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Tetrazolium-based Assays for Cellular Viability: A Critical Examination of Selected Parameters Affecting Formazan Production

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ABSTRACT

The hydrogen acceptor 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) is commonly utilized to estimate cellular viability in drug screening protocols. The present investigation was prompted, in part, by observations that reduction of MTT to its colored reaction product, MTT formazan, varied between cell lines and with culture age. A correlation was established between the D-glucose concentration of the culture medium at the time of assay and the production of MTT formazan for cell lines representing seven tumor histologies. A decrease in the concentration of D-glucose from culture medium was accompanied by a decrease in MTT specific activity (MTT formazan/μg cell protein) for a number of cell lines. Cells which extensively metabolized D-glucose exhibited the greatest reduction in MTT specific activity. Further evidence that the D-glucose concentration of the culture medium played an important role in MTT reduction was provided by experiments which demonstrated that transfer of cells to a glucose-free medium (L-15) was accompanied by an immediate decrease in MTT reduction which was pH independent. These studies suggested that cellular transport and constant metabolism of glucose were required for maximum MTT reduction. Decreases in the cellular concentration of the reduced pyridine nucleotides NADH and NADPH were accompanied by concomitant decreases in MTT formazan production. MTT formazan varied significantly among cell lines in both the kinetics of its formation and the degree of saturability exhibited. Apparent IC_{50} values for Adriamycin varied, in a cell line-specific manner, with MTT exposure time. These results indicate that MTT specific activity is significantly influenced by a number of parameters and suggest that assay conditions should be established which minimize their effects.

INTRODUCTION

An intensive effort to discover new selective antitumor agents has been initiated by the Developmental Therapeutics Program of the Division of Cancer Treatment, National Cancer Institute (1). This disease-oriented preclinical drug discovery strategy utilizes panels of human tumor cell lines for the initial *in vitro* screening of candidate agents. Panels represent a broad range of malignancy and currently include central nervous system, colon, lung, melanoma, renal, ovarian, and leukemia cancer cell lines. An integral component of this process is that each compound is tested against each cell line at multiple concentrations. Cells are exposed to the agent for a fixed time, and, at the conclusion of the assay period, the viability of the cell population is estimated.

Two hydrogen acceptor reagents, MTT[2] (2) and XTT (3), have been extensively evaluated as indicators of cell viability in this high-flux *in vitro* antitumor drug screening effort. The validity of both of these assays depends, in part, on the assumption that only viable cells reduce tetrazolium salts to colored formazan derivatives which can be quantitated spectrophotometrically.

During the course of this effort it became apparent that both assays presented unique difficulties associated with, in part, the magnitude of the drug screening effort. These problems, initially discovered in a fully operational drug screening mode, prompted a series of studies, described herein, to investigate formazan production in selected cell lines.

MATERIALS AND METHODS

MTT, NAD, NADH, NADP, NADPH, α-ketoglutarate, glucose 6-phosphate, semicarbazide, glutathione, bovine liver glutamate dehydrogenase (28 mg protein/ml; 43 units/mg protein), yeast alcohol dehydrogenase (340 units/mg), and yeast glucose-6-phosphate dehydrogenase (300 units/mg protein; 1.2 mg protein/ml) were obtained from the Sigma Chemical Company (St. Louis, MO). XTT was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute.

Growth of Human Tumor Cell Lines. Human tumor cell lines (Table 1 were obtained from the tumor repository (Frederick, MD) and routinely grown in RPMI 1640 (Quality Biological, Gaithersburg, MD) containing 5% fetal bovine serum (HyClone Laboratories, Logan, UT). Cultures were maintained in 75-cm^2 Costar culture flasks in a humidified atmosphere of 95% air/5% carbon dioxide and were passaged at 85–90% of confluency. Cells were removed by trypsinization and subcultured according to the following procedure. Medium was decanted, and the cells were rinsed once with 2 ml of 0.05% trypsin containing 0.1% EDTA (Quality Biological). The cells were then overlaid for 2–5 min with 1.5 ml of a trypsin solution which was prepared by adding 1 ml of a 1% trypsin solution (Sigma type III; twice recrystallized) in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline to 50 ml of 0.05% trypsin containing 0.05% EDTA. Cells were plated in 75-cm^2 Costar culture flasks at a concentration of 1×10^4 cells/cm^2 in 30 ml RPMI 1640 containing 5% fetal bovine serum. All cell counts were performed on a Model ZM Coulter Counter. A series of experiments, described below, was designed to investigate the possible role of medium D-glucose concentration in formazan production. The studies utilized both glucose-free L-15 medium (Quality Biological) and PDRG basal growth medium (Quality Biological), a new medium designed for propagation of cells in atmospheric CO_2 (4).

Medium D-Glucose, Cellular Proliferation, and MTT Specific Activity. Selected cell lines (Table 1) were used. Adherent lines were trypsinized as described above and seeded, in a volume of 200 μl, into 96-well plates at concentrations of 10,000 and 20,000 cells/well with the exception of K 562 and HT 29 which were seeded at 5,000 and 10,000 cells/well, respectively.

