The Mechanism of Action

of

Methotrexate

by

Elizabeth Ann Jacobson

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"THE MECHANISM OF ACTION

OF METHOTREXATE"

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E. ANN JACOBSON

A dissertation submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

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TABLE OF CONTENTS

LIST OF CHARTS AND TABLES

CHART

TABLE

ABBREVIATIONS USED ARE:

MTX, methotrexate; DHFR, dihydrofolate reductase enzyme;

^{3}H-UdR, deoxyuridine tritiated in position 6;

^{3}H-TdR, thymidine tritiated in the methyl group;

^{3}H-dUMP, deoxyuridine monophosphate tritiated in position 5;

FH_2, dihydrofolate; FH_4, tetrahydrofolate;

TS, thymidylate synthetase enzyme; AdR, deoxyadenosine

SUMMARY

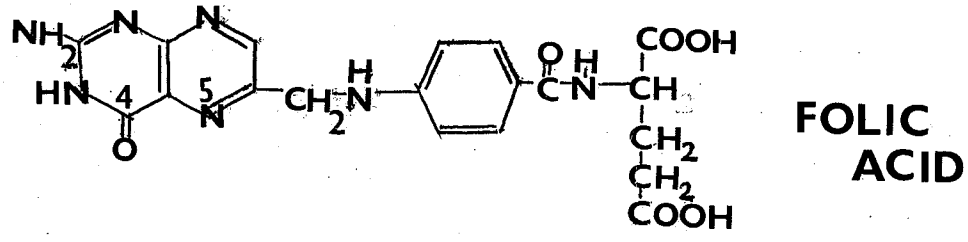
The 'calculated tritiated deoxyuridine rate' has previously been correlated with a methotrexate (MTX)-induced thymineless death as measured by cloning in L5178Y cells. This study extends the correlation to mid-log and resting cultures of CHO cells. The correlation could not be shown in early log CHO cultures due to the presence in fetal calf serum of dialyzible substances that protect against the cytocidal effects of MTX. Mid-log CHO cultures could also be protected against MTX by supplementing the medium with additional fetal calf serum at the time of drug treatment.

A MTX-induced drop in thymidylate synthetase activity was observed in extracts of both log and resting cultures of L5178Y cells. The addition of an exogenous purine source prevented the MTX-induced suppression of protein synthesis and prevented the drop in thymidylate synthetase activity. MTX may inhibit dihydrofolate reductase, rapidly deplete the L5178Y cells of reduced folates and thus produce a thymineless and purineless state. The purineless state, by inhibiting the synthesis of thymidylate synthetase may facilitate the deepening of the thymineless state.

An increased sensitivity to sonication of thymidylate synthetase activity from log cultures over resting cultures of L5178Y cells was detected. The recognition of this effect along with the discovery of protecting factors in fetal calf serum casts doubt upon many of the conclusions from previous studies of the mechanism of action of MTX where these variables may not have been adequately controlled.

HISTORICAL REVIEW

Megaloblastic anemia has been treated with folic acid conjugates with great success since 1943 (9). Soon after the development of this therapy, folates were used in an unsuccessful attempt to treat the anemia of acute lymphoblastic leukemia. Since the leukemic process was in fact enhanced by the folate, producing an 'acceleration phenomenon', it occurred to Farber that an anti-folate might be of value in the treatment of patients with acute leukemia (30). Following his suggestion, Subbarow and colleagues developed a series of folic acid analogs in 1948. The first analog was aminopterin which was folic acid with an amino group substituted for the oxygen at position C_4 of the pteridine ring.



FOLIC ACID

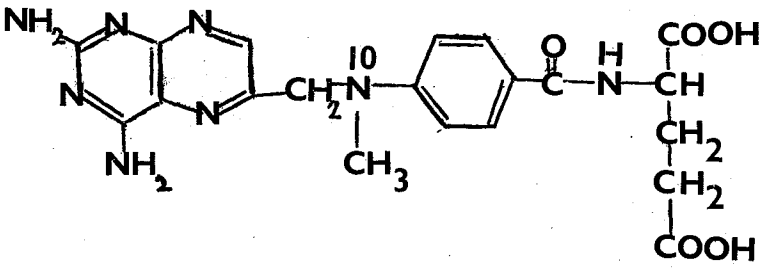
PTERIDINE

P-AMINO BENZOIC ACID

GLUTAMIC ACID

Ten out of 16 leukemic children treated with aminopterin by Farber exhibited rapid remission of signs and symptoms of their disease. However, eventually all relapsed and died despite continued treatment. This failure was not necessarily due to the drug but

rather the choice of dosage and scheduling. (60). Methotrexate (MTX), or amethopterin another analog, is aminopterin with an additional methyl group at position N_{10}.



MTX

Because aminopterin caused more gastrointestinal toxicity in animals with no increase in tumour cell kill, MTX is used more extensively in the clinic. MTX therapy has been found to cure some malignant diseases, and produce long survivals in others. However, in most solid tumours, it has been of limited usefulness.

One aspect of the action of MTX is interesting. If the malignancy is sensitive to MTX even in vitro, that malignancy is amenable to other forms of chemotherapy. For example, the degree of sensitivity to MTX in vitro, of cells from patients with acute myeloblastic leukemia (AML) appears to correlate with survival even though those patients are treated with drugs other than MTX (62). Thus, MTX appears to detect some vulnerability in the malignancy that makes it amenable to treatment. This susceptibility to MTX, if defined, might be exploited in the treatment of the malignancy. In order to understand how MTX might exert its antitumour activity, it is first necessary to review some basic concepts in chemotherapy of malignancy. One main theoretical scientific approach to the drug

treatment of malignancy was first suggested 15 years ago with the development of the cell kill hypothesis.

CELL KILL AND CANCER CHEMOTHERAPY

The concept of cell kill and its relationship to successful antitumour chemotherapy was first investigated by Skipper and Bruce (18,102,103). From their work evolved a more rational approach to drug therapy.

As defined by Skipper and Bruce, drug-induced cell kill was achieved when a cell's metabolic processes were perturbed to the point where it was unable to replicate itself even when no longer exposed to the drug. A cell might be intact physically and be capable of metabolism, e.g., limited protein synthesis, but still be 'dead', in the sense of being incapable of giving rise to clones or colonies.*

Skipper based his studies upon the ability of L1210 murine leukemia cells to proliferate in BDF_1 mice. Bruce and his colleagues developed a model to measure the differences in the effect of chemotherapeutic agents on normal marrow or spleen cells and malignant lymphoma cells in AKR mice.

The concepts developed with the use of these two model systems may be summarized as follows:

1. Injection of a single L1210 leukemic cell in a BDF_1 mouse would result in the death of the animal.
2. The larger the number of cells injected, the sooner the animal died.

*Clonable cells are assayed by transplanting small numbers of cells in drug-free medium on petri dishes, into soft agar, or suitable mice, and counting the number of colonies formed. The number of colonies is linearly related to the number of viable proliferating cells.

3. The time of death of the host could be predicted from a knowledge of the number of cells injected and their doubling time because these factors determined when the tumour cell population reached the critical size required to kill the animal.

4. Chemotherapy reduced the number of clonable cells remaining. Prolongation of survival was attributable to the percentage of cells killed.

5. Indefinite survival resulted when all the tumour cells were eradicated.

6. Skipper noted that for cells with equivalent growth rates, a given dose of drug killed a constant percentage of cells regardless of the tumour load. A single dose that reduced the leukemic cell population by 99.9999% (6 logs of kill) 'cured' none of a group of animals bearing 1×10^9 cells, 40% of those bearing 1×10^6 cells or all bearing 1×10^4 cells.

7. Three classes of dose-response curves were noted for the chemotherapeutic agents tested:

(A) For nitrogen mustard and gamma radiation, cell kill was exponential with respect to dose. The sensitivity of normal and lymphoma cells was identical. These agents killed cells in all portions of the cell cycle. Sensitivity did not depend upon the proliferative state of the cells.

(B) In the second class of chemotherapeutic drugs, were ^{3}H-thymidine (^{3}H-TdR), vinblastine, and MTX. The lymphoma cells were more sensitive than the normal cells. The survival decreased as a function of dose but eventually a dose was reached whereat further dose increments did not kill any more cells. It was suggested that

these drugs acted at one portion of the cell cycle and those cells not killed were in non-sensitive portions of the cell cycle. On the average, lymphoma cells complete a generation cycle about every 10 hours. If the agents kill during a particular portion of the cell cycle, i.e., DNA synthesis as for ^{3}H-TdR, exposure of the cells to the cytotoxic agent for 24 hours would result in substantial loss of the proliferative capacity. On the other hand, the normal hematopoietic colony-forming cells pass through the cycle less frequently; most are thought to be in a non-proliferative or G_o state, are not as susceptible to the same drug, and therefore fewer are killed per unit time of drug treatment.

(C) Treatment with agents of the third class, 5-fluorouracil, actinomycin D and cyclophosphamide, resulted in cell survival curves which decreased exponentially with dose. There were marked differences between the kill curves for malignant and normal cells. Since there was no nadir with increasing doses, it was hypothesized that the cells were sensitive to these agents throughout their generation cycle. It was suggested that the differences in sensitivity were a direct consequence of the differences in proliferative rate. Thus, Skipper and Bruce hypothesized that the prolongation of life span with chemotherapy was due to the cytocidal effects on the tumour cell population. The delay in host death was the time required for the tumour cells surviving chemotherapy to proliferate to a lethal number and was not thought to be consistent with a drug-induced lag phase or change in generation time (103).

With these cell models, it was possible to investigate the differences in the effect of chemotherapeutic agents upon the normal

and malignant cells and to devise regimens using combinations of drugs which allowed the killing of all tumour cells yet sparing the host from lethal toxicity.

It should be noted that this hypothesis does not take into consideration drug-induced or spontaneous mutations in tumour cells resulting in resistance to chemotherapy or the possibility of viral re-induction of new tumours after the original tumour has been eradicated. G_o was a theoretical concept that has not been verified. For example cells said to be in G_o could be a subpopulation of cells still in cycle but very resistant to drugs for biochemical reasons. A change in dose and scheduling of drug therapy might result in increased kill beyond the plateau reached by the schedules used in the experiments of Skipper and Bruce. Besides, not all tumour cells are equally exposed to the drugs, since not all agents can pass the blood-brain barrier (85). Furthermore the sensitivity of L5178Y lymphoblasts to nitrogen mustard, an agent of the first class has been shown to be a function of the proliferative state of the cells (38). Despite these reservations, the cell kill hypothesis has been useful framework within which to study chemotherapeutic agents. Based upon the assumption that the antitumour activity of MTX is related to its ability to kill cancer cells, the aim of the present research is to investigate how MTX kills cells. From this it is hoped one could predict which cells would be sensitive and which resistant to the drug.

MTX AS A CHEMOTHERAPEUTIC AGENT

MTX has been useful in managing acute leukemia in children, osteogenic sarcoma, squamous cell carcinoma of the head and neck, choriocarcinoma in the female, African Burkitt's lymphoma and breast cancer. It is of limited value in adult leukemias and most solid tumours.

Cell kill by MTX cannot be measured in human malignant cells directly sampled from humans, since they are not readily clonable with high efficiency. However, if one could derive biochemical correlates of cell kill from a clonable cell line, one might then predict the cytocidal effects of MTX on non-clonable cells (such as those from patients with acute leukemia) and, therefore, might predict survival of the patient after treatment.

"Predictive procedures are important for rapidly assessing probable drug responsiveness. In AML, unless remission is achieved rapidly, the patient may die before a second empirical therapy can be tried. It is important to avoid the use of an ineffective drug or drug combination, since the success of any chemotherapeutic program depends upon the rapidity with which the most appropriate drugs can be selected" (20). However, attempts at predicting the results of chemotherapy on the basis of in vitro tests have met with limited success.

The in vitro effect of 5-fluorouracil, an inhibitor of thymidylate synthetase, on ^{3}H-TdR incorporation in gastrointestinal tumour and normal tissue was almost identical and therefore could not be regarded as a valuable in vitro test even though the drug is useful in the treatment of gastrointestinal cancer (118).

In patients with acute nonlymphoblastic leukemia, the highest percentage of marrow cells labelled with thymidine (TdR) was found in patients who later went into complete remission. The rate of incorporation of C^{14}-TdR after incubation with combinations of cytotoxic drugs was found to be depressed in cells of patients who later were found to be responders to that combination (121).

Some correlation was found between clinical response to the

drug and uptake by leukemic cells of MTX during short term in vitro culture. This correlation was found only if extracellular drug levels were kept sufficiently low (72). This work has not yet been confirmed by other researchers. When the effect of 1×10^{-6}M MTX on ^{3}H-TdR incorporation was studied after 4 hour treatment of suspensions of human acute leukemia blasts in vitro, there was in general, a correlation between sensitivity to MTX in vitro and duration of survival of the patients regardless of whether these patients were treated with MTX, or other agents (62). However, this test did not predict responsiveness of each individual patient with a high degree of accuracy. Thus, the search for a test predicting MTX responsiveness continues. In designing such a test, attention should be focused upon those pathways affected by the drug. These pathways relate to folate biochemistry.

FOLATE BIOCHEMISTRY

Folic acid is a nutritional factor comprised of residues of glutamic acid, p-aminobenzoic acid and pterin. A large portion of the folic acid in food is protein-bound. It exists in the polyglutamate form with linkage in the gamma position and deconjugation by intestinal conjugases is necessary to facilitate absorption. The precise mechanism of absorption is unknown but appears to be energy-requiring at low concentrations and occurs at the level of the small intestine. Vitamin B_{12} is necessary for the effective transport of folates into the liver and probably across other cell membranes (37).

Once in the cell, folic acid is reduced to the dihydro (FH_2) compound and subsequently to the tetrahydro form (FH_4) by the action of dihydrofolate reductase (5,6,7,8-tetrahydrofolate NAD(P) oxido-

reductase (E.C. 1.5.1.3) (DHFR) (9). The nitrogen atoms at positions 5 and 10 are the sites for the subsequent reactions in which FH_4 acts as a carrier of one carbon-containing groups at three levels of oxidation: methanol, formaldehyde, and formic acid as well as the formimino group.

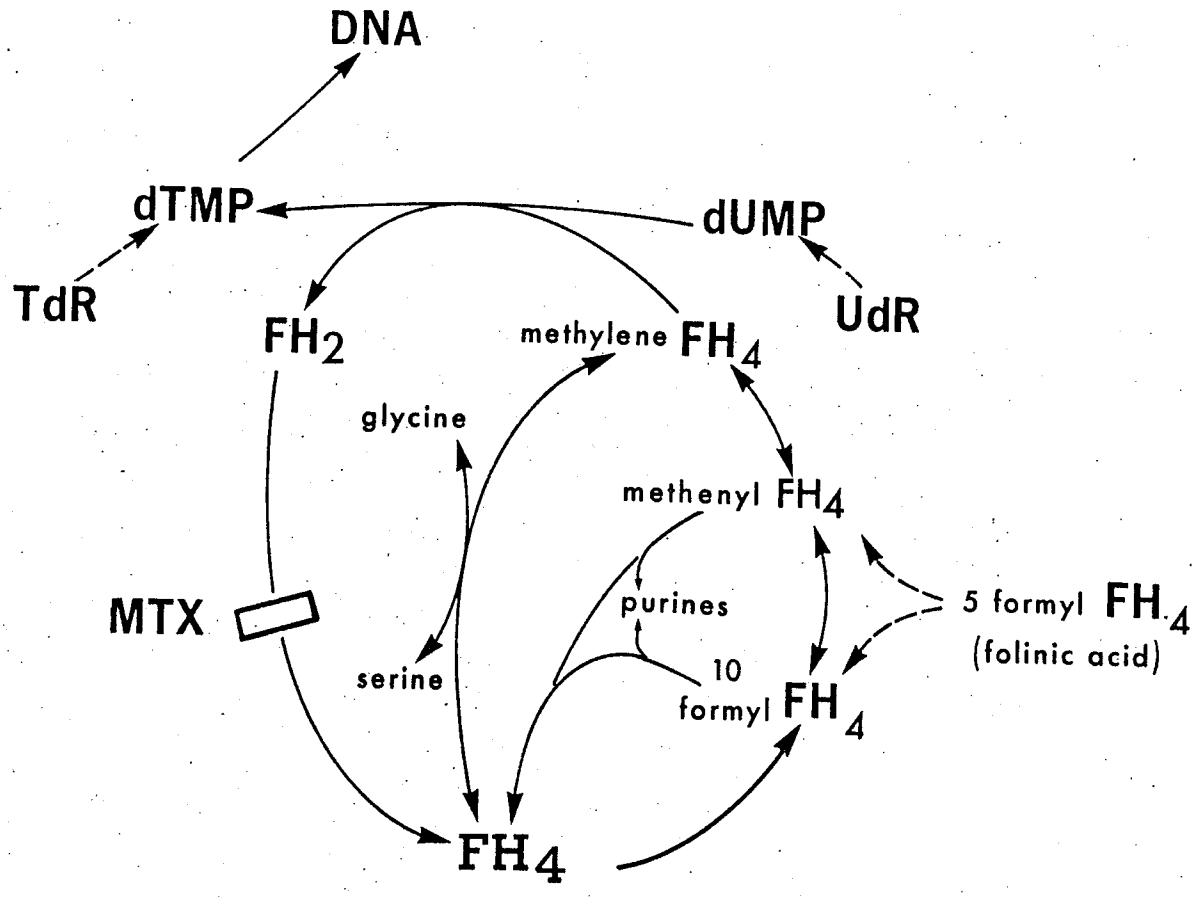
FH_4 is converted to N^5, N^{10} methylene FH_4 with the production of glycine by serine hydroxymethyltransferase. The methyl group can then be transferred to deoxyuridine monophosphate (dUMP) by thymidylate synthetase (TS), to form thymidylic acid (dTMP). In this reaction, FH_2 is formed and must be reduced to FH_4 by DHFR. Thymidylate can also be formed from thymidine by thymidine kinase (TK), a scavenger reaction.

