SYNCHRONIZED L1210
CELLS TREATED WITH ψ-OX

Cell samples for FMF profiles were taken at 72-56 hours after treatment of synchronized cells for 2 hours with ψox at the times after TdR release shown. Profiles show treated cells normalized to control cell numbers.

——— ψ-ox

---- Control


0hr
2hr
4hr
6hr
8hr
10hr
12hr
14hr
16hr
RELATIVE NUMBER OF CELLS
RELATIVE DNA CONTENT

increase in the G2 peak compared to the control. Thus the maximum effect of Ψox appears to be on cells in late G2 or at the G2/M boundary. Cells exposed in Ψox during the latter stages of mitosis and early G1 (8-10 hours after TdR release) seemed to have less effect both on the cell growth (Figure 18) and on the pattern of growth shown by FMF (Figure 19). Although a large proportion of the cells are blocked in G2, the presence of the G1 peak indicates cycling cells not blocked to the same degree as those exposed at 6-8 hours and the FMF profile 62 hours after removal of Ψox shows a build up of cells in S. As seen in Figure 14 there are some cells in S during this time. At 10-12 hours cells were mostly in mid and late G1. At 12-14 hours cells were again in late G1 and early S and the FMF profile is very similar to that for the 0-2 hour exposure. Surviving cells exposed from 14-18 hours (late G1/early S) again show characteristic build-up in the G2 + M peak.

Thus, Ψox appears to kill cells in all phases of the cell cycle, however, those surviving become blocked in G2 primarily when exposed during late G2. Cells exposed to Ψox during S are slowed down in their progression through S and later become blocked in G2. This indicates that the synthesis of some component necessary for mitosis may be inhibited during S and again that it, or another component, is inhibited or not present in late G2. Lower concentrations of Ψox may produce the G1/S effect seen with thymox and inox.

Table 8 shows the effect of thymox on the proliferation of cells at the various cell cycle phases when cells were evaluated at the times after removal of TdR as shown by the bracketed numbers. Thymox is not

TABLE 8

THE EFFECT OF THYMOX ON SYNCHRONIZED L1210 CELL PROLIFERATION

Synchronized cells were treated with 200 uM thymox for two hours at two hour intervals after release of the TdR block. Cells were counted at the times shown in brackets and expressed at percent of control counts.

THE EFFECT OF THYMOX ON SYNCHRONIZED L1210 CELL PROLIFERATION

Time After TdR release (Hr)	Cell Proliferation					
	Cell/ml	% of Control	Cells/ml	% of Control	Cells/ml	% of Control
0	109,700 (0)	100	304,000 (48)	64	566,000 (72)	67
2	104,200 (0)	99	214,000 (46)	50	416,000 (70)	45
4	103,000 (0)	99	246,000 (44)	52	460,000 (68)	64
6	102,300 (0)	96	244,000 (42)	58	432,000 (66)	53
8	117,580 (0)	98	246,000 (40)	48	448,000 (64)	47
10	144,760 (0)	99	248,000 (38)	42	462,000 (62)	54
12	160,560 (0)	100	210,000 (36)	51	374,000 (60)	46
14	156,880 (0)	96	184,760 (34)	31	330,000 (58)	30
16	166,680 (0)	101	312,000 (32)	67	574,000 (56)	62

as toxic as Wox, but this may be due to differential permeability of the cells to the drugs during the two hour exposure time.

Figure 18 shows the effect of thymox on cells at specific cell cycle phases. Thymox produced a decrease in cell growth when added at 0-2 hours (during late G1/early S). Since thymidine causes blocking of cells of the G1/S boundary this is not surprising and the decrease in cell number compared to the control may in part be due to that. A second, though smaller, effect was seen when thymox was added to cells at 6-8 hours (during late G2 and peak M). Cells in mitosis and early G1 were less affected (8-10 hours). When cells again reached late G1/early S (12-14 hours) there was a sharp decrease in cell number. Once the majority of cells were in S there was little selective effect of thymox (14-18 hours).

The FMF profiles for thymox, Figure 20, at 0 through 8 hours were similar and resembled profiles for asychronously growing non-treated controls with the exception that there seems to be a slowing in S for cells treated from 6-8 hours (during G2). This is difficult to explain, but may be due to death of the affected or blocked cells by the 72-56 hour sampling time or to loss of the parasynchronous proliferation pattern of the control at the same sampling time. Perturbation of the FMF profile was seen in cells treated at 12 and 16 hours with thymox (during late G1 and S). Control cells for the 16 hour time point seem to be traversing the cell cycle in a parasynchronous wave.

Cells treated with inox at a 200 uM concentration for 2 hours at the 2 hour interval after TdR release were not markedly reduced in number

FIGURE 20

FMF PROFILES OF SYNCHRONIZED L1210
CELLS TREATED WITH THYMOX

Cell samples for FMF profiles were taken at 72-56 hours after treatment of synchronized cells for 2 hours with thymox at the times after TdR release shown. Profiles show treated cells normalized to control cell numbers.

—— Thymox

---- Control

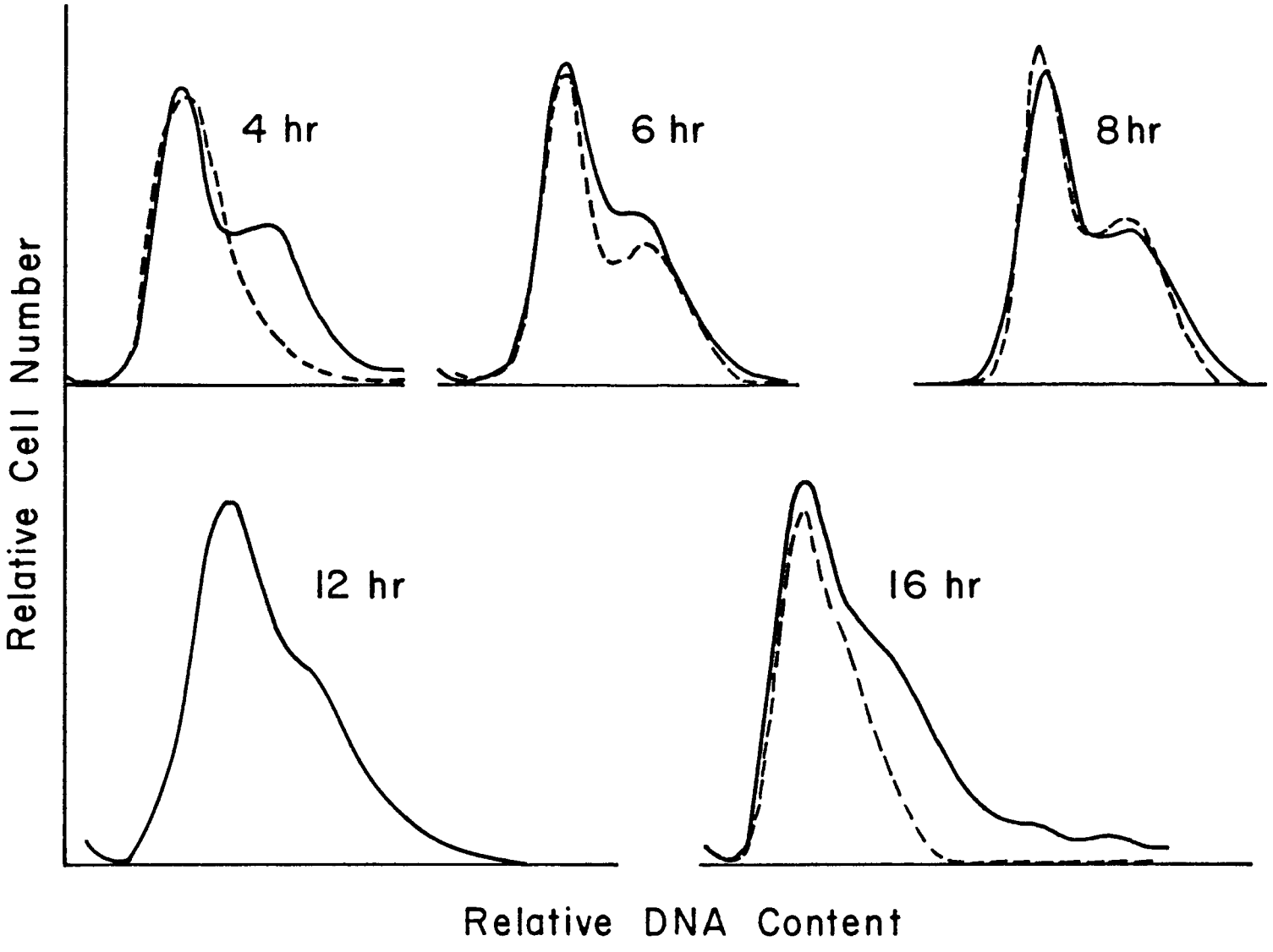

4 hr
6 hr
8 hr
12 hr
16 hr
Relative Cell Number
Relative DNA Content

TABLE 9

THE EFFECT OF INOX ON SYNCHRONIZED L1210
CELL PROLIFERATION

Synchronized cells were treated with 200 uM inox for two hours at two hour intervals after release of the TdR block. Cells were counted at the times shown in brackets and expressed as percent of control counts.

THE EFFECT OF INOX ON SYNCHRONIZED L1210 CELL PROLIFERATION

Time After TdR release (Hr)	Cell Proliferation					
	Cells/ml	% of Control	Cells/ml	% of Control	Cells/ml	% of Control
0	109,700 (0)	100	368,000 (48)	78	760,000 (72)	80
2	105,400 (0)	96	308,000 (46)	71	634,000 (70)	68
4	102,400 (0)	98	392,000 (44)	83	824,000 (68)	88
6	102,600 (0)	96	384,000 (42)	91	718,000 (66)	88
8	113,980 (0)	95	380,000 (40)	75	738,000 (64)	78
10	152,480 (0)	104	382,000 (38)	81	676,000 (62)	79
12	153,780 (0)	96	396,000 (36)	97	720,000 (60)	88
14	162,220 (0)	100	390,000 (34)	65	752,000 (58)	70
16	165,800 (0)	101	324,000 (32)	70	662,000 (56)	71

over the test period (Table 9). Compared to controls, inox treated cells had reached from 68-88% of control cell numbers by the time final samples were evaluated at 72-56 hours. The plot of this data (Figure 18) shows that as in the case of thymox, inox had maximum effects on cell numbers when cells were treated during late G1 and early S (0-2 and 12-14 hours). However, during mid-G1 (10-12 hours), thymox treated cells decreased in number whereas inox treated cells increased; but, at 14-16 hours inox treated cells did not increase as did those treated with thymox. Further experimentation would be necessary to determine if this is a real effect or due to experimental variaton. During the period 6-8 hours (late G2 and early M) both inox and thymox reduced proliferation. There appeared to be less effect of inox than thymox at 4-6 hours (during G2), however this may be due to concentration dependent effect and lower reactivity of inox.

FMF patterns for inox (Figure 21) for cells exposed at 6-8 and 8-10 hours (during late G2 and M) showed an increase in the S peak compared to controls. There was an increase in the S peak for cells exposed 12-14 hours during late G1 and early S. At 16 hours both inox and thymox treated cells (treated at 16-18 hours during S) showed a broader profile indicating that cells were slowing down in S and some were accumulating in G2. It is possible that higher doses of these drugs are necessary in experiments of this type in order to see the normal G2 + M peak accumulation that was seen with Ψox. However, the cell number data for inox and thymox is reliable since it represents a true decrease in cell due to drug treatment.

