THE TOXICITY OF 5-BROMO 2'-DEOXYURIDINE TO MALIGNANT LYMPHOID CELLS

BY

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ABSTRACT

THE TOXICITY OF 5-BROMO 2'-DEOXYURIDINE TO MALIGNANT LYMPHOID CELLS

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In this study malignant lymphoid cells of murine and human origin are shown to be much more sensitive to the cytotoxic effects of bromodeoxyuridine than are most other cell types. The spectrum of BrdU*-sensitivity includes malignant plasma cells and lymphocytes of both B and T cell origin. Thymidine protects these cells from the cytotoxic effects of BrdU by competing with the analogue for both uptake into the cell and incorporation into DNA.

BrdU treatment of malignant lymphoid cells causes the appearance of large intracellular structures that can be seen by either phase or electron microscopy. The appearance of these intracytoplasmic inclusions precedes the effect of BrdU on the growth of these cells. Cells that are resistant to BrdU's cytotoxic effects do not show the morphological changes seen in the sensitive cells.

BrdU enters cells by two kinetically distinguishable transport systems: one which is saturable and temperature sensitive, and a second which has the characteristics

of pure diffusion. Thymidine competes with BrdU for the facilitated uptake system. BrdU-resistant clones of malignant lymphoid cells do not concentrate the nucleoside, however, they do allow the analogue to enter by passive diffusion. BrdU resistant clones also do not concentrate thymidine.

BrdU is rapidly phosphorylated when it enters BrdU-sensitive, but not resistant, cells since the resistant cells are deficient in thymidine kinase activity. This is due to a lack of functional enzyme rather than an inhibitor. In BrdU-sensitive "wild-type" cells intracellular free BrdU is found mostly as its mono and triphosphate.

Deoxycytidine protects several cell types from the toxicity of BrdU. It has been suggested that this is because BrdU triphosphate is a strong negative allosteric effector of ribonucleotide reductase, creating a deoxypyrimidineless state which can be alleviated by deoxycytidine. However, it is unlikely that this explanation can account for the toxicity of low concentrations of BrdU to malignant lymphoid cells for the following reasons: 1) BrdU does not reduce the intracellular concentration of deoxy derivatives of radiolabeled cytidine, 2) BrdU does not prevent the utilization of ribonucleoside for DNA synthesis, 3) cytidine, as well as deoxycytidine, protects malignant lymphocytes from the

effects of BrdU, and 4) deoxycytidine does not protect malignant plasma cells from BrdU.

BrdU and deoxycytidine do not share the same transport system, nor is there competition between them for uptake and incorporation into DNA. Thus, the protection of malignant lymphocytes, by cytosine nucleosides, against the toxic effects of BrdU is not due to a decreased uptake of the latter in the presence of the former.

Because of the sensitivity of malignant plasma cells to low concentrations of BrdU, and the protection of other cell types from even large doses of the analogue by deoxycytidine, a BrdU/deoxycytidine regimen for the treatment of plasma cell malignancies is proposed. In vivo experiments indicate that such a regimen might be effective if pharmacological difficulties are overcome. Approaches to this are discussed.

List of abbreviations:

BrdU, 5-Bromo 2'-deoxyuridine; BU, Bromouracil; BU-DNA, BrdU-Substituted DNA; CD, Circular dichroism; CS, chondroitin sulfate; CS-SS, Hank's balanced salt solution containg 15% fetal calf serum; Cyd, cytidine; DMSO, Dimethyl sulfoxide; EBV, Epstein Barr virus; TAT, Tyrosine aminotransferase; TCA, Trichloroacetic acid; TK, Thymidine Kinase; IdU, 5-Iodo 2'-deoxyuridine; PCA, Perchloric acid.

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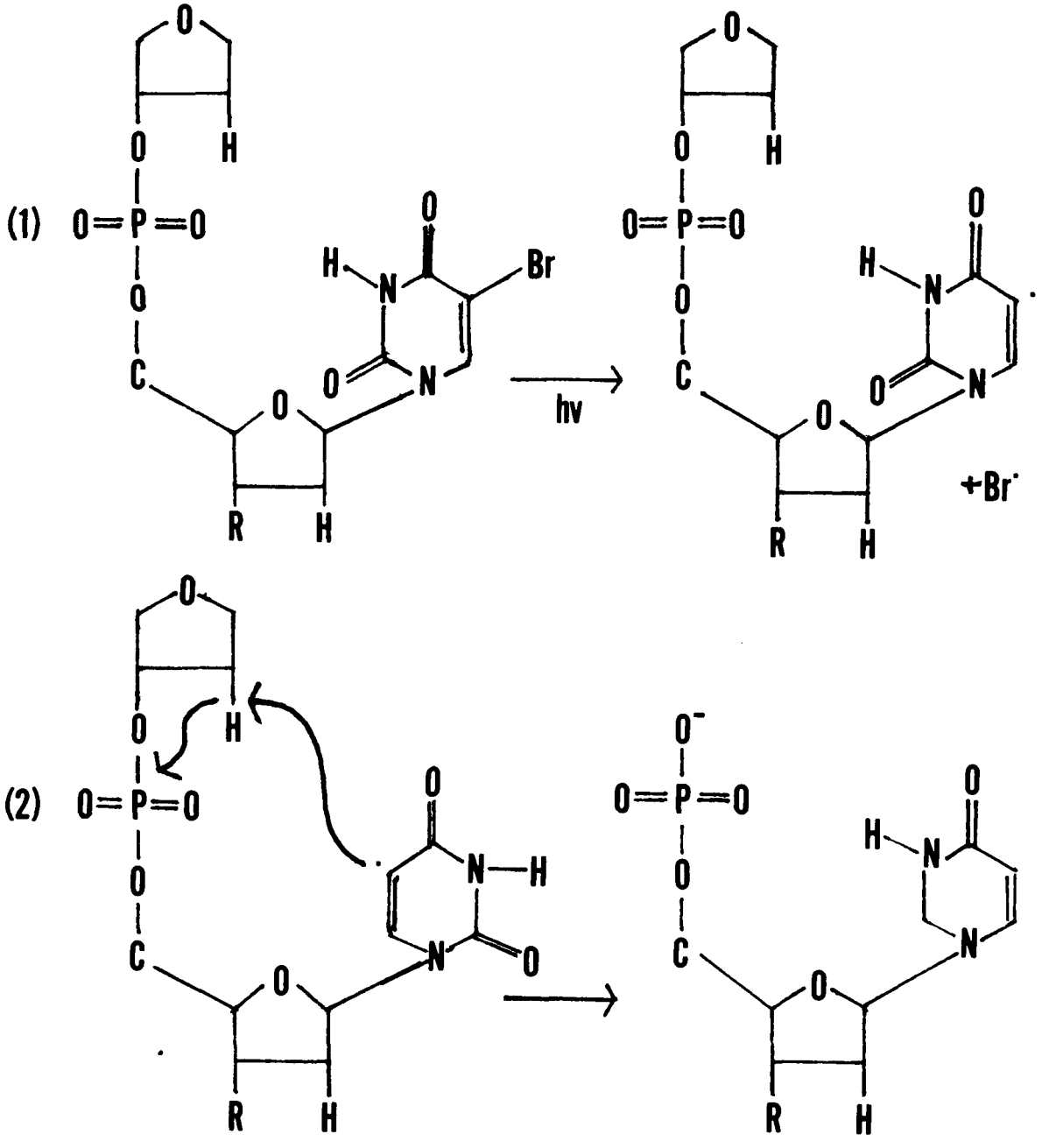
INTRODUCTION

5-Bromo 2'-Deoxyuridine (BrdU) has diverse chemical and biological properties. Irradiation of bromouracil, or its derivatives, with radiation of energy higher than that of violet light results in photochemical reactions which proceed with high quantum efficiency. Incubation of cell cultures, shielded from light, with BrdU causes many phenotypic changes which occur in the absence of detectable photochemical degradation of the analogue. The major phenotypic effects are the induction of the synthesis of previously latent viruses, inhibition of the expression of tissue specific traits, and reduction of the oncogenic potential of malignant cells.

BrdU is a chemical analogue of thymidine, the 5 position of the pyrimidine ring being occupied by a bromine atom instread of a methyl group. BrdU's effects may derive from this close structural relationship to thymidine. The Van der Vaals radius of bromine (1.95 A) is quite close to the 2.00 A radius of a methyl group. BrdU is utilized in place of thymidine in many biological processes, and its effects may therefore derive from substitution in place of thymidine in DNA. There is also evidence, described below, which suggests that the genome is not the only locus of action of BrdU.

Figure 1 - Photolytic Single-Strand Scission of BU-DNA. The product on the 5' end of the Break is Uncertain, Depending upon the Reaction Conditions.


(1)
hν
+Br·
(2)

THE PHOTOBIOCHEMISTRY OF BrdU

Interest in the photolability of BrdU substituted DNA (BU-DNA) originated with the observation by Greer and Zamenhof (1957) that E. coli grown in BrdU are profoundly photosensitive. Similar observations have been made on DNA viruses (Stahl et al., 1961), mammalian cells (Djordjevic et al., 1960) and bacterial transforming DNA (Opara-Kubinska, 1961).

Irradiation of BU-DNA results in the photo-dissociation of the bromine atom generating the highly reactive uracilyl radical (Figure 1, reaction number 1) (Danziger et al., 1968), which extracts a hydrogen atom from the deoxyribose on its 5' side, causing a single-stranded break (Figure 1, reaction 2) (Wacker et al., 1962; Wacker, 1961; and Smith, 1964). BU-DNA is photolabile in the ultraviolet, as is all DNA. However, the lability of BU-DNA extends into the low wavelength end of visible light, with an absorbtion of 313 nm, a wavelength at which unsubstituted DNA is unaffected.

INDUCTION OF VIRUS PRODUCTION BY BrdU

BrdU activates the production of previously latent viruses in many murine cell types (Lowy et al., 1971; Aaronson et al., 1971; Weiss et al., 1971; Silagi et al., 1972). By measuring induced reverse transcriptase activity, it has been found that maximal virus induction occurs with 20μg BrdU/ml for 24 hrs. The level of RNA-directed DNA polymerase

activity in the culture fluid and viral antigens in the cells peaks at about three days after BrdU treatment, followed by a rapid decrease. Electron microscopic examination reveals a large increase in both the proportion of cells containing incomplete virus particles and the number of such particles per cell two to three days after treatment. A high level of virus production can be maintained for at least 15 days by continuous culture of the cells in 4 μg BrdU/ml. Under these conditions BrdU reverses the rounded, transformed appearance of cells, causing them to have a flat, fibroblastic morphology (Margalith et al., 1975).

Unlike most mouse cell types, mouse myeloma cells cannot be provoked to produce virus by treatment with BrdU. Stewart et al. (1975) has reported that virus production by these cells can be encouraged by halogenated pyrimidines only in the presence of 2% DMSO. However, some of the data to be presented here suggests that the enzymatic activity produced by this treatment of mouse myeloma cells is not a true reverse transcriptase.

Halogenated pyrimidines have also been shown to enhance C type particle production in rat (Klement et al., 1972; Donner et al., 1974; Verwoerd, 1973), cow (Chan et al., 1974) and guinea pig (Rim et al., 1973) cells.

Incorporation of BrdU or 5-Iodo 2'-deoxyuridine (IdU) is a prerequisite to their action as inducers of latent virus (Teich et al., 1973; Ihle et al., 1974; and Greenberger and Aaronson, 1975).

Induction of virus synthesis by BrdU is not limited to RNA viruses. Treatment of human lymphoid cell lines with BrdU results in the production of Epstein-Barr virus (EBV) associated antigens (Gerger, 1972). Studies on synchronized Burkitt Lymphoma cells reveal that, following IdU treatment, there is synthesis of EBV early antigen and viral structural antigen (Hampar et al., 1974).

While the induction of virus synthesis by BrdU and IdU provides an excellent system for studying the molecular events in this process, the theoretical aspects of this phenomena are perhaps even more important. A major controversy in tumor virology has been whether oncogenesis involves the infection of a cell providing it with viral information, or is it the activation of information, possibly the viral genome, which is already present. This latter model suggests that latent viral information, known as a "pro-virus ", exists in all cells whether or not they have undergone malignant transformation. Moreover, evidence for the presence of the pro-virus should even be found in embryo cells. Induction of virus synthesis by halogenated pyrimidines has provided a

probe for the existence of viral information in "non producer" cells. Evidence, as summarized above, is accumulating, suggesting that at least in the mouse, and probably in other species, at least some viral information is present in all cells as part of their genetic endowment.

THE EFFECT OF BrdU ON PHENOTYPIC DIFFERENTIATION

The bulk of research done on BrdU involves studies on its effect on phenotypic expression. In various systems described below the following generalities emerge: 1) BrdU inhibits the expression of tissue-specific differentiated traits; 2) this inhibition does not extend to general "non-luxury" cellular functions such as growth rate, glycolytic enzyme levels, etc; 3) the phenotypic dedifferentiation occurs at concentrations of BrdU which are non-toxic; 4) the effect can involve inhibition of differentiation or dedifferentiation of mature cells. The various systems studied are given in Table 1.

The most extensively studied system of the effects of BrdU on phenotypic expression is the inhibition of the production of chondroitin sulfate by cultured chondrocytes in the presence of the analogue (Abbott and Holtzer, 1968; Shulte-Holthausen et al., 1969). The effect of BrdU on chondrogenesis is prevented by the simultaneous presence of uridine, thymidine (Lasher et al., 1969), cytosine arabanoside or cycloheximide (Mayne et al., 1973).

Table 1

Cell Type	Phenotypic Effect	References
Chondrocytes	↓ Chondroitan Sulfate Synthesis ↑ Surface Area	Abbott, 1968; Shulte Holthausen et al 1969; and Lasher et al, 1969.
Mesenchymal Cells	↓ Chondrogenic differentiation	Levitt et al, 1975.
Amnion Cells	↓ Hyaluronic Acid Synthesis ↑ Surface Area	Bischoff et al, 1968.
Presumptive Myoblasts	↓ Maturation	Stockdale et al, 1961; Coleman et al 1966; and Coleman et al 1969; Rogers et al, 1975.
Post-mitotic Myoblasts and Myotubes	No morphological effect ↑ Ca^{++}-Mg^{++} ATPASE	Bischoff and Holtzer, 1970 Loetzee and Gevers, 1977.
Yolk sac RBC precursors	↓ Erythroid differentiation	Wilt, 1974.
Erythroleukemic cells	↓ DMSO stimulated differentiation	Friend et al, 1971. Scher et al, 1972; Bick, 1977.
Bone marrow cells	↓ Colony stimulating factor induced differentiation	Kinkade, 1974.
Melanoma cells	↓ Pigment production ↑ Surface Area	Silagi and Bruce, 1970; Silagi, 1971.
Embryo pigment cells	↓ Pigment production ↑ Surface area	Zimmerman et al, 1974.
Mammary gland cells	↓ Prolactin Stimulated Casein and Alpha lactal-bumin synthesis	Turkington et al, 1971.
Pancreas	↓ Responsiveness to Inducers of Zymogen granule formation	Wessells, 1964; Walther et al, 1974.
Adrenal tumor cells	↓ Steroidogenesis	Wishnow et al, 1974.
Neuroblastoma	↑ differentiation	Schubert and Jacob, 1970.
Embryo retina	↓ Histogenesis	Morris, 1973.

Cell Type	Phenotypic Effect	References
Glial tumor	↓ Responsiveness to epinephrine	Schwartz et al, 1973.
Newborn cerebellum	↓ myelination	Younkin and Silberberg, 1973.
	↓ enzymatic differentiation	Latovitski and Silberberg, 1975.
Cervical carcinoma	↑ Alkaline phosphatase (not by de Novo synthesis)	Goz and Walker, 1976.
Hepatoma	↓ TAT inducibility	Stellwagen & Tomkins, 1971.
Definitive streak stage chick embryo	↓ brain and somite development	Lee et al, 1974.
Pre-Implantation Mouse embryo	Arrest of development	Golbus and Epstein, 1974
Tobacco plant	↓ Cytokinin dependence	Meins, 1976
Lymph Node	↓ Antibody production	Dutton et al, 1960
Mouse (IN VIVO)	↑ Antibody production	Giswold et al, 1975.
B.subtilis	↓ sporulation	Coote, 1977.

Like chondroitin sulfate, hyaluronic acid production is also inhibited by BrdU (Abbott et al., 1972).

Myogenesis is inhibited by BrdU in a manner similar to the effects of connective tissue cells. BrdU-treated presumptive myoblasts do not progress to become myoblasts and myotubes (Stockdale et al., 1961). This effect is competitively prevented by thymidine (Coleman et al., 1966) and is reversible (Coleman et al., 1969). Deoxycytidine and deoxyuridine reverse the inhibition of myogenesis by BrdU without affecting its incorporation into the genome.

Several studies on the effect of BrdU on phenotypic differentiation involve its modification of erythroid development. Embryonic erythroid differentiation is inhibited by BrdU (Wilt, 1974).

The most useful systems for studying erythroid differentiation are murine virus induced erythroleukemic cell lines. In these cells BrdU suppresses DMSO-induced erythropoiesis (Friend et al., 1971). Thymidine, but not uridine, prevents its effect (Scher et al., 1972). The susceptibility of various clones to BrdU-induced dedifferentiation correlates directly with their ability to incorporate BrdU into DNA (Ostertag et al., 1973). BrdU acts by inhibiting the induction by DMSO of hemoglobin mRNA (Priesler et al., 1973).

Accompanying the DMSO-induced differentiation is a

transformation of Friend cells to an erythropoietin responsive state (Priesler and Zanjani, 1974).

There has been a large body of research done on the dedifferentiation of melanin producing cells (melanocytes) by BrdU. Silagi and Bruce (1970) have shown that pigment production by mouse melanoma cells is dramatically reduced by treatment with BrdU. Coincident with this is a flattening of the cells. Changing to a BrdU-free medium causes the cells to again synthesize melanin (Silagi, 1971). In these cells tyrosinease shows progressively decreasing activity during BrdU treatment. Part of this is attributed to the appearance of a dialyzable tyrosinease inhibitor (Wrathall et al., 1973).

Studies with chick embryo pigment cells yield results which are fundamentally the same as those described above for melanoma cells (Zimmerman et al., 1974). Turkington et al. (1971) have provided evidence that mammary gland organ cultured in the presence of BrdU cannot be induced by prolactin to produce casein and α-lactalbumin, as can control tissue.

Wessells (1974) has shown that treatment of embryonic pancreatic cells with BrdU before the point when they stop dividing results in a non-responsiveness to inducers of zymogen granule formation. Walther et al. (1974) have shown

that although the rate of DNA, RNA and protein synthesis is unaffected by BrdU, that of chymotrypsin, amylase, ribonuclease and procarboxypeptidase A and B production is sharply reduced. rRNA synthesis is unaffected by BrdU. No effect on the synthesis of phospholipids, sphingolipids or various other glycoproteins were observed. There is also no effect of BrdU on acetate incorporation into steroid or fatty acids. The BrdU effects are prevented by thymidine which causes a decrease incorporation of BrdU into DNA. The effect of BrdU on pancreatic rudiments is reversible following transfer to BrdU-free medium.

Incubation of mouse adrenal tumor cells with BrdU causes an inhibition in both basal and ACTH-stimulated steroidogenesis. The inhibition is reversible and is inhibited by thymidine. Incorporation of BrdU into DNA appears to be necessary for its effect on steroid synthesis. General adrenal cellular RNA and protein synthesis is relatively unaffected by BrdU (Wishnow et al., 1974).

Neuroblastoma cells, unlike the various cell types previously described, can be induced to become more specifically differentiated by treatment with BrdU (Schubert and Jacob, 1970). In this study it is concluded that incorporation into the genome is not a necessary pre-requisite for induction of neuroblastoma differentiation by BrdU since

it occurs in the presence of BrdU and cytosine arabinoside or mitomycin C. However, it is now known that inhibition of DNA synthesis can cause differentiation of cultured neuroblastoma cells (Prasad and Kumar, 1974).

Non-transformed neural cells are affected by BrdU in ways similar to that described above for neuroblastoma cells. BrdU irreversibly inhibits histogenesis in embryonic chick neural retina cells. Glutamine synthetase, a marker enzyme for differentiation in this tissue, cannot be induced in BrdU treated cells. These effects of BrdU are irreversible, but they can be prevented by the simultaneous addition of excess thymidine (Morris, 1973). BrdU decreases the elevation of cAMP by norepinephrine in glial tumor cells (Schwartz et al., 1973). 45μg BrdU/ml inhibits myelination of the developing cerebellum, although morphologically the oligodendrocytes appear normal (Younkin and Silberberg, 1973).

BrdU's effect on enzyme induction in the well characterized rat hepatoma (HTC) cells has also been studied. The synthesis of the inducible enzyme tyrosine aminotransferase (TAT) is dramatically reduced when these cells are grown in BrdU. The rates of cell growth and general protein and RNA synthesis are unaffected by the analogue. The effect of BrdU on TAT are counteracted by the addition of thymidine (Stellwagen and Tomkins, 1971).

There have been several studies on the effect of BrdU on lymphoid cells. BrdU inhibits antibody production in vitro by these cells. This inhibition occurs without any effect on the rate of protein or RNA synthesis (Dutton et al., 1960). Paradoxically, IdU stimulates the response of mice to injected sheep erythrocytes (Griswold et al., 1975).

MODULATION OF ONCOGENICITY BY BrdU

The earliest, and probably the best studied system, of the inhibition of oncogenicity by BrdU is cultured melanoma cells. Mouse melanoma cells grown in BrdU flatten out and become a contact inhibited monolayer. When injected into the appropriate host mouse they do not give rise to a tumor (Silagi and Bruce, 1970). When cultured in BrdU-free media the tumorigenicity of these cultures gradually returns. Mice injected with BrdU-treated melanoma cells are immune to later challenge with tumorigenic melanoma cells (Silagi, 1971). It has been suggested (Silagi et al., 1972) that the immunity conferred by BrdU treated cells is due to a high concentration of surface viral antigens. The reduction in tumorigenicity of mouse melanoma cells by BrdU also correlates with a decrease in detectable cellular plasminogen activator (Christman et al., 1975).

Murine neuroblastoma and adrenal cortical cells also show a decrease in tumorigenicity when grown in BrdU

containing media (Silagi, 1971). Associated with this phenomenon is the expression of a "differentiation antigen" on the surface of these cells (Brown, 1971).

As described in the section on phenotypic effects of BrdU, there is an extensive body of knowledge relating to the effect of BrdU on Friend erythroleukemic cells. DMSO treated Friend cells give rise to less malignant tumors than control cells. This is correlated with the DMSO induced differentiation of these cells which is reversed by BrdU (Friend et al., 1971).

Chronic treatment of virus transformed "non-producer" BALB/3T3 cells with BrdU causes them to produce oncornoviral particles continuously. During this treatment the rounded, transformed appearance of the cells changed to a flat fibroblastic morphology, similar to the parent 3T3 line (Margalith et al., 1975).

When cells are transformed by the DNA tumor virus SV-40 a characteristic nuclear antigen, known as the T antigen, appears. BrdU treatment of SV-40 transformed cells causes a suppression of this "transformation" antigen as measured both by complement fixation and immunofluorescence.

SV-40 transformed cells deficient in thymidine kinase (TK^-) show a decrease in tumorigenicity when cultured in the presence of BrdU. However, these cells still contain BrdU-substituted DNA, presumably due to low levels of

residual TK activity. Nevertheless, substitution of BrdU into DNA may not be significantly related to the decreased oncogenicity of SV-40 transformed cells since growth of these cultures in BrdU free media until no analogue could be detected in DNA by density gradient analysis does not result in a full return of normal tumorigenicity. In fact, TK^- cell lines that have never been treated with BrdU are also less tumorigenic (Rothschild and Black, 1973), suggesting that this enzyme may play a role in the BrdU sensitive modulation of malignant potential.

Polyoma virus transformed cells have a sharply reduced oncogenicity when cultured with BrdU. Associated with this decrease in oncogenicity is a reduced ability to form colonies on either soft-agar or on monolayers of non-transformed cells (Grady and North, 1974).

A Syrian hamster malanoma cell line with a mutation called "BrdU dependence" requires the analogue for optimum growth rate and can substitute 100% of its thymidine by BrdU without any ill effects. Paradoxically, this line is contact inhibited only in the absence of BrdU. Correlated with the contact inhibited state is a transition from rounded, typically transformed cells, to spindle shaped melanocytes. In addition to being contact inhibited, these cells, when grown in the absence of BrdU, have a

higher serum requirement, grow less well in soft agar, and are less agglutinable by wheat germ agglutinin than cultures grown in BrdU. Thus, by several criteria these cells appear to not display characteristics of the transformed state in the absence of BrdU. However, the dependent cells grown in BrdU are much less tumorigenic than those grown in the absence of the analogue (Horn and Davidson, 1975).

Attempts have been made to assess the effect of BrdU on myeloma cells. These have been severely hampered by the extreme sensitivity of these cells to BrdU. When this has been controlled for by calculating the percentage of viable cells injected it appears that their oncogenicity may be reduced (Pettengill and Sorenson, 1974). However, interpretation of these results is made difficult by the large correction made necessary due to the excessive toxicity and the uncertain fate of "viable" cells. Our own studies on this subject will be presented.

MECHANISM OF ACTION OF BrdU

The preceding literature review highlights the many biological properties of BrdU. Although these have been studied, catalogued and characterized, there is a great deal of conflict surrounding the question of its mechanism of action.

BrdU has been shown to substitute for DNA thymidine in all natural systems studied. The earliest studies, for example those of Zamenhof and Griboff (1954) and Zamenhof (1959), showed that this substitution was highest in thymine requiring strains of E.coli in which there was no dilution of the BrdU-thymidine pool by endogenous thymidine synthesis. Similarly, aminopterin, which blocks cellular thymidine synthesis, increases BrdU substitution into DNA.

Studies on mammalian cells have revealed the same general pattern of substitution by BrdU for thymidine as described above for bacteria. Eidenoff and his co-workers (1959) have shown that when human cervical carcinoma H.ep.1 cells are incubated with 50μg BrdU/ml in the presence of aminopterin, nearly half of the thymidine in the DNA is replaced by the BrdU. That the actual moiety going into the DNA is bromouradylate has been shown by the identification of the hydrolysis products of DNA from cells chronically grown in BrdU-containing media. The molar amount of BrdU plus thymidine is equivalent to the amount of DNA adenine (Littlefield and Gould, 1960).

There is considerable interest in the possibility of regional localization of BrdU in DNA. Such a heterogeneous distribution might explain why the phenotypic effects of BrdU are so selective for non-essential functions. However,

human cell line D98/AG, after having been grown in BrdU for over a year, had a homogeneous substitution of BrdU for thymidine in its DNA, as judged by $CsCl_2$ ultracentrifugation (Djordjevic and Szybalski, 1960). It is possible that the effects of BrdU are not the result of its incorporation into DNA. Strong evidence that BU-DNA is fully functional comes from the demonstration by Szybalski that Bacillus subtilis DNA, in which nearly 100% of the thymidine has been replaced by BrdU, has a specific transforming ability indistinguishable from that of unsubstituted DNA (Szybalski et al., 1960). Further evidence for a possible extragenomic action of BrdU comes from the data of Hsu and Somers (1962) on BrdU-resistant mouse L cells, which heavily incorporate the analogue into DNA despite insensitivity to its effects. Similarly, Zamenhof et al. (1956) could not find a correlation between the amount of IdU incorporated into DNA and its effect when the analogue was used in sufficiently high concentrations to cause inhibition of growth.

In contrast to the studies just described are a series of experiments suggesting that incorporation into the genome is a necessary prerequisite for at least some of BrdU's actions. For example, some studies on the effect of BrdU on CS synthesis in chondrocytes show a requirement for cell proliferation for the phenotypic effect of the analogue (Mayne et al., 1973).

While the exact level at which BrdU exerts its various effects is controversial, one generality which emerges is its competitive relationship with thymidine. In the many systems described in earlier sections of the Introduction, thymidine competitively prevents the effects of BrdU.

Several enzymatic processes have been implicated as part of the locus of action of BrdU. A study by Prusoff et al. on a variety of cell types, revealed that when incubated with iododeoxyuridine, the rate limiting enzyme for thymidine utilization varies. It is possible that the inhibitory effects are due to the analogue acting similar to thymidine phosphates, which are known to be physiological inhibitors of several enzymatic reactions. For example, thymidine triphosphate (dTTP) has been shown to inhibit, among other enzymes, thymidine kinase (Ives et al., 1962).

One of the most interesting metabolic effects of BrdU is its inhibition of deoxypyrimidine production. dTTP is a natural negative allosteric effector of ribonucleotide reductase (Reichard et al., 1960). dBUTP, an analogue of dTTP, is a very potent inhibitor of this enzyme causing a "deoxypyrimidineless state" (Meuth and Green, 1974). These authors have reported that 3T6 mouse fibroblasts can be grown in approximately 50μg BrdU/ml without any effect on

viability provided that an equivalent amount of deoxycytidine was also present in the growth media. This protection occurs despite extensive incorporation of BrdU into DNA. This explains the early reports that deoxycytidine improves growth of human sternal marrow cells in the presence of BrdU (Szybalski, 1961), and L5178Y cells in the presence of thymidine (Morris and Fischer, 1960). The possibility that the toxic effect of high concentrations of BrdU may be due to inhibition of deoxypyrimidine synthesis explains why the BrdU-resistant cells often incorporate high levels of BrdU into the genome without any evidence of toxicity (Hsu and Somers, 1962). However, these studies do not prove that the protection by deoxycytidine is due to circumventing the block of ribonucleotide reductase. It is unlikely that ribonucleotide reductase inhibition can explain all the effects of thymidine or BrdU on cell proliferation or phenotypic expression, since several of these phenomena are not prevented by the simultaneous presence of deoxycytidine (for example, Kelman et al., 1975).

BU-DNA exhibits some alteration in physical properties, both as free nucleic acid and as part of chromatin. Radiosensitization has been described earlier. Additionally, halogen substituted DNA has heightened sensitivity to hydrodynamic shear, and an increased melting temperature

(Tm) (Szybalski, 1961). The increased Tm of BU-DNA is also observed in BU-substituted chromatin (Simpson and Seale, 1974; Augenlicht et al., 1974). This thermostability approaches that of condensed chromatin. The increased Tm of BU-chromatin is reduced to control values by 1% DMSO, suggesting that this may be related to the inhibition of DMSO effects by BrdU (Bekhor and Lapeyre, 1975). BU-DNA is more sensitive to thermal degradation than is unsubstituted DNA (Szybalski, 1961). BrdU has been reported to cause alterations in chromosomal structure. Hsu and Somers (1961) have found chromatin abnormalities in mouse L cells. BrdU-resistant clones of L cells do not show these chromosome breaks when cultured in the presence of the analogue, despite extensive incorporation of it into DNA (Hsu and Somers, 1962).

Growth of HeLa cells in BrdU does not affect the amount or composition of chromosomal proteins (Simpson and Seale, 1974). In a similar study, Case et al. (1975) found that BrdU does not alter the synthesis of DNA binding proteins.

Circular dichromism (CD) studies on both free DNA and chromatin reveals conformational differences between control and BU-substituted samples (Simpson and Seale, 1974; Augenlicht et al., 1974). The alteration of elipticity in the BU-chromatin is such as to suggest that it is conformationally

similar to condensed chromatin. DMSO tends to nullify these changes (Bekhor and Lapeyre, 1975), suggesting that conformational changes may be responsible for the opposite effects of DMSO and BrdU in many systems.

Studies by Hill and Baserga (1975) have indicated that BU-chromatin causes a relative increase in GMP and AMP incorporation into RNA transcribed in vitro. Based on this data Baserga has hypothesized that altered template properties of BU-chromatin are related to conformational changes as evidences by CD measurements.

Lin and Riggs (1972) have approached the question of the ability of BU-DNA to serve as a template for inducible RNA synthesis by studying the binding of the lac repressor to BU-DNA. While poly (d(A-T) binds the represser, Poly (dA-BU) binds it 40 times more effectively. Thus, they speculate, BrdU may modulate specific RNA synthesis by tight binding of regulatory proteins by BU-DNA. In a more physiologically meaningful experiment they have shown that BU-substituted lac operator binds the lac repressor 10 times tighter than normal lac operator DNA (Lin and Riggs, 1974). Similarly, histones bind BU-DNA more strongly than unsubstituted DNA (Lin et al., 1976).