In purine biosynthesis, carbons 2 and 8 come from FH_4 cofactors. Glycinamide ribonucleotide transformylase catalyses the transfer of a carbon atom from N^5, N^{10} methenyl FH_4 for the C_8 of the purine. C_2 is inserted by a transformylase acting on N^{10} formyl FH_4. N^{10} formyl FH_4 results from the hydrolysis by cyclohydrolase ofN^5, N^{10} methenyl FH_4. This compound is formed by the oxidation of N^5, N^{10} methylene FH_4 by methylene FH_4 dehydrogenase. Histidine is involved in folic acid metabolism since it utilizes a portion of an existing purine nucleus in bacteria.

Methylene FH_4 reductase reduces N^5, N^{10} methylene FH_4 to give N^5 methyl FH_4, the main transport and storage form of reduced folates. This methyl group is transferred intact for the de novo synthesis of methionine, and is also used in the synthesis of choline, sarcosine, and dimethyl glycine.

Reduced folates can also arise from the action of N^5 formyl FH_4 isomerase on folinic acid, which is present in small amounts in

the diet, to give N^5, N^{10} methylene FH_4 or N^{10} formyl FH_4.



Folate coenzymes function in the synthesis of RNA directly through the synthesis of purine bases and indirectly through the contribution to DNA synthesis. Protein synthesis is influenced by the interconversion of serine from glycine as well as by the contribution of folate coenzymes to RNA synthesis. In prokaryotic systems, folate coenzymes are necessary in the formylation of methionine esterified to transfer RNA (tRNA). N formyl methionyl

tRNA provides the N-terminal in the growing polypeptide chain, but an absolute requirement for the formyl group has not been shown in mammalian cells even though eukaryotic mitochondrial protein synthesis is initiated with N formyl methionyl tRNA. Formylation of labelled methionine continues in the presence of MTX indicating that the formyl group may not be contributed by an FH_4 derivative (111).

CHEMISTRY, PHARMACOLOGY AND TOXICITY OF MTX

MTX, a weak organic acid, is readily absorbed from the alimentary tract but may be given parenterally. At low doses the drug does not pass the blood-brain barrier in sufficient amount to give clinically significant concentrations in the cerebro-spinal fluid (85). It can be injected intrathecally as prophylaxis or to treat already established leukemic involvement of the central nervous system (49,83). Plasma levels of the drug are dose-related with a mean plasma half-life of 2 hours. The antifolate distributes itself to total body water within 1 hour following an intravenous or small oral dose. 50 to 90% of absorbed MTX is excreted unchanged in the urine within 48 hours (51).

The tissues that retain the largest amounts of MTX are the organs that contain the highest content of DHFR and of folate coenzymes (68, 120). These are the kidney, liver, gut and marrow.

When rats were given intraperitoneal injections of MTX and then sacrificed, the intracellular localization of the drug in the liver was determined by homogenizing the tissue and fractionating by differential centrifugation. The bulk of the drug was lost with a half-life of 120 to 360 hours and enzyme activity partially reappeared when the supernatant was dialyzed against sucrose (0.25 M).

The rest was displaced when the samples were dialyzed against sucrose containing folate (2×10^{-3}M). This resulted in complete reactivation of DHFR. Thus a significant portion of MTX is tightly bound to DHFR (114).

The rapidly exchangeable intracellular MTX not bound to DHFR is likely present in the cytoplasm in an osmotically active state. A small amount of the exchangeable fraction is loosely adsorbed at or near the cell surface (7).

In the rat liver, MTX appears to follow the biosynthetic routes of folic acid in being raised to the poly-gamma-glutamyl derivatives (3). The rate of disappearance of the polyglutamate forms of MTX from the liver and kidney was 8 times faster than that of unconjugated MTX (115). In S. faecium, increasing numbers of glutamyl residues led to decreasing inhibition of bacterial growth possibly due to reduced penetration of the drug or reduced inhibition of DHFR (84).

After administration of MTX to humans, some persists in the liver bound to DHFR and is released only slowly over a period of months. This most likely is due to slow dissociation from the enzyme which it stabilizes against degradation. The slow release could also be due to metabolism to a form with less affinity for DHFR or to the eventual degradation of the enzyme. However, at the usual doses used, the drug is metabolized to an insignificant degree, initially all appearing in the urine in unchanged form.

Toxic effects can be seen 3 to 4 days after administration of a large single dose to a normal human. The major lesion occurs in the proliferating tissues, i.e., intestinal tract, bone marrow,

and hair follicles. Ulceration of the oral mucosa is the first to occur. Bone marrow toxicity is manifested by pancytopenia and may be fatal. The loss of hair occurs frequently but surprisingly the hair regrows despite continued administration of the drug. With high doses, histological changes occur in the convoluted tubules with impaired renal clearance. Extensive desquamation of epidermal cells can occur in severe toxic reactions (9).

MTX TRANSPORT

The rate of transport of MTX is a critical determinant of its cytotoxicity. The higher the free intracellular MTX level acheived and the longer it is sustained, the longer the interval over which the DHFR will be inactivated.

Influx of MTX into cells follows Michaelis-Menten kinetics, has marked oxygen, pH and temperature dependence and is abolished by p-chloromercurobenzoate, a sulfhydryl reactive reagent which may inhibit active transport. An electrochemical potential gradient for free intracellular MTX can be demonstrated at low levels of extracellular MTX. Folinic acid, (N^5 formyl FH_4) when added to the extracellular medium competitively inhibits the influx and promotes the efflux of MTX, indicative of counter-transport. In addition, when the cells are preloaded with folates and MTX added to the extracellular medium at a steady state, there is enhancement of the efflux of folates. This suggests that the naturally occurring folates not only inhibit the interaction between MTX and a carrier but at least in part utilize the same carrier. Thus, MTX might exert a cytotoxic effect by first inhibiting the cellular uptake of N^5methyl FH_4 the main reduced folate extracted by cells from the extracellular compartment, and

then inhibiting DHFR intracellularly. In this way, MTX would doubly deprive the cell of circulating reduced folates (39,41,45).

The uptake of MTX is inhibited by high levels and stimulated by low levels of dibutryl cyclic AMP. High concentrations of dibutryl cyclic AMP inhibit the uptake of N^5 methyl FH_4 and inhibit thymidine incorporation into DNA but do not inhibit the uptake of folic acid by the cell (56). Cyclic AMP might affect MTX entry by affecting energy production required for active transport or it might have a more direct effect on the MTX transport mechanism.

In vitro studies suggest that chemotherapeutic agents and antibiotics given concurrently with MTX may significantly alter its cellular transport (19). For example, the vinca alkaloid, vincristine, inhibits the efflux of MTX from human leukemic cells while not affecting the influx (4,44). In animals such interactions may affect the anti-tumour effect of antifolate therapy but whether this also occurs in humans is not known.

INHIBITION OF DHFR BY MTX

The primary target of action of MTX is DHFR. MTX-DHFR has a dissociation constant of 3×10^{-11}M which implies an affinity of DHFR for MTX more than 100,000 times greater than that for folic acid at pH 6 and 20,000 times greater than that for FH_2 at pH7.5 (117). Inhibition of DHFR is pH dependent and is essentially stoichiometric at acidic pH, i.e., practically all the enzyme is bound to inhibitor. In this situation, Michaelis-Menten kinetics cannot be applied since at low levels of inhibitor the amount of inhibitor combined with the enzyme is not negligible in comparison to the free inhibitor present. Therefore, a Lineweaver-Burk plot does not determine whether or not

the inhibitor is competitive. The following equation applies:

$$\frac{1}{V} = \frac{1}{V_{MAX}} \left[\frac{1}{1 - \frac{I}{E}} \right] \left[1 + \frac{K_S}{S} \right]$$

in which E is the concentration of the inhibitor-binding sites on the enzyme and I is the total concentration of inhibitor (46,114). However, the relationship is in part competitive, since some reversibility can be shown at pH 7.5 with low inhibitor, low enzyme and high substrate concentrations, i.e., 10,000 times MTX. Under these conditions, a maximal value of Ki of approximately 6.7×10^{-10}M can be derived. The enzyme activity increases when the inhibitor-enzyme mixture is diluted. The enzyme can be titrated with increasing amounts of MTX to a concentration of inhibitor equal to a stoichiometric amount of enzyme (7).

The rate of transport of MTX is reduced 4 to 16 fold in some sublines of cells resistant to MTX (31). In one subline of L1210 cells for example, kinetic data shows an increased K_m with no appreciable change in the V_{max} which may indicate a change in affinity of the carrier for the drug (97). Other sublines selected for resistance to MTX have normal transport but have increased levels of DHFR, as a genetically stable trait. Thus, of 24 different L1210 lines resistant to MTX all had elevated levels of DHFR activity but were karyotypically heterogenous. Fifty-three mutations in resistant pneumococcus have been mapped and appear to be in the structural gene for DHFR which is in the same I locus. Enzyme induction or adaptation can also result in acquired resistance (67). This is the probable mechanism of resistance in human malignant cells (8).

As a consequence of the inhibition of DHFR, no further reduction of folates can occur. The pool of reduced folates are depleted by the dUMP to dTMP conversion, and the rate of this synthesis decreases. As reduced folates are further depleted, the cells lack even the coenzyme amounts necessary for de novo purine synthesis.

SECOND SITE OF ACTION OF MTX

Inhibition of ^{3}H-UdR incorporation does not occur until intracellular accumulation of MTX is well in excess of that required to inhibit the content of DHFR (98). This suggests MTX has a second site of action.

The suppression of DNA synthesis in host and tumour tissues by MTX was monitored in mice by determining the in vivo incorporation of ^{3}H-UdR (21). The duration of inhibition of ^{3}H-UdR incorporation into the tissues was related to the dose of MTX and was a direct function of the drug concentrations in plasma or ascitic fluid.

Intracellular MTX not bound to DHFR has a variable efflux rate. The differential effect of MTX on normal and leukemic cells has been studied by comparing the efflux of MTX from murine tumour cells with that from intestinal mucosal cells. A correlation was found between the level of persistent 'free' drug and response of the particular tissue being studied. In the normal cells, free intracellular MTX lasted only 3 hours. In the L1210 leukemic cells in the peritoneum of the same animal however, the drug level stayed above that required to bind all DHFR for 18 to 20 hours after treatment. The leukemic cells were killed but the normal cells were not.

The rate of loss of MTX bound to the enzyme in various types of tumour cells was similar. Thus, the greater sensitivity of some

cells might be due to prolonged retention of 'free' MTX and this may also occur with the other antifolates, methasquin, aminopterin, and 5-Cl-deaza-AM (99,100).

At high doses of MTX, complete inhibition of ^{3}H-UdR incorporation into DNA of L cells was observed despite the presence of folinic acid. If the sole site of action of MTX were DHFR, folinic acid should have permitted the conversion of dUMP to dTMP by bypassing the MTX block of DHFR and thus supplying the reduced folates necessary for the reaction. The failure of folinic acid to reverse this inhibition strongly suggests that MTX was inhibiting at another site. Similar results were found in hamster and mouse embryo cultures (13).

McBurney and Whitmore found no accumulation of FH_2 or change in the distribution of the intracellular folates during MTX treatment of CHO cells (79). This would not have been expected if inhibition of DHFR were the principle site of action of MTX. However, during the 24 hours of exposure to MTX, the CHO cells were supplemented with TdR, AdR, and glycine, and grew exponentially. Since under these conditions, no de novo synthesis of these compounds would have been required of the cells, no folate coenzymes need have been used. This could have effectively masked any changes found in cells treated with MTX and the results might not be relevant to conditions more closely mimicking the clinical situation.

The second site of action of MTX, if there is one, might be the inhibition by the free MTX of a low-affinity DHFR (43) or direct inhibition of TS or of one of the other folate enzymes.

There is indirect evidence that TS is the target of the free intracellular MTX. This can be summarized as follows:

1. MTX is a relatively weak inhibitor of TS extracted from

Erhlich ascites carcinoma cells. TS is inhibited by MTX uncompetitively with respect to dUMP and competitively with respect to FH_4 cofactors. During MTX therapy, intracellular folate levels should be depleted, and might be reduced to levels sufficiently low so that the relatively weak inhibition of TS by free MTX could become significant (13).

2. TS activity is increased 200 to 500 times in MTX resistant Lactobacillus casei while DHFR is increased 100 fold (27,28).

3. In vivo, the synthesis of dTMP is more inhibited than the synthesis of purines or of serines (2,11). The inhibition of the incorporation of the carbon into position 2 is greater than the inhibition of incorporation of the carbon atom into position 8 of the purine nucleus. The same enzyme form is used in the synthesis of both dTMP and serine and all the other folates cofactors should be easily interconvertible.

EFFECTS OF MTX ON CELLULAR METABOLISM

i. Thymineless Death

Following inhibition of DHFR by MTX and depletion of reduced folates, synthesis of purines and of thymidylate are inhibited. Thymidylate deficiency can lead to the inhibition of DNA synthesis resulting in the cessation of cellular proliferation and cell death.

That thymine (thymidylate) deficiency can result in cell death was first established in bacterial cells by Cohen and Barner in 1954 (24,25). In these cells, death results from unbalanced growth, i.e., DNA synthesis is inhibited but RNA and protein synthesis continue. As the unbalanced growth proceeds, the bacterial cells begin to lyse and have a high rate of mutation. Continued protein synthesis is a critical requirement for thymineless death in bacterial cells. Rolfe showed that when thymine requiring mutants of E. Coli were grown with-

out thymine, a concentration of actinomycin D just sufficient to inhibit RNA synthesis and therefore protein synthesis, but not DNA synthesis, blocked thymineless death. However, when actinomycin D was added late after thymine deprivation, thymineless kill was not stopped, indicating that some essential step, possibly the synthesis of some specific lethal protein, had been completed. Rolfe suggested this may be the induction of a defective prophage. In the same system, chloramphenicol, puromycin, and arginine starvation, all of which inhibit protein synthesis, also prevent a thymineless death (91).

In mammalian HeLa cells Ruekert and Mueller showed that MTX produced an unbalanced growth similar to that seen in bacteria. Upon exposure to the antifolate, HeLa cells rapidly ceased to divide but continued to increase in size. There was a limited increase in DNA-like material which was unstable and eventually lost. RNA and protein synthesis continued at normal rates for about 16 hours, but after 36 hours RNA synthesis ceased completely while protein synthesis continued slowly. Viability of the cells was lost as judged by plating in medium enriched by serine, glycine, adenosine, and thymidine (93).

Thymidine protected L cells from MTX while deoxyadenosine (AdR), a purine source, potentiated cell kill (11,12). In direct contrast, L5178Y cells were not protected by thymidine (TdR) alone while purines along prevented cell kill (59).

Tattersall et al. assessed the effect of MTX in a variety of cell lines by measuring inhibition of cell proliferation rather than the reduction of cloning efficiency. Five cell lines with varying sensitivity to MTX were studied: the murine leukemic lines L1210 and L5178Y, Yoshida sarcoma, human lymphoblasts (W1-L2) and mouse fibroblasts (L cells).

If the cells were dying a thymineless death, the addition of TdR should 'rescue' the cells from the effects of MTX. TdR rescued only the Yoshida line with little to no protection shown in the other lines. Similarly, when AdR was added, there was rescue only of the Yoshida line and no rescue of the L5178Y or L1210 cells. Potentiation of the cytotoxic effect by AdR was shown in the W1-L2 and L cells.

In the study of Tattersall et al. the ratio of the activities of N^5, N^{10} methylene FH_4 dehydrogenase (DH) and thymidylate synthetase (TS) seemed to correlate with the ability of TdR to reverse the antifolate effects. The decrease in ability of TdR to reverse the effect of MTX seemed to be reflected in a low ratio of DH to TS. It was postulated that if the DH were active, folate cofactors would be diverted to thymidylate biosynthesis.

Total folate pools also seemed to correlate with the ability of TdR to 'rescue' the cells. It was hypothesized that where the reduced folate pools are large, purine biosynthesis would be sustained longer in the presence of MTX and TdR would be more able to rescue treated cells (108).

It must be remembered that these conclusions were drawn from studies of changes in total cell numbers resulting from treatment. The inhibition of proliferation of cells may not be a true reflection of the cytocidal action of the drug. As a result of treatment, cells may proliferate slowly but may not be killed and may resume normal proliferation after the drug is removed. If the cell kill hypothesis is correct, these cells could still kill the host when drug treatment was discontinued. Furthermore, in the study of Tattersall and Harrap,

cells were grown in undialyzed fetal calf serum (FCS). As will be discussed later, FCS contains substances which can mask biochemical changes and protect against the cytocidal effects of MTX.