A 10-μl aliquot of medium was removed at appropriate intervals following cell inoculation and utilized for determination of D-glucose using the Sigma hexokinase reagent kit. MTT was added for a period of 4 h according to a previously published procedure (2), the resulting formazan was extracted with dimethyl sulfoxide, and the absorbance was read at 540 nm. The medium was removed from replicate cultures, the cells were solubilized in 0.2 N NaOH, and protein was determined according to the method of Bradford (5).

Quantitation of Cellular NADH and NADPH. The SN12K1 renal cell carcinoma was trypsinized as described above. K 562 leukemic cells

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[2] The abbreviations used are: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H* tetrazolium hydroxide; IC_{50}, drug concentration resulting in a 50% decrease in MTT formazan compared to control; PDRG, Program Development Research Group.

Table 1 *Histology and doubling times of human tumor cell lines*

The doubling times (h) for these cell lines are: XF 498 (41); SNB 56 (45); M 19 MEL (27); OVCAR 5 (24); HOP 62 (29); EKVX (26); SN12K1 (18); HT 29 (32); and K 562 (17). Doubling times were calculated as described in Ref. 4.

Cell line	Histology
XF 498	Central nervous system (glioblastoma)
SNB 56	Central nervous system (glioblastoma)
M 19 MEL	Melanoma
OVCAR 5	Ovarian adenocarcinoma
HOP 62	Lung adenocarcinoma
EKVX	Non-small cell lung adenocarcinoma
SN12K1	Renal cell carcinoma
HT 29	Colon adenocarcinoma
K 562	Chronic myelogenous leukemia

were harvested from their respective growth media by centrifugation at $300 \times g$ for 6 min. Cells (3×10^8) were resuspended in 2 ml of −20°C 0.5 N KOH in 50% ethyl alcohol in a small glass tube and heated to 90°C in a water bath for 5 min. The alkaline extract was cooled to 0°C in an ice bath for 5 min and slowly neutralized, with intermittent cooling and stirring, to pH 8 with a buffer/substrate solution (approximately 1 ml) composed of 5 mM α-ketoglutarate, 0.5 M triethanolamine, 30 mM NH_4Cl, and 0.5 M potassium phosphate (6). The substrate-cofactor solution was warmed to 37°C, and NADH and NADPH were immediately oxidized to their stable analogues by addition of 10 μl of glutamate dehydrogenase/ml for 15 min. The reaction mixture was acidified by addition of 0.2 ml 3 N $HClO_4$/ml extract, and protein was removed by centrifugation at $14,000 \times g$. The resulting solution was neutralized, at 4°C, to pH 7.2 with 1 N KOH. $KClO_4$ was removed by centrifugation, and the supernatant, containing NAD and NADP, was divided into 2 equal aliquots and lyophilized to dryness. NADH formation was monitored at 340 nm using an alcohol dehydrogenase-catalyzed reaction (3 units) following reconstitution in the following buffer system: 2.5 ml sodium pyrophosphate (0.1 M, pH 9.0); 0.1 ml semicarbazide·HCl (250 mg/ml, pH 6.5); 0.1 ml ethyl alcohol; and 0.01 ml glutathione. NADPH formation was similarly quantitated at 340 nm using glucose-6-phosphate dehydrogenase in the following buffer system: 2.4 ml triethanolamine (0.1 M, pH 7.6); 0.02 ml 0.1 M $MgSO_4$; 0.1 ml glucose 6-phosphate (10 mg/ml); and 10 μl glucose-6-phosphate dehydrogenase.

Effect of MTT Concentration and Incubation Time on MTT Formazan Production and Adriamycin Cytotoxicity. The SNB 56 glioblastoma and the M 19 melanoma cell lines were utilized to examine the effect of varying the concentration and the length of exposure to MTT upon both the formation of MTT formazan and adriamycin cytotoxicity. Cell lines were inoculated into 96-well plates (10,000 cells/well) and allowed to attach for 24 h. MTT was added to a final concentration of 0.075 to 1 mM, and MTT formazan was quantitated (2) following an exposure time of 0.5 to 24 h. The effect of MTT concentration and incubation time upon Adriamycin cytotoxicity to each cell line was similarly investigated. Cells were inoculated into 96-well plates (10,000 cells/well) on day 0. Adriamycin was added 24 h later, and MTT formazan was quantitated 48 h following drug addition according to previously published procedures (2).