One widely quoted property of BrdU is its ability to acts as a mutagen. However, an anlysis of the actual data reveals that while it is mutagenic in certain circumstances,

this is not a term that should be used in a general sense.

Phage T_4 is sensitive to the mutagenic effects of BrdU (Litman and Pardee, 1956). These BrdU-induced mutations are probably point mutations since they are easily reverted. The mechanism of this mutagenesis is suggested to be a mistake in base pairing with BU since the electonegative bromine atom would increase the likelyhood of the base being in the less favored enol form which might then base pair with guanine. The net result of this would be a genetic "transition", that is, a point mutation arising by replacement of a purine with a purine or a pyrimidine (Freese, 1959).

Rudner (1960) has examined the question of BrdU induced transitions by studying the reversion of Salmonella typhimurium from tryptophan dependence (Try^-) to independence (Try^+). Mutants to prototrophy appear only after a round of DNA synthesis is permitted. Stable mutants require 3 rounds of replication. A model is presented in which the first round of DNA synthesis allows BU incorporation; mispairing occurs during the second round; and a stable transition occurring during the third round when the mispaired guanine templates for cytosine in the new strand of DNA being synthesized. The net results being a transition from (T-A) to (C-G).

It has been suggested that ionization of a base in DNA,

rather than tautomerism, may play a role in point transitional mutagenesis. Figure 2 shows the possible base pairing of ionized BU with guanine, in which 2 hydrogen bonds are formed without distortion of the DNA helix, compared to the one hydrogen bond that would form between ionized BU and adenine. That this may be the case is suggested by the finding that the pKa of BrdU is 8.1, compared to 9.8 for thymidine (Lawley and Brookes, 1962). Others such as Zamenhof (1959), showed that the mutation frequency in bacteria grown in high concentrations of BrdU was very high. The sensitivity of bacteria to BrdU mutagenesis depends largely on their ability to remove mismatched bases (Rydberg,1977).

While high concentrations of BrdU are mutagenic to bacteria and their phages, the situation with mammalian cells is very different. As described earlier, Szybalski (1961) has grown human sternal marrow cells to very high replacement of thymidine by BrdU without any toxic effect as would be expected if the analogue was acting as a mutagen. More dramatically, Bick and Davidson (1974) have isolated a BrdU dependent Syrian hamster melanoma cell line that grows chronically with almost complete replacement of DNA thymidine with BrdU without any greater mutation frequency than would be expected from spontaneous basal mutagenesis. Additionally, it is unlikely that the phenotypic effects of

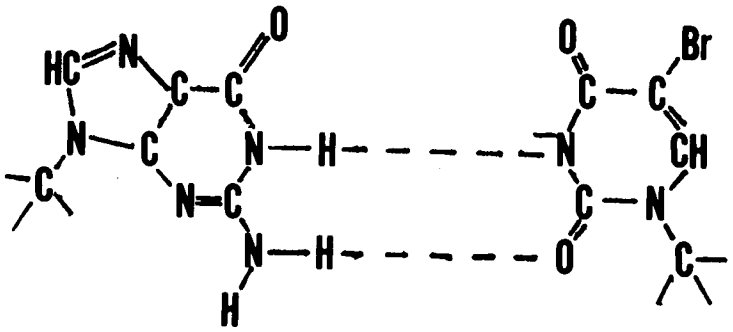


Figure 2

Base pairing of ionized bromoracil with guanine.

BrdU are due to mutagenesis since most of them have been shown to be completely reversible, as reviewed earlier. In addition, when looked for, no altered proteins could be detected following BrdU treatment (Walther et al., 1974).

It has been suggested that the effects of BrdU could be due to selection and enrichment of pre-existing cells in the population. Freese (1959) showed that this was not the case in his study of r_{II} mutants of phage by the fluctuation test. Similarly, in a clonal study of muscle cell differentiation (Coleman et al., 1969), it was shown that individual clones can recover from BrdU induced suppression of differentiation. Essentially, all the clones observed recovered. In general, the ease of reversibility of the phenotypic effects of BrdU make selection a very unlikely explanation.

CYTOTOXIC EFFECTS OF BrdU

The last property of BrdU to be considered is its cytotoxic effect. As shall be shown these are very dramatic in cell lines originating from malignant lymphoid diseases (myelomas, lymphomas, and leukemias).

The earliest studies of the toxic properties of BrdU (Cohen and Barner, 1956) showed that while E. coli can grow normally in 500μg BrdU/ml, thymine requiring strains can

be killed by 3μg/ml. BU has little effect on mammalian cells, but the deoxyriboncleoside BrdU, in sufficient concentration is toxic to these cells. Hakala (1959) has shown that a variety of human cell lines grown in BrdU plus aminopterin are killed at concentrations of the analogue ranging from 3-10μg BrdU/ml. Similar results on BrdU toxicity at higher analogue concentrations in the absence of aminopterin were simultaneously published by Littlefield and Gould (1960), and Cheong, Rich and Eidinoff (1960).

BrdU is rapidly phosphorylated when it enters the cell. Cells resistant to the cytotoxic effects of BrdU are often deficient in thymidine kinase (TK^-).

Haploid frog cell lines adapted to growth in gradually increased doses of BrdU show a two-step pattern of resistance. At low concentrations of the analogue a transport deficiency occurs, which upon exposure to higher concentration becomes supplemented by a TK^- mutation (Freed and Mezger-Freed, 1973).

It has been suggested that chromosomes, abnormalities may be associated with BrdU cytotoxicity. Hsu and Somers (1961) found that treatment of mouse L cells with BrdU causes sister chromatid exchanges. Similar results have been reported by others (Ikushima and Wolf, 1974).

One mechanism postulated for BrdU toxicity independent of an effect at the level of DNA is the inhibition of

ribonucleotide reductase described earlier. This hypothesis derives from the work of Morris and Fischer (1960) who showed that deoxycytidine prevents the inhibition by thymidine of mouse cell lines. Similarly, some of the phenotypic effects of BrdU are reversed by deoxycytidine or deoxyuridine (reviewed earlier). Meuth and Green (1974) have shown that dBUTP is a powerful negative allosteric effector of ribonucleotide reductase and "can therefore kill cells by starving them for deoxycytidine nucleotides."

Several other enzymatic processes have been implicated as being affected by BrdU and thus may play a role in its cytotoxic effects (Delamore and Prusoff, 1962; Kim et al., 1967; Prusoff and Chang, 1968). However, other than ribonucleotide reductase, no enzyme has been found to be inhibited by BrdU in more than just one or a few closely related cell types.

The previously described studies of Mezger-Freed on the emergence of BrdU-resistance in frog haploid cells yields some possibly important insights for the present study. Using the haploid system she can unveil recessive mutants that would be masked by multiploidy. Noting the high frequency of emergence of BrdU resistance through a decrease in membrane transport Mezger-Freed has suggested a non-genetic origin of this state, since the rates of alteration

to BrdU resistance are similar in haploid and pseudodiploid lines. These results are postulated to be due to effects on cell differentiation rather than mutation (Mezger-Freed, 1972). An alternative explanation is a BrdU resistant dominant mutation which would be expressed independent of ploidy. However, this does not explain the unexpectedly high frequency of appearance of BrdU resistant cells. In addition, chemical mutagenesis does not increase the frequency of emergence of resistant cells in this population.

BrdU-resistant plant cells have been isolated and crossed with parental wild type cells sensitive to 30μg BrdU/ml. The distribution of phenotypes produced by these crosses suggest that BrdU resistance in these cells is determined by a simple Mendelian factor (Marton and Maliga, 1975).

The work to be presented shows that malignant lymphoid cell lines are unusually sensitive to the cytotoxic effects of BrdU compared to most other cell types. One other general class of BrdU sensitive tissues are those in embryos. Several studies on these tissues reveal that they are sensitive to the cytotoxic effects of BrdU at concentrations of the analogue not generally harmful to other cells (Karnofsky and Basch, 1960; Gontcharoff and Mazia, 1967; Tencer and Brachet, 1973; Rizkiet al., 1972; Skalko et al., 1971

Ruffolo and Ferm, 1965; Garner, 1974; Sherman and Biatlevza, 1975).

The cytotoxic effects of BrdU have been attempted to be utilized in the treatment of various conditions. Since the present work involves malignant lymphoid cells, prior studies with BrdU in the treatment of these diseases will be reviewed. The earliest paper studying the effect of BrdU on malignant lymphoid cells reported that when mice bearing lymphomas were treated with BrdU there was a reduced incorporation of ^{14}C-formaldehyde or ^{14}C-formate into DNA. Associated with this was a slight increase in the survival time of these mice (Kit et al., 1958). Shortly after this was published Prusoff noted (Mathias et al., 1959), as part of his investigation of thymidine metabolism in murine lymphomas, that IdU immediately killed these cells. More recently Pettengill and Sorenson (1974) have reported the unusual BrdU sensitivity of one line of mouse myeloma cells. T lymphoma cells are known to be killed in vitro by thymidine (Ralph et al., 1973). However, it is unclear whether this is related to the very dramatic sensitivity of lymphoma and myeloma cell to BrdU that will be described in the Results section, since myeloma cells do not appear to be affected by thymidine.

In a National Cancer Inst. study in which no data was

given BrdU was shown to be "active" against chronic myelocytic leukemia (Slavik, 1975). In another study Papac et al. (1962) presented evidence to show that very low doses of IdU can cause responses in several kinds of tumors including leukemias and myelomas, including one patient with acute leukemia who showed a very dramatic response after just one course of therapy.

There have been several attempts to use the radiosensitizing properties of BrdU in conjunction with radiotherapy in the treatment of solid tumors (Bagshaw et al., 1967; Stone, 1974).

BrdU, and in congener IdU, have found application in anti-viral chemotherapy (Haynes et al., 1973; Pavan-Langston; 1975; Pavan-Langston et al., 1975; Martenet, 1975) and for herpes zoster (Simpson, 1975).

THE PRESENT WORK

The work described herein involves a study of the unusually dramatic cytotoxicity of low concentrations of BrdU to malignant lymphoid cells. Specifically, the following questions were considered:

1) is BrdU more cytotoxic to malignant lymphoid cells than it is to other cell types?
2) is this cytotoxicity effected by hormonal manipulation?
3) does BrdU also cause an alteration in the malignancy of

these cells?

4) is BrdU's cytotoxic effect to these cells related to viral activation?

5) how is BrdU handled by these cells?

   a) what is the mechanism of entry of BrdU into the cells?

   b) what happens to it once it gets into the cells?

6) what is the difference between BrdU sensitive malignant lymphoid cells and BrdU-resistant clones of these cells?

7) is this dramatic toxic effect of BrdU related an inhibition of ribonucleotide reductase?

8) Can the sensitivity of these cells to BrdU form the basis for an approach to chemotherapy of malignant lymphoid disease?

MATERIALS AND METHODS

CELL LINES

The characteristics and histories of the cell lines used in this study are given below.

MOPC 315

MOPC 315 is a murine myeloma that was given to our laboratory in 1972 by Dr. H. Eisen. We have maintained it in continuous culture since 1973.

MPC 11

MPC 11 is a murine myeloma that has been in continuous culture in our laboratory since its receipt from Dr. M. Scharff in 1972.

CCRF-CEM

CCRF-CEM is a lymphoblastoid cell line established in 1964 by Dr. G. E. Foley (Foley et al., 1965), from the buffy coat of the peripheral blood of a four year old Caucasian female with acute lymphoblastic leukemia.

S49.1

S49.1 is a theta-positive lymphoma (Harris, 1970) was received from the Salk Institute in 1974, and has been in continuous culture in our laboratory ever since.

S49.1TB.2

S49.1TB.2 is a clone of S49.1 selected for growth in 30μg BrdU/ml. It was cloned from a mutagenized population

on soft BrdU-containing agar.

EL4

EL4 is a C57BL murine lymphoma (Gorer, 1950) which carries the theta antigen (Boylston, 1973).

EL4BU

EL4BU is a BrdU-resistant clone of EL4 which was received in our laboratory in 1975.

L929

L929 was established by Dr. W. R. Earl et al. (Sanford et al., 1948) from mouse L cells in their 95th subculture generation (Earl, 1943).

RAG

RAG is a clone from BALB/c renal adenocarcinoma established by Drs. Ruddle, Kleb and Chen (Kleb et al., 1970).

HeLa

HeLa is a cell line derived from human cervical carcioma in February 1951 (Gey et al., 1952).

MMT

MMT is from a spontaneous mammary carcinoma in a C57BL x AF F_1 hybrid female mouse. It was originally cultured by Dr. D. J. Sykes in 1962.

CULTURE TECHNIQUES

All cell lines were carried in 75 cm^2 Falcon tissue culture flasks with a gas phase of 5% CO_2/95% air. Suspension cultures were routinely seeded at 0.8 to 1.0 x 10^5 cells/ml, in 10 ml of media, and diluted down to that level 2 or 3 times a week. When cells were being prepared for experimental use they were grown in 20 ml media/flask. Once every week the cells were reseeded in 10 ml of media in fresh flasks. Adherent cell lines were removed from the plastic vessels once a week with 5 ml proteolytic enzyme solution. 0.2ml of the resulting suspension was reseeded into fresh flasks containing 10 ml of media. The removal of the cells from the plastic was accomplished by shaking at 37°C with 0.25% trypsin in Hanks' balanced salt solution for all cells except RAG, which required 0.25% Pronase (Calbiochem, San Diego, California) for quantitative recovery of the cells. In addition to these passages the media above the adherent cultures was changed one or two times per week.

The growth media used for the various cell lines is given in Table 2.

SURVEILLANCE AGAINST MYCOPLASMA

Surveillance against mycoplasmal contamination of the cultures used in this study was carried out by three techniques: direct culture from the growth media (Hayflick, 1965),

Table 2

Growth Media Used in the Cultivation of
Cell Lines Used in These Studies

Line	Media	Serum
MOPC 315	D'MEM	20% H.I. Horse
MPC 11	D'MEM	20% H.I. Horse
CCRF/CEM	D'MEM	20% Fetal Calf
S49.1	D'MEM	20% H.I. Horse
S49.1TB.2	D'MEM	20% H.I. Horse
EL4	D'MEM	20% H.I. Horse
EL4 BU	D'MEM	20% H.I. Horse
L929	MEM	10% Fetal Calf
HeLa	MEM	10% Human
MMT	MEM	10% Calf

H.I. (heat inactivated) indicate that the serum was heated for 30 minutes at 56°C prior to use.

electron microscopy (Anderson et al., 1965), and uridine phosphorylase assay (Levine, 1972).

FREEZING AND DEFROSTING OF CELL LINES

Samples of all lines carried in culture were frozen every 60 days and stored in both a -90°C deep freeze and in liquid nitorgen (-197°C).

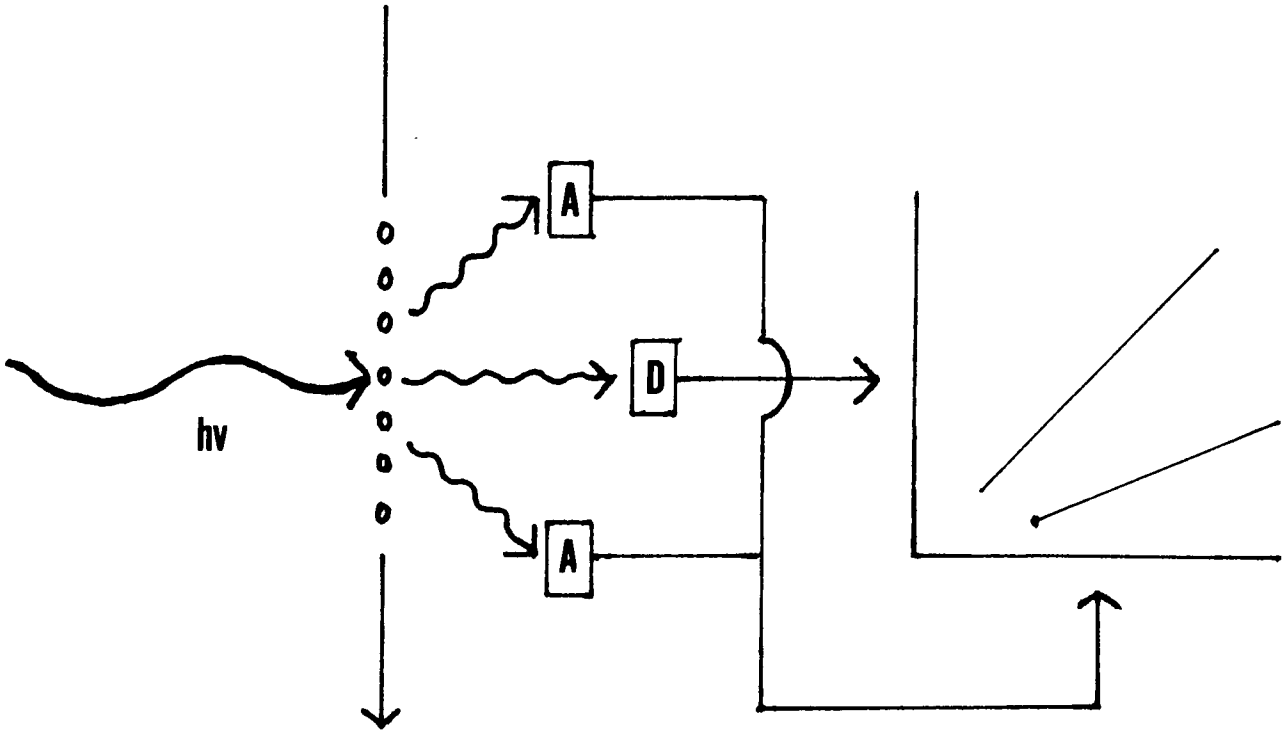
CELL QUANTITATION

Cell counting was done with either a hemocytometer or a Biophysics 6300A Cytograf. Viability was determined by using the trypan blue exclusion test. The technique for cell counting by hemocytometer is given by Absher (1973).

Most of the cell enumeration data in this study was obtained with the Cytograf 6300A. This instrument focuses a laser-light beam on a jet propelled stream of cells and detects scattering with the use of a direct sensor, 180° from the laser source, and two angle sensors, as illustrated in Figure 3. The output of the sensing devices for a cell population is a series of points with a constant direct to angle signal ratio, the magnitude of each signal being proportional to the size of the scattering particles. Uptake of trypan blue changes the signal ratio, thereby separating the electrical output into two populations, one excluding and one including the dye. Hence, two lines which can be electronically discriminated are separated on

Figure 3

Diagrammatic representation of the conversion of sensor signals to an oscilloscope output. Laser light is incident on a stream of cells and is both transmitted and scattered. Cells containing trypan blue have reduced scattering properties, thus reducing the ratio of input into the angle (A) detectors to the direct (D) detectors. The trypan blue accepting cell population therefore gives a second line of lower slope than that of the dye excluding cells.


A
D
A
hv

a monitor. By setting the electronic discriminator to differentiate between the two populations it is possible to count the total cell number in the 0.1ml sample, and the proportion of cells which are trypan blue including.

Figure 4 shows the correlation between cell counts made by hemocytometer verses those made by cytography.

Cell counts on the Cytograf are made by filtering 200μl of cells with 1.0 ml of 0.05% freshly filtered trypan blue in Hank's balanced salt solution or physiological saline to give a final trypan blue concentration of 0.04%. In order to prevent coincidence error in counting, if there are anticipated to be more than 4×10^5 cells/ml in diluted sample, the original cell suspension is diluted further before it is aliquoted into the 0.5% trypan blue.

RADIOISOTOPIC STUDIES ON WHOLE CELL POPULATIONS

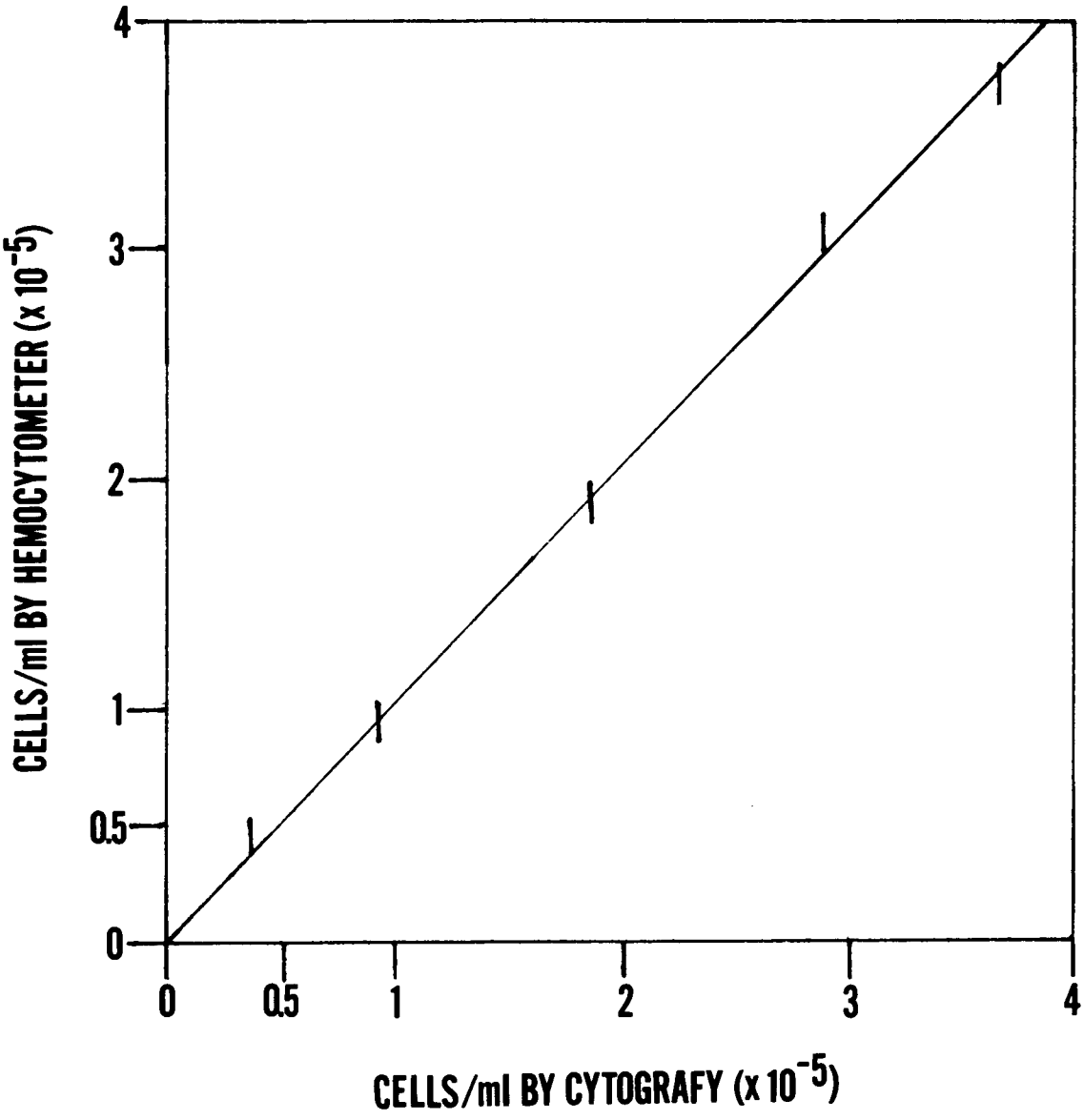
General Consideration

The protocol used in these studies was developed in order to accomplish the following goals: 1) wash the cells free of external radioactivity with a minimum loss of cell or intracellular material, 2) quantitate intracellular nucleoside in both free and polymeric forms. These goals were fulfilled by determining the conditions for each step in the assay.

The general protocol used in the experiments herein is

Figure 4

Comparison between cell counts obtained with a hemocytometer and those obtained by a Biophysics Cytograf 6300 A. Counts were made on aliqouts of the same cell suspensions.


4
3
2
1
0.5
0
0
0.5
1
2
3
4
CELLS/ml BY HEMOCYTOMETER (x 10^{-5})
CELLS/ml BY CYTOGRAFY (x 10^{-5})

described in Figure 5. The data upon which the design of this procedure rests are given in the Results section.

Liquid Scintillation Counting

Experiments involving the quantitation of radioactivity were done using a Packard 3380 Liquid Scintillation Spectrometer. Generally, 100μl of aqueous solution to be counted was solubilized in 10ml of either Triton-X toluene, Bray's solution, or ACS^{tm}.

CHROMATOGRAPHIC STUDIES

Thin-layer Chromatography

Nucleoside and nucleoside derivatives were separated and identified by thin-layer chromatographic techniques employing a variety of resins and solvents. The choice of the chromatographic systems for each experiment was determined by selecting those with strong capabilities of resolving the particular compounds of interest. Table 3 gives the various resins and solvents used in these studies. Chromatographic identity was considered to be established when the substance of interest could be identified unambiguously in at least three different thin-layer systems capable of resolving compounds closely related to the one being studied.

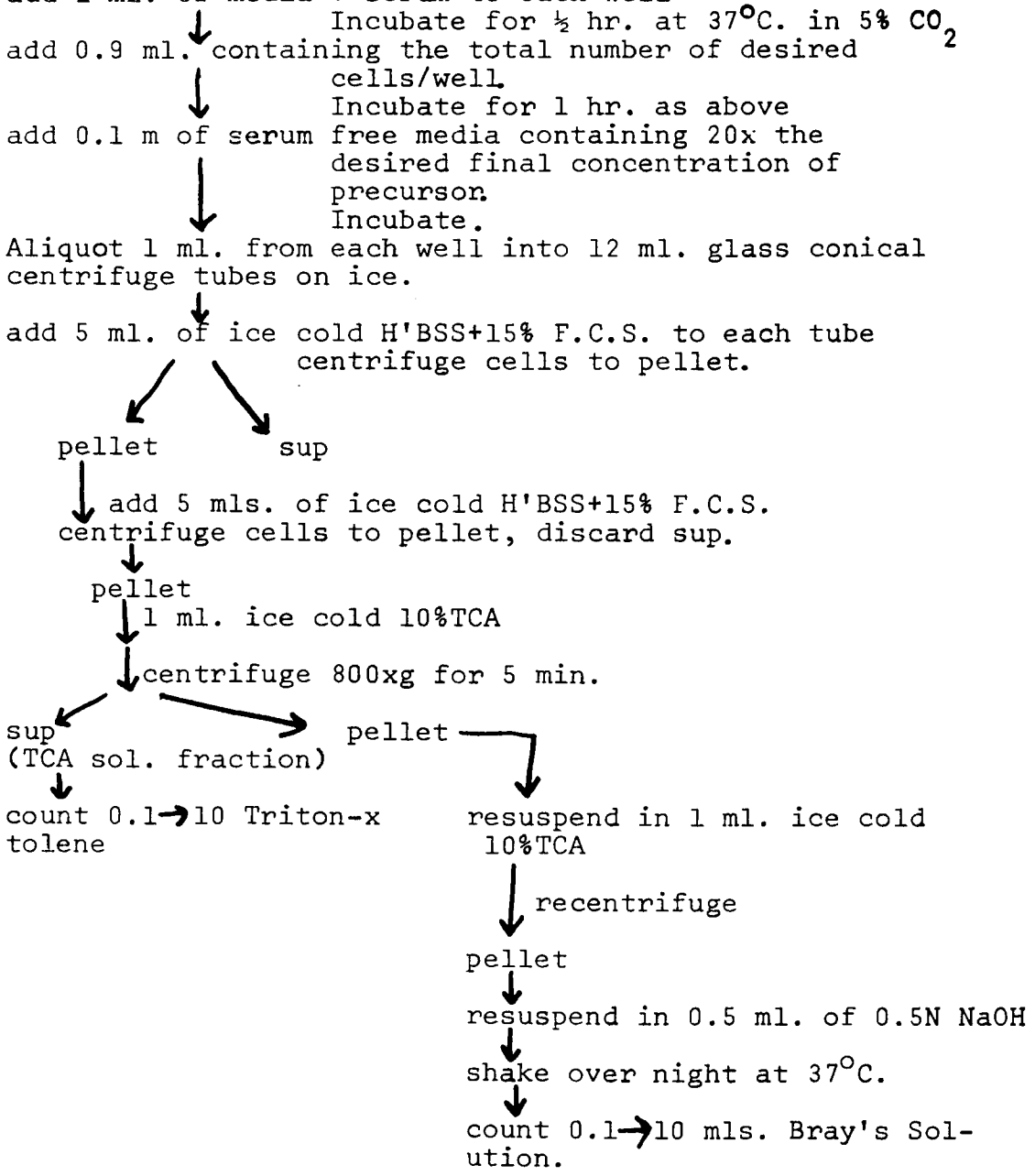
Spotting and Running Thin-Layer Chromatograms: If the samples were applied directly to the resin they were spotted

Table 3 Thin Layer Chromatographic Resins and Solvent Systems Used in These Studies

Resins	Solvent Systems
Silica	Water
Cellulose	1M LiCl
DEAE - Cellulose	Acetonitrile 10:n-Bu-OH60:0.1M NH_4 AcO 20: 28% NH_4OH 10
Polyethylenimine-Cellulose (PEI)	Acetonitrile 30:n-Bu-OH40:0.1M NH_4 AcO 20: 28% NH_4OH 10
	40% n-Butanol
	80% isopropanol, 10^{-3} M EDTA
	2M Ammonium formate, pH 4.4
	n-Bu-OH 2: Acetic Acid 1: Water 1
	1M LiCl, 0.5M Ammonium sulfate
	1M LiCl, 1M Ammonium formate, pH 4.4

Figure 5

GENERAL PROTOCOL FOR RADIO-ISOTOPIC STUDIES



on X's marked with a No. 2 lead pencil 2 cm above the base of the plate. Different samples were spotted at least 1.5 cm from each other. One μl volumes were spotted successively.

If the thin-layer plate had a pre-absorbant area, the samples were spotted directly on these in 5 μl aliquots. Thin-layer chromatograms were run until the solvent front reached approximately 3/4 of the way of the plate. All thin-layer plates used in this study were 20 cm long.

Locating Substances on Thin-Layer Chromatographic Plates

The location of compounds on the thin-layer plate was determined either by ultraviolet absorption, by direct radioscanning, or by liquid scintillation counting of eluted 2 mm fractions of the plate. The eluteants used are given in Table 4.

Gel Chromatography

Gel chromatography of TCA soluble nucleotides was accomplished with the use of 46 x 0.9 cm BioGel P-2 (Bio-Rad) column. The resin was hydrated in water and "fines" were repeatedly discarded by decantation. The column was prepared

Table 4 Solvents Used to Elute Nucleosides and Nucleotides From Thin Layer Chromatographic Plates

Species	Elutriants	Resin	% recovery
Nucleosides	Methanol	PEI	96%
	Randerath's Elutriant	Cellulose	104%
	"	PEI	102%
	"	DEAE-Cellulose	107%
	"	Silica	102%
Nucleotides	Randerath's Elutriant	Cellulose	91%
	"	PEI	100%
	"	DEAE-Cellulose	98%
	"	Silica	95%
	Ammonical alcohol	PEI	88%
	"	Silica	93%

Nucleosides and nucleotides were eluted from the TLC plates by scraping 2 mm of resin into a scintillation vial followed by the addition of 0.5ml elutriant. The vial was then rocked for 2 hrs and the contents counted in Triton-x toluene. Randerath's elutriant consists of 0.7M $MgCl_2$, 0.2M Tris-HCl, pH 7.4. Ammonical alcohol is composed of 10% ethanol and 5% ammonium hydroxide.

by slowly pouring the degassed P-2 suspension down the column fitted with a funnel on top, the suspension packing onto a nylon net on the bottom of the column. Polyethylene tubing was connected from the column outlet through the measuring cell of an LKB conductylizer and an LKB Uvicord II optical system.

Columns were run at room temperature at a flow rate of 10-30 ml per hour. Most of the salt was obtained between the nucleotide and nucleoside peaks. Fractions from the P-2 column were analyzed by thin-layer chromatography as described earlier.

DEAE-Cellulose Chromatography

Prior to the adaptation of the gel chromatographic technique described above, nucleotides were separated by DEAE-cellulose anion exchange chromatography.