Added purines may potentiate MTX-induced cell kill. This is consistent with a thymineless death mechanism requiring the synthesis of a lethal protein. Thus, if RNA and protein synthesis were interrupted by MTX-induced inhibition of de novo purine synthesis, the addition of a source of preformed purines would allow resumption of RNA and protein synthesis, making the cells more susceptible to a thymineless death.

A thymineless state results in accumulation of an increased number of breaks in DNA strands (56,82). This may be due to inhibition of synthesis of the ligase or polymerase necessary to repair these breaks or the depletion of thymidylate for DNA or both. Accumulation of a critical number of strand breaks might kill the cell (82).

ii. Purineless State, Purineless Death

There are situations where the addition of purines, instead of potentiating cell kill, prevent cell kill. Hryniuk et al. showed that purines protected L5178Y cells against kill by MTX for 4 hours. Thus, these cells were dying by a purineless death (59).

During a search for a biochemical correlate of cell kill by MTX, it was noted that when L5178Y cells were grown at different rates and treated with the antifolate, suppression of ^{3}H-TdR incorporation into DNA occurred in some cells. The suppression was most marked in the most rapidly proliferating cells, and the degree of suppression correlated with cell kill as judged by cloning. This biochemical correlate, the suppression of ^{3}H-TdR incorporation, was a reflection of the purineless state because added purines immediately reversed the suppression. ^{3}H-

uridine and ^{3}H-leucine incorporation into RNA and protein respectively were also inhibited during this period and both recovered with added purines. Despite inhibition of protein synthesis due to the purineless state, cell kill still occurred in contrast to other cell lines where thymineless kill requires continued protein synthesis. The added purines also temporarily prevented cell kill by MTX giving rise to the concept that MTX could produce purineless death as well as thymineless death. The mechanism of purineless death is unknown, but may be due to the inhibition of synthesis of such proteins as DHFR or other log phase enzymes.

After 12 hours of exposure of L5178Y cells to MTX, there was a reversal of the purineless state. This reversal was not due to the resumption of de novo purine synthesis but was due to the re-expansion of the acid-soluble purine pools (63). There was recovery of ^{3}H-TdR incorporation into DNA but not of ^{3}H-leucine incorporation into protein. The cells continued to die.

Coincident with the spontaneous reversal of the purineless state, the addition of hypoxanthine no longer prevented cell kill. Therefore at this point, the cells were not dying by a purineless mechanism. Presumably, thymineless death predominated (59). Thus, suppression of ^{3}H-TdR incorporation no longer could be used as a correlate of cell kill and a biochemical parameter to measure thymineless kill was sought.

iii. Thymineless State, Thymineless Death: Biochemical-Cytocidal Correlate

The lethal thymineless state results from the inhibition of the conversion by thymidylate synthetase (TS) of dUMP to dTMP, either by the depletion of reduced folates or direct inhibition of

TS by MTX. This causes thymineless death.

The difference between different tissues in susceptibility to thymineless death can be exploited in chemotherapy. When mice bearing a transplantable leukemia are given TdR along with MTX, the normal cells of the mice are protected from the toxic effects of the drug by the TdR while the tumour cells continue to die. Presumably the tumour cells are dying primarily by a purineless mechanism (105). Thus, the use of TdR together with MTX increases the therapeutic index in mice.

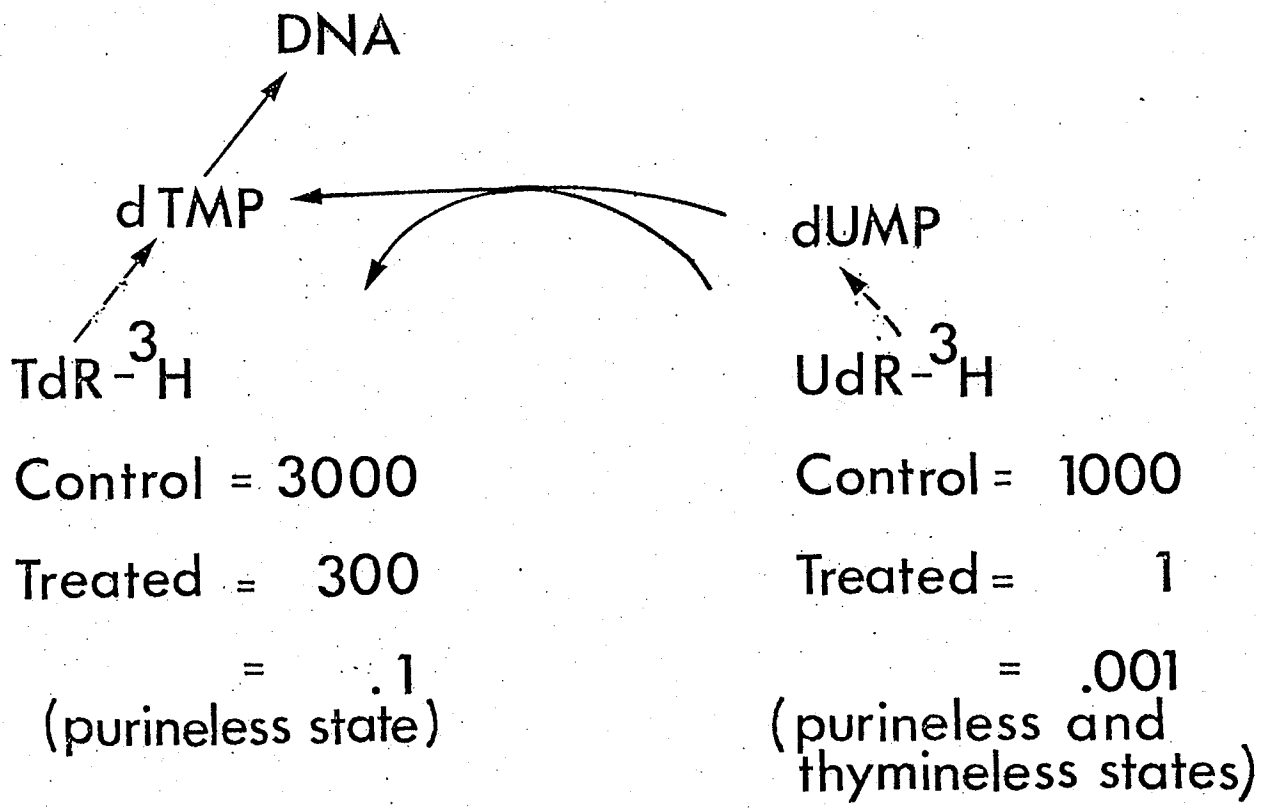
Conversely, when allopurinol, a xanthine oxidase inhibitor which elevates serum levels of hypoxanthine, is given concurrently with MTX to tumour bearing animals, the antitumour effect of MTX is abolished while the normal cells continue to die (47).

It is not known whether human malignant cells treated with MTX are killed primarily by a purineless mechanism or a thymineless mechanism or both. High levels of TdR can prevent MTX toxicity in humans, but whether the therapeutic index is thereby increased remains to be seen.

An improvement in the therapeutic index of MTX has also been achieved in animals and humans with the use of leucovorin (N^5 formyl FH_4, folinic acid, citrovorum factor) (39,74), but the mechanism is not understood (20).

The block by MTX of the conversion of dUMP to dTMP suppresses the incorporation of ^{3}H-UdR into DNA, while ^{3}H-TdR bypasses this block. The incorporation of both labels is suppressed due to the lack of de novo purine synthesis. It is possible to isolate the effect of MTX on the conversion of dUMP to dTMP thus monitoring the

thymineless state, the predominant mechanism of cell kill during the latter stages of treatment of L5178Y cells.



$$\text{Calculated UdR rate} = \frac{.001}{.1} = .01 \text{ (thymineless state)}$$

For example, if the MTX-induced purineless state causes a 10 fold decrease in the ^{3}H-TdR rate, this can be applied to the decrease in the ^{3}H-UdR rate. If during MTX treatment, the ^{3}H-UdR rate is suppressed 1000 fold then the suppression due to the purineless state is 10 fold and that due to the thymineless state is 100 fold. This measurement of the thymineless state can also be expressed as a calculated ^{3}H-UdR rate of 0.01 of the rate of conversion of dUMP to

dTMP in the untreated culture. This calculated rate coincides with the ^{3}H-UdR rate actually observed under conditions where MTX treatment does not suppress ^{3}H-TdR incorporation, i.e., when hypoxanthine is added to the MTX-treated culture. Conversely when MTX stimulated ^{3}H-TdR incorporation a similar stimulation of ^{3}H-UdR incorporation occurs. As a result, the calculated ^{3}H-UdR rate is less than the observed ^{3}H-UdR incorporation rate.

In the L5178Y line the calculated ^{3}H-UdR rate bore a close relationship to cell kill when both parameters were plotted on log scale for the 7th to 12th hour of MTX treatment. This was the time when purines no longer rescued the cells and they were dying primarily by a thymineless mechanism. A log-log plot was used on the assumption that the degree of biochemical disturbance should correlate to the degree of cell kill for a given dose of drug.

In the present study, it was decided to examine the correlation between the calculated ^{3}H-UdR rate and cell kill in cell types other than L5178Y lymphoblasts. If the correlation did hold in a variety of situations, the calculated ^{3}H-UdR rate might serve as a biochemical substitute for the measurement of cell kill due to the thymineless state in non-clonable cells (such as those from patients with acute leukemia).

In addition, TS activity was measured in extracts from cells treated with MTX. This was to see if there was any decrease in enzyme activity and to see if such a decrease could account for the slow decline in the ^{3}H-UdR rate observed during antifolate treatment. If a decrease in TS activity were observed and could account for the slow decline in the ^{3}H-UdR rate this could contribute to thymineless death in addition to the role played by depletion of reduced folates.

MATERIALS AND METHODS

MTX (sodium salt) was a gift from Cyanamid (Lederle) of Canada, Montreal, Canada; Fischer's Medium for Leukemic Cells of Mice, Minimal Essential Medium, horse serum and fetal calf serum were obtained from Grand Island Biological Company, Grand Island, New York. Alpha modification of Eagle's Medium was obtained from Flow Laboratories, Rockville, Md.

Noble agar was obtained from Difco Laboratories, Detroit, Michigan; hypoxanthine and thymidine from Nutritional Biochemicals Corporation, Cleveland, Ohio. 2-Mercaptoethanol was purchased from Eastman Organic Chemicals; Tris buffer and Darco 60 activated carbon from Fisher Scientific Co., 2'(5-^{3}H) deoxyuridine-5' monophosphate (10 Ci/mmole), ^{3}H-Thymidine (1.9 Ci/mmole) and ^{3}H-deoxyuridine (21 Ci/mmole) were obtained from Amersham-Searle, Amersham, England. Hyamine hydroxide was obtained from Nuclear-Chicago Corporation, Des Plaines, Illinois. A solution of dl L-5, 10-methylenetetrahydrofolate (1×10^{-2}M) was prepared by dissolving 25 mg of dl, L-tetrahydrofolate in a solution containing 7×10^{-2}M HCHO, 5×10^{-2}M $NaHCO_3$, and 0.25M 2-mercaptoethanol (28). This solution was stable for 2 weeks frozen under N_2. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer model 3375 with correction for efficiency and quenching with automatic external standardization. Calculations of rates and standard deviations were performed on a table-top Olivetti Programma computer model P602 using standard statistical formulae (104). Cell counts were made with a hemocytometer or on a Coulter Counter Model B. Proteins were measured by a modified Lowry method (76).

Cells and Culture Techniques

Chinese hamster ovary cells (CHO) with a doubling time of 12 hours in Alpha medium with 10% fetal calf serum were obtained from H. Ceri, Department of Cell Biology, University of Manitoba and grown as a monolayer in glass bottles. Human esophogeal KB cells, with a doubling time of approximately 23 hours, obtained from Dr. C. Hannan, Department of Medical Microbiology, were grown as monolayer cultures in Minimal Essential Medium with either 10% fetal calf or horse serum. Diploid L5178Y mouse lymphoblasts were from suspension cultures with a doubling time of 12 hours at 37°C in Fischer's Medium and 10% horse serum. L5178Y and CHO cells were maintained in an atmosphere of 5% CO_2; KB cells were grown in room air. After the medium was decanted from the monlayer cultures, and a cell count taken, trypsin (0.25%) was added (15 to 30 minutes at 37°) to detach the cells from the glass. A cell count was taken, the suspension was centrifuged for 5 minutes at 500 g and the pellet washed once in serum-free medium. The L5178Y cells were simply centrifuged. Cell pellets were used as a source of TS or resuspended in fresh medium and either pulsed with tritiated precursors to measure the rate of incorporation into macromolecules or cloned to measure cell kill.

Cloning

L5178Y cells were cloned into soft agar to form colonies. 3 ml of medium at 44°C containing 0.2% agar and 15% serum were added to culture tubes containing 2×10^{-5}M hypoxanthine and 1×10^{-5}M TdR, neither of which affected cloning efficiency, to protect against any intracellular MTX remaining after treatment.

2 ml of growth medium with 15% serum containing sufficient cells to produce about 40 colonies were added to each tube to give a final concentration of 0.12% agar. After gentle mixing, the tubes were placed in ice for 5 minutes and then removed and incubated at 37° for 7 to 10 days when the colonies were counted in a Quebec colony counter (23). CHO and KB cells were cloned by seeding cells onto plastic plates (Falcon) or glass bottles in medium containing hypoxanthine and TdR. After 14 days, the medium was decanted, the colonies adherent to the glass fixed with methanol, stained with Giesma and counted.

Trypan Blue Exclusion

To determine the intactness of cell membranes, the ability of cells to exclude trypan blue was measured after 5 minutes exposure to a solution with a final concentration of 0.2% trypan blue.

Dialysis

The dialysis tubing was boiled for 20 minutes in 1% $NaHCO_3$ and then washed with distilled water. The fetal calf serum was dialyzed against 30 to 40 times its volume of diphosphate buffered saline (pH 7.5) twice for 2 to 4 hours.

Thymidylate Synthetase Preparation

To extract the enzyme, the cell suspension was centrifuged for 5 minutes at 500 g and sonicated in 0.5M Tris buffer (pH 7.5) for varying lengths of time using a Bronwell-Blacksone Biosonik Ultrasonic Probe at 20,000 cycles per second. Alternatively, the cells were lysed for 15 minutes in water, and then the pH and molarity were rapidly adjusted to pH 7.5 and 0.5M Tris. The preparation was centrifuged for 5 minutes at 1000 g. Sucrose was added to the supernatant to a concentration of 5%.

To be sure that all the activity was released on sonication, the unspun sonicate was assayed, along with a 100,000 g sonicate. The activities were comparable indicating that the main activity was released into the supernatant on sonication. The specific activity was dependent upon the amount of cell debris removed by centrifugation.

Chromatography of ^{3}H-dUMP

The impurities in the commercially obtained ^{3}H-dUMP used in the TS assay varied between 1 to 6% in different lots. When the higher figure was approached, repurification was necessary.

To purify the ^{3}H-dUMP, the sample was applied to Whatman #1 paper and run in a system consiting of isopropanol-concentrated ammonium hydroxide - 0.1 M boric acid (6:1:3). After chromatography, a guide strip was cut into 1 cm sections and dissolved in 0.5 ml of a toluene; hyamine hydroxide mixture in glass vials for 1 hour and after adding 7 ml of scintillation fluid, counted for tritium. When the main peak was located, the label was eluted with water and lyophilized.

Thymidylate Synthetase Assay

Assays were carried out in a total volume of 0.3 ml in 5 ml plastic tubes (Falcon). The standard reaction mixture for the TS assay contained 1.6×10^{-3} M methylene FH_4, 2.1×10^{-3} M ^{3}H-dUMP, 0.25 M 2-mercaptoethanol, 0.1 M $MgCl_2$, varying amounts of TS solution and 0.1 M Tris-HCl pH 7.5. Reactions were started by the addition of ^{3}H-dUMP at 37^{o} and terminated by the addition of 1.0 ml of a 10% Darco 60 activated carbon suspension in 0.05 M Tris HCL (pH 7.5). After adsorption of nucleotides to the activated charcoal, the suspension was filtered through a Millipore filter (GSWP 1300). 0.5 ml of filtrate was added to 20 ml scintillation fluid and the tritium released to water was

counted in the Packard scintillation spectrometer (88,69).

Using 3mM dUMP, with a specific activity of 2.87 Ci/mole, the reaction was linear with respect to time for up to 35 minutes or enzyme volume up to 150 microlitres of extract. The enzyme activity was shown to increase with increasing dUMP concentration to saturation, and was strictly dependent upon the availability of methylene FH_4. This assay entailed several problems. Insensitivity: to increase the sensitivity the concentration of unlabelled dUMP was decreased to 0.05 mM and the specific activity was increased to 530 Curies per mole. The use of label purified by chromatography resulted in increased sensitivity and apparent increased activity suggesting that the impurities might have inhibited the reaction. The amount of label displaced into the blank increased with the amount of 2-mercaptoethanol present, increasing the blank value as reported by Kawai and Hillcoat (69).