RESULTS

Medium D-Glucose Depletion, Cellular Proliferation, and MTT Specific Activity. Initial studies were undertaken with the SN12K1 renal carcinoma, the XF 498 glioblastoma, the HOP 62 lung adenocarcinoma, and the HT 29 colon adenocarcinoma. Proliferation of these cell lines was accompanied by a progressive decline in the concentration of D-glucose in the culture medium (Fig. 1). The SN12K1 renal carcinoma had depleted nearly 90% of D-glucose in the culture medium by 4 days following inoculation while the XF-498 glioblastoma, a more slowly growing tumor, had depleted only 15% of the sugar during the same interval. The decrease in medium D-glucose concentration in cultures of the SN12K1 renal carcinoma was accompanied by a marked decrease in MTT specific activity (MTT formazan produced/μg cell protein). The specific activity of MTT decreased 4-fold from day 1 to day 2 at a time when cells were proliferating rapidly and were 100% viable. A similar decrease in medium D-glucose and MTT specific activity was observed in the HOP 62 lung adenocarcinoma and the HT 29 colon adenocarcinoma. In contrast, the specific activity of MTT in the XF 498 glioblastoma remained nearly constant during the 4-day growth period. These contrasting results prompted a similar investigation with a more diverse series of tumor cell lines. These additional results (Fig. 2) indicated that metabolism of D-glucose by the cell lines examined varied markedly and showed a good correlation between the concentration of D-glucose in the culture medium and the specific activity of MTT. The cell lines examined seemed to cluster in 2 groups, those in which medium D-glucose concentrations were <50% of initial values (HT 29, HOP 62, SN12K1) and those which were >50% of initial values (M 19 MEL, EKVX, OVCAR 5, XF 498, K 562).

Medium Carbohydrate Composition and MTT Specific Activity. In order to more fully investigate the role of D-glucose concentration in the reduction of MTT, a series of experiments was undertaken with the K 562 myelogenous leukemia cell line. The experimental approach taken was to examine MTT specific activity in cells grown in a D-glucose-containing growth medium (RPMI 1640) and L-15, a glucose-free medium originally described by Leibovitz (7) which contains D-galactose and pyruvate as the principal carbohydrates. The results of these experiments (Fig. 3) indicated that MTT reduction by the K 562 leukemia cell line was decreased 7-fold by growth in L-15 medium. Comparative experiments with PDRG basal growth medium, a D-glucose-containing medium designed for growth of cells at atmospheric CO_2 (4), indicated that the observed reduction in MTT specific activity in L-15 medium was not due to propagation of cells in atmospheric CO_2. The specific activity of K 562 leukemic cells propagated in PDRG medium in atmospheric CO_2 was identical to that of cells grown in 5% CO_2 in RPMI 1640 (Fig. 3). In order to more fully examine the role of medium carbohydrate composition on MTT specific activity, additional experiments were performed, and these indicated that transfer of K 562 leukemic cells to glucose-free L-15 medium was accompanied by an immediate reduction in MTT specific activity (Fig. 3).

Cellular NADH and NADPH Concentrations in K 562 Leukemia and SN12K1 Renal Carcinoma Cells in Glucose-containing and Glucose-free Media. The results described above (Fig. 2) suggested that a relationship existed between MTT reduction and the concentration of D-glucose in the culture medium. MTT reduction in cell lines which metabolized the carbohydrate extensively, *e.g.*, SN12K1, HOP 62, and HT 29, was affected most. These observations prompted initiation of a series of experiments designed to examine the effect of D-glucose deprivation on cellular concentrations of the reduced pyridine nucleotides, NADH and NADPH. The results of these experiments with the K 562 leukemia line (Fig. 4) indicated that propagation of cells in the D-galactose-containing L-15 medium resulted in a decrease in cellular NADH which was observable 24 h following subculture. NADH concentrations in cells grown in L-15 medium continued to decline during the 7-day growth period. In contrast, NADH concentrations in cells grown in RPMI 1640 remained relatively constant during the entire growth period. The failure to observe a decline in NADH for these cells grown in D-glucose-containing RPMI 1640 was not