DEAE-cellulose (Whatmann DE52) was equilibrated with a particular buffer used for absorbtion, for example, 0.3M ammonium formate, pH 4.4. The fines were decanted at least five times and the remaining resin was loaded into a Pasteur pipet with glass wool packed into the distal end. One-half ml samples were applied to the column and the flow through of 5 column volume washes were assayed. Nucleotides were found to quantitatively stick to the resin. Components were eluted from the column with a discontinous salt gradient of

from 5-50mM. However, the salt in the eluate rendered the sample difficult to analyze by subsequent thin-layer analysis. Hence, analysis of nucleotide pools was carried out by P-2 desalting followed by TLC.

ENZYME ASSAYS

Thymidine Kinase Assays

Assays for thymidine kinase (TK) are all based on the general conditions described by Bollum and Potter (1959). These involve the incubation of the 100,000 x g supernatant of broken cells and 37° with ATP, 3-phosphoglycerate, magnesium and potassium chlorides, radiolabeled thymidine, and 2-mercaptoethanol in a tris buffer. Assays differ mostly by the method of analyses of the products. These fall into two broad classes, those chromatographically separating all of the products from each other, and those just separating thymidine nucleotides from the nucleoside by the ability of nucleotides to stick to DEAE filter paper.

Complete separation of thymidine nucleotides has been accomplished using Dow XI column chromatography, paper chromatography (Bollum and Potter, 1959; Kit et al., 1963) and TLC (Furner and Mellet, 1975). Gross separation of nucleosides from nucleotides has been reported with DEAE cellulose, for example Adams (1969), Brent (1971), Arima et al. (1972), and Furner and Mellet (1975). The latter authors compared filter paper adsorption of total

nucleotides and thin-layer chromatographic separation of all products. Both techniques gave comparable values for TK activity.

Protocol for T.K. Assay

Approximately 50 ml, containing approximately 5 x 10^7 cells, of cell suspension was centrifuged and the cell pellets were resuspended in a total volume of 3 ml of ice cold homogenization buffer (HB) composed of 0.15M KCl, 0.01M Tris, pH 8.0, 3mM 2-mercaptoethanol. The cell suspension was sonnicated at maximum tune on a Kontes sonicator at 4°C for 20 seconds. This amount of sonication was sufficient to disrupt the entire cell population. A high speed supernatant was made from the sonicate by centrifugation at 105,000 x g for one hour at 4°C in an SW50.1 rotor. The pellet was found to be devoid of activity. Samples of the high speed supernatent were taken for protein determination and an immediate protein estimate was made spectrophotometrically. Assays were done at 37°C in a water bath in 1.5 ml snap cap tubes. The assay mix consisted of:

50 µl of 105,000 x g sup (or BSA in Tris KCl to the same protein concentration by OD).

25 µl of substrate mixture (20mM ATP, 1µC ^{3}H-nucleoside, 24mM 3-phosphoglycerate, 20mM magnesium).

25 µl of 0.2M Tris, pH 8.0.

Reactions were terminated by the addition of 10μl of ice cold 100% TCA. Protein was precipitated by centrifugation and TCA was removed from the soluble fraction by two extractions of 200 μl ethyl ether each. After the extraction the aqueous phase was de-etherized by degassing in vacuo.

Products of the reaction were analyzed either by TLC as described previously, or by adsorption on DEAE filter paper. This was done using Whatmann DE81 DEAE-cellulose 2.5 cm circles. 50 μl of each sample was spotted onto 4 discs. Two of these were placed directly into scintillation vials for determination of total radioactivity. The other two were washed two times with 100cc of water while gently rocking. This removes non-phosphorylated products. To each vial one ml of 0.2M KCl in 0.1N HCl was added in order to elute the bound nucleotide. Recovery of radioactivity was over 90%.

DNA Polymerase Assays

Polymerase activity was assayed in 100 μl reaction mixtures containing 0.4 μg synthetic template, 5 μmole Tris-HCl, pH 8.0, 0.6 μmole $MgCl_2$ or 0.025 μmole $MnCl_2$, 0.1 μmole DTT, and the enzyme preparation. 5 nMoles ^{3}H-dTTP (5000 cpm/pmole) was used in reactions templated by oligo dT:polyrA or oligo dT:poly dA. 5 nMoles ^{3}H-dGTP (5000 cpm/pmole) was used in reactions templated by

oligo dG:poly rC, oligo dG:poly rCm, or oligo dG. Reactions using the latter primer assess terminal deoxynucleotydyl - transferase activity. $MgCl_2$ was used in those reactions incorporating dTTP and $MnCl_2$ was used in the dGTP containing mixtures.

Reactions were incubated at 37°C and the acid insoluble product was assayed by precipitation onto nitrocellulose filters.

Synthetic oligo and polynucleotides and deoxyribonucleoside triphosphates were purchased from P-L Biochemicals. ^3H-labeled nucleotides were the products of Amersham-Searle.

NUCLEOSIDE METABOLISM

TCA soluble fractions were de-acidified by two extraction of two volumes of ethyl ether each. Non-radioactive carrier nucleotides were added to the aqueous phase of these extractions, and the resulting solutions were degassed *in vacuo*. The nucleosides were then desalted by application to a 46 cm x 0.9 cm BioGel P2 column which was eluted with degassed distilled water. The desalted fractions were evaporated to dryness by shaking *in vacuo* at 40°C.

The desalted residues were resuspended in 40μl of distilled water and 10 μl samples were applied to TLC plates by successive 1 μl aliquots, as described earlier. After

development the TLC plates were divided into small sections and the resins from each section scraped into a scintillation vial. Nucleotides and nucleosides were eluted and the radioactivity of the eluate was determined.

Specific Determination of Precursor Incorporation into RNA and DNA

Incorporation of radiolabeled precursors specifically into DNA and RNA was determined by the technique of Schmidt and Thannhauser (1945) as described by Monro and Fleck (1966).

Perchloric acid (PCA) precipitates of cell suspensions were made as described earlier for a TCA precipitations. 0.5 ml of 0.02N PCA were pipetted over the washed cell pellets. The PCA precipitates were then washed two times with 0.5ml of absolute ethanol. The radioactivity of the precipitated fractions represent the total counts in both RNA and DNA. The ethanol-washed PCA precipitates were shaken for 15 hours at 37°C in 1N KOH. The supernatant fractions from this digestion consists of counts originally present in RNA. These were assayed by cooling the KOH digest to 0°C and acidifying to 0.02N acid by the addition of 0.5ml of 1.4 N PCA. The PCA insoluble DNA was precipitated as described above for TCA precipitations. The counts derived from RNA, present in the PCA soluble fraction, were assayed as described above for TCA soluble material.

MICROSCOPIC STUDIES ON BrdU-TREATED MALIGNANT LYMPHOID CELLS

Phase Microscopy

Phase photomicrographs were made with a Nikon inverted microscope equipped with a Wilde camera and automatic exposure meter. Kodak Panatomic-X film was used.

Electron Microscopy

Cell pellets were fixed for two hours at 4°C in 3% glutaraldehyde, after which they were rinsed four times (for a total of 45 min) in 0.1 M sodium cacadylate. Samples were then post-fixed in 1% OsO_4 (made fresh from 2% OsO_4) for two hr at 4°C after which they were rinsed 3-4 times (for 15 min) in 0.1 M sodim cacadylate. The post-fixed samples were then dehydrated and epon embedded.

Samples were stained with 5% uranyl acetate, counterstained with 4% lead citrate, and examined with an RCA EMU 3H electron microscope.

PROTEIN DETERMINATION

Direct Spectrometry

Protein estimations were done spectrophotometircally as described by Warburg and Christian (1942).

Lowry

Protein determinations were performed using the technique described by Lowry et al. (1951).

AUTORADIOGRAPHY

Autoradiographic studies were done by centrifuging cell suspensions onto a glass slide followed by a PCA precipitation *in situ*, washing off unprecipitated nucleoside, and coating with a liquid emulsion NTB-2 (Kodak). Slides were examined with a light microscope after development and staining.

PCA Precipitations

Slides were immersed for 20 min in ice cold 1% perchloric acid, followed by a 30 min rinse in water. Slides were then dried for 24 hr in a box containing Drierite.

Coating with Emulsion

All handling of emulsion was done in the dark room at least 4 feet from a Kodak safelight 1A filter over a 15 Watt light bulb. Before using an emulsion in an experiment, a sample of it was first applied to a test slide and developed 24 hr later. Grain counts over 100/field were considered indicative of an unacceptable emulsion.

RESULTS

GROWTH OF CELL LINES IN THE PRESENCE OF BrdU

Concentrations of BrdU which are commonly used to study phenotypic differentiation of normally proliferating cells are acutely cytotoxic to malignant lymphoid cell lines. This cytotoxic effect is defined by three criteria which are always simultaneously satisfied in these studies. These are reduction of growth rate, increase in the number of trypan blue including cells, and loss of subculturability.

Toxicity of BrdU to MOPC 315

Figure 6 is a growth curve of MOPC 315 in 1.0 and 0.1 μg BrdU/ml showing an acute toxic effect by days 3 and 4, respectively. Figure 7 shows the decrease in viability which occurs in the presence of BrdU. As shown in Figure 8, longer exposure to MOPC 315 to even lower concentrations of BrdU causes a similar cytotoxic effect, the time necessary for this to become manifest being inversely proportional to the dosage. As can be seen from the figure, the average time necessary to cause a toxic effect is eleven days at 0.01 μg BrdU/ml and eighteen days at 0.001 μg BrdU/ml. The toxicity of these latter concentrations of BrdU become evident after several subculturings in the presence of the analogue. The kinetics of cell killing given here are for cultures grown completely in the dark and manipulated

Figure 6

Effect of 1.0 and 0.1 μg Brdu/ml on the growth of MOPC 315. Triplicate 2 ml cultures were seeded at an initial cell density of 8×10^4 cells/ml. One plate was counted each day and discarded. Culture conditions were as described in Materials and Methods. 0 (O), 1.0 (□), and 0.1 (●) μg BUDR/ml.

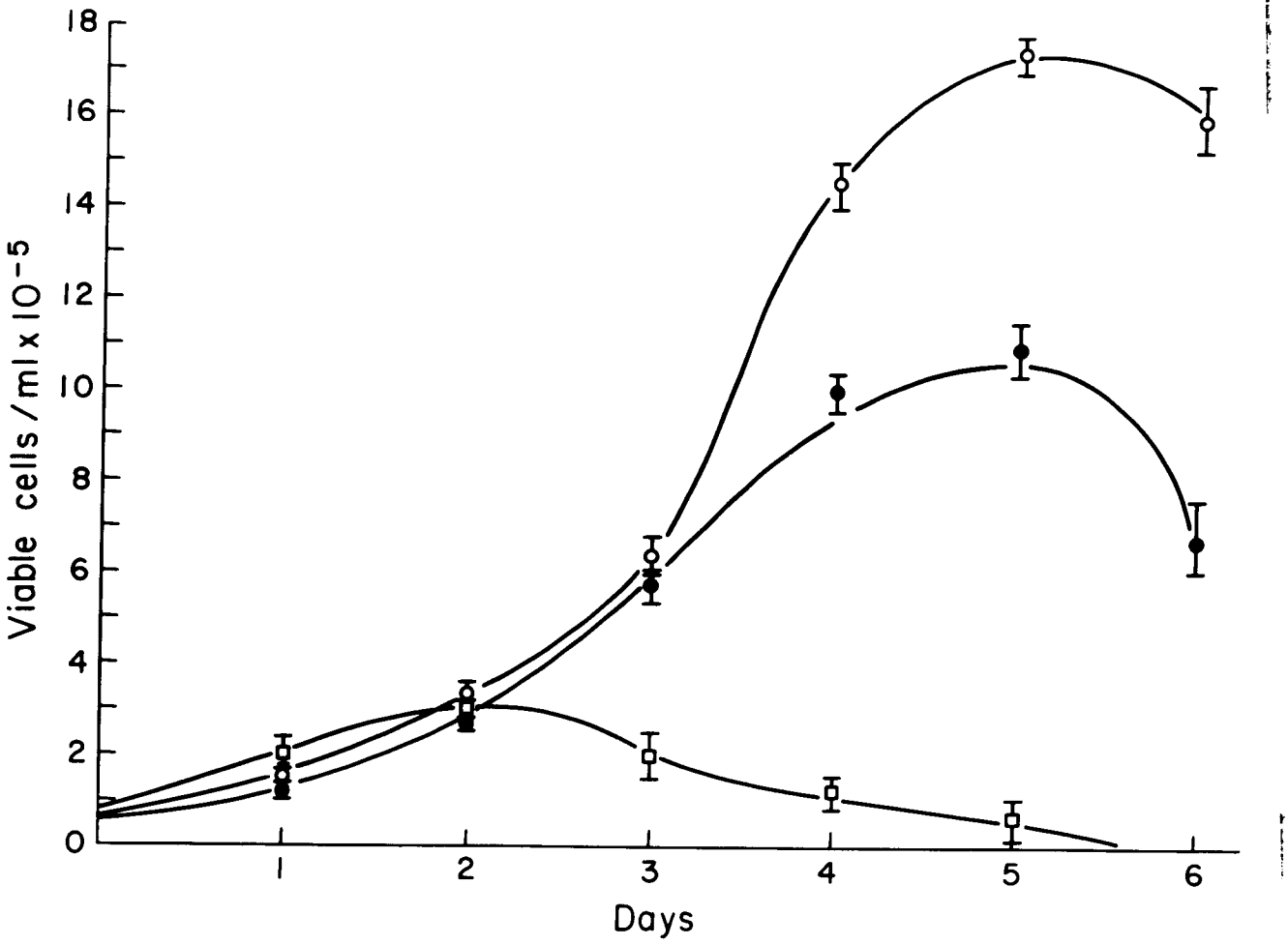

Viable cells / ml x 10-5
0
2
4
6
8
10
12
14
16
18
1
2
3
4
5
6
Days

Figure 7

Loss of viability of MOPC 315 cells grown in one μg BrdU/ml. 8 x 10^5 cells/ml were seeded in 2ml linbro plates as described in Materials and Methods. One plate was removed for assay each day and discarded. Viability was determined by the trypan blue exclusion test. The points given are the means of three replicates.

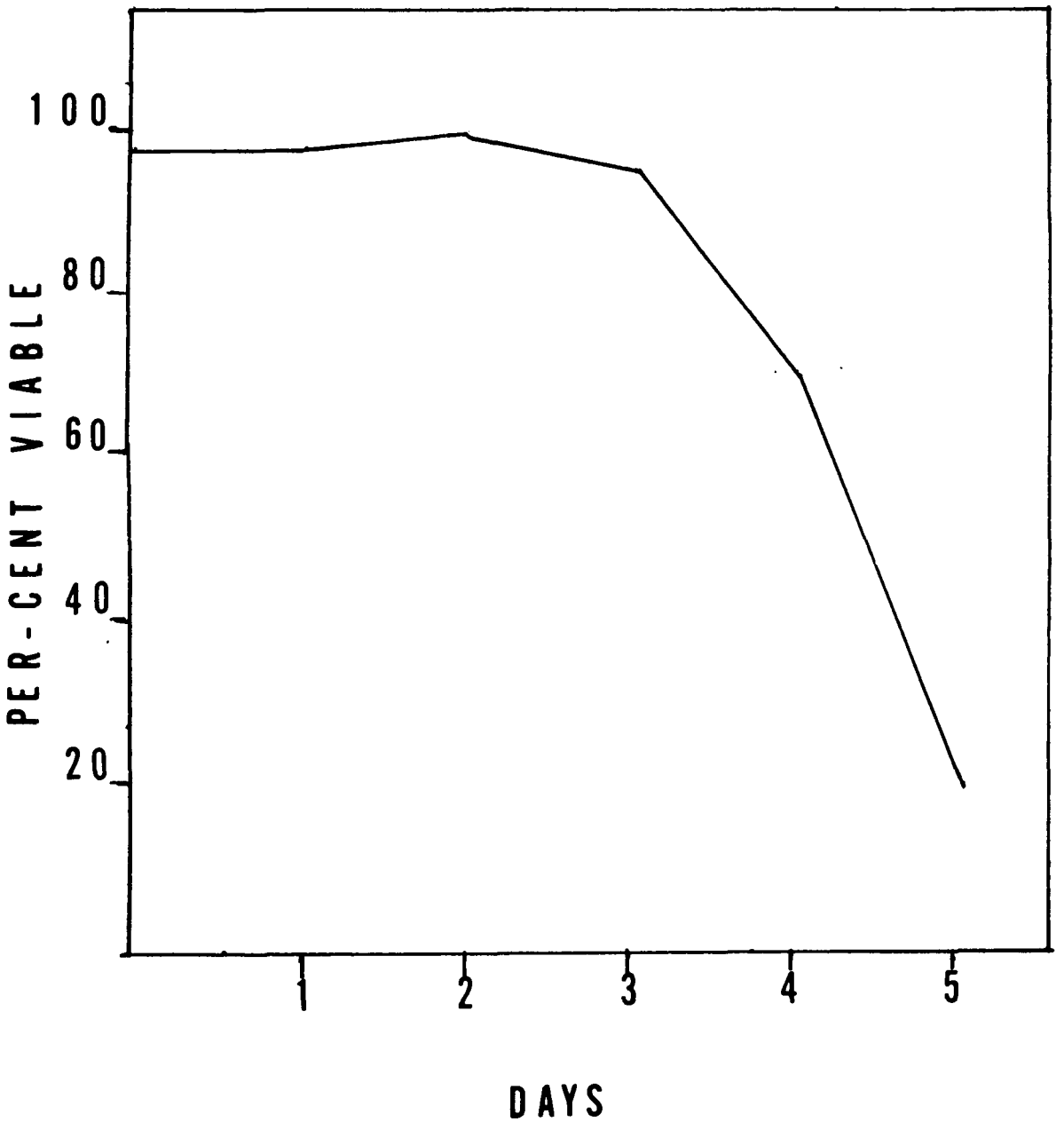
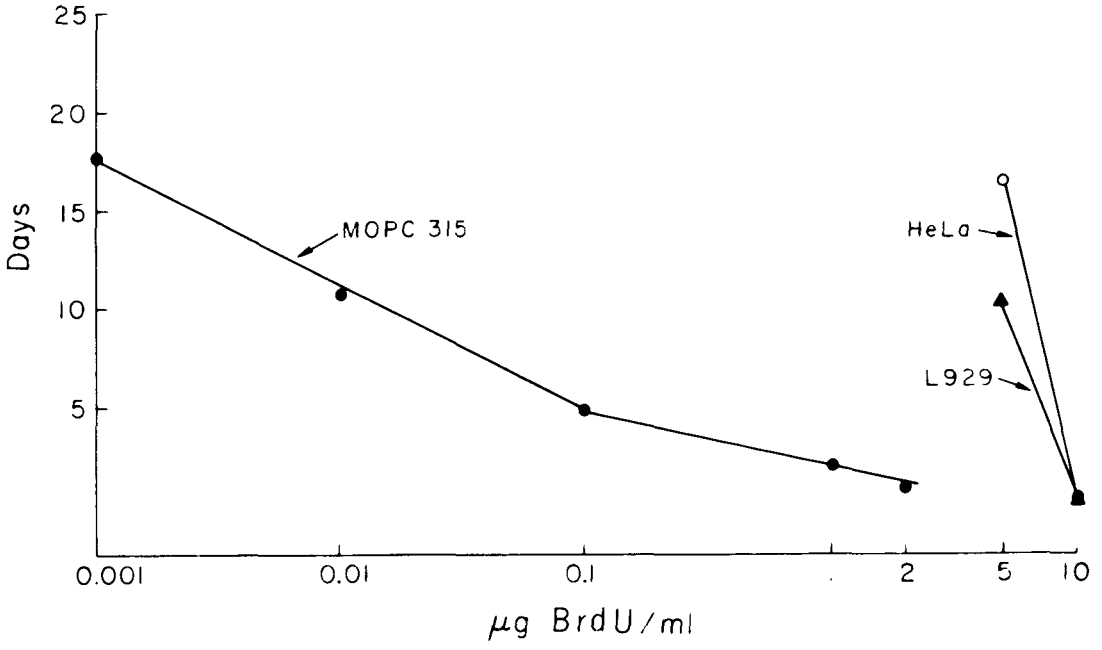

100
80
60
40
20
PER-CENT VIABLE
1
2
3
4
5
DAYS

Figure 8

Times necessary for the toxic effect of BrdU to MOPC 315 (●), HeLa (○), and L929 (▲). Times are mean values calculated from several growth experiments. Toxicity is defined as statistically significant inhibition of cell proliferation as compared to control cultures.

TIME NECESSARY FOR THE TOXICITY OF BrdU
TO MOPC 315



only under Kodak 1A photographic red safelights. Exposure of the cultures to fluorescent lights potentiates the killing.

As shown in Table 5, the dose of BrdU needed to cause a 50% reduction in proliferation of MOPC-315 cells in 4 days is 0.09 μg/ml. Autoradiographs reveal that all of the cells in the culture incorporate the analogue into their nuclei.

Toxicity of BrdU to Another Murine Myeloma, MPC 11

Attempts to grow mouse myeloma MPC-11 in the presence of BrdU results in a pattern of toxicity similar to that described above for MOPC 315. As can be seen in Figure 9, acute toxicity of 1 and 0.1 μg BrdU/ml is evident by 3 and 4 days, respectively. Loss of viability, as measured by the trypan exclusion test, parallels the reduction in growth rate of the culture.

The LD_{50} for MPC 11, defined here as the concentration of BrdU that will cause a 50% inhibiton of cell proliferation by 4 days, is 0.20 μg/ml. As seen in Table 5, this is similar to the LD_{50} of 0.09 μg/ml for MOPC 315.

Toxicity of BrdU to Malignant Lymphocytes

The kind of cytotoxic effect of BrdU to murine myelomas described above is also seen when malignant lymphocytes of mouse or human origin are grown in the presence of the analogue. Table 6 shows that when mouse lymphoma S49.1

TABLE 5 CONCENTRATION OF BrdU REQUIRED TO CAUSE A 50% INHIBITION OF CELL PROLIFERATION IN 4 DAYS (LD_{50}).[a]

Group	Cell line	LD_{50} (μg/ml)
I	MOPC 315	0.09
	MPC 11	0.20
	CCRF/CEM	0.31
	S49.1	0.16
	S49.1TB.2	87.0
II	RAG	3.70
	L929	6.23
	HeLa (monolayer)	3.14
	HeLa (suspensions)	4.20
	MMT	4.25

[a]The 50% Lethal doses (LD_{50}) of BrdU were determined from a graph of inhibition vs dose for 4 days of growth in the presence of the analog.

Figure 9

Growth of MPC 11 in BrdU
Triplicate 2 ml cultures were set up as described in Materials and Methods. Cell titers are given as the mean of 3 replicate determinations. The numbers refer to μg BrdU/ml.

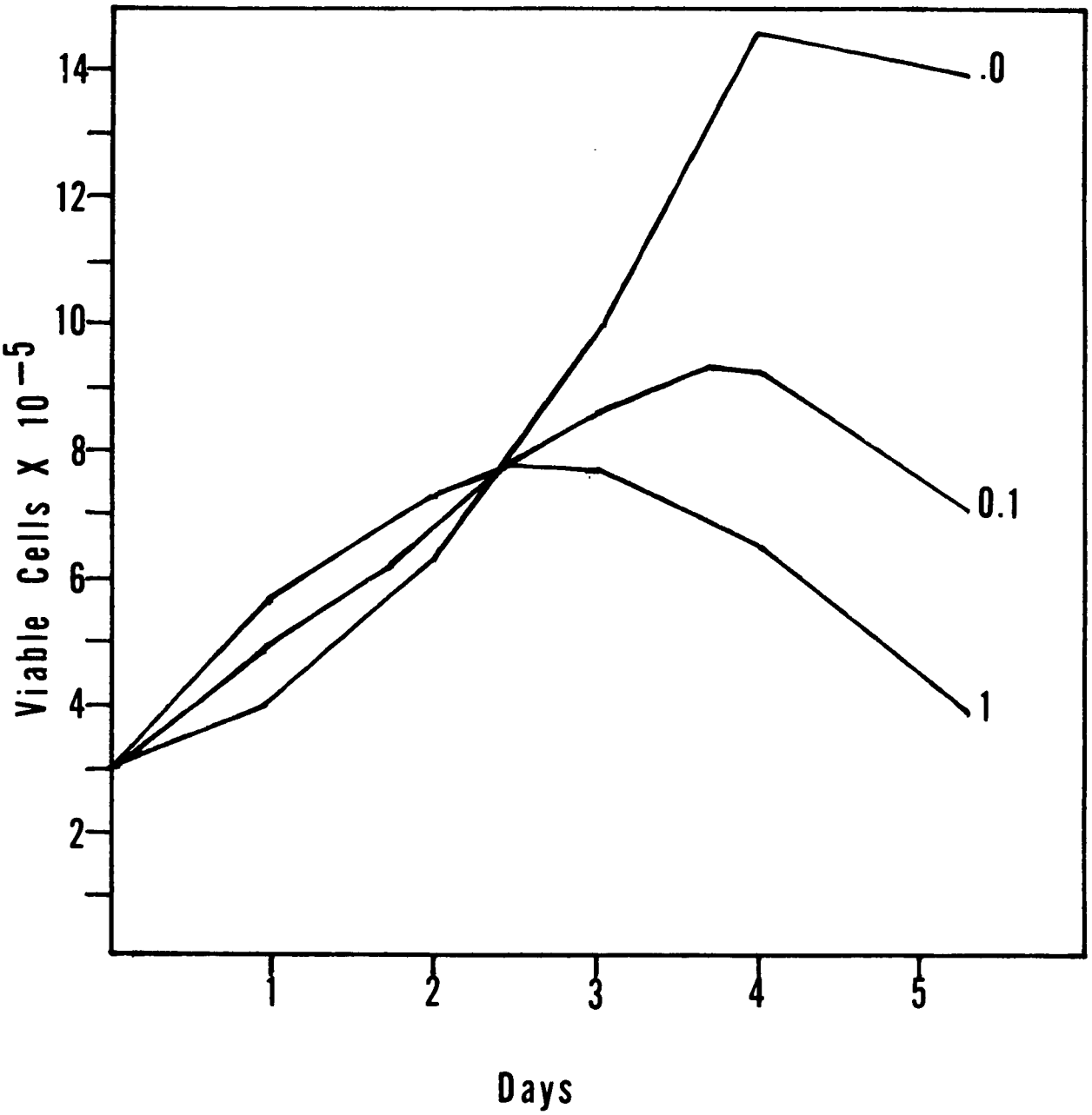

Viable Cells X 10−5
14
12
10
8
6
4
2
1
2
3
4
5
Days
.0
0.1
1

Table 6

PROLIFERATION OF VARIOUS CELL LINES IN THE PRESENCE OF BrdU. DATA IS GIVEN AS % CONTROL AFTER 4 DAYS.

Line	Description	µg BrdU/ml	% Control
MOPC 315	Mu. Myeloma	1	28*
		0.1	34*
MPC 11	Mu. Myeloma	1	14*
		0.1	22*
CCRF/CEM	Hu. A.L.L.	1	47*
		0.1	49*
s49.1	Mu. Lymphoma	1	61*
		0.1	69*
RAG	Mu. Renal Adenoca.	1	87
MMT	Mu. Mammary Ca.	1	111
L929	Mu. Fibroblast	2	87
		1	91
HeLa	Hu. Cervical Ca.		
a) Monolayer		2	108
		1	111
b) Suspension		1	111

* = $p < 0.05$

cells are grown in BrdU, significant growth inhibition occurs by 4 days at concentrations on the order of 1 or 0.1 μg/ml. As seen in Table 5, the LD_{50} for S49.1 is 0.16 μg/ml, which is similar to that of myeloma cells.

S49.1TB.2 is a BrdU-resistant clone of S49.1. The LD_{50} of 87.0 μg/ml for this line is more than 500 times higher than that of the parental sensitive lines.

Growth of human acute lymphoblastic leukemic lymphocytes CCRF/CEM in BrdU follows a pattern generally similar to those described above for MOPC 315, MPC 11, and S49.1. The decline in growth and loss of both viability and subculturability occur at BrdU concentrations and times comparable to that of these other malignant lymphoid cell lines. Table 6 shows the inhibition of growth by 4 days of BrdU at the 1 and 0.1 μg/ml level. As seen in Table 5 the LD_{50} for this line is 0.31 μg BrdU/ml.

Effects of BrdU on Various Other Cell Lines

The data given above describes the cytotoxic effect BrdU on malignant lymphoid cell lines at concentrations of the analogue which, as reviewed in the Introduction, are not generally harmful to most other cell lines. We have verified this relative insensitivity to BrdU of several cell lines of diverse origin as compared to the vulnerability of malignant lymphoid cells. These studies all include a

simultaneous assay of the effect of the BrdU containing growth media on MOPC 315. In all of these experiments there was a cytotoxic effect of the BrdU to MOPC 315 comparable to that described in the section relating to the growth of MOPC 315 in the presence of the analogue.

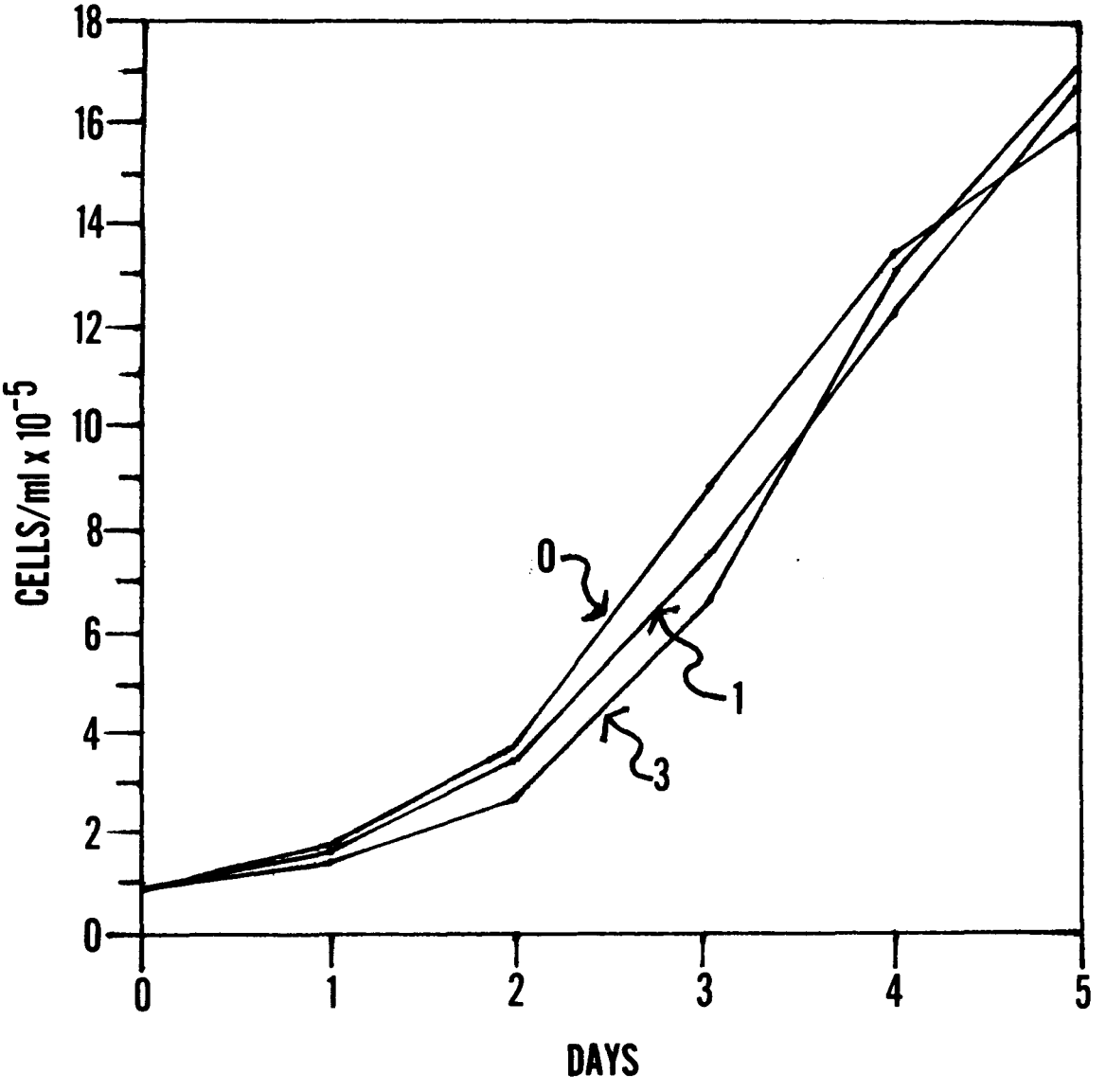
LD_{50}s for the various cell lines studied are given in Table 5. As seen in the table these range from 3.1 μg/ml for HeLa to 6.2 μg/ml for L929 averaging 4.3 μg/ml. In sharp contrast to these relatively high BrdU concentrations are the values for cell lines of malignant lymphoid origin, ranging from 0.1 μg/ml for MOPC 315 to 0.3 μg/ml for CCRF/CEM, averaging 0.2 μg/ml.

