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THE DELAYED CYTOTOXIC REACTION OF 6-MERCAPTOPURINE

by

© DAVID M. TIDD

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "THE DELAYED CYTOTOXIC REACTION OF 6-MERCAPTOPURINE" submitted by David M. Tidd in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Pulse exposure to 6-mercaptopurine (MP) induced a delayed cytotoxic reaction in cultures of L5178Y cells and L1210 cells. L5178Y cells continued to proliferate during, and for 1 - 2 doublings after, exposures to MP that were ultimately lethal to 98 - 99% of the cells. The delayed cytotoxic reaction was characterized by "unbalanced growth" in which cell volume, and cellular content of RNA and protein increased above normal values. Subsequently, cell proliferation ceased in MP-treated cultures and enlarged cells lysed. Pulse exposures to 6-thioguanine (TG), β-2'-deoxythioguanosine and ethyl methanesulfonate induced similar delayed cytotoxic reactions. Brief exposure to 5-bromodeoxyuridine elicited a delayed cytotoxic effect although cells did not enlarge before disintegrating.

Inhibitions of purine nucleotide synthesis were apparently unrelated to delayed cytotoxic effects of MP and TG. Intracellular purine ribonucleotide pool sizes were reduced during pulse exposures to MP, but returned to normal values after exposures were terminated; the pool sizes of these metabolites in large degenerating cells were similar to those in untreated control cells. Thymidine partially protected cells against delayed cytotoxic

effects of MP, but did not affect reductions in purine ribonucleotide pool sizes. TG-induced reductions in purine ribonucleotide pool sizes were only slight. Inhibitions of purine nucleotide synthesis were probably responsible for increased cell population doubling times during MP exposure periods. Intracellular pools of acid-soluble MP derivatives readily declined after drug exposures were terminated.

MP was incorporated as TG nucleosides in internal 3',5'-phosphodiester linkages of DNA and RNA chains. A relation was observed between the extent of this incorporation and the delayed cytotoxicity of MP. Mycophenolic acid protected cells against the delayed cytotoxic reaction of MP and suppressed incorporation into nucleic acids. Cell cultures which spontaneously developed partial tolerance to MP exposure also exhibited a reduced capacity for incorporation of MP as TG into DNA and RNA. 6-Methylthioinosine potentiated cytotoxic effects of MP in a partially tolerant cell culture line and stimulated incorporation into DNA and RNA. Nucleic acid-incorporated TG was the major thiopurine derivative persisting in MP-sensitive cells at the time of the delayed cytotoxic reaction to MP exposure.

Cytotoxic effects of MP may be mediated by incorporation of TG anabolites into DNA. It is likely that the delayed cytotoxic reaction of MP is in reality that

of TG. Consistent with the hypothesis that incorporation into DNA is responsible for cytotoxic effects of MP was the observation that cells were most sensitive to MP during the mid DNA-synthetic period of the cell cycle.

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List of Abbreviations

MP	6-Mercaptopurine
TG	6-Thioguanine
TX	6-Thioxanthine
TG sulfinate	2-Aminopurine-6-sulfinate
A	Adenine
G	Guanine
U	Uracil
R	Ribosyl group at purine 9-position
dR	Deoxyribosyl group at purine 9-position
P	Monophosphate group (at pentosyl 5'-position if unspecified)
PP	Diphosphate group at pentosyl 5'-position
PPP	Triphosphate group at pentosyl 5'-position
3'-	Substituent (monophosphate) at pentosyl 3'-position
5'-	Substituent (monophosphate) at pentosyl 5'-position
FGAR	α-N-Formylglycinamide ribonucleotide
AIC	Aminoimidazole carboxamide
PRPP	5-Phosphoribosylpyrophosphate
DNase I	Pancreatic deoxyribonuclease
EAC	Ehrlich ascites carcinoma

FOOTNOTES

1. Based on a review by A. R. P. Paterson and D. M. Tidd. 6-Thiopurines: a. Basic Aspects. In: Handbook of Experimental Pharmacology Volume 38 "Antineoplastic and Immunosuppressive Agents", Part 2 (Sartorelli, A. C. and Johns, D. G., ed.). Springer-Verlag, in press.

2. Figures 5, 9, 15, 19 and 20 have been published in Cancer Research, 32: 317-322, 1972.

3. J. F. Henderson

I. INTRODUCTION

If progress is to continue in the design and development of cancer chemotherapeutic agents and procedures, it is important that the mechanisms of action of existing drugs be understood. Specific features of drug-cell interactions may then be exploited in a rational approach to the control of malignant disease by chemicals. 6-Mercaptopurine (MP) was first synthesized over 20 years ago by Elion et al. (52) and has since found extensive use in the therapy of human acute leukemia [see reviews by: Henderson (78); Goldin et al. (66)]. However, during the intervening years there has been no satisfactory biochemical explanation for the antitumor activity of MP. Rapidly proliferating cell populations appear to be most sensitive to MP (4,18,101,125,158), and this characteristic may be at least partially responsible for the chemotherapeutic selectivity achieved with this drug. As with other antimetabolites, MP toxicity toward the host involves the normal proliferating elements of bone marrow and intestinal epithelium (28,29,141). Quantitative biochemical differences and differences in cell generation time and growth fraction between normal and malignant tissues may provide the margin for chemotherapeutic success. MP also exhibits immunosuppressive (5,18,89,91,163)

and anti-inflammatory (18,62,83,131,132) activities and the explanation for these properties may lie in the destruction by MP of specific populations of proliferating normal cells.

The current understanding of MP metabolism and metabolic effects of the major anabolites of MP are reviewed below[1] in order to provide a background for discussion of the present results which concern the action of MP in mouse lymphoma L5178Y cells. In addition, the metabolism and metabolic effects of 6-thioguanine (TG) are reviewed since it would appear that TG nucleotides are also anabolites of MP.

The biochemical properties of the 6-thiopurines have been reviewed frequently; for example: Brockman (21); Brockman and Anderson (23); Elion et al. (54); Henderson and Mandel (81); Hutchison (93); Elion and Hitchings (51); Stock (170); Elion (49); Balis (12); Montgomery (125); and Roy-Burman (149).

The metabolism of MP and related thiopurines is summarized in Figure 1., which refers to transformations that take place in animal cells. Abbreviations used in this diagram are explained in the text. Figure 1 presents findings from a variety of mammalian cell types; particular cells may be unable to effect some of the conversions indicated. The concept that analogs of the natural purine bases become active as antimetabolites after conversion to

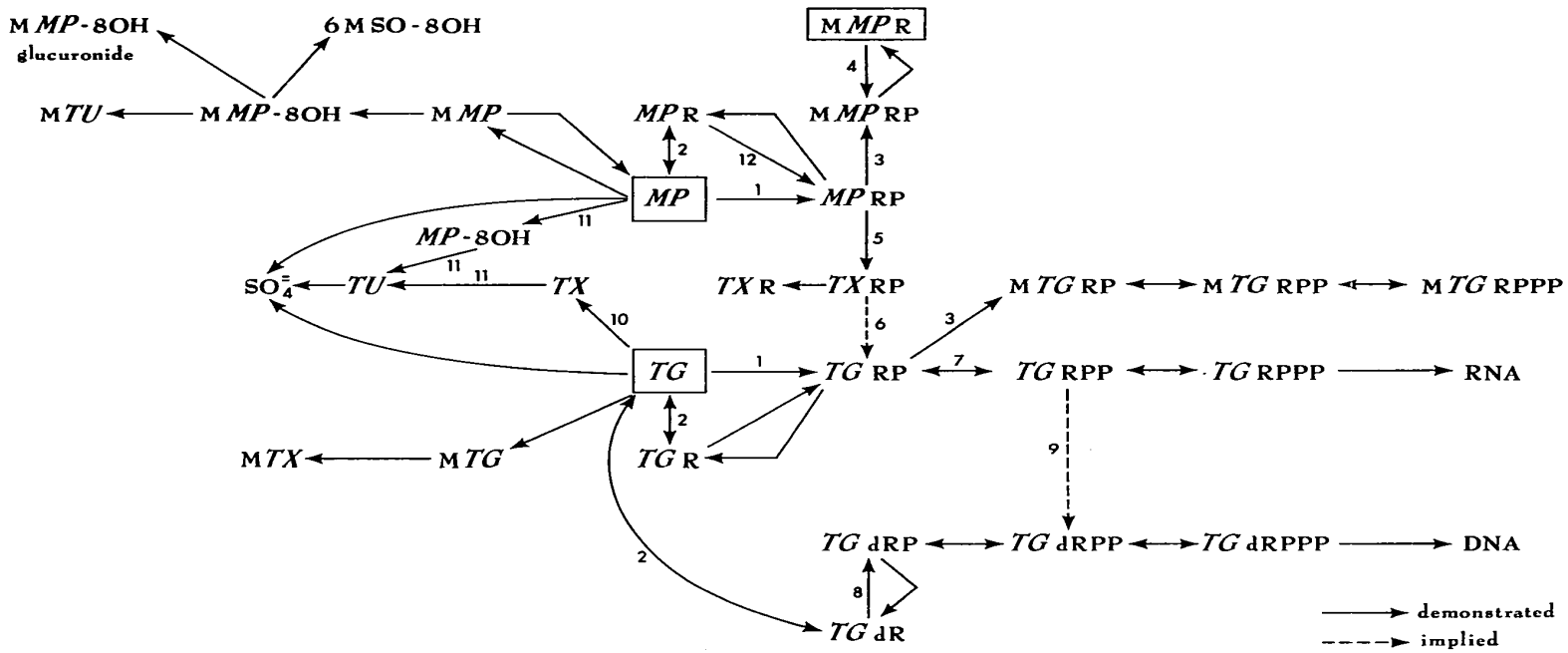

M MP-8OH
glucuronide
6 M SO-8OH
M MP R
M TU
M MP-8OH
M MP
MP R
M MP RP
MP
MP RP
MP-8OH
SO4=
TU
TX
TX R
TX RP
M TG RP
M TG RPP
M TG RPPP
TG
TG RP
TG RPP
TG RPPP
RNA
M TX
M TG
TG R
TG dRP
TG dRPP
TG dRPP
DNA
TG dR
demonstrated
implied

FIGURE 1. THIOPURINE METABOLISM

ENZYMES

1. Hypoxanthine-guanine phosphoribosyltransferase
2. Purine nucleoside phosphorylase
3. Methyltransferase
4. Adenosine kinase
5. Inosinate (IRP) dehydrogenase
6. Xanthylate (XRP) aminase
7. Guanylate (GRP) kinase
8. Deoxynucleoside kinase
9. Ribonucleotide reductase
10. Guanase
11. Xanthine oxidase
12. Inosine kinase

KEY TO ABBREVIATIONS

MP	6-Mercaptopurine
TG	6-Thioguanine
MMPR	6-Methylthioinosine
TX	6-Thioxanthine
MP-8OH	8-Hydroxy-6-mercaptopurine
TU	6-Thiouric acid
6MSO-8OH	6-Methylsulfinyl-8-hydroxypurine
SO_4=	Inorganic sulfate
R	Ribosyl group at purine 9-position
dR	Deoxyribosyl group at purine 9-position
M	Methyl group on 6-thiol of 6-thiopurine
P	Monophosphate group at pentosyl 5'-position
PP	Diphosphate group at pentosyl 5'-position
PPP	Triphosphate group at pentosyl 5'-position

ribosyl 5'-phosphate derivatives appears to have originated in the suggestion by Kidder and Dewey (98) that 8-azaguanine acted in this way. It has since been established that this process is the first essential step in the anabolism and mechanism of action of MP and the related thiopurines, 6-thioguanine (TG) and 6-methylthioinosine (MMPR).

A. Metabolism of 6-Mercaptopurine

1. Anabolism

(a). 6-Thioinosinate. The initial reaction in the anabolism of 6-mercaptopurine (MP) is conversion to 6-thioinosinate (MPRP) (see Figure 1) by hypoxanthine-guanine phosphoribosyltransferase (enzyme 1, Figure 1) (25,118). This step was recognized as essential to the inhibitory action of MP in experiments which showed that certain MP-resistant sublines of rodent neoplasms were devoid of this particular enzyme activity [see review by Brockman (21)].

The term "activation" has been used to describe this step; however, with hindsight, it now appears that earlier reference to 6-thioinosinate as the "active" form of the drug may have been misleading because, in addition to enzymatic inhibitions directly attributable to 6-thioinosinate, further metabolites of this compound are probably involved in MP cytotoxicity. The di- and triphosphate

derivatives of 6-thioinosine have not been identified as MP metabolites; however, it is possible that these may be among the uncharacterized minor metabolites of MP detected by Caldwell (30).

(b). 6-Methylthioinosinate. MP and other 6-thiopurine derivatives are substrates for an S-adenosylmethionine-requiring methyltransferase activity (enzyme 3, Figure 1) in animal cells (146,147). 6-Methylthioinosinate (MMPRP) is formed by methylation of 6-thioinosinate and is evidently responsible for certain metabolic effects of MP (see below). This compound is a major metabolite of MP in several cell types (2,15,30,138).

6-Methylthioinosinate is also the principle metabolite of 6-methylthioinosine (MMPR) in several cell types, including the human erythrocyte (114). 6-Methylthioinosine is a substrate for adenosine kinase (enzyme 4, Figure 1) and is converted thereby to the 5'-monophosphate ester (16,31[see also references 5-11, Henderson et al.(82)]).

6-Methylthioinosinate does not appear to be a substrate for nucleoside monophosphate kinases because the di- and triphosphate derivatives have not been detected in several cell types where formation of the monophosphate has been demonstrated. Accordingly, incorporation of 6-methylthioinosinate into nucleic acids would not be expected. Intracellular 6-methylthioinosinate persists for long

periods in Ehrlich ascites carcinoma cells and in human erythrocytes (115,138), but in cultured L5178Y cells, this compound has a half-time of less than one hour (C. T. Warnick and A. R. P. Paterson, unpublished results).

(c). 6-Thioxanthylate. 6-Thioinosinate is a substrate for inosinate dehydrogenase (enzyme 5, Figure 1) from Aerobacter aerogenes (74); the product of this reaction, 6-thioxanthylate (TXRP) (see Figure 1), is a major metabolite of MP in 3 mouse neoplasms, the Ehrlich ascites carcinoma, lymphoma L5178Y and Leukemia L1210 (7,30,122,138).

(d). Other Anabolites of 6-Mercaptopurine. By anion exchange chromatography on columns of DEAE Sephadex, Caldwell (30) showed that MP-treated Ehrlich ascites carcinoma cells contain a number of MP metabolites (perhaps 8) other than those mentioned above. These compounds have not been characterized.

(e). Incorporation into Nucleic Acids. A clue to the identity of some of the uncharacterized MP metabolites mentioned in the preceeding paragraph may be found in the work of Scannell and Hitchings (156), who demonstrated that DNA from an MP-treated tumor contained deoxythioguanosine (TGdR). It is implicit in this finding that nucleotide derivatives of 6-thioguanine (TG) are formed from MP. Although this has not been studied directly, it may be presumed that amination of 6-thioxanthylate by xanthylate aminase (enzyme

6, Figure 1) is a step in the formation of the 6-thioguanosine phosphates from MP and that deoxythioguanosine phosphates are derived therefrom. These compounds have not been demonstrated as metabolites of MP; however, their implied existence is likely because TG is also anabolized by these sequences (see Figure 1 and below). The implied existence of thioguanosine phosphates as MP anabolites suggests that MP would be incorporated into RNA as well as into DNA and this is confirmed in the present investigation (see below). The incorporation of TG into RNA nucleotides in mouse tumor cells has been demonstrated by LePage (107). Incorporation of MP into RNA has been reported (20), however characterization of this material was not rigorous. Hansen and coworkers presented evidence which suggested that MP may be bound firmly to RNA by forming metal mercaptides with divalent ions associated with the nucleic acid (75,76). The amount of MP bound to RNA was within the order of magnitude of that reported as "incorporated" by Bieber et al. (20).

(f). 6-Thioinosine. 6-Thioinosine (MPR) (see Figure 1) is a substrate for purine nucleoside phosphorylase (enzyme 2, Figure 1) (100); Ehrlich ascites carcinoma cells cleave this compound phosphorolytically, with liberation of the free base (137). However, phosphorylation of 6-thioinosine has been demonstrated in extracts of a thiopurine-

resistant subline of the Ehrlich ascites carcinoma which lacked hypoxanthine-guanine phosphoribosyltransferase (enzyme 1, Figure 1) (145). The enzyme responsible has been termed "inosine kinase" (enzyme 12, Figure 1) (144); previously, the existence of this enzyme activity had been doubted (23). "Inosine kinase" activity may not be significant in the intact cell since thiopurine-resistant tumors which lack hypoxanthine-guanine phosphoribosyltransferase are cross resistant to thioinosine.

2. Catabolism

The urinary excretion products of the 6-thiopurines are derived by oxidation and methylation processes. In the mouse, MP is converted to 6-thiouric acid (TU) by xanthine oxidase (enzyme 11, Figure 1), probably by way of the intermediate 6-thio-8-hydroxypurine (MP-8OH). In the mouse, both MP and 6-thiouric acid are excreted in the urine; a portion of the 6-thiouric acid may be further degraded by uricase, with the ultimate formation of sulfate (49,54,55). In man, MP, 6-thiouric acid, and sulfate are also MP excretion products; the route of sulfate formation is unknown, but does not involve uricase since this enzyme is absent in man. 6-Methylsulfinyl-8-hydroxypurine (6MSO-8OH) and several other methylated oxidation products (see Figure 1) have also been identified as urinary excretion products of

MP in man (49). 6-Methylthiopurine (MMP) occurs in rat urine after administration of MP (152), but has not been found in mouse or human urine (49).

The xanthine oxidase inhibitor, allopurinol (4-hydroxypyrazolo[3,4-d]pyrimidine), inhibits the oxidation of purines and of MP *in vivo* and *in vitro* by both mouse and human tissues; the toxicity of MP, and its potency as an antineoplastic agent, are increased several-fold in the presence of allopurinol in both mouse and man (3,54,87). Very little oxidative catabolism of MP occurs within tumor tissue; however, in leukemia L1210 cells there is a small amount of direct dethiolation of MP, presumably with the production of hypoxanthine (122).

B. Metabolism of 6-Thioguanine

DNA isolated from MP-treated cells has been shown to contain deoxythioguanosine (see Chapter I, Section A. I. (e) and Chapter IV). This implies that thioguanine nucleotides are anabolites of MP and therefore the metabolism of TG is included in this discussion.

1. Anabolism

(a). 6-Thioguanosine Phosphates. The initial step in the anabolism of TG is conversion to the ribonucleoside

monophosphate derivative, 6-thioguanylate (TGRP) (see Figure 1). As with MP, this reaction is catalyzed by hypoxanthine-guanine phosphoribosyltransferase (enzyme 1, Figure 1). This became apparent when particular mouse tumors, which were resistant to MP through deletion of this enzyme, were found to be cross resistant to TG and also to lack the ability to convert TG to 6-thioguanylate (56,102,171). As with MP, the formation of the ribonucleoside monophosphate derivative is essential to the cytotoxic action of TG, but metabolites beyond 6-thioguanylate are involved in cytotoxicity. 6-Thioguanylate is a prominent metabolite of TG in mouse tumor cells; metabolites tentatively identified as thioguanosine di- and triphosphates (TGRPP and TGRPPP) have also been detected (107,123, 126). This is in accordance with the demonstration by Miech et al. (123) that 6-thioguanylate is a substrate for the highly specific guanylate kinase (enzyme 7, Figure 1) of Sarcoma 180 cells; 6-thioguanylate behaves as an alternative substrate for this enzyme, but the reaction has a low maximum velocity relative to that with guanylate.

(b). Deoxythioguanosine Phosphates. Although free deoxyribonucleoside phosphate derivatives of TG have not been isolated, they are evidently formed in cells because TG is incorporated into nucleotide linkage of DNA (104,111, 112).

(c). Incorporation into Nucleic Acids. TG is incorporated into DNA (see Figure 1) in internal nucleotide positions (104,111,112). In demonstrating the latter, DNA was degraded with deoxyribonuclease and phosphodiesterase, and the incorporated analog was recovered in the form of a mononucleotide; this has been demonstrated in several mouse tumors and in normal mouse tissues (112). The incorporation of TG into DNA nucleotides has also been demonstrated with bone marrow from leukemic patients and patients with solid tumors (111). That TG also becomes incorporated into RNA (Figure 1) is apparent in the recovery of TG nucleotides from RNA isolated from TG-treated mouse tumor cells (107).

(d). Other Anabolites of 6-Thioguanine. Allan and Bennett (1) have demonstrated that 6-methylthioguanylate (MTGRP) (see Figure 1) is an anabolite of TG in H.Ep.No.2 cells grown in culture, and is apparently formed by the methylation of 6-thioguanylate involving the previously mentioned methyltransferase (enzyme 3, Figure 1) (147). The di- and triphosphates of 6-methylthioguanosine were also tentatively identified.

(e). 6-Thioguanosine and β-2'-Deoxythioguanosine. One route by which β-2'-deoxythioguanosine (TGdR) is anabolized in normal and neoplastic cells is by way of a kinase-catalyzed phosphorylation (enzyme 8, Figure 1) with eventual incorporation into DNA (94,112,140). A

kinase activity present in Ehrlich ascites carcinoma cells also catalyzes the phosphorylation of 6-thioguanosine (TGR) (145). The corresponding di- and triphosphate derivatives of 6-thioguanosine and its deoxyribosyl homologue (see Figure 1) have been demonstrated in acid extracts of tumor cells which were treated in vivo with the analog nucleosides (107). α-2'-Deoxythioguanosine is also converted to mono-, di-, and triphosphate derivatives and incorporation into the terminal nucleoside positions of RNA and DNA in a mouse tumor has been shown. In contrast, β-2'-deoxythioguanosine was incorporated into internal nucleotide positions in DNA (107).