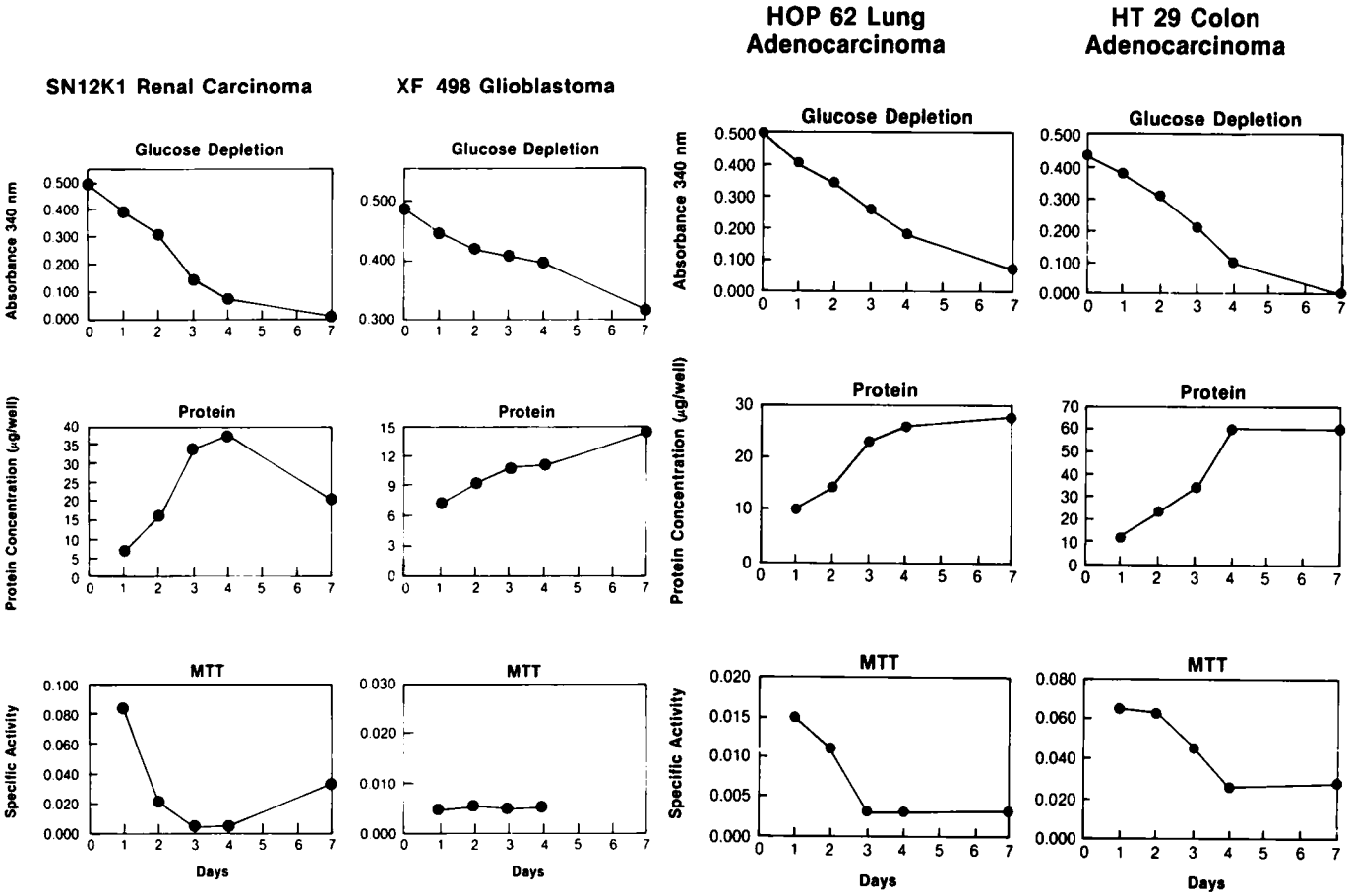

Fig. 1. Medium D-glucose depletion, cellular proliferation, and MTT specific activity in the SN12K1 renal carcinoma, the XF 498 glioblastoma, the HT 29 colon adenocarcinoma, and the HOP 62 lung adenocarcinoma. Cells were trypsinized and seeded into 96-well plates on day 0 as indicated in "Materials and Methods." MTT reduction (2), cell protein determinations (5), and medium D-glucose concentrations were made at 24-h intervals as described in "Materials and Methods." Experimental values were determined from individual wells.

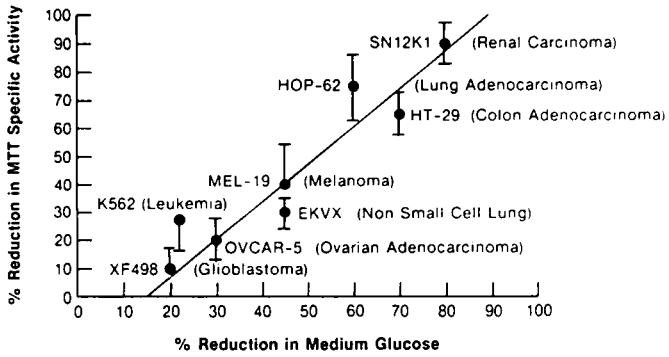

Fig. 2. Medium D-glucose depletion and MTT specific activity in selected human tumor cell lines. Cells were seeded into 96-well plates as described in "Materials and Methods." MTT reduction (2), cell protein determinations (5), and medium D-glucose concentration were determined 4 days later. *Points*, means for 3 separate determinations; *bars*, SD. The line of best fit was derived by linear regression.

unexpected because K 562 does not extensively metabolize the sugar and does not exhibit a marked decrease in MTT specific activity during the course of the assay (Fig. 2). The concentration of NADPH in K 562 cells grown in L-15 medium for 24 h was similar to that of cells grown in RPMI 1640. Thereafter the cellular content of NADPH declined in cells grown in both media and, at 4 days, was 2-fold less in cells grown in L-15 medium.