Incorporation of Precursors

To measure incorporation of precursors, 1 ml aliquots of cell suspension of sufficient number to provide an accurate rate while maintaining a linear relationship between cell number and rate of incorporation, were delivered into 5 ml plastic tubes (Falcon Plastics, Oxnard, California). These were placed in a Dubnoff shaking water bath at 37°C and, in the experiments with CHO or L5178Y cells, the atmosphere over the cell suspension in the tubes was adjusted to approximately 5% CO_2 by breathing several times into each tube before stoppering. After 5 minutes of warmup incubation, either ^{3}H-TdR (1.9 Ci/mole) at a final concentration of 6×10^{-7}M or ^{4}H-UdR (3.1 Ci/mmole) at a final concentration of 9×10^{-7}M was added. The incorporation was stopped at varying times by the addition of 10 ml of ice-cold 5% perchloric acid (PCA)

precipitating the cells and serum proteins. The precipitates were washed 3 times to remove acid soluble radioactivity. The radioactivity remaining from DNA was extracted into 5% PCA by heating at 85°C for 15 minutes and measured by liquid scintillation techniques (32).

^{3}H-Leucine was also incorporated by the same methods as ^{3}H-TdR, except that ^{3}H-leucine (4716 Ci/mole) was added to a final concentration of 3.5×10^{-7}M. The incorporation was stopped by adding 0.9 ice-cold saline. The pellets were washed twice with 5% PCA to remove any acid soluble radioactivity, again with cold saline, dissolved in 0.5 ml NCS tissue solubilizer (Amersham-Searle), added to scintillation fluid and counted.

Incorporation of precursors into macromolecules was calculated by plotting counts per minute per million cells against the time of incubation with the label and computing the slope of the regression line to give an incorporation rate. Sufficient cells, specific activity of label, and incubation time were used to ensure that the standard deviation of the rate was kept to less than 20% of the absolute value of the rate. Thus the error is kept proportional to the absolute value of the measurement. When this requirement is fulfilled, plotting results on a log scale is statistically justified (104).

The rate was expressed as picomoles of tritiated precursors incorporated into the macromolecules per minute of incubation per million cells. Rates in the presence of MTX were expressed as decimal fractions of the control rates, and were designated as "fractional rates".

The calculated ^{3}H-UdR rate was determined by mathematically correcting the ^{3}H-UdR rate, observed during treatment with MTX alone, for the simultaneous effects of MTX on ^{3}H-TdR incorporation, e.g., the fractional ^{3}H-UdR rate was divided by the fractional ^{3}H-TdR rate.

Experimental Design

All cell cultures were treated with 1×10^{-6}M MTX. Drug treatment of CHO cells was started at varying times after seeding 5.0×10^{4} rapidly proliferating cells into culture bottles. Early log cultures were treated 45 to 48 hours after seeding when the culture density was approximately 1×10^{6} cells per bottle. Mid-log cultures were treated with MTX 80 to 85 hours after seeding when the culture density was approximately 5×10^{6} cells per bottle while in resting cultures, treatment was started when the culture density was greater than 2.0×10^{7} cells per bottle.

Similarly KB cells were treated with MTX 48 hours after seeding with 5.0×10^{5} cells per bottle when the culture density was approximately 1.8×10^{6} cells per bottle. In the thymidylate synthetase experiments, log cultures of L5178Y cells had a density of 7.0 to 12.0×10^{4} cells per ml. Resting cultures were defined as cultures where the density was greater than 400,000 cells per ml and no further increase in density had occurred for 12 hours.

The period of drug treatment varied according to the experimental design.

RESULTS

The initial aim of this research was to investigate the generality of the correlation between the calculated ^{3}H-UdR rate and cell kill as measured by cloning. Therefore, mid-log cultures of CHO cells were studied.

The nature of cell kill in this line was investigated by the addition of exogenous purines and/or TdR at the time of treatment with MTX (Chart 1). By 2 hours there was a statistically significant ($p < .01$) drop in clonable cells, then a gradual decrease up to 16 hours of MTX treatment where 32% of the cells were still clonable. From then on, a sharp loss of cloning ability occurred. The addition of hypoxanthine as a purine source gave little significant protection against the cytocidal effects of MTX. On the other hand, the addition of TdR afforded significant protection with 56% of the cells remaining viable after 24 hours of exposure to 1×10^{-6}M MTX as compared with 3% for the unrescued culture (Chart 1). Complete protection was provided by the addition of both hypoxanthine and TdR (not shown).

The effect of MTX on the incorporation of ^{3}H-TdR was studied as well. As shown in Chart 2, there was significant stimulation of ^{3}H-TdR incorporation throughout the period of MTX treatment. ($p < .05$ at 12, 16) Three hours of MTX treatment markedly suppressed ^{3}H-UdR incorporation with not much more suppression occurring with 16 hours of exposure to the drug. The calculated ^{3}H-UdR rate was determined as outlined as the 'Introduction', and the results shown in

Chart 3. After an initial fast drop, the calculated ^{3}H-UdR rate paralleled the loss of cloning ability from 3 to 16 hours of treatment. (No significant difference between slopes). After this period, cell kill outstripped the calculated ^{3}H-UdR rate.

MTX treatment markedly stimulated ^{3}H-TdR incorporation in cells from resting cultures (Chart 4). In the resting cultures, the ^{3}H-UdR rate showed marked fluctuations due to inknown factors that could not be controlled, possibly (1) variation in plateau culture density (2) numbers of cells used in assay. Incorporation rates were lower when cells were in a high state of contact inhibition and were based on the numbers of cells added to the assays, and not on the number of viable cells added. Therefore, the rates were falsely low when large numbers of dead cells had been added.

Although the calculated ^{3}H-UdR rate appeared to parallel cell kill during MTX treatment (Chart 5), nevertheless, the correlation is obscured by the fluctuations in the calculated ^{3}H-UdR rate. There was no significant difference between the slopes of the cloning efficiency and the calculated ^{3}H-UdR rate for 2 to 8 hours of MTX treatment and 8 to 24 hours of treatment. Despite the scatter, is is evident that these cells were more resistant to the cytocidal effects of MTX when compared to cells from log cultures (Compare Charts 3 and 5).

Early log cultures have a higher proliferative rate than mid-log cultures and the cells should be more susceptible to the cytocidal effects of MTX (61, 64). In Chart 6, the MTX-induced change in ^{3}H-TdR incorporation was statistically insignificant but there was marked suppression of ^{3}H-UdR incorporation into DNA occurring during

MTX treatment significant at each time point. Surprisingly, however, these cells resisted the cytocidal effects of MTX as judged by cloning and no correlation was found between the markedly suppressed calculated ^{3}H-UdR rate and the slight degree of cell kill (Chart 7) ($p < .0001$) It seemed possible that resistance to cell kill was conferred by some substance in the fresh culture medium in which the early log cells were treated which was depleted by the time the mid-log cells were ready for treatment. The medium was known to contain nothing likely to protect against the cytocidal effects of the drug.

The most likely source of protection was the fetal calf serum (FCS). To test this possibility, this FCS was dialyzed before being used to supplement the medium in which the cells were grown. Under these conditions, one log of cell kill was seen (Chart 8). Correlation was apparent between the calculated ^{3}H-UdR rate and cell kill, since only 2 time points were studied. Correlation might have been detected during early MTX treatment, but these studies were not done because culture growth in dialyzed FCS required repeated sub-culturing to obtain acceptable cultures with 12 hour doubling times.

If mid-log cultures were supplemented with undialyzed FCS just prior to MTX treatment, they too became resistant to the cytocidal effects of the drug for 10 hours (Chart 9). Less resistance was seen after this time presumably due to depletion of the protecting substances in FCS.

The 3 main candidates for this depletable substance in FCS were (1) a large amount of reduced folates, (2) purines,

(3) thymidine.

The levels of reduced folates were measured in horse serum and FCS and were very low (10^{-8}M). The concentration in medium (10^{-9}M) would be too low to account for the protection seen in early log CHO cultures. As a result it was not thought worthwhile to try to overcome the technical difficulties involved in measuring the levels of reduced folates in medium from early log and mid-log cultures. If a significant concentration had been found it would have been necessary to determine if the loss of resistance could be correlated with a depletion of folate concentration found with culture growth.

While attempts were being made to measure the FCS content of thymidine, reports indicated that large amounts of purines and thymidine were present (17, 81).

Concurrent with the experiments on CHO cells, the calculated ^{3}H-UdR rate was measured in KB cells grown in medium supplemented with either FCS or horse serum (Charts 10 and 11). No correlation could be found between the calculated ^{3}H-UdR rate and cell kill in this system. However, the cell kill data was inaccurate since the cloning was with very low efficiency. Furthermore, rates of incorporation of both ^{3}H-UdR and ^{3}H-TdR varied with cell concentration in the assay. This was not seen in mid-log CHO cultures and was not tested in resting CHO cultures. No significant difference was seen between the effect of MTX on cells grown in horse serum or FCS. If the growth in this system had been more reliable, any differences between horse serum and FCS in the concentrations of protecting factors might have been detected.

Correlation could be made between cell kill as judged by cloning and the calculated ^{3}H-UdR rate under conditions where serum factors did not interfere with the cytocidal effects of MTX, e.g., L5178Y (58), mid-log CHO cells and possibly resting CHO cells. It was therefore decided to look directly at the step isolated by the calculated ^{3}H-UdR rate, that is the conversion of dUMP to dTMP. The increase in the block of dUMP conversion to dTMP could be due to a direct effect of MTX on TS. Therefore, the enzyme from MTX treated L5178Y lymphoblasts was measured in extracts of these cells.

TS activity in extracts from log cultures was not suppressed after 4 hours of treatment but was suppressed to 32% of control after 12 hours (Chart 12).

In view of the fact that MTX induces a purineless state in L5178Y cells, thereby suppressing RNA and protein syntheses, it was considered that the MTX-induced inhibition of TS activity could be due to the suppression of enzyme synthesis. Protein synthesis was inhibited to 42% of control in log cultures and to 73% in resting cultures, after 12 hours of MTX treatment (Table 1). An exogenous purine source protected L5178Y cells completely from this inhibition of protein synthesis and prevented the MTX-induced suppression of TS activity in log cultures (Compare Table 1 and Chart 12).

The decrease in TS activity did not correlate with the decrease in the calculated ^{3}H-UdR rate in log cells where no purineless state occurs, i.e. with the addition of hypoxanthine.

During the course of these experiments, the TS activity in extracts of log cultures was found to decrease upon sonication at a much faster rate than that from resting cultures. To investigate this phenomenon, sonication time was varied and the resultant

activity of the extract assayed and expressed as a percentage of the activity obtained by lysis. A 5 fold difference in sensitivity to sonication was seen with 4 x 30 second bursts of sound (Chart 13). With that extraction method, the activity from sonicates of resting cultures ended up being higher than that from log cultures. When sonication was used to extract the enzyme, the drop in activity after treatment with MTX of the whole cell was less than that seen in experiments where the enzyme was extracted by hypotonic lysis. Moreover, the difference in activity of hypotonic controls was significant ($p<.05$) while the means of the MTX-treated samples were not significantly different. Therefore, the effect of MTX on the activity obtained by lysis was greater in log cells than in resting. The loss in activity due to sonication was much greater in extracts from untreated log cultures than in the MTX-treated samples.

DISCUSSION

Previous studies had shown that during MTX treatment of L5178Y cells, the calculated ^{3}H-UdR rate, a measure of the thymineless state, correlated with cell kill as judged by cloning for 24 hours of MTX treatment. In the present study, this is true for mid-log and possibly resting cultures of CHO cells. As in the L5178Y system, the calculated ^{3}H-UdR rate, after an initial fast drop, progressively decreases during MTX treatment. This reflects the deepening thymineless state, presumably the condition which causes the predominant mechanism of cell death for CHO cells, since only when thymidine (TdR) is added during MTX treatment, the cells are protected. In contrast, in the L5178Y system, TdR alone did not protect the cells.

In CHO cells, MTX stimulates ^{3}H-TdR incorporation suggesting that purines are not lacking for DNA synthesis. This suggestion is strengthened by the observation that added purines provide little protection against MTX-induced cell kill. The stimulation seen in incorporation may be due to one or more of (1) an increase in activity of thymidine kinase activity (derepression or activation), (2) constriction of intracellular pools of thymidylate or (3) cell synchrony. The latter possibility is most likely in resting cultures where the greatest stimulation of ^{3}H-TdR incorporation is observed. Resting CHO cultures are also the most resistant to MTX. This tends to support previous observations made on acute leukemic cells (62). While the synthesis of DNA in L5178Y cells is restricted for lack of purines, ^{3}H-TdR incorporation in CHO cells treated with MTX could be due to the availability of large amounts of preformed purines inside or outside of the cells.

In previous studies in the L5178Y cell system, there was no close correlation between the calculated ^{3}H-UdR rate and cell kill for the first 7 hours of MTX treatment because cell death was occurring by both a purineless and thymineless mechanism while the calculated ^{3}H-UdR rate described only the thymineless state. The breakdown in

the correlation in mid-log CHO cells seen after 16 hours of MTX treatment does not appear to be due to the increasing importance of a purineless state since hypoxanthine does not protect against the cytocidal effects of the drug at that time and no suppression is seen in the ^{3}H-TdR incorporation rate. Thus, after such prolonged MTX treatment, some additional mode of cell death may be operational such as the synthesis of a lethal protein, or the more rapid accumulation of lethal chromosome breaks (82).

The hypothesis that the calculated ^{3}H-UdR rate correlates with thymineless death as judged by cloning is supported by the study of mid-log and, to a limited extent, resting cultures of CHO cells. This is not the case in early log cultures of CHO cells. There is no correlation between the marked decrease in the calculated ^{3}H-UdR rate on the one hand, and the relative resistance of the cells to the cytocidal effects of MTX on the other. This suggests the presence, in the fetal calf serum (FCS), of substances protecting the cells from the effects of MTX.

This is confirmed when dialysis of the FCS renders the cells susceptible to the cytocidal effects of MTX. This protection can be afforded also to mid-log CHO cultures upon the addition of undialyzed FCS just prior to MTX treatment. These dialyzible protecting substances appear to be utilized in the growth of the culture since normally relatively less protection is found in mid-log cultures.

A high concentration of reduced folates was a possible source of protection against MTX. In the present study, the levels of reduced folates found in FCS are too low to account for the protection found in early log cultures of CHO.

The importance of TdR or purines as protecting substances depends upon not only their concentration but also the relative

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importance of the thymineless and purineless states in killing the cell type. The rate of depletion of purines and thymidine or reduced folates from the medium will depend upon the proliferative rate of the cells.

The presence of these protecting substances may have clinical significance, i.e., high plasma levels of these factors may be a mechanism of resistance for human tumours. In turn, the levels of such substances may be subject to influence from factors independent of tumour type, e.g., the nutritional status of the individual, use of other chemotherapeutic agents, location of the tumour etc.

The presence of possible substances such as purines, TdR, or reduced folates in serum which protects cells from MTX effects was established in other systems while the present studies were being conducted (17,81). The presence of a depletable factor(s) can, as in this study, prevent correlation of biochemical changes with cytocidal effects. Previously, many studies have been reported on the effect of MTX on cells grown in culture medium supplemented with undialyzed FCS, but in view of our findings, conclusions drawn from such studies of the mechanism of action of MTX may be seriously questioned. For example, Tattersall and Harrap investigated the changes in deoxyribonucleoside triphosphate pools following MTX treatment of L5178Y cells grown in medium supplemented with 10% undialyzed FCS. No consideration was taken of the possible effects of levels of nucleotides in the serum on pool sizes (107,108).

Furthermore, the effect of MTX on ^{3}H-TdR incorporation into DNA by human leukemic blasts (62), is affected by the serum chosen for supplementing the medium in even brief <u>in vitro</u> experiments. MTX

treatment *in vitro* suppresses incorporation of ^{3}H-TdR into DNA in some leukemic cells suspended in medium supplemented with horse serum or human serum. These cells come from patients whose disease subsequently responds to treatment with MTX or other chemotherapeutic agents. The same cells, when treated in medium supplemented with FCS exhibit no MTX-induced suppression of ^{3}H-TdR incorporation into DNA (57). Suppression is restored when the cells are treated in medium supplemented with extensively dialyzed FCS (57). Thus, *in vitro* tests of sensitivity to MTX of human leukemic cells provide useful clinical information if the tests are carried out in medium supplemented with horse serum (62), but not if done in the presence of undialyzed FCS.

The calculated ^{3}H-UdR rate may be a valid *in vitro* correlate for cell kill when tested in medium supplemented with horse serum, as was seen in L5178Y cells. However, the generality of the correlation cannot be examined in the presence of FCS if protection against MTX is afforded by exogenous factors or in systems with low cloning efficiency, i.e., KB cells.

In cultures with a low cloning efficiency, a large number of cells may be present in the population with biochemical characteristics entirely different from those of the low number of clonable cells. It is the clonable cells that are presumably responsible for tumour growth and are of interest in experiments measuring the cytocidal effects of the drug. No clinical significance has yet been demonstrated for the calculated ^{3}H-UdR rate as predictor of sensitivity of acute leukemic cells to chemotherapy (57).

Inhibition of ^{3}H-UdR incorporation does not occur until intracellular accumulation of MTX is well in excess of that required to

inhibit the content of DHFR, suggesting a secondary site of action of MTX (98). The increase in the slow component of the block in the calculated ^{3}H-UdR rate is consistent with both hypothesized secondary sites of action of MTX; a low affinity DHFR or TS.