The actual growth curves, as typified in Figure 10 for HeLa cells, reveals two features which emerge as being qualitatively constant, but yet quantitatively variable, for the relatively BrdU-resistant cell lines.

During the first two to three days in culture, the cell lines undergo a slight depression in growth rate as compared to the non-treated control cultures. This reduction in growth rate is not accompanied by an increase in the proportion of tyrpan blue accepting cells. Shortly after this transient reduction in growth, which is rarely more than a 30% inhibition, the BrdU-treated cells undergo a transition to a slightly stimulated rate of growth compared to the untreated cultures.

Figure 10

Growth of HeLa cells in 0, 1, 3 μg BrdU/ml, triplicate 2 ml wells were seeded at an initial cell titer of 8×10^4 cells/ml and cultured and quantitated as described in Material and Methods.


18
16
14
12
10
8
6
4
2
0
CELLS/ml x 10⁻⁵
0
1
2
3
4
5
DAYS
0
1
3

This stimulation of proliferation in BrdU is dose dependent, being most evident in the range of 1-3 μg/ml.

Figure 8 contrasts the kinetics of the appearance of the cytotoxic effects of BrdU at various dosages for the sensitive line MOPC 315 and the two insensitive lines, HeLa and L929.

EFFECTS OF HORMONES ON THE TOXICITY OF BrdU TO MOPC 315

Various studies on our laboratory have shown that anti-proliferative treatment of murine myeloma cells can be reversed by certain hormones. We thus tested a variety of hormonal substances for an affect on the toxicity of BrdU to MOPC 315. The data in Table 7 shows that neither 0.5 units insulin/ml nor 0.025 μg dihydrotestosterone (DHT) per ml, alone or together, have any affect on the toxicity of BrdU to MOPC 315. The insulin concentration used in this experiment was that reported to reverse the anti-proliferative effects of cAMP on murine plasmacytoma cells (Naseem and Hollander, 1973). DHT concentrations used in these studies were on the order of physiological levels. Previous studies in our laboratory have shown that testosterone, or its dihydro derivative, may exert a proliferative affect on murine myeloma cultures. However, these do not seem to be relevant to the BrdU effect on these cells.

Table 7

EFFECT OF INSULIN AND DHT ON THE TOXICITY OF BrdU TO MOPC 315

Condition (2 lines)	Cells/ml ± SEM on day "0" (x 10^{-4})	Cells/ml ± SEM on day 3 (x 10^{-5})
Control	6.2 ± 0.6	4.8 ± 0.1
BrdU	6.8 ± 2.2	1.8 ± 0.4
INSULIN	3.5 ± 0.7	2.9 ± 1.1
DHT	8.9 ± 3.1	3.5 ± 0.7
BrdU + INSULIN	6.4 ± 1.4	2.3 ± 0.3
BrdU + DHT	7.6 ± 1.2	1.1 ± 0.5
BrdU + DHT + INSULIN	7.2 ± 1.2	2.6 ± 0.8
0.008% ETHANOL	9.0 ± 2.0	6.1 ± 0.8

Duplicate 4ml Cultures were set up at the indicated initial cell titers. Oncogenicity was determined by injection 10^4 or 10^3 cells in 0.1ml sub-cutaneously into female BAlb/c mice. BrdU was at 1 ug/ml, Insulin was 0.5 unit/ml and DHT was 0.025 ug/ml. All DHT containing cultures were 0.008% in Ethanol.

GROWTH OF MALIGNANT LYMPHOID CELLS IN BROMOURACIL

Unlike BrdU, bromouracil (BU) does not cause an acute toxic effect on cultures of malignant lymphoid cells, as shown in Table 8.

EFFECT OF THYMIDINE ON THE TOXICITY OF BrdU TO MALIGNANT LYMPHOID CELLS

As reviewed in the Introduction, thymidine and BrdU show a competitive relationship with each other. Thus, an excess of thymidine usually prevents the various effects of BrdU. Based on this we investigated the effect of thymidine on BrdU's toxicity to malignant lymphoid cell lines.

Figure 11 is a graph showing the toxicity of BrdU to MOPC 315 in the presence of a range of thymidine concentrations. 10μg thymidine/ml completely protects MOPC 315 cells against the toxicity of 1 μg BrdU/ml. 1 μg thymidine/ml or less has a progressively less protective effect on these cells.

Table 9 summarizes the effect of thymidine on the toxicity of BrdU to murine myeloma and lymphoma cells. From the Table it can be seen that 1 μg thymidine/ml completely protects lymphoma and partially protects myeloma cells from the toxicity of 1 μg BrdU/ml.

Table 8

EFFECT OF BROMOURACIL ON THE GROWTH OF MALIGNANT LYMPHOID CELL LINES

Cell line	Conditions	cells/mlx10^{-5} on day 0	cells/mlx10^{-5} on day 4	%control	Sig
MOPC 315	Control	0.8	4.92 ± 0.19		
	1 μg BU/ml	0.8	7.52 ± 0.90	135	-
MPC11	Control	0.8	10.6 ± 0.4		
	1 μg BU/ml	0.8	14.9 ± 3.23	114	-
549.1	Control	0.8	11.2 ± 3.1		
	1 μg BU/ml	0.8	20.5 ± 1.7	138	-
EL4	Control	0.8	6.98 ± 1.87		
	1 μg BU/ml	0.8	7.11 ± 0.92	102	-
EL4Bu	Control	0.8	14.0 ± 4.14		
	1 μg BU/ml	0.8	20.2 ± 0.47	148	-

Triplicate 2ml cultures were prepared, grown, and quantitated as described in materials and methods. Sig. refers to statistical significance by the Student's "t" test.

Figure 11

Reversal of the toxicity of BrdU to MOPC 315 by thymidine. All point were calculated based on the toxicity of 1 μg BrdU/ml after 4 days. The ordinate gives the percent toxicity of BrdU/ thymidine compared to BrdU alone.

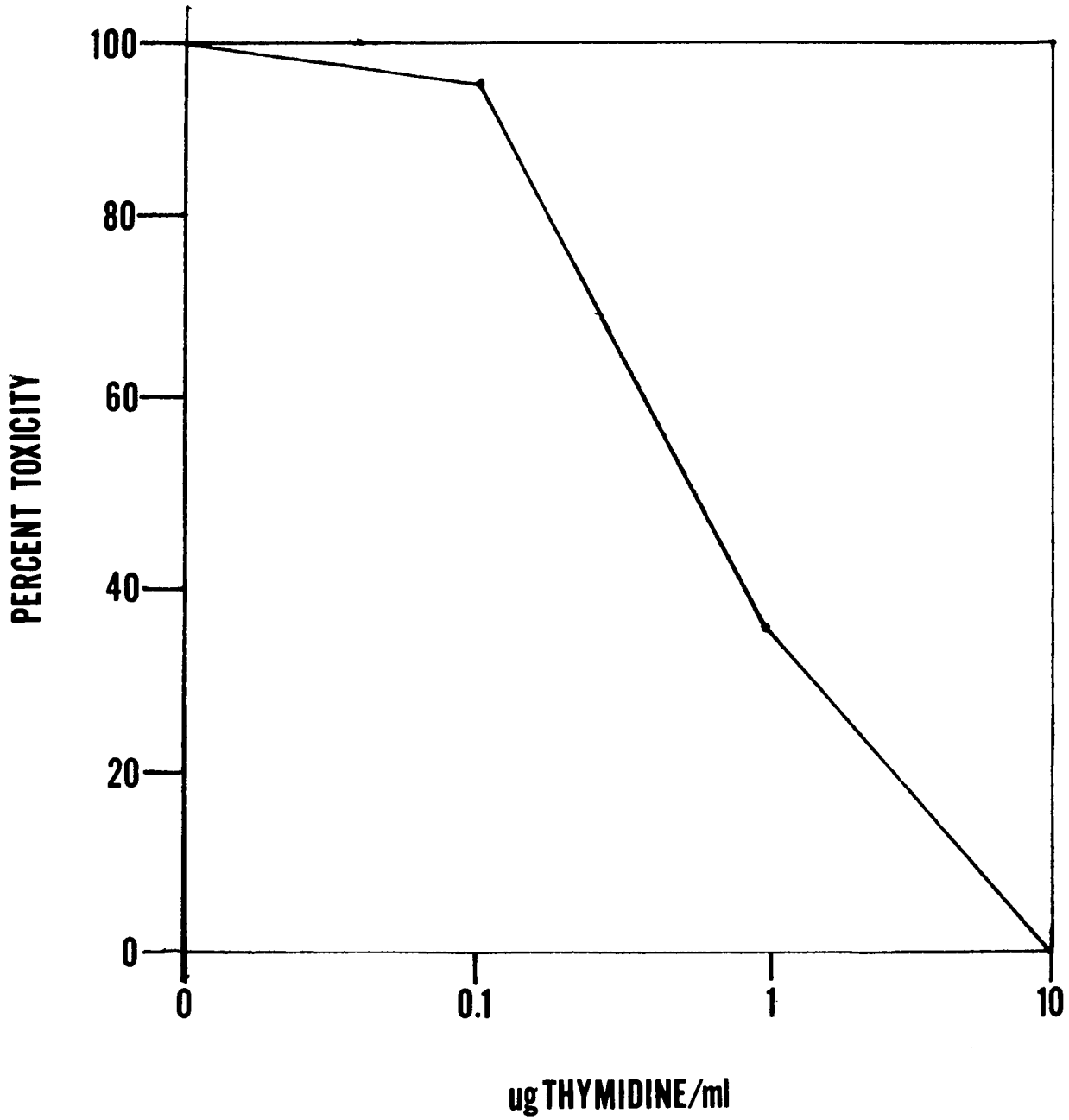

100
80
60
40
20
0
PERCENT TOXICITY
0
0.1
1
10
ug THYMIDINE/ml

Table 9

Protection by Thymidine Against the Toxicity of 5-Bromo 2' -Deoxyuridine to Malignant Lymphoid Cell Cultures

Condition	MOPC 315	MPC 11	S49.1	S49.1TB.2
Control	100	100	100	100
1 ugBrdU/ml	37.2[a]	45.8[a]	64.1[a]	101
(1 ugBrdU + 1 ug Thymidine)/ml	77.7[a]	83.6[b]	104	113[c]
1 ug Thymidine/ml	95.0	109	107[a]	120[c]
(1 ugBrdU + 10 ug Thymidine)/ml	85.7	104	103	113[b]
10 ug Thymidine/ml	81.4	110[d]	103	119[a]

a) $p < 0.001$; b) $p < 0.005$; c) $p < 0.01$; d) $p < 0.025$

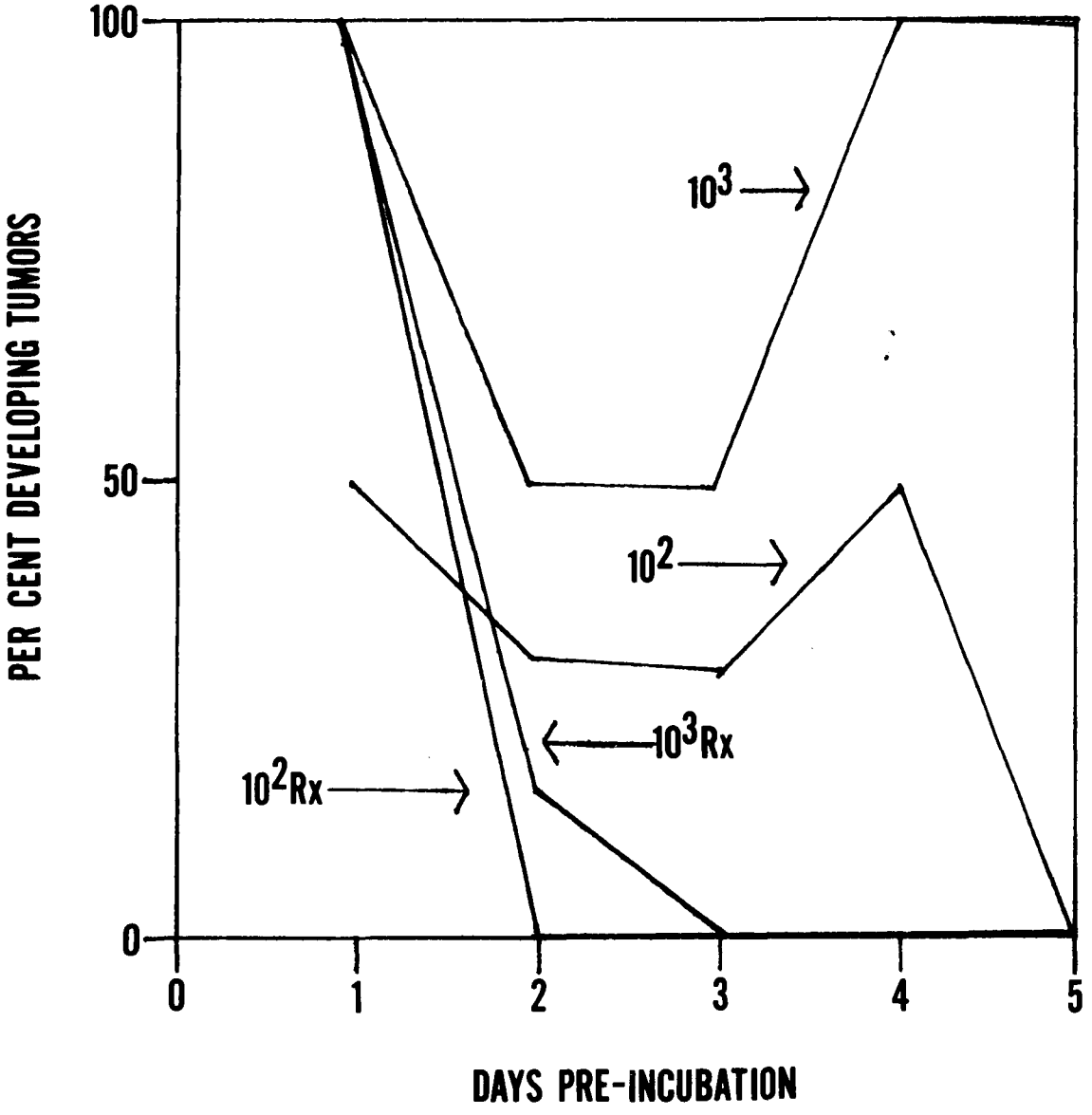
Cells were grown and quantitated as described in Materials and Methods. Data is given as per-cent control after 4 days of growth.

ONCOGENICITY OF MOPC 315 CELLS PRETREATED WITH BrdU

BrdU has been reported to reduce the tumorigenicity of cells cultured in its presence. The data supporting this assertion has been reviewed in the Introduction. Examination of this parameter is very difficult using malignant lymphoid cell lines because of the superposition of the acute toxicity of the analogue to these cells. However, we have examined this phenomena by pre-treating cells for times less than those necessary for BrdU to have an adverse effect on the growth of the cells. The data is shown in Figure 12. This experiment used 10^3 cells, a dose expected to give a high rate of tumor take, but usually less than 100%. A 10-fold dilution of this dose was also given to evaluate experiments where the 10^3 dose gave a 100% rate of tumor development, thereby reducing the sensitivity of the assay. As seen in the figure, all points after the injection of the indicated number of viable tumor cells preincubated with BrdU for more than 1 day shows them to be somewhat less tumorigenic than in comparable number of control cells. Following 2 days incubation with 1 μg BrdU/ml, doses of 10^3 and 10^2 MOPC 315 cells were markedly less tumorigenic than control cells. By day 2 of incubation, 10^2 treated cells had no tumorigenic potential and 10^3 treated cells gave rise to

Figure 12

Oncogenicity of MOPC 315 cells injected into BALB/c female mice. Cells were grown for 1-5 days in the presence, (Rx) or absence of 1 μg BrdU/ml. After the incubation 10^3 or 10^2 trypan blue exluding cells were inoculated into the mice.


100
50
0
PER CENT DEVELOPING TUMORS
10^3
10^2
10^3Rx
10^2Rx
0
1
2
3
4
5
DAYS PRE-INCUBATION

tumor in only 12% of the animals inoculated, compared to the 33 and 50% for control cells, respectively. By day 3 of inoculation, the 10^3 treated cells were oompletely non-tumorigenic.

BrdU-containing cell-free tissue culture media, in which cells have been grown for 2 or 4 days, is non-tumorigenic when injected into newborn BALB/c mice. 0.1ml of the media was injected into sibling mice. As shown in Table [10] no tumors developed in mice watched for 20 months.

MORPHOLOGICAL STUDIES ON BrdU TREATED CELLS

Phase Microscopy

Phase micrographs of untreated live MOPC 315 cells show them to be rounded with a prominent nucleus. As seen in Figure 13A, the nuclei of these cells has the "spoke wheel" type of appearance characteristic of plasma cells. The cytoplasm of these cells is free of very prominent collections of organelles. Approximately 15% of the cells of an untreated mid-log phase culture contain 1 or 2 small (approximately 1 micron) highly refractile cytoplasmic structures. The cultured cells are morphologically indistinguishable from plasma cells from the parental MOPC 315 tumor *in vivo*.

MOPC 315 cells treated with BrdU contain significantly more of the cytoplasmic refractile structures than the

Table 10

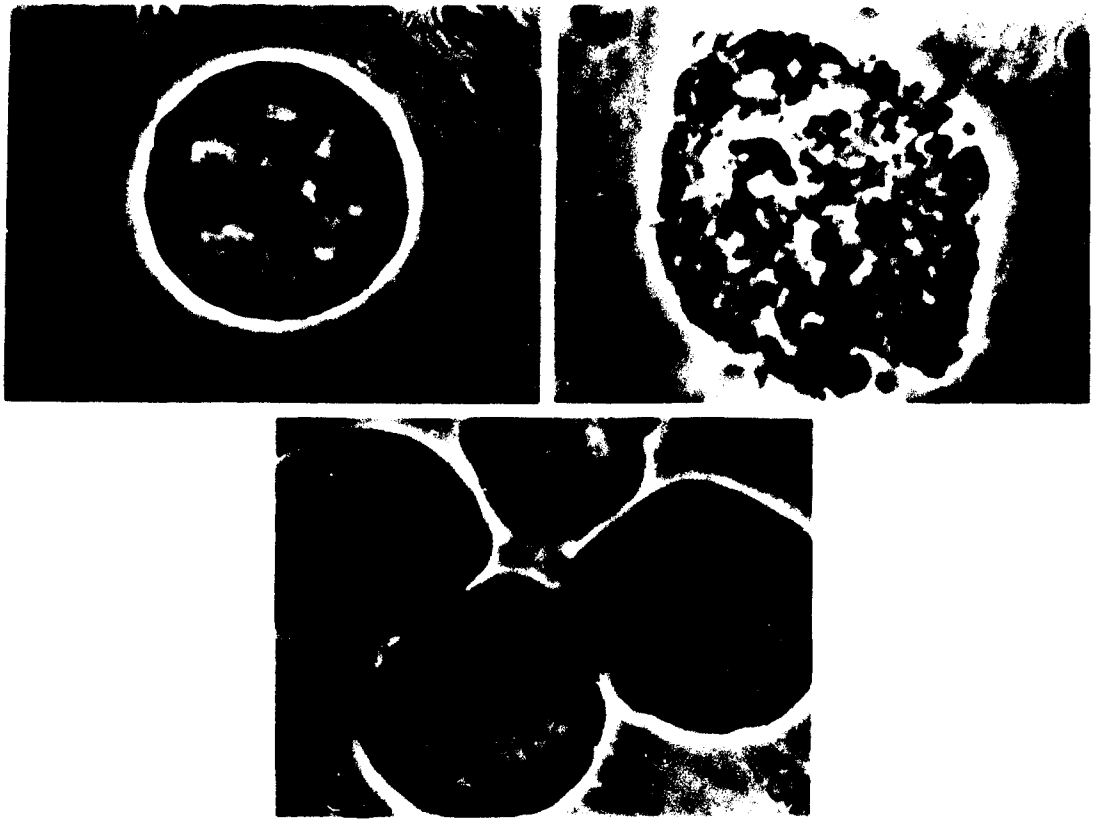
ONCOGENICITY OF CULTURE MEDIA IN WHICH
BrdU TREATED CELLS HAVE BEEN GROWN

Days of Exposure	1 ug BrdU/ml	% Developing Tumor
2	-	5
	+	0
4	-	0
	+	0

MOPC 315 cells were inoculated at $1x10^5$ cells/ml and grown as described in Materials and Methods. After 2 or 4 days the cells were centrifuged out of suspension and 0.1 ml of the culture supernatent was injected IP into newborn BAlb/c mice. 1-2 litters were used for each determination.

Figure 13

1250X phase photomicrograph of a MOPC 315 cell (upper left), MOPC 315 cell grown in 0.1 μg BrdU/ml for 3 days (upper right), and S49.1TB.2 cell grown in 10μg BrdU/ml for 8 days. Phase photomicrographs were taken with a Wild photoautomat on a Nikon phase microscope as described in Materials and Methods.



untreated controls. Figure 13B shows a typical treated cell. At all doses studied, the appearance of these structures preceded the gross effect of BrdU on cell proliferation. Figure 14 gives the kinetics of the accumulation of MOPC 315 cells containing cellular inclusions. A cell containing an increased number of inclusion is defined here as one with 4 or more of such structures per cell. Figure 15 shows the increase in the number of particles per cell. The abscissa is plotted as the percent of the time necessary to achieve statistically significant growth inhibition.

A similar picture of the accumulation of refractile cytoplasmic inclusions occurs when MOPC 315 cells are allowed to grow passed their saturation density of over 1.8 x 10^6 cells/ml.

The appearance of cytoplasmic inclusions of BrdU-treated S49.1 lymphoma cells follows a pattern similar to that seen for MOPC 315. Up to 20% of these cells contain 1 or 2 particles per cell in untreated mid-log phase cultures. The percentage of cells containing significantly more particles increases during BrdU treatment with kinetics that are qualitatively the same as those described above for MOPC 315.

S49.1TB.2, a BrdU-resistant clone of S49.1, does not show any increase in the cytoplasmic structures following

Figure 14

Kinetics of the accumulation of MOPC 315 cells containing cellular inclusions. As described in the text, a cell was considered positive for inclusion if it contained at least 4 separate structures per cell. Each point was determined by counting 100 cells at 1250 x magnification using phase optics.

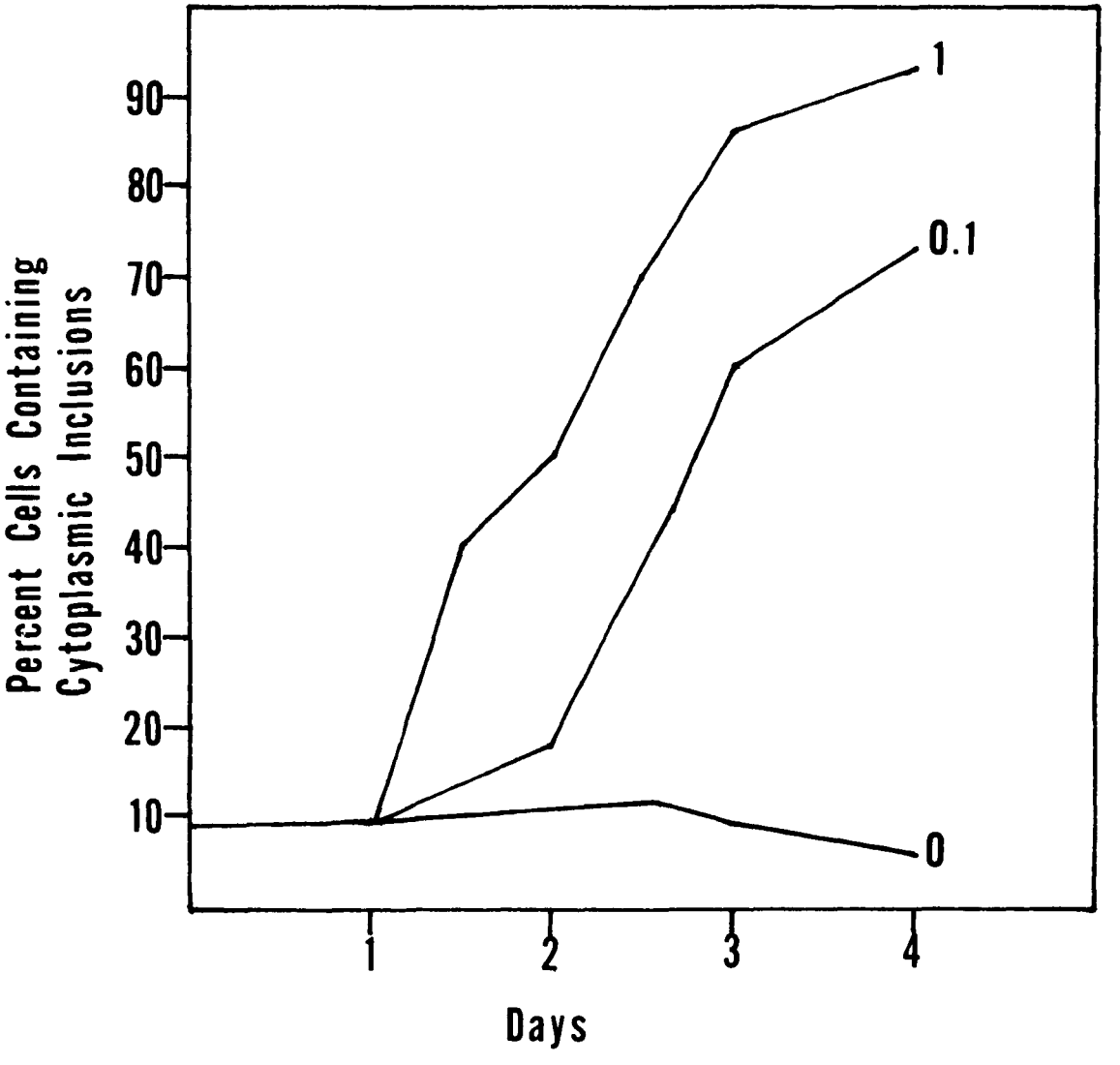
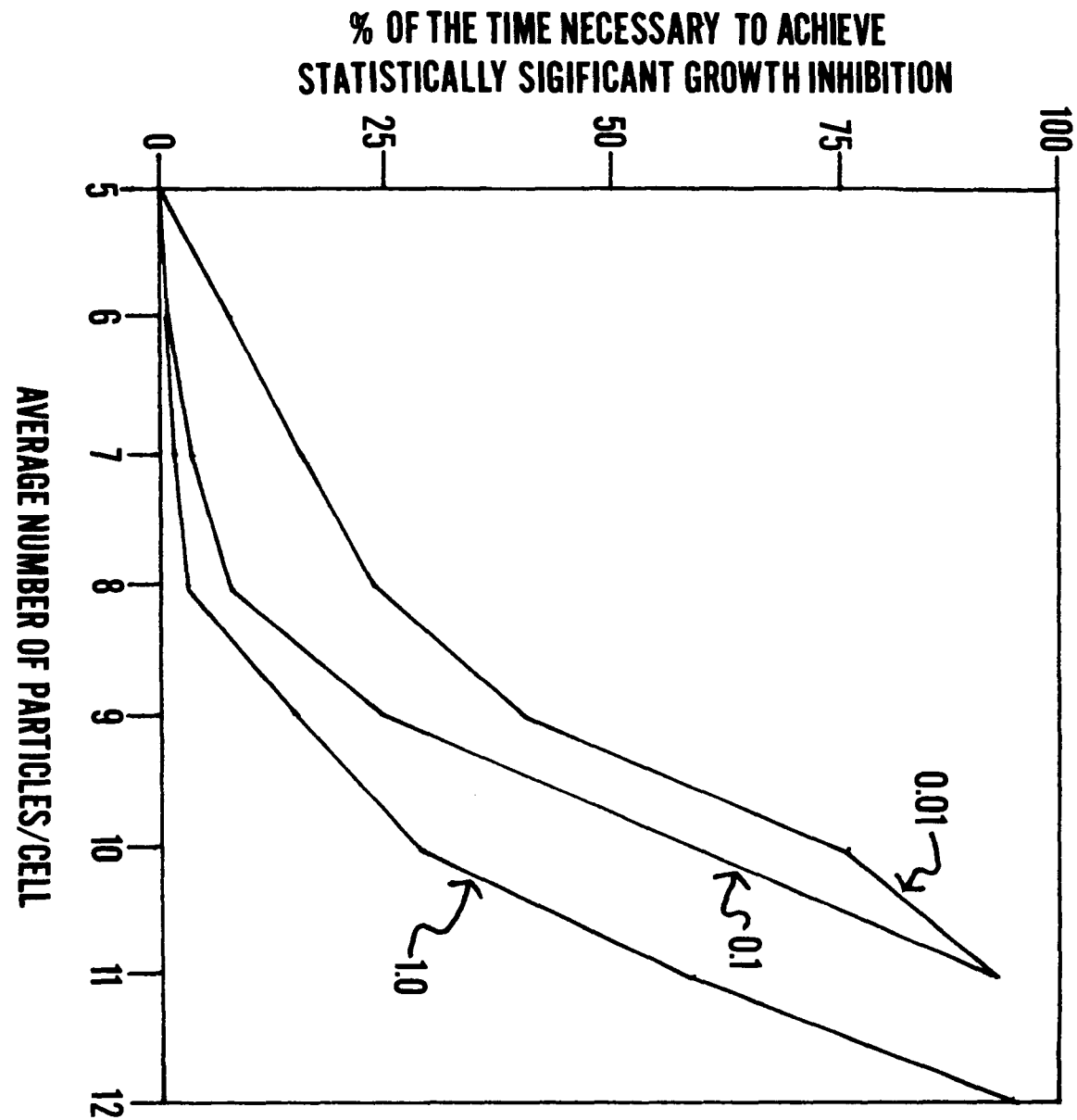

Percent Cells Containing Cytoplasmic Inclusions
90
80
70
60
50
40
30
20
10
1
0.1
0
1
2
3
4
Days

Figure 15

Effect of BrdU treatment on intracellular particle appearance.

MOPC 315 cells were grown in the indicated concentrations of BrdU (in μg/ml). Particles were counted as described in the legend to the proceeding figure.


% OF THE TIME NECESSARY TO ACHIEVE
STATISTICALLY SIGIFICANT GROWTH INHIBITION
0
25
50
75
100
AVERAGE NUMBER OF PARTICLES/CELL
5
6
7
8
9
10
11
12
0.01
0.1
1.0

growth in the presence of BrdU. Figure 13C shows S49.1TB.2 cells after 8 days of growth in 10 μg BrdU/ml. These cells are morphologically indistinguishable from untreated S49.1-TB.2 or S49.1 cells.

Electron Microscopy

The structural abnormalities of BrdU-treated malignant lymphoid cells observed by phase microscopy as described above, were examined by electron microscopy. Figure 16 shows a field of MOPC 315 cells. These cells have eccentricly placed indented nuclei, often with areas of highly electron dense chromatin near the indentation. At this low power, the many mitochondria in the cytoplasm of these cells are readily seen. High power electron micrographs, such as those seen in Figure 17, reveal that these cells contain, in addition to the features described above, virus-like particles which are often found budding from, or near, the plasma membrane. Both rough and smooth endoplasmic reticulum are seen.