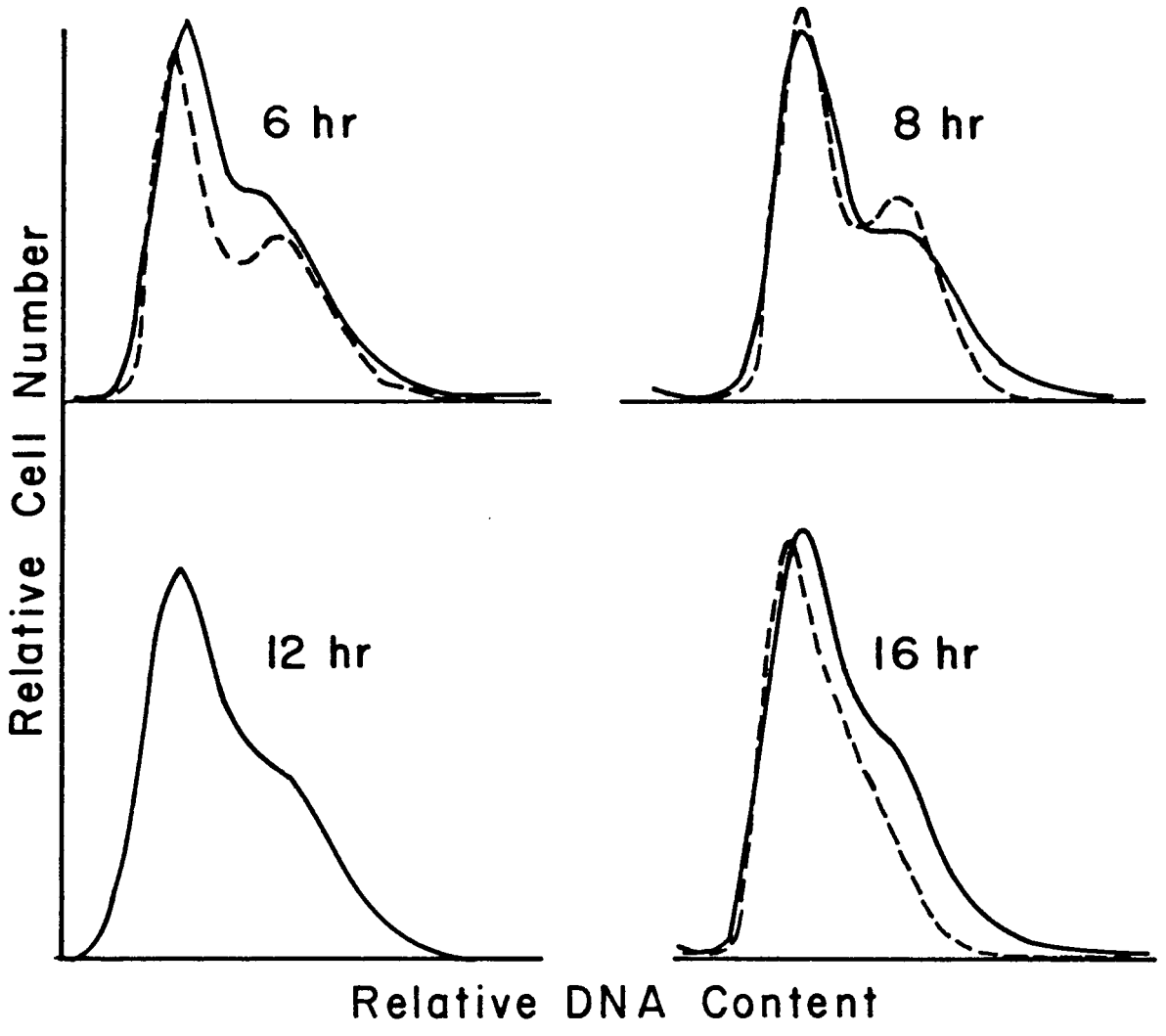
FIGURE 21

FMF PROFILES OF SYNCHRONIZED L1210
CELLS TREATED WITH INOX

Cell samples for FMF profiles were taken at 72-56 hours after treatment of synchronized cells for 2 hours with inox at the times after TdR release shown. Profiles show treated cells normalized to control cell numbers.

——— Inox

---- Control


6 hr
8 hr
12 hr
16 hr
Relative Cell Number
Relative DNA Content

To summarize cell cycle specific activity for these drugs, it appears that although the drugs reduce the number of cells in the population regardless of the phase when drugs were administered, the deleterious effect is greatest when drugs are added during late G1/early S and a lesser effect is seen in late G2/early M for thymox and inox. For Ψox the greatest effect is observed after treating cells in S and especially in late G2. Cells in late G1/early S were also affected. These effects are probably dose dependent for all three drugs. VBL exerts its greatest effect in late G1 and early S but not in G2 as do Ψox, thymox and inox.

Section I

DISCUSSION

The time and concentration dependent effect on cell proliferation and viability of the oxidation product drugs reported by other researchers (32) was confirmed by similar results in L1210 cells treated with ψox and thymox. The greater effectiveness of ψox over inox or thymox may be in part due to differences in permeability of the cells to the different drugs. Cysyk and Adamson (44) studied membrane reactivity of [^{14}C]-inox in human erythrocytes and found that after 30 minutes, 23.2% of added inox was inside the cells. Membrane fractions contained only 0.23% of the total inox added. They suggest that the positive Coomb's reaction seen in patients receiving inox (53) may be due to membrane bound drug. Microscopic examination and observation of cell clumping in my experiments confirms their observation. The higher reactivity of ψox may also be due to spacial relationships in binding to cellular constituents. Possibly the intracellular metabolism of ψox with its carbon-carbon glycosidic linkage is more rapid than that of thymox and inox with their carbon-nitrogen linkage. Though 5-Aza-cytidine with the N-C glycosidic linkage was less stable than pseudo-cytidine with a C-C linkage (54). Studies with thin layer chromatography of these drugs after incubation in tissue culture medium were inconclusive, therefore no statement can be made concerning the form of the drugs or metabolites within the cells.

Ψox loses biological activity rapidly when incubated in FGM at 37°C. Thymox and inox are less rapidly inactivated. Cysyk and Adamson (44) report that inox reacts rapidly with glycine and other amines resulting in reduced toxicity to cells. Thymox and inox appear to be more stable than Ψox, a fact which requires further investigation. Ψox and thymox are stable when stored at 0°C in the lyophilized state for more than one year.

Blocking of cells in G2 is consistent with reports of Plagemann et al. for inox (32). Some FMF profiles showed a broadening at the base of the G2+M peak. This probably was due to polyploidy in some drug treated cells (40).

The cell cycle specific activity of Ψox and thymox was consistent with the report of Bhuyan and Fraser that inox is most active against cells in late G1 or early S (55). They also report little activity in cells in M, early G, mid-S and G2. Drugs which cause accumulation of human lymphocyte cells in G2 are adriamycin, bleomycin, L-phenyl-alanine, rubidazone, 4'-demethyl-epipodophyllotoxin 9-(4, 6-O-ethylidene-B-D-glucopyranoside) and 3,3'-iminodi-1-propanol, dimethanesulfonate (ester) hydrochloride (Yoshi 864) (56). Mechanisms of action of these drugs are varied. Some act as alkylating agents and some intercalate or otherwise bind to DNA. The most sensitive cell cycle phase for induction of a G2 block was different for these drugs suggesting differences in mechanisms of action. For instance, adriamycin added to cells in any phase of the cell cycle caused the G2 block. This is

similar to the non-phase specificity seen with Ψox. However, bleomycin produced a G2 block when administered during G2, which is also consistent with phase specific activity of Ψox.

Knowledge of phase specificity allows designing of chemotherapeutic regimens. It appears that due to cytotoxicity during all phases of the cycle, Ψox and thymox would be drugs of choice in treating solid or slow growing tumors, and since there is some phase specific action, these same drugs would also be effective in treating fast growing malignancies.

SECTION II

BIOCHEMICAL MECHANISM OF ACTION STUDIES

INTRODUCTION

The various mechanisms of action of the periodate-oxidation product drugs include inhibition of specific enzymes such as ribonucleotide reductase (13, 32, 57), DNA and RNA polymerases (12, 30, 58), DNA dependent RNA polymerase (58, 59), and thymidylate kinase (30). The cross linking of proteins (44), and the formation of Schiff's bases with the e-amino group of lysine in active centers (60) of these enzymes or functionally critical moieties accounts for some inhibitions of these enzymes. Enzymes which do not appear to be affected by inox are uridine, thymidine, UMP, dTMP and dTDP kinases and hypoxanthine-guanine phosphoribosyltransferase (32). Enzymes involved in thymidine and guanine incorporation and deoxycytidine deaminase in Ehrlich tumor cells are not affected by inox or the oxidation product of IMP (57). Using E. coli RNA polymerase Cysyk and Adamson, found no inhibition of this enzyme by inox at a concentration of 1×10^{-3} M (44). Cory et al. reported no inhibition of RNA polymerase activity in nuclear extracts of inox-treated Ehrlich tumor cells (61).

Inox inhibits DNA, RNA and protein synthesis in a time and concentration dependent manner and causes arrest of cells in the G2 phase of the cell cycle (32). The inhibition of DNA and RNA synthesis is about

equal in Ehrlich and L1210 cells treated with inox (57). Protein, DNA and RNA synthesis are also inhibited by the oxidation product of β-D-ribosyl-6-methylthiopurine in L1210 cells in mouse ascites (11).

The specific molecular mechanisms by which these inhibitions are produced by the oxidation product drugs have not been clearly elucidated. Inox cross-linked proteins and formed complexes with lysine, glysine, histidine and bovine serum albumin (BSA) are reported by Cysyk and Adamson (44). Both aldehyde groups were functional. Cross-linking occurred with inox between glycine and BSA indicating that small molecules could be linked to larger ones. There was no interaction with the nucleic acid bases or nucleosides or nucleic acids when [^{14}C]-inox labelled in the purine ring was used. Cysyk and Adamson concluded that inox could react with all amino acids with primary amino groups. Acetylation of primary amino groups or complexing with BSA resulted in loss of inox cytotoxicity. The periodate-oxidation products of adenosine, cytosine and methyl ribose also interacted with BSA. The bifunctional aldehydes, glyoxal and malondialdehyde interacted with DNA and the nucleic acid bases cytidine and guanine (62). George and Cory (63) stated that inox (U-^{14}C] was taken up by the tumor cells without degradation to hypoxanthine and that within the cell, inox was stable and was present in the nucleoside and nucleotide pools of the tumor cells. They suggest that inox is incorporated into RNA, especially the small molecular weight RNA's. In these experiments, inox was statistically uniformly labelled in the inosine part of the molecule and appeared to be incorporated as such.

In searching for a specific cause of the G2 arrest, there is a possibility that a specific mitotic promoter protein may be absent in oxidation product treated cells. Credence is given to the idea that these drugs may inhibit synthesis of a particular protein by the finding of Cronenberger and Kimball (64). In studies with methylthioinosine periodate-oxidation product (MMPR-OP), they showed that the drug blocked the intracellular biosynthesis of an immunoglobulin protein chain of the MOPC-46 plasma cell tumor which secretes a k light chain. MMPR-OP also inhibited the intracellular incorporation of glucose and galactose into the polysaccharide moiety of the k chain. MMPR-OP did not prevent the secretion of previously synthesized protein chains. If such oxidation-product drug inhibition of specific protein synthesis is possible, and progression of cells into mitosis depends on this protein, microtubule protein may be involved in the G2 block.

The importance of mictotubules in mitosis was suggested by Ledderberg and Porter in 1963 (65). Based on the cell fusion experiments by Rao and Johnson (66), mitosis may occur only when a critical level of a mitotic inducer accumulates in G2. Since mitosis depends on the assembly of previously synthesized microtubule protein (67), and the basic protein component of microtubules accumulates continuously throughout interphase as shown by Robbins and Shelanski with HeLa cells (68), it is reasonable to assume that levels of microtubule protein below those necessary for polymerization might be one factor which would prevent cells from entering mitosis and hence result in a G2 block.

The description of Borisy (69) of a rapid quantitative method for determining the amount of microtubule protein in cell extracts has made available an assay for detecting changing levels of this protein as a function of treating cells or cellular extracts with drugs. The method takes advantage of the specific binding of the mitotic spindle "poison", colchichine, to the dimer subunit protein of microtubules (70) and the affinity of microtubule protein for DEAE ion exchangers under conditions of moderate ionic strength and neutral pH. The filter stack method of Borisy which employs Whatman DE81 cellulose impregnated filters has been used to determine the effects of podophyllotoxin and the vinca alkaloids on the binding of tubulin to colchicine (71) and involvement of microtubulin in secretory processes (72).

Various investigators (73, 74, 75) have defined the parameters of colchichine binding to tubulin. The colchicine-tubulin complex is tightly bound, has characteristics of a bimolecular reaction, with non-covalent binding, and colchicine appears to bind by a hydrophobic reaction in a nonpolar pocket of the tubulin molecule. Colchicine is not chemically altered by binding and there is one binding site per dimer tubulin molecule. The rate of binding is strongly temperature dependent with no significant binding at 0°C. However, once formed, the complex is stable at 0°C. Optimal binding occurs at 37°C. The binding reaction itself is not affected by pH ranges of 5.5-8.5 or by ionic strengths of 0.1-0.5. The decay rate of the complex is temperature, pH and ionic strength dependent. The stability of the complex is related to the stability of the protein per se. Decay rates differ with different

tissue sources of the protein and with different concentrations of microtubule protein available in the reaction mixture. Klevecz and Forrest (76) have shown that tubulin may comprise 5 to 15% of the soluble protein in cultured cells and that it is synthesized primary in late S and G2 phases of the cell cycle.

The binding of colchicine to the dimer subunit prevents additional binding of dimer subunits and thus effectively prevents polymerization of microtubules. Podophyllotoxin binding to mictotulin protein is competitive with colchicine and possibly inhibits colchicine complex formation by binding at an allosteric site (71). The mechanism of podophyllotoxin binding is probably different from that of colchicine since the binding of podophyllotoxin is more rapid.

Section II describes the effect of Ψox, and thymox on macromolecular synthesis of DNA, RNA and protein; the direct effect of the drugs on DNA and RNA polymerases in crude cellular extracts; and the effect on tubulin levels in drug treated cells. Other mechanisms of action are suggested.