The deepening thymineless state reflected in the decrease in the calculated ^{3}H-UdR rate may be due to a decrease in TS activity or progressive depletion of intracellular folate coenzymes. A decrease in TS activity may be due to direct intracellular inhibition by MTX, to increased degradation of the enzyme from lack of substrate stabilization during MTX treatment, or to inhibition of synthesis of a rapidly turning-over enzyme.

In the present studies of the L5178Y system, MTX treatment of cells in culture results in a decreased activity of TS in extracts from the cells. The substantial decrease is due to a large extent to the inhibition of protein synthesis caused by the MTX-induced purine-less state. The decrease in TS activity is greater than the degree of inhibition of protein synthesis as judged by leucine incorporation.

Our initial hypothesis was that the inhibition of TS activity should correlate with the calculated ^{3}H-UdR rate in the presence of hypoxanthine where no pure thymineless state occurs. However, the addition of hypoxanthine completely prevents the drop in TS activity of the cellular extracts; i.e., the purineless state, by inhibiting protein synthesis, inhibits the synthesis of TS. In effect then, the purineless state deepens the thymineless state beyond that caused by the depletion of reduced folate coenzymes. However, exogenous purines were previously shown to have little effect on the MTX-induced progressive decrease in the calculated ^{3}H-UdR rate, i.e., the steadily deepening thymineless state induced by MTX (59), but as shown in the

present study, exogenous purines completely prevent the drop in TS activity in extracts of treated cells. Thus, the deepening thymineless state cannot be entirely due to inhibition of synthesis of TS. Furthermore, the mechanisms of purineless death cannot be due entirely to accentuation of the thymineless state by inhibition of synthesis of TS: the addition of TdR alone did not prevent purineless death.

It might be of value to investigate the effect of MTX on TS enzyme in CHO cells. No purineless state appears to be present and no inhibition of TS synthesis would be expected. The observed deepening of the thymineless state might come only from progressive depletion of reduced folates in this system as well as decreased TS activity.

MTX may also directly inhibit TS intracellularly, apart from the decline in TS activity induced by the purineless state. As yet, no kinetic studies have been attempted to examine the MTX-induced inhibition of TS under conditions of limiting amounts of reduced folates, due to the problems of obtaining large amounts of purified stable enzyme.

MTX may modulate TS activity by its effect on DHFR. Kawai and Hillcoat investigated the synergism of TS and DHFR *in vitro* and *in vivo*. TS was shown to be inhibited by FH_2, a substrate of DHFR, and DHFR by methylenetetrahydrofolate, a substrate of TS. The activity of TS decreased with increasing concentrations of DHFR at pH 7.5 while the reverse occurred at pH 8.5. This study suggested the importance of *in vivo* interactions of substrates and enzyme concentrations on the apparent activity of TS. Since these factors vary from cell type to cell type, it is therefore impossible to extrapolate conclusions from one cell type

to another or more importantly from the cell-free assay system to the intact cell (69).

The susceptibility of the TS enzyme of L5178Y cells to sonication as demonstrated in the present study, is of considerable interest. The loss of TS activity by extracts of log cultures upon sonication seems to be due to the sonication energy itself, since loss is seen even with several short bursts of sound separated by periods in ice. The increased sensitivity to sonication of TS activity in extracts from log cultures over that from resting cultures may represent an increased enzyme lability and may be due to the presence of a stabilizing factor in the resting cultures.

Alternatively, there may be different enzyme structures in cells from log and resting cultures. TS in extracts of pig thymus (48) or from L. casei (27) exists in subunit form. If this were true in L5178Y log cells, sonication might disrupt the quaternary structure to the lower activity form. The lower activity enzyme in resting cells may be primarily in a monomer form and therefore not susceptible to sonication.

Finally, sonication might dissociate TS from the membrane, and thereby deactivate the enzyme, while lysis might not. However, there is no evidence that membrane association is necessary for maximum activity of the TS enzyme.

The extreme instability of the enzyme from L5178Y cells must be remembered using any extraction method. This factor may not have been adequately controlled by other workers, even though the disruption of cells by sonication to release TS is a standard technique.

Roberts and Loehr found an increase in TS activity in sonicated extracts of CCRF-CEM cells from resting cultures compared

to log cultures (90). This was also seen in the present study when sonication was used to extract TS from L5178Y cells. However, the higher activity of TS from log cultures over resting cultures that was found with hypotonic lysis extraction is more consistent with the fact that TS is a log enzyme, and probably represents a closer approximation to the state of affairs in intact cells.

Freisheim et al. sonicated Streptococcus faecium for 20 minutes to release TS (34). The 60 fold increase detected in TS activity in the resistant compared to the sensitive strain might have been much different if a differential sensitivity to sonication between TS of sensitive compared to resistant strains were present and were corrected for by control over the extraction procedure.

In the early studies of the properties and kinetics of inhibition of TS by fluorinated pyrimidines, TS was obtained by sonication for 4 minutes at 20 kilocycles per second from Ehrlich ascites carcinoma cells. The results of inhibition studies could depend very much on the final form of TS extracted, and no assurance was given that this was not a factor of concern (87).

A 2 fold elevation in TS activity was found by Roberts on the addition of 10 micromolar MTX to resting cultures of CCRF-CEM cells (90) in comparison to the 3 fold decrease found in the present study. The difference may be due to the different cell line used or the 10 fold higher MTX concentration which may stabilize the enzyme as suggested. The conclusions in the study must be questioned however since (1) the enzyme was extracted by sonication (conditions unstated); (2) cultures were treated in fresh medium supplemented with undialyzed FCS.

A similar stabilization of TS activity by MTX was found

in the Bonney and Maley study (10) with cultures of parenchymal cells isolated from regenerating rat liver. Again the interpretation of the data is difficult, since these cells were cultures in medium containing 15% undialyzed FCS and treated with concentrations of MTX in excess of 10^{-5}M.

MTX treatment may result in an increased intracellular degradation of the more sensitive enzyme from log L5178Y cells. Alternatively, MTX may inhibit synthesis of new enzyme while the normal rate of degradation is allowed to proceed.

The increase in inhibition of TS by MTX detected in extracts obtained by lysis over sonication is consistent with both possibilities. However, the fact that hypoxanthine prevented the MTX-induced decline in TS activity suggests that the main mechanism is inhibition of synthesis of new enzyme without alteration in the rate of degradation. In that case, the enzyme from cells in log cultures would have a much higher rate of turnover than TS of cells in resting cultures. Since the drop in activity during MTX treatment is less in the extracts of the sonication-resistant resting cultures and in the sonicates of log cultures than that in the log culture lysates, the sonication resistant enzyme is probably a more stable form intracellularly with a slower rate of turnover. This may have clinical implications since a difference in turnover rates may account for the different responses of patients to MTX therapy.

LEGENDS TO CHARTS

Chart 1. Hypoxanthine and thymidine protection from cytocidal effects of MTX on CHO cells in mid-log cultures. Cells were treated with MTX alone (▼) or MTX plus 3 x 10^{-5}M hypoxanthine (O) or MTX plus 2 x 10^{-5}M thymidine (X). At intervals during treatment, cells were cloned and the efficiency was expressed as a percent of that in untreated control cultures. Cloning efficiency in control cultures was 20 to 80%. Shown are geometric means of 2 to 6 experiments at each time point. Bars, ±1 S.E.

Chart 2. Effects of MTX on ^{3}H-TdR (●) and ^{3}H-UdR (▲) incorporation into DNA by CHO cells in mid-log cultures. At intervals during treatment with MTX, cells were pulsed and the rate of incorporation expressed as a percent of the rate in the untreated culture. Shown are the geometric means of 2 to 5 separate experiments at each time point. Bars, ±1 S.E.

Chart 3. Effect of MTX treatment on cloning efficiency correlated with calculated ^{3}H-UdR rate in CHO cells. Cells from mid-log cultures were treated with MTX and at intervals, the cloning efficiency was measured as a percent of untreated cultures (▼) or the calculated ^{3}H-UdR rate was determined (See "Materials and Methods') from the data in Chart 2. Shown are geometric means of 2 to 6 experiments at each time point. Bars, ±1 S.E.

Chart 4. Effect of MTX treatment on ^{3}H-UdR (▲) and ^{3}H-TdR (●) incorporation into DNA by CHO cells in resting cultures. Experimental design as in legend to Chart 2 except that cells were treated in resting cultures. Shown are geometric means for 2 to 5 experiments at each time point. Bars, ±1 S.E.

Chart 5. Effect of MTX treatment on cloning efficiency (▼) correlated with calculated ^{3}H-UdR rate (O) in CHO cells from resting cultures. Experimental design and calculation of results as in legend to Chart 3 except that cells treated were from resting cultures and the calculated ^{3}H-UdR rate was determined from data from Chart 4. Shown are geometric means of 2 to 5 experiments at each time point. Bars, ±1 S.E.

Chart 6. Effect of MTX treatment on ^{3}H-UdR (▲) and ^{3}H-TdR (●) incorporation into DNA by CHO cells from early log cultures. Experimental design as in legend to Chart 2 except that early log cultures were heated. Shown are geometric means for 2 to 4 experiments at each time point. Bars, ±1 S.E.

Chart 7. Effect of MTX treatment on cloning efficiency (▼) correlated with ^{3}H-UdR rate (O) in CHO cells from early log cultures. Experimental design and calculation of results as in legend to Chart 3 except that early log cultures were treated, and the calculated ^{3}H-UdR rate was determined from data in Chart 6. Shown are geometric means for 2 to 3 experiments at each time point. Bars, ±1 S.E.

Chart 8. Effect of MTX treatment on cloning efficiency (▼) correlated with the calculated ^{3}H-UdR rate (O) in CHO cells from early log cultures grown in medium supplemented with dialyzed fetal calf serum. Experimental design and calculations of results as in Chart 3 except that cells were grown in medium supplemented with dialyzed fetal calf serum for 48 hours prior to treatment and the calculated ^{3}H-UdR rate was determined from data derived as in legend to Chart 2. Shown are geometric means for 3 experiments at each time point. Bars, ± 1 S.E.

Chart 9. Fetal calf serum protection from cytocidal effects of MTX on CHO cells in mid-log cultures. At the time of treatment with MTX, a supplemental 10% fetal calf serum was added to the cultures. At 12 or 24 hours after treatment, cytocidal effects (●) were measured as in legend to Chart 1. The cloning efficiency of cultures treated without the addition of supplemental fetal calf serum is replotted from Chart 3 for comparison (▼). Shown are geometric means for 2 to 3 experiments at each time point. Bars, ±1 S.E.

Chart 10. Effect of MTX treatment on cloning efficiency (▼) with the calculated ^{3}H-UdR rate (O) in KB cells. Cells from early log cultures in medium supplemented with horse serum were treated with MTX, and cytocidal effects measured as in legend to Chart 1. The cloning efficiency in control cultures was 4 to 15%. The calculated ^{3}H-UdR rate was determined from data derived as in legend to Chart 2. Shown are geometric means for 2 experiments at each time point.

Chart 11. Effect of MTX treatment on cloning efficiency (▼) correlated with the calculated ^{3}H-UdR rate (O) in KB cells from early log cultures grown in medium supplemented with fetal calf serum. Experimental design and calculation of results as in legend to Chart 10. Shown are geometric means for 2 experiments at each time point.

Chart 12. Hypoxanthine protection from effect of MTX on thymidylate synthetase activity in L5178Y leukemia cells. Thymidylate synthetase activity was measured as described in 'Materials and Methods". Activity was expressed as a percent of activity in untreated control lysates. Shown are geometric means ± 1 SE of 2 to 14 experiments at each point 1×10^{-6}M MTX alone (X), MTX plus 2×10^{-5}M hypoxanthine (O). Activity in controls was 4 to 11 x 10^{-12} moles of tritium exchanged

to H_2O per min. per mg. protein.

Chart 13. Effect of sonication on L5178Y thymidylate synthetase activity. Cells from log (O) or resting cultures (X) were sonicated for a varying number of varying periods or lysed in H_2O. Activity was expressed as a percent of that in concurrently assayed lysates. Shown are geometric means ± 1 SE for 3 to 7 experiments at each time point. 100% activity of the lysates varied from 6 to 17 x 10^{-12} moles/min/mg. for log cultures and 2 to 5 x 10^{-12} moles/min/mg. for resting cultures.

Chart 14. Comparison of effect of MTX treatment on thymidylate synthetase activity from L5178Y cells as extracted by sonication or hypotonic lysis. Sonication was by 4 x 30 second bursts at 20 kilocycle per second and hypotonic lysis was a described in 'Materials and Methods'. Experiments were done on both log (O) and resting cultures (X). Shown are geometric means ± 1 SE of 4 to 10 experiments at each time point.