Figure 18 shows a field of MOPC 315 cells grown for 3 days in 0.1 μg BrdU/ml. A collection of intracytoplasmic inclusion bodies can be seen in one of these cells. Higher magnification, as shown in Figure 19, reveals that these cells are similar in overall appearance to the untreated cells, except for the inclusion bodies which can be seen

Figure 16

Electron micrograph of cultured MOPC 315 cells.

Figure 17

Untreated MOPC 315 cell. 23,131x magnification of a cell from the micrograph shown in figure 16.

Figure 18

Low power (7,653x) electron micrograph of cultured MOPC 315 cells treated for 3 days with 0.1 μg BrdU/ml.

Figure 19

23,131 x magnification of a cell shown in the micrograph in figure 18.

to have an ultrastructure of their own. Figure 20 shows these inclusions at still higher magnification. At this magnification, it can be seen that these structures have both a peripheral membrane-like ensheathment and an inner membranous reticulum. Figure 21 shows a cell treated for 5 days with 0.001 μg BrdU/ml containing a particularly well developed membranous inclusion.

BrdU-treated cultures often show disintegrating cells containing abundant inclusions that are both smaller and less highly developed than those described above. Similar structures are seen in cultures grown to high density in the absence of the analogue. An example of this is shown in Figure 22.

DEVELOPMENT OF ISOTOPIC METHODOLOGY

Most of the studies to be described in the balance of these results deal with experiments involving the fate of radiolabeled precursors. A general methodology was thus developed so that a uniform and valid protocol may be followed for all of these studies. The general procedure ultimately followed is given in Figure 5 . What follows is a resume of the data upon which this protocol rests.

Cell Centrifugation

Cell suspensions were washed free of extracellular isotope by successive centrifugation in Hank's balanced salt solution plus 15% fetal calf serum (CS-SS). The cell pellets

Figure 20

Higher power electron micrograph of a cell shown in figure 19. Magnification is 38,098x.

Figure 21

23,131 x magnification of a MOPC 315 cell grown for 5 days in 0.001 μg BrdU/ml.

Figure 22

Electron micrograph from a culture of high density untreated MOPC 315 cells.

were centrifuged to a state which, although packed, was easily dispersed.

Number of Washings

Two washes following the initial pelleting of suspended cells is sufficient to remove 99.9% of the radioactivity originally present in the culture media. This reduces the radioactivity to well below that of the intracellular pools in these studies.

TCA Precipitations

Acid insoluble material was precipitated by resuspending the washed cell pellet in 0.5 ml of 10% TCA and centrifuging for 10 min at 770 x g. The supernatant fraction from this precipitation is referred to as the TCA soluble fraction.

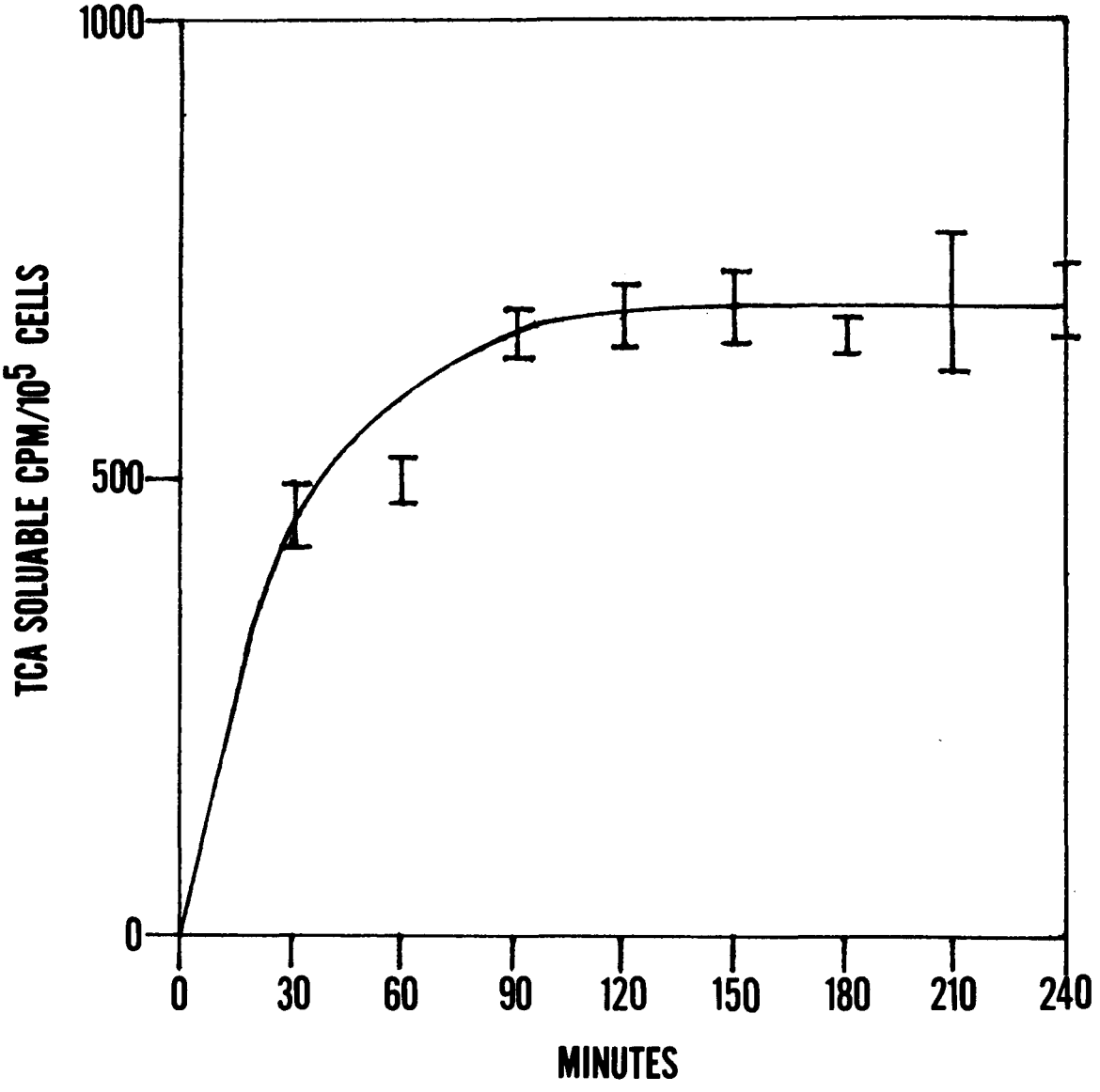
UPTAKE AND INCORPORATION OF BrdU AND THYMIDINE BY MALIGNANT LYMPHOID CELL LINES

Uptake of BrdU

As shown in Figure 23 there is a rise in internal nucleoside for the first 90 minutes, after which the TCA soluble fraction remains constant. As will be described later, during incubations of this length, there appears to be no reutilization of radiolabeled molecules via salvage pathways from degraded polynucleotides. Thus, the steady-steady

Figure 23

Uptake of BrdU (0.325 uM) by S49.1 cells. Cells were pulsed with 1μC ^{3}H-BrdU/ml and the radioactivity assayed as reviewed in Materials and Methods. Similar curves were obtained for MOPC 315. MPC 11, and CCRF/CEM cells. Points are given as the mean $\pm$ SEM.


1000
500
0
TCA SOLUABLE CPM/10^5 CELLS
0
30
60
90
120
150
180
210
240
MINUTES

observed here derives from influx from the extracellular compartment and outflow into DNA.

As seen in Figure 24, the rate of uptake of extracellular nucleoside is dependent on nucleoside concentration. This dependency is greatest at concentrations up to 50 μM, at which point the slope of the curve describing rate become much less dramatic. The slower component of uptake is non-saturable, continuing unchanged up to 130 μM, the highest concentration used. Figure 25 shows the same data plotted as a lineweaver-burk transform. From this, as from the Michealis-Menten curve shown in Figure 24, a 2 component system is possible : a fast facilitated uptake seen at low substrate concentrations and a slow diffusional component evident at higher nucleoside concentrations. The identity of these two types of transport systems is suggested by their kinetic behavior. That the low Km saturable system, but not the slower non-saturable component, may be facilitated suggested in Figure 26, in which the faster component is eliminated by the metabolic inhibitor pCMB. Further evidence for the facilitated nature of the saturable transport process comes from its relatively strong dependency on temperature . At 22°C the rate of uptake at 5 and 10 μM BrdU is reduced to under 50% of the rate at 37°C. The slope of the rate vs. concentration curve above 50 μM is the same at 22°C and at

Figure 24

Concentration dependency of BrdU uptake by S49.1 cells. See legend to Figure 23.

UPTAKE OF BrdU BY LYMPHOMA S49.1 AND
A BUDR RESISTANT CLONE S49.1 TB.2

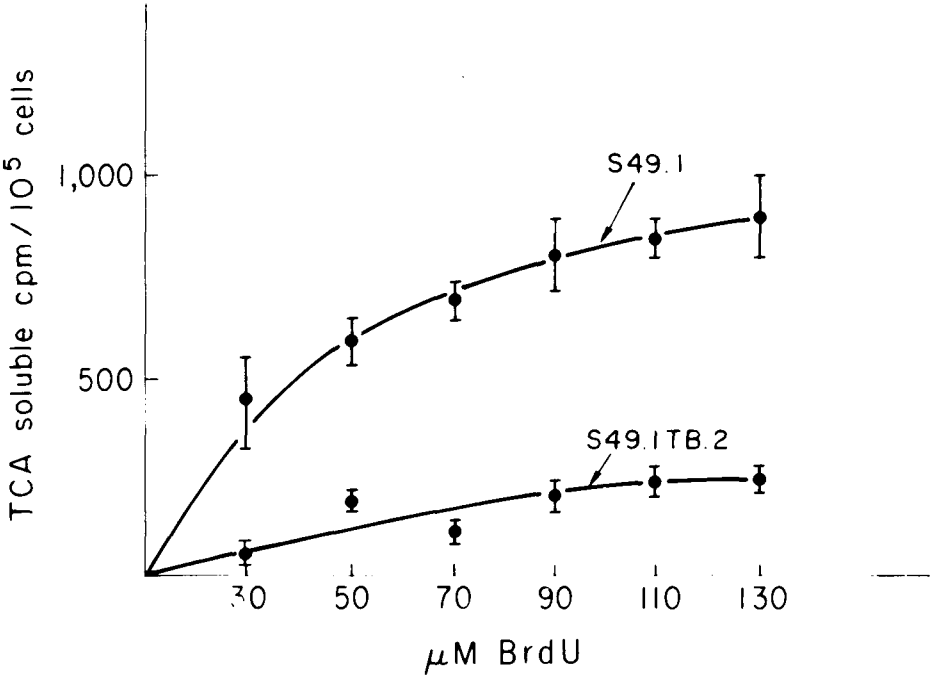


Figure 25

Lineweaver-Burk transform of the data given in Figure 24. See legend to Figure 23.

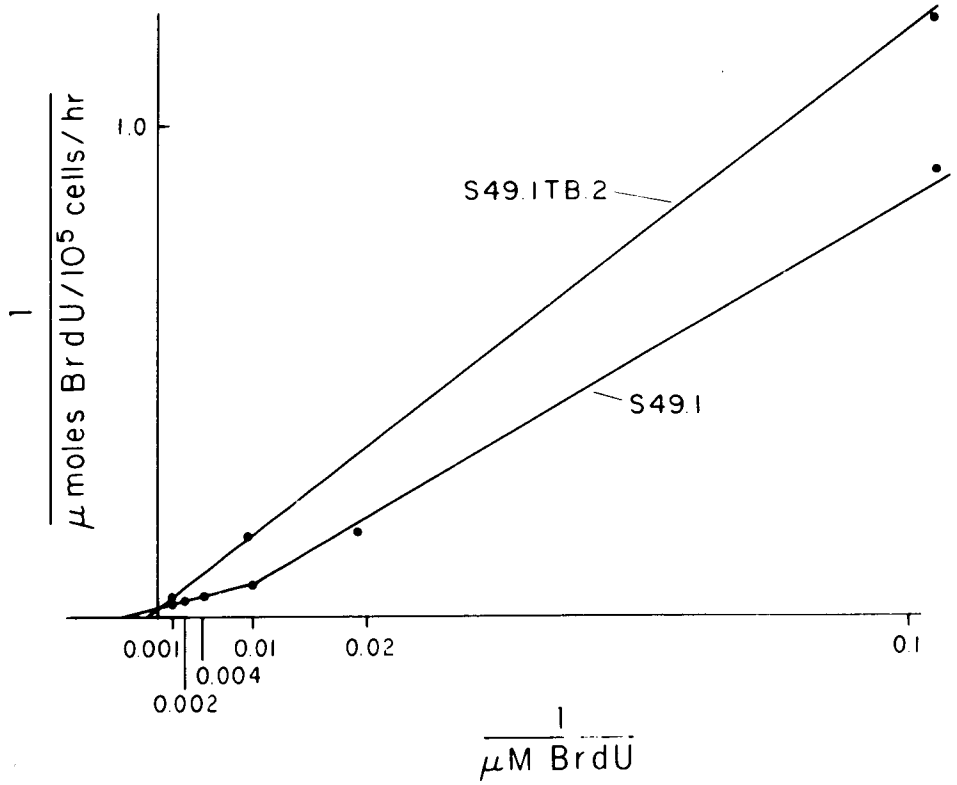
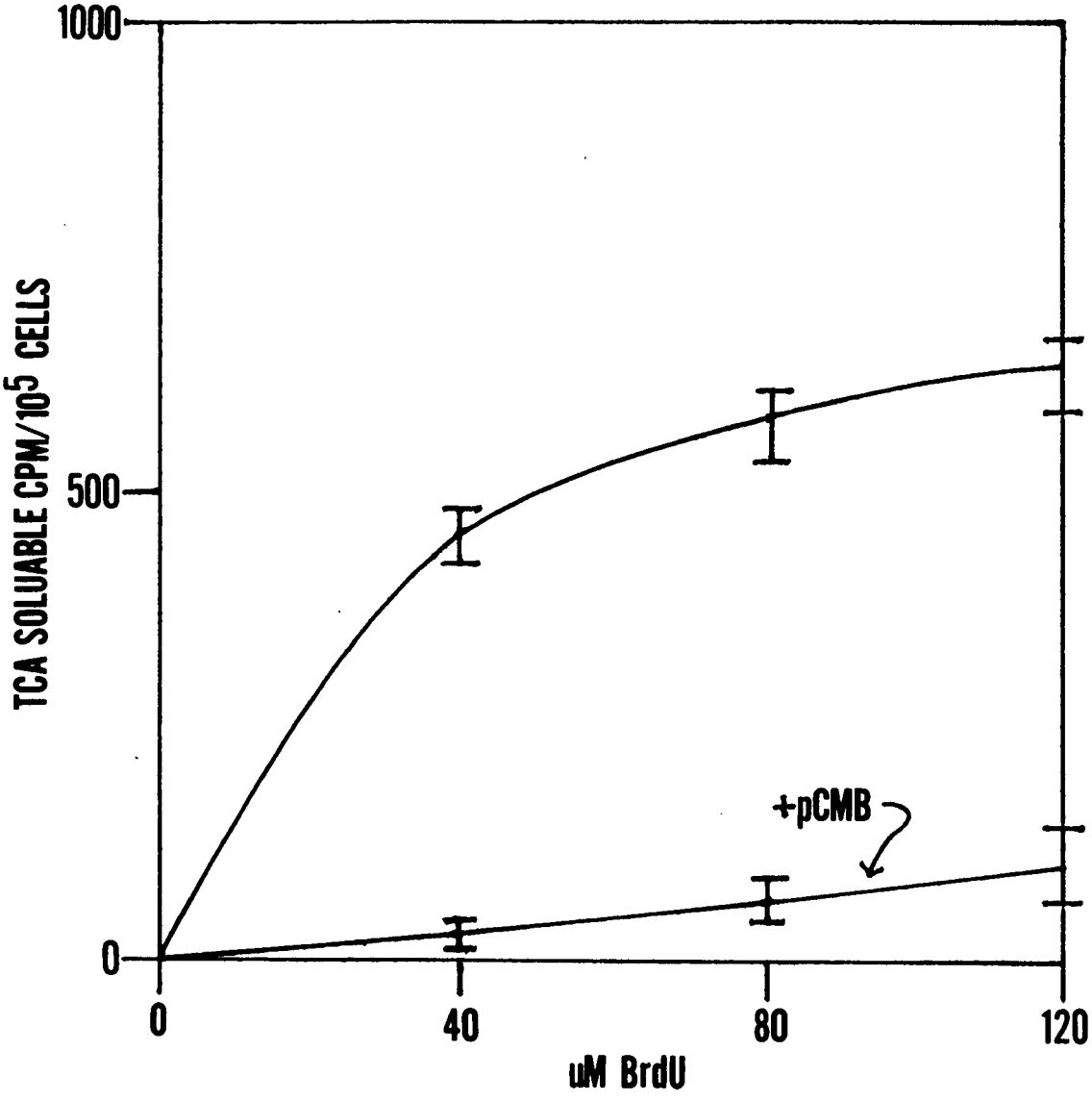

1
μ moles BrdU/10⁵ cells/hr
1.0
S49.1TB.2
S49.1
0.001
0.004
0.002
0.01
0.02
0.1
1
μM BrdU

Figure 26

Effect of 100 μM pCMB on the facilitated uptake of BrdU by S49.1 cells. Cells were pre-incubated for 1 hr in pCMB before being pulsed with BrdU as described in the leged to Figure 23.


1000
500
0
TCA SOLUABLE CPM/10^5 CELLS
+pCMB
0
40
80
120
uM BrdU

37°C.

Table 11 shows the uptake of BrdU and thymidine from 2 clones of BrdU-resistant cells, one from lymphoma S49.1 and the other, known as EL4.BU derived from leukemia EL4. From the table it can be seen that BrdU-resistant clones, lacking the facilitated uptake system for BrdU, also do not actively transport thymidine. Evidence to be presented shortly indicates that both BrdU and thymidine are transported by the same facilitated system.

Unlike the TCA soluble free nucleoside pools, the TCA insoluble macromolecular labeled fraction increases with time for all incubation periods in this study. Figure 27 shows the increase in TCA insoluble cpm for lymphoma S49.1 cells labeled with ^{3}H-BrdU. The increase with time shows considerable deviation from linearity, showing an initial lag period followed by a parabolic increase in counts. The regressions of this curve fit best to a line described by $y = a + bx + cx^2$; $y = ax^b$ and $y = ae^{bx}$ also give fairly close fit. The initial lag period is caused by two factors. First, the increase in nucleoside incorporation into nucleic acid increases as free radiolabeled nucleoside pools expand during the first 100-110 minutes of incubation. There is also a lag resulting from the time necessary to metabolize underivatized nucleoside to the corresponding triphosphate

Table 11

Uptake and incorporation of Thymidine and BrdU by EL4 and EL4 B.U; and S49.1 and S49.1TB.2

Cell Line	Isotope	TCA sol. CPM/10^5 Cells	TCA Insol. CPM/10^5 Cells
EL4	BrdU	255 ± 6.49	2,800 + 192
EL4BU	BrdU	49.0± 1.1*	21.7± 15.2*
EL4	Thymidine	357± 10.7	2,020± 470
EL4BU	Thymidine	2.77± 0.04 *	3.96± 0.53 *
S49.1	BrdU	166± 20	2,980± 470
S49.1TB.2	BrdU	6.3± 2.0*	0.0± 0.0*
S49.1	Thymidine	189± 11.0	2,563± 110
S49.1TB	Thymidine	4.4± 4.4*	6.23± 0.24*

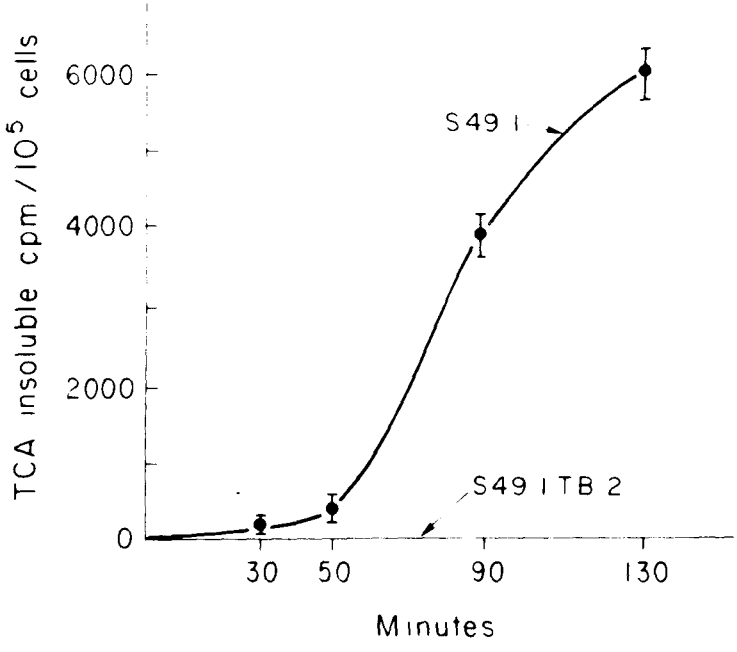
*p≤ 0.001

Uptake and incorporation of luC ^{3}H-BrdU or Thymidine for a 1 hour incubation. Experiments were performed as described in Materials and Methods, Results, and Figure 5 . Counts are given as the Mean± S.E.M.

Figure 27

Incorporation of BrdU into TCA the insoluble fraction of S49.1 cells. See legend to Figure 23.

INCORPORATION OF BUDR IN S49.1
AND S49.1 TB.2



prior to utilization in polynucleotide synthesis.

Competition Between BrdU and Thymidine for Uptake

As shown in Figure 28, there is a competitive relationship between the amount of BrdU taken up by the cells and the thymidine in the incubation media. There is a dose dependent reduction of BrdU uptake at increasing external thymidine concentrations. The competition is also evident at the level of incorporation as measured by the amount of BrdU utilized for DNA synthesis as shown in Figure 29.

The Question of Efflux

The assertion that the small amount of labeled nucleoside in the BrdU-resistant cells is due to a transport defect requires that the following be considered. The BrdU-resistant cells are TK^-, as will be described shortly. Thus, should nucleoside be transported into the cells, it would remain non-phosphorylated. This pool of intracellular free nucleoside, should one exist, might undergo efflux from the cell during the assay procedure described in Figure 5 . If this were the case, determination of the intracellular nucleoside after such a procedure would give an erroneously low value. The experiments described in this section are intended to determine if the rate of efflux of nucleoside from BrdU-resistant TK^- lymphoma cells is such as to significantly affect the quantitation of transport rate.

Figure 28

Uptake of ^{3}H-BrdU by mouse myeloma MPC 11 cells. Cells were incubated with 1μC ^{3}H-BrdU/ml in the presence of the indicated amount of thymidine as described in Materials and Methods. The results given are for a 15 minute pulse. Similar curves obtained with MOPC 315, S49.1 and CCRF/CEM.

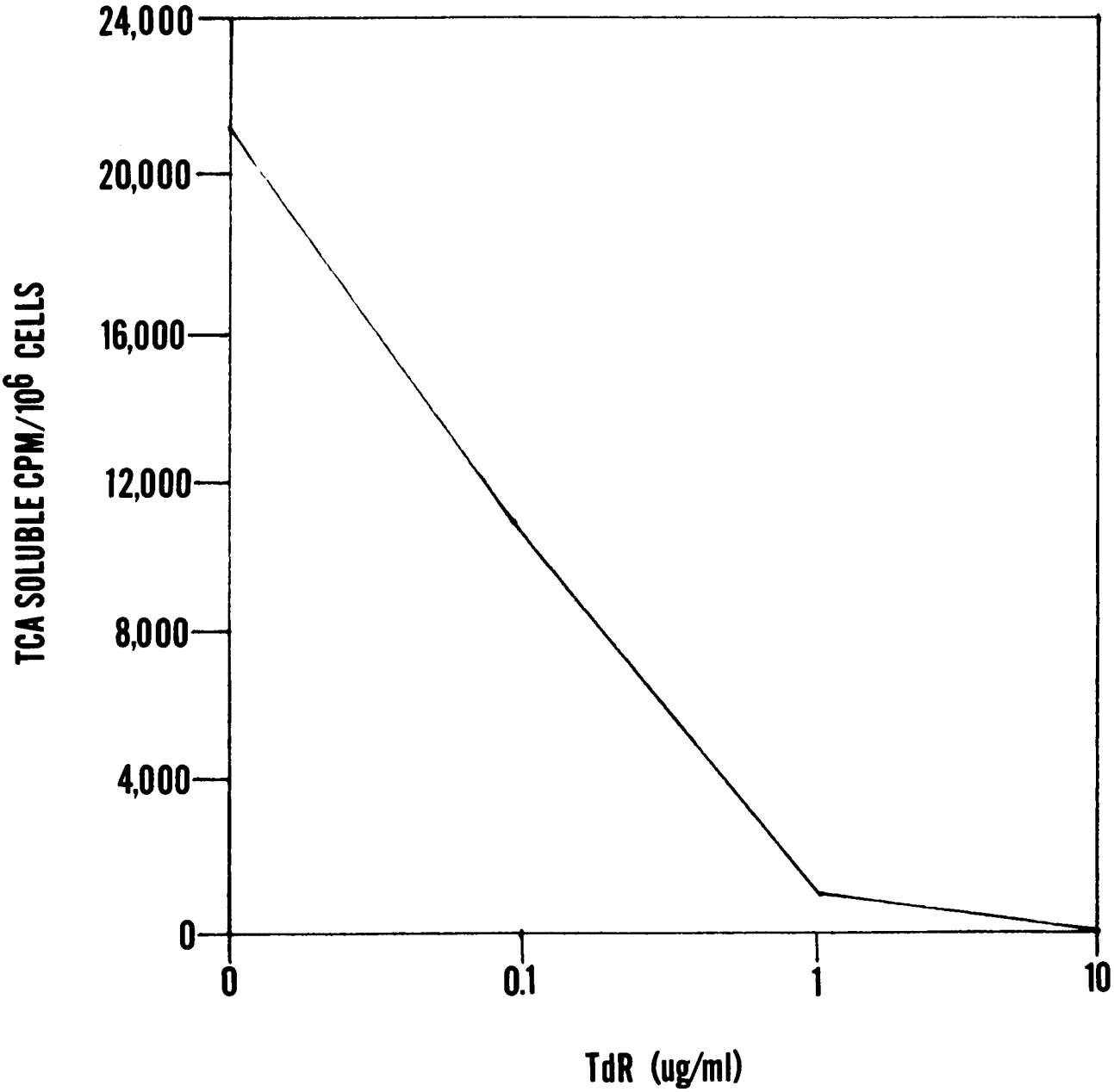
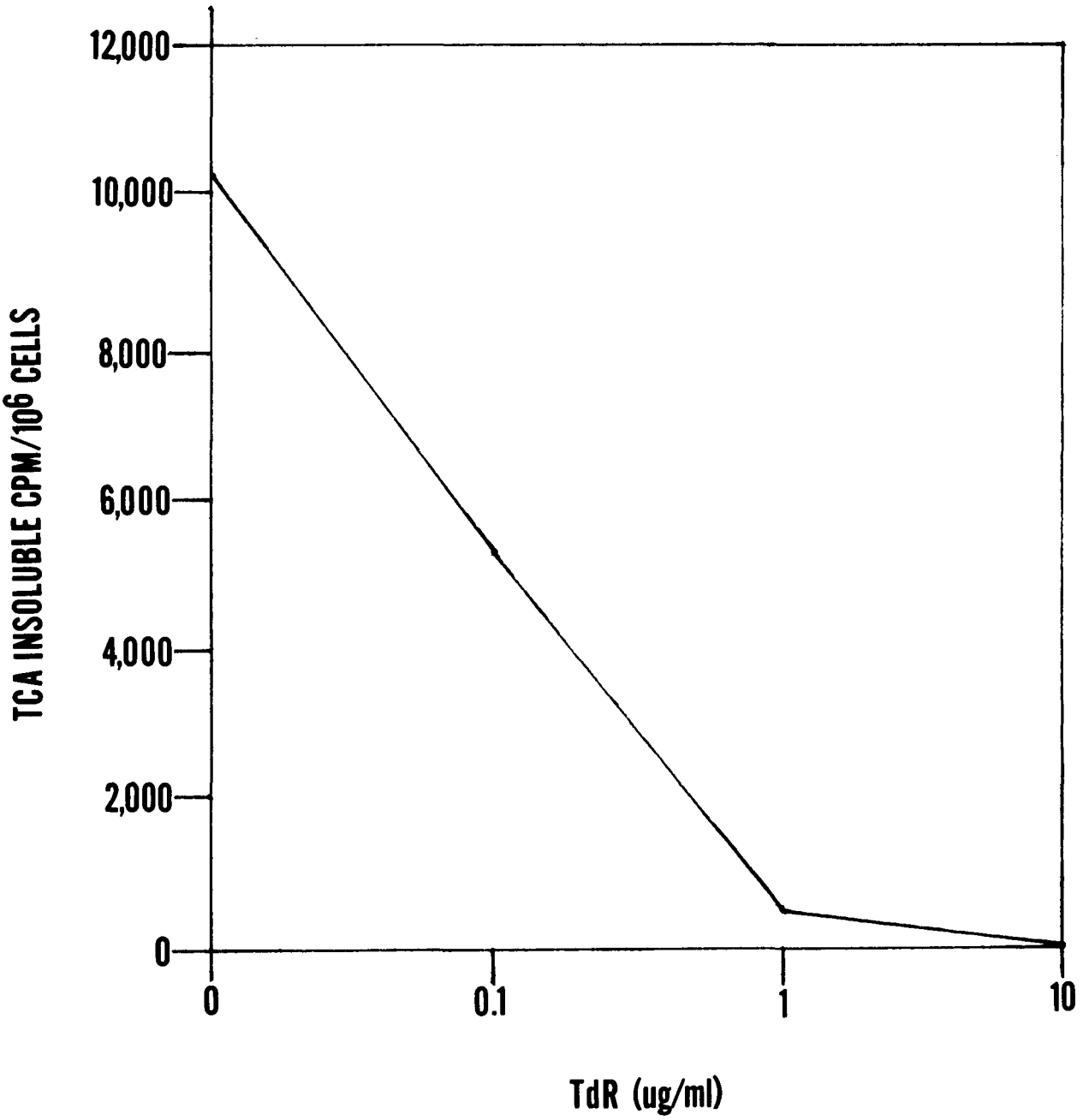

24,000
20,000
16,000
12,000
8,000
4,000
0
TCA SOLUBLE CPM/10^6 CELLS
0
0.1
1
10
TdR (ug/ml)

Figure 29

Incorporation of ^{3}H-BrdU into DNA of mouse myeloma MPC 11 in the presence of the indicated amount of thymidine. See legend to Figure 28.


12,000
10,000
8,000
6,000
4,000
2,000
0
TCA INSOLUBLE CPM/10^6 CELLS
0
0.1
1
10
TdR (ug/ml)

In order to determine if there is significant efflux of nucleoside from cells during our assay procedure the following two questions were asked:

1) Is there a loss of TCA soluble counts when the cells are subjected to repeated centrifugation?

2) Is there an efflux of counts from the TCA soluble fraction when cells are suspended in CS-SS at 0°C?

The data relating to possibility no. 1 is given in Table 12A in which it is seen that there is a 6.7% loss of counts during centrifugation for S49.1. The decrease in TCA soluble counts during centrifugation for S49.1TB.2 is 12.5%. Table 12B shows that there is a time-dependent loss of counts from the TCA soluble compartment of both S49.1 and S49.1TB.2 cells. The rates of decrease of counts are 7.2% per 30 min for S49.1 and 33.2% per 30 min for S49.1TB.2. Thus, the rates of efflux of nucleoside from the resistant cells during our assay procedure is much too small to prejudice our transport experiments.