MATERIALS AND METHODS

Drugs and Reagents

The periodate oxidation product drugs are synthesized and prepared for testing as described in Section I. Deoxynucleotides for DNA polymerase assays and nucleotides for RNA polymerase assays were purchased from the Sigma Chemical Co. Tritiated TTP (thymidine 5'-triphosphate, [methyl-^{3}H], specific activity 60 Ci/mmole) and tritiated UTP (uridine 5'-triphosphate, [5,^{3}H], specific activity 27 Ci/mmole) were purchased from ICN. The reagents for the colchicine binding assay came from various sources. The [ring C-methoxyl-^{3}H] colchicine, specific activity 10 mCi/mg, was from the Amersham/Searle Corporation. Etopside (Bristol Laboratories) was a gift from Dr. T. Li Loo of the M. D. Anderson Hospital and Tumor Institute. Tritiated leucine, [4,5-^{3}H], (specific activity 61 Ci/mmole) was from Swartz/Mann. Fluorescein isothiocyanate (FITC) (J. T. Baker Company), was made up at 0.05 mg/ml in 0.5 M sodium bicarbonate (Fisher Scientific Company) at pH 8.0. The PK-GTP buffer consisted of 0.067 M sodium phosphate buffer (pH 6.8), containing 0.1 M KCl (Fisher Scientific Company) and 0.1 mM guanosine 5'-triphosphate (GTP)(Sigma Chemical Company). Trichloloacetic acid (TCA), Scintanalyzed toluene, (Fisher Scientific Company), and Insta-Gel (Packard Instrument Company) were used in radioactive labeling experiments. The toluene scintillation cocktail was prepared by addition of 24g of PPO (2,5-diphenyloxazole) (INC) and 3g POPOP (1-4-bis-2-5-phenyloxazolyl) benzene (New England Nuclear) to 4 liters of Scintanalyzed toluene.

Cells and Cellular Homogenates

Extracts of L1210 tumor cells grown in BDF_1 female mice were prepared as follows. Ascities cells were removed from the abdominal cavity of mice 5 days after intraperitoneal injection with 200,000 cells. Cells were washed repeatedly with Dulbecco's phosphate buffered saline (PBS) without Mg^{++} and Ca^{++} (GIBCO) to remove red blood cells. The cells were then swollen by addition of 2 volumes of ice cold double distilled water to the cell pellet. Cells were dispersed by pipetting and allowed to stand in an ice-water bath for 10 minutes. An equal volume of 2X PK-GTP buffer was added to swollen cells and cells were homogenized with 40 strokes of the teflon pestle of an Elvehjem motor driven tissue homogenizer. Cellular debris was removed by centrifugation at 1470 x g for 10 minutes. Subsequent centrifugation in a Beckman model LS-65 ultracentrifuge at 100,000 x g for one hour or at 16,000 x g for 30 minutes provided the supernatant extract used for microtubulin assays and for crude enzyme extracts for the DNA and RNA polymerase assays respectively. Total protein was determined by the Lowry method (77). Extracts were prepared for in vitro cultured L1210 cells in a similar manner except that cells were washed only 3 times with Dulbecco's PBS. Protein in extracts of tissue culture cells ranged from 0.2 to 0.8 mg/ml.

Proliferation of tissue culture cells was monitored with a Model ZB1 Coulter counter (Coulter Electronics). Exclusion of trypan blue by cells indicated presumptive viability at the time the cells were sampled (See Section I).

DNA Synthesis

The effect of drug treatment on the rate of macromolecular synthesis in L1210 cells in vitro was evaluated by modification of the method of Cass and Au-Yeung (36). Suspension cell cultures with a doubling time of 20±4 hours were diluted on the day prior to drug addition of drugs to a concentration of 1-1.5 X 10^5 cells/ml. The periodate-oxidation product drugs were made up in Fischer's growth medium (FGM), filtered through a Millex 0.45 um pore size filter and added in 0.03 volumes in logarithmically growing cells at 2-3 x 10^5 cells/ml. At scheduled intervals 10 ml aliquots of drug treated and control cells were pipetted into flasks held in a Dubnoff metabolic shaker water bath (Precision Scientific) at 37°C. Tritiated thymidine [methyl-^{3}H] (Swartz/Mann) at a final concentration of 0.2 μM (1 uCi/ml) was added to duplicate samples and cells were incubated with gentle shaking. At 10-20 minute intervals 1 ml aliquots of cells were pipetted into tubes containing 10 ml of 100 uM unlabelled ice-cold thymidine (TdR) in 0.9% saline. Cells were then collected on RAWP 1.2 um pore size filters (Millipore) in a Millipore manifold (model 1225) and extracted 3 times with 10 ml of cold 5% TCA. Filters were dried, placed in scintillation vials with 10 ml of toluene scintillation cocktail. Radioactivity was determined in a Beckman LS 9000 Scintillation Counter.

RNA Synthesis

The procedure for determining the effect of drugs on total RNA synthesis was the same as that for DNA synthesis except that [5-^{3}H]-

uridine (Schwatz/Mann) was used instead of tritiated thymidine. After 10-20 minutes incubation with [^{3}H]-uridine, 1 ml aliquots of cells were pipetted into tubes containing ice cold 100 uM uridine in 0.9% saline. The procedure for assaying incorporated radioactivity was the same as that for DNA synthesis.

DNA Polymerase Assay

Crude enzyme extracts (16,000 x g supernatant extracts of cell homogenates) were made from L1210 cells grown in DBA/2 or BDF_1 mice as described in Materials and Methods. The supernatant homogenate contained 4.6 mg/ml protein as determined by the Lowry method (77). DNA polymerase activity was evaluated by a modification of the method of Vander Velde et al. (12). Each reaction tube, in duplicate, contained the following reagents:

	volume/tube	reagent
1.	50 ul	1 M tris-HCl buffer at pH 8.0 containing 0.1 M $MgCl_2$
2.	50 ul	Creatine kinase, 1.5 mg/ml, 100 mM creatine phosphate and 20 mM ATP.
3.	100 ul	dATP, dGTP, dCTP and [^{3}H]-(CH_3)-dTTP specific activity 1 uCi/umole.
4.	50 ul	Calf thymus DNA, 2 mg/ml in double distilled water, denatured by boiling in a water bath for 5 minutes and immediatley cooling in an ice bath.
5.	150 ul	Crude enzyme extract.
6.	100 ul	Drug or water.

Tubes containing all reagents, except the deoxynucleotide substrates, were incubated in a 37°C water bath (Thelco 183, Precision Scientific Company) for 10 minutes, then cooled to 3°C in an ice bath. Substrates were added and the reaction tubes were incubated at 37°C for an additional 20 minutes. The reaction was stopped by adding 50 ul of cold 50% TCA. Tubes were placed in an ice bath and 5 ml of cold 5% TCA were added to each tube. DNA was collected by pouring contents of these tubes onto RAWP 1.2 um pore size membranes supported in a Millipore manifold. Filters were washed three times with 10 ml volumes of cold 5% TCA to remove unbound label. Membranes were dried, placed in scintillation vials and incorporated radioactivity was counted in 10 ml of toluene scintillation cocktail in a Beckman LS 9000 Liquid Scintillation Counter.

RNA Polymerase Assay

RNA polymerase activity in crude enzyme extracts of L1210 cells derived from ascities in mice as described was assayed by a modification of the method of Jendrisak as described in Worthington Enzymes 1978 (78). Each reaction tube, in duplicate contained the following:

1. 2.5 umoles tris-HCl (pH 7.9)
2. 3.5 umoles manganese chloride
3. 5 umoles magnesium chloride
4. 31.3 umoles ammonium sulfate
5. 100 nmoles each of adenosine 5'-triphosphate guanosine 5'-triphosphate, cytosine 5'-triphosphate

6. 100 pmoles [5-^{3}H]-uridine 5'-triphosphate, specific activity 1 uCi/100 pmoles

7. 50 ug heat denatured calf thymus DNA

The crude enzyme extract was incubated with drugs or water for 10 minutes before addition of the above reagents. The incubation was carried out at 25°C. After addition of reagents and substrates, the tubes were vortexed and incubated an additional 15 minutes. Addition of 0.5 ml ice-cold 50% TCA stopped the reaction. Tubes were placed in an ice water bath for 10 minutes. Acid isoluble precipitates were collected by filtration of the mixture through 0.45 um pore size filters supported in a Millipore manifold. Each tube was washed five times with 3 ml volumes of cold 5% TCA and each wash was filtered. Filters were then washed with an additional 5 ml volume of 5% TCA, removed from the holder and air dried. Dried membranes were placed in scintillation vials containing 10 ml of toluene scintillation cocktail and incorporated radioactivity was counted in a Beckman LS 9000 Liquid Scintillation Counter.

Tubulin-Colchicine Binding Assay

Colchicine binding to tubulin receptor protein in 100,000 x g supernatant extracts of cells was assayed by slight modification of the filter stack method described by Borisy (69). One ml aliquots of cellular extracts were incubated with 0.1 ml [^{3}H]-colchicine (final concentration of 2 X 10^{-6}M, approximately 10^{6} CPM) for 1-2 hours in the 37°C water bath. During the incubation period, 2.5 cm diameter DE81 filter discs (Whatman) pre-wet in PK buffer (without GTP) were placed

in a Millipore 122S sampling manifold. Each stack consisted of 4 filter discs. After incubation, tubes containing the reaction mixture were placed in an ice-water bath to stop binding. The mixture was then poured onto the filter stacks. Tubes were rinsed with 5 ml of PK (without GTP) buffer which was then poured onto the appropriate stacks. Vacuum was applied so that the material filtered slowly to allow colchicine-bound tubulin to adsorb to the filter stack. Each stack was washed 5 times with 10 ml of PK buffer and allowed to run dry for a minimum time of 10 seconds after the last wash. Stacks were removed from the holder, placed in scintillation vials with 10 ml of Instagel (Packard Electronics), held overnight, and bound radioactivity was counted in a Beckman LS 9000 Scintillation Counter.

Reaction of Ψox, Thymox, Inox and Etopside with Tubulin

The 100,000 x g supernatant extracts of ascites cells were prepared in PK-GTP buffer as described. One ml aliquots were preincubated with drugs for 1 hour at 37°C and then incubated for 2 hours with [^{3}H]-colchicine. Bound colchicine was assayed as decribed.

Effect of Drugs on Cellular Tubulin Levels

L1210 cells were subjected to drugs for 18 and 24 hours. The 100,000 x g supernatant extracts were prepared as described and the amount of tubulin was determined by incubation of one ml of the supernatant extracts with 0.1 ml of [^{3}H]-colchicine for 1 hour at 37°C. Bound colchicine was assayed as described.

Protein Synthesis

Protein synthesis in drug treated and control L1210 cells was evaluated by [^{3}H]-leucine uptake. Tritiated leucine, final concentration 1 uCi/ml was added to duplicate 10 ml samples of cells in flasks placed in a Dubnoff metabolic shaker water bath at 37°C. One ml aliquots were removed at 10-15 minute intervals and mixed with 10 ml ice-cold 10^{-4} M leucine made up in 0.9% saline. Cells were then collected on RAWP 1.2 um pore size filters in a Millipore filter holder and extracted with ice-cold 5% TCA. Filters were dried and placed in scintillation vials with 10 ml of toluene scintillation cocktail. Radioactivity was determined in a Beckman LS 9000 scintillation counter.

RESULTS

The Effect of Ψox, Thymox and inox on DNA Synthesis

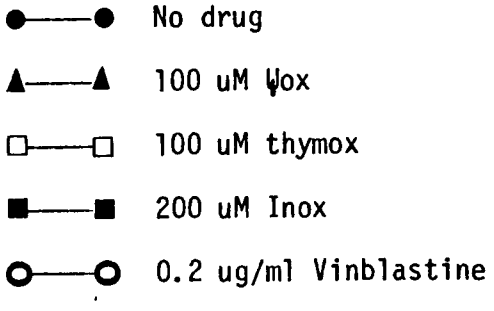
The incorporation of thimidine into the acid insoluble pool of cells treated with Ψox (100 um), thymox (100 um) and inox (200 um) was reduced by approximatley 62%, 30% and 15% respectively after 4 hours of exposure to the cells (Figure 22). After 26 hours of exposure (Figure 23) a greater reduction was observed (75%, 86% and 81%) indicating the time dependent inhibition. This time and concentration dependent inhibition of DNA synthesis is consistent with the results of other researchers in the same and in different cell systems (13, 30, 32, 57). The differences in percentages of inhibition between 4 and 26 hour incubation is greater for thymox and inox than for Ψox, however, the magnitude of inhibition is about the same by 26 hours for all three drugs. Though it appears that Ψox exerts a greater effect earlier this effect is possibly due to differential permeability of cells to the drugs. That cells treated with Ψox show less reduction of DNA synthesis at 26 hours than for thymox or inox is not surprising since Ψox was shown in Section I to lose activity when incubated in FGM after 5 hours. The rapidity of inhibition by Ψox, and the magnitude of inhibition at 26 hours indicate an effect on the DNA of cells at any stage of the cell cycle. The inhibition of all three drugs appears to be irreversible.