Similar experiments were undertaken with the SN12K1 renal carcinoma, a cell line which extensively metabolizes D-glucose and exhibits a marked reduction in MTT specific activity as metabolism of the sugar occurs (Figs. 1 and 2). These results indicate that NADH concentrations in cells grown in RPMI 1640 progressively decline over the course of the experiment and contrast with the results with K 562 leukemia cells. NADPH concentrations of cells grown in RPMI 1640 were also lower than those observed in the K 562 leukemia. NADH and NADPH concentrations in SN12K1 renal carcinoma cells grown in L-15 medium were less than in cells grown in RPMI 1640.

Effect of MTT Concentration and Incubation Time on the Production of MTT Formazan in Selected Human Tumor Cell Lines. Reduction of MTT to its corresponding formazan is preceded by cellular internalization of the tetrazolium (11), *e.g.*, transport. Since transport processes exhibit many of the same kinetic parameters as enzymatic reactions, *i.e.*, temperature sensitivity, saturability, sensitivity to metabolic inhibitors, and time-dependent formation of end product, a detailed investigation into several of these parameters was undertaken in an attempt to determine their effect on the production of MTT formazan. A subset of cell lines representing the various panels in the National Cancer Institute antitumor drug screen (Table 1) was selected, and the effect of both substrate (MTT) concentration and incubation time on the reduction of MTT was examined. The results of these studies for 2 cell lines, the M 19 melanoma and the SNB 56 glioblastoma (Fig. 5), illustrated both similarities and differences in the kinetics of MTT formazan production in these 2 cell lines and were representative of

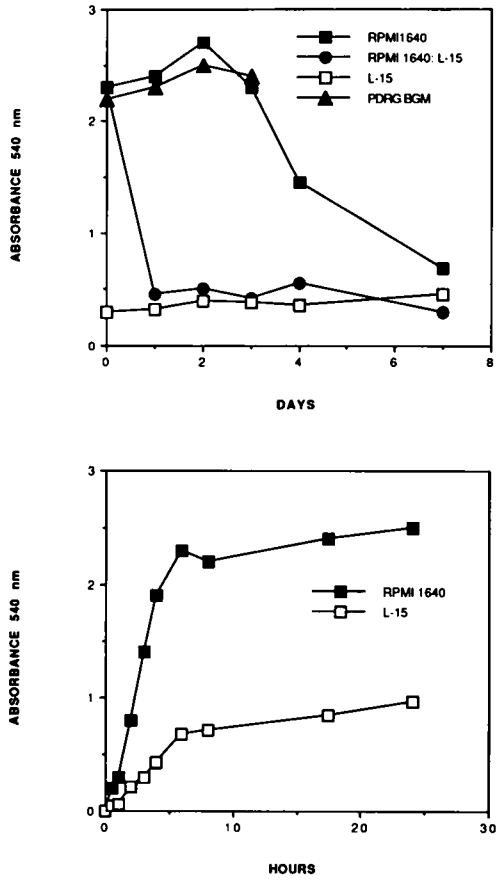

Fig. 3. Influence of medium carbohydrate composition on MTT specific activity in K 562 leukemia cells. *Top*, MTT reduction (2) was quantitated on day 0 for cells growing in RPMI 1640 medium (■) in 5% CO_2, and in either L-15 (□) or PDRG basal growth medium (▲) for cells growing in atmospheric CO_2. MTT reduction was determined at 24-h intervals in the respective media as well as in cells which had been transferred from RPMI 1640 to L-15 medium (●). *Bottom*, K 562 leukemia cells, in RPMI 1640, were centrifuged and resuspended in either RPMI 1640 (■) or L-15 (□) media. MTT reduction (2) was measured at the indicated time points. Experimental values were determined from a single experiment.

the results found in other cell lines examined.

In both cell lines the formation of MTT formazan at low concentrations (0.075–0.1 mM) exhibited a lag phase. Increasing concentrations of MTT were accompanied by increased production of MTT formazan, the magnitude of which was 2-fold greater in the M 19 melanoma than in the SNB 56 glioblastoma. Examination of the kinetics of MTT formazan production also revealed differences among cell lines. The formation of MTT formazan by M 19 melanoma cells was linear for approximately 30 min while it was linear for 60 min in the SNB 56 glioblastoma. Longer exposure to MTT in both cell lines was characterized by a nonlinear increase in MTT formazan. An additional difference in the kinetics of MTT formazan production by these 2 lines was that no evidence of saturation was found in the SNB 56 glioblastoma. In contrast, MTT concentrations of 0.75 and 1.0 mM resulted in nearly identical formation of MTT formazan in the M 19 melanoma cell line.