BrdU METABOLISM

Extracellular

Figure 30 shows the rate of degradation of extracellular BrdU by a culture of MOPC 315 cells grown in medium containing 1 μg/BrdU/10 μc/ml. The only product detected was BU. The rate of degradation of BrdU was 18%/24 hours for MOPC 315. Similar rates were obtained with S49.1, S49.1TB.2 and L929. The degradation of BrdU appears to be only partially biological since BrdU in cell free media

Table 12

Effect of The Number of Cell Washings on

The Recoverey of TCA Soluble Counts

A	CPM/10^5 Cells After 3rd Wash	CPM/10^5 Cells After 4th Wash
s49.1	362 ± 12.5	338 ± 11.0
s49.1TB.2	28.1 ± 3.0	24.6 ± 0.42

Effect of Incubation in Media At 0° on

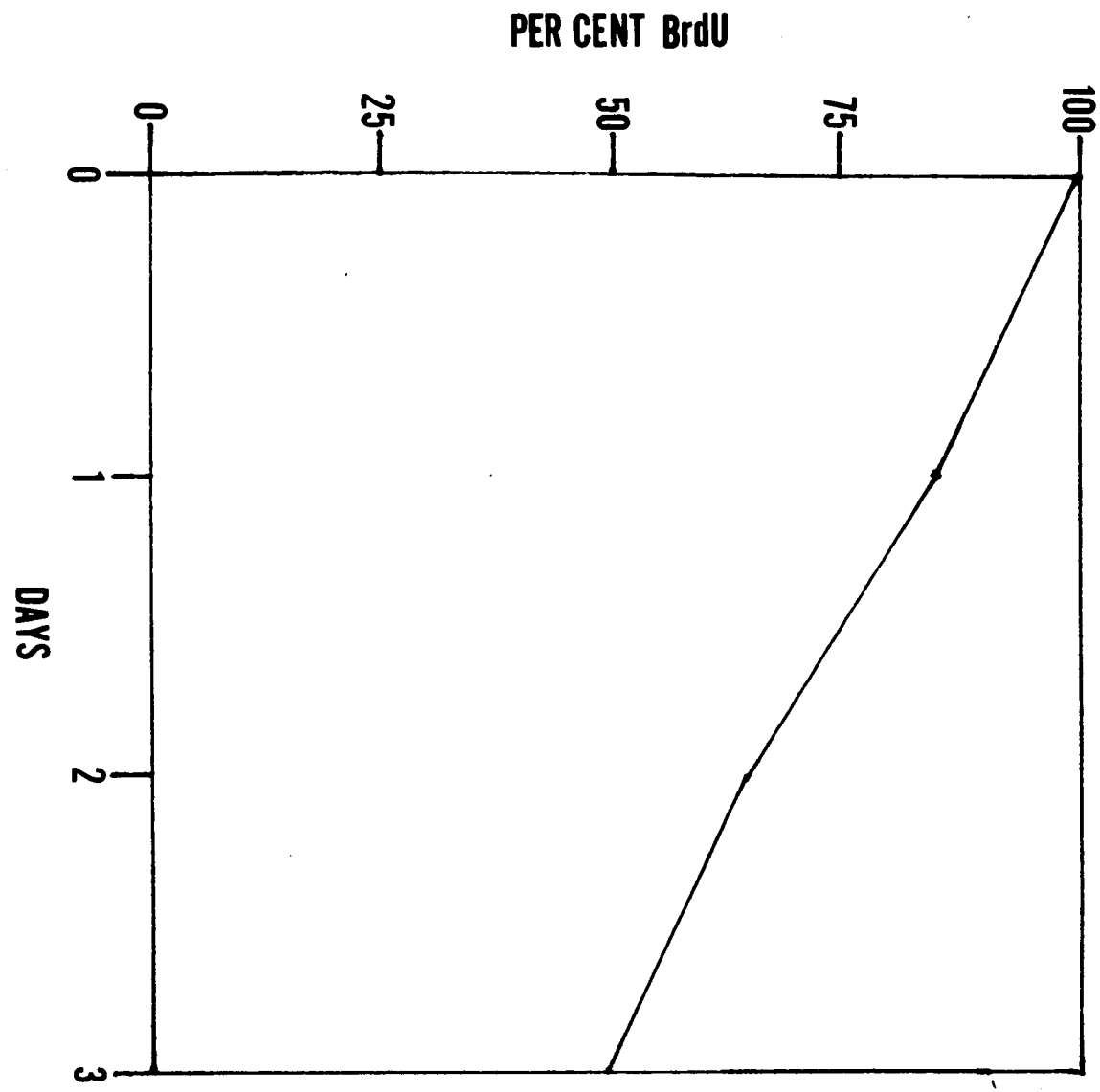
The Recovery of TCA Soluble Counts

B	CPM/10^5 Cells 0 Minutes	CPM/10^5 Cells 10 Minutes	CPM/10^5 Cells 20 Minutes	CPM/10^5 Cells 30 Minutes
s49.1	362 ± 12.5	355 ± 18.6	341 ± 8.5	336 ± 9.0
s49.1TB.2	28.1 ± 3.0	25.3 ± 3.5	21.1 ± 4.0	18.6 ± 1.8

Cells were pre-loaded with ^{3}H-BrdU and subjected to the assay procedure described in Figure 5. After washing the cells free of extracellular isotope they were either subjected to further washing (A) or left to incubate in CS-SS (B).

Figure 30

Rate of degradation of BrdU in a culture of MOPC 315 cells. Cells were seeded at an initial density of 1×10^5 cells/ml, containing 1 μg BrdU/10 μC/ml, and grown as described in Materials and Methods. The conversion of BrdU to BU, the only product found, was measured as described earlier.


PER CENT BrdU
0
25
50
75
100
DAYS
0
1
2
3

is converted to BU at a rate of 10%/24 hours.

Intracellular

BrdU, like thymidine, is quickly phosphorylated upon entry into the cell. Thus, all the intracellular BrdU is found either polymerized into polynucleotides or as free nucleotide. Table 13 shows the steady-state concentration, in terms of per-cent total free radioactivity, of BrdU metabolites. BrdU is present mostly as its triphosphate, less as the monophosphate, and least as its diphosphate. Figure 31 shows the rates of accumulation of BrdU metabolites until the steady state shown in Table 13 is achieved.

ENZYME ASSAYS

Thymidine Kinase

As described in Materials and Methods, two different types of assay systems were used to determine TK activity. Table 14 compares the results of the filter and the TLC type of assays. From the table it can be seen that these two techniques give roughly comparable results provided that a correction is made for the somewhat lower efficiency of counting in the filter paper assay.

In the data presented below we define one unit of TK activity as that quantity of enzyme which converts 1 pmole of thymidine to dTMP/min. The specific activity

Table 13

Distribution of Intracellular Nucleosides in Mouse Myeloma MOPC 315 and Mouse S49.1 Cells

Cell Line	Nucleosides	% Total Radioactivity Present As									
		CMP	CDP	CTP	dCMP	dCDP	dCTP	dBUMP	dBUDP	dBUTP	dTTP
S49.1	^{3}H-Cyd	8.4	3.2	79.6	-	-	8.5	-	-	-	4.1
"	^{3}H-Cyd+BrdU	9.0	3.3	84.4	-	-	4.4	-	-	-	1.7
"	^{3}H-CdR	-	-	5.6	21.6	16.4	59.6	-	-	-	13.0
"	^{3}H-CdR+BrdU	-	-	4.0	16.5	12.0	55.8	-	-	-	22.2
"	^{3}H-BrdU	-	-	-	-	-	-	24.1	3.6	68.9	
"	^{3}H-BrdU+CdR	-	-	-	-	-	-	28.8	2.0	78.6	
MOPC 315	^{3}H-Cyd	9.9	4.5	86.3	-	2.7	5.4	-	-	-	2.2
"	^{3}H-Cyd+BrdU	7.5	2.1	77.0	-	-	11.1	-	-	-	5.1
"	^{3}H-CdR	-	-	7.1	18.5	2.2	67.1	-	-	-	9.1
"	^{3}H-CdR+BrdU	-	-	4.2	16.0	5.8	71.1	-	-	-	6.6
"	^{3}H-BrdU	-	-	-	-	-	-	28.2	11.5	69.9	-
"	^{3}H-BrdU+CdR	-	-	-	-	-	-	21.7	8.7	73.0	-

Cultures were grown for 1 hour in 1uC/ml of radioactive nucleoside with 1ug BrdU/ml where indicated. Chromatography of TCA-soluble fractions were carried out as described in Materials and Methods.

Figure 31

Rate of accumulation of BrdU metabolites by MOPC 315 cells. See legend to Table 13.

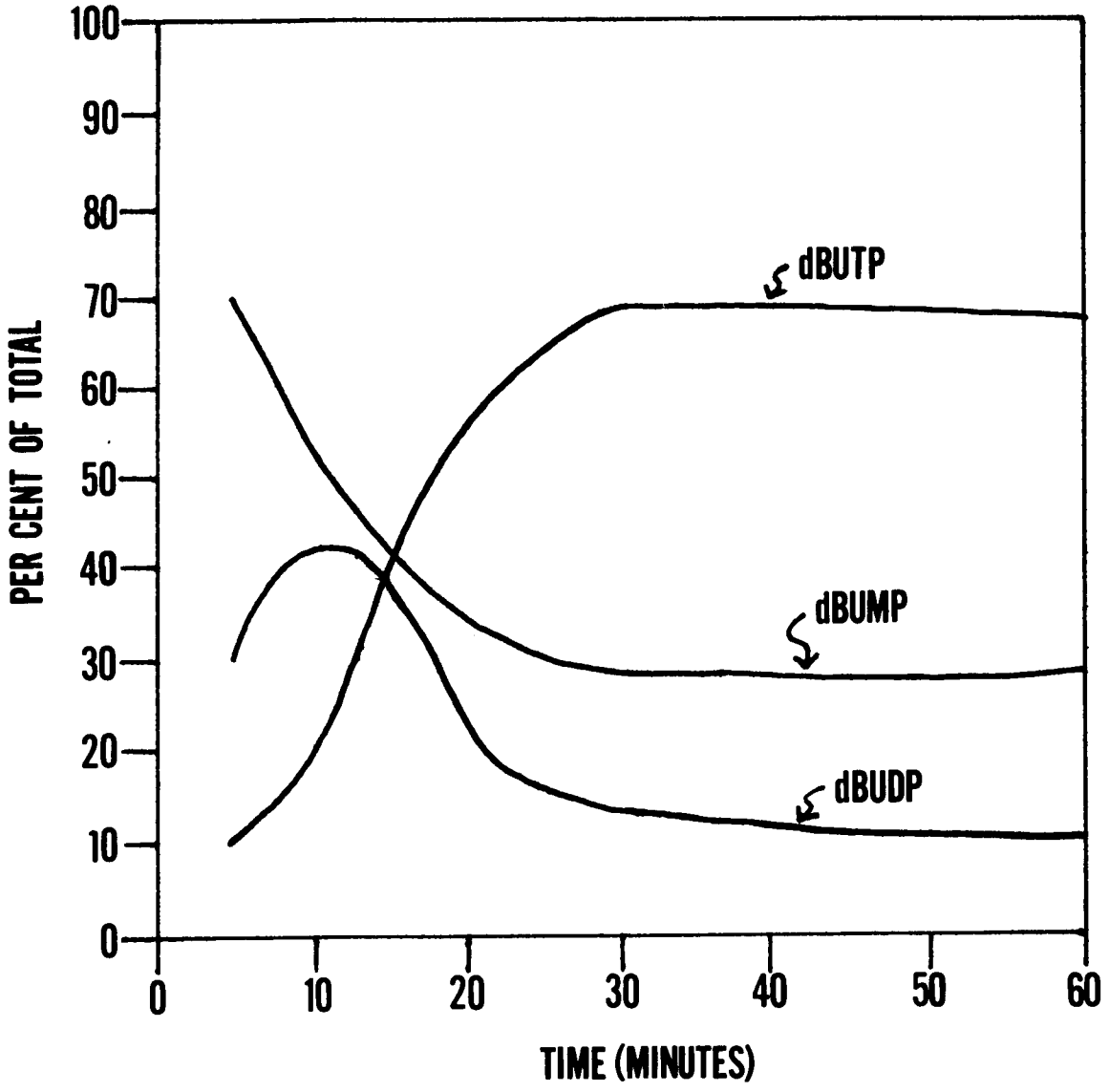

100
90
80
70
60
50
40
30
20
10
0
PER CENT OF TOTAL
dBUTP
dBUMP
dBUDP
0
10
20
30
40
50
60
TIME (MINUTES)

TABLE 14

COMPARISON BETWEEN FILTER AND TLC ASSAYS

Enzyme Source	Type of Assay	% TdR	%dTNP
S49.1	TLC	32.7	67.3
	Filter	28.7	71.3
S49.1TB.2	TLC	90.7	9.3
	Filter	90.2	9.7
B.S.A.	TLC	95.2	4.8
	Filter	92.3	7.6

Cells were disrupted and high-speed supernatants were prepared and assayed as described in Materials and Methods. The reaction was stopped with 0.5ml of 10% TCA which was then extracted twice with 1ml ethyl ether each. Samples were assayed by either PEI TLC or DE 81 DEAE-cellulose paper circles. 20 ul was applied to a PEI plate which was developed in Butanol, Acetic acid, water; or 50ul was applied to DE 81 circles which were eluted with 0.1n^{HC}l 0.2MKCl directly or after 2 washes with 100mls water each. dTNP refers to the total amoun of phosphorylated nucleoside.

is defined as units per mg of protein.

TK activity was constant for at least the first 60 min for MOPC 315 and S49.1, as shown in Figure 32. Figure 33 shows that BrdU is also a substrate for TK. It should be noted that the enzyme handles both substrates similarly. That the phosphorylating activity of thymidine and BrdU is due to the same enzyme is shown in Table 15 , which shows that they mutually compete with each other for phosphorylation while deoxycytidine has no effect on this activity.

The amount of TK activity determined by this assay is directly proportional to the amount of added enzyme. As shown in Figure 34 the specific activity of TK is constant at least up to 10 mg protein while the TK activity increases proportional to the amount of added enzyme.

As shown in Table 16 , most of the dTMP or dBUMP produced by the TK reaction is phosphorylated to the corresponding diphosphate. 10-20% of this is further phosphorylated to the triphosphate.

Table 17 gives the TK activity for MOPC 315 and S49.1. As seen in the table, the activity of the BrdU-resistant clone S49.1TB.2 is nil, the same amount of nucleoside being phosphorylated as in reactions containing BSA. This lack of activity appears to be due to a deficiency of functional enzyme rather than to the presence of an inhibitor of TK

Figure 32

Linearity with time of TK activity from MOPC 315 and S49.1 cells. TK was prepared and assayed by the filter paper method as described in Materials and Methods.

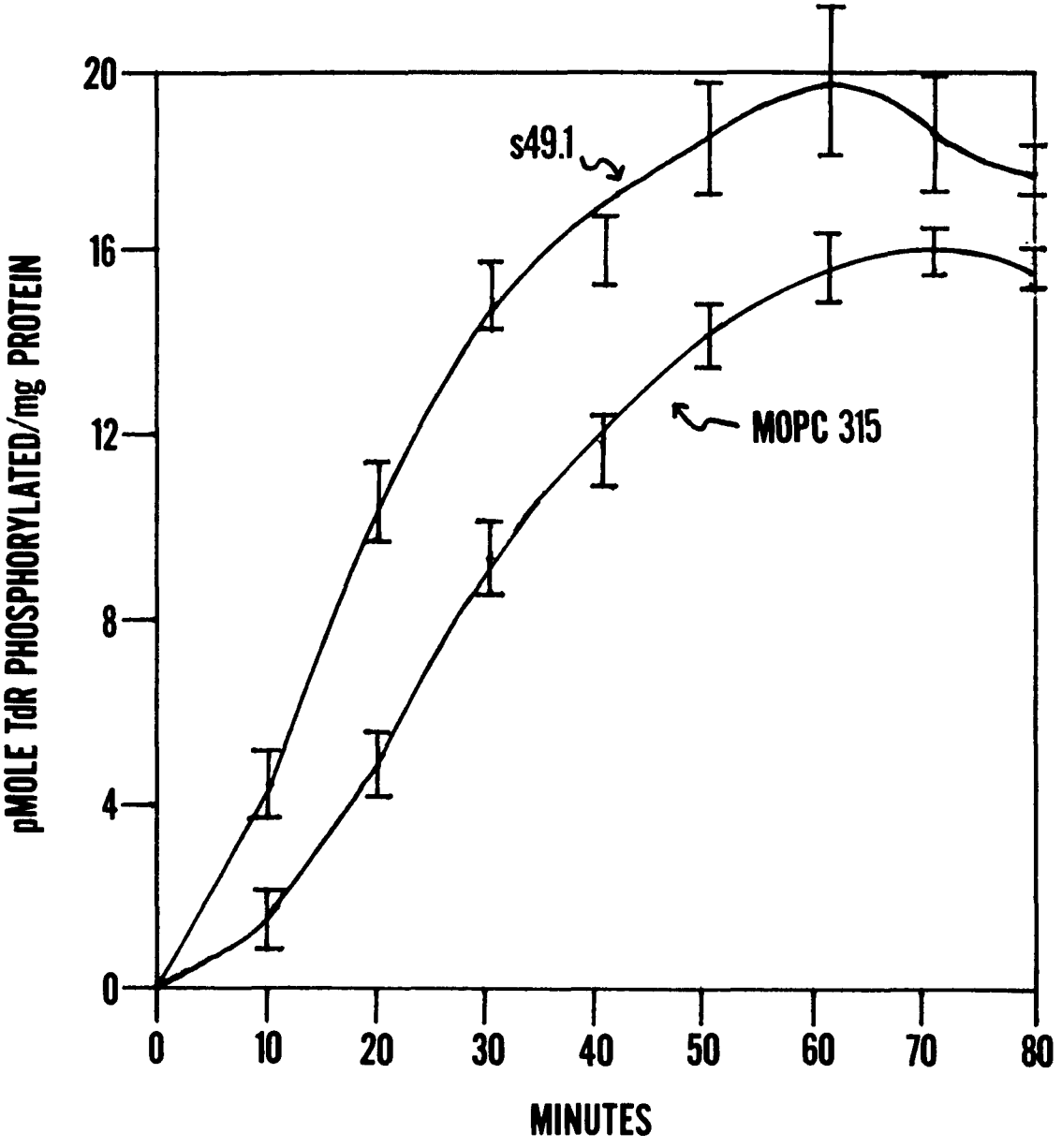

20
16
12
8
4
0
pMOLE TdR PHOSPHORYLATED/mg PROTEIN
s49.1
MOPC 315
0
10
20
30
40
50
60
70
80
MINUTES

Figure 33

Utilization of BrdU by TK. The reactions were run exactly as those represented in Figure 32 except that BrdU was used as the substrate.

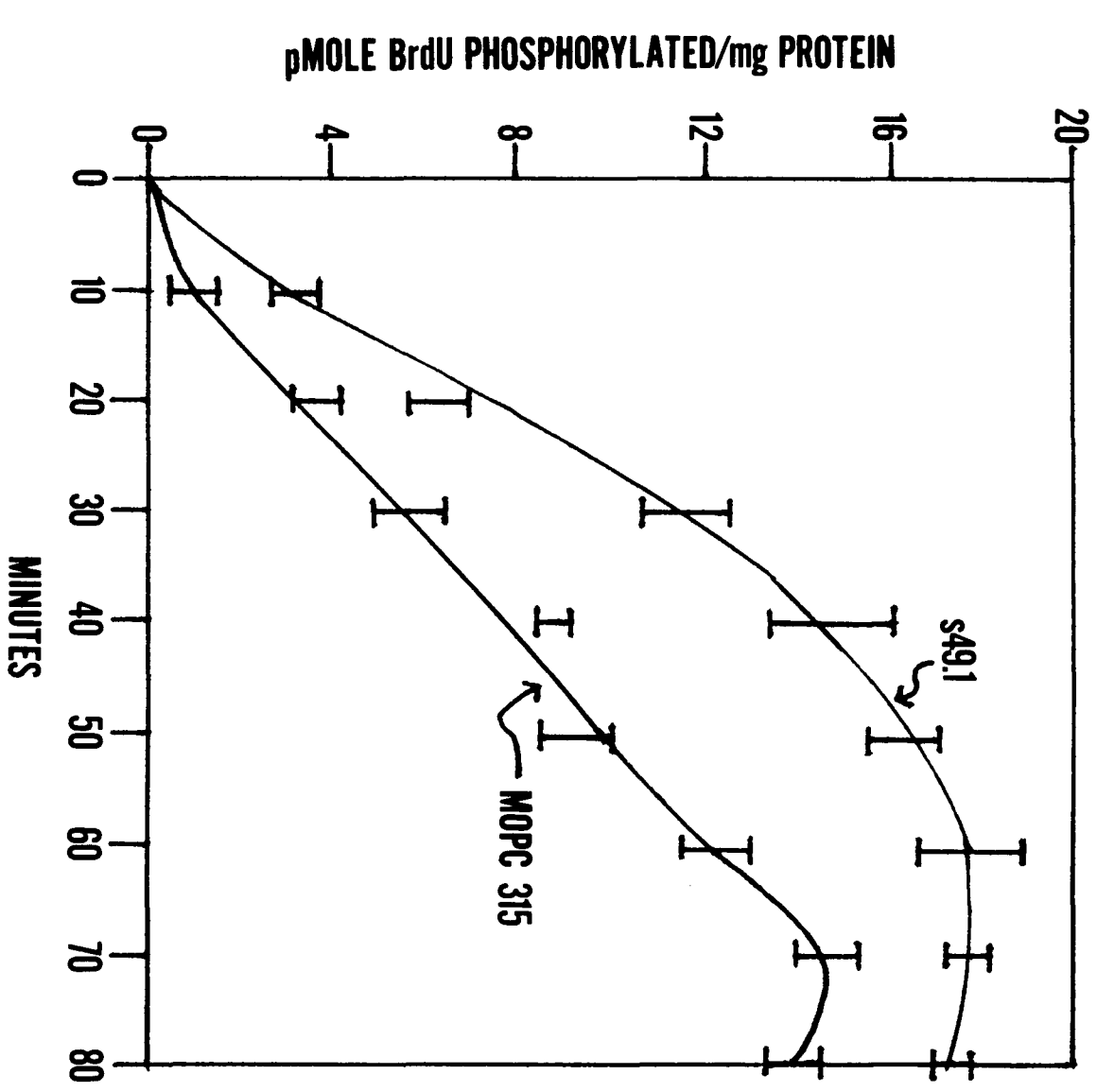

pMOLE BrdU PHOSPHORYLATED/mg PROTEIN
0
4
8
12
16
20
MINUTES
0
10
20
30
40
50
60
70
80
S49.1
MOPC 315

Table 15

COMPETITION FOR TK BY Brdu AND TdR

Label	Competitor	CPM
^{3}H-BrdU	--	539,805
^{3}H-BrdU	0.1uM TdR	4,853
^{3}H-BrdU	1uM TdR	592
^{3}H-BrdU	10uM TdR	71
^{3}H-BrdU	.1mM TdR	0
^{3}H-BrdU	1mM TdR	0
^{3}H-BrdU	10mM TdR	0
^{3}H-BrdU	1mM CdR	499,303
^{3}H-TdR		701,640
^{3}H-TdR	0.1uM BrdU	8,150
^{3}H-TdR	1uM BrdU	901
^{3}H-TdR	10uM BrdU	102
^{3}H-TdR	.1mM BrdU	15
^{3}H-TdR	1mM BrdU	0
^{3}H-TdR	10mM BrdU	0
^{3}H-TdR	1mM CdR	669,222

Figure 34

Linearity with enzyme of TK activity. Various amounts of enzyme preparation were added to the reaction mixture described in Materials and Methods. The enzyme activity was assayed by the filter paper technique.

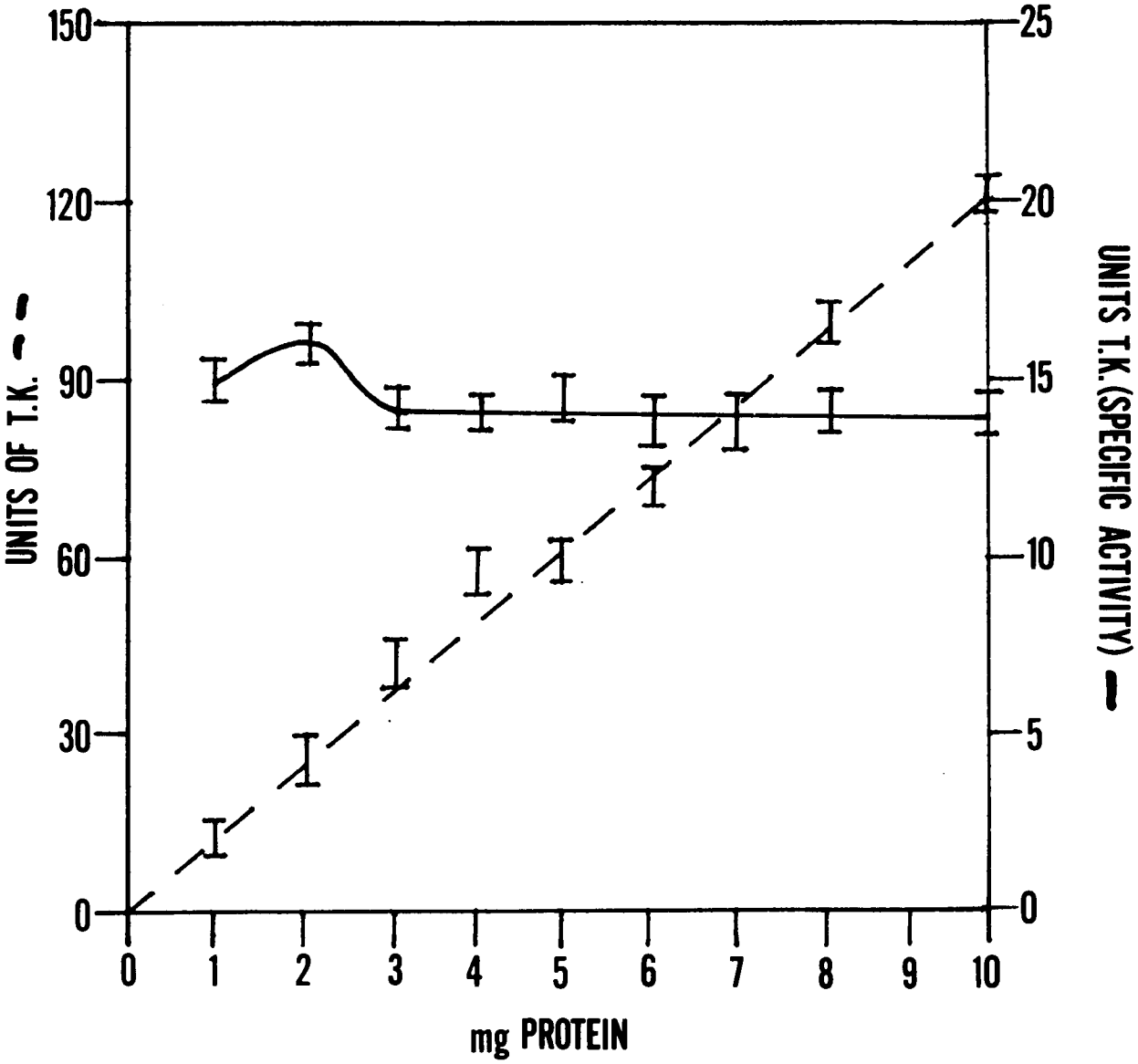

150
120
90
60
30
0
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10
mg PROTEIN
UNITS OF T.K. -
UNITS T.K.(SPECIFIC ACTIVITY) -

Table 16

DISTRIBUTION OF PRODUCTS OF TK REACTION

Enzyme Source	Product	CPM
S49.1	dTMP	9,705
	dTDP	277,500
	dTTP	35,400
	dBUMP	12,258
	dBUDP	282,318
	dBUTP	18,052
S49.1TB.2	dTMP	823
	dTDP	18,930
	dTTP	4,740
	dBUMP	1,360
	dBUDP	20,245
	dBUTP	1,001
MOPC 315	dTMP	8,450
	dTDP	381,214
	dTTP	21,622
	dBUMP	7,440
	dBUDP	441,077
	dBUTP	7,650
BSA	dTMP	1,805
	dTDP	2,445
	dTTP	2,823
	dBUMP	962
	dBUDP	871
	dBUTP	496

Tk Assays were performed as described in Materials and Methods. The products were analyzed on PEI TLC as described in the legend to Table 14.

TABLE 17

SPECIFIC ACTIVITIES OF THYMIDINE KINASE

Enzyme Source	Specific Activity
MOPC 315	14.5
MPC 11	16.7
S49.1	19.5
s49.1TB.2	0.4
EL 4	15.0
EL 4 B.U.	1.1
CCRF/CEM	18.9
HeLa	13.1
L 929	12.8
MMT	13.6

TK was assayed in the 105,000xg supernatent as described in Materials and Methods. The substrate was ^{3}H-TdR and the products were analyzed using the DEAE-Cellulose paper method.

activity since a combination of enzyme preparations from S49.1 and S49.1TB.2 have the activity predicted by the S49.1 component.

Reverse Transcriptase Assays

As reviewed in the Introduction BrdU has been shown to provoke the production of viruses by many cell types. Mouse cells can be almost universally encouraged to synthesize oncorna virus-like particles by treatment with BrdU or its cogener IdU. Since malignant lymphoid cells are unusual in their particularly marked sensitivity to BrdU it is tempting to speculate whether this vulnerability is related to the unusual reluctance of cells of this origin to be induced to produce viral particles. However, IdU in combination with DMSO has been reported to cause murine myeloma cultures to produce virus particles (Stewart et al., 1975).

We have examined the question of virus induction by BrdU in our cultures by several criteria, including reverse transcriptase assay. BrdU does not cause MOPC-315 cells to produce viral particles. However, 1% DMSO causes murine myeloma cultures to produce particles that can be labeled with ^{3}H-leucine but only slightly labeled with ^{3}H-uridine. These particles band at 1.17 g/ml in a sucrose gradient. 1 μg BrdU/ml plus 1% DMSO causes the production of approximately 20 times as much viral material. This data is summarized

in Table 18.

Reverse transcriptase assays on disrupted viral particles induced under a variety of conditions are shown in Table 19. Although there is some activity induced under the conditions of Stewart et al. (1975) the patterns of activity suggests that the enzyme is not a true reverse transcriptase but is instead, probably a DNA dependent DNA polymerase.

EFFECT OF DEOXYCYTIDINE ON THE TOXICITY OF BrdU TO MALIGNANT LYMPHOID CELLS

As reviewed in the Introduction, it has been reported that deoxycytidine can protect several cell types from the cytotoxic effects of BrdU. It was therefore of considerable interest to determine if the toxicity of low levels of BrdU to malignant lymphoid cells was at all affected by the presence of deoxycytidine.

Growth of MOPC 315 and BrdU and Cytidine Nucleosides

Deoxycytidine was found to have no effect on the toxicity of BrdU to MOPC 315. As shown in Figure 35 MOPC 315 grown in 50 μg deoxycytidine/ml grows at a rate slightly faster than that of cells growing in nucleoside-free medium. Cells grown with 1 μg BrdU/ml exhibited the kind of kinetics of cytotoxicity described earlier. 1 μg BrdU/ml and a 50-fold excess of deoxycytidine causes the same kind of toxic effect on MOPC 315 as does BrdU alone. Cytidine has no effect on

Table 1 8

Labelling of MOPC 315 Cell Culture Produced Viruses

Inducer(s)	Label	CPM/10^8 cells/24 hours
--	^{3}H-Leucine	4,250
BrdU		3,080
DMSO		9,000
BrdU + DMSO		159,300
--	^{3}H-Uridine	301
BrdU		451
DMSO		1,100
BrdU + DMSO		6,620

10^8 MOPC 315 cells were grown in, when indicated, 1 ug BrdU/ml, 1% DMSO, 20 uC ^{3}H-Leucine/ml or 20 uC^3 H-uridine/ml. After labelling the media was centrifuged 2,000 RPM for 5 minutes and 10,000 RPM for 10 minutes. The supernatent of this centrifugation was centrifuged for 1 hr at 25,000 RPM. All centrifugations were done at 4^{o}C. The particles pelleting in this centrifugation were resuspended in TNE (10mM Tris, pH 8.0, 15 M NaCl, 10mM EDTA) and counted by liquid scintillation counting.