The Effect of the Drugs on RNA Synthesis

Incorporation of [^{3}H]-uridine into the RNA acid-insoluble pools was also inhibited by these drugs (Figures 24 and 25). RNA synthesis

FIGURE 22

DNA SYNTHESIS 4 HOURS AFTER ADDITION OF DRUGS TO ASYNCHRONOUSLY GROWING L1210 CELL CULTURES



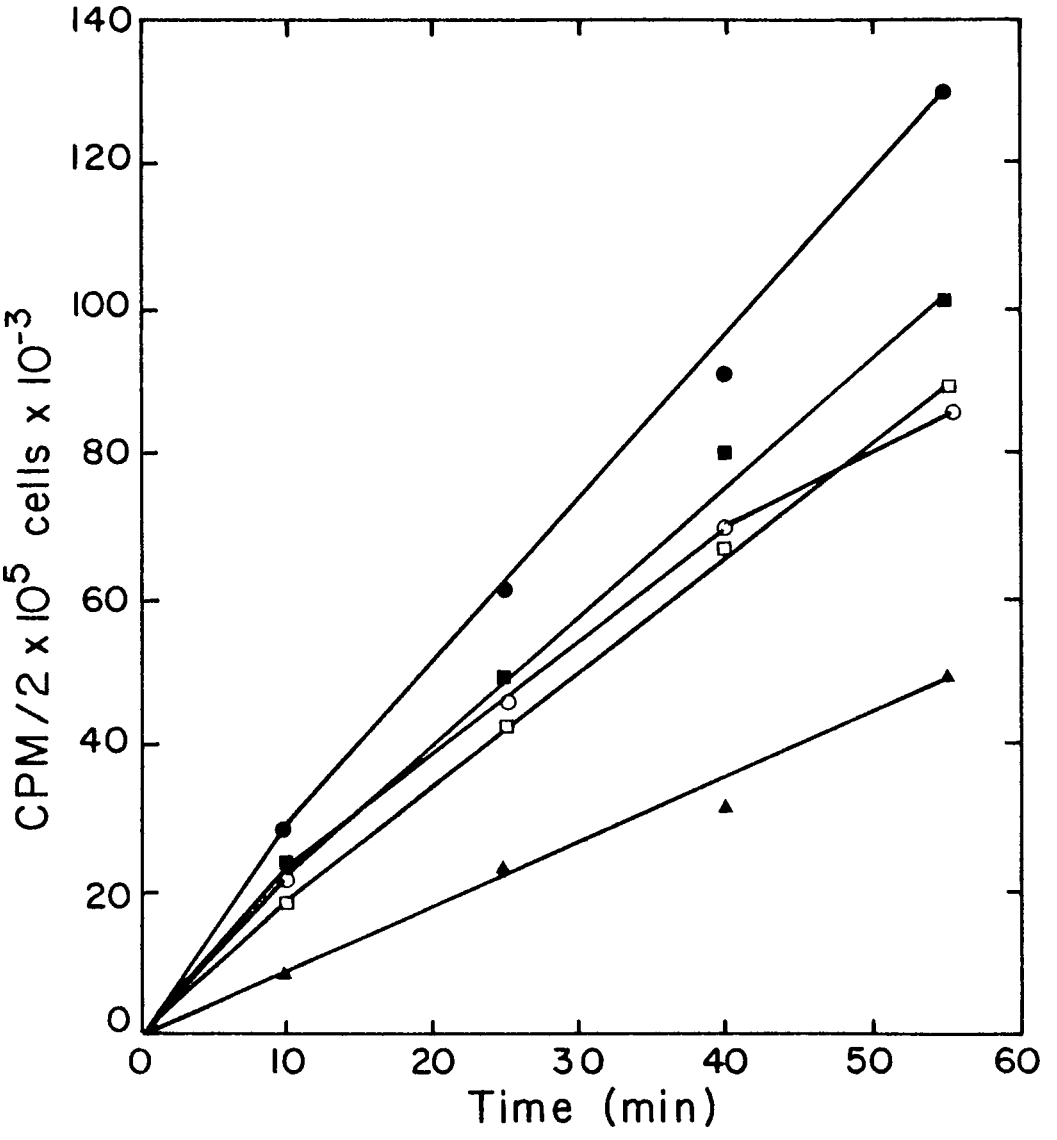
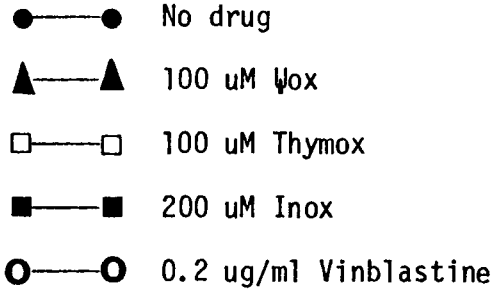

140
120
100
80
60
40
20
0
CPM/2 x 10^5 cells x 10^-3
0
10
20
30
40
50
60
Time (min)

FIGURE 23

DNA SYNTHESIS 26 HOURS AFTER ADDITION OF DRUGS TO ASYNCHRONOUSLY GROWING L1210 CELL CULTURES



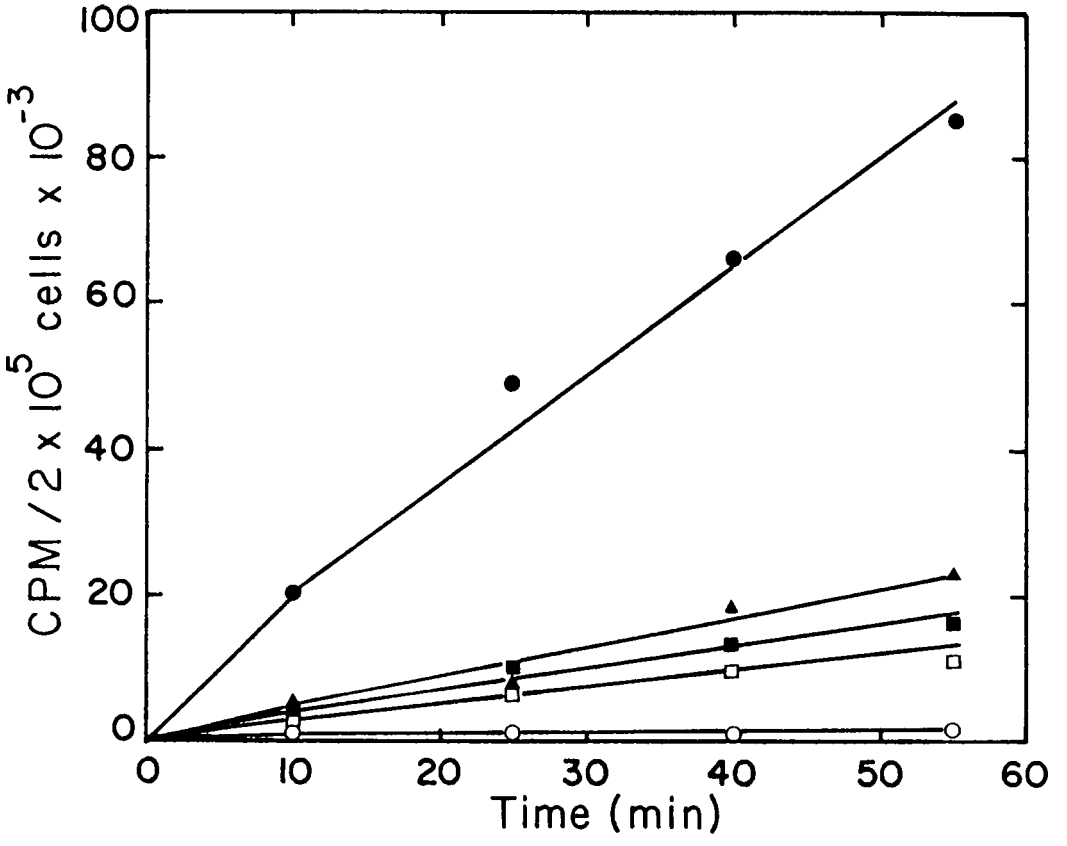
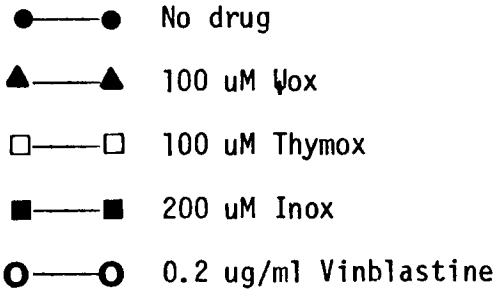

100
80
60
40
20
0
CPM / 2 x 10^5 cells x 10^-3
0
10
20
30
40
50
60
Time (min)

FIGURE 24

RNA SYNTHESIS 4 HOURS AFTER ADDITION OF DRUGS TO ASYCHRONOUSLY GROWING L1210 CELL CULTURES



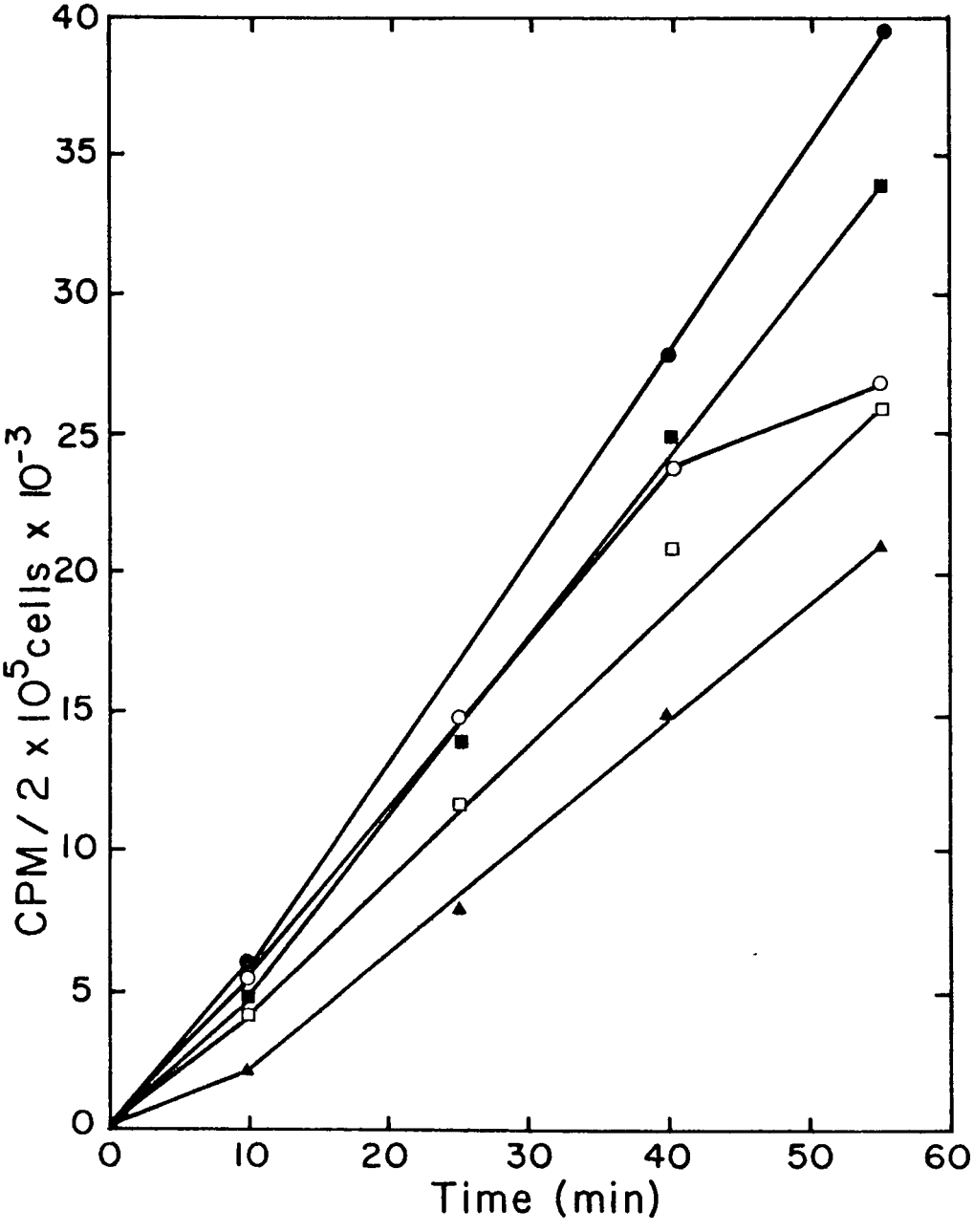
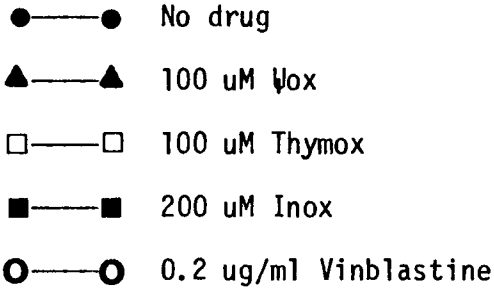
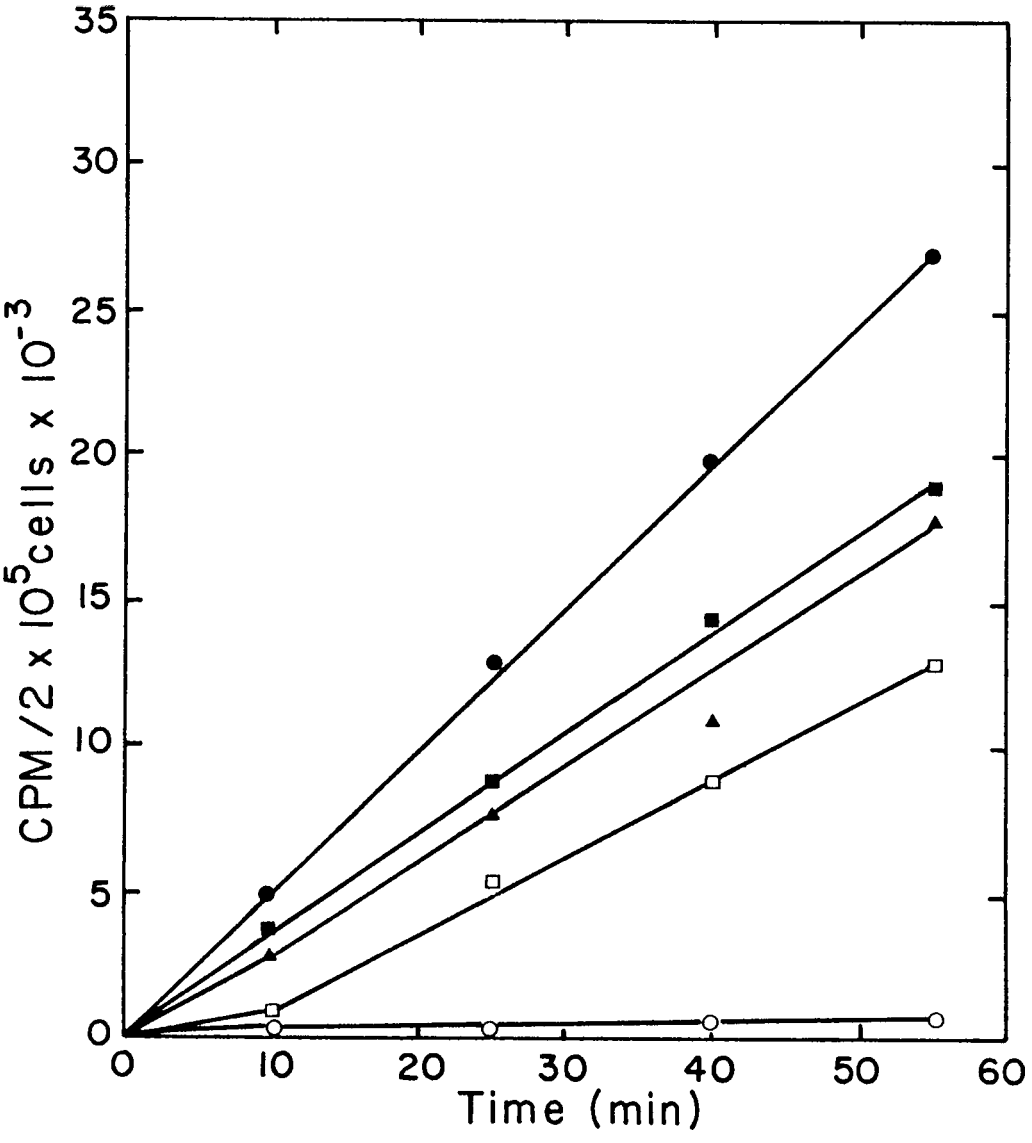