In an alternate anabolic route, the ribosyl and deoxyribosyl derivatives of TG are cleaved by phosphorolysis (enzyme 2, Figure 1) with release of the base which is then available for conversion to nucleotide by hypoxanthine-guanine phosphoribosyltransferase (enzyme 1, Figure 1). Phosphorolysis of the ribosyl and β-2'-deoxyribosyl derivatives of TG has been demonstrated in tumor cell extracts, but α-2'-deoxythioguanosine is not cleaved by purine nucleoside phosphorylase (107).

2. Catabolism

TG is a substrate for guanase (enzyme 10, Figure 1) and the deamination product is 6-thioxanthine (TX) (see

Figure 1) (42). The latter is oxidized by xanthine oxidase (enzyme 11, Figure 1) and the reaction product is 6-thiouric acid (TU). 6-Thioxanthine and 6-thiouric acid have been identified as catabolites of TG in mouse tissues (126). 6-Thioxanthine and 6-thiouric acid are major catabolites of TG in patients with tumors of the head and neck (103). However, degradation of TG by this route occurs only to a small extent during therapy of human leukemias (49). Methylated excretion products of TG are more abundant in these patients and 2-amino-6-methylthiopurine (MTG) (see Figure 1) is a prominent urinary constituent (53). Lefkowitz et al. (103) suggested that these differences in man may be related to the type of disease. Sulfate is also a major endproduct in the catabolism of TG. Small amounts of TG undergo direct dethiolation in tumor tissue and the products have been detected as nucleic acid purines, principally as guanine (155).

C. Metabolic Effects of 6-Mercaptopurine

Efforts to elucidate the biochemical mechanisms of MP cytotoxicity have involved searching for biochemical disturbances produced by the drug and its metabolites which could account for the drug's biological effects. Elion and Hitchings (51) have summarized some of the metabolic effects

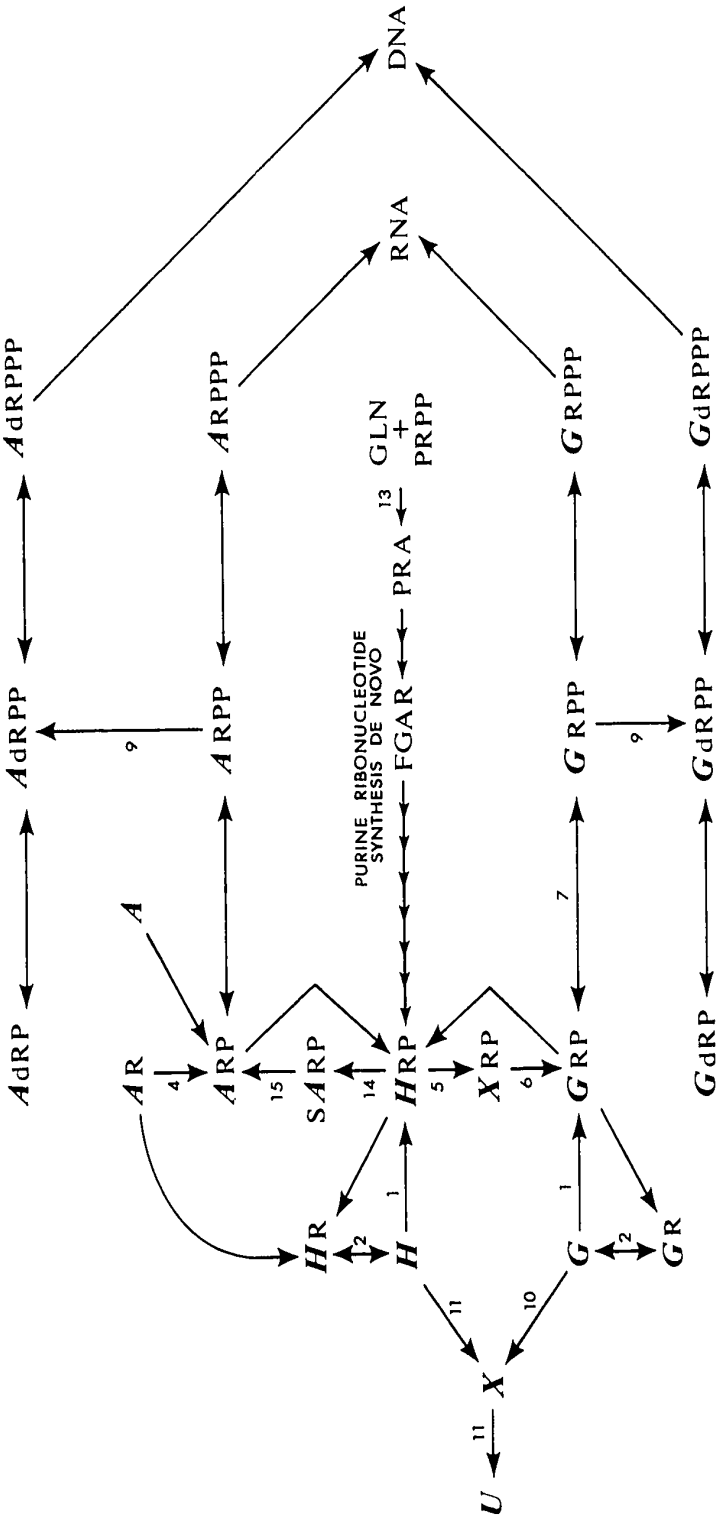
of MP anabolites; the present discussion of these effects does not attempt to be comprehensive and is admittedly selective. The metabolism of normal purines is summarized in Figure 2, and effects of MP anabolites at the level of purine nucleotide synthesis and interconversions are discussed in relation to inhibitions of enzyme catalyzed reactions represented in this diagram.

1. The Free Base, 6-Mercaptopurine

MP has been shown to inhibit hypoxanthine-guanine phosphoribosyltransferase (enzyme 1, Figure 2) (6) and xanthine oxidase (enzyme 11, Figure 2) (164); in both inhibitions the analog is a competitive substrate. Since conversion to 6-thioinosinate is the first step essential to the cytotoxic activity of MP [see review by: Brockman (21)], these inhibitory effects of the free base are of doubtful chemotherapeutic significance.

2. Nucleotide Anabolites

Studies of MP "mechanisms of action" have concentrated upon metabolic effects of the anabolites, 6-thioinosinate and 6-methylthioinosinate (see Figure 1); enzymatic inhibitory effects of possible importance in the present context are not known for 6-thioxanthylate, the other major anabolite of MP. The isolation of


DNA
RNA
AdRPPP
ARPPP
GLN
+
PRPP
GRPPP
GdRPPP
AdRPP
ARPP
PRA
GRPP
GdRPP
PURINE RIBONUCLEOTIDE SYNTHESIS DE NOVO
FGAR
AdRP
A
AR
ARP
SARP
HRP
XRP
GRP
GdRP
HR
H
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GR
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FIGURE 2. PURINE METABOLISM

ENZYMES

1. Hypoxanthine-guanine phosphoribosyltransferase
2. Purine nucleoside phosphorylase
4. Adenosine kinase
5. Inosinate (IRP) dehydrogenase
6. Xanthylate (XRP) aminase
7. Guanylate (GRP) kinase
9. Ribonucleotide reductase
10. Guanase
11. Xanthine oxidase
13. Glutamine-phosphoribosylpyrophosphate (PRPP) amidotransferase
14. Adenylosuccinate synthetase
15. Adenylosuccinate lyase

KEY TO ABBREVIATIONS

H	Hypoxanthine
A	Adenine
G	Guanine
X	Xanthine
U	Uric acid
PRA	5-phosphoribosylamine
FGAR	α-N-formylglycinamide ribonucleotide
PRPP	5-phosphoribosyl pyrophosphate
GLN	Glutamine
SARP	Adenylosuccinate
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
R	Ribosyl group at purine 9-position
dR	Deoxyribosyl group at purine 9-position
P	Monophosphate group at pentosyl 5'-position
PP	Diphosphate group at pentosyl 5'-position
PPP	Triphosphate group at pentosyl 5'-position

deoxythioguanosine from DNA of MP-treated cells (156) revealed that TG nucleotides are formed in the metabolism of MP. TG nucleotides would appear to be derived by way of 6-thioinosinate conversion to 6-thioguanylate, presumably via 6-thioxanthylate. It is at least theoretically possible that MP-treated cells will express the superimposed metabolic effects of several MP anabolites, including TG nucleotides. The nature of the MP effect would depend upon the relative proportion of the MP anabolites. The following discussion will be concerned mainly with the metabolic effects of 6-thioinosinate and 6-methylthioinosinate.

(a). Inhibition of Purine Ribonucleotide Synthesis de Novo. It was originally demonstrated that MP inhibited the incorporation of ^{14}C-labeled formate or glycine into acid-soluble purines (59) and nucleic acid purines of experimental neoplasms (68,77,108,166). The inhibition by MP of incorporation of phosphate into nucleic acids (44) could be interpreted in terms of these observations, which suggested that MP inhibited either purine ribonucleotide synthesis de novo or purine ribonucleotide interconversions, or both of these processes (see Figure 2).

MP also inhibited the accumulation of α-N-formylglycinamide ribonucleotide (FGAR) in azaserine-treated tumor cells, either in vivo or in vitro (22,24,109). Azaserine,

a glutamine analog, inhibits the glutamine-requiring amidation of FGAR; flux through the preceding steps of purine synthesis continues and FGAR accumulates in azaserine-treated cells. Thus, the inhibition by MP of FGAR accumulation indicated that MP inhibited an early step in purine ribonucleotide synthesis de novo (see Figure 2). Brockman (22) showed that MP did not inhibit the accumulation of FGAR in thiopurine-resistant cells that lacked the capacity to form 6-thioinosinate, in agreement with earlier results of LePage and Jones (109) which suggested that a nucleotide metabolite of the drug was responsible for the inhibition of the purine pathway. Natural purines also inhibited accumulation of FGAR (79) and it seemed that mammalian cells were similar to E. coli. cells in which purine ribonucleotide synthesis de novo was subject to feedback regulation and in which thiopurine metabolites evidently mimicked natural purine nucleotides as feedback inhibitors of an early enzyme step (67). On the basis of similar experiments with intact tumor cells, 6-methylthioinosine (as the 5'-monophosphate) was shown to be a very potent inhibitor of the de novo pathway of purine synthesis (80).

6-Thioinosinate and 6-methylthioinosinate have been reported to inhibit various preparations of glutamine-phosphoribosylpyrophosphate (PRPP) amidotransferase (enzyme 13, Figure 2), the first enzyme unique to the pathway of

purine ribonucleotide synthesis de novo (35,84,120,121,174). In this effect, the thiopurine nucleotides mimicked the effect of natural purine nucleotides and consequently these analog ribonucleotides were described as "pseudo-feedback inhibitors" of the enzyme. 6-Methylthioinosinate is the most potent of the reported nucleotide inhibitors of the amidotransferase (84,174), and it has been suggested that intracellular 6-methylthioinosinate, formed as a metabolite of MP (see Figure 1), is responsible for most of the inhibition of purine synthesis de novo observed in MP-treated cells (15).

Bennett et al. (17) concluded that for three mouse neoplasms treated in vivo with MP, interference with an early step in purine synthesis de novo was of greater significance in inhibition of purine nucleotide synthesis than inhibition of purine ribonucleotide interconversions. In these experiments, MP markedly inhibited the incorporation of ^{14}C-formate or ^{14}C-glycine into nucleic acid purines, but had little or no effect on the incorporation of ^{14}C-aminoimidazole carboxamide (AIC). Similarly, Brockman and Chumley (24) found that incorporation of AIC into purine ribonucleotides in drug-sensitive cells was not inhibited at concentrations of MP which strongly inhibited the azaserine-induced accumulation of FGAR. Hakala and Nichol (71) reported that AIC partially prevented MP inhibition

of tumor cell growth in culture. They suggested that a block prior to the formation of AIC ribonucleotide on the de novo purine pathway was at least partially responsible for growth inhibition in this system in which cell proliferation was totally dependent upon endogenous formation of purines. In contrast, LePage and coworkers found no correlation between tumor growth inhibition and feedback inhibition of purine ribonucleotide synthesis de novo by MP (68,109). Also Hitchings and Elion (90) reported that MP elicited feedback inhibition of purine synthesis de novo to the same extent in lines of Adenocarcinoma 755 which were MP-sensitive and MP-resistant. Thus, inhibition of purine synthesis de novo by a metabolite of MP is an important biochemical effect of the drug; however, a relationship between this effect and cytotoxicity has not been established.

An argument against the idea that MP cytotoxicity derives from inhibition of purine synthesis de novo may be found in the fact that therapeutic effects are enhanced synergistically when MP and several inhibitors of purine synthesis de novo are used in combination in chemotherapy of various rodent tumors. The glutamine analogs, azaserine and 6-diazo-5-oxo-L-norleucine inhibit de novo purine synthesis, and both synergize with MP (38, 86,173). As well, it has been demonstrated that

6-methylthioinosine potentiates the cytotoxic activity of MP, apparently by inhibiting glutamine-PRPP amidotransferase and thereby increasing the availability of PRPP for phosphoribosyltransferase-catalyzed reactions; in this way, the anabolism of MP is enhanced (136,138,139,159,162,177). It is difficult to imagine how combinations of these agents with MP would result in synergistic enhancement of cytotoxic effects, if MP toxicity were due solely to inhibition of PRPP-amidotransferase. However, inhibition of glutamine-PRPP amidotransferase and the concomitant accumulation of PRPP would appear to be the basis of the "self-enhancement" effect of MP, in which the ability of cells to convert MP to 6-thioinosinate is enhanced by prior exposure to MP (122,133,134).

Hitchings and Elion (90) concluded that potentiation of azaserine by MP was inconsistent with the concept that the purine analog acts primarily on de novo purine synthesis. They suggested that effects on purine ribonucleotide interconversions may play an important role in tumor inhibition.

(b). Inhibition of Purine Ribonucleotide Interconversions. Experiments with microorganisms provided the first evidence that thiopurines inhibited purine ribonucleotide interconversions [see reviews by: Elion and Hitchings (50); Skipper and Bennett (167); Brockman (21); Brockman and

Anderson (23); Balis (12)]. Hakala and Nichol (70) and Davidson (43) found that MP, presumably as the nucleotide, inhibited the conversion of inosinate (HRP) to adenylate (ARP) (see Figure 2) in intact tumor cells, and Simpson et al. (165) reported that formation of guanylate (GRP) from inosinate (see Figure 2) by Ehrlich ascites cells in vivo was also inhibited by MP and was apparently more susceptible to inhibition by MP than formation of adenylate. Cell-free preparations of Sarcoma 180 exhibited reduced capacity to convert inosinate to adenylate and xanthylate (XRP) (see Figure 2) when the tumor was obtained from animals which had been treated with MP (150). In contrast, Baker and Bennett (11) concluded that the concentration of 6-thioinosinate required to inhibit the conversion of inosinate to adenylate in a cell-free system from Adenocarcinoma 755 was sufficiently high that this inhibition was not likely to be of primary significance in vivo. However, inosinate concentrations in cells are low and Elion (49) has suggested that the 6-thioinosinate concentrations achieved in MP-treated cells would likely inhibit inosinate metabolism, despite the fact that 6-thioinosinate has lower affinity than inosinate for inosinate dehydrogenase (enzyme 5, Figure 2) and adenylosuccinate synthetase (enzyme 14, Figure 2). Whether or not these metabolic effects of 6-thioinosinate contribute to the

therapeutic result is still an open question because intracellular pools of 6-thioinosinate have a transient existence only (12,133).

Inosinate dehydrogenase (enzyme 5, Figure 2) catalyzes conversion of inosinate to xanthylate; preparations of this enzyme from pigeon liver (151) and from Ehrlich ascites tumor cells (8) were inhibited by 6-thioinosinate. Adenylosuccinate synthetase (enzyme 14, Figure 2) catalyzes conversion of inosinate to adenylosuccinate (SARP), and adenylosuccinate lyase (enzyme 15, Figure 2) is responsible for the conversion of adenylosuccinate into adenylate. Atkinson et al. (9) reported that both enzymes (partially purified from Ehrlich ascites tumor cells) were inhibited by 6-thioinosinate and concluded from a comparison of the activities and kinetic parameters of inosinate dehydrogenase, adenylosuccinate synthetase, and adenylosuccinate lyase that the first of these enzyme activities was probably most sensitive to inhibition by 6-thioinosinate in intact Ehrlich ascites tumor cells. Salser and Balis (150) demonstrated that the intrinsic capacity for synthesis of adenylate and xanthylate was lower in Sarcoma 180 than in normal mouse liver and suggested that the lower enzyme activities in tumors might account for the selective cytotoxic action of MP toward neoplastic tissue. In spite of these observations, a causal relationship between

inhibition of purine nucleotide interconversions and MP cytotoxicity has not been established. Further, it has not been established that temporary constriction of the production of purine nucleotides is necessarily lethal to the cell.

(c). Incorporation into DNA. Scannell and Hitchings (156) demonstrated that deoxythioguanosine was present in DNA isolated from a MP-resistant subline of Adenocarcinoma 755 after treatment with MP. The amount of deoxythioguanosine associated with the DNA of the resistant tumor was twice that found in the sensitive parent line and was of the same order of magnitude as that reported for incorporation of TG into DNA under comparable conditions (106). Although it appears that incorporation of TG into DNA is centrally involved in the cytotoxic activity of TG (see below), the mechanism by which the incorporated analog exerts its toxic effect is not evident. It would seem that in some resistant mutants toxic effects of the DNA-incorporated analog are suppressed in some manner.

(d). Resistance to 6-Mercaptopurine. In a number of instances, MP resistance in experimental tumors has been associated with loss of the ability to convert MP to 6-thioinosinate [see review by Brockman (21)]. This has usually involved reduced activity of or deletion of hypoxanthine-guanine phosphoribosyltransferase. However,

it is clear that resistance to MP may be acquired by other means, as will be apparent in the following examples. A subline of the Ehrlich ascites carcinoma selected for MP resistance (135) possessed MP phosphoribosyltransferase activity comparable to that of the MP-sensitive parent line. Wolpert et al. (180) suggested that enhanced breakdown of thiopurine nucleotides by alkaline phosphohydrolase may be at least partially responsible for the insensitivity of a subline of Sarcoma 180 to these agents.

Bieber et al. (20) have described a MP-resistant line of Adenocarcinoma 755 which apparently incorporated at least as much MP into acid-soluble nucleotides and into the nucleic acids as the MP-sensitive line. Scannell and Hitchings (156) demonstrated that deoxythioguanosine was present in DNA of this resistant tumor after treatment with MP (see above).

What little is known about thiopurine resistance in human neoplastic disease also indicates that mechanisms other than phosphoribosyltransferase deletion are operative; for example, assay of phosphoribosyltransferase activities of leukocytes from leukemic patients who had become resistant to therapy with MP after initially responding, revealed a deficiency of hypoxanthine phosphoribosyltransferase in cells of only one of the fifteen subjects tested (45).

(e). Summary. The intracellular presence of the MP anabolites, 6-methylthioinosinate and 6-thioinosinate, results in inhibition of purine ribonucleotide synthesis and interconversions. The specific enzymatic effects are inhibition of glutamine-PRPP amidotransferase by 6-thioinosinate and 6-methylthioinosinate, and inhibitions by 6-thioinosinate of inosinate dehydrogenase, adenylosuccinate synthetase and adenylosuccinate lyase. Conversion of MP to TG nucleotides introduces the possibility of still further loci of action for MP. There has not been sufficient evidence to attribute the lethal effects of MP exposure to the enzymatic inhibitions discussed above.

D. Metabolic Effects of 6-Thioguanine

DNA isolated from MP-treated cells has been shown to contain deoxythioguanosine (see Chapter I, Section A. 1. (e) and Chapter IV). This implies that thioguanine nucleotides are anabolites of MP and therefore the metabolic effects of TG are included in this discussion.

Treatment with TG inhibits the growth of a number of transplantable rodent tumors, including Adenocarcinoma 755, leukemia L1210, Ehrlich ascites carcinoma

and others (153,160,176,179). Useful therapeutic responses were obtained in clinical trials of TG in treatment of acute lymphocytic leukemia and chronic myelogenous leukemia (57,58,72,129). TG and MP appeared to have the same therapeutic spectrum, and TG was not effective in leukemias resistant to MP (72). It has been a general opinion among clinical investigators that TG and MP were similar in metabolism and that therapeutic responses to each were similar, although TG was more toxic on a molar basis. Of the two, MP has been the most studied.

On a molar basis, TG is about 25 times more toxic to the mouse than MP (168). The principal feature of TG toxicity in mammals is the highly selective action against bone marrow (141,143). In man toxic effects include leukopenia and thrombocytopenia with some gastrointestinal disturbances (142). Marrow depression is also the principal toxicity associated with the combination of TG and arabinosylcytosine (63).

1. The Free Base, 6-Thioguanine

TG was shown to inhibit the incorporation of guanine into nucleic acids of ascites tumor cells, and it was suggested that this effect might be partly attributable to competition between TG and guanine for hypoxanthine-guanine phosphoribosyltransferase (154).

2. Nucleotide Anabolites

6-Thioguanylate (TGRP) (see Figure 1) is a major metabolite of TG in normal tissues of the mouse, guinea pig bone marrow, and in several ascitic tumors of the mouse (107,123,126). 6-Thioguanosine di- and triphosphates (see Figure 1) have also been tentatively identified as TG metabolites in mouse ascites tumors. In Ehrlich ascites carcinoma cells which were treated in vivo with TG, intracellular 6-thioguanylate persisted for substantial periods (after 24 h, 6-thioguanylate concentrations were about 20% of those at 1 h (126)), suggesting that this metabolite may turn over more slowly than 6-thioinosinate (133). It was established that formation of 6-thioguanylate is essential to the cytotoxic action of TG as a result of experiments with thiopurine-resistant sublines of mouse tumors which were devoid of hypoxanthine-guanine phosphoribosyltransferase activity and were therefore unable to anabolize TG (21). This finding focused attention on the effects of 6-thioguanylate on the intermediary metabolism of purine nucleotides. However, it was also apparent that the involvement of 6-thioguanylate in TG cytotoxicity did not preclude the involvement of metabolites beyond 6-thioguanylate. In attempts to relate TG cytotoxicity to the biochemical effects of TG metabolites, three areas have been investigated: (a) purine nucleotide synthesis de novo,

(b) interconversions of purine nucleotides (see Figure 2), and (c) incorporation of TG into DNA. Studies on the first two areas have attempted to identify inhibitory actions of 6-thioguanylate and to relate these to growth inhibition.