Table 19

DNA Polymerase Activities from DMSO and BrdU Treated MOPC 315 Cell Cultures

Enzyme Source	Culture Treatment	Template	CPM-Bkg ± SEM
Rausher Leukemia Virus		oligo dT:Poly rA	63,840 ± 2080
		oligo dT:Poly dA	651 ± 197
		oligo dG:Poly rC	57,280 ± 396
		oligo dG	964 ± 36
1 Liter MOPC 315 Media		oligo dT:Poly rA	4,340 ± 671
		oligo dT:Poly dA	6,250 ± 975
		oligo dG:Poly rC	1,010 ± 101
		oligo dG	1,621 ± 161
	BrdU	oligo dT:Poly rA	18,450 ± 1000
		oligo dT:Poly dA	16,660 ± 1110
		oligo dG:Poly rC	2,250 ± 197
		oligo dG	999 ± 41
	BrdU + DMSO	oligo dT:Poly rA	32,450 ± 2,210
		oligo dT:Poly dA	35,160 ± 1,610
		oligo dG:Poly rC	1,800 ± 123
		oligo dG	1,310 ± 197
	DMSO	oligo dT:Poly rA	4,270 ± 113
		oligo dT:Poly dA	4,610 ± 497
		oligo dG:Poly rC	973 ± 51
		oligo dG	2,020 ± 519
1 liter MOPC 315 media high speed pellet		oligo dT:Poly rA	2,750 ± 73
		oligo dT:Poly rA	3,410 ± 146
		oligo dG	1,974 ± 151
	BrdU	oligo dT:Poly rA	3,750 ± 171
		oligo dT:Poly dA	4,110 ± 514
		oligo dG	745 ± 44
	BrdU + DMSO	oligo dT:Poly rA	62,300 ± 14,400
		oligo dT:Poly dA	73,180 ± 8,190
		oligo dG	1,970 ± 518
	DMSO	oligo dT:Poly rA	5,780 ± 131
		oligo dT:Poly dA	3,810 ± 711
		oligo dG	942 ± 180

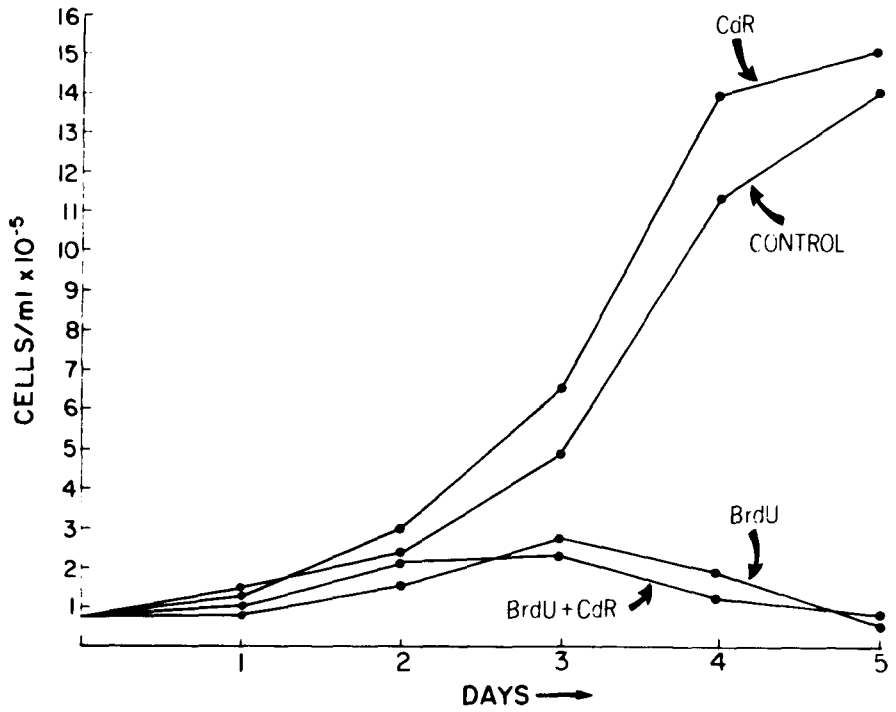
MOPC 315 cells were grown as described in materials and methods in, where indicated, 1 ug BrdU/ml and/or 2% DMSO. Media was assayed 36 hours after the start of the incubation. Total media was precipitated at 50% Ammonia Sulfate (V/V) containing 5000 units of trasylol/ml. The precipitate was dissolved in buffer consisting of 50 mM sodium borate pH 8.0, 5mM DTT, 0.4 MKCl, and 0.6% NP-40; and dialyzed against 10mM potassium phosphate, PH 7.2, 1 mM DTT, 0.2% NP40, and 20% glycerol.

High speed pellets of cell growth media were made by first centrifuging the media 2000 RPM for 5 min and 10,000 RPM for 10 minutes after which it was centrifuged for 1 hr at 25,000 RPM in an SW 27 rotor, the pellets were resuspended in 50 mM sodium borate ph 8.0, 5mM DTT, 0.4M KCl, 0.6% NP40, and 20% glycerol. 10 Microliters of this was used in each 100 microliter reaction.

Figure 35

Growth of MOPC 315 in BrdU and deoxycytidine. Triplicate 2ml cultures were initially seeded at 8 x 10^4 cells/ml in, where indicated, 1μg BrdU/ml and/or 50μg deoxycytidine/ml. Cells were grown and quantitated as described in Materials and Methods.

GROWTH of MOPC 315 in BrdU and DEOXYCYTIDENE



the toxicity of BrdU to MOPC 315. However, as shown in Figure 36, 50 μg of cytidine/ml alone has an inhibitory influence on MOPC 315.

Figure 37 shows the titration of the effect of both deoxycytidine and cytidine on the toxicity of BrdU to MOPC 315. As can be seen from the figure, deoxycytidine alone has a general stimulatory effect on MOPC 315, maximally evident at 20 μg deoxycytidine/ml, causing a growth rate of over 50% more than that of control cells. At deoxycytidine concentrations over 20 μg/ml growth stimulation gradually declines until approximately 35 μg/ml, at which point it tapers off at a stimulation of 120% of control values. The stimulation of the growth of MOPC 315 by deoxycytidine remains constant up to 75 μg/ml, the highest concentration tested. 45 μg deoxycytidine/ml does not even reverse the toxicity of 0.1 μg BrdU/ml.

As seen in both Figure 36 and Figure 37 cytidine causes a dose dependent inhibition of growth of MOPC 315 cells. This effect of cytidine is linear with increasing cytidine concentrations, causing an inhibition of growth to under 50% of growth values for 75 μg cytidine/ml, the highest concentration studied. The effect of cytidine in combination with BrdU was completely indistinguishable from that of BrdU alone.

Figure 36

Growth of MOPC 315 in BrdU and Cytidine. The experiment is essentially the same as that described in Figure 35, except that the cells were grown, where indicated, in 1 μg BrdU/ml and/or 50 μg cytidine/ml.

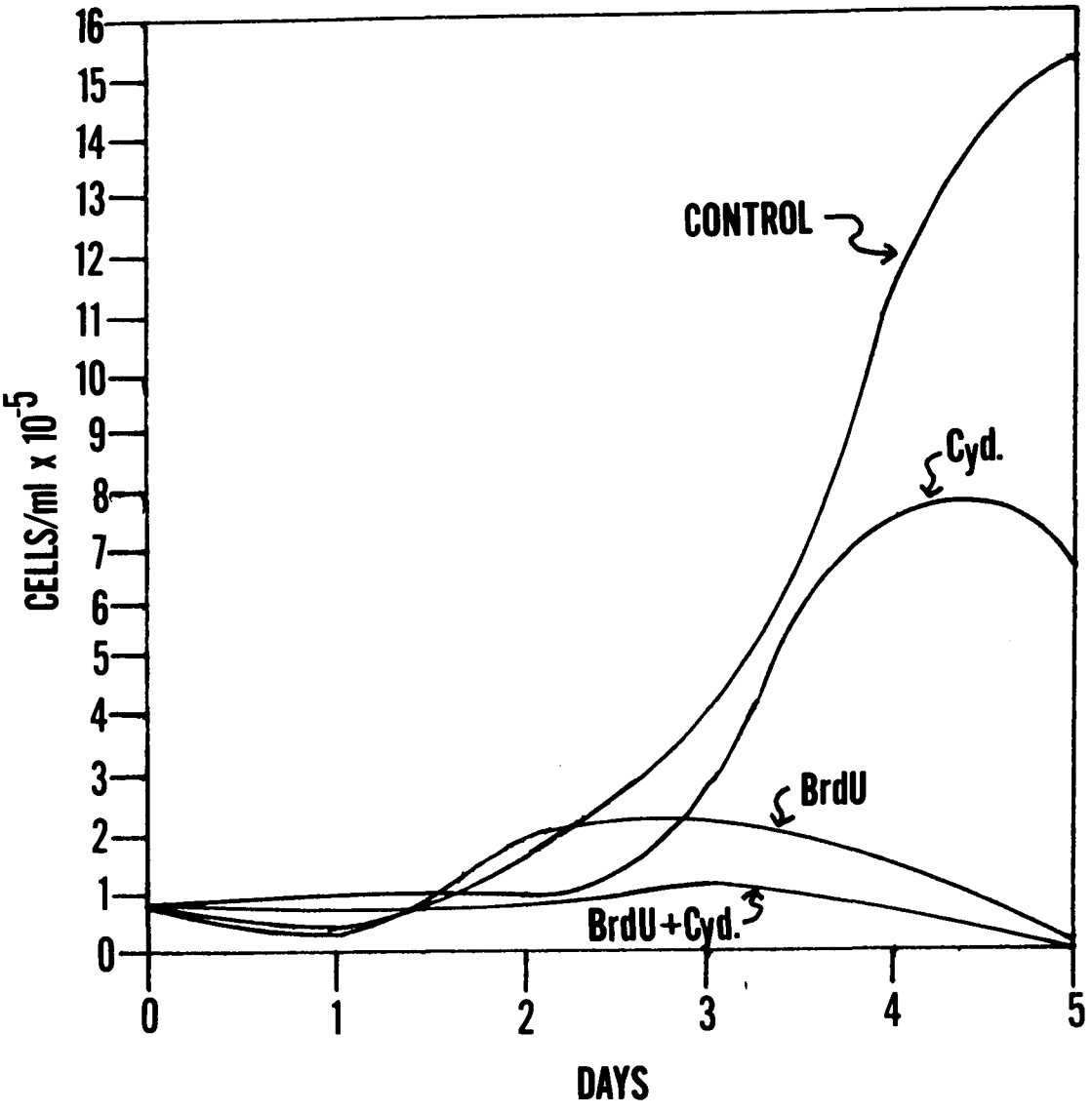
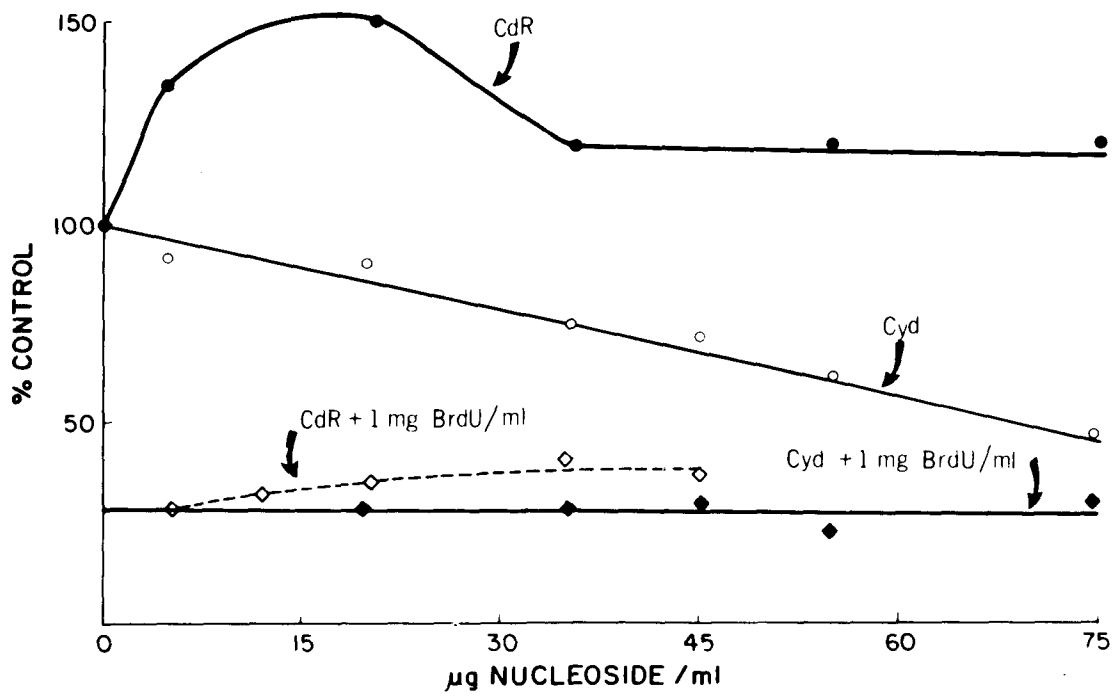

16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
CELLS/ml x 10-5
CONTROL
Cyd.
BrdU
BrdU+Cyd.
0
1
2
3
4
5
DAYS

Figure 37

Triplicate 2 ml cultures were set up as described in Materials and Methods. The data given are calculated on the basis of viable cell counts after 4 days of growth.

EFFECT of BrdU and CYTOSINE NUCLEOSIDES on MOPC 315 CELLS



Growth of MPC 11 in BrdU and Cytidine Nucleosides

Like MOPC 315, myeloma MPC 11 is vulnerable to the toxic effects of BrdU, even in the presence of up to 45 μg deoxycytidine/ml. However, as shown in Table 20, deoxycytidine offes some protection. Cells grown in 45 μg deoxycytidine and 1 μg BrdU/ml grow to a titer of about 75% of control values in 4 days, compared to 47.4% for cells treated with BrdU alone. Cytidine, however, appeared to offer no protection to MPC 11 from the effect of BrdU, the cells in the presence of both nucleosides growing to only 45.1% of control titers. However, unlike the situation for MOPC 315, cytidine alone did not have any toxic properties. Cells grown in 45 μg cytidine had growth rates almost identical to that of untreated control cells.

Growth of S49.1 in BrdU and Cytidine Nucleosides

While deoxycytidine does not offer any protection to mouse myeloma cells against the destructive effects of BrdU, the situation with mouse lymphomas is quite different. As seen in Table 20 cells grown in 1 μg BrdU/ml for 4 days have viable counts of only 31% of that of control cultures. 50 μg of deoxycytidine/ml protects against this toxicity, restoring the cells proliferation to 103% of control values. 50 μg deoxycytidine alone is without significant effect. The toxicity of 50 μg BrdU/ml is not prevented by 50 μg

Table 20

Effect of Cytosine Nucleosides on the Toxicity of BrdU to mouse myeloma MPC 11 and mouse lymphoma S49.1

Cell Line	Nucleoside	% Control on day 4	P
MPC 11	--	100	---
"	1ug BrdU/ml	47.4	<0.01
"	1ug BrdU/ml + 45ugCdR/ml	75.1	<0.05
"	45ugCdR/ml	112	<0.05
"	1ug BrdU/ml + 45ug Cyd/ml	45.1	<0.01
"	45ugCyd/ml	100	N.S.
"	0.1ug BrdU/ml	55.6	<0.01
"	0.1ug BrdU /ml + 45ugCdR/ml	78.8	<0.01
S49.1	--	100	--
"	1ug BrdU/ml	31.0	<0.001
"	1ug BrdU/ml + 50ugCdR/ml	103	N.S.
"	50ugCdR/ml	105	N.S.
"	50ug BrdU/ml	18.0	<0.001
"	50ug BrdU/ml + 50ugCdR/ml	19.0	<0.005
"	1ug BrdU/ml + 50ugCyd/ml	103	N.S.
"	50ugCyd/ml	120	<0.005
"			
S49.1TB.s	--	100	--
"	1ug BrdU/ml	101	N.S.
"	1ug BrdU/ml + 50ugCdR/ml	100	N.S.
"	50ugCdR/ml	106	N.S.
"	1ug BrdU/ml + 50ugCyd/ml	124	<0.001
"	50ugCyd/ml	126	<0.001

Triplicate 2 ml cultures were grown containing the indicated amounts of BrdU, deoxycytidine (CdR), and cytidine (Cyd). Initial cell titers were 7.2, 8.4 and 8.1 x 10^4 cells/ml for MPC 11, S49.1 and S49.1TB.2. Titers of control cultures on day four were 5.99 x 10^5, 1.2 x 10^6 and 9.9 x 10^5 cells/ml for MPC 11, S49.1 and S49.1TB.s. S49.1TB.2 is a BrdU resistant clone of S49.1.

deoxycytidine/ml. It is important to note that 50 μg cytidine per ml also reverses the toxicity of 1 μg BrdU/ml to S49.1 cells. Cytidine alone causes a slight stimulation if S49.1 cell proliferation. Figure 38 shows a titration of the prevention of BrdU toxicity to S49.1 at various doses of deoxycytidine. As little as 10 μg deoxycytidine/ml will protect the cells from the toxicity of 1 μg BrdU/ml. Higher doses of deoxycytidine actually cause the BrdU treated cultures to have growth rates exceeding that of untreated control. Thus, unlike the situation with mouse myloma cells, cytosine nucleosides protect lymphoma cells from the cytotoxic effects of BrdU.

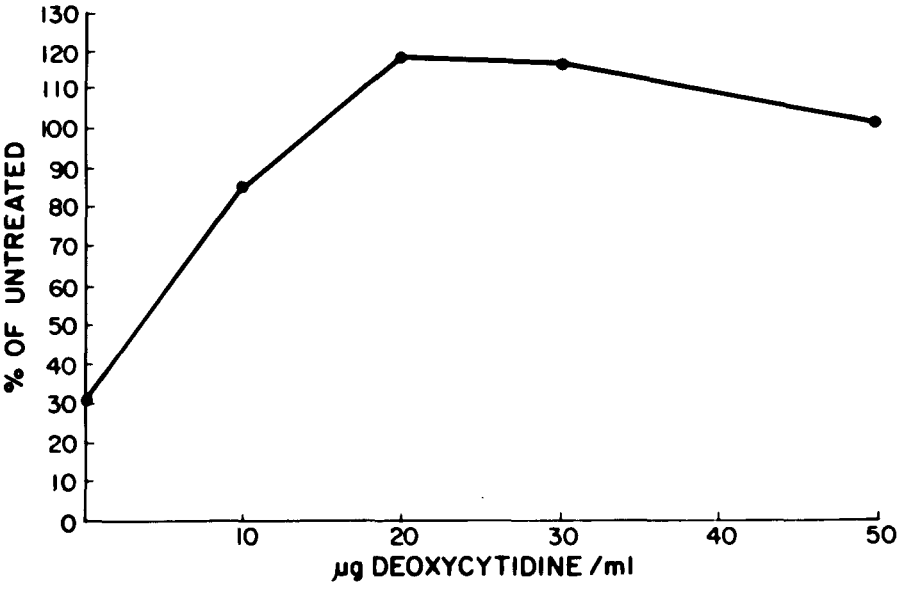
As seen in Table 20, neither deoxycytidine nor BrdU has any effect on the growth of S49.1TB.2. However, 50 μg cytidine, with or without BrdU present, causes a 25% increase in proliferation of these cells.

UPTAKE AND INCORPORATION OF CYTOSINE NUCLEOSIDES BY MALIGNANT LYMPHOID CELL LINES

Since deoxycytidine prevents the toxic effects of BrdU to lymphoma S49.1 cells, it was of interest to determine if the nucleoside interfered with either BrdU transport into the cell, or incorporation into DNA. As seen in Table 21, a 10,000 fold excess of deoxycytidine only inhibits BrdU uptake by 30%. Incorporation of BrdU into DNA under these circumstances is reduced by 60%. The reciprocal experiment

Figure 38

Reversal of the toxicity of 1 μg BrdU to S49.1 by deoxycytidine. Each point represents the mean of replicate cultures as determined after 4 days of growth in the nucleoside.


% OF UNTREATED
130
120
110
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
µg DEOXYCYTIDINE /ml

reveals a similar pattern. Excess BrdU inhibits deoxycytosine uptake by 13%. Deoxycytosine incorporation into the genome is reduced by 28%.

THE METABOLISM OF CYTOSINE NUCLEOSIDES BY MOPC 315 and S49.1

The major metabolic products of cytosine and deoxycytosine are shown in Table 13 . The major intracellular metabolite of cytidine is its triphosphate in both MOPC 315 and S49.1. Under 10% is present as deoxycytidine nucleotides. In addition to these, approximately one-third of the total reduced nucleotides are in the form of dTTP. BrdU treatment causes an approximately 50% decrease in the conversion of cytidine to deoxypyrimidine nucleotides in S49.1, but actually increases cytidine reduction in MOPC 315 cells.

INCORPORATION OF CYTIDINE INTO THE DNA OF MALIGNANT LYMPHOID CELL LINES

BrdU does not inhibit the incorporation of cytidine via ribonucleotide reductase, into DNA. As shown in Table 22 incubation of trace amounts of cytidine with 1 μg BrdU/ml does not reduce the amount of ribonucleoside utilized for DNA synthesis in either myeloma MOPC 315 or lymphoma S49.1. The data given in Table 22 is for a one hour incubation. This pattern, however, remains unchanged for 8 hours, the

Table 21

Uptake and Incorporation of BrdU and Deoxycytidine by Lymphoma S49.1 Cells

Nucleoside	TCA sol. CPM/10^6 cells±S.E.M.	p	TCA insol. CPM/10^6 cells±S.E.M.	p
^{3}H-BrdU	1140 ± 110		5770 ± 52.9	
^{3}H-BrdU + 30ugCdR/ml	765 ± 30.0	≤0.05	2330 ± 14	0.005
^{14}C-CdR	76.6 ± 1.50		192 ± 18	
^{14}C-CdR + 1ugBrdU/ml	66.4 ± 1.90	≤0.05	138 ± 17	N.S.

Triplicate 2ml cultures were grown and treated as described in Materials and Methods. Cells were pulsed with 1uC ^{3}H-BrdU or 0.2uC ^{14}C-CdR per ml. Each well contained 2 x 10^6 cells and incubation was terminated 15 minutes after addition of the nucleosides.

Table 22

Incorporation of Cytosine Nucleosides in DNA or RNA

Cell Line	Nucleosides	CPM DNA/10^6 cells	p	CPM RNA/10^5 cells	p
MOPC 315	^{3}H-Cyd	8760 ± 405	-	67,630	
	^{3}H-Cyd+1ugBrdU/ml	11300 ± 562	N.S.	80,100	N.S.
	^{14}C-CdR	3430 ± 101	-	380	
	^{14}C-CdR+1ugBrdU/ml	2610 ± 115	N.S.	153	0.005
S49.1	^{3}H-Cyd	5510 ± 201		62,820	
	^{3}H-Cyd+1ugBrdU/ml	6990 ± 426	N.S	91,504	$<$0.05
	^{14}C-CdR	3270 ± 112		161	
	^{14}C-CdR+ugBrdU/ml	5400 ± 282	$<$0.005	102	N.S.

Triplicate 2ml Cultures were set up and treated as described in Materials and Methods. Cells were pulsed with 1uC ^{3}H-Cyd or 0.2uC ^{14}C-CdR and incubated for 1 hour. Each well contained between 1 and 2 x 10^6 cells/well.

longest incubation studied. The incorporation of cytidine into RNA is unaffected by BrdU for MOPC 315 and slightly stimulated for S49.1. Counts from deoxycytidine ultimately incorporated into RNA were reduced by coincubation with BrdU.

EFFECT OF BrdU ON MOPC 315 IN VIVO

Because of the possible chemotherapeutic utility of BrdU in the treatment of lymphoid tumors it was of interest to see if BrdU alone, or in combination with deoxycytidine, is effective against mouse tumors. Since the most likely type of tumor for chemotherapeutic success is a myeloma, MOPC 315 was used for these studies. Myeloma cells, like malignant lymphocytes, are extremely sensitive to the toxic effects of BrdU. However, unlike lymphocytes, the plasmablasts are not protected by cytosine nucleosides. Hence, a BrdU/deoxycytosine combination could be a powerful regimen, being cytotoxic to the myeloma cells while other cell types are protected.

Figure 39 shows the effects of dose on the toxicity of BrdU to normal female BALB/c mice on a "seven days on five days off" regimen. From the figure it can be seen that 50 mg BrdU/day was toxic to 100% of the mice. 10mg/day, on the other hand, was much less toxic, the mortality rates of this treatment being 11%.

Figure 39

Effect of does on the toxicity of BrdU to Balb/c mice. Mice were given 0, 10, 25, 50, 75 or 100 mg BrdU/day intraperitoneally on a seven days on - five days off regimen. At 50mg/ml the mean time to death was 18 days.

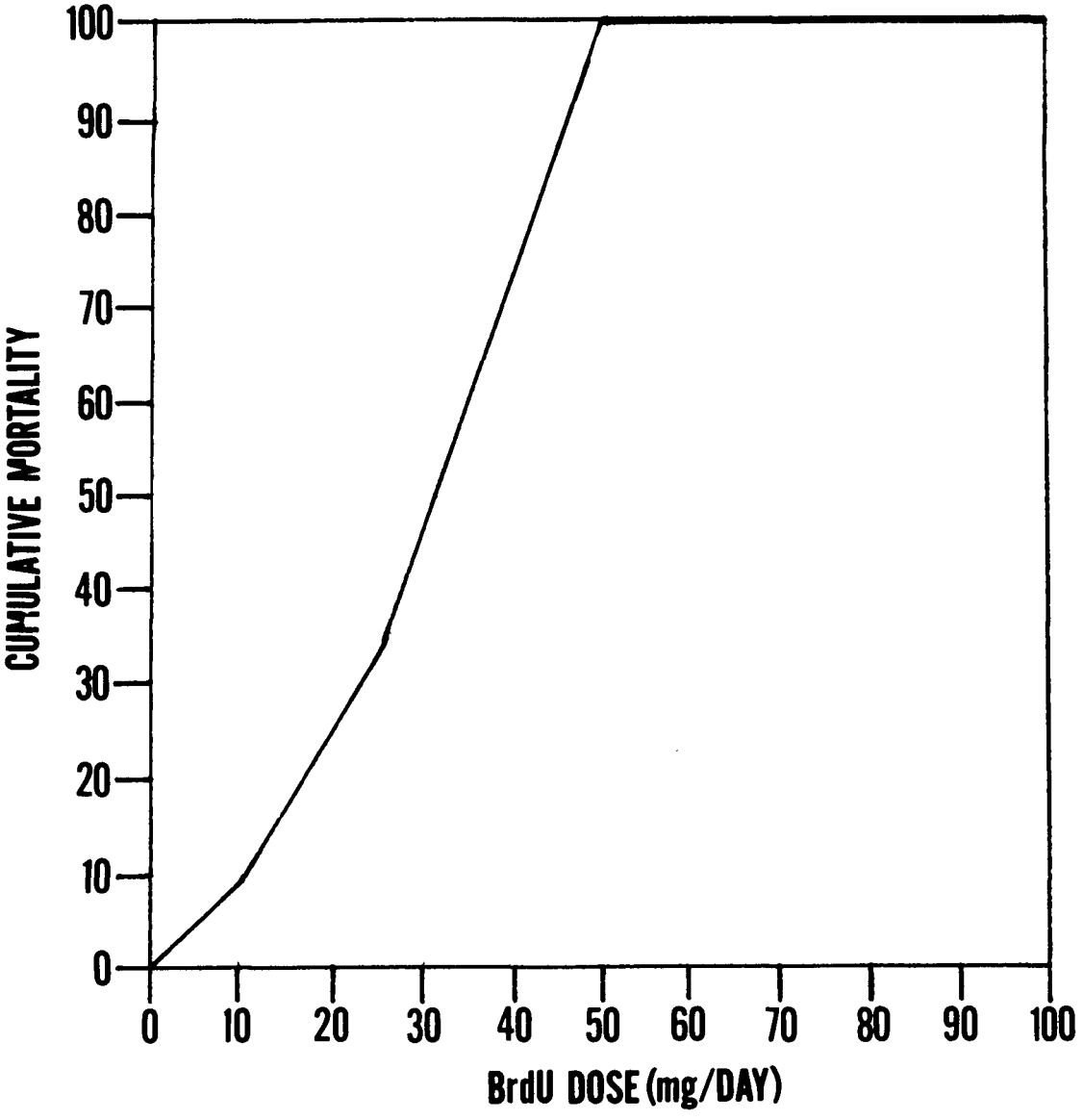

CUMULATIVE MORTALITY
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
80
90
100
BrdU DOSE (mg/DAY)

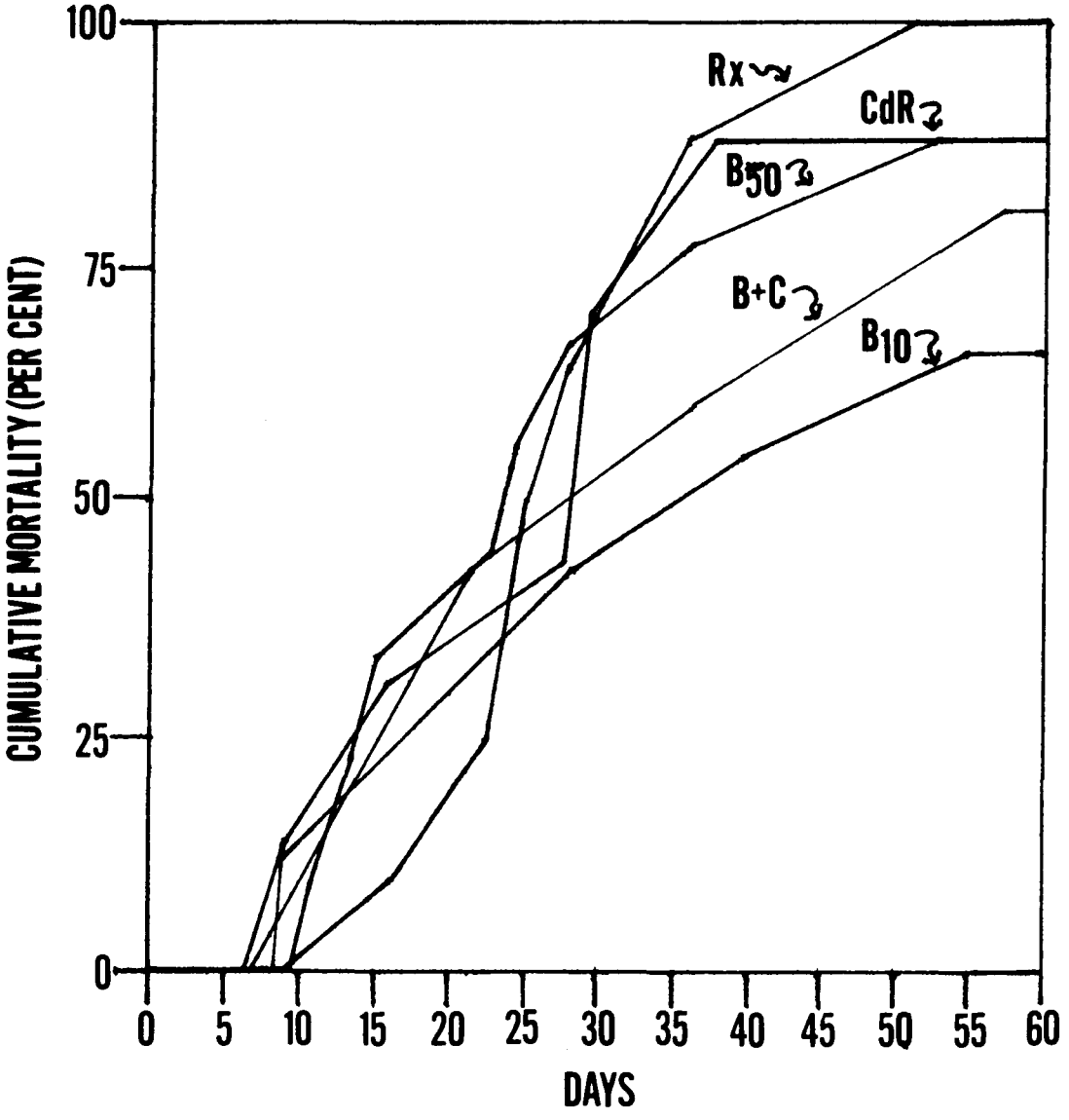
Figure 40 shows a chemotherapeutic experiment on mice bearing MOPC 315 receiving either saline, 50 mg BrdU/day, 50 mg BrdU + 50 mg deoxycytidine/day, 50 mg/deoxycytidine/day, or 10 mg BrdU/day. From this experiment it can be seen that 50 mg BrdU/day is fatal if given to tumor bearing as well as normal mice. Simultaneous administration of deoxycytidine seems to protect the mice from the toxic effects since mice receiving the double regimen had a mortality rate less than that predicted by the tumor incoulation alone. Curiously, deoxycytidine alone had some protective effect.