CHART 1

CHO CELLS (MID-LOG)
HYPOXANTHINE AND THYMIDINE RESCUE

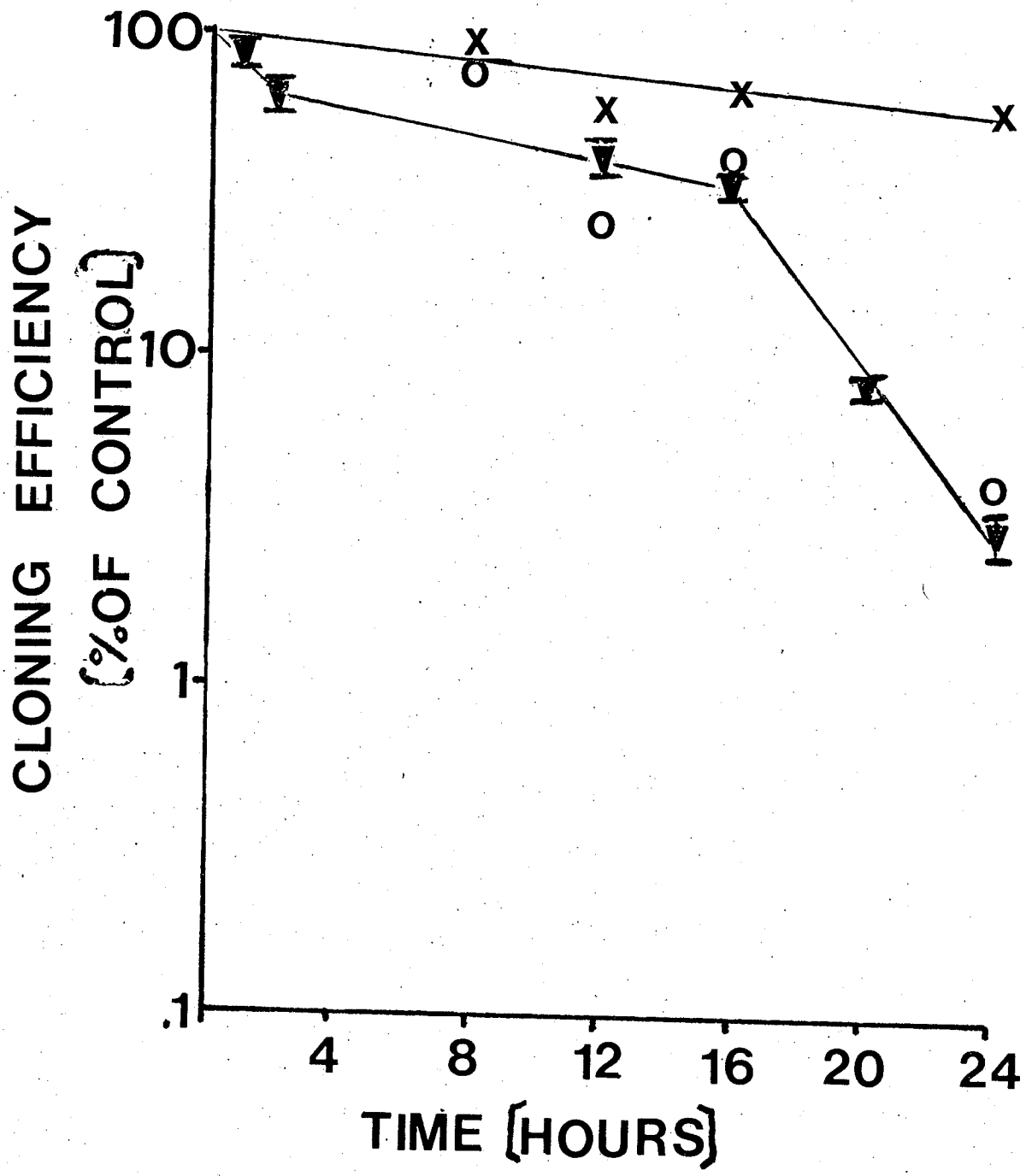

CHART 2

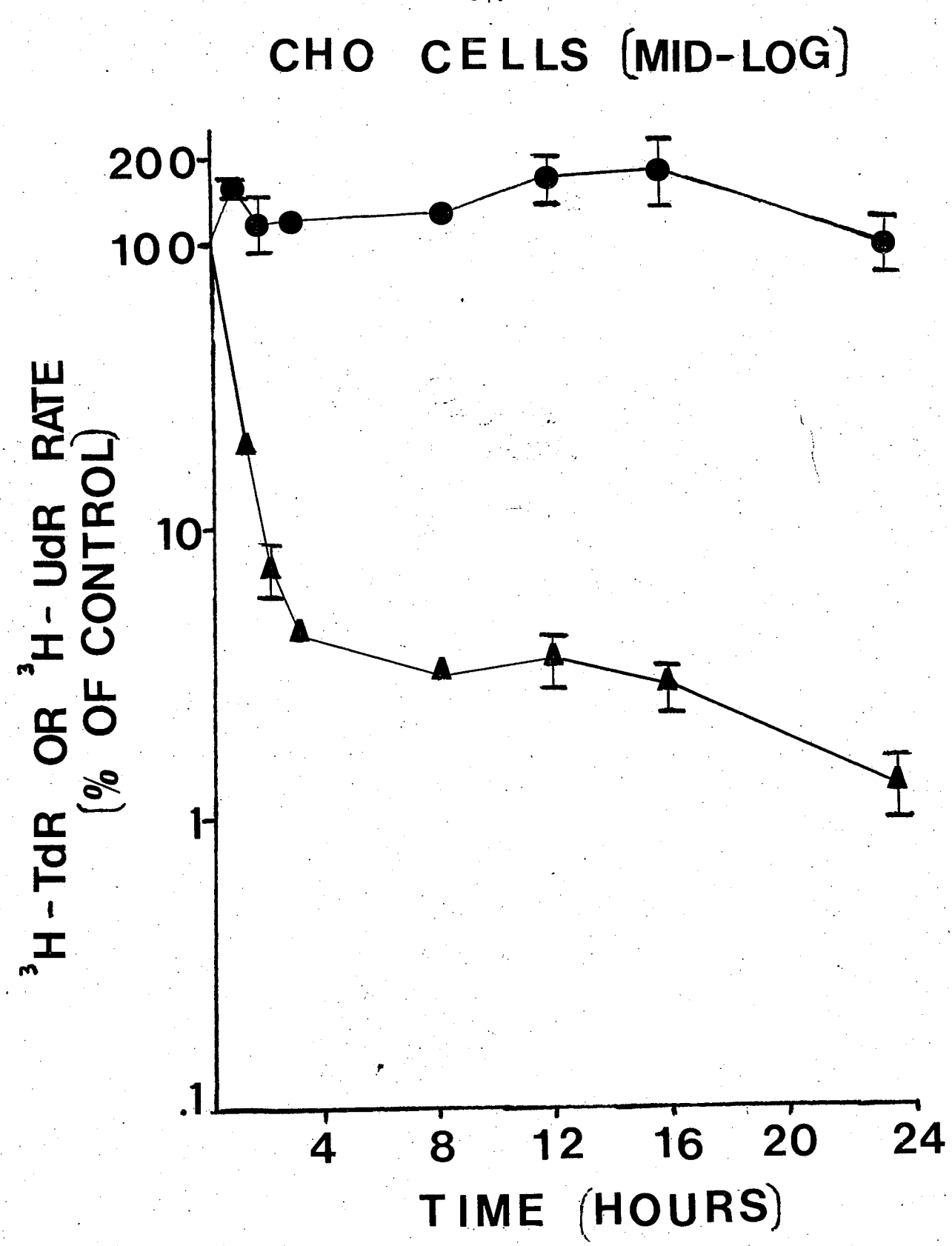
CHO CELLS (MID-LOG)
200
100
10
1
.1
^{3}H-TdR OR ^{3}H-UdR RATE (% OF CONTROL)
4
8
12
16
20
24
TIME (HOURS)

CHART 3

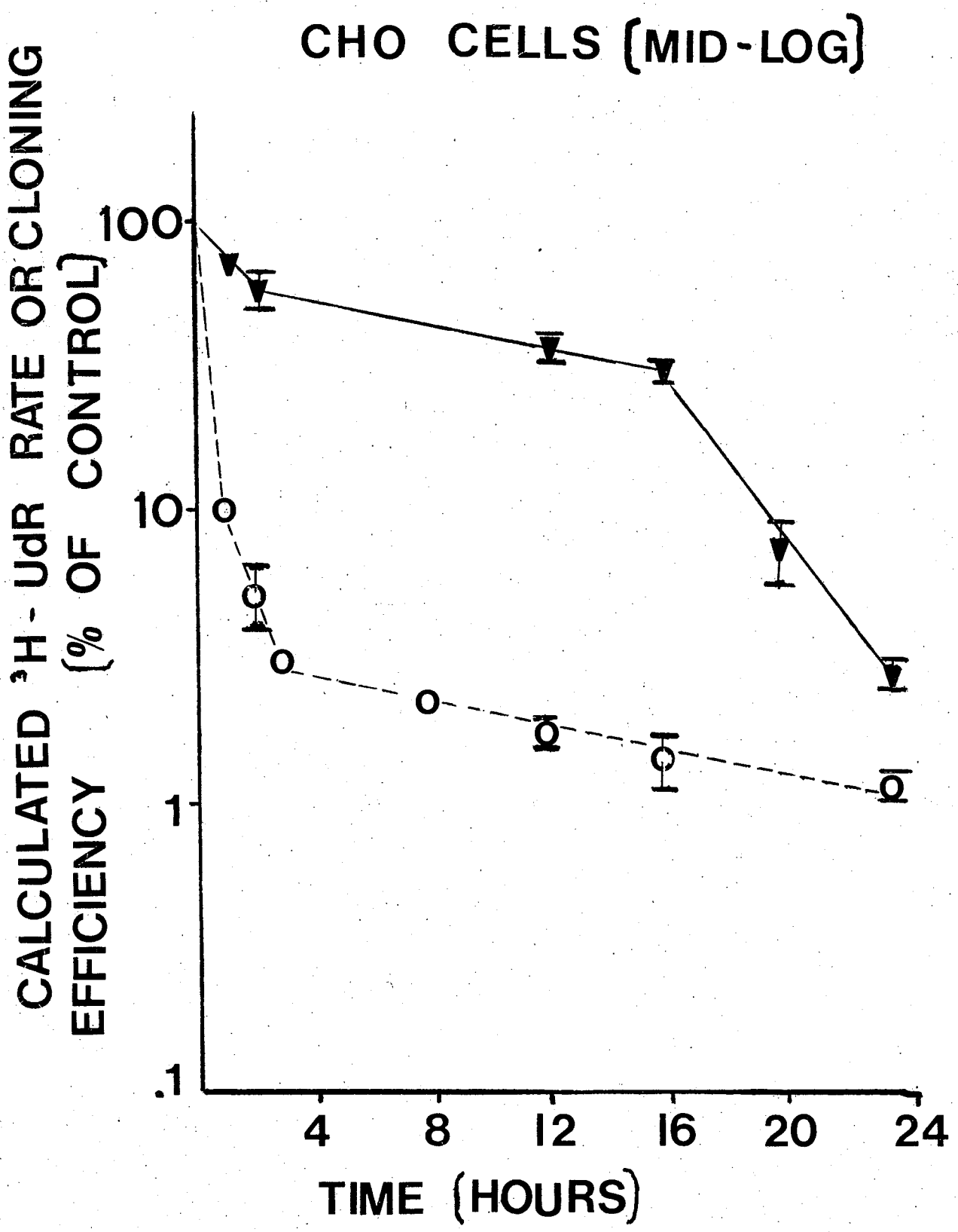
CHO CELLS (MID-LOG)
CALCULATED ³H-UdR RATE OR CLONING EFFICIENCY (% OF CONTROL)
100
10
1
.1
4
8
12
16
20
24
TIME (HOURS)

CHART 4

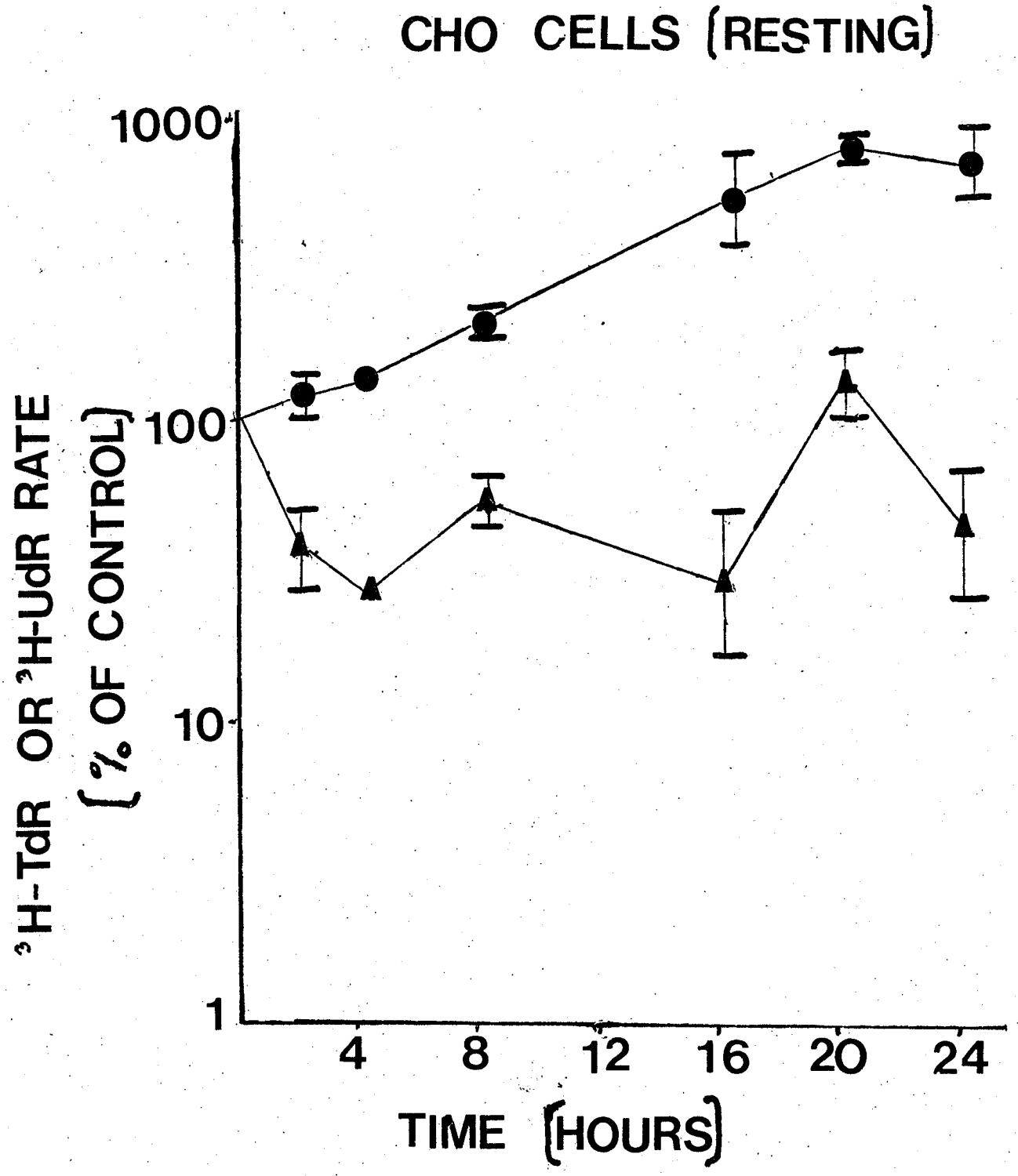
CHO CELLS (RESTING)
1000
100
10
1
4
8
12
16
20
24
TIME (HOURS)
^{3}H-TdR OR ^{3}H-UdR RATE
(% OF CONTROL)

CHART 5

CHO CELLS (RESTING)

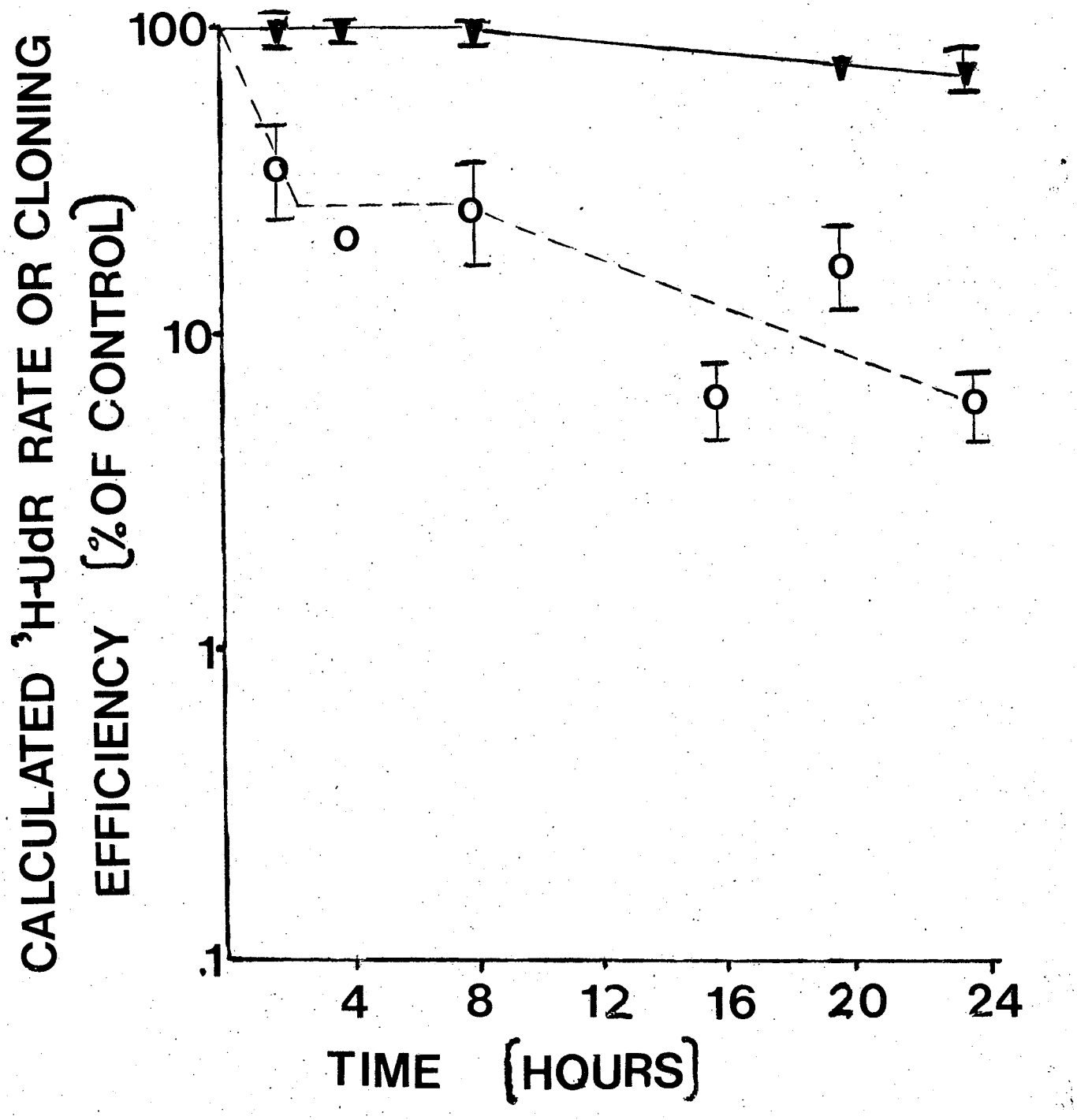

CHART 6

CHO CELLS (EARLY LOG)