(a). Inhibition of Purine Ribonucleotide Synthesis de Novo. The presence of 6-thioguanylate in cells has a number of direct consequences. Glutamine-PRPP amidotransferase (enzyme 13, Figure 2), the first enzyme in the reaction sequence by which purine nucleotides are synthesized de novo, is apparently subject to allosteric regulation by purine nucleotides, and is inhibited by several nucleotide derivatives of thiopurines (121). Allan and Bennett (1) have compared the abilities of 6-thiopurine ribonucleotides to inhibit partly purified PRPP amidotransferase from mouse tumor cells; the results (expressed in terms of the concentration (mM) required for 50% inhibition of the amidotransferase) are as follows: 6-thioinosinate (1.11), 6-thioguanylate (0.27), 6-methylthioinosinate (0.09), and 6-methylthioguanylate (1.15). From the concentrations of 6-thioguanylate achievable in tumor cells (about 0.1 - 0.2 mM (123,126)), it would appear that this metabolic effect of 6-thioguanylate may be operative in TG-treated tumor cells. Consistent with these facts are the demonstrations that TG blocks (a) the first portion of the de novo pathway

of purine synthesis and (b) the incorporation of glycine-^{14}C into the purine bases of the nucleic acids of tumor cells (105). The formation of 6-methylthio derivatives of the thioguanosine phosphates (see Figure 1) probably does not contribute to the inhibition by TG of purine synthesis de novo (1).

(b). Inhibition of Purine Ribonucleotide Interconversions. Inosinate dehydrogenase (enzyme 5, Figure 2) is inhibited by 6-thioinosinate and 6-thioguanylate, apparently because the analogs form disulfide bonds with an enzymic sulfhydryl group at the inosinate reaction site (8,74). The inactivation by thiopurine nucleotides of the inosinate dehydrogenase activities of Aerobacter aerogenes and of Sarcoma 180 cells is reversible by sulfhydryl reagents (74,123). 6-Thioguanylate is an inhibitor of guanylate kinase (enzyme 7, Figure 2) from Sarcoma 180 cells and from hog brain. The analog behaves as a competitive inhibitor of the substrate guanylate with a K_i of about 6×10^{-5}M and is itself a substrate for the enzyme, although the maximum velocity of the reaction is low (124).

Miech et al. (123,124) have proposed that the presence of 6-thioguanylate in tumor cells should impair the synthesis of guanylate through inhibition at the 2 sites noted above, glutamine-PRPP amidotransferase (enzyme 13, Figure 2) and inosinate dehydrogenase

(enzyme 5, Figure 2). The authors suggest that these inhibitions, together with that of guanylate kinase (enzyme 7, Figure 2), might result in a general lowering of intracellular concentrations of guanine nucleotides, a situation which might have serious consequences for the cell. It remains in the realm of speculation whether a temporary constriction in the synthesis of guanosine phosphates would have lethal consequences for cells.

(c). Incorporation into DNA. On the basis of investigations into the metabolic effects of TG, LePage concluded that TG incorporation into DNA (see Figure 1) consistently correlated with tumor inhibition and was therefore a likely basis for TG cytotoxicity (104,106, 109,110,154). A number of TG-sensitive mouse tumors incorporated TG into DNA, whereas such incorporation was much less in the TG-insensitive C_3HED and Mecca lymphosarcomas (106,109). In contrast, inhibition of the de novo pathway of purine synthesis by 6-thioguanylate did not correlate with tumor sensitivity to TG; this effect of TG was operative in the TG-insensitive lymphomas, as well as in the sensitive neoplasms (109). The postulate that TG cytotoxicity was due to entry into DNA structure suggested that β-2'-deoxythioguanosine might be an effective antitumor agent with some advantage over TG in that fewer metabolic steps would be required for its incorporation

into DNA. The α- and β-anomers of 2'-deoxythioguanosine were found to have antitumor activity and were inhibitory to TG-resistant tumor sublines and to the naturally insensitive Mecca lymphosarcoma (112). Mecca cells incorporated the β-anomer into DNA at a much higher rate than TG; the α-anomer also entered DNA, partly in the form of chain termini (110).

LePage and Whitecar (111) have shown that bone marrow of patients with acute leukemia responded rapidly to TG treatment with the disappearance of a large fraction of the original cells. A course of 5 daily treatments projected the surviving cells into a proliferative state, and in those cells, one-half to essentially all DNA guanine was replaced by TG. The specific toxicity of TG for bone marrow may be attributable to the ability of other rapidly proliferating tissues to deaminate the analog (see Figure 1).

(d). Summary. 6-Thioguanylate inhibits the de novo pathway of purine synthesis and purine nucleotide interconversions; the enzymes inhibited by the analog nucleotide are glutamine-PRPP amidotransferase, inosinate dehydrogenase and guanylate kinase. However, there is reasonable doubt that the cytotoxic effects of TG can be attributed to these inhibitions. Incorporation of TG into DNA correlates with drug lethality, but the mechanism by

which the incorporated analog exerts its effect is not apparent.

E. Objectives in this Investigation

Experiments performed in this laboratory indicated that there was a delay between exposure to MP and effects of this drug treatment on proliferation of EAC cells *in vivo* and mouse lymphoma L5178Y cells *in vitro*. A search of the thiopurine literature revealed that similar effects of MP had been reported in the early work on the antitumor properties of MP. However, these observations were evidently overlooked during subsequent investigations of the biochemical mechanisms responsible for cytotoxic effects of MP. MP-induced inhibitions at the level of purine nucleotide synthesis have been offered as explanations for MP cytotoxicity. Such inhibitions would be expected to elicit immediate effects on cell proliferation rate.

The present investigation was undertaken as part of a research effort directed towards elucidating the biochemical interactions responsible for the delayed cytotoxic activity of MP in cultured mouse lymphoma L5178Y cells. The metabolism and metabolic effects of MP were examined under conditions in which the effects of the

drug on cell proliferation were also monitored.

Similar mechanisms may be responsible for the antineoplastic activity of MP in man and an aim of this project is to provide an understanding of MP action in human cells which will aid in the development of improved cancer chemotherapeutic agents and procedures.

II. THE DELAYED CYTOTOXIC REACTION[2]

A. Introduction

Paterson and Moriwaki (136) described a delay in the cytotoxic response of cultured cells of mouse lymphoma L5178Y during continuous exposure to a low concentration of MP. Further experiments in this laboratory demonstrated that treatment of EAC cells with MP in vivo resulted in a delayed effect on cell proliferation; tumor cell numbers increased for 4 to 5 days after the final MP treatment of a schedule which produced 85% 50 day survivors amongst the host mice. Similarly, cultured L5178Y cells proliferated during and for limited periods after a pulse exposure to MP; delayed cytotoxic effects were observed in these cultures.

Hirshaut et al. (85) cultured human malignant leukocytes in the presence of moderate levels of MP and noted that cell multiplication continued for several days before lethal effects were apparent. Similar reports of delayed cytotoxic effects of MP were found in the early work on the antitumor properties of MP, but it was apparent that these observations had been overlooked in subsequent discussions of the biochemical mechanism of action of MP.

Bases (14) originally observed that MP-treated

HeLa cells continued to divide when all but 1 or 2% were "sterile". He pointed out the similarity between this effect of MP and that of low doses of X-irradiation on cultured HeLa cells. Tomizawa and Aronow (175) reported that brief exposure to MP caused a "delayed" cytotoxicity in cultures of Earle's L strain of mouse fibroblast. They postulated that the delayed cytotoxic effect might be caused by incorporation of MP into DNA. This type of cell death could possibly explain why MP gave a negative result (on the borderline of scoring as a potential cancer chemotherapeutic agent) in early screening against Sarcoma 180 (88). Clarke et al. (37) subsequently demonstrated that the majority of Sarcoma 180 transplants from MP-treated animals failed to grow in their new hosts despite poor apparent responses to therapy with MP. The appearance of large cells was a characteristic feature of the delayed cytotoxic effect of MP in vitro (14) and this was also described for MP-treated Sarcoma 180 (37). Berenbaum (18) has discussed these effects of MP in terms of the concept of "reproductive death".

In the present investigation, cultured mouse lymphoma L5178Y and L1210 cells exhibited a delayed cytotoxic reaction following a pulse exposure to MP. Toxic effects of MP were observed at a time when the drug was no longer available to the cells. Delayed cytotoxic

reactions in cultures of L5178Y cells were also observed for pulse exposures to TG, β-2'-deoxythioguanosine, 5-bromodeoxyuridine and ethyl methanesulfonate. Normal purine nucleotide synthesis is essential for continued cell proliferation and consequently inhibitions by MP anabolites at the level of purine nucleotide synthesis (see Chapter I) would be expected to produce an immediate effect on cell multiplication. Therefore, it seemed likely at the outset of the present investigation that this type of mechanism was not responsible for the delayed cytotoxic reaction of MP.

B. Materials and Methods

1. Chemicals

MP hydrate was purchased from Sigma Chemical Co., St. Louis, Mo. Stock solutions of MP (2 - 3 mM) were prepared by dissolving MP hydrate in sterile 0.9% NaCl solution. Initially, heating was used to effect total dissolution of MP and in experiments with MP-8-^{14}C (Schwarz BioResearch Inc., Orangeburg, N.Y.) it was shown that this procedure resulted in approximately 1% decomposition of MP to hypoxanthine. Therefore, alternative methods for preparing MP stock solutions were employed. In one method, MP hydrate was added to sterile 0.9% NaCl and dissolved by addition

of 1.2 equivalents of sodium hydroxide (final pH approximately 10). Solutions were prepared in this manner 6 - 8 h before use, sterilized by filtration through a sterile 0.45 μm pore size Millipore filter (Millipore Filter Corp., Bedford, Mass.) and stored at -20° until required. Stock solutions of MP (3 mM) were also prepared by dissolving MP hydrate in sterile twice concentrated Fischer's tissue culture medium (without horse serum and antibiotic supplements) at pH 8.4 - 8.9. These solutions were prepared 6 - 8 h before use and were stored at 4° until required. There was no detectable decomposition of MP-8-^{14}C in such solutions during storage at 4° for 3 days. However, approximately 10% of the MP reacted reversibly with a component of the medium; the addition of mercaptoethanol reversed this reaction in samples of the stock solution removed for concentration measurements by spectrophotometer. Mercaptoethanol was also added to reduce MP disulfide in samples of MP stock solutions in 0.9% NaCl that were removed for concentration measurements. Samples of stock solutions were diluted x50 in 0.05 M potassium chloride-hydrochloric acid buffer, pH 2, containing 0.2% v/v mercaptoethanol and optical densities of the dilutions were measured at 323 nm ($\varepsilon = 21.3 \times 10^3$).

TG was purchased from the Sigma Chemical Co., St. Louis, Mo., and stock solutions (1 mM) were prepared

in sterile 0.9% NaCl by heating or by addition of 1.2 - 1.4 equivalents of sodium hydroxide. TG solutions prepared with sodium hydroxide were sterilized by filtration through a sterile 0.45 μm pore size Millipore filter. Solutions were prepared 6 - 8 h before use and were stored at -20° until required. Concentrations were determined by diluting TG solutions with HCl (with or without 0.2% v/v mercaptoethanol) to a final concentration of 1 N HCl and measuring optical density at 345 nm ($\varepsilon = 17.0 \times 10^3$). β-2'-Deoxythioguanosine was generously donated by Dr. G. A. LePage. Stock solutions of β-2'-deoxythioguanosine (200 μM) were prepared in sterile 0.9% NaCl at 37°. Concentrations were determined by diluting β-2'-deoxythioguanosine solutions with 0.1 M sodium acetate buffer, pH 4.5, and measuring optical density at 341.5 nm ($\varepsilon = 24.8 \times 10^3$). Stock solutions of β-2'-deoxythioguanosine were stored at -20° until required. 6-Methylthioinosine was purchased from the Sigma Chemical Co., St. Louis, Mo. Stock solutions of 6-methylthioinosine (100 μM) were prepared in sterile 0.9% NaCl at 25°. Concentrations were determined by measuring optical density of 6-methylthioinosine solutions at 292 nm ($\varepsilon = 16.4 \times 10^3$). Stock solutions of 6-methylthioinosine were prepared 6 - 8 h before use and were stored at -20° until required. 5-Bromodeoxyuridine (P-L Biochemicals, Inc., Milwaukee, Wis.) was dissolved in sterile 0.9% NaCl

at 37° and concentrations were determined by measuring optical density at 278 nm ($\varepsilon = 8.8 \times 10^3$, pH 7). Stock solutions of 5-bromodeoxyuridine were stored at -20° until required. Ethyl methanesulfonate (Eastman Kodak Co., Rochester, N.Y.) was added directly to cell cultures.

MP-8-^{14}C, specific activity 24 mCi/mmole was purchased from Schwarz BioResearch Inc., Orangeburg, N.Y. Bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was used as a standard for protein determinations in cultures of L5178Y cells. Yeast ribonucleic acid (Calbiochem, La Jolla, Calif.) was used to construct standard response curves for determination of RNA in cultures of L5178Y cells by the orcinol reaction.

2. Cell Culture Procedures

Mice bearing the L5178Y lymphoma were provided initially by Dr. G. A. Fischer, Brown University, Providence, R.I. The lymphoma has been maintained in BDF_1 mice obtained from Microbiological Associates, Inc., Bethesda, Md., and University of Alberta Laboratory Animal Breeding Service by weekly i.p. transplantation of 10^7 cells obtained from ascitic fluid. During the early part of this investigation each cell culture experiment was initiated with a separate inoculum of lymphoma cells obtained from ascitic

fluid of mice bearing the in vivo-passaged cell line. However, cells obtained in this manner varied in sensitivity to MP in culture. In order to avoid this complication, an explant of the in vivo-passaged cell line was plated in soft agar medium (see below) and, after incubation at 37.5° for 10 days, a single cell colony was selected, established in culture, and stocks therefrom were maintained in frozen storage. Thus cloned, cells were removed from frozen storage and unless otherwise stated were passaged in culture for no more than six weeks; after this time fresh cultures were initiated from the frozen stock.

Lymphoma cells were cultured at 37.5° in stoppered, stationary tubes (16 x 100 mm) containing 6 ml of Fischer's medium (60) (Grand Island Biological Co., Grand Island, N.Y.) supplemented with sodium bicarbonate (1.125 g/liter), 10% horse serum, streptomycin (100 µg/ml), and penicillin (100 units/ml). When larger culture volumes were required (for example, for cell volume and RNA determinations), stoppered, soda glass bottles were used as culture vessels.

Mice bearing the L1210 lymphocytic leukemia were provided by Dr. W. R. Laster, Jr., Southern Research Institute, Birmingham, Ala. The lymphocytic leukemia has been maintained in DBA/2 mice obtained from University of Alberta Laboratory Animal Breeding Service by weekly i.p. transplantation of 10^5 cells obtained from ascitic fluid. A

line of L1210 cells was established in culture with an inoculum of lymphocytic leukemia cells obtained from ascitic fluid of mice bearing the in vivo-passaged cell line. These cells were cultured in Fischer's medium under conditions that were identical to those used for L5178Y cell culture.

Unless otherwise stated, cell numbers were determined with a Coulter Model F electronic particle counter. In two experiments involving pulse MP exposures, intact lymphoma cells were recognized by their ability to exclude trypan blue stain (0.4% in Hanks' balanced salt solution). Nonstaining cells were counted in a hemocytometer at intervals throughout the experiments and cell counts determined in this manner were compared with those obtained by the Coulter counter.

Exponentially proliferating L5178Y cells were treated with MP in the following manner. Appropriate volumes of stock solutions of MP (2 - 3 mM in 0.9% NaCl solution or 3 mM in twice concentrated Fischer's medium) were added to cultures to achieve the MP concentrations specified; control cultures received identical volumes of 0.9% NaCl solution or twice concentrated Fischer's medium. Cell densities did not exceed 100,000 cells/ml when MP was added. MP treatment was terminated by changing the medium; the cells were collected by centrifugation, washed

once in warm drug-free medium, and then resuspended in the latter. During subsequent incubation, exhaustion of the medium was avoided by diluting measured volumes of the untreated control cultures daily with fresh, warm medium; treated cultures were diluted once or twice during the period of abortive proliferation between removal of the drug and onset of the delayed cytotoxic reaction; further dilution was not necessary until the surviving population became appreciable. Cell densities reported in the figures are hypothetical values derived from actual cell counts and cumulative dilution factors. Similar procedures were followed in demonstrating delayed cytotoxic reactions of TG, β-2'-deoxythioguanosine, 5-bromodeoxyuridine and ethyl methanesulfonate.

3. Cell Volume Determinations

A Coulter Model F electronic particle counter was used to monitor cell volume distributions. The counter was calibrated for volume determinations with a suspension of ragweed pollen grains of known dimensions (Hollister-Stier Laboratories, Spokane, Wash.). The pollen grains were wetted with 2 - 3 drops of isopropanol, suspended in 0.9% NaCl and allowed to equilibrate for 15 min.

For determination of cell volume distributions, culture samples were diluted 20-fold with 0.9% NaCl and allowed to equilibrate for 15 min. Small changes in cell

volume occurred after dilution with saline, but these were essentially complete by 15 min. Cell volume distributions were measured by counting cell suspensions at progressively higher pulse height threshold values. This procedure was repeated three times and the average cell count at each threshold value was used to define the distribution. Median cell volumes were determined by locating the threshold values which gave one half of the total cell counts.

4. Protein Determinations

Total cellular protein in L5178Y cell cultures was assayed by the procedure of Lowry et al. (116). Cells ($1 - 4 \times 10^5$) in 1 ml culture samples were washed with 0.9% NaCl and dried; dried cell pellets were resuspended in alkaline tartrate/copper sulfate solution, incubated at 25° for 20 min, and then sampled for protein determination. Bovine serum albumin was used to construct standard response curves for this procedure. The average cell content of protein in normal, exponentially proliferating cultures was 0.17 - 0.23 ng protein/cell.

5. RNA Determinations

Total RNA in L5178Y cell cultures was estimated by the orcinol reaction (161). Cells ($1 - 2 \times 10^7$) were washed with 0.9% NaCl at 4° and extracted with 1 ml cold 10% trichloracetic acid. The acid insoluble material was

washed three times with 2 ml portions of cold 10% trichloracetic acid, resuspended in 0.5 ml 1 N potassium hydroxide solution and incubated for 20 h at 37°. DNA and protein were then precipitated by the addition of 0.1 ml 6 N hydrochloric acid and 0.5 ml 5% trichloracetic acid. The supernatant solution containing RNA breakdown products was removed and the precipitate was washed with 0.5 ml 5% trichloracetic acid. Supernatants were combined and the total volume adjusted to 2.0 ml by addition of 5% trichloracetic acid; samples of the solution were assayed by orcinol reaction (161). Yeast RNA was used to construct standard response curves for this procedure.

6. Cloning Assay for Cell Survival

The surviving cell fraction in MP-treated L5178Y cultures was estimated by a method which involved the ability of such cells to produce macroscopic colonies in soft agar medium. Measured numbers of cells were suspended in 20 ml Fischer's medium supplemented with 0.14% w/v Noble's Agar (Difco Laboratories, Detroit, Mich.) and containing 10% v/v horse serum (Grand Island Biological Co.), 10% v/v conditioned medium ("spent" Fischer's medium obtained from 10-day cultures of L5178Y cells), streptomycin (100 µg/ml), and penicillin (100 units/ml). 5.0 ml volumes of the cell suspension were dispensed into tubes

(16 x 100 mm) which were then plugged with cotton and incubated at 37.5° in a 5% CO_2-air atmosphere for 10 days. After incubation the contents of each tube were carefully poured into plastic petri plates (60 x 15 mm, Falcon Plastics, Los Angeles, Calif.) and macroscopic colonies were counted under a stereo microscope. Untreated control cells were suspended in soft agar medium at a density of 20 cells/ml and produced 50 - 70 macroscopic colonies per tube. MP-treated L5178Y cells were "plated" at a density sufficient to produce 10 - 70 colonies per tube.

C. Results

1. Characteristics of the Delayed Cytotoxic Reaction of 6-Mercaptopurine

The delayed cytotoxic reaction of MP is illustrated by the experiments of Figures 3 and 4. In the experiment of Figure 3, exponentially proliferating L5178Y cultures were exposed to various concentrations of MP (20 - 100 μM) for 13 h. "Medium change" indicates that the exposure to MP was terminated; cells were washed and resuspended in fresh, warm, drug-free medium at this time. Cells continued to divide during the period of exposure to MP and for approximately 24 h after the drug was removed. Further cell division (1 - 1.5 population doublings) occurred after MP exposure was terminated. Cell

proliferation ceased approximately 24 h after the drug was removed and thereafter cells disintegrated. Repopulation of cultures by surviving cells occurred after a time interval which was dependent upon the concentration of MP to which the cultures were originally exposed. In the experiment of Figure 4, exponentially proliferating L5178Y cultures were exposed to 25 μM MP for various periods of time (0.3 - 25 h). MP-treated cells were washed and resuspended at 0 h and effects of MP exposure on subsequent proliferation were monitored in these cultures. Cells continued to divide during the periods of exposure to MP (data not shown) and further limited proliferation of the "doomed" cells was observed after the drug was removed. As in the experiment of Figure 3, surviving cells eventually repopulated MP-treated cultures. It was apparent that the time taken for surviving cells to repopulate these cultures was also dependent upon the duration of the original exposure to MP (Figure 4).