40
35
30
25
20
15
10
5
0
CPM / 2 x 10^5 cells x 10^-3
0
10
20
30
40
50
60
Time (min)

FIGURE 25

RNA SYNTHESIS 26 HOURS AFTER ADDITION OF DRUGS TO ASYCHRONOUSLY GROWING L1210 CELL CULTURES




35
30
25
20
15
10
5
0
CPM/2 x 10^5 cells x 10^-3
0
10
20
30
40
50
60
Time (min)

appeared to be inhibited to a lesser extent than DNA synthesis. At 4 hours inhibitions of incorporation for tritiated UTP for Ψox, thymox and inox were approximately 46%, 33% and 13% respectively and at 26 hours inhibitions were 33%, 52% and 30%.

Cells treated with Ψox for 26 hours showed less inhibition of RNA synthesis than at 4 hours. This may be due to the fact that Ψox is 50% inactivated after 5 hours incubation with FGM (Section I). Cellular RNA synthesis and processing mechanisms may promote recovery from the Ψox block and thus circumvent the effects of the drug. Thymox was 50% active after 24 hours incubation in FGM (Section I) and continued to exert its effect on RNA synthesis. This might account for the increased inhibition at 26 hours. Inox showed the time dependent inhibition of RNA synthesis as reported by Plagemann et al. for inox in Novikoff rat hepatoma cells (32) and by Cory et al. in Ehrlich tumor cells (61).

A comparison of inhibitions of DNA and RNA synthesis by these drugs would indicate that both are rapidly inhibited to some extent by 4 hours. DNA synthesis of Ψox at 4 hours was inhibited more than RNA synthesis (62% for DNA and 46% for RNA). DNA and RNA synthesis were inhibited to about the same extent for thymox and inox at 4 hours (30% and 15% for DNA, and 33% and 13% for RNA). At 26 hours, however, the RNA synthesis rate was much less affected by all 3 drugs than DNA synthesis (33%, 52% and 30% for RNA; and 75%, 86% and 81% for DNA) for Ψox, thymox and inox respectively. This indicates that the drugs appear to have a primary effect on DNA not RNA synthesis. Also, even

though cells were blocked in G2 at 26 hours (see FMF profiles, Section I), they were still synthesizing RNA and at a faster rate than DNA. It appears that presence of the drug is required to inhibit RNA synthesis, whereas the inhibition of DNA synthesis seems to be irreversible.

Vinblastine (VBL) was included in these studies because its mechanism of action is known. It affects cells in S and causes a block of cell cycle progression in G2 (52, 56). In these studies VBL inhibited DNA and RNA synthesis at 4 hours to about the same extent as the oxidation product drugs. The viability by TBE at 26 hours showed that few cells were viable and this accounts for the 98% and 93% inhibitions of DNA and RNA synthesis respectively.

The Effect of the Drugs on DNA Polymerase Activity

Crude enzyme extracts of L1210 cells from BDF_1 mice were prepared and the drugs, dissolved in double distilled water, were incubated with the extracts as described in Materials and Methods. The protein concentration in the lysate was 4.5 mg/ml by the Lowry method (77). Results are shown in Table 10. These oxidation product drugs inhibit the enzyme 60%, 43% and 31% for Wox, thymox and inox respectively at drug concentrations of 400 uM. The inhibition is concentration dependent. Differences in activities of these drugs are not due to permeability of cells in this case since cellular extracts were used, however, purified enzymes would be ncessary for more quantitative evaluations of drug effect on DNA polymerase per se. In similar

TABLE 10

INHIBITION OF DNA POLYMERASE BY Ψ-OX, THYMOX AND INOX

Crude enzyme extracts (4.5 mg/ml protein) were incubated with drugs or water for 10 minutes then cooled to 3°C and substrates were added and incubation was continued for 20 minutes. Total reaction volume per tube was 0.5 ml. Incorporation of [^{3}H]-thymidine into DNA was evaluated as described in Materials and Methods.

INHIBITION OF DNA POLYMERASE BY Ψ-OX, THYMOX AND INOX

DRUG	CONC. uM	EXP. 1 CPM	EXP. 1 % I	EXP. 2 CPM	EXP. 2 % I	Avg. % I
Ψox	100	63824	20	38278	17	19±2
	400	30520	62	20090	57	60±3
Thymox	100	58053	27	43990	5	16±11
	400	40490	49	31748	31	40±9
Inox	100	72027	10	38374	17	14±3
	400	54893	31	32189	30	31±0
Control	---	79403	0	46228	0	0

experiments, Vander Velde et al. (12) reported inhibition of DNA polymerase by dimethyladenosine dialdehyde in L1210 cells and Kimball et al. reported inhibition of DNA polymerase by the periodate oxidation product of β-D-Ribosyl-6-methylthiopurine (30).

The Effect of the Drugs on RNA Polymerase Activity

In a series of experiments conducted on similar enzyme preparations, Spoor et al (59) reported that methylthioinosinedialdehyde (MMPR-OP) inhibited RNA polymerase from Ehrlich ascites cells. The percent inhibition was 44.5% at a MMPR-OP concentration of 500 uM. Results of research reported herein (Table 11) show that Ψox, inox and thymox also inhibit RNA polymerase at 400 uM by 44%, 21% and 34% respectively. Other researchers (44, 61) report that RNA polymerase is not inhibited by inox. Inhibition of RNA polymerase may be affected by the preparation and source of the enzyme. For example, Cory et al., using isolated nuclei or nuclear extracts of Ehrlich ascites cells concluded that the inhibition of RNA synthesis was not the result of selective inhibition of RNA polymerases (61). Using E. coli RNA polymerase Cysyk and Adamson also report that inox did not inhibit the enzyme (44). Since enzyme preparations used in this research were not purified and were from total cellular RNA polymerases, other factors which may affect the reactivity and inhibition may be present in the preparation. Under these conditions, however, it is safe to assume that the activity of RNA polymerase is affected by these drugs as shown by these techniques.

TABLE 11

INHIBITION OF RNA POLYMERASE BY Ψ-OX, THYMOX AND INOX

Drugs or water were incubated with crude enzyme extracts of L1210 cells (4.5 mg/ml protein) for 10 minutes at 25°C. Substrates and reagents were added and incubation was continued for 15 minutes as described in Materials and Methods. Total reaction volume was 0.25 ml. Incorporation of [^{3}H]-uridine was assayed as described.

INHIBITION OF RNA POLYMERASE BY ψ-OX, THYMOX AND INOX

DRUG	uM	EXP. 1 CPM	EXP. 1 %I	EXP. 2 CPM	EXP. 2 %I	Avg. % I
ψox	100	792	15	1502	48	32±16
	400	563	40	1494	48	44±4
Thymox	100	758	19	1881	34	27±8
	400	725	22	1590	45	34±12
Inox	100	734	21	2284	20	21±0
	400	613	34	ND	ND	34
Control	---	931	0	2869	0	0

It may be postulated that in the intact cell the activity of RNA polymerase may be inhibited.

Detection of Colchicine Binding Activity in Cellular Extracts

The 100,000 x g supernatant extracts of cells obtained from ascites tumors of 3 BDF_1 mice were prepared as described in materials and methods. The extracts, containing 4.5 mg/ml protein, were diluted with PK-GTP buffer to contain varying protein concentrations. One ml aliquots were mixed with 0.1 ml [^{3}H]-colchicine and incubated for 1 hour at 37°C. Bound colchicine were detected by the filter stack assay as described. Counts were adjusted for background which accounted for only 0.06% of the input activity. Results (Figure 26) show that optimal concentrations of protein for colchicine binding activity range from 100 ug/ml to 1.5 mg/ml. In subsequent tests, assays were done using protein concentrations within these ranges.

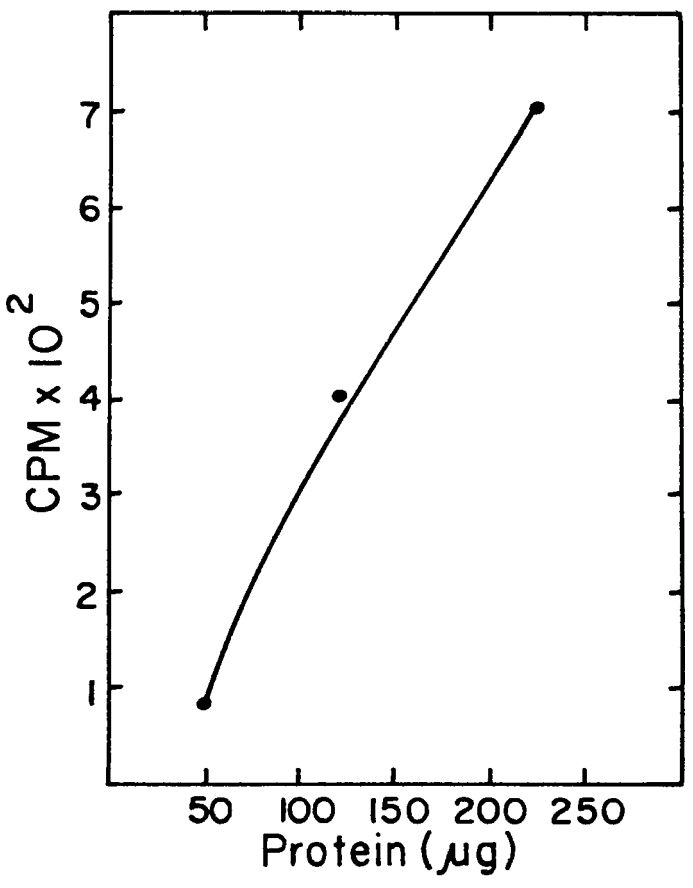
Effect of Wox, Thymox, Inox and Etopside and Colchicine Binding Activity in Vitro

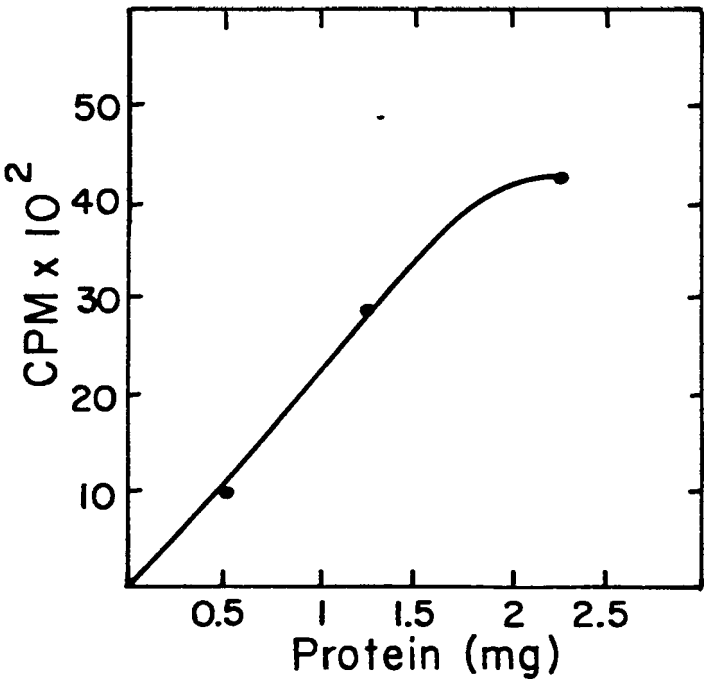
The 100,000 x g supernatant extracts prepared from ascites tumors in DBA/2 mice were tested immediately or stored at -87°C and used within 2 weeks. Periodate oxidation product drugs were made up in distilled water immediately before testing. Tubes containing 1 ml aliquots of cellular extract (1 mg of protein/tube) were preincubated with 100 ul of drugs for 1 hour at 37°C. [^{3}H]-colchicine was added to each reaction tube and the mixture was incubated for two additional

FIGURE 26

COLCHICINE BINDING ACTIVITY IN L1210
CELL EXTRACTS

One ml volumes of the 100,000 x g supernatant extracts of BDF_1 ascites tumor cells diluted in PK-GTP buffer to contain varying concentrations of protein were incubated for 1 hour at 37°C with 0.1 ml (^3H)-colchicine (final concentration 2 X 10^{-6}M, approximately 10^6 CPM). Final volume per tube was 1.1 ml. Bound colchicine was assayed by the filter stack method.