Effect of MTT Concentration and Incubation Time on Apparent Adriamycin IC_{50}s in the M 19 Melanoma and SNB 56 Glioblastoma. The results of the above-described experiments on the kinetics of MTT formation by the M 19 melanoma and SNB 56 glioblastoma cell lines prompted experiments which were designed to examine whether the quantitation of drug cytotoxicity was influenced by either MTT concentration or the length of exposure to the tetrazolium (Tables 2 and 3). These results indicated that the apparent IC_{50}s for Adriamycin in both cell lines were relatively insensitive to variations in MTT concentration from 0.075 to 1.0 mM. In contrast, a striking difference between these two cell lines was observed when the length of exposure to MTT was varied. The apparent IC_{50}s for Adriamycin against the M 19 melanoma were not influenced by the length of exposure to MTT. However, in the SNB 56 glioblastoma, the apparent IC_{50}s increased with increased exposure time to MTT. For example, at an MTT concentration of 2 mM, the apparent IC_{50} for Adriamycin increased from 2.1 μM for a 30-min exposure to MTT to 23.8 μM for an MTT exposure of 24 h.

DISCUSSION

Tetrazolium salts have long been utilized to quantitate cellular reductive capacity. These salts accept electrons from oxidized substrates or appropriate coenzymes, including NADH and NADPH, which results in their reduction to a colored formazan product. MTT, first described by Beyer and Pyl (8), is easily reduced ($E_0' = -0.11$ V) (9) by electron donors such as NADH and NADPH ($E_0' = -0.317$ V). Early studies (10) with the succinate dehydrogenase system in rat liver homogenates indicated that MTT is reduced at the ubiquinone and cytochrome *b* and *c* sites of the mitochondrial electron transport system. However, there is no substantive evidence to indicate that MTT reduction is confined to mitochondria. The fact that tetrazolium salts are widely utilized in histochemistry for demonstration of specific nonmitochondrial enzymes (11) suggests that MTT reduction may occur at multiple cellular sites. MTT was utilized by Mosmann (12) to quantitate cellular growth and cytotoxicity and subsequently was investigated by the National Cancer Institute in 1986 (13) for feasibility of use in its *in vitro* disease-oriented drug discovery screen under development.

The present investigation was prompted by the observation that MTT reduction varied significantly between cell lines and that the magnitude of MTT reduction, in some cell lines, appeared to decrease with increasing culture age. The results described here indicated that MTT reduction correlated well with the D-glucose concentration of the culture medium at the time of assay (Fig. 2). Cell lines which metabolized the sugar extensively (SN12K1 renal carcinoma, the HOP 62 lung adenocarcinoma, and the HT 29 colon adenocarcinoma) exhibited the greatest decrease in the production of MTT formazan. Additional evidence that MTT reduction was dependent upon maintenance of a threshold D-glucose concentration was provided (Fig. 3) by experiments in which cells were grown in L-15 medium (7). This D-glucose-free growth medium contained D-galactose and pyruvate as the principal carbohydrates. Transfer of cells from RPMI 1640, a D-glucose-containing medium, to L-15 resulted in a 7-fold decrease in MTT specific activity after 24 h. Further experiments (Fig. 3) indicated that a decrease in MTT specific activity occurred immediately upon transfer to D-glucose-free medium and was independent of pH (Fig. 3). We interpreted these results to indicate that transport and constant intracellular metabolism of D-glucose were required for optimal MTT reduction. Evidence in support of this conclusion derives from data which indicate that addition of D-glucose to nutrient-deficient culture medium immediately prior to assay results in only a minimal increase MTT reduction (data not shown). Reduction in MTT specific activity was also accompanied by a decrease in cellular reduced pyridine nucleotides