The experiments of Figure 3 and Figure 4 indicated that L5178Y cells are capable of limited abortive proliferation after an ultimately lethal exposure to MP. This delayed cytotoxic reaction to MP exposure was most clearly demonstrated when cells were exposed to MP for a period (13 h) that was slightly in excess of the normal doubling times (10 - 12 h) for cells in the exponential phase of culture growth. In some experiments, two abortive

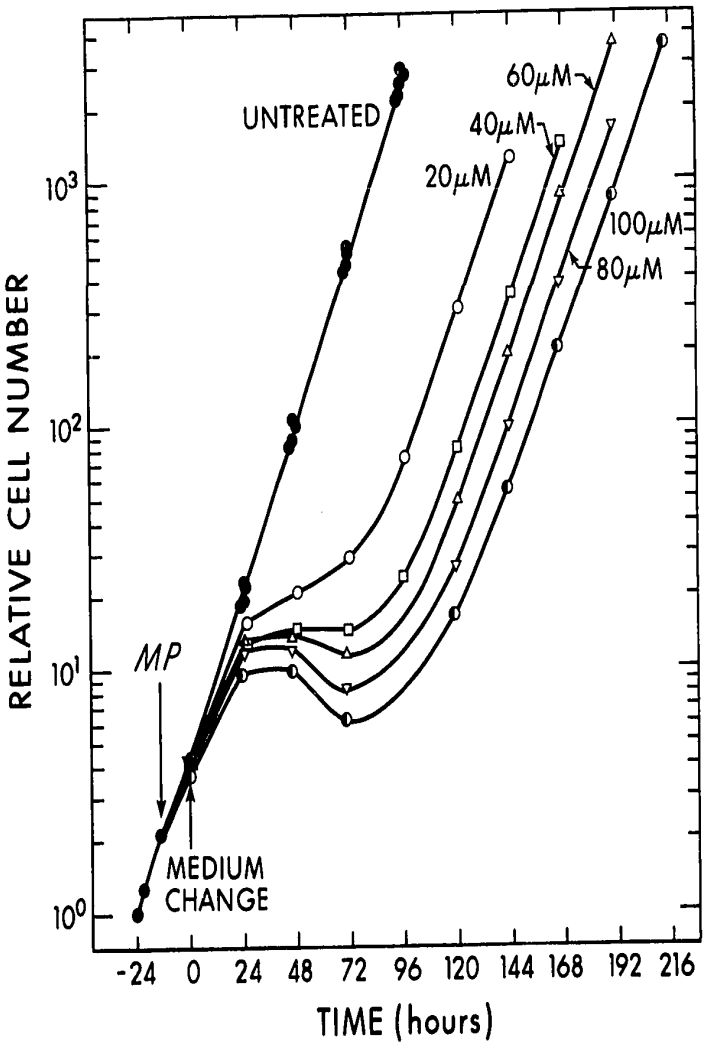

Figure 3. The effect on cultured L5178Y cell proliferation of exposure for a fixed interval (13 h) to various concentrations of MP. The drug was removed at 0 h and cell numbers are relative to those at -24 h. Concentrations of MP during exposure are indicated on the figure. All cultures were derived from the same explant of the in vivo passaged lymphoma.

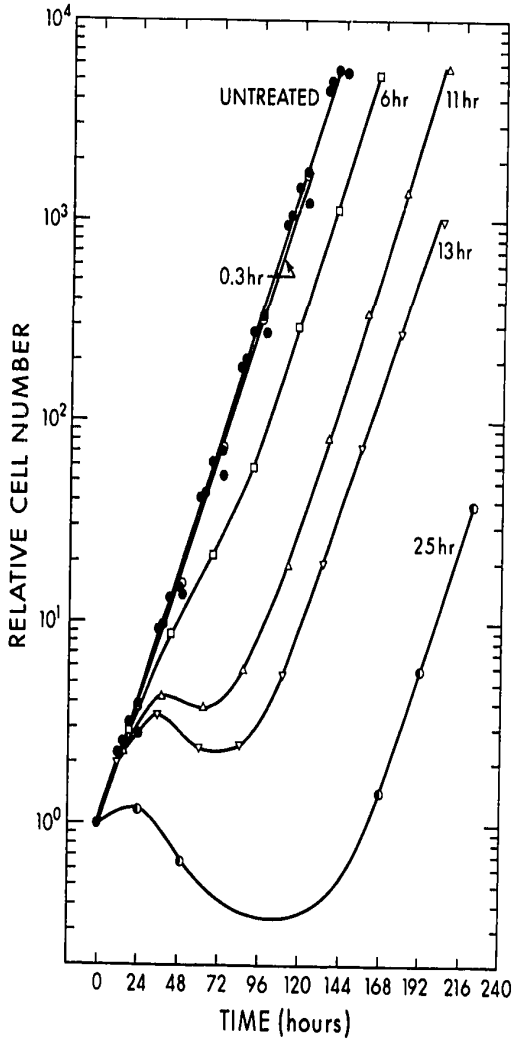

Figure 4. The effect on cultured L5178Y cell proliferation of exposure for various intervals to a fixed concentration (25 μM) of MP. The drug was removed at 0 h and cell numbers are relative to those at this time. The durations of preceeding MP exposures are indicated on the figure. All cultures were derived from the same explant of the in vivo passaged lymphoma.

doublings of the cell populations occurred after MP exposure under these conditions. Cells surviving MP exposure established proliferation rates which did not differ significantly from those of untreated controls (Figures 3 and 4). In other words, the exponential portions of survivor growth curves (log cumulative cell number versus time) paralleled the exponential straight-line plots for the corresponding control cultures. The extent of the axial displacements of such curves from the control curves were related to both the concentration of MP and the duration of MP exposure.

In the experiments of Figures 3 - 5, cell numbers were determined with a Coulter Counter, but in that of Figure 5 cell numbers were also determined by hemocytometer. The latter experiment was designed to test the validity of the electronic counter in the assessment of cell numbers in drug-treated cultures. Cultured L5178Y cells were exposed to MP (50 μM) for 25 h and then washed and resuspended in drug-free medium. At various times, samples were removed for cell enumeration by the two methods. For hemocytometer counts, 0.1 ml of trypan blue solution was added to 0.5 ml of culture and the sample was incubated for 5 min at 25°. Both chambers of the hemocytometer were filled with this cell suspension and non-staining, intact cells were counted over the entire calibrated field in both chambers. It is apparent in Figure 5 that the two

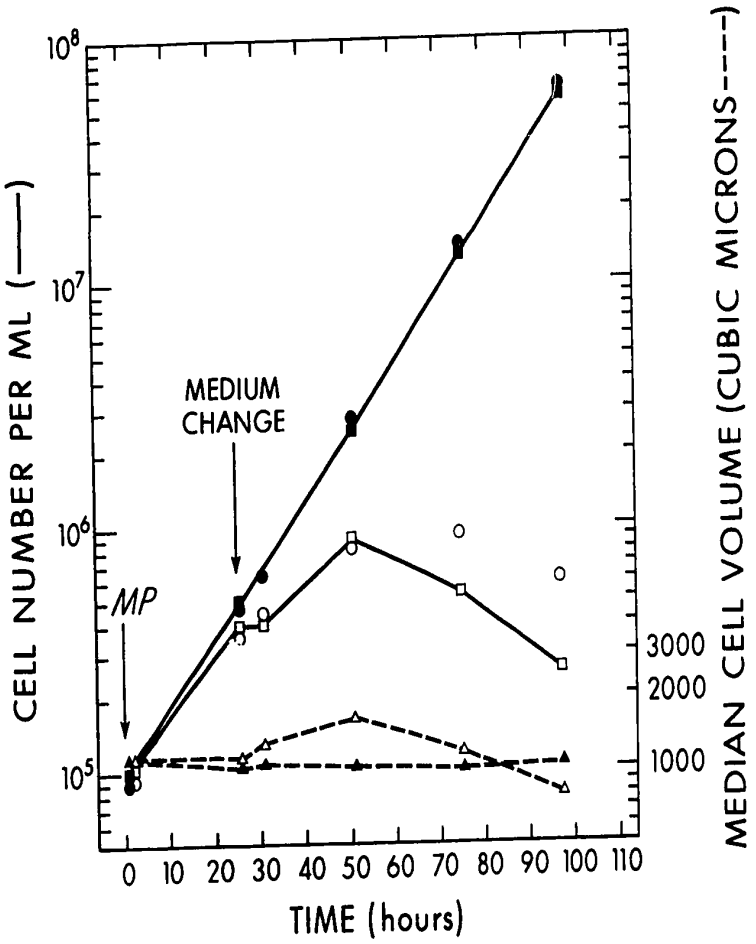

Figure 5. The effect of exposure to MP (50 μM) on the proliferation of cultured L5178Y cells. Cultures were treated for 25 h with 50 μM MP, and cell numbers per ml of culture were measured by an electronic particle counter or by hemocytometer counts of cells that excluded trypan blue. Measurements of median cell volume were made concurrently. Cell number determined by Coulter counter: o, treated; ●, control. Number of nonstaining cells per ml: □, treated; ■, control. Median particle volume: △, treated; ▲, control.

methods of cell enumeration gave similar results for untreated control cultures. For MP treated cultures, the two methods of enumeration agreed closely during both MP exposure and the period of limited proliferation which followed MP treatment; however, they diverged after cell division had stopped, evidently because trypan blue-staining cells and cell debris accounted for a substantial portion of the counts recorded by the electronic particle counter. Similar results were obtained for cells exposed for 13 h to 50 μM or to 100 μM MP. It was concluded that the post-exposure proliferation was real and that the Coulter counter gave a valid estimate of cell numbers in MP-treated cultures during most of the period of abortive proliferation.

Figure 5 shows that the median cell volume of MP-treated cells gradually increased after removal of the drug and was maximal at about the time that cell numbers began to decline. Microscopic examination of cell samples taken at this time showed that cells were large and non-staining (trypan blue). Large cells with blebs and globular cell fragments (many of which stained with trypan blue) became abundant in the cultures as cell disintegration proceeded. Cell fragments registered by the electronic particle counter were responsible for the progressive decrease in the apparent median cell volume to values below those of control cells (Figure 5).

All cultures treated with MP by the procedures described above contained cells destined to survive drug exposure. These surviving cells were able to produce macroscopic cell colonies in soft agar medium. However, since cells doomed to die were capable of limited proliferation after MP exposure, it was apparent that the plating efficiency (i.e., the ratio of number of macroscopic colonies formed to number of cells originally plated) of MP-treated cultures would be approximately constant during the period of abortive proliferation. In the experiment of Figure 6, culture samples were transferred to soft agar medium at various intervals after 12 h exposure to 100 μM MP (see Figure 7 for effect of this treatment on cell proliferation). Cells were plated in sufficient numbers to produce 30 - 100 macroscopic colonies per tube and each point in Figure 6 represents the mean plating efficiency of 4 - 16 replicate tubes. During the period (0 - 31 h, Figure 6) of abortive proliferation after MP was removed, the plating efficiency of the MP-treated culture increased from 0.77% to 1.13%. Cell number tripled in the MP-treated culture during this interval (Figure 7). When the plating efficiency of the untreated control is compared with that of the MP-treated culture, it is apparent that only about 1% of the cells in the MP-treated culture were destined to survive at the time that the drug was removed. Therefore, cells doomed to die were mainly

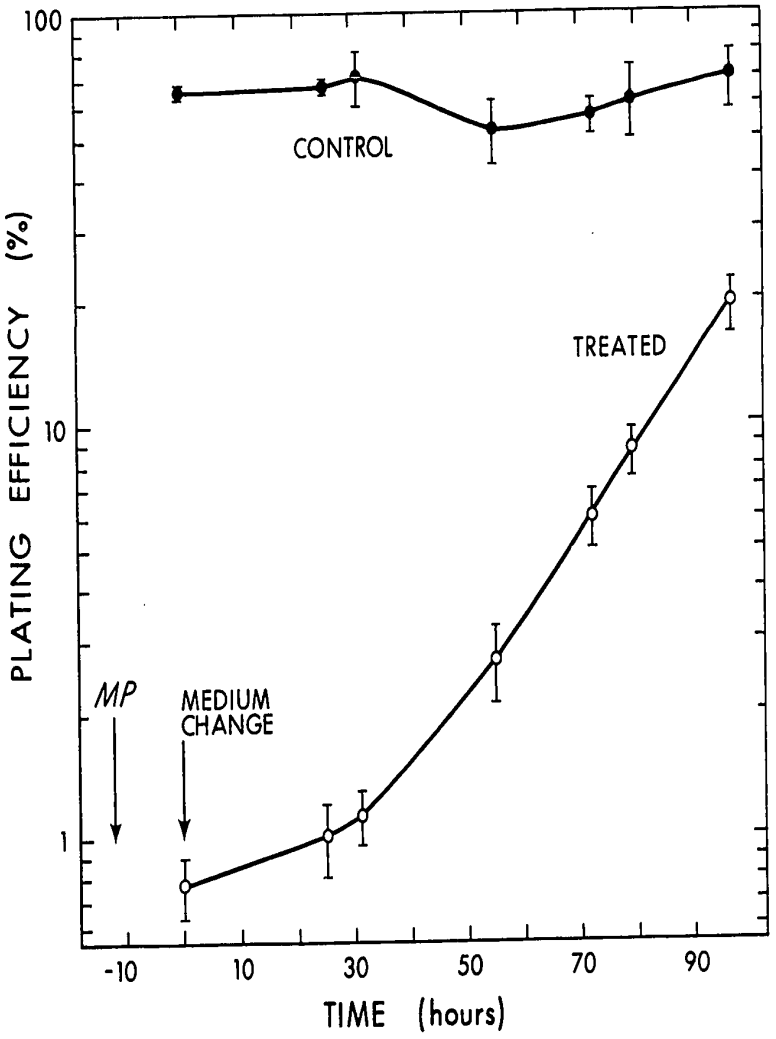

Figure 6. Progressive changes in plating efficiency of cultured L5178Y cells after 12 h exposure to MP. Cultures were treated for 12 h with 100 µM MP; at 0 h, cells were collected by centrifugation, washed and resuspended in fresh, warm, drug-free media. Samples of the cultures were removed at various intervals thereafter and cells were suspended in soft agar medium. After incubation at 37.5° for 10 days macroscopic cell colonies were counted. Plating efficiencies were calculated as the ratio of number of colonies produced to number of cells "plated". Mean plating efficiencies ± S.D. (vertical bars) for 4 - 16 tubes are plotted against the times at which the cell samples were taken from cultures. o, MP-treated cells; ●, untreated control cells. Corresponding effects of this MP exposure on cell proliferation are presented in Figure 7.

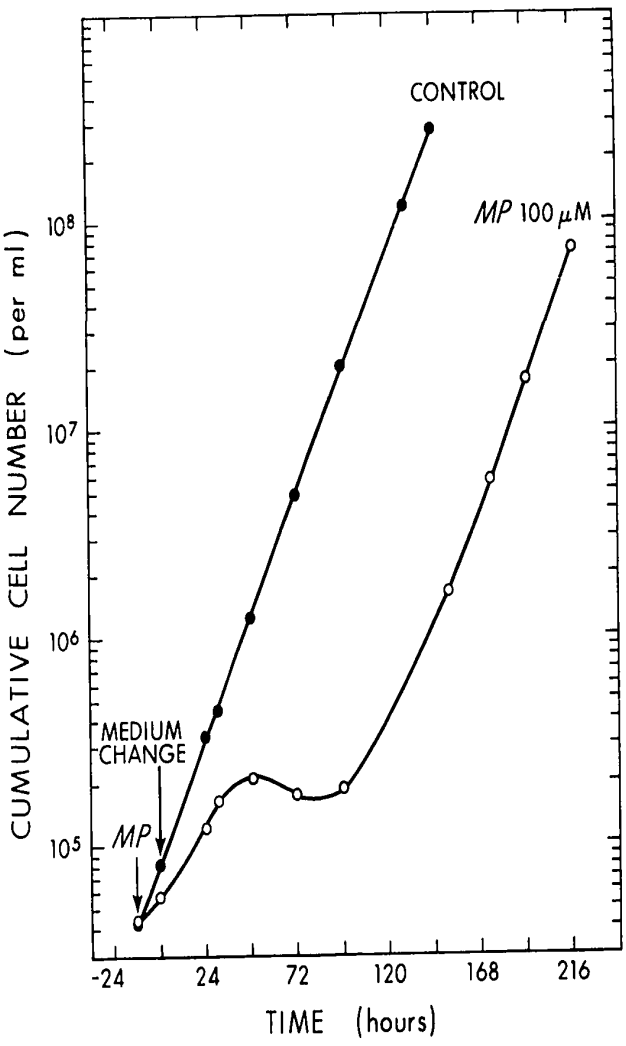

Figure 7. The effect of 12 h exposure to 100 μM MP on the proliferation of cultured L5178Y cells. These are data from the experiment of Figure 6. o, MP-treated cells; ●, untreated control cells.

responsible for the further 1.9 doublings of the cell population which occurred during the initial 49 h after MP was removed (Figure 7). The lower relative increase in plating efficiency compared with increase in cell number is compatible with this conclusion. Proliferation ceased in the MP-treated culture after 49 h and cells lysed. Beyond 31 h, plating efficiency increased rapidly in the MP-treated culture (Figure 6) and plating efficiencies comparable to those of the untreated control were observed by 145 h (data not shown).

Increased cell volume was a characteristic of the delayed cytotoxic response to MP (see Figure 5). This characteristic was investigated in greater detail by monitoring the effects of MP on the distribution of cell volumes in L5178Y cell cultures. The volume distribution of exponentially proliferating L5178Y cells in control cultures was logarithmic normal. This type of distribution yields a straight line when the data are plotted on logarithmic probability graph paper. Rosenberg and Gregg (148) have used this type of representation to detect small changes in cell volume distribution and have referred to distribution plots on logarithmic probability paper as "volume spectra". The volume spectra of MP-treated cells (13 h, 100 μM) were identical to those of control cells both during the period of MP-exposure and for as long as 14 h after the drug was removed. Thereafter, the spectrum of

the treated cells shifted progressively to higher volumes and became biphasic (Figure 8, left, 26 h after termination of MP exposure); the higher volume portion of the plot remained parallel to the control cell line. The biphasic spectrum probably reflects the presence of 2 populations: one population in which cells are growing unusually large as a result of the delayed cytotoxic reaction (upper portion of curve, Figure 8, left) and the other population in which the delayed cytotoxic reaction has not commenced and which retains the untreated cell volume distribution (lower portion of curve, Figure 8, left). The latter conclusion was derived from the observation that if the data specifying the lower portion of the MP-treated cell volume spectrum (Figure 8, left) are processed independently of the remainder of the distribution, the resultant volume spectrum is superimposable upon that of untreated control cells. If the data specifying the upper portion of the MP-treated cell volume spectrum are processed independently of the remainder of the distribution, the resultant volume spectrum is a straight line parallel to the control distribution, but shifted to higher cell volumes. In the experiment of Figure 8, cell debris began to accumulate at 30 h after MP exposure and obscured measurement of the cell volume distribution of the remaining intact cells. The median cell volume increased to approximately 160% of the control value before the accumulation

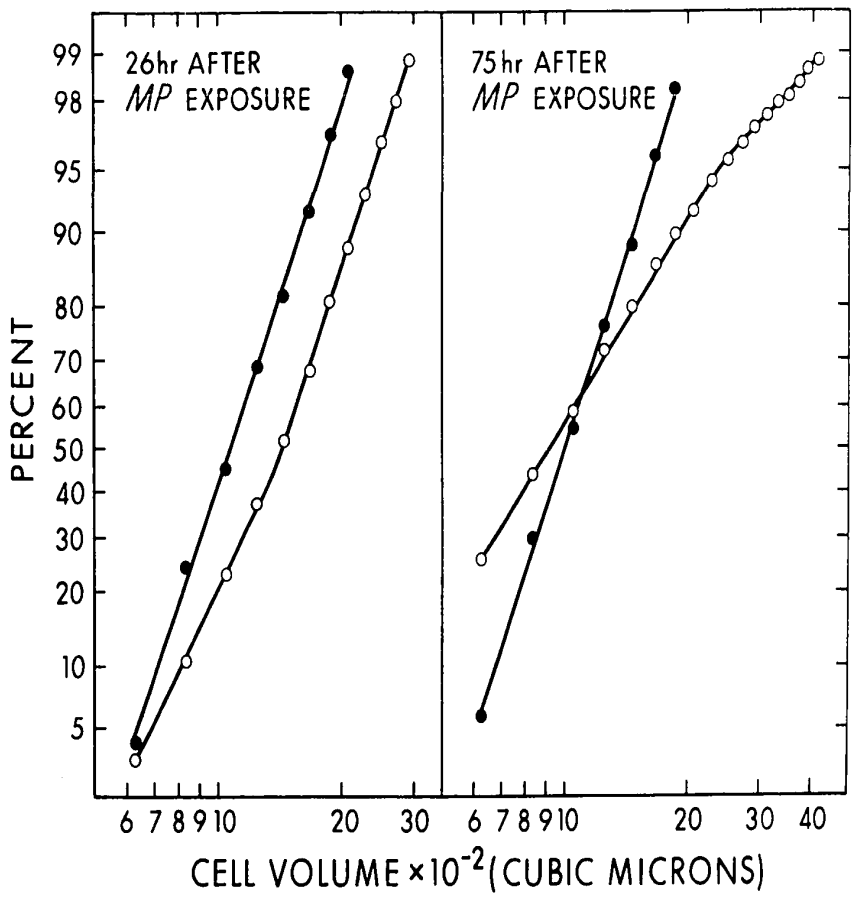

Figure 8. Volume spectra of L5178Y cells following exposure (13 h) to MP (100 μM). The scale is logarithmic-probability. The numbers of cells with volumes less than or equal to those specified along the abscissa, are expressed as percentages of the total cell population. Treated cells (o); control cells (●).

of debris obscured the spectrum. At 75 h after MP exposure (Figure 8, right) the volume spectrum indicated that large cells and an appreciable amount of cell debris were present in the MP-treated culture.