CPM x 10²
7
6
5
4
3
2
1
50
100
150
200
250
Protein (µg)


CPM x 10²
50
40
30
20
10
0.5
1
1.5
2
2.5
Protein (mg)

hours. Bound colchicine was assayed by the DE81 filter stack method described. Results are shown in Table 12.

Podophyllotoxin at a concentration of 10^{-5} moles per liter effectively prevents colchicine binding to chick embroy brain supernatant extracts (71). As shown in Table 12A etopside, an analog of podophyllotoxin, was a less potent inhibitor. It reduced colchicine binding 25% and 48% at concentrations of 3.4×10^{-4} and 3.4×10^{-3} respectively. No significant inhibition of binding was observed after treatment of the 100,000 x g supernatant extracts with ψox, thymox or inox. The apparent 14% increase in binding in the presence of thymox may reflect slight stabilization of binding as seen with VBL (71).

To show the effect of drugs on the preformed tubulin-colchicine complex, tubes containing 1 ml of the 100,000 x g supernatant extract were preincubated with [^{3}H]-colchicine for 2 hours at 37°C before addition of etopside. After addition of etopside, incubation was continued for an additional hour. As shown (Table 12B) etopside had no significant effect on the preformed colchicine-tubulin complex.

Effect of Drug Treatment on Cells Used for Tubulin Extraction

Since the oxidation product drugs had no significant direct effect on tubulin-colchicine binding (Table 12), experiments were done to determine if the amount of tubulin in the cells was altered after 18 and 24 hours exposure to the drugs.

L1210 cells with a population doubling time of 24±2 hours were diluted with growth medium to a concentration of $5\text{-}6 \times 10^5$ cells/ml

TABLE 12

EFFECT OF ψOX, THYMOX, INOX AND ETOPSIDE ON COLCHININE BINDING

A. Tubes containing 1 ml aliquots of 100,000 x g supernatant extract (1.0 mg protein) were preincubated with 100 ul volumes of drugs for 1 hour at 37°C. [^{3}H]-colchicine, final concentration 2 x 10^{-6}M, approximately 10^6 CPM, (0.1 ml) was added to each tube and incubation was continued for two hours. Total volume in each tube was 1.1 ml and bound colchicine was determined by the filter stack method.

B. Tubes containing 1 ml of the 100,000 x g supernatant extract were preincubated with 0.1 ml [^{3}H]-colchicine for two hours then with etopside for an additional hour and assayed as described.

EFFECT OF Ψ-OX, THYMOX, INOX AND ETOPSIDE ON COLCHICINE BINDING

DRUG	CONCN. Moles/Liter	CPM	INHIBITION %	INCREASE %
A)				
Etopside	3.4×10^{-3}	4820	48	--
	3.4×10^{-4}	6899	25	--
	3.4×10^{-5}	9511	--	3
Ψox	1.0×10^{-4}	9745	--	5
	4.0×10^{-4}	8713	6	-
Thymox	1.0×10^{-4}	10697	--	14
	4.0×10^{-4}	10690	--	14
Inox	1.0×10^{-4}	9034	2	--
	4.0×10^{-4}	9772	--	5
Control	--	9238	0	-
B)				
Etopside	3.4×10^{-3}	8979	3	--
	3.4×10^{-4}	8344	9	--

and the drugs contained in 0.03 volumes of medium were added to 120 ml volumes of cell suspension. The drugs, 100 uM final concentration were made up in FGM and filtered through 0.45 um pore size Millex filters immediately before addition to cell suspensions. Results are shown in Table 13. Cells treated with ψox and thymox decreased in number by 23 and 22% respectively over the 18 hour period in experiment I, and by 11 and 3% in the 24 hour period of experiment II. Inox treated cells increased in number by 1% in experiment I and 1.2% in experiment II. Differences in cell numbers between experiments may reflect populations with a faster generation time in experiment II as well as the 24 hour testing period. Control cells increased only 1.1 times in experiment I compared to 1.9 times in the second experiment.

At the time cells were harvested for tubulin extractions, the viability of ψox treated cells was 85% and 77% for the two experiments. Thymox and inox treated cells were essentially as viable as control cells.

Mitotic indices of ψox treated cells were slightly lower than for controls. There was no significant difference in the mitotic index for thymox and inox treated cells compared to the control.

To confirm that cells were accumulated in G2, FMF profiles were run (data not shown). There was a typical marked increase in the number of cells in the peak representing the G2+M phase of the cell cycle for the drug treated cells.

TABLE 13

PROLIFERATION, VIABILITY AND MITOTIC INDEX OF L1210 CELLS TREATED WITH Ψ-OX, THYMOX, AND INOX

Cells, 120 ml volumes, were treated for 18 and 24 hours with drugs contained in 0.03 volumes of growth medium to give a final drug concentration of 100 uM. Samples were removed and evaluated as described in Materials and Methods.

PROLIFERATION, VIABILITY AND MITOTIC INDEX OF L1210 CELLS TREATED WITH ψ-OX, THYMOX AND INOX

SAMPLE TIME	DRUG	CELLS/ml ($X\ 10^5$)	VIABILITY %	MITOTIC INDEX %
Experiment I. (18 hour)				
0 hr.	ψox	5.5	ND	ND
	Thymox	5.6	ND	ND
	Inox	5.5	ND	ND
	Control	5.5	ND	ND
18 hr.	ψox	4.2	85	3
	Thymox	4.4	96	7
	Inox	5.7	98	3
	Control	6.2	98	8
Experiment II. (24 hour)				
0 hr.	ψox	6.2	ND	ND
	Thymox	6.0	ND	ND
	Inox	6.0	ND	ND
	Control	6.0	ND	ND
24 hr.	ψox	5.5	77	3
	Thymox	6.1	91	8
	Inox	7.2	95	6
	Control	11.9	94	7

Effect of Drug Treatment on Total Protein Content of L1210 Cells

Table 14 shows the amount of total protein in drug treated cell extracts. When the amount of total protein was adjusted to values for 100,000 cells/ml, the difference in the total amount of protein in extracts of ψox treated and control cells was 17% in experiment I and 3% in experiment II. In experiment I, thymox and inox treated cells had the same amount of protein as the controls, however, in experiment II, thymox and inox treated cells had 30% more protein than control cells. Accumulation of protein due to a longer incubation period may correlate with observed microscopic appearance of enlarged cells after thymox and inox treatment. ψox treated cells do not appear significantly enlarged.

Since these Lowry determinations do not reflect the protein content distribution in cells in the population, FMF analysis of these cells was done. Whole cells were stained with FITC as described. Figure 27 shows FMF profiles of total protein distribution of cells exposed to drugs for 18 hours in experiment I. The control cell population protein distribution shows two peaks representing one large population with greater protein mass and a smaller population with less protein. Distributions for ψox, thymox and inox treated cells show a shift of the cell population containing large protein mass to the population with less protein. The shape of the large mass population, however, suggests that many cells contain a greater amount of protein than those in the large peak of the control.

TABLE 14

TOTAL PROTEIN IN 100,000 X g SUPERNATANT EXTRACTS

Cells were exposed to 100 uM concentrations drugs for 18 and 24 hours for Experiments I and II respectively. The 100,000 X g supernatant extracts were prepared as described in Materials and Methods and the total protein was determined by the Lowry method.

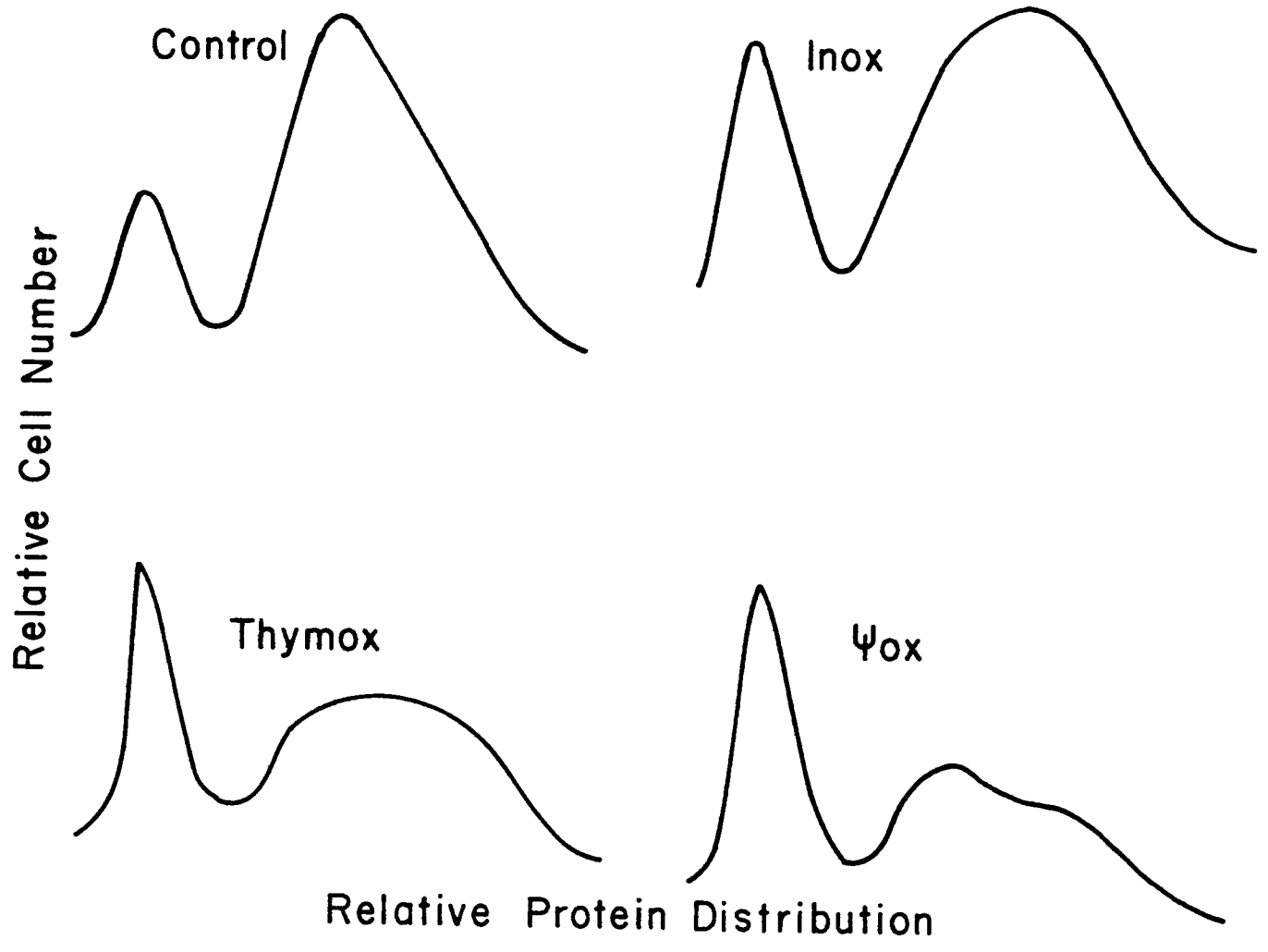
TOTAL PROTEIN IN 100,000 x g SUPERNATANT EXTRACTS

DRUG	CONCN. ug/ml	TOTAL PROTEIN ug/100,000 cells	% of Control
Experiment I.			
Wox	294	69	17
Thymox	382	86	104
Inox	474	83	100
Control	518	83	--
Experiment II.			
Wox	350	64	3
Thymox	574	94	142
Inox	669	93	141
Control	782	66	--

FIGURE 27

FMF PROTEIN PROFILES OF CELLS TREATED FOR
18 HOURS WITH DRUGS

Cells were treated for 18 hours with the drugs and stained with FITC as described in Materials and Methods.