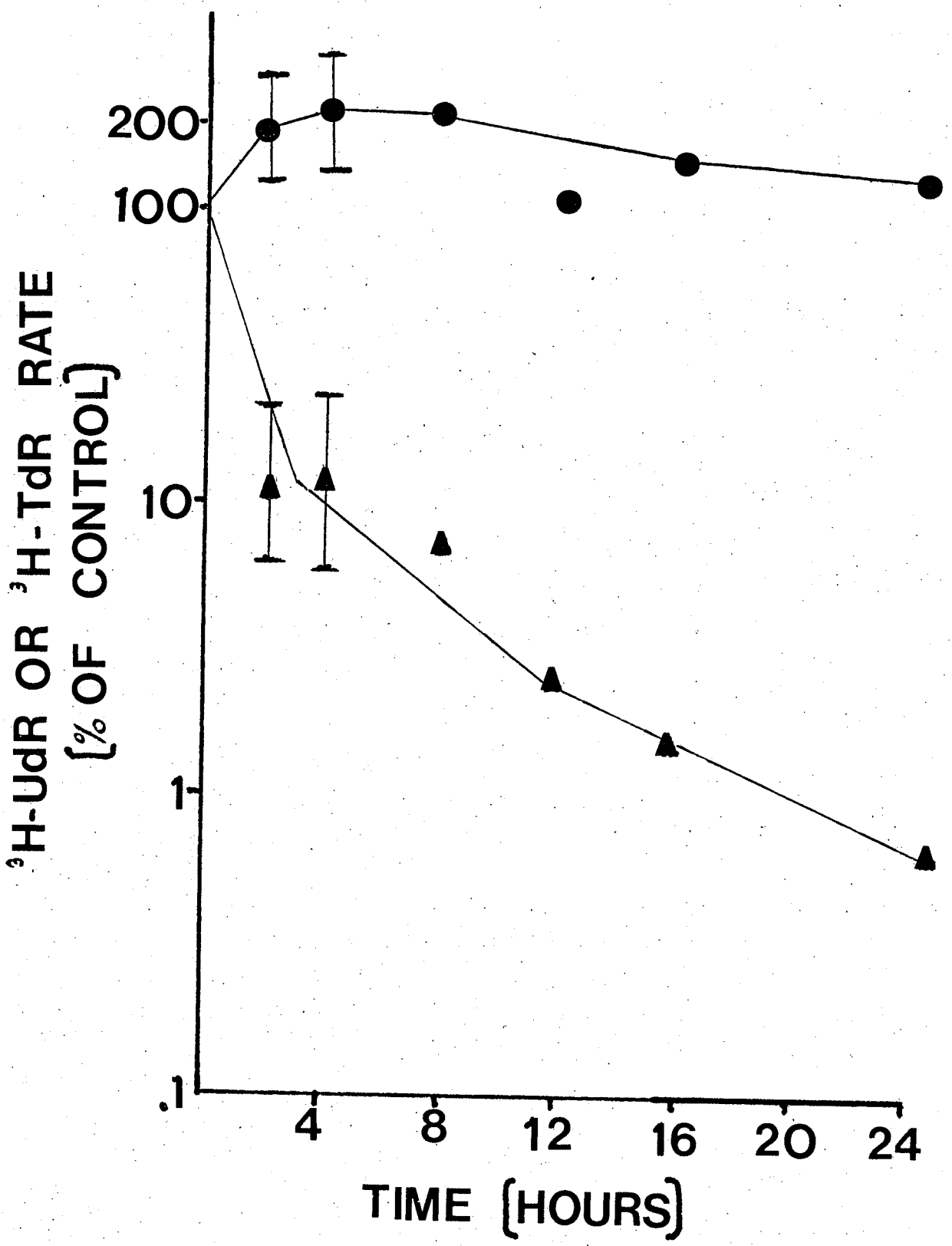

CHART 7

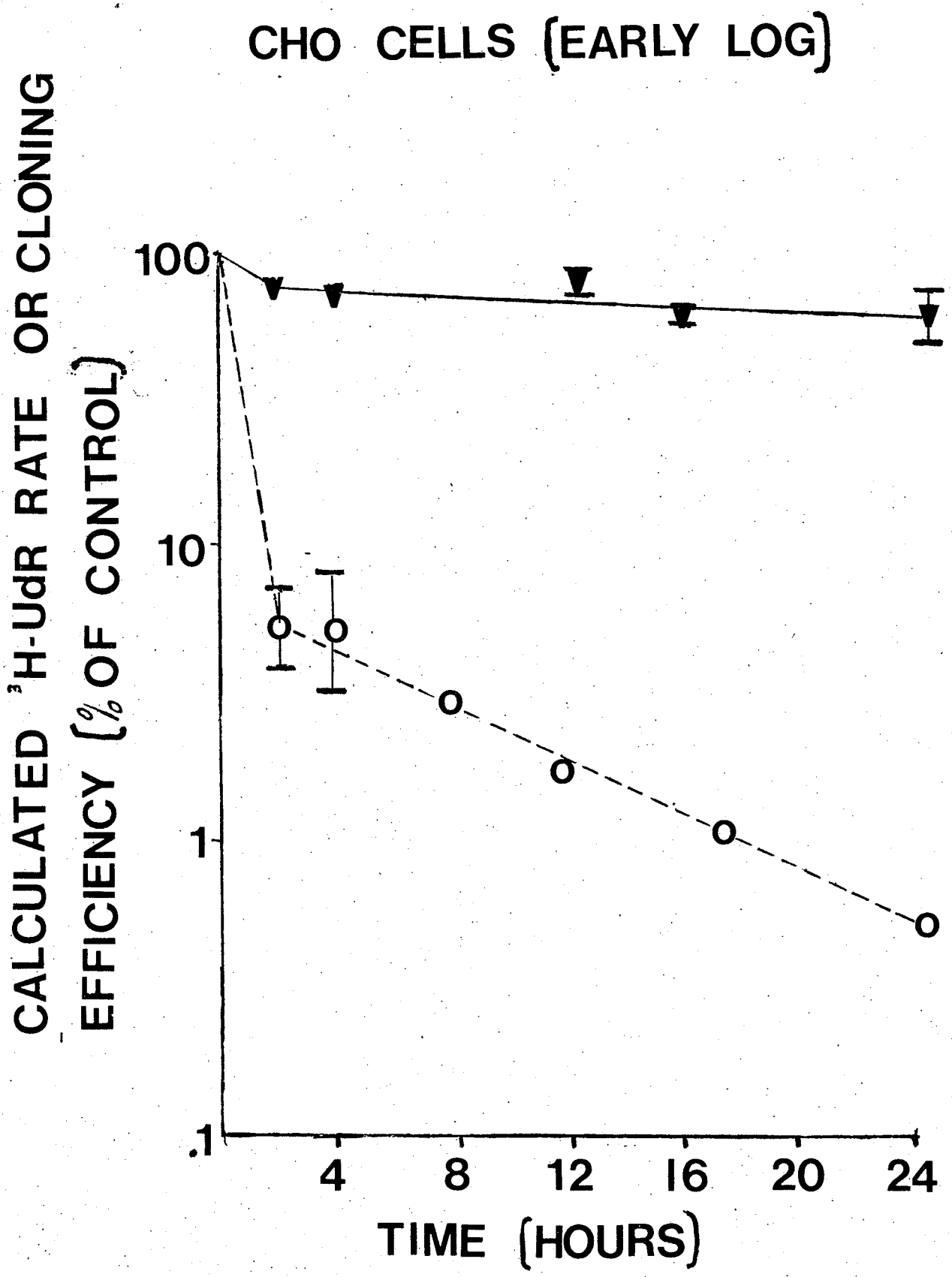
CHO CELLS (EARLY LOG)
CALCULATED ³H-UdR RATE OR CLONING EFFICIENCY (% OF CONTROL)
100
10
1
.1
4
8
12
16
20
24
TIME (HOURS)

CHART 8

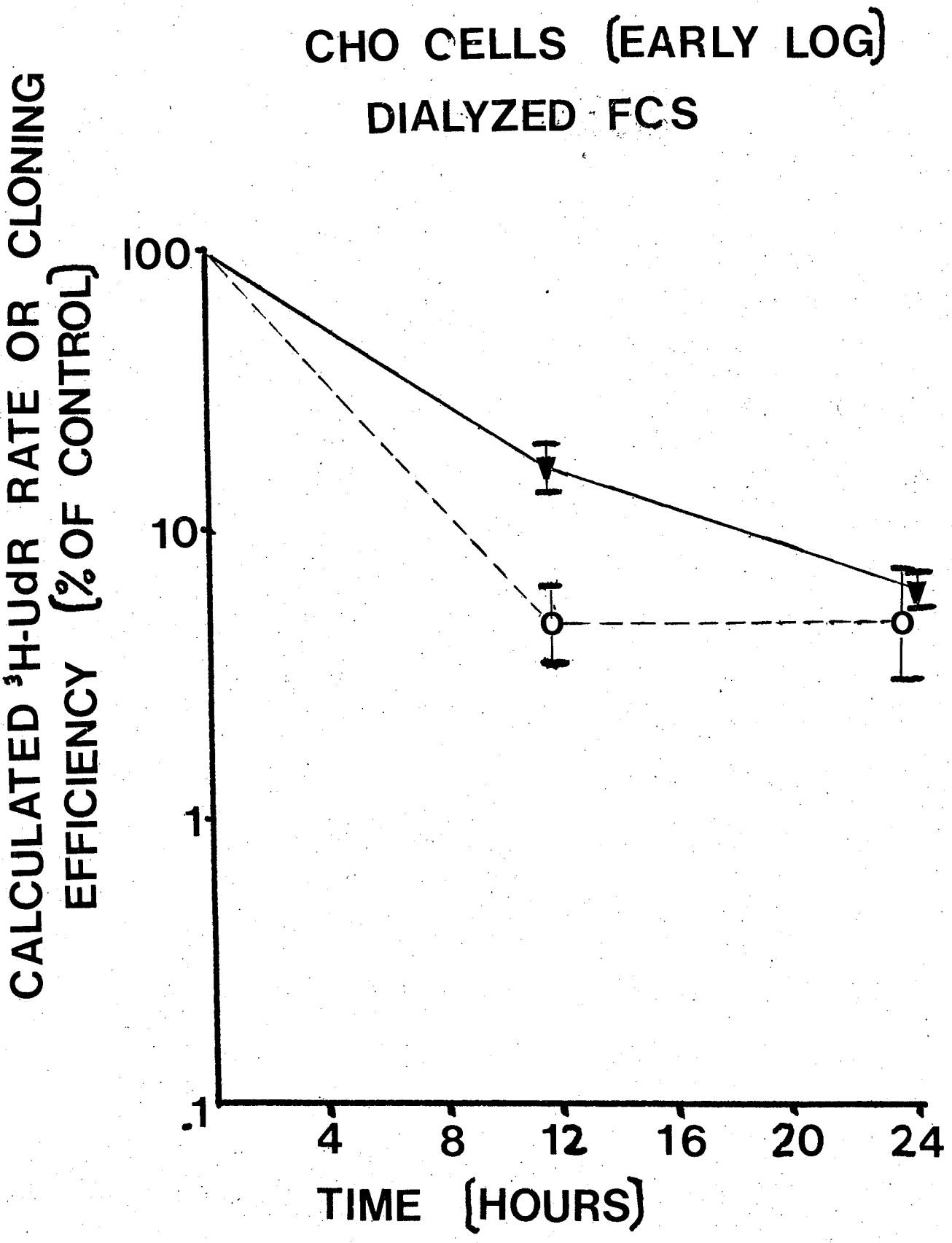
CHO CELLS (EARLY LOG)
DIALYZED FCS
CALCULATED ³H-UdR RATE OR CLONING EFFICIENCY (% OF CONTROL)
100
10
1
.1
4
8
12
16
20
24
TIME (HOURS)

CHART 9

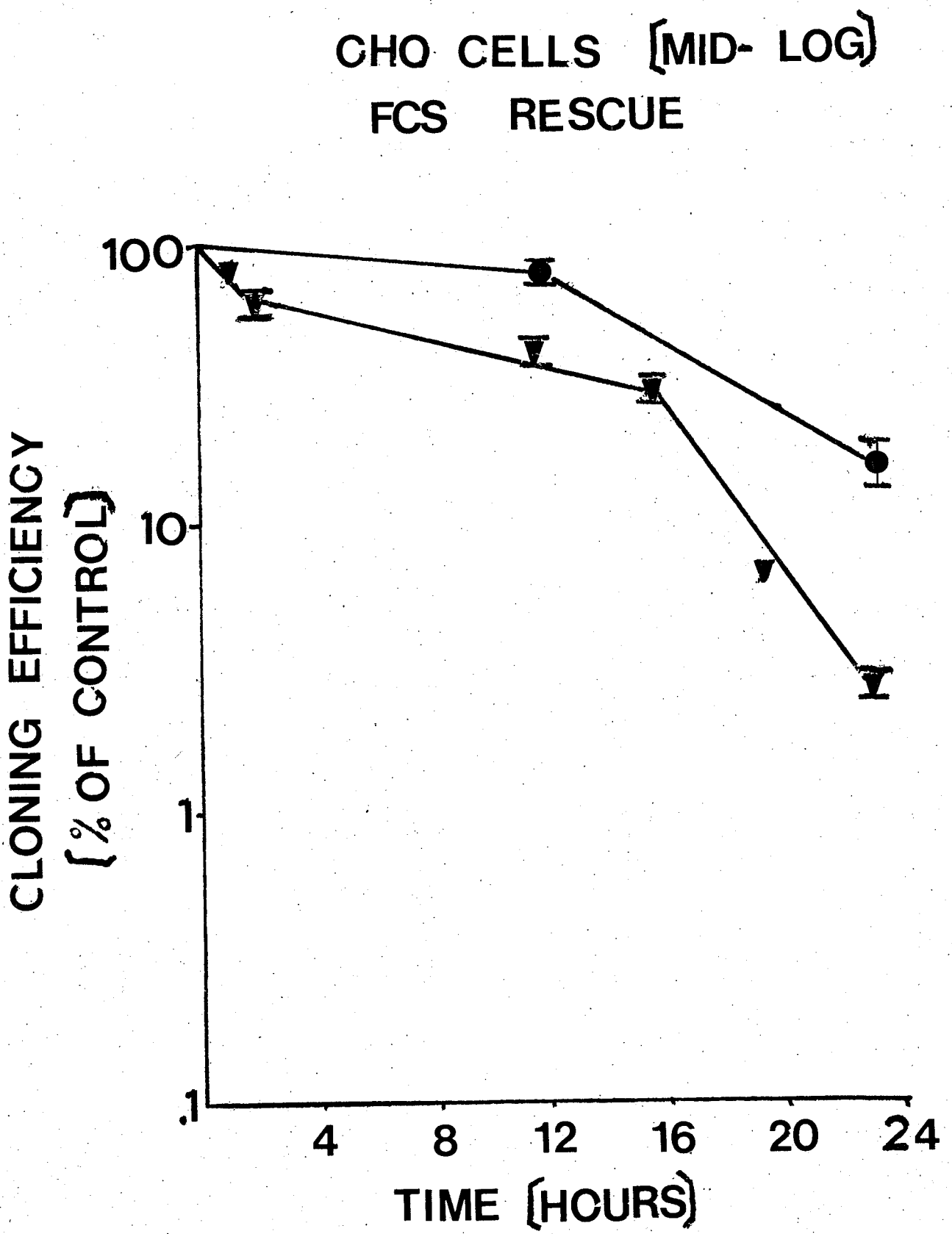
CHO CELLS (MID- LOG)
FCS RESCUE
100
10
1
.1
CLONING EFFICIENCY
(% OF CONTROL)
4
8
12
16
20
24
TIME (HOURS)

CHART 10

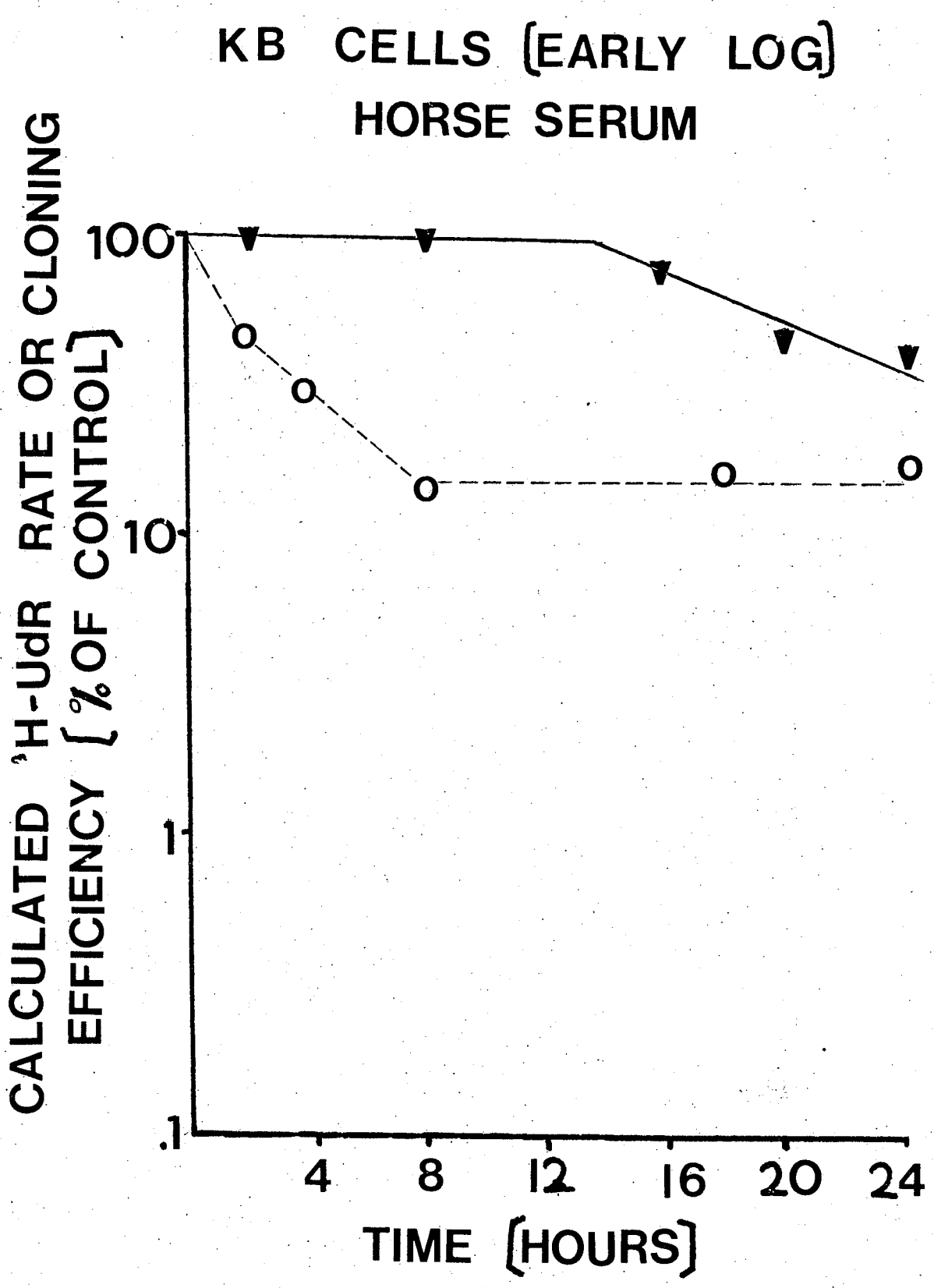
KB CELLS (EARLY LOG)
HORSE SERUM
CALCULATED ³H-UdR RATE OR CLONING EFFICIENCY [% OF CONTROL]
100
10
1
.1
4
8
12
16
20
24
TIME (HOURS)