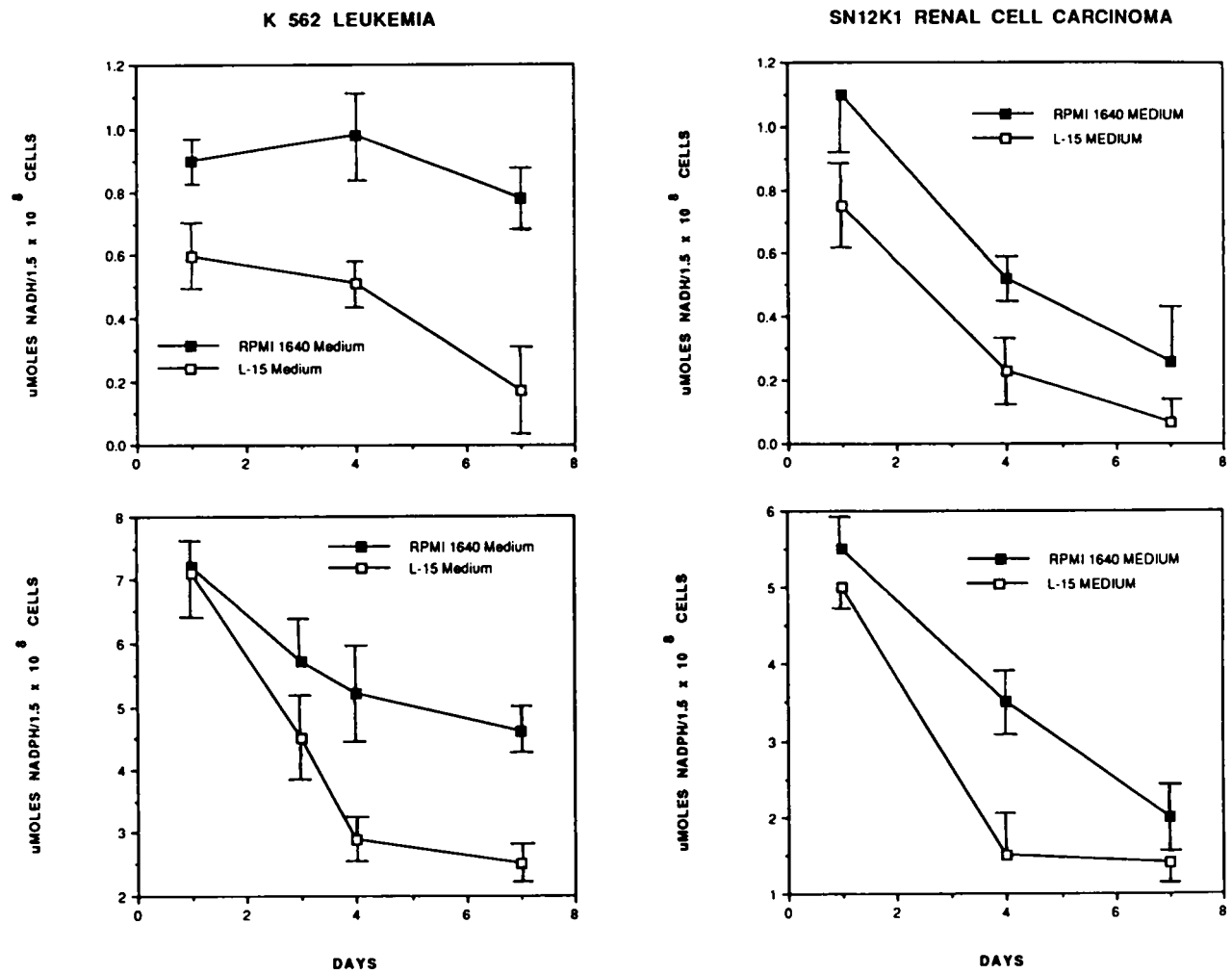

Fig. 4. NADH and NADPH concentrations in K 562 leukemia and SN12K1 renal carcinoma cells grown in D-glucose-containing (RPMI 1640) and glucose-free (L-15) media. Cells were harvested from either RPMI 1640 medium (■) or L-15 medium (□) and subcultured into the same medium. Cells were harvested at the indicated time points, and NADH and NADPH concentrations were determined as described in "Materials and Methods." The pH of each medium was 7.4. *Points*, means for 2 separate determinations; *bars*, SD.

(Fig. 4). Cellular NADH concentrations appeared to be most sensitive to D-glucose deprivation because a decrease in this pyridine nucleotide occurred 24 h following transfer of cells to L-15 medium. The decrease in cellular NADPH concentration occurred later than that of NADH. Several other recent studies (14, 15) also suggested that pH played a role in MTT reduction. Our results (Fig. 3) indicated that a reduction in MTT specific activity occurred immediately when cells were transferred to D-glucose-free medium with an identical pH. However, it may be, as suggested by Jabbar *et al.* (15), that other factors present in conditioned culture medium can accentuate the effect of pH on MTT reduction. Further study will be required to resolve this point. The present results (Fig. 5) are consistent with previously published data (14) which indicate that MTT formazan production was dependent upon the MTT concentration in the culture medium. In addition, the present findings indicate that both the kinetics of MTT formazan production and the degree of saturation vary in a cell line-specific manner. In addition, quantitation of drug cytotoxicity may be profoundly influenced by the length of exposure to MTT (Table 2).

These and several additional considerations (16) have recently resulted in the adoption of a protein-based end point assay (17) to replace MTT and XTT (3), a second generation tetrazolium salt, in the disease-oriented drug screening program of the National Cancer Institute. These include both the elimination of time-dependent steps such as the length of exposure to the tetrazolium and, for MTT, the necessity to extract the formazan with an organic solvent such as dimethyl sulfoxide. In addition the sulforhodamine B protein assay more effectively quantitates, for some compounds, drug effects in the extreme levels of growth inhibition (16). The formazan of XTT, in contrast to MTT formazan, is water soluble and thus can be quantitated in culture medium without the necessity for extraction with organic solvents. However, XTT presented several unique problems associated with high-flux drug screening including the inability of many cell lines to metabolize the tetrazolium in the absence of an added electron transfer reagent such a phenazine methosulfate (3). In retrospect, the observed increase in aqueous solubility provided by the sulfonate groups on XTT most probably resulted in limiting cellular penetration of the tetrazolium salt requiring the presence of the electron transfer agent. Several studies (18, 19) indicated that disulfonate compounds, such as the amino-reactive agent 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid, do not penetrate the plasma membrane and thus are used as probes for the external surface of the plasma membrane. In addition, alkalinization of growth medium which occurred during seeding of assay plates, drug treatment, and assay termination resulted in