Control
Inox
Thymox
Ψox
Relative Cell Number
Relative Protein Distribution

FMF profiles for Experiment II are shown in Figure 28. Cells were sampled at 24 hours in this experiment and only one major protein peak was seen for control cells. The observation of two peaks in Experiment I and one peak in Experiment II may simply be an expression of cells in different cell cycle phases due to sampling times. For asychronously growing L1210 cells, Crissman et al. observed only one protein peak (20). As seen in Figure 28, FMF profiles for thymox and inox treated cells were drastically different from the control. Populations of cells with the larger protein mass predominated. The difference in sampling times for the two experiments makes it difficult to compare the profiles directly. However, in both experiments, the protein distribution shows a broadening and flattening of the peak for cells with greater protein mass. It is safe to conclude from these FMF profiles that the drugs dramatically alter the protein content of the cells. This is consistent with the idea that thymox and inox treated cells become enlarged and that ψox cells also become enlarged but to a lesser degree.

Effect of Drug Treatment on the Rate of Protein Synthesis in L1210 Cells

After 4 hours of exposure to drugs, cells were sampled for protein synthesis as a function of [^{3}H]-leucine uptake as described in Materials and Methods. There was a reduction in the rate of synthesis (Figure 29) of 54%, 40% and 22% respectively for ψox, thymox and inox after 45 minutes of incubation with the labeled precursor.

FIGURE 28

FMF PROTEIN PROFILES OF CELLS TREATED FOR
24 HOURS WITH THYMOX AND INOX.

Cells were treated with 100 uM drugs and stained for FMF analysis with FITC.

——— Control cells

– – – – – – Thymox

·· – ·· – ·· Inox

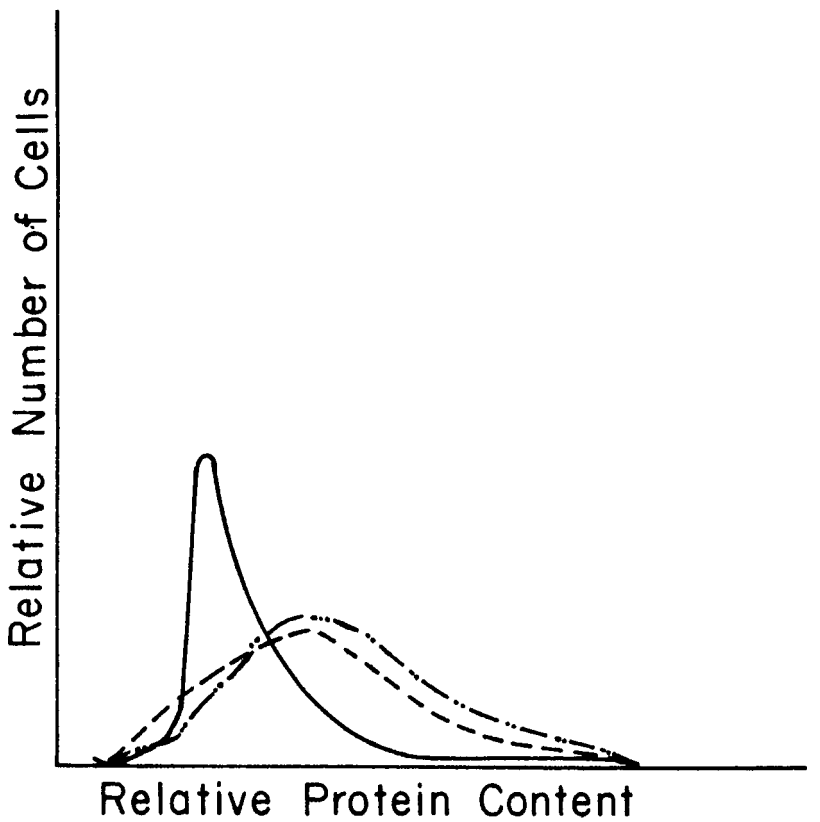
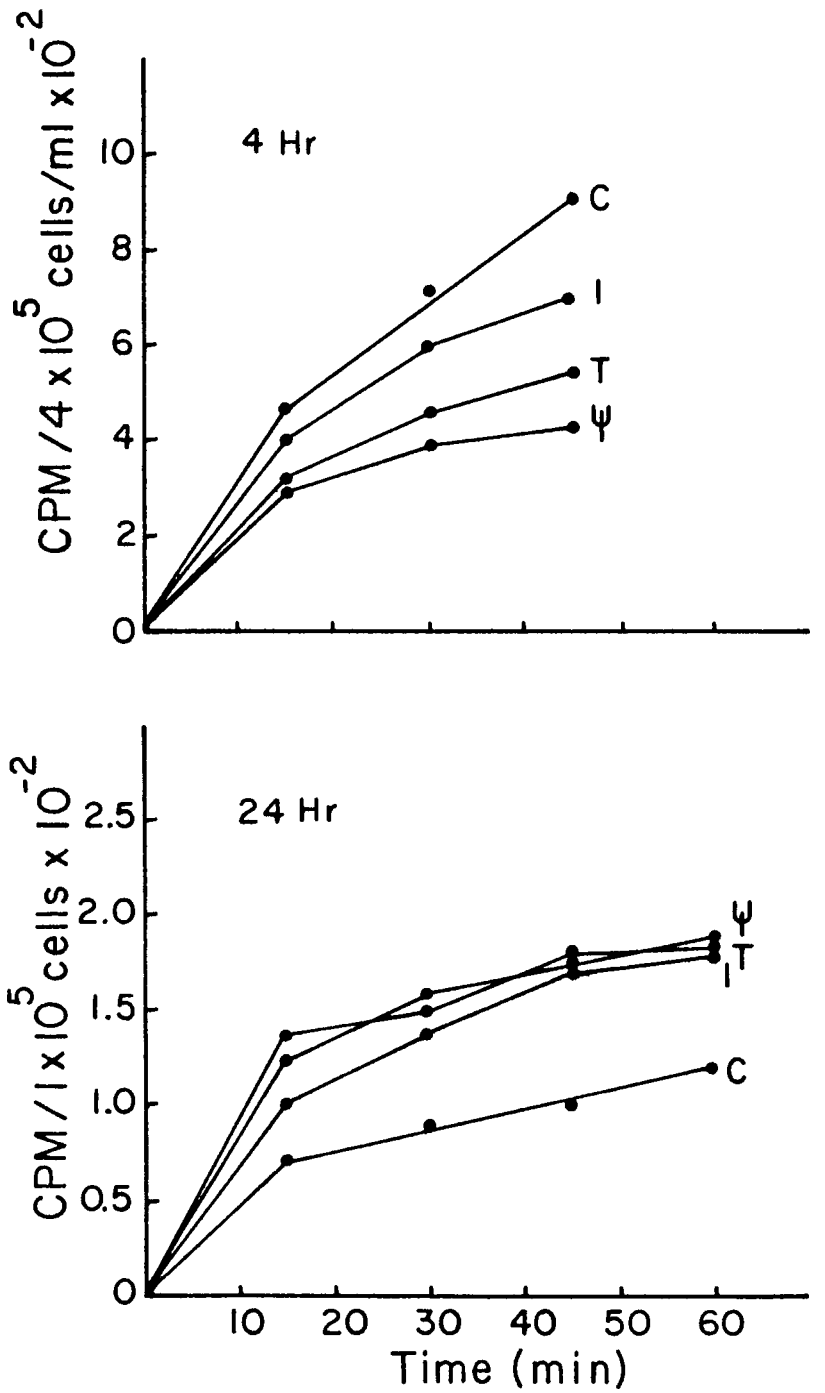

Relative Number of Cells
Relative Protein Content

FIGURE 29

THE RATE OF PROTEIN SYNTHESIS AT 4 AND 24 HOURS IN
Ψ-OX, THYMOX AND INOX TREATED CELLS

Cells were treated with drugs at 100 uM for 4 and 24 hours. Ten ml aliquots were removed at 15 minute intervals and (^{3}H)-leucine incorporation was measured as described in Materials and Methods.


4 Hr
CPM/4 x 10^5 cells/ml x 10^-2
0
2
4
6
8
10
C
I
T
Ψ
24 Hr
CPM/1 x 10^5 cells x 10^-2
0
0.5
1.0
1.5
2.0
2.5
Ψ
T
I
C
10
20
30
40
50
60
Time (min)

After 24 hours of exposure to the drugs, the cells were still able to synthesize protein, but at a much reduced rate. This indicates that a portion of the cells in the population is unaffected. While treated cells have fewer cycling cells (see Table 14, Experiment II), Figure 29 shows that the rate of protein synthesis appears to be greater per unit number of cells than that of the control. This is partially due to the fact that control cells had reached a growth slow-down because of population density (1.2×10^6 cells/ml). The total cell counts at 24 hours for Ψox, thymox and inox expressed as percent of control were 56%, 51% and 61% respectively, whereas the total cell protein synthesis rate at 60 minutes for the drugs was 66%, 73% and 86% of the control respectively. This suggests that some blocked cells may continue to synthesize protein.

Based on the findings that protein synthesis is inhibited, that there is an effect on protein content of the cells as shown by FMF, that total protein detected by the Lowry method in extracts of populations of drug treated cells is not significantly reduced in Experiment I at 18 hours, and that cells accumulate a G2 amount of DNA and do not reach mitosis, it is reasonable to speculate that tubulin is one protein selectively inhibited by these drugs.

Tubulin Levels in Drug Treated Cells

The effect of drugs on the amount of tubulin available for colchicine binding is shown in Table 15. The amount of colchicine binding activity in extracts of Ψox treated cells is significantly reduced

TABLE 15

EFFECT OF DRUGS ON TUBULIN LEVELS IN L1210 CELLS

One ml of the 100,000 x g supernatant extract of cells treated with 100 uM concentrations of drugs was incubated with 0.1 ml [^{3}H]-colchicine (final concentration 2 x 10^6 M, approximately 10^6 CPM) for one hour at 37°C. Tubulin bound colchicine was evaluated by the filter stack method as described.

EFFECT OF DRUGS ON TUBULIN LEVELS IN L1210 CELLS

DRUG	[^{3}H]-COLCHINE INCORPORATED pmoles/ug protein	INHIBITION %
Experiment I		
Ψox	7.25	81
Thymox	19.20	50
Inox	33.8	12
Control	38.3	0
Experiment II		
Ψox	6.16	72
Thymox	15.5	29
Inox	21.4	2
Control	21.8	0

(81% and 72%). Since the amount of total protein per 100,000 cells (Table 15) decreased by an average of only 10% for the two experiments, there is an apparent selective reduction in amount microtubule protein as a result of ψox treatment. For cells treated with thymox for 18 and 24 hours, the average inhibition of colchicine binding was approximately 40% even though total cellular protein was increased by an average of 18% compared to controls in the two experiments. Thus, there is a marked decrease in the amount of microtubule protein in cells as a result of treatment for 18 and 24 hours with ψox and thymox. There was no significant effect on tubulin levels in inox treated cells. This may be due to the low concentration of inox used in these tests (100 uM) or less reactivity in the cells as a result of steric effects due to molecular structural differences between the purine and pyrumidine bases.

Section II

DISCUSSION

The effects of ψox, thymox and inox on macromolecular synthesis of DNA, RNA and protein are consistent with reports of others (11, 32, 57, 61) with the exception that these results show more inhibition of DNA than RNA synthesis. These differences may be partially due to experimental methods employed. Inhibition of DNA synthesis of ψox was greater at 4 hours than for thymox or inox possibly due to differences in cell permeability to the drugs. After 26 hours, DNA synthesis for all 3 drugs is drastically inhibited. RNA synthesis is inhibited less at 26 hours than at 4 hours for ψox. Since ψox has a half-life of approximatele 5 hours in FGM, it appears that the presence of the drug is needed to maintain the level of initial effect. Inhibition of RNA synthesis may in part be reversible with time. This is suggested by the fact that DNA synthesis is inhibited to a much greater degree at at 26 hours and appears therefore to be cumulative and irreversible. The half-life of mRNA in HeLa cells is 6-7 hours for about 60% of the mRNA and 24 hours for 40% (79). In L cells, mRNA turns over about once per generation (80). Crissman (20) states that 66.5% of asynchronously growing cells are in S at any given time. The greater effect seen on DNA synthesis may be due in part to this fact. This data shows clearly, however, that the primary effect is on DNA synthesis rather than RNA synthesis.

Cysyk and Adamson (44) reported that there was no inhibition of RNA polymerase enzymes purified from E. coli and Cory et al. stated a lack of inhibition of RNA polymerase in nuclei of Ehrlich tumor cells (61). My research showed some inhibition of both RNA and DNA polymerases in crude cellular extracts. These inhibitions may be due to the presence in the preparations of other factors which enhanced the inhibition and, indeed, may reflect the process in intact cells. Other researchers using crude extracts also demonstrated inhibition of DNA and RNA polymerases with other oxidation product drugs (12, 30, 59). Miller, using purified E. coli RNA polymerase, showed inhibition of this enzyme by Ψox (81).