CHART II

KB CELLS (EARLY LOG)
FCS

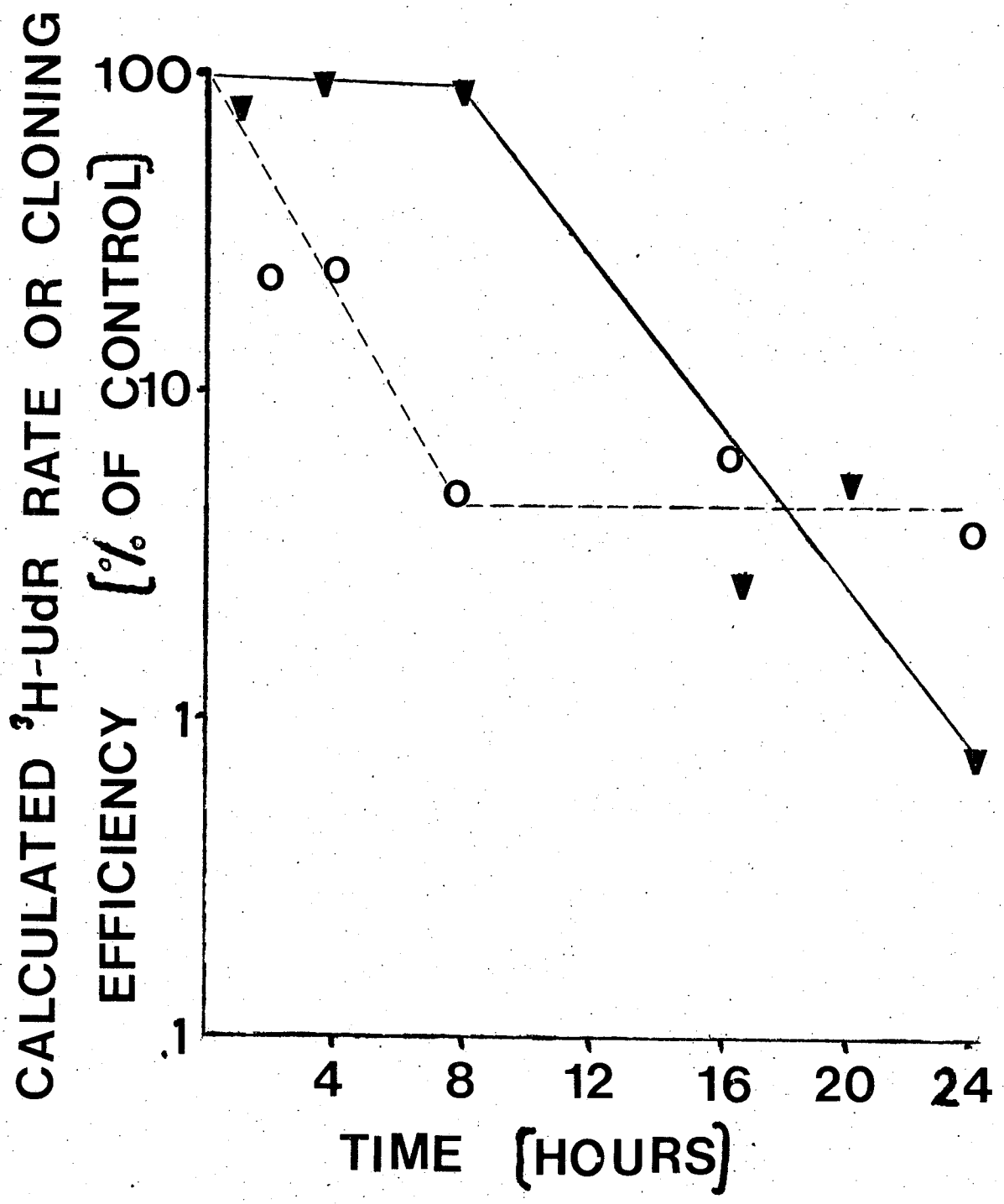

CHART 12

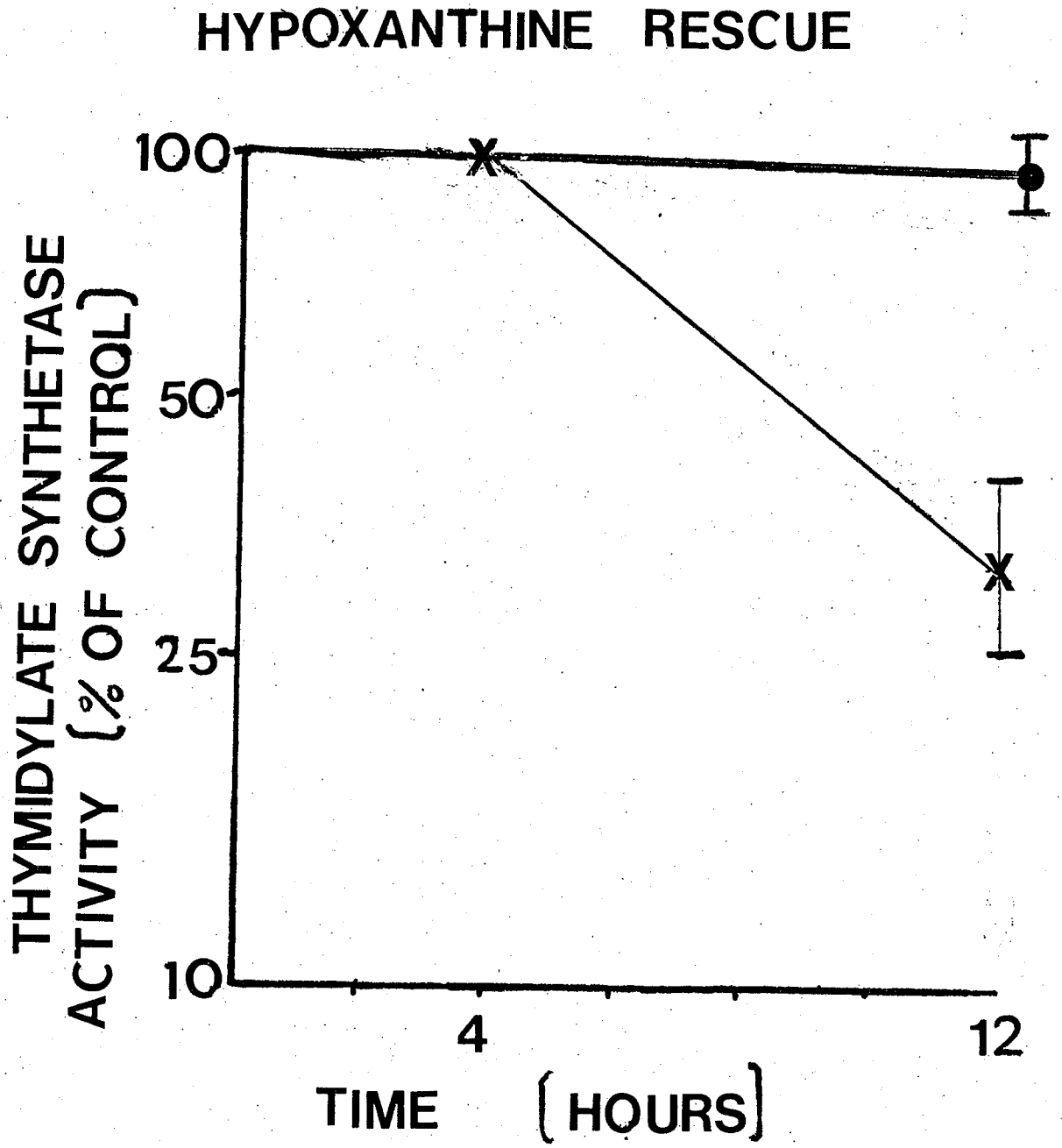
HYPOXANTHINE RESCUE
THYMIDYLATE SYNTHETASE ACTIVITY (% OF CONTROL)
100
50
25
10
4
12
TIME (HOURS)

CHART 13

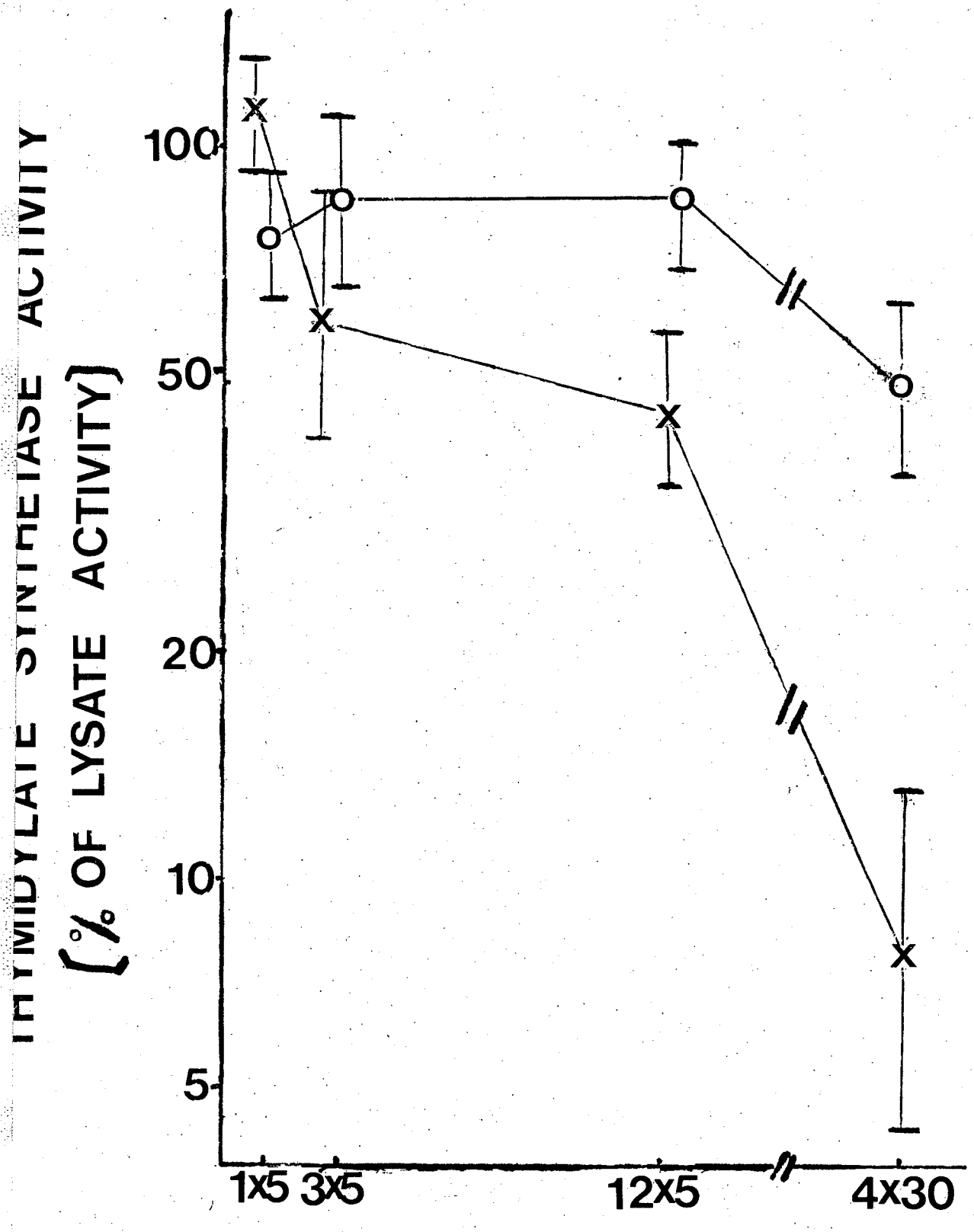
INTIMIDYLATE SYNTHETASE ACTIVITY
[% OF LYSATE ACTIVITY]
100
50
20
10
5
1x5
3x5
12x5
4x30
SONICATION TIME
[NUMBER BURSTS X SECONDS]

CHART 14

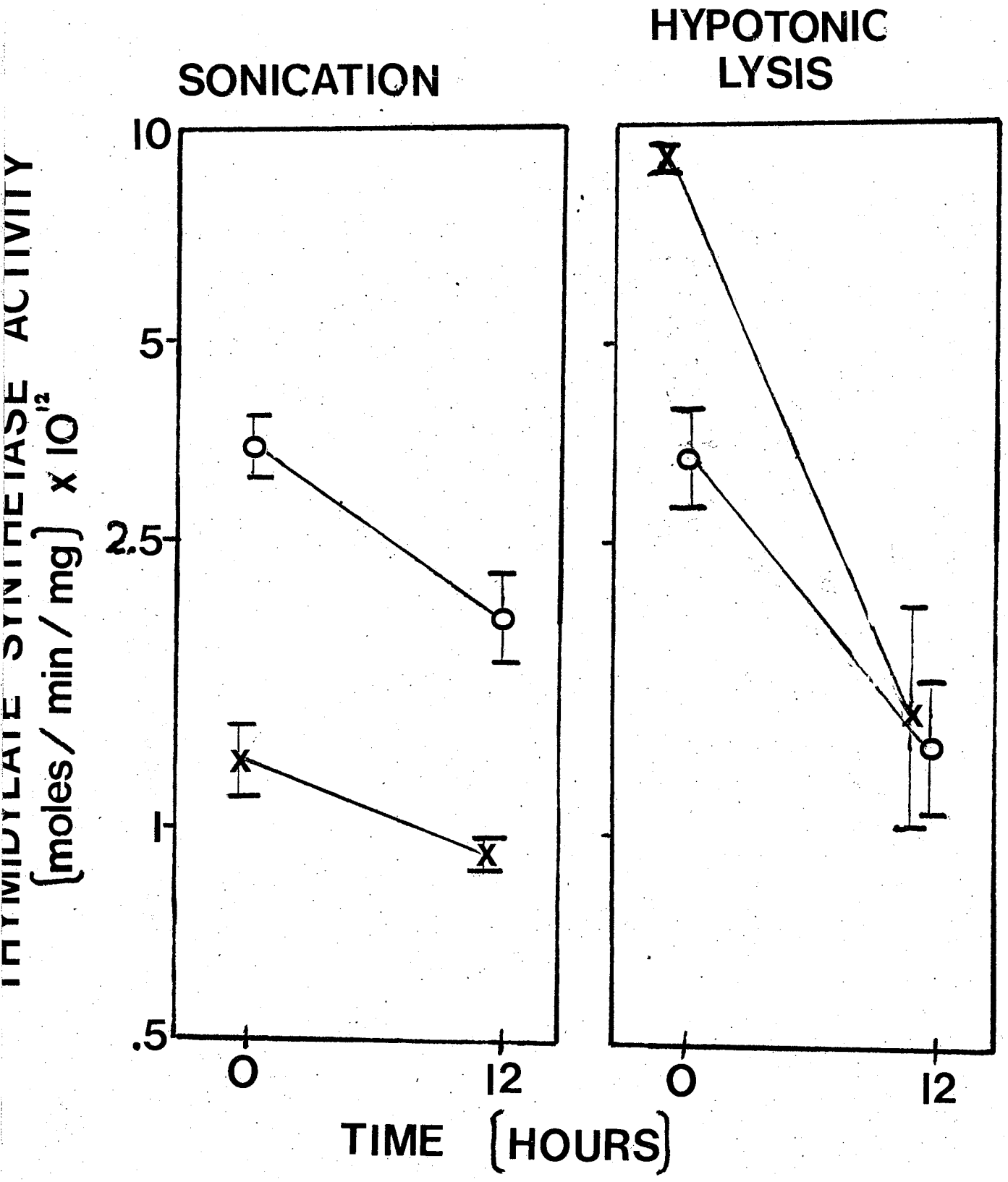
SONICATION
HYPOTONIC LYSIS
10
5
2.5
1
.5
0
12
0
12
TIME (HOURS)
(moles/min/mg) x 10¹²

TABLE 1

THE EFFECT OF MTX ON PROTEIN SYNTHESIS IN L5178Y CELLS

Culture Condition	Rate of ^{3}H-leucine Incorporation (pmoles/min/10^{9} cells)
Log	
No Additives	81.1
+ MTX(1)	34.4
Hypoxanthine(2)	73.3
Hypoxanthine + MTX(3)	83.4
Resting	
No Additives	55.8
+ MTX(1)	41.0

This is representative of 3 experiments. Cells were incubated for 12 hours then removed and pulsed for 5 to 30 minutes with ^{3}H-leucine.

(1) 1×10^{-6}M MTX

(2) 3×10^{-5}M Hypoxanthine

(3) 3×10^{-5}M Hypoxanthine + 1×10^{-6}M MTX

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