Large cell formation may result from the condition of "unbalanced growth" or, alternatively, through osmotic swelling of degenerating cells. Distinction between these possibilities can be made on the basis that the former process is characterized by accumulation of cell protein and RNA above normal levels. Accordingly, cell protein (Figure 9) and RNA (Figure 11) were measured in L5178Y cell cultures at various intervals after MP-exposure to determine which mechanism was operative. Figure 9 demonstrates that, in untreated cultures of the lymphoma cells, the relationship between cell numbers and the total cellular protein did not change with time; on the relative logarithmic scale used in this figure, growth curves for protein and cell number were superimposable. In contrast, the relationship between these parameters was not constant in cultures that had been treated with MP; these data indicate that the cellular protein content increased during expression of the delayed cytotoxicity of MP.

Under regular culture conditions in untreated cultures of the lymphoma cells, the cell content of RNA was found to vary over the range $1.35 - 2.00 \times 10^{-5}$ µg per cell.

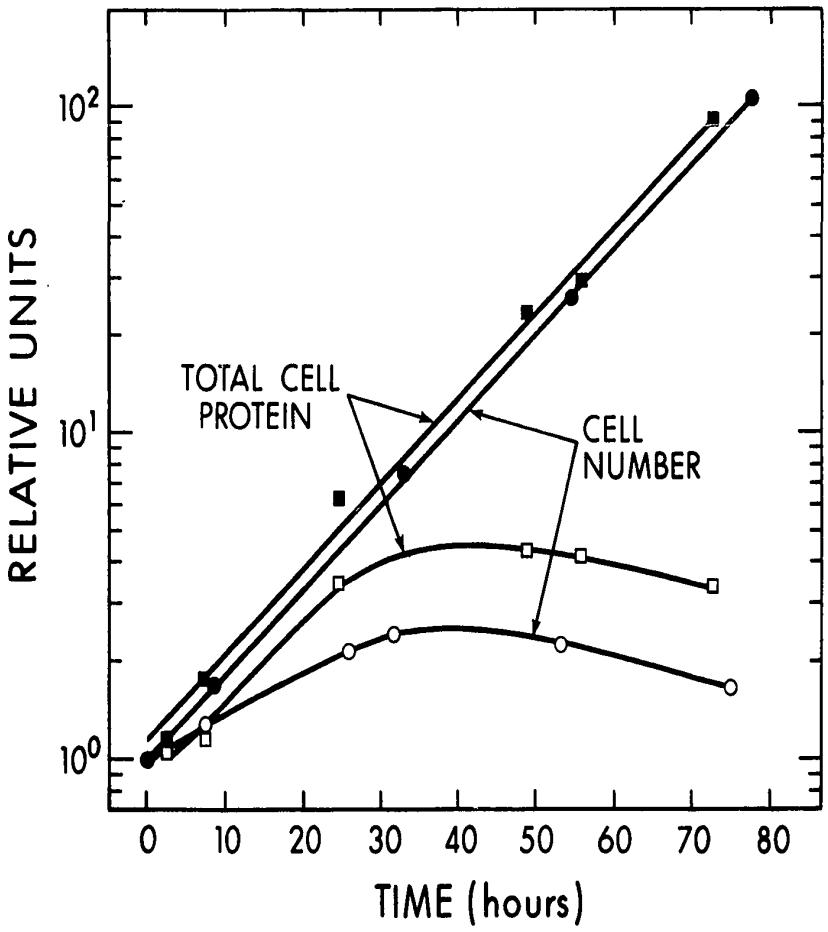

Figure 9. The effect of exposure to MP on protein content of L5178Y cells proliferating in culture. Cultures were treated for 13 h with 100 μM MP; at 0 h, culture fluids were replaced with drug-free media. Total cellular protein and cell numbers were determined at the indicated times; data are expressed relative to their zero time values. Treated cells: □, total protein, o, cell number. Untreated control cells: ■, total protein; ●, cell number.

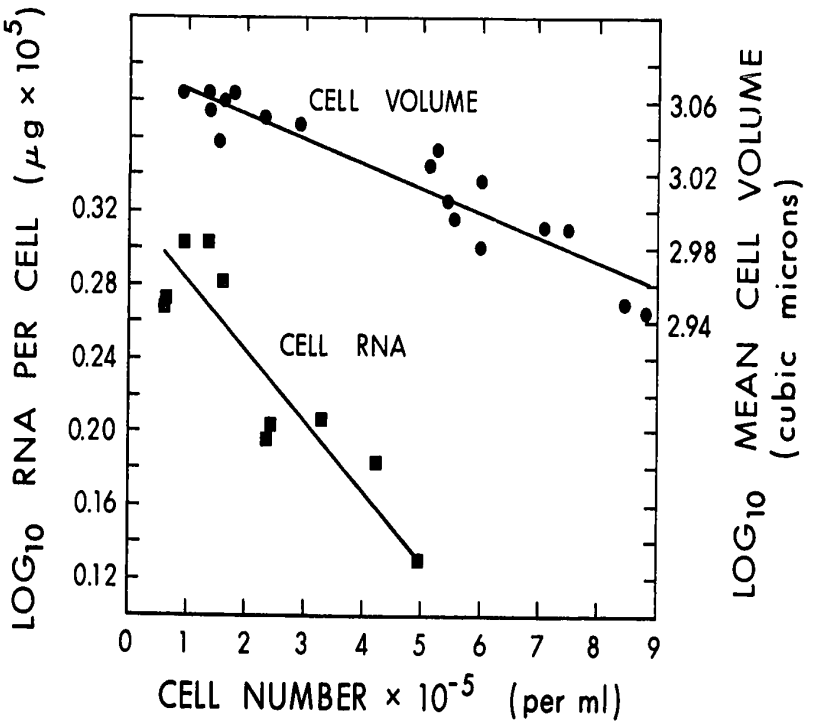

Figure 10. The relationship between cell population density and cell volume and RNA in normal, exponentially proliferating, L5178Y cell cultures. Mean cell volume and cellular RNA content were determined for cultures of different cell densities in which cells were proliferating at the same exponential rate. For RNA determinations, samples containing 1.5×10^7 cells were taken from cultures of different cell density, cells were collected by centrifugation and washed with 0.9% NaCl. The RNA content of these cell samples was measured by orcinol reaction. ●, $\log_{10}$ mean cell volume (cu μm), right ordinate. ■, $\log_{10}$ RNA per cell (μg x 10^5), left ordinate.

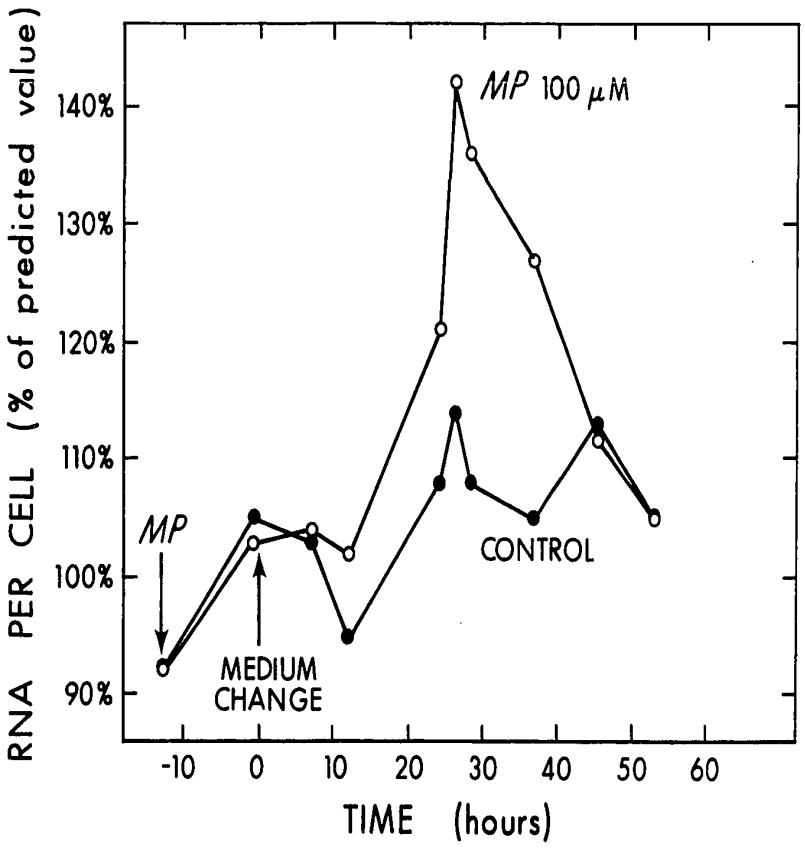

Figure 11. The effect of exposure to MP on the RNA content of L5178Y cells proliferating in culture. Cultures were treated for 13 h with 100 μM MP; at 0 h, culture fluids were replaced with drug-free media. Cellular RNA was determined at the indicated times; data are expressed as percentages of the values predicted from the cell RNA regression equation of Figure 10 on the basis of the corresponding cell population densities. o, MP-treated cells; ●, untreated control cells.

The data of Figure 10 demonstrated that a relationship exists between cell RNA content and cell population density which is independent of proliferation rate; these data were obtained with cultures which exhibited identical rates of proliferation (i.e., doubling times were identical). Each point represents RNA determined in cell samples of 1.5 x 10^7 cells (approximately 15 mg wet weight) derived from cultures of different cell density. The decrease in RNA with increase in cell population density may be related to a similar decrease in cell volume (Figure 10). However, the decrease in cell RNA was proportionately greater than the decrease in cell volume in the range of culture cell densities (1 - 4 x 10^5 cells/ml) chosen for drug experiments. Therefore, population density-related effects on cellular RNA represented a serious source of variability in determinations of drug effects on the cellular content of this nucleic acid. Further, it would seem likely that the "condition" of the culture medium was responsible for changes in cell RNA in untreated cultures of the lymphoma cells. Cell population density is a poor measure of this variable, since the medium condition will depend upon the time and the population density of the preceding culture dilution. However, in the absence of a more precise indicator, cell population density was used to correct RNA values for MP-treated cells. Figure 11 demonstrates the effect of MP exposure

(13 h, 100 μM) on cell RNA. RNA values per cell are expressed as percentages of the values predicted from the regression equation of the data of Figure 10 on the basis of actual cell population densities. Cellular RNA levels were similar for MP-treated cells and untreated controls both during and for approximately 12 h after MP exposure (Figure 11). Beyond this stage, RNA increased significantly in MP-treated cells and was maximal at about 26 h after MP was removed. The increase in cell RNA corresponded to an increase in cell volume in the MP-treated culture. These data indicate that the cellular RNA content increased during expression of the delayed cytotoxic reaction of MP.

Thus, the increase in cell volume, characteristic of the delayed cytotoxic reaction of MP, was accompanied by parallel increases in cell RNA and protein and, therefore it would appear that MP-treated cells grow large rather than swell osmotically and that the condition of "unbalanced growth" is responsible for this increase in cell volume.

2. Delayed Cytotoxic Reactions Induced by Other Agents

TG and β-2'-deoxythioguanosine produced delayed cytotoxic reactions in L5178Y cell cultures, which were identical to those induced with MP. The delayed cytotoxic

reactions to β-2'-deoxythioguanosine and TG exposures (13 h) are illustrated in the data of Figures 12 and 13. The effects of various concentrations (0.5 - 3.0 μM) of β-2'-deoxythioguanosine and TG (0.5 - 4.0 μM) are compared with the effect of MP (13 h, 100 μM). It is apparent that β-2'-deoxythioguanosine and TG induced delayed cytotoxic reactions when present at concentrations which were about 0.01 times that required for a comparable effect with MP. The delayed cytotoxic reactions of TG and β-2'-deoxythioguanosine were characterized by cell volume increases similar to those induced by MP. In a detailed investigation of changes in cell volume distribution induced by TG exposure (13 h, 3 μM), the volume spectra of TG-treated cells were seen to alter in a similar manner to that observed for MP-treated cells (K. Horakova, D. M. Tidd and A. R. P. Paterson, unpublished results). At the time of the delayed cytotoxic reaction of TG, the volume spectrum of TG-treated cells was biphasic and, as with MP-treated cells, the upper portion of the curve was parallel to the untreated cell volume spectrum, but shifted to higher volumes (see Figure 8, left, for volume spectrum of MP-treated cells).

5-Bromodeoxyuridine also produced delayed cytotoxic reactions in L5178Y cell cultures. The delayed cytotoxic reaction to 5-bromodeoxyuridine exposure (13 h)

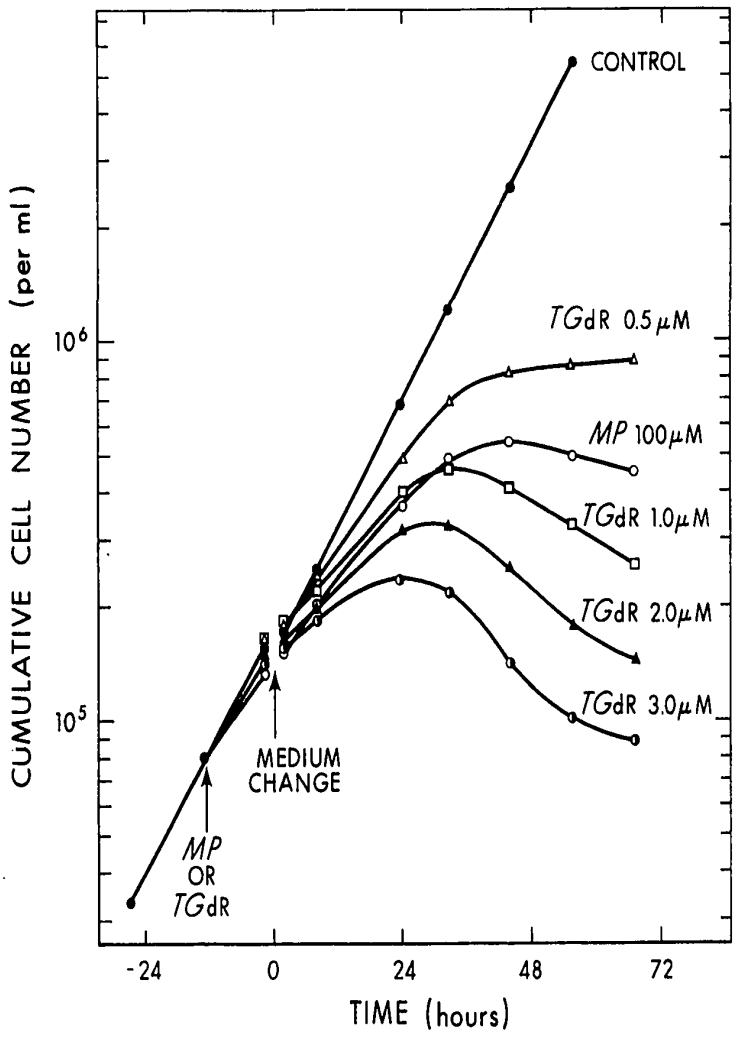

Figure 12. Comparison of the effects of MP and β-2'-deoxythioguanosine (TGdR) exposure on the proliferation of cultured L5178Y cells. Cultures were treated for 13 h with MP (100 μM) or β-2'-deoxythioguanosine (0.5 μM, 1.0 μM, 2.0 μM, 3.0 μM); at 0 h, culture fluids were replaced with drug-free media. Concentrations of β-2'-deoxythioguanosine and MP during exposure are indicated on the figure. All cultures were prepared from the same parent culture at -27.5 h.

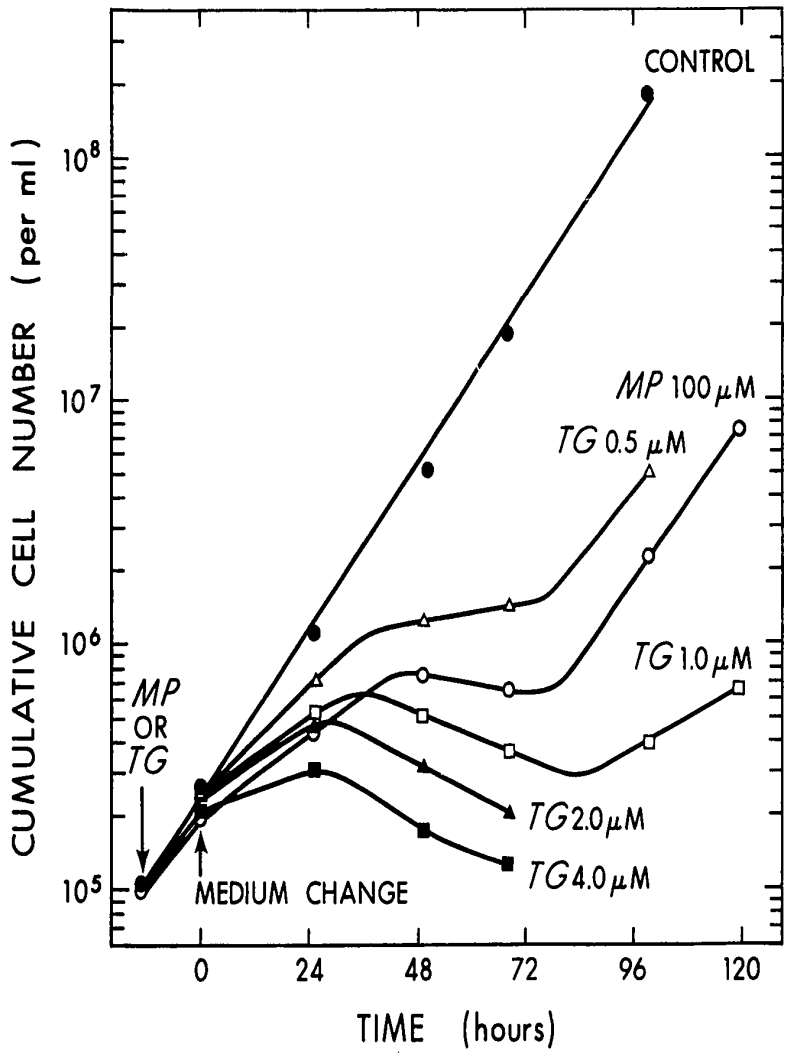

Figure 13. Comparison of the effects of MP and TG exposure on the proliferation of cultured L5178Y cells. Cultures were treated for 13 h with MP (100 μM) or TG (0.5 μM, 1.0 μM, 2.0 μM, 4.0 μM); at 0 h, culture fluids were replaced with drug-free media. Concentrations of MP and TG during exposure are indicated on the figure. All cultures were prepared from the same parent culture at -26 h.

is illustrated in the data of Figure 14. Cell cultures exposed to 5-bromodeoxyuridine (50 - 100 μM) for 13 h underwent further proliferation (1 - 1.5 doublings) after drug exposure was terminated. Cell proliferation ceased in the 5-bromodeoxyuridine-treated cultures at approximately 22 h after the drug was removed and widespread cell lysis then occurred. Measurements of median cell volume were made throughout the experiment of Figure 14. At the end of the 13 h exposure period, cell volumes were elevated above control values in cultures treated with 75 μM and 100 μM 5-bromodeoxyuridine; cell volumes were the same as control values in cultures treated with 50 μM 5-bromodeoxyuridine. In contrast to the delayed cytotoxic reaction of MP, the delayed response to 5-bromodeoxyuridine exposure was not characterized by an increase in cell volume (Figure 14). At the time of the delayed cytotoxic effect, the median volume of cells previously exposed to 50 μM 5-bromodeoxyuridine was the same as that of untreated control cells. Residual effects of the cell enlargements which occurred during drug exposure were probably responsible for slightly higher cell volumes in the cultures treated with 75 μM and 100 μM 5-bromodeoxyuridine. The 5-bromodeoxyuridine-treated cells lysed without the preliminary increase in cell volume characteristic of the delayed cytotoxic reaction of MP, TG and β-2'-deoxythioguanosine.

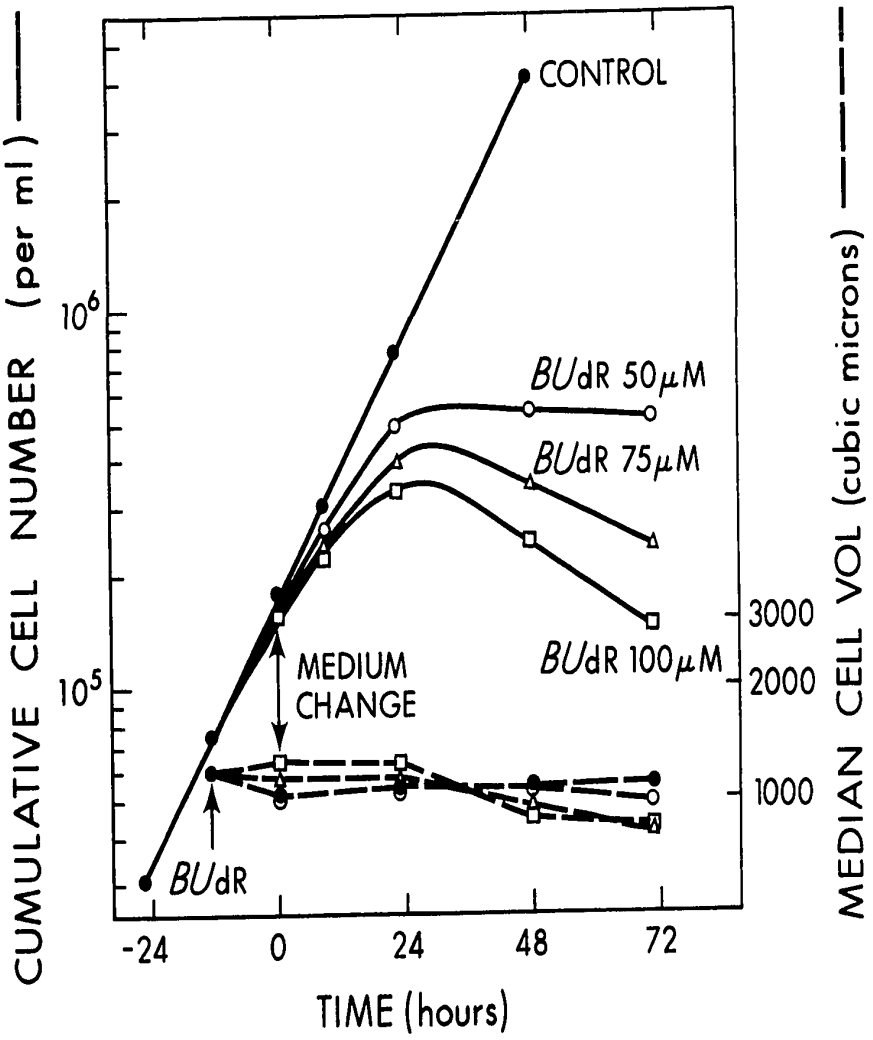

Figure 14. The effect of exposure to 5-bromodeoxyuridine (BUdR) on the proliferation of cultured L5178Y cells. Cultures were treated for 13 h with 50 μM, 75 μM and 100 μM 5-bromodeoxyuridine; at 0 h culture fluids were replaced with drug-free media. Measurements of median cell volume were made throughout the experiment. Cumulative cell number (————), median cell volume (------). Concentrations of 5-bromodeoxyuridine during exposure: 50 μM (o); 75 μM (Δ); 100 μM (□). Untreated control cells (●). All cultures were prepared from the same parent culture at -26 h.