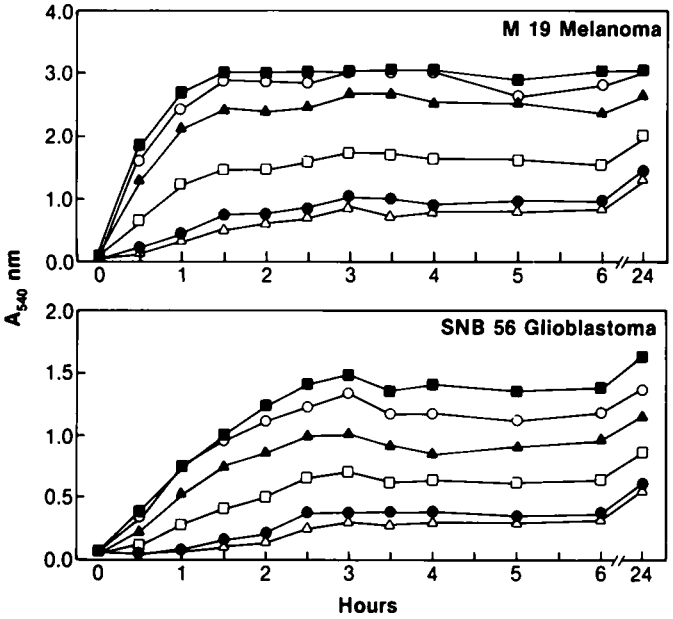

Fig. 5. Effect of MTT concentration and incubation time on MTT reduction by M 19 melanoma and SNB 56 gliosarcoma cells. Cells were seeded into 96-well plates and cultured for 48 h. MTT was added, and the incubation was continued for the appropriate time. MTT formazan was quantitated following extraction with dimethyl sulfoxide as previously described (2). MTT concentrations (mM): 0.075 (△); 0.1 (●); 0.25 (□); 0.5 (▲); 0.75 (○); 1.0 (■). Experimental values were determined from individual wells.

Table 2 *Effect of MTT concentration and incubation time on Adriamycin IC_{50} (μM) in SNB 56 tumor cells*

Cells were inoculated into 96-well plates (10,000 cells/well) on day 0. Adriamycin was added 24 h later, and MTT reduction was measured 48 h following drug addition according to previously published procedures (2).

MTT exposure (h)	MTT (mM)							
	0.075	0.10	0.25	0.50	0.75	1.0	1.5	2.0
0.5		1.8	1.2	3.1	0.9	0.9	1.2	2.1
1			1.7		3.3	3.7	3.1	4.7
2		4.3	2.6	3.0	5.8	5.2	6.8	6.1
3	3.5	3.8	10.5	7.1	9.3	9.2	8.1	8.8
4	4.2	12.1	11.7	10.1	12.0	9.9	13.0	8.2
5	17.4	21.9	16.0					23.8

Table 3 *Effect of MTT concentration and incubation time on Adriamycin IC_{50} (μM) in M 19 melanoma cells*

Cells were inoculated into 96-well plates (10,000 cells/well) on day 0. Drug treatment was as described in Table 2.

MTT exposure (h)	MTT (mM)							
	0.075	0.10	0.25	0.50	0.75	1.0	1.5	2.0
0.5	0.9	0.7	0.3	0.4	0.3	0.3	0.3	0.4
1	0.5	0.3	0.4	0.3	0.3	0.3		0.4
2	0.4	0.4	0.3	0.3	0.4	0.4	0.3	0.50
3	0.3	0.4	0.3	0.3	0.4	0.3	0.3	0.5
4	0.5	0.1	0.4	0.3	0.5	0.4	0.4	0.5
5	0.4		0.2	0.7	0.5	0.5		0.6

the occasional formation of crystalline material in the XTT assay which caused erratic absorbance measurements. Subsequent studies (20) indicated that crystal formation was significant at pH 7.8 to 8.0 and was attributed to reaction of phenazine methosulfate (E_0' = +0.08 V) with the cellular nucleophile glutathione (E_0' = −0.24 V). The presence of a positively charged nitrogen atom on phenazine methosulfate would facilitate a direct interaction of a negatively charged sulfhydryl group at alkaline pH.

It is apparent from the results described here and elsewhere (14, 15) that MTT reduction is affected by metabolic and other factors which may, in turn, substantially affect the quantitation of cell viability. It would seem prudent to establish, for each cell line, assay conditions which minimize their effect. These would include utilizing initial cell inoculation densities and an assay length which do not result in depletion of nutrients from the medium as well as establishing optimum concentrations and exposure times for MTT.

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