Mice treated with 10 mg BrdU/day shows a slightly lower mortality than those treated with saline alone. Based on the results above, it is possible that a regimen of 10 mg BrdU + 50 mg deoxycytidine/ml will result in an even better response to the treatment.

The various factors disposing against a more dramatic therapeutic result in this study and other approaches to the use of BrdU, are considered in the Discussion.

Figure 40

Chemotherapy of MOPC 315 with 50 mg BrdU/day (B_{50}), 10 mg BrdU/day (B_{10}), 50 mg BrdU + 50 mg CdR/day (B + C), or 50 mg CdR/day (CdR) on the seven days on - five days off regimen. Rx means mice received only physiological saline. Each group consisted of from 7-11 mice. Treatment was started when tumors were palapble.


100
75
50
25
0
CUMULATIVE MORTALITY (PER CENT)
Rx
CdR
B50
B+C
B10
0
5
10
15
20
25
30
35
40
45
50
55
60
DAYS

DISCUSSION

SENSITIVITY OF MALIGNANT LYMPHOID CELL LINES TO BrdU

We have shown that malignant lymphoid cell lines are unusually sensitive to the cytotoxic effect of BrdU. The cells are killed at concentrations of the analogue that do not affect most other cell types. Ranges of sensitivity to BrdU for a large variety of cell types are known, both from the published scientific literature, as reviewed in the Introduction, and from our own studies, as discussed below.

We define a cytotoxic effect here to be one that satisfies the three criteria of reduction in growth rate, increase in the proportion of trypan-blue accepting cells, and loss of subculturability. These criteria were satisfied in all studies described herein establishing cytotoxicity. The cell line initially used for these studies, which therefore served as a paradigm for BrdU sensitivity, is mouse myeloma MOPC 315. Growth of this line in 1.0 or 0.1 μg BrdU/ml results in an acute toxic effect, satisfying the criteria described above in three and five days, respectively. MOPC 315 grown in 0.01 and 0.001 μg BrdU/ml can be subcultured several times before cytotoxic manifestations become apparent. However, even at these very low BrdU concentrations the cultures eventually deteriorate, experiencing a protracted but nevertheless lethal course of growth.

This chronic cytotoxic effect is evident, with some variation from experiment to experiment, on days 17 and 20 for 0.01 and 0.001 μg BrdU/ml, respectively. A graph of toxicity vs BrdU concentration reveals an LD_{50} of 0.09 μg/ml for 4 days growth. The design of experiments evaluating various other cell lines was such as to search for a pattern of acute cytotoxicity as described above MOPC 315, and determine and compare LD_{50}s for the cells studied.

The cytotoxic manifestations of BrdU to MOPC 315 were found not be restricted to that particular line of myeloma cells. Studies on another mouse myeloma, MPC 11, reveal a pattern of acute toxicity caused by BrdU, with kinetics similar to that described above for MOPC 315. Similarly, the LD_{50} for MPC 11 is 0.20 μg/ml which is not dramatically different from the LD_{50} of 0.09 μg/ml for MOPC 315.

Studies with murine lymphoma S49.1 and human acute lymphoblastic leukemia CCRF/CEM reveal that the cytotoxic effects of BrdU to malignant plasma cells extends to various other malignant cells of lymphoid origin. These cells, when grown in BrdU, show a pattern of acute cytotoxicity kinetically similar to that described above for mouse myelomas. The LD_{50}s for the effect of BrdU in malignant lymphocytes is 0.16 μg/ml for S49.1 and 0.31 μg/ml for CCRF/CEM, which are quite similar to the values for myeloma cells.

The discrepancy between the extreme cytotoxicity observed by us in studies on malignant lymphoid cells, and the less pronounced killing at the same BrdU concentrations for other cell types as reported in the cumulative literature could be explained in three alternative ways. Studies on phenotypic modulation of cells grown in BrdU are based on an argument that requires the assertion that these effects are not simply associated with cell killing. For example, statements that BrdU causes chondrocytes to stop producing CS are of trivial significance if this is simply a concommitant of a generalized toxicity associated with BrdU treatment. Thus, studies with BrdU may have been carried out only on those cell types for which culture in its presence had been feasible. This kind of selection of cell types can serve to suggest in the scientific journals that most cell types are relatively unharmed by the analogue. A second possible way to explain the apparent unusual sensitivity of malignant lymphoid cells to BrdU is that there is some factor in the expermental procedure that creates the mistaken impression that BrdU treatment <u>per se</u> was responsible for the unexpected toxicity. For example, a radiation leak, which could even be from the fluorescent light source in the area in which the cells were grown, could cause a cytotoxic effect secondary to the radiosensitization by BrdU. Cell killing under these

conditions would be expected for any cell type. The third plausible explanation for these effects is that cell lines of malignant lymphoid origin are more susceptible to cytotoxicity by BrdU than are most other cell types. To distinguish between these alternative possibilities we surveyed a variety of cell lines, of varying origin, for susceptibility to the toxic effects of BrdU. These experiments were done exactly the same way as those on malignant lymphoid cell lines. MOPC 315 was always run as a parallel control.

The data given in the Results section indicate that the concentrations of BrdU that are acutely cytotoxic to malignant lymphoid cells do not affect other cell types. None of the three criteria for cytotoxicity were satisfied in these cell lines at BrdU concentrations under at least 3 μg BrdU/ml. L929 and HeLa cells were grown for 20 and 25 days, respectively, with no affect on cell proliferation. A comparison of the LD_{50}s of malignant lymphoid cell lines and those of various other origins, as shown in Table 5, show that they fall into two distinctly different groups. The LD_{50}s of cells of malignant lymphoid origin average 0.19 μg BrdU/ml, ranging from 0.09 for MOPC 315 to 0.31 for CCRF/CEM. In contrast, cells of alternative origin have LD_{50}s ranging from 3.14 to 6.23 μg BrdU/ml for HeLa and L929, respectively, with a mean value of 4.30. Two consistent effects of BrdU on the growth of the latter set of relatively insensitive lines were observed

from their growth curves. The first of these occurs during the first 2 to 3 days in culture with the analogue when there is often a slight inhibition of growth rate compared to their non-treated counterparts. This transient inhibition of proliferation is followed by the second of the noticable effects, which is a slight stimulation of growth compared to control cultures. From what has been shown a picture emerges indicating that cells of malignant lymphoid origin are distinctly different from those other cell types in their susceptibility to killing by BrdU. Species differences do not appear to be significant. Although mouse cells were used for most studies, the pattern observed seems to apply to human cells as well. CCRF/CEM is a human acute leukemic cell line with an LD_{50} of 0.31 μg BrdU/ml which indicates a considerable susceptibility to BrdU's cytotoxic effect. In contrast, human cervical carcinoma HeLa, with and LD_{50} in the range of 4 μg BrdU/ml is not BrdU sensitive.

The cells in the BrdU sensitive group all grow as suspension cultures while most of the resistant group grow as adhereht cultures. However, this difference does not appear to bear any relevance to the pattern of toxicity which we have observed because HeLa cells, whether in suspension or monolayer cultures are insensitive to BrdU's toxic properties. In fact, HeLa suspension cultures have an

LD_{50} of 4.2, which is higher than the 3.1 of the monolayer cultures. Additionally, although less poignantly, S49.1TB.2, which is quite resistant to BrdU with an LD_{50} of 87.0 μg/ml, grows as a suspension culture.

The specific toxicity of BrdU to malignant lymphoid cell lines extend to cells of both B and T cell origin. The murine lymphoma S49.1, which we have shown to be quite sensitive to BrdU, is derived from T lymphocytes as indicated by the presence of the theta antigen on its surface, a property diagnostic for T cell identity. In contrast to the T cell malignancies, myelomas are neoplastic proliferations of plasma cells which are the end product of B lymphocyte differentiation. Thus, the sensitivity of the myelomas MOPC 315 and MPC 11 to BrdU indicate that cells of B cell derivation are also vulnerable to BrdU.

HORMONAL EFFECTS ON THE TOXICITY OF BrdU TO MOPC 315

For several years the laboratory in which this work was carried out has had a very active interest in the effects of hormones on cell proliferation. It was thus natural to consider whether certain hormones could have any effect on the toxicity of BrdU to malignant lymphoid cell lines.

Insulin has been emerging as a hormone exerting a generalized permissive effect for cell proliferation in a large variety of systems (Banerjee, 1976). This growth permitting effect of insulin may derive from its tendency

to depress intracellular cAMP levels (Goldfine, 1977). The relationship between insulin and cAMP was particularly interesting to study in our system, as Coffino et al. (1975) have shown that elevated cAMP levels may cause cytolysis of lymphoma cells and BrdU has been shown to elevate cAMP in certain cells (Schwartz et al., 1973). Studies in our laboratory on the effect of cAMP on murine myeloma cells have shown that the nucleotide is growth inhibitory, but not cytotoxic, at concentrations up to 300 nM. The inhibition is reversible and is prevented by insulin (Naseem and Hollander, 1973). That cAMP did not cause cell toxicity and cell death to murine myeloma cells, suggests that the toxicity of BrdU to these cells is not due to elevated intracellular cAMP levels.

The concentration of insulin used in these studies was 0.5 units/ml, which is somewhat higher than physiological (insulin is present in the plasma at 10-100 μunit /ml). However, for experimental purposes, the higher insulin concentration was used since this was the amount of hormone required to prevent the inhibition of growth by cAMP to murine myeloma cells. As can be seen in Table 7, after 3 days of growth MOPC 315 cells increased by a factor of 8. In the presence of 1 μg BrdU/ml they increased to only 2.5 times their original titer. This decreased proliferation

is only slightly reversed by insulin, in which the growth increment is 4.0. With insulin alone, the cells increased by a factor of 8.

The incidence of plasmacytoma in Balb/c mice following intraperitoneal injection of mineral oil is much higher in male than in female mice. This increased incidence is reduced if the males are castrated and increased in the females if they are treated with testosterone (Hollander et al., 1968). That testosterone may promote plasma cell proliferation has also been observed in vitro (Weisband and Hollander, unpublished data). Our studies show that DHT does not reverse the inhibition by BrdU, in fact, it may compound BrdU's harmful effect as cells treated with BrdU plus DHT proliferated only half as much.

MORPHOLOGICAL STUDIES ON BrdU-TREATED MALIGNANT LYMPHOID CELLS

Phase Microscopy of BrdU-treated Cells

Cultured MOPC 315 cells, when viewed in the phase microscope, appear to be typical myeloma cells, as seen in Figure 13. A small percentage of the cells contain some highly refractive cytoplasmic organelles. BrdU treatment causes an increase in both the number of cells containing such particles and in the average number of structures per cells. This increase in cytoplasmic structures, precedes the increase in the proportion of trypan blue accepting cells or the diminished growth rate caused by the

BrdU. It is improbable that the occurrence of intracytoplasmic structures is specifically related to BrdU treatment. Rather, it is more likely representative of the general toxicity associated with exposure to the analogue. That this is the case is suggested by the observation that if the cells are grown to saturation density and not passed to a lower dilution a similar kind of accumulation occurs.

A similar pattern of BrdU-induced intracytoplasmic inclusions is seen in lymphoma cells S49.1. Thus, the development of these structures is probably a general feature of the toxic effect of BrdU to malignant lymphoid cells. This notion is supported by the observation that S49.1TB.2, which is a clone of S49.1, is refractory to BrdU's toxicity and does not show any of these structures when cultured with BrdU, nor do the relatively insensitive HeLa cells.

Electron Microscopy of BrdU-treated Myeloma Cells

Electron microscopic examination of murine myeloma cells show them to be typical myeloma cells often containing A type particles (Karpes and Cawley, 1972). These are heterogeneous, intracellular ring shape particles of approximately 70 mμ diameter. C type particles are seen infrequently in murine myeloma cells. The profile of virus particles in murine myeloma cells does not change by treatment with BrdU (Pettengill and Sorenson, 1974).

This property distinguishes myeloma cell from other mouse cells. As reviewed in the Introduction, BrdU almost universally induced viral protein production in mouse cells. The relationship between the sensitivity of myeloma cells to BrdU and the non-inducibility of virus particles is obscure, but nevertheless it is an important question.

Treatment of human lymphoblastoid cell lines, established from patients with infectious mononucleosis, with 20 μg BrdU/ml for 72 hours causes the formation of 20-25 nm diameter tubuloreticular structures within the cells' ER. Although these lines were infected with Epstein-Barr virus, herpes nucleocapsids or virions were not induced by BrdU. A small percentage of non-infected cells contain these structures (Grimly et al., 1973). It is unclear what relationship the BrdU-induced tubuloreticular structures have to the refractile intracytoplasmic inclusions seen in BrdU-treated myeloma and lymphoma cells.

Myelin-like structures have been reported in melanoma cells exposed to 15-30μg BrdU/ml. Like the accumulation of inclusions we have seen in myeloma and lymphoma cells, these electron micrographically identifiable structures are found also in older untreated cultured (Endo and Hu, 1974). Similarly, Zimmerman et al. (1974) have described "membranous-whirls" similar to those structures we see in

electron micrographs of BrdU-treated myeloma cells..

Our micrographs show that there are two types of identifiable intracellular structures associated with BrdU treatment of malignant lymphoid cells. One of these, the larger membranous structures similar to those described by Endo and Hu (1974) and Zimmerman et al. (1976), are seen in Figures 18-21. The other type of structure is somewhat small and seems to fill the cytoplasm of degenerating cells, as shown in Figure 22. These are similar to the kind of structures reported previously in myeloma cells (Azar et al., 1972). It should be noted that untreated cells also contain some of both kinds of structures.

RELATIONSHIP OF BrdU TOXICITY TO VIRAL INDUCTION

As reviewed in the Introduction, BrdU has been widely used in many systems to induce the production of latent viruses. Synthesis of DNA viruses usually results in concommitant cell death. Conversely, production of RNA viruses does not generally coincide with cellular degeneration. However, under the influence of BrdU, which when used to provoke virus synthesis is usually present at concentrations of 10-200 μg/ml, cell toxicity does occur.

Is the toxicity of low doses of BrdU to malignant lymphoid cells related to viral induction? As already reviewed, some mouse myeloma cells are known to contain

A and C type particles. The C particle produced by myeloma cells is known as the murine myeloma associated virus (MMAV). It is an NB tropic agent. MMAV shows a density of 1.16 grams/cc on an isopycnic sucrose gradient and has reverse transcriptase activity (Kreuger, 1975).

The A type particle found in mouse myeloma cells resides in an intracysternal location. Purified A particles contain a reverse transcriptase similar to that of known oncorna viruses. It is not clear whether these A type particles are actually distinct from the C type particles, or whether they are C particle cores. Curiously, the RNA of these A type particles is small (5-15S) unlike the 35 or 70S components characteristic of other oncorna viral particles (Robertson et al., 1975).

As reviewed previously, mouse myeloma cells have been the unusual exception among mouse cells in that they are not susceptible to virus induction by BrdU. Stewart et al. (1975) have found that a concentration of 20μg IdU/ml plus 2% DMSO induces some kind of particles. DMSO alone has inductive properties. It is difficult to precisely identify what their induced particles are since they do not give distinct peaks in sucrose density gradients and have no detectable reverse transcriptase activity. These are two of the major criteria which must be satisfied before the diagnosis of an oncorna virus can be made.

We have shown that 1% DMSO induces some particles that can be labeled with ^{3}H-leucine but incorporate much less ^{3}H-uridine. Although BrdU has no effect on the production of these particles, the analogue in conjunction with DMSO causes a more pronounced synthesis. The induced particles band at 1.16-1.17/ml in a sucrose density gradient. However, since the polymerization templated by oligo dT:poly rA is of the approximate magnitude of that promoted by oligo dT: poly dA it appears that the induced activity is not a true reverse transcriptase, but possibly is a DNA dependent DNA polymerase. Further evidence for the designation of DNA dependent DNA polymerase for the activity associated with those particles comes from the low oligo dG : poly rC templated synthesis. Thus, the particles may be contaminated with cellular debris containing cellular DNA polymerase activity that should not be confused with a viral reverse transcriptase.

As BrdU alone does not induce virus production in malignant lymphoid cell lines, it is unlikely that its toxic properties be due to virus synthesis. As shown earlier, the toxicity of BrdU extends to malignant lymphocytes of human origin. Unlike the situation in mouse cells, it has proven very difficult to induce viral particles from human cells. This too speaks against the notion of viral

induction by BrdU as a causal factor in its toxicity to malignant lymphoid cells.

As shown in Table 10, tissue culture media from murine myeloma cels is not infectious when injected intraperitoneally into two newborn BALB/c mice. This result is in agreement with the data of Karpas (1973) in a similar experiment. However, it must be considered that this may not be the appropriate bioassay for a myeloma inducing agent as host factors may be critical in determining susceptibility to tumorigenesis. Specifically, a granulomatous environment such as that produced by mineral oil injection may be a prerequisite to tumorigenicity (Potter, 1973). Furthermore, a specific hormonal environment with androgens at a concentration higher than that found in the newborn mouse may be required (Hollander et al., 1968). It is well known that myeloma is not a childhood disease.

Our electron microscopic studies, described earlier, show that untreated myeloma cells have many viral-like particles in their cytoplasm. BrdU treatment does not obviously affect this pattern.

RESISTANCE TO THE CYTOTOXIC EFFECTS OF BrdU

The loci of resistance to the cytotoxic effects of BrdU has generally been thought to be the enzyme thymidine kinase (TK). We have shown that the same enzyme catalyzes

the initial 5'phosphorylation of thymidine or BrdU, to yield the nucleoside monophosphate. TK^- cells cannot phosphorylate BrdU and thus render the analogue harmless because of the rapid efflux of non-phosphorylated nucleosides from the intracellular pools and the inability of the cells to utilize free BrdU for metabolic processes. Similarly, resistance to the effect of other cytotoxic nucleosides, for example cytosine arabanoside (Ara-C), has also been attributed to a deficiency of the appropriate phosphorylating enzymes (Coleman et al., 1975).

The literature on BrdU reveals that resistance to a cytotoxic effect may occur independent of the status of TK. In fact, some BrdU-resistant cells even incorporate BrdU into DNA in amounts comparable to wild-type cells (Hsu and Somers, 1962; Dubbs and Kit, 1970; Kaufman and Davidson, 1977).

There has been a recent suggestion that the toxic effects of BrdU may be due to allosteric inhibition of the enzyme ribonucleotide reductase (Meuth and Green, 1974). Should this be a general explanation for BrdU's cytotoxic effect, resistance to BrdU would derive from either short circuiting the block of deoxypyrimidine production, or an altered ribonucleotide reductase refractory to inhibition by dBUTP. Neither of these two possibilities has yet been adequately explored for enough cell types to draw general

conclusions. However, the data presented here on malignant lymphoid cells reveals several points which speak against the ribonucleotide reductase mechanism for the toxicity of these cells. The evidence against ribonucleotide reductase inhibition as a mechanism of toxicity of BrdU is:

1) no decrease in the intracellular deoxypyrimidine metabolites of ribonucleoside precursors following treatment with BrdU;
2) no inhibition by BrdU of the utilization of ribocytidine for DNA synthesis;
3) protection of lymphoma cells by cytidine; and
4) lack of protection of myeloma cells by deoxycytidine.

The data upon which these observations are based are discussed in the next section. The work of Mezger-Freed (1972) has shed some insight into the mechanism of BrdU-resistance of haploid frog cells. She has found that the emergence of a BrdU-resistant cell populations follows a 2 step pattern: a transport defect followed by a TK deficiency. Our data yield similar results on BrdU-resistant clones of malignant lymphoid cells.

We have characterized the BrdU-resistant clone of murine lymphoma S49.1, known as S49.1TB.2. These cells

have a double loci of resistance to BrdU. One of these is at the level of TK, and the other is a transport deficit. We obtained similar results with EL4BU, a BrdU-resistant clone of murine leukemia EL4.

The TK deficiency, shown in Table 17, is evidenced by the failure of high speed supernatant fractions from BrdU-resistant malignant lymphoid cells to phosphorylate BrdU or thymidine. This appears to be due to a lack of TK rather than the presence of an inhibitor since a combination of preparations from S49.1 and S49.1TB.2 had the activity predicted by the amount of S49.1 enzyme present.

The transport defect in S49.1TB.2 is seen in Figures 24 and 25, and in Table 11. These data reveal a two component transport system. One of them with a K_m of 30μm appears to be a facilitated uptake system which is sensitive to sulfhydryl inhibitors and temperature. This system is absent in S49.1TB.2. The other system, which is present in both sensitive and resistant cells probably represents passive diffusion. Subtraction of the transport rate of S49.1TB.2 cells from that of S49.1 cells yields a parallel line suggesting that the uptake by S49.1TB.2 represents a passive diffusional system common to both cell types. A defect of similar magnitude is seen in EL4VU, a BrdU-resistant clone of lymphoma El4, as shown in Table 11. However, experiments using pCMB are difficult to interpret since it may bind sulfhydral groups of T.K. and therefore cause an increase in the intracellular free nucleoside pool.

Since the S49.1TB.2 cells are TK^-, there are certain potential problems inherent in studying nucleotide transport in these cells. Intracellular BrdU, being predominantly non-phosphorylated in TK^- cells, may be lost from the TCA soluble pools by the possibly rapid efflux of this unchanrged moiety. To evaluate the influence of this factor on the experiments designed to quantitate the intracellular pool of BrdU or thymidine in the TK^- clones we determined the rate of efflux of non-phosphorylated nucleoside from cells subjected to our standard protocol. The results show that the rate of efflux of BrdU from pre-loaded S49.1TB.2 cells, using our assay procedure, is much too low to account for the decreased intracellular nucleoside concentrations found after our incubations.

The protection of cells by thymidine against the toxic effect of BrdU appears to be largely due to competition for uptake between the two nucleosides. In contrast, as we shall discuss below, the protection of malignant lymphoma cells by cytosine nucleosides is unrelated to transport.

EFFECT OF CYTOSINE NUCLEOSIDES ON THE TOXICITY OF BrdU TO MALIGNANT LYMPHOID CELLS

The information contained in the Introduction section speaks to the relationship between BrdU toxicity and pyrimidine metabolism. Deoxynucleotides are synthesized from

ribonucleosides at the ribonucleoside diphosphate level. Ribonucleotide reductase reduces CDP to dCDP, which can then be metabolized to the direct substrates for DNA synthesis, dTTP or dCTP. dTTP, in a typical feedback control system, is a negative allosteric effector of ribonucleotide reductase (Reichard et al., 1960). BrdU triphosphate, which we have shown to be a significant metabolite of BrdU in malignant lymphoid cells, is a particularly potent inhibitor of this enzyme (Meuth and Green, 1974). Thus, it is possible that the toxic effects of BrdU are related to the inhibition of this enzyme, causing a "deoxypyrimidine-less" state. That this may indeed by the case has been suggested by Meuth and Green (1974), who have shown that the toxicity of 47 μg BrdU/ml to mouse fibroblasts is prevented by the simultaneous addition of 46 μg CdR/ml to the growth medium. The literature on BrdU research, when viewed in retrospect, anticipates this notion. For example, Szybslski (1961) found that human sternal marrow cells, growing in BrdU in order to label replicating DNA, grow better when the media is supplemented with CdR.

Since one locus of BrdU action is ribonucleotide reductase, it was of great interest to consider: 1) does supplementation of the growth medium with CdR alter the toxicity of low levels of BrdU to malignant lymphoid cells?

and 2) if there is such an effect does it occur because BrdU prevents the ultimate utilization of cytidine for DNA synthesis?

The data presented on cytosine nucleosides and the toxicity of BrdU to malignant lymphoid cells reveals several interesting features. As shown in Figure 38 and Table 20, deoxycytidine protects lymphoma cells from BrdU's fatal effects. As little as 10 μg deoxycytidine/ml protects them against 1 μg BrdU/ml. Deoxycytidine itself is without any apparent affect on cell growth. It is particularly interesting to note that cytidine also protects lymphoma cells, suggesting that inhibition of ribonucleotide reductase is not responsible for BrdU's toxicity to malignant lymphoid cells. Other evidence, to be discussed shortly, from studies on cytidine metabolism in BrdU-treated lymphoma cells, also speaks to the implausibility of the ribonucleotide reductase explanation. It is not surprising that BrdU does not inhibit ribonucleotide reductase since the very low concentrations of BrdU used in these studies do not give intracellular BrdU pools large enough to inhibit the enzyme. It is actually not clear whether the deoxycytidine protection against toxicity at higher levels of BrdU to various cell types can be attributed to inhibition of ribonucleotide reductase, since most studies do not

make a point of documenting the lack of effect of cytidine. There is some gross stimulation by cytidine as shown in Table 20, but this is hardly large enough to account for the reversal of BrdU's toxicity to lymphoma cells.

In contrast to the results given above for lymphoma cells is the data on deoxycytidine and BrdU's affects on myeloma cells. Even a 500-fold excess of deoxycytidine to BrdU does not protect MOPC 315 cells. The vulnerability of myeloma cells to BrdU in the presence of deoxycytidine probably does not absolutely extend to all myelomas as MPC11, unlike MOPC 315, is slightly protected by deoxycytidine.

Deoxycytidine alone has a dose dependent growth stimulating effect on myeloma cultures, maximally seen at about 20 μg/ml at which concentration the cells grow at a rate 50% faster than controls. The growth stimulation by deoxycytidine is obliterated by BrdU since cells treated with both nucleosides have growth rates similar to those treated with BrdU alone. That deoxycytidine does not significantly protect myeloma cells from BrdU's effects suggests that in these cells, as in the lymphoma cells described above, induction of a deoxypyrimidineless state by inhibition of ribonucleotide reductase is not the explanation for BrdU's toxicity.

Incubation of mouse myeloma cells with cytidine reveals an interesting aspect of the nucleosides effect. Cytidine causes a linearly dose dependent inhibition of cell growth, causing an over 50% reduction at 75 μg cytidine/ml. The cytidine in combination with BrdU causes a substantial toxic effect, exactly the same as that obtained with BrdU alone.

One possible mechanism whereby cytidine nucleosides could nullify the affects of BrdU on lymphoma cells is competition for uptake such that the nucleoside reduces the amount of BrdU transported into the cells. However, the data in Table 21 suggests that this could not be the complete explanation as an over 1000-fold excess of deoxycytidine reduced BrdU uptake by only 33%. This amount of inhibition may represent the saturation by deoxycytidine of a common carrier system which represents only one component of the transport mechanism whereby BrdU can enter the cells. That this may indeed be the case is seen in a reciprocal experiment. Where S49.1 cells are incubated with an excess of BrdU over deoxycytidine, deoxycytidine uptake is reduced by only 15% (Table 21). Evidence discussed earlier suggested that BrdU was transported into the cells by the thymidine uptake system. This transport system is separate from the deoxycytidine or cytidine one. However,

there are relatively non-selective facilitated uptake systems, with high Kms, that may account for the slight competition between BrdU and deoxycytidine (Hauschka, 1973).

THE EFFECT OF BrdU ON THE UTILIZATION OF CYTIDINE FOR DNA SYNTHESIS

BrdU's toxicity to malignant lymphocytes is prevented by the simultaneous presence of deoxycytidine. This supports the notion that BrdU prevents the utilization of ribonucleotides for DNA synthesis. Two questions arise from this. Since deoxycytidine does not protect myeloma cells, is BrdU's mechanism of action on that cell type completely different from that of lymphoma cells? Does BrdU really prevent utilization of ribonucleotides for DNA synthesis at the low levels of the analogue used in these studies?

The data is shown in Table 22. It is obvious from the data that BrdU does not decrease the utilization of cytidine for BrdU synthesis. In fact, the utilization of ribonucleosides for DNA synthesis is slightly increased by co-incubation with BrdU. This finding, combined with the other data on deoxycytidine and cytidine, suggests that inhibtion of ribonucleotide reductase is not the mechanism of action of low doses of BrdU on malignant lymphoid cells.

METABOLISM OF BrdU AND CYTIDINE NUCLEOSIDES

The metabolism of BrdU reflects its rate of mono-, di-, and tri-phosphorylation and the utilization of the triphosphate for DNA synthesis. The steady-state metabolic products of BrdU are given in Table 13.

The TCA soluble pools of BrdU metabolites are constant at a given fixed extracellular analogue concentration. For all times after the 30 minutes required to attain steady-state nucleotide values, the TCA soluble pools are much smaller than the insoluble pools. The rapid outflow from the free nucleoside pool into DNA is probably a major factor in the early establishment of the steady-state.

BrdU in the incubation media is hydrolized to BU at the rate of 18%/24 hours (Figure 30) which is in the general range of nucleoside hydrolysis of non-mycoplasma infected mammalian cell cultures. Cultures infected with these subtle, yet troublesome, organisms degrade nucleoside at a much faster rate owing to the presence of the nucleoside phosphorylase activity of mycoplasma.

Table 13 also shows the distribution of deoxycytidine metabolites in S49.1 and MOPC 315 cells. Their distribution and rate of metabolism to thymidine nucleotides are unaffected by BrdU. The rate of conversion of cytidine to deoxycytidine is only slightly reduced by BrdU for

S49.1 cells and not reduced at all for MOPC 315 cells. This data suggests that at the concentrations of the analogue used in these studies, BrdU does not inhibit nucleotide reduction via ribonucleotide reductase.

CHEMOTHERAPEUTIC TRIALS WITH BrdU

The work presented on the selective toxicity of BrdU to malignant lymphoid cells suggests its possible utility in the chemotherapy of neoplasms of these cells. There have been a large number of studies on the possible use of BrdU, or its cogener IdU, as a radiosensitizing agent, hopefully making tumor cells particularly susceptible to radiotherapy. As discussed in the Introduction, these studies have not yielded promising results.

Chemotherapeutic studies using BrdU on lymphoid tumors have not been done, except for those reported here. The lack of a dramatic effect of the analogue on these tumors is not surprising since it is known that BrdU rapidly disappears from the blood (Kriss et al., 1963), making it difficult to maintain therapeutically efficacious blood levels. Several approaches to this problem are summarized below.

Our studies on the BrdU/deoxycytidine combination show that deoxycytidine can protect mice from a regimen utilizing what would be well above the toxic dose of BrdU if the

analogue were used alone. This protection, indicated by experiments *in vitro* and confirmed *in vivo* may make the successful treatment of mouse myelomas with BrdU possible.

Prospects

There are three possible approaches to the problem of using BrdU therapeutically:

1) find an analogue to the analogue. 5-substituted deoxyuridines with methyl sized substituents having an electron withdrawing nature may act similar to BrdU;

2) inhibit the enzyme systems responsible for dehalogenation of BrdU;

3) infuse BrdU so there is a constant influx into the vascular compartment. One example of this was the attempt by Mark and Calabresi (1962) to treat head and neck tumors by intraarterial infusions of IdU. Alternatively, the "infusion *in situ*" approach, such as the implantation of BrdU adsorbed to activated charcoal as described by Russev and Tsanev (1975), might be useful. Another possible way of infusing BrdU into the blood *in situ* is to couple it to a polycation. Woodman (1966) showed increased incorporation of IdU into C_3H mouse mammary tumors if it was administered as part of a water soluble complex with the polycation polyethylenime. Pellets of cholesterol and Idu implanted s.c. into mice have been shown to result in

sustained high IdU blood levels (Lee et al., 1976). This approach is also of possible utility in the chemotherapy of malignant lymphoid tumors with BrdU.

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