5-Bromodeoxyuridine is a thymidine analog and high external concentrations of thymidine specifically inhibit DNA synthesis in cultured cells. This effect is apparently produced by elevated intracellular thymidine nucleotide pools which restrict the synthesis of deoxycytidine nucleotides required for DNA synthesis (128). Inhibition of DNA synthesis without parallel inhibitions of RNA and protein synthesis may result in the condition of "unbalanced growth". It is likely that 5-bromodeoxyuridine mimics this effect of thymidine and that this is responsible for cell volume increases during drug exposure.

Ethyl methanesulfonate elicited a delayed cytotoxic reaction in cultures of L5178Y cells. Cell proliferation continued during exposure (13 h) to ethyl methanesulfonate (0.4 mg/ml culture) and a further doubling of the cell populations occurred after exposure was terminated. At the end of exposure, cell volumes were normal, but cell volume increased during the period of abortive proliferation after the drug was removed. The delayed cytotoxic reaction of ethyl methanesulfonate was characterized by increases in cell volume above normal values and in this respect was very similar to the delayed cytotoxic reactions induced by MP, TG and β-2'-deoxythioguanosine.

3. Cell Survival Curves for 6-Mercaptopurine and Factors Affecting Cell Survival

Surviving cells eventually repopulated MP-treated cultures and established exponential proliferation rates which did not differ significantly from those of untreated controls (see Figures 3 and 4). In other words, the exponential portions of the survivor growth curves (log cumulative cell number versus time) paralleled the exponential, plots for the corresponding control cultures. The displacement (measured in the ordinate direction) of the exponential portion of the survivor curve from the control plot was used as a measure of the fraction of cells that survived the drug treatment. In so doing, the simplifying assumption is made that the generation time of cells destined to survive was unaltered during their recovery from drug exposure. This provides only a minimum estimate of the surviving fraction. The real circumstances are undoubtedly complex, and the true surviving fraction may well be larger. Similar assumptions have been used in estimating the fraction of tumor cell populations killed in vivo by chemotherapeutic treatment (157); the data yielded are qualitatively similar to those obtained by clonal methods (27). The fraction of L5178Y lymphoma cells that survived MP treatment, as estimated by the above method, was dependent upon the duration of the exposure to MP and upon the

concentration of MP present during exposure. Estimates of minimal surviving fraction are plotted against the corresponding periods of exposure in Figure 15A. The data indicate that a constant fraction of tumor cells were killed during consecutive equal periods of exposure to a fixed concentration of MP. This response occurred over a logarithmic range of approximately 3 and at several concentrations of MP. Kill kinetics of this sort have been described as empirically first order with respect to concentration of cells (169). In a separate experiment it was shown that MP concentrations in the medium did not decline appreciably during the period of cell treatment; a 60 μM medium concentration of MP-8-^{14}C was not significantly reduced in a 25 h incubation during which cell density increased from 100,000 to 370,000 cells/ml.

Figure 15B presents dose-response data for a fixed period (13 h) of exposure to MP and demonstrates that the rate of cell kill was not first order with respect to the concentration of the drug. Successive equal increments in drug concentration contributed progressively less to the overall fractional kill. Qualitatively similar results were obtained when surviving fractions were estimated from the ability of MP-treated cells to produce macroscopic cell colonies in soft agar medium (Figure 16). In the experiment of Figure 16, exponentially proliferating L5178Y cells were

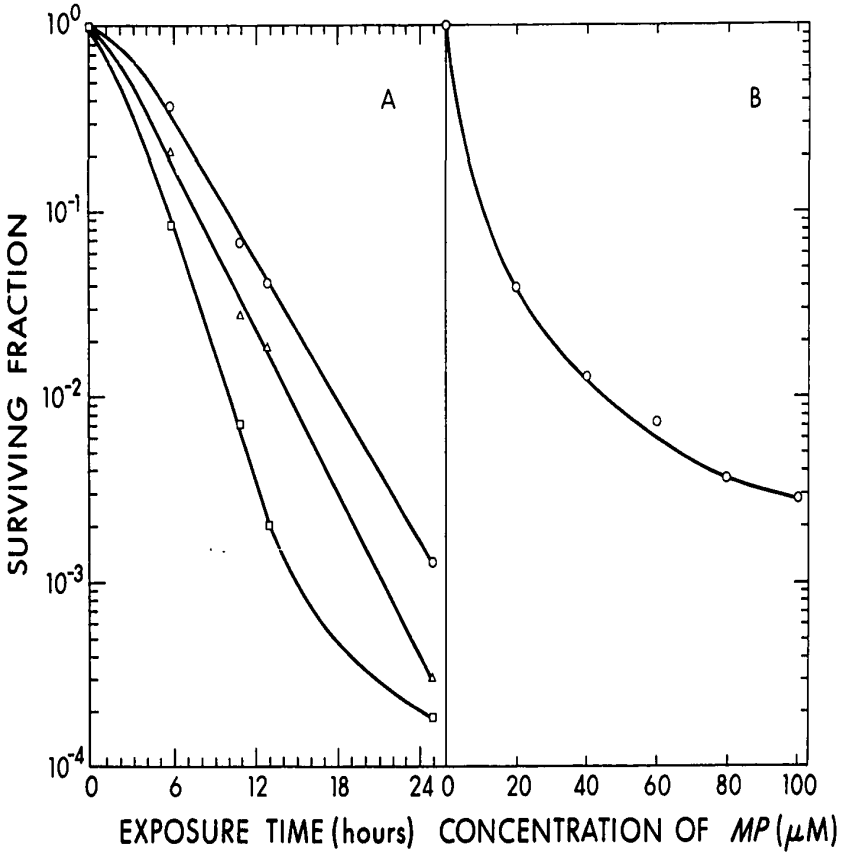

Figure 15. A: Exposure-survival curves for L5178Y cells treated with MP. Surviving fractions are minimal values derived from exponential survivor growth curves. MP concentrations: o, 48 μM; Δ, 77 μM; □, 109 μM. Curves, data obtained with different explants of the in vivo-passaged lymphoma. B: Dose-survival curve for L5178Y cells exposed to MP. Duration of exposure was 13 h. Surviving fractions are minimal values derived from exponential survivor growth curves. All cultures were derived from the same explant of the in vivo-passaged lymphoma.

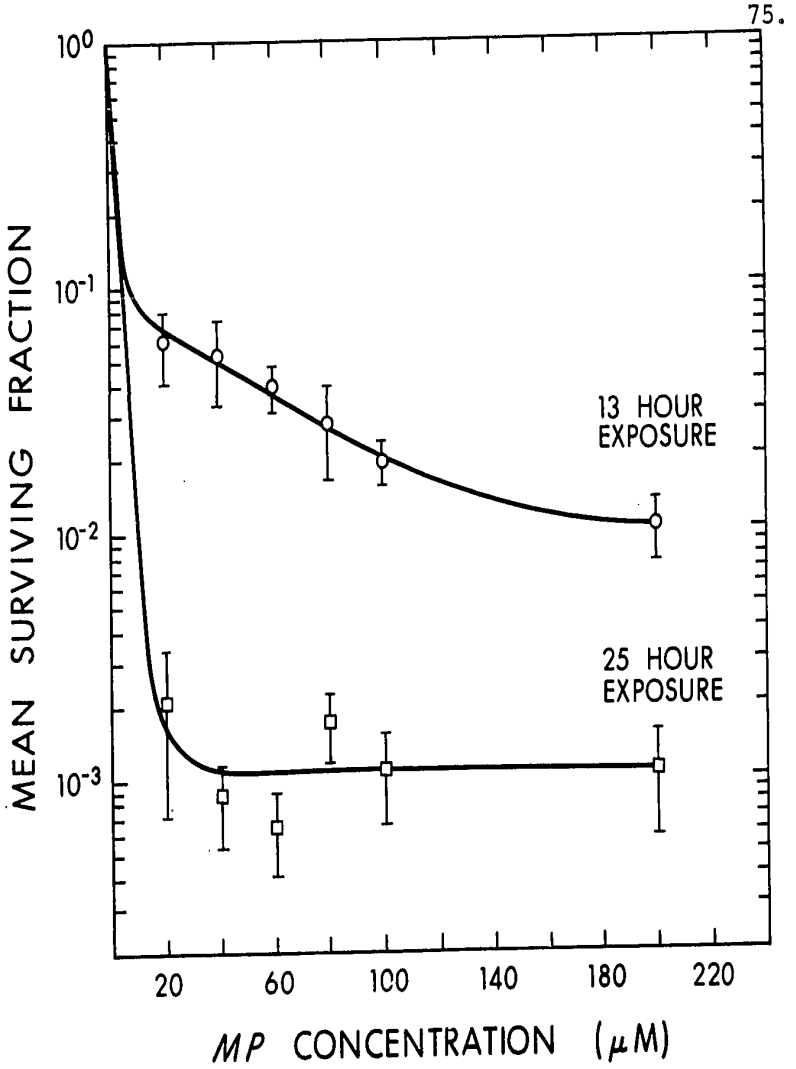

Figure 16. The effect of increasing concentrations of MP on cell survival when exposure period is constant. Cultured L5178Y cells were exposed to various concentrations of MP (20 - 200 μM) for fixed intervals (13 h or 25 h) and then washed, resuspended in fresh drug-free medium and "plated" in soft agar medium. Macroscopic cell colonies were counted after 10 days incubation at 37.5°. These data are presented as mean surviving fraction ±S.D. (vertical bars) calculated from the mean plating efficiency ±S.D. of MP-treated cells (4 tubes) and the mean plating efficiency ±S.D. of the corresponding untreated controls (4 tubes) for each MP concentration and exposure period. Proportionate standard deviations from the mean plating efficiency for corresponding MP treatments and controls were summed in this calculation. All cultures were derived from the same explant of the in vivo-passaged cell line.

exposed to various concentrations of MP (20 - 200 μM) for a fixed period of time (13 h or 25 h). Drug exposure was terminated by washing the cells in drug-free medium. Directly after resuspension, measured numbers of cells were plated in soft agar medium; after incubation for 10 days at 37.5°, macroscopic colonies were enumerated and plating efficiencies were determined. Estimates of the surviving fraction in MP-exposed cultures were calculated by dividing the plating efficiency for such cells by the corresponding value for untreated control cells. It is apparent in the data of Figure 16 that for 13 h exposure the response of the lymphoma approached a maximal value as the MP concentration was increased, and further cell kill was achieved only by increasing the length of the exposure period. This type of behaviour has been associated with agents that are toxic to cells only in a particular phase of the cell cycle (27). Cells that do not pass through the sensitive phase of the cell cycle during drug exposure are spared the cytotoxic effects of even the highest concentration of the drug. As the exposure period is increased, progressively greater cell kill is achieved since more cells pass through the sensitive phase and incur lethal damage. In the case of MP, the situation may be more complicated than this. Paterson and Wang (138) have demonstrated that conversion to thioinosinate is a rate limiting

step in the cytotoxic action of MP. Availability of PRPP is apparently the limiting factor and agents such as 6-methylthioinosine, which produce elevated PRPP pools, simultaneously increase the capacity of cells to anabolize MP, and in so doing, potentiate the cytotoxic activity of the drug (see Chapter I). The data of Figure 17 demonstrate the potentiation of the delayed cytotoxic reaction of MP by 6-methylthioinosine. 6-Methylthioinosine was added to L5178Y cell cultures at concentrations which induced no inhibition (0.05 μM, 0.1 μM) or less than 20% inhibition (0.2 μM) of cell proliferation rate (178). 6-Methylthioinosine was added 3 h before addition of MP (100 μM) and was present throughout the 13 h MP exposure period. MP and 6-methylthioinosine were removed at the same time when cells were washed and resuspended in drug-free medium. On the basis of the displacement of the survivor curves, it is apparent that substantial increases in cell kill were achieved in cultures treated with the combination. Treatment with 6-methylthioinosine alone under these conditions did not elicit a delayed cytotoxic reaction; a higher concentration (0.5 μM) of 6-methylthioinosine inhibited cell proliferation rate by 66% during 13 h exposure, but this effect was reversed completely when the drug was removed (see Figure 24, Chapter III).

Returning to the dose-survival data of Figure 16,

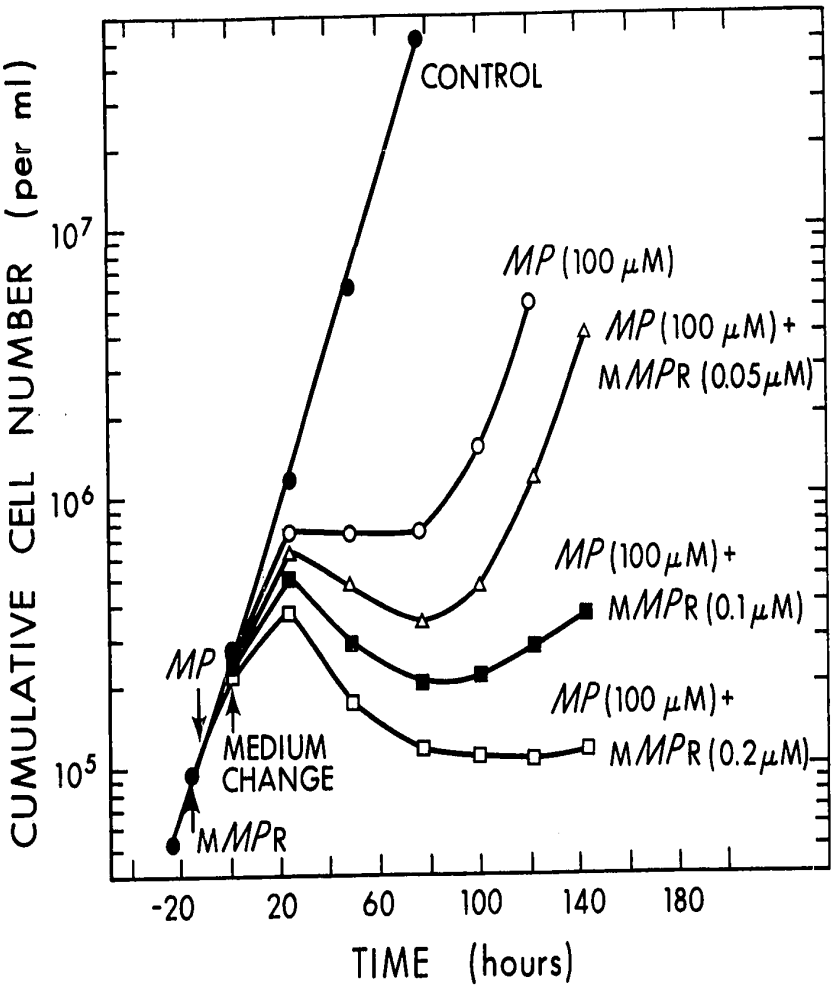

Figure 17. Potentiation of the delayed cytotoxic reaction of MP in L5178Y cell cultures by simultaneous exposure to non-toxic concentrations of 6-methylthioinosine (MMPR). Cultures were treated with 6-methylthioinosine (0.05 μM, 0.1 μM, 0.2 μM) for 3 h prior to addition of MP (100 μM) and throughout the 13 h period of MP exposure. Both agents were removed at 0 h when culture fluids were replaced with drug-free media. Effects of exposure to the combination on proliferation of cultured L5178Y cells are compared with the effect of 13 h exposure with 100 μM MP alone. Concentrations of drugs during exposure are indicated on the figure. All cultures were prepared from the same parent culture at -24 h.

it would seem that these limiting kill curves may partially reflect a saturation of hypoxanthine-guanine phosphoribosyltransferase by MP. The data are still indicative of a mechanism of drug action involving a sensitive phase of the cell cycle. However, cells surviving at saturating concentrations of MP are not necessarily cells that did not pass through a sensitive phase during drug exposure; some survivors may be cells that were sublethally damaged during such a passage. Through stimulation of MP anabolism, 6-methylthioinosine would be expected to increase the number of cells killed as they passed through a MP-sensitive phase of their mitotic cycles. The time dependence of cell kill would indicate that cells may accumulate lethal damage in successive passages through a sensitive phase. The postulate of a MP-sensitive phase was confirmed in experiments with L5178Y cell cultures that were synchronized for mitosis by sequential thymidine and colcemid arrest (see Chapter V). A wave of mitosis occurred in such cultures when colcemid was removed and these cultures exhibited maximum sensitivity to MP (1.5 h exposure) during the third quarter or mid-S phase of the subsequent cell cycle.

Cultured cells of mouse lymphocytic leukemia L1210 also exhibited a delayed cytotoxic reaction to MP exposure

(13 h, 50 - 200 μM) (Figure 18). Displacement of survivor growth curves from untreated control cell growth curves indicated a dose saturation of cell kill similar to that observed for L5178Y cells. However, saturation apparently occurred at higher surviving fractions than with L5178Y cells exposed for the same period of time. L5178Y cells and L1210 cells exhibited similar proliferation rates in culture and therefore the L1210 cells may have a lower capacity than L5178Y for anabolism of MP or alternatively a greater ability to dephosphorylate 6-thioinosinate once formed.

L5178Y cells in the stationary phase of culture growth were considerably less sensitive to TG exposure (13 h, 3 μM) or MP exposure (13 h, 100 μM) than were the same cells in the exponential phase of culture growth. MP or TG was added to L5178Y cell cultures when cell numbers had become static, following a period of exponential proliferation. Exposure was terminated by washing and resuspending the cells at low population density in drug-free medium. Normal rates of exponential proliferation were established in untreated control cultures by 24 h after resuspension at low population density. Drug-treated cultures exhibited low levels of cell kill and surviving cells rapidly repopulated these cultures after resuspension at low population density. Viable cells in stationary phase

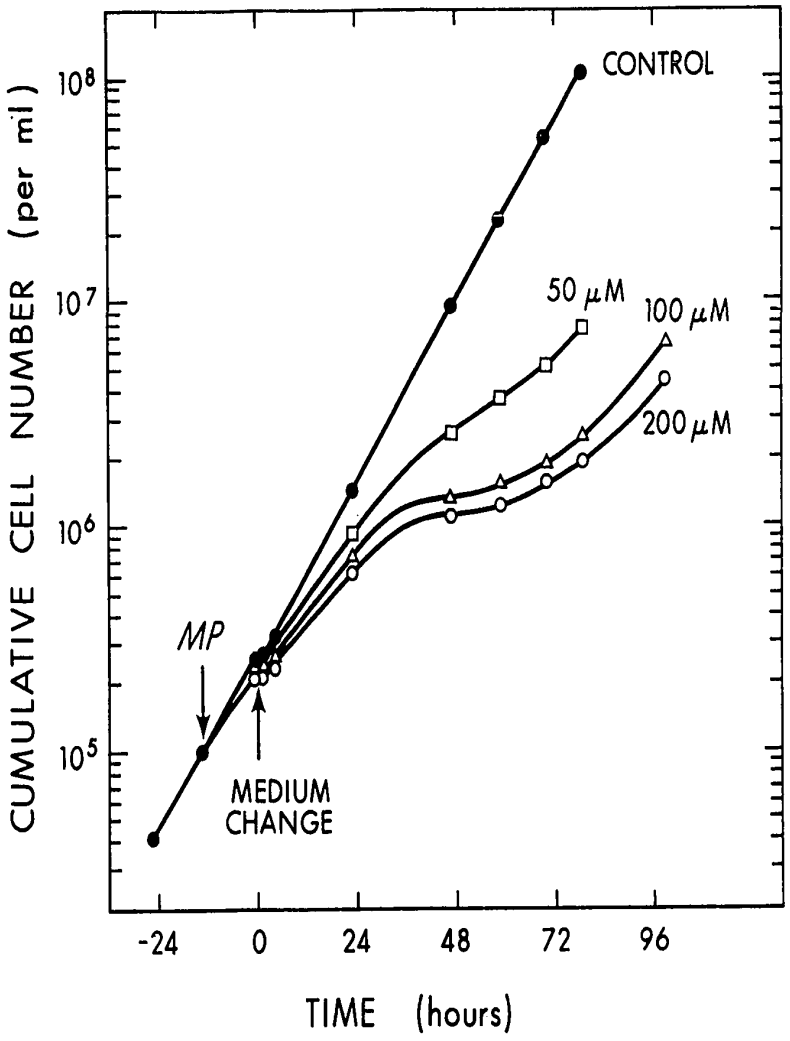

Figure 18. The effect of 13 h exposure to MP on the proliferation of cultured L1210 cells. Concentrations of MP during exposure are indicated on the figure. All cultures were prepared from the same parent culture at -25.5 h.

cultures may have a reduced capacity to anabolize MP and also many of the cells may not enter the MP sensitive period of the cell cycle during exposure.

L5178Y cell populations that survived exposure to MP were substantially less sensitive to further challenge with the drug; this is exemplified in the data of Figure 19, in which it is seen that cells which survived a 25 h exposure to 200 μM MP were less sensitive to 50 μM MP than were untreated cells from the same explant. It appears that the 25 h exposure to 200 μM MP significantly enriched the population with resistant cells.