The effect of the drugs on protein synthesis is immediate, as for DNA and RNA synthesis inhibitions. Studies at 4 hours after addition of drugs to cells showed inhibition of DNA > protein > RNA synthesis. However, as shown in Figure 29, protein synthesis occurred after 24 hours of drug exposure and it appeared that some blocked cells continued to synthesize protein. The continued synthesis of protein and RNA while DNA synthesis is greatly reduced would results in unbalanced growth and lead to cell death. This is consistent with appearance of enlarged cells and viability studies reported in Section I. In Experiemnt II, thymox and inox treated cells contained more protein than in Experiment I. This is probably due to the longer test period (24 hours versus 18 hours), and is consistent with the continued synthesis of total cellular protein. Apparently, however, the level of tubulin was selectively reduced in these cells (Table 16).

That levels of certain proteins, possibly the nonhistone proteins, in cells may influence entry of cells into mitosis has been previously suggested (66). The level of microtubule protein in the cell might also influence the progression of cells from G2 into mitosis. Formation of the mitotic spindle, a factor in the promotion of cells from G2 into mitosis, occurs in a sequential manner and is dependent on the previous synthesis of the components of spindle fibers and the replication of the centrioles as well as the assembly of tubulin into microtubules and orientation of the spindle (82). The assembly of tubulin into microtubules apparently is not affected by Ψox, thymox and inox since these drugs do not prevent or compete with colchicine binding to tubulin in cellular extracts (Table 13). Therefore, a mechanism of action of these drugs is not prevention of polymerization of pre-synthesized tubulin as in the case of podophyllotoxin and colchicine (71, 83). Etopside, an analog of podophyllotoxin, though less effective, inhibits colchicine binding (Table 13).

Since the oxidation product drugs had no effect on the binding of colchicine to tubulin in vitro, experiments were done to determine the effect on cellular tubulin levels by exposing cells to drugs for the time required for one population doubling. Cells harvested after 18 and 24 hours of exposure to the drugs showed reduced colchicine binding activity presumably indicating a reduction in the amount of tubulin in drug treated cells (Table 16). The drug, taxol, blocks cells in late G2 or M by promoting microtubule assembly and by preventing the disassociation of polymerized microtubules (84). Thus it effectively

reduces the level of free tubulin necessary for assembly of the mitotic apparatus. Though the oxidation product drugs do not promote assembly, it appears that they effectively reduce the amount of microtubule protein. Reduction of tubulin below the critical level needed for microtubule assembly might account for the G2 block. Also, tubulin associated protein (TAP) is necessary for 80% of assembly promoting activity of tubulin (85). Whether the activity of the periodate oxidation-product drugs is directly on the synthesis of microtubule protein or its associated proteins, or on the function or synthesis of a tubulin associated protein, remains to be determined. The following experiments would provide further evidence of the involvement of tubulin as a target for these drugs: premature chromosome condensation studies to determine if blocked cells contain G2 type chromatin and if it is damaged; electron microscopic examination of cell preparations to determine if centrioles have formed and replicated and to visualize microtubules; and microscopic examination to find if cells are multi-nucleated indicating faulty cytokinesis.

The basic question still remains. What is the primary biochemical mechanism of action of the periodate oxidation product drug? Since the drugs were shown to have cell cycle phase specific activity as well as non-specific activity, I propose that the drugs are first altered by rapid irreversible binding (probably Schiff's base reactions) to many cellular components which causes cleavage of the molecule thus providing more than one moiety for activity with cellular components.

Khym and Cohn (86) reported a high preference (90-95% yield) for the sodium borohydride reduction product of one aldehyde group, the one distal to the purine or pyrimidine base. This occurs in slightly acid medium conditions (perhaps such as inside cells). The resulting monoaldehydes then react with phenylhydrazine to give the free base, glyoxal and glycerol. As stated the dialdehyde moiety reacts rapidly with glycine, lysine, other primary amines and proteins (44, 87). The glyoxal compounds are reactive with DNA and the nucleic acid bases cytidine and guanosine (62). If this is the case inside the cell, then at least two classes of reactive moities are available, the nucleic acid base and a glyoxal. These are in addition to reactivity of the oxidation product molecule per se.

Martin Apple (88) has proposed four classes of mechanism by which the currently effective anticancer drugs seem to act. They are action at an enzyme regulatory center, action at an enzyme catalytic center, blocking of enzyme-substrate complex by action on the substrate, and action on non-enzymatic protein receptors. Using this model and the proposed chemical modifications of the drugs described above I have attempted to fit my experimentally obtained data to these mechanisms and to propose reasonable mechanisms of action for Wox and thymox.

The oxidation product drugs may function to inhibit specific enzymes, as stated by this and other researchers, by exerting effects on allosteric sites of enzymes if free bases are released by reactions of the oxidation products within the cell. For instance, thymidine

is a feedback inhibitor in the pathway to DNA. MMPR acts as a false feedback inhibitor in the synthesis of AICR (89).

Action on enzyme active centers by reacting with e-amino groups of lysine could be due to reactivity of the aldehyde moities, possibly resulting in cleavage of the glycosidic bond of the drug and freeing the base purine or pyrimidine.

Examples of action on non-enzyme protein receptors are steroid-specific transposition-effector proteins and tubulin protein (88). Drugs like colchicine, vinblastine, and maytansine react with tubulin protein and impair its function (90). Since I showed that Ψox, thymox and inox had no direct effect on tubulin binding in vitro, this model does not apply unless the mechanism is more complicated and involes gene regulation. In the case of the steroid receptor protein, the receptor has two subunits. After formation of a steriod-receptor complex, the complex is transposed to the chromosomes where a nonhistone chromosomal protein combines with one subunit of the steroid receptor complex. The other steroid receptor is released and combines with DNA which creates an RNA polymerase initiator site (91). This model illustrates the function of one protein in conjunction with nonhistone proteins in gene regulation. Involvement of the oxidation product drugs with nonhistone proteins and gene regulation cannot be ruled out.

The last of Apple's proposed possibilities, that of blocking a reaction because of the drug forming a complex with a substrate is especially appealing. Examples of these reactions include intercalation and alkylation. There is probably little reason to suspect that the

oxidation product drugs act by intercalation because of the high reactivity of the dialdehyde groups. There are more than a dozen alkylating agents in use as chemotherapeutic drugs and as yet no distinctive relationship linking their differing structures to their activity in alkylation have been confirmed. These drugs may transfer alkyl groups to functionally important cell components and thus impair function. The reactive cellular molecule may be amino, carboxyl, sulfhydryl, phosphate or any base (88). Some alkylations are repaired; but those occurring in purine bases are not repaired if interchain linking or linking of alkylated guanine to a chromosome-bound histone or nonhistone protein occurs. The phosphate backbone of nucleic acid strands may be alkylated on the phosphate backbone and thus induce strand breakage (92). Binding of [^{14}C]-inox to phosphate or to DNA was not shown by Cysyk and Adamson (44). This was probably because the moiety that binds is not the base but rather the cleaved glyoxal moiety. The effects of Ψox and thymox are irreversible on DNA synthesis and cell proliferation. If interchain linking of DNA or linking of alkylated guanine to an essential protein occurs, the effect would be irreversible. Using [^{14}C] labeled inox, Cysyk and Adamson failed to show binding of inox to nucleic acids or bases, but if the glycosidic bond is broken and the alkylating moiety resides in the glyoxyl part of the molecule, then their method would not detect the true effect and binding may really occur. Miller (81) found that Ψox cross-links histones but at

a non-physiological concentration. However, inside the cell, the drugs may be concentrated or metabolized to a form which would coincide with this mechanism.

Additonal evidence that the oxidation product drugs may produce their major activity by reacting with DNA comes from consideration of the action of bleomycin, a DNA binding drug. Bleomycin causes cells to accumulate in G2 after a prolonged S phase (93) in the same manner as Ψox, thymox and inox. The mechanism of DNA strand interaction by bleomycin was proposed by Müller et al. in 1972 (94). Sulfhydryl groups enhance bleomycin scission of DNA (not yet shown for our drugs), thymidine is released from cut DNA and this is in conjunction with generation of free aldehyde groups. Possibly the aldehyde formed from ring-opening of deoxyribose is a result of hydrolysis of thymine N-glycosidic bonds. Phosphodiester bond cleavage is associated with generation predominantly of 5'-phosphoryl-ended DNA fragments (95), and the breaks may not be repaired by DNA ligase. In L cells cut DNA was isolated and 10 single strand breaks for every double strand break were found (96). This is consistent with Miller's report of single strand scission being more common than nicks of double strands with PM2 DNA by Uox (81). Miller's suggestion that damage to DNA may be due to depyrimidation is consistent with these observations with bleomycin.

Based on these observations and the action of alkylating agents in substitution reactions (alkylation of bases), cross-linking reactions (intra- or inter-strand cross-linking), and strand breaking reactions, it is reasonable to assume that the activity of the oxidation product

drugs is due to their action as alkylating agents. This evidence is indirect and should be proven by chemical studies and by use of drugs labeled on the ribose moiety as well as the base.

The effect on tubulin levels in drug treated cells is consistent with the hypothesis since alkylation could result in an irreversible block of some regions of DNA which perhaps transcribe the message for tubulin. Evidence shows that cell cycle traverse in eukaryotic cells is regulated by gene expression (97). Replication of DNA follows cycle sequence during characteristic parts of S and the order depends partially on frequency of repetition of certain nucleotide sequences (98). For instance, some types of highly repeated sequences are replicated preferentially in early or in late S. DNA for one kind of moderately repeated sequence, that for ribosomes (rDNA), is replicated mostly during early S in CHO cells. The role for the moderately repeated nucleotide sequences probably have regulatory functions for transcription. If drug binding occurs at a time when the moderately repeated nucleotide sequences are being transcribed, then the regulation of transcription of certain mRNA, thus specific protein synthesis, could be impaired. This might account for the specific reduction of certain proteins like tubulin since cells in late G1/early S are affected by Ψox and thymox. Irreversible binding of drugs at this time could prevent unwinding and transcription of certain messages while other mRNA and protein synthesis would be unaffected. It should be stated that other proteins needed for mitosis may

be affected as well. These would include the histone and nonhistone proteins and tubulin associated proteins. Cyclic AMP also should be considered.

To confirm whether the oxidation products affect the gene for tubulin per se, application of the technique reported by Meza (99) using isolated tubulin mRNA could be used. Cory et al. have stated that activity of inox may be due to template function impairment or RNA chain termination (61). By using the isolated message for tubulin such a question could be solved for Wox and thymox.

SUMMARY

The periodate-oxidation products of pseudourudinedicarboxaldehyde (ψox) and thymine ribosidedicarboxaldehyde (thymox) inhibited the proliferation of L1210 cells in vitro in a time and concentration dependent manner. At equimolar concentrations (100uM) ψox was more cytotoxic. The biological activity half-life of ψox and thymox, when incubated in cell culture medium at 37°C was 5 and 24 hours respectively. ψox is stable for at least one year stored lyophilized at 0°C and for at least 5 days dissolved in water. The minimum effective exposure time of cells to drugs was 30 minutes for ψox and 2 hours for thymox to produce a 30% reduction in proliferation rate. FMF profiles for DNA distribution show that both durgs blocked cell cycle progression in G2+M and mitotic index studies showed that the arrest was in G2. In synchronized L1210 cells the cell cycle phase specific activity for both ψox and thymox was on cells in late G1-early S and G2. ψox activity also increased dramatically in cells treated during S and late G2. The drugs showed some cytotoxicity throughout all phases of the cell cycle. ψox was more toxic than thymox.

Biochemical mechanisms of action include time and concentration dependent inhibition of DNA and RNA synthesis. DNA synthesis was inhibited more than RNA synthesis. DNA and RNA polymerases in crude cellular extracts were inhibited to about the same extent. Protein synthesis was inhibited to a greater degree than RNA synthesis after 4 hours of exposure of cell to drugs. Blocked cells continued to

synthesize some RNA and protein at 24 hours of exposure. FMF profiles of protein at 18 and 24 hours show severe perturbations on the distribution of protein in drug treated cells. Subjecting the cells to Ψox and thymox for 18 and 24 hours reduced the amount of cellular tubulin detected by the colchicine binding assay The drugs did not inhibit colchicine binding to tubulin in vitro as did an analog of podophyllotoxin (etopside). Therefore assembly of microtubules was presumably not affected by Ψox and thymox. The effect of Ψox and thymox in reducing cellular tubulin levels appears to be the inhibition of synthesis of microtubule protein. The lack of tubulin in cells may account, in part, for the G2 arrest of cells.

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APPENDIX A

Giemsa Stain and Stock Buffers

Preparation of Giemsa Stain and Stock Buffers

A. Giemsa Stain Stock Solution

1. add 0.6 g giemsa powder to 50 ml methanol
2. dissolve on magnetic stirrer
3. add 25 ml glycerol
4. let stand at room temperature in a brown bottle for 2-3 days
5. filter through any large pore size filter paper

B. Buffered Giemsa Solution

4.5 ml KH_2PO_4 stock

5.5 ml Na_2HPO_4 stock

3.0 ml filtered giemsa stock

30.0 ml distilled H_2O

C. Buffered H_2O

4.5 ml KH_2PO_4 stock

5.5 ml Na_2HPO_4 stock

30.0 ml distilled H_2O

D. KH_2PO_4 stock (0.15M)

4.637 g KH_2PO_4

500 ml distilled H_2O

E. $NaHPO_4$ stock (0.15M)

4.733 g Na_2HPO_4 or 8.94 g $Na_2HPO_4 \cdot 7H_2O$

500 ml distilled H_2O