D. Discussion

Cultured mouse lymphoma L5178Y cells exhibited a delayed cytotoxic reaction to brief exposure with MP. Cells continued to divide during the MP exposure period and a further 1 - 2 abortive doublings of the cell populations occurred after the drug was removed. In a time lapse microphotographic study of cell division events following lethal MP exposure (12 h, 100 μM), 50 individual MP-treated cells were observed during the period of post-exposure proliferation (A. R. P. Paterson, S. C. Kim and D. M. Tidd, unpublished results). In agreement with the results described above, 90% of these 50 MP-treated cells underwent at least

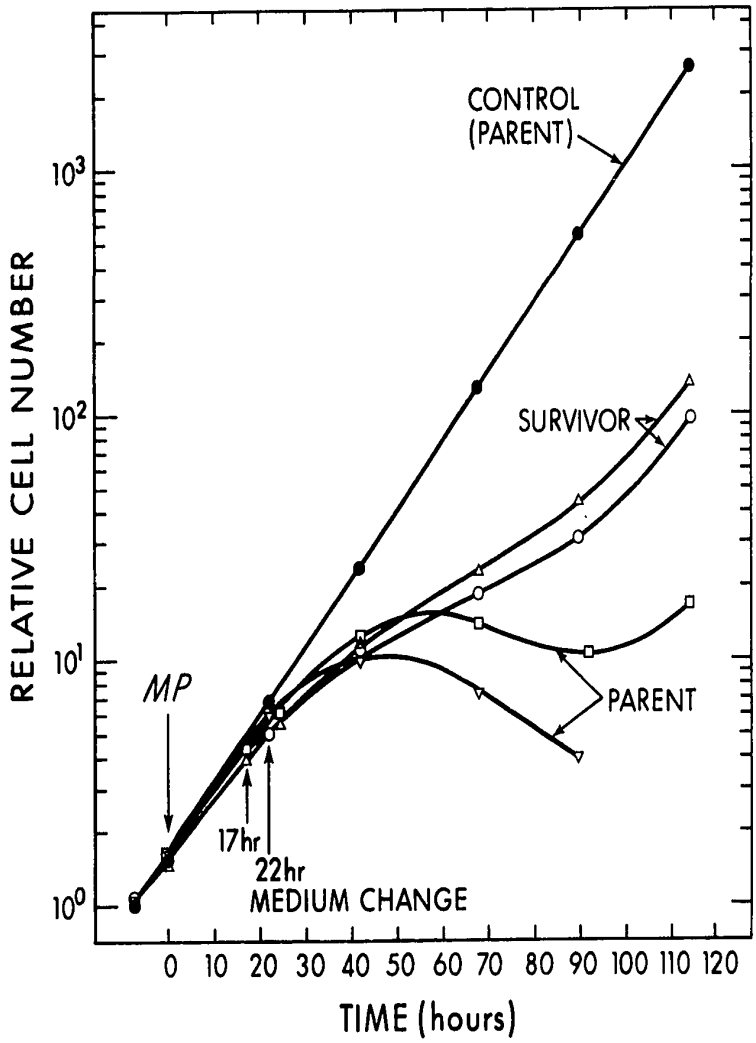

Figure 19. Sensitivity of surviving lymphoma cells to further challenge with MP. Cells that survived treatment for 25 h with MP (200 μM) were exposed to MP (50 μM) for 17 (△) and 22 (○) h. Untreated cells from the same explant (i.e., "parent" line) were cultured with MP (50 μM) for 17 (□) and 22 (▽) h, as well as without MP (●, control). Time in h is relative to the time the drug was added, and cell numbers are relative to those at -8 h. Arrows labeled 17 and 22 h, termination of MP exposure periods.

one division and 52% of the 50 "colonies" contained cells which underwent a second division before disintegrating.

The delayed cytotoxic reaction to MP was characterized by "unbalanced growth" in which cell volume, cell protein and cell RNA increased above normal values. Subsequently, cell division ceased and the enlarged cells lysed. The culture medium used in experiments with the lymphoma provided no content of purines and pyrimidines (apart from that which might be present in serum) and accordingly, the lymphoma cells were entirely dependent upon their own capacity to synthesize these compounds. It might therefore be reasoned that these cells would be very sensitive to inhibitions at the level of purine nucleotide synthesis (see Chapter I). However, this type of mechanism would be expected to produce an immediate effect on cell multiplication. Effects of MP at the macromolecular level would offer a more likely possibility to explain the delay in expression of MP lethality. Nevertheless, the occurrence of "unbalanced growth" as an integral part of the delayed reaction indicates that at least the gross capacity for RNA and protein synthesis is not impaired at this time.

The cytotoxic action of MP on L5178Y cells is apparently "radiomimetic" since the effects of the drug are analogous to the effects of X-rays on cultured cells (14,99). At low dosage, X-rays give rise to "mitotic

death" defined by the characteristic that cells divide several times before death and undergo "giant" cell formation (10). The extension of the concept of mitotic death to chemotherapeutic agents (65) is useful since a common type of biochemical mechanism may be involved. Outright cell death, called "interphase death" (10), may be possible by other mechanisms at much higher doses of these particular agents, but mitotic death is probably predominant when they are used in experimental or clinical cancer therapy (65).

TG, β-2'-deoxythioguanosine, 5-bromodeoxyuridine and ethyl methanesulfonate were shown to elicit delayed cytotoxic reactions or mitotic death in L5178Y cell cultures. TG and β-2'-deoxythioguanosine may be essentially different ways of presenting the same drug to a cell. The cytotoxic effects of both compounds are thought to result from incorporation of TG into internucleotide linkage in DNA (see Chapter I). Consistent with the idea that TG incorporation into DNA has lethal consequences are the experiments of Barranco and Humphrey (13) which showed that cultured Chinese hamster ovary cells are sensitive to β-2'-deoxythioguanosine during the early and middle portions of the S-phase or DNA-synthetic period of the mitotic cycle. Similarly, there is strong evidence to suggest that incorporation of 5-bromodeoxyuridine into DNA is responsible for the cytotoxic effects of this agent (36,69,96,113).

Ethyl methanesulfonate is a monofunctional alkylating agent. Alkylation of DNA is generally considered to be the mechanism responsible for the cytotoxic action of alkylating agents. Kao and Puck (97) concluded that chromosomal aberrations rather than mutational events were the main factor involved in cell death induced by X-radiation and ethyl methanesulfonate. They presented data which indicated that specific single gene mutations do not contribute appreciably to the killing of Chinese hamster cells since the probability of inducing single gene mutations at any locus is so small. "A cell would have to be heterozygous or hemizygous for approximately 10^4 to 10^5 recessive lethal genes, or would require an equal number of dominant lethal genes before single gene mutations could contribute to cell killing in a fashion approximating that of chromosomal aberrations". Another alkylating agent, dimethyl myleran, has also been shown to induce mitotic death in cultures of L5178Y cells (65).

The similarity between the delayed cytotoxic reaction of MP and the delayed cytotoxic reactions of agents which are known to affect the integrity of DNA, suggests that reaction with or incorporation into DNA is involved in MP cytotoxicity. It is noteworthy that chromosomal breaks and bizarre chromosomal rearrangements and structures were observed in MP-treated Chinese hamster ovary cells in culture (K. Horakova and A. R. P. Paterson,

unpublished results).

E. Summary

Pulse exposure to MP induced a delayed cytotoxic reaction in cultures of L5178Y cells and in cultures of L1210 cells. Abortive proliferation occurred after lethal MP exposure and the delayed cytotoxic reaction was characterized by "unbalanced growth" in which cell volume, RNA and protein increased above normal values. Subsequently, division ceased and enlarged cells disintegrated. Similar delayed cytotoxic reactions were observed for TG, β-2'-deoxythioguanosine, and ethyl methanesulfonate. Pulse exposure to 5-bromodeoxyuridine induced a delayed cytotoxic effect in cultures of L5178Y cells, although 5-bromodeoxyuridine-treated cells did not enlarge before disintegrating. The similarities suggested a mechanism of action for MP involving reaction with or incorporation into DNA.

III. THIOPURINE EFFECTS ON CELLULAR NUCLEOTIDE POOLS

A. Introduction

Evidence presented in Chapter II suggested that reaction with DNA or incorporation into DNA might be involved in the delayed cytotoxic reaction of MP. However, effects of MP on purine nucleotide synthesis (see Chapter I) could not be completely discounted. For this reason the effects of brief MP exposure on purine nucleotide pool sizes and the effects of brief purine starvation on cell survival were investigated. Warnick and Paterson (178) measured cell survival and reductions of intracellular purine ribonucleotide pools in 6-methylthioinosine-treated L5178Y cell cultures. As part of the present work, perturbations in cellular purine ribonucleotide pools induced by non-toxic 6-methylthioinosine treatment were compared with those induced by cytotoxic TG and MP exposures.

B. Materials and Methods

The preparation of drug solutions and L5178Y cell culture and drug exposure procedures are described in Chapter II, Sections B.1. and B.2. Cell volume determination is described in Chapter II, Section B.3.

1. Chemicals

Thymidine was purchased from the Sigma Chemical Co., St. Louis, Mo. and solutions of thymidine (38 mM) in 0.9% NaCl were sterilized by filtration through a sterile, 0.45 μm pore size Millipore filter (Millipore Filter Corp., Bedford, Mass.). Adenosine was purchased from Pabst Laboratories, Milwaukee, Wis. and inosine was supplied by Calbiochem, La Jolla, Calif.

2. Preparation of Acid Extracts of Cultured L5178Y Cells

Immediately before acid extraction, L5178Y cultures (approximately 120 ml containing 2 - 4 x 10^7 cells (or 0.02 - 0.04 ml of packed cells)) were sampled, and total cell number and median cell volume were determined. These parameters were used to calculate the total volume of tissue extracted; nucleotide concentrations were subsequently related to tissue volume rather than to cell number since cell volume increased during expression of the delayed cytotoxic reaction of MP and TG. Cultures at 37° were transferred to centrifuge bottles (140 ml capacity, conical bottoms, Bellco Glass, Inc., Vineland, N.J.), cooled to 4° in ice, and the cells then collected by centrifugation. Cells from MP-8-^{14}C-treated cultures were washed twice with 100 ml portions of ice-cold Fischer's medium before extraction. It was found essential to cool

cultures before centrifugation, since extensive degradation of cellular nucleoside triphosphates occurred when cells were pelleted at 37°. Perchloric acid (20 µl, 42% w/v) was added to cell pellets (at 4°) and the precipitated material was suspended in the residual supernatant (approximately 0.2 ml). The suspension and a 0.05 ml 4.2% w/v perchloric acid rinse of the bottle tip were transferred to a 12 ml conical centrifuge tube, allowed to stand for 15 minutes at 4°C and then centrifuged. The supernatant was combined with a 0.1 ml 4.2% w/v perchloric acid wash of the precipitate and the extract was neutralized with 7 N potassium hydroxide at 4°. Extracts were analyzed immediately or stored at -20° for no longer than 24 h before analysis.

3. Nucleotide Determination in L5178Y Cell Extracts

Nucleotide concentrations were determined in the acid extracts by anion exchange chromatography using a high pressure, liquid resin system and a Varian Aerograph LCS-1000 instrument, as described by Brown (26). Generally 5 - 10 µl of the extract were placed on the column; therefore the nucleotide content of about 5 - 10 x 10^5 cells (approximately 5 - 10 x 10^{-4} ml packed cells) was measured. The absorbance of the eluate at 254 nm was monitored continuously and transmitted to a pen recorder. Nucleotide

concentrations were calculated from peak areas in the absorbancy trace of the column effluent. Response factors for individual nucleotides were determined with standard solutions of the nucleotides. Peak areas were determined by planimeter, by cutting out and weighing Xerox copies of individual peaks or by measurement of peak height and width at half height. The three methods gave similar results.

Expressed as μmoles/ml tissue, the intracellular content of ribonucleoside triphosphates in normal, exponentially proliferating cultures of L5178Y cells were as follows: adenosine triphosphate, 2.5; guanosine triphosphate, 0.8; uridine triphosphate, 1.0; cytidine triphosphate, 0.6. The relative pool sizes of adenosine triphosphate, adenosine diphosphate and adenosine monophosphate were about 25:3:1, respectively. The relative pool sizes of guanosine triphosphate and guanosine diphosphate were approximately 8:1. The following experiments suggested that these ratios were representative of the distribution of nucleotides within the three levels of phosphorylation in the proliferating cell. Cultured cells were collected by centrifugation at 37^{0} and resuspended at high cell density (3×10^{7} cells/ml) in a small volume (9 ml) of fresh, warm Fischer's medium. The cell suspension was incubated with gentle shaking at 37^{0} for 20 min. A 0.1 ml sample of the cell suspension was removed and returned to conventional

culture conditions whereupon these cells proliferated at their usual rate. The remainder of the cell suspension was extracted directly with perchloric acid without prior chilling or centrifugation of the cells. The relative amounts of adenosine mono-, di- and triphosphate, and guanosine di- and triphosphate in this extract were similar to those in extracts where cultured cells were chilled and centrifuged before extraction. In another experiment, the medium was carefully syphoned out of vessels containing exponentially proliferating cells, without disturbing the cell sediments. This operation was performed at 37^{0} and perchloric acid was added directly to the small amount of residual medium. The relative amounts of adenosine mono-, di- and triphosphate, and guanosine di- and triphosphate in these extracts were similar to those in extracts where cultured cells were chilled and collected by centrifugation before extraction. The foregoing results indicate that degradation of nucleoside triphosphates did not occur during chilling and centrifugation of cells and the relative nucleotide concentrations in extracts of cells collected in this manner are probably representative of those in the proliferating cell.

C. Results

1. 6-Mercaptopurine Effect on Cellular Nucleotide Pools

Although cultured L5178Y cells continued to proliferate during pulse exposures to cytotoxic concentrations of MP, increases in doubling times were observed (see Chapter II, Figures 3, 5, 7 and 19). This "acute" effect of MP would appear to be more compatible with drug induced inhibitions of purine nucleotide synthesis (discussed in Chapter I) than would the delayed cytotoxic action of MP. The experiment of Figure 20 indicated that inhibition of cell division by MP was not immediately lethal to these cells. Cell populations that had become static in the presence of a high concentration of MP (200 μM) (Figure 20) remained so for 7 h and then, upon removal of the drug, resumed division. Figure 20, lower curve, shows changes in cell number that occurred between 0 and 25 h in the presence of 200 μM MP. Cell multiplication ceased 6 h after MP was added and cell numbers did not change significantly during the remaining 19 h of exposure. However, when the drug was removed at 6, 11 or 13 h, proliferation resumed. The delayed cytotoxic effects of MP were manifested in these cultures about 40 h after MP removal (data not shown). Cells did not resume proliferation after 25 h of MP exposure, but did increase in volume after the drug

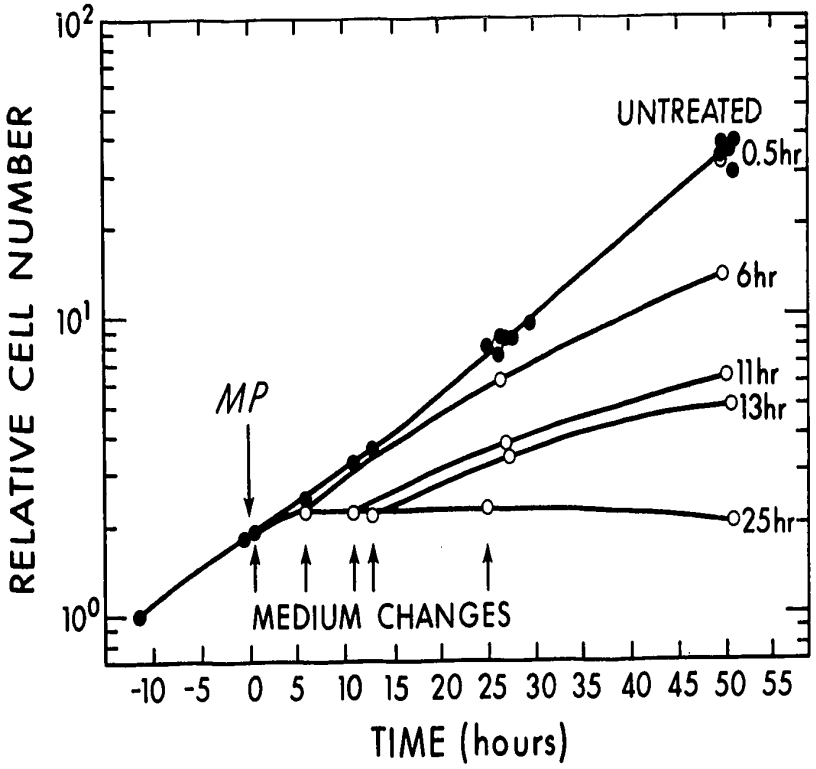

Figure 20. Growth curves of L5178Y cultures exposed to MP for various intervals. MP (200 μM) was added at zero time, and cell numbers are relative to those at -11.5 h. The periods of exposure to MP are indicated. o, treated cells; •, untreated controls.

was removed. Increased doubling times during exposure to lower concentrations of MP may represent minor manifestations of this same effect. It would seem that different mechanisms were responsible for this acute inhibition of proliferation and for the delayed lethal activity of MP. Acute inhibition of cell division induced by MP may result from inhibitions by MP anabolites at the level of purine nucleotide synthesis. In support of this conclusion was the observation that hypoxanthine (10 μM) or adenine (10 μM) partially alleviated the inhibition of cell proliferation when present in the medium to which cells were transferred after MP exposure (K. Horakova and A. R. P. Paterson, unpublished observations). This effect was only observed during the early stage of abortive proliferation. The post-exposure cultivation of MP-treated cells in medium containing adenine or hypoxanthine did not prevent the eventual onset of the delayed cytotoxic reaction of MP. Thus, these purines did not reverse the cytotoxic effects of prior exposure to MP, but to a certain extent did relieve the inhibition of cell division during the early part of post-exposure proliferation.

Cultured L5178Y cells were protected or partially protected against the delayed cytotoxic action of 100 μM MP by simultaneous exposure (13 h) to MP and 20 μM

concentrations of any of the following physiological purine derivatives: hypoxanthine, inosine, guanosine, adenine and adenosine (K. Horakova and A. R. P. Paterson, unpublished observations). Guanine did not protect cells against the delayed cytotoxic reaction of MP. However, horse serum used in the culture medium contained a high guanase activity which catalyzed the rapid hydrolysis of guanine to the poorly metabolized derivative, xanthine (J. F. Henderson, personal communication). Protection against MP by these purine derivatives might suggest a lethal, drug-induced inhibition of purine ribonucleotide synthesis which was alleviated by anabolism of the purines added to the culture medium. However, conversion to 6-thioinosinate is the first step essential to the cytotoxic activity of MP (see Chapter I) and also appears to be a rate-limiting factor for cell kill (138,162,177) (see Chapter II). Hypoxanthine-guanine phosphoribosyltransferase is the enzyme responsible for conversion of MP, hypoxanthine and guanine to nucleotides. Therefore, protection by hypoxanthine could be the result of competition with MP for the phosphoribosyltransferase. The major route for anabolism of inosine and guanosine is probably by way of intracellular phosphorolysis to hypoxanthine and guanine; the latter would likely compete with MP for the phosphoribosyltransferase. Cultured L5178Y cells readily deaminated

extracellular adenosine; paper chromatography of medium samples demonstrated that 1.5 mM adenosine was converted to inosine within 6.5 h by a suspension of 1.5×10^6 cells/ml. Hypoxanthine derived intracellularly from adenosine would probably compete with MP for hypoxanthine-guanine phosphoribosyltransferase. Paterson and coworkers have demonstrated that intracellular availability of PRPP is a rate-limiting factor affecting conversion of MP to 6-thioinosinate (136,138,177). PRPP is consumed during anabolism of adenine and it is possible that adenine would inhibit phosphorylation of MP indirectly through competition for available PRPP. Therefore, hypoxanthine, adenine, inosine, guanosine and adenosine may protect cells against MP at least partially by inhibiting the formation of 6-thioinosinate. Further, intracellular purine nucleotide pools may be elevated when preformed purine derivatives are supplied exogenously (F. F. Snyder and J. F. Henderson, personal communication) and at increased concentrations, purine nucleotides may inhibit enzymes involved in "activation" of MP beyond the initial stage of 6-thioinosinate formation.

Acute and delayed effects of MP on cellular nucleotide pools were investigated in an attempt to establish whether drug effects at the level of purine nucleotide synthesis bore any relation to the delayed cytotoxic

reaction of MP. No such relation was observed. In the experiment of Figure 21, cultured L5178Y cells were exposed for 13 h to 100 µM MP alone or in combination with thymidine at high concentration (1 mM). This concentration of thymidine was expected to retard the passage of cells through the S-phase of the cell cycle and was also used in experiments where division of cultured L5178Y cells was synchronized by the method of sequential thymidine and colcemid arrest (A. R. P. Paterson, S. C. Kim and D. M. Tidd, unpublished results). Cell division was reduced by 35% in L5178Y cell cultures during 7 h exposure to 1 mM thymidine alone and proliferation ceased completely during 24 h exposure. At the end of the 13 h exposure period in the experiment of Figure 21 there was no significant difference in cell numbers for cultures treated with MP alone or MP in combination with thymidine. The reason for this is unclear. However, cells exposed to thymidine plus MP exhibited the characteristics of unbalanced growth induced by selective thymidine inhibition of DNA synthesis and the median volume of these cells was 1,460 cu µm when the 13 h exposure period was terminated. In contrast, the median volume (1,170 cu µm) of cells exposed to MP alone did not differ significantly from the median volume (1,130 cu µm) of untreated control cells at this time.

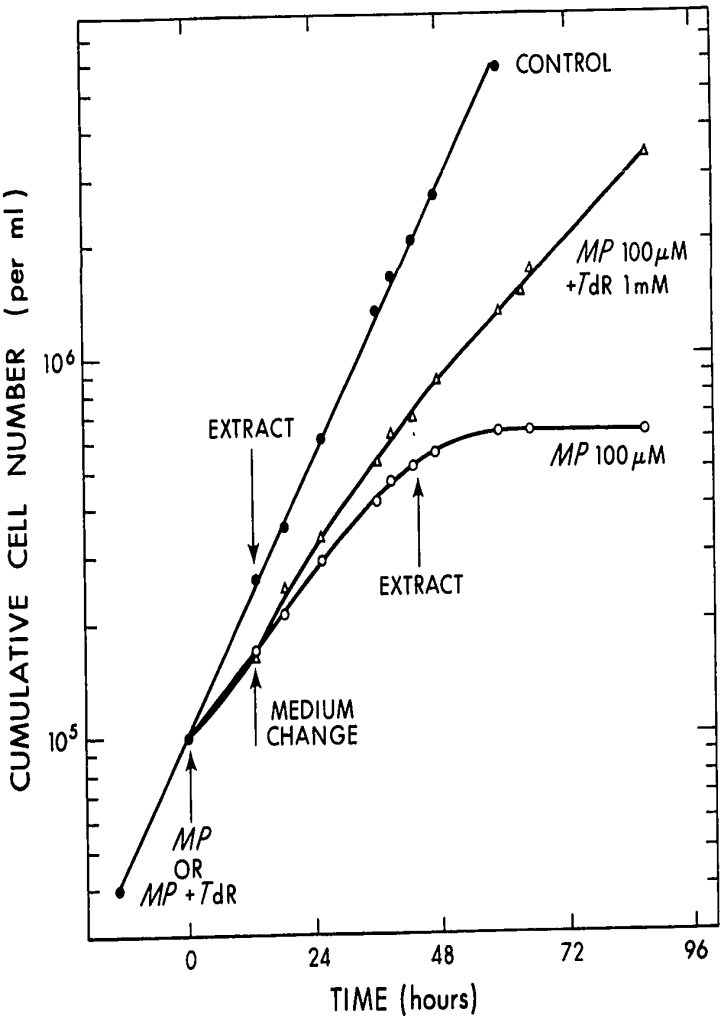

Figure 21. The effects of 13 h exposure to MP and MP plus thymidine (TdR) on the proliferation of cultured L5178Y cells. At 13 h, culture fluids were replaced with drug-free media. Concentrations of agents during exposure are indicated on the figure. Arrows labeled EXTRACT indicate times at which acid extracts were prepared from cells in treated and untreated cultures. Intracellular nucleotide pool sizes were determined by chromatographic analysis of these extracts and are presented in Figure 23.

It is apparent in Figure 21 that simultaneous exposure to thymidine and MP partially protected cells against the delayed cytotoxic reaction of MP. Acid extracts of cultures were prepared immediately prior to termination of drug treatment and 31 h later (at the time of the delayed cytotoxic reaction of MP). Analysis of these extracts demonstrated that thymidine did not affect MP-induced changes in purine nucleotide pools, suggesting that thymidine did not protect cells by inhibiting MP-anabolism.

An acid-soluble profile of normal, exponentially proliferating L5178Y cells is presented in Figure 22. Drug-induced changes in the concentrations of adenosine triphosphate and guanosine triphosphate were always accompanied by similar changes in the concentrations of the corresponding diphosphates. Variations in the concentration of uridine triphosphate were accompanied by approximately coordinate variations in the concentration of uridine diphosphate-sugar derivatives. Adenosine monophosphate, guanosine monophosphate, uridine monophosphate and uridine diphosphate comprised only small fractions of the total ribonucleotide pools for these bases. Thus, variations in the pool sizes of uridine triphosphate, adenosine triphosphate or guanosine triphosphate were representative of gross changes in the total ribonucleotide pool for the corresponding base.

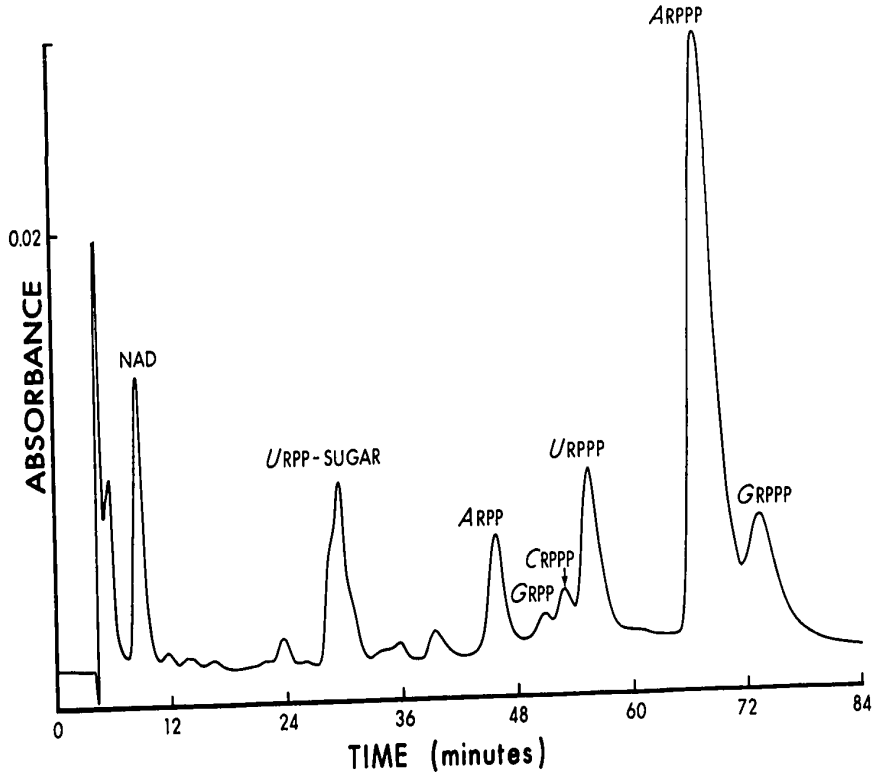

Figure 22. The acid-soluble profile of normal, exponentially proliferating L5178Y cells. Nucleotides in an acid extract of cultured cells were separated by anion-exchange chromatography; presented is the absorbancy (254 nm) trace of the column effluent. The identity of the major components is indicated on the figure: NAD, nicotinamide adenine dinucleotide; URPP-SUGAR, uridine diphosphate-sugar derivatives; ARPP, adenosine diphosphate; GRPP, guanosine diphosphate; CRPPP, cytidine triphosphate; URPPP, uridine triphosphate; ARPPP, adenosine triphosphate; GRPPP, guanosine triphosphate.

The effects of treatments with MP and MP plus thymidine (Figure 21) on intracellular pools of uridine triphosphate, adenosine triphosphate and guanosine triphosphate are shown in Figure 23. In deriving the data of Figure 23, nucleotide pool sizes for drug-treated cells were measured as μmoles/ml of tissue and are expressed as percentages of the pool sizes in untreated control cells. The abscissa in Figure 23 corresponds to the time scale of Figure 21. 13 h exposure to 100 μM MP alone had no significant effect on the intracellular content of uridine triphosphate; in contrast, adenosine triphosphate was reduced by approximately 30% and the guanosine triphosphate pool was 60% lower than that in untreated control cells. These effects are consistent with inhibitions that 6-thioinosinate is known to exert on purine nucleotide synthesis (see Chapter I). However, in spite of these effects, the cells continued to proliferate, albeit at a reduced rate (see Figure 21) and when MP was removed, these cells regained normal amounts of adenosine triphosphate and guanosine triphosphate (Figure 23). At 44 h, 31 h after MP was removed, cells treated with 100 μM MP alone were enlarged (median cell volume, 1,760 cu μm) and dying (see Figure 21) and yet they had normal pools of purine ribonucleotides (Figure 23). Thymidine (1 mM) present during 13 h

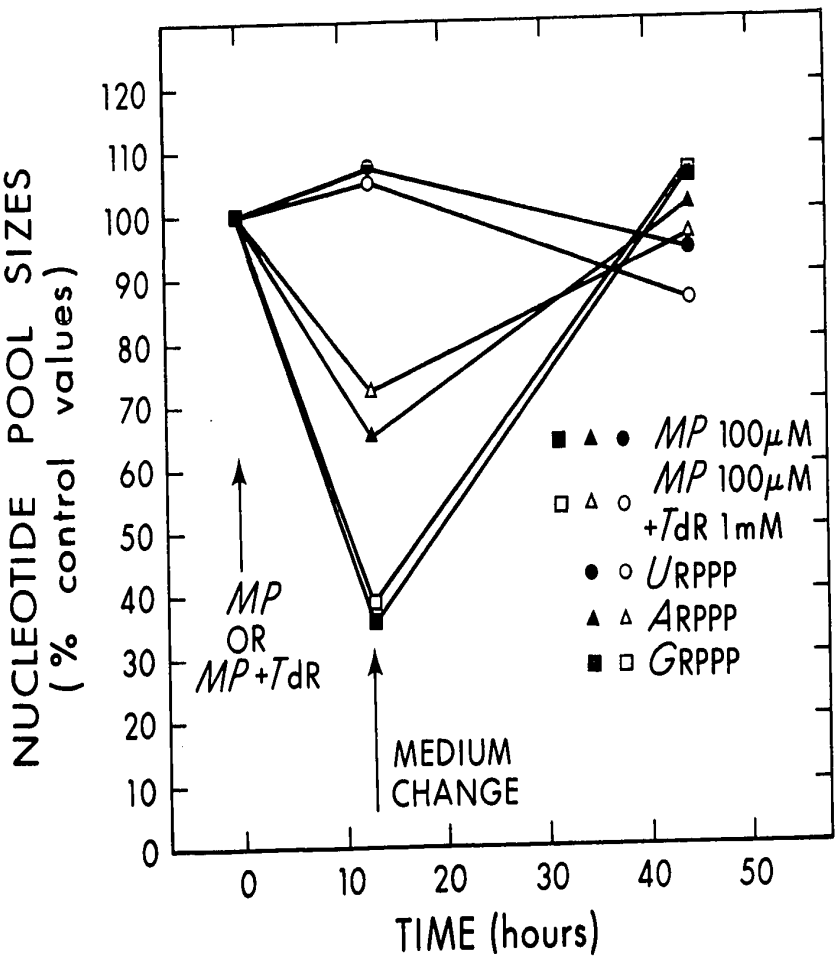

Figure 23. The effect of 13 h exposure to MP and MP plus thymidine (TdR) on the intracellular nucleotide pool sizes of cultured L5178Y cells. At 13 h, culture fluids were replaced with drug-free media. The corresponding effects on cell proliferation are presented in Figure 21. Acid extracts of cells in treated and untreated cultures were prepared at 13 h, before medium replacement, and at 44 h. Intracellular nucleotide pool sizes were determined by chromatographic analysis of these extracts; nucleotide pool sizes in treated cells are presented as percentages of the values for untreated control cells. Concentration of agents during exposure: 100 μM MP, filled symbols; 100 μM MP plus 1 mM thymidine, open symbols. Nucleotides: adenosine triphosphate (▲△); guanosine triphosphate (■□); uridine triphosphate (● ○).

exposure to 100 μM MP did not alter these effects of MP on cellular purine ribonucleotide pools; however, thymidine did partially protect the cells against the delayed cytotoxic reaction of MP (see Figure 21). At 44 h the median cell volumes were: MP plus thymidine-treated cells, 1,420 cu μm; untreated control cells, 1,250 cu μm. It would appear that thymidine did not inhibit conversion of MP to 6-thioinosinate, since it did not alleviate MP-induced reductions in cellular purine ribonucleotide content and, therefore, some other mechanism must be responsible for protecting cells against MP cytotoxicity. In addition, since thymidine-protected cells experienced the same reductions in purine ribonucleotide pools as unprotected cells, it is unlikely that the preceding period of purine starvation was responsible for the subsequent delayed cytotoxic reaction in the latter. MP-8-^{14}C was used in the experiment of Figures 21 and 23. Radioactivity in acid extracts prepared at the end of the 13 h MP-8-^{14}C exposure period corresponded to intracellular pools of acid soluble radioactivity equivalent to 0.109 μmoles ^{14}C-labeled derivatives/ml tissue for cells treated with 100 μM MP-8-^{14}C alone, and 0.126 μmoles/ml tissue for cells treated with 100 μM MP-8-^{14}C + 1 mM thymidine. 31 h later, at the time of the delayed cytotoxic reaction of MP, the amount of acid soluble radioactivity remaining was

equivalent to 0.002 μmoles/ml tissue for cells originally treated with MP-8-^{14}C alone, and 0.001 μmoles/ml of tissue for cells originally treated with MP-8-^{14}C plus thymidine. Thus, only 1 - 2% of the original intracellular content of acid soluble ^{14}C-labeled derivatives remained at the time of the delayed cytotoxic reaction of MP.

2. Comparison of Effects of 6-Mercaptopurine, 6-Thioguanine and 6-Methylthioinosine on Cellular Nucleotide Pools

During a study of cellular nucleotide concentrations in 6-methylthioinosine-treated L5178Y cell cultures, Warnick and Paterson (178) demonstrated that 6-methylthioinosine (0.1 μM) reduced intracellular purine ribonucleotide pools by 40% without affecting proliferation rates throughout a 6-day period. An 18 h exposure with 0.5 μM 6-methylthioinosine reduced intracellular purine ribonucleotide content by about 70% and proliferation rates by about 30%. This treatment did not affect cell viability as determined by formation of macroscopic cell colonies in drug-free, soft agar medium. Proliferation rates and purine ribonucleotide pools returned to normal values after culture fluids were replaced with drug-free media (C. T. Warnick and A. R. P. Paterson, unpublished observations). In the present work, a similar experiment was conducted in which effects on cellular purine ribonucleotide pools

of 13 h exposure to 0.5 μM 6-methylthioinosine were compared with effects of 13 h exposure to cytotoxic concentrations of MP or TG.

The data of Figure 24 illustrate the effects of 13 h exposure to MP, TG or 6-methylthioinosine on the proliferation of cultured L5178Y cells. During the drug-exposure period, cell proliferation was inhibited to a greater extent by 100 μM MP than by 3 μM TG. These data show that cells continued to proliferate for a limited period after MP or TG was removed and delayed cytotoxic reactions were observed. Cell proliferation was inhibited to the greatest extent during drug exposure in cultures treated with 0.5 μM 6-methylthioinosine; however, this inhibition was completely reversed by washing and resuspending the cells in drug-free medium. Median cell volumes at the end of the 13 h drug exposure period and 30 h later were, respectively, 1,130 cu μm and 1,630 cu μm in MP-treated cultures; 1,130 cu μm and 920 cu μm in 6-methylthioinosine-treated cultures; 1,090 cu μm and 1,840 cu μm in TG-treated cultures; 1,090 cu μm and 1,090 cu μm in untreated control cultures.

In the experiment of Figure 24, acid extracts of untreated and treated cells were prepared immediately prior to removal of the drugs and 30 h after drug exposures were terminated; intracellular ribonucleotide pool sizes were determined by chromatographic analysis of these extracts. Drug-induced changes in cellular adenosine triphosphate and guanosine triphosphate pools are shown in Figures 25 and 26. Nucleotide pool sizes were measured as μmoles/ml tissue and are expressed as percentages of the values for untreated control cells. The abscissae of Figures 25 and 26 correspond to the time scale of Figure 24. Figure 25 compares the effects on cellular adenosine triphosphate and guanosine triphosphate pools of 13 h exposures with 100 μM MP and 0.5 μM 6-methylthioinosine. The effects of MP were similar to those observed in the experiment of Figure 23 (see above). An exposure of 13 h to 100 μM MP reduced the cellular content of adenosine triphosphate by approximately 20%, whilst the content of guanosine triphosphate was over 60% lower than that observed in untreated control cells. Concentrations of these nucleotides increased after MP exposure was terminated and at 43 h, the time of the delayed cytotoxic

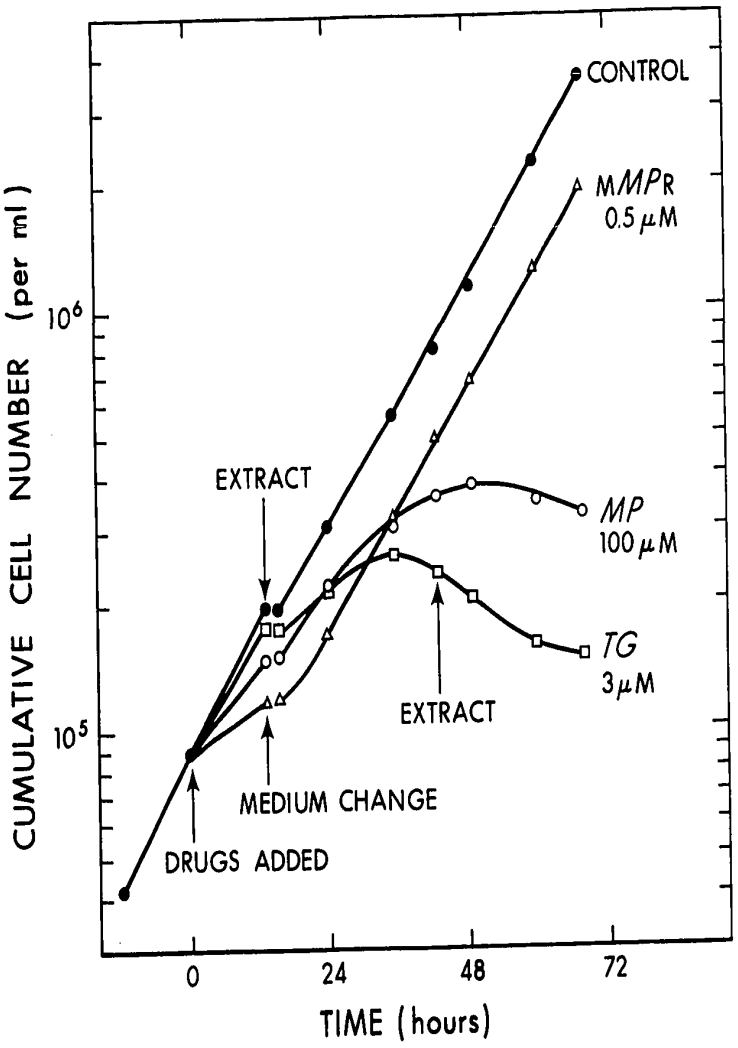

Figure 24. The effect of exposure to MP, TG or 6-methylthioinosine on the proliferation of cultured L5178Y cells. Cultures were treated for 13 h with 100 μM MP, 3 μM TG or 0.5 μM 6-methylthioinosine; at 13 h, culture fluids were replaced with drug-free media. Concentrations of drugs during exposure are indicated on the figure. Arrows labeled EXTRACT indicate times at which acid extracts were prepared from cells in treated and untreated cultures. Intracellular nucleotide pool sizes were determined by chromatographic analysis of these extracts and are presented in Figures 25 and 26.

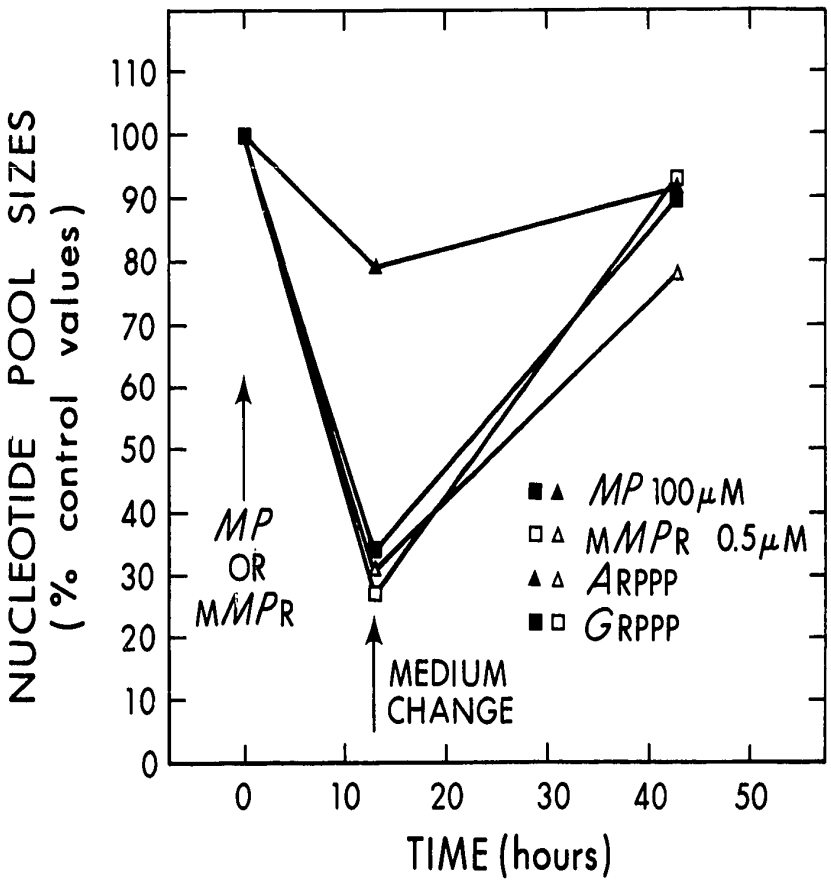

Figure 25. The effects of 13 h exposure to MP and 6-methylthioinosine on the intracellular purine ribonucleotide pool sizes of cultured L5178Y cells. At 13 h culture fluids were replaced with drug-free media. These are data from the experiment of Figure 24. Acid extracts of cells in treated and untreated cultures were prepared at 13 h, before medium replacement, and at 43 h. Intracellular nucleotide pool sizes were determined by chromatographic analysis of these extracts; nucleotide pool sizes in treated cells are presented as percentages of the values for untreated control cells. Concentration of drugs during exposure: 100 μM MP, filled symbols; 0.5 μM 6-methylthioinosine, open symbols. Nucleotides: adenosine triphosphate (▲△); guanosine triphosphate (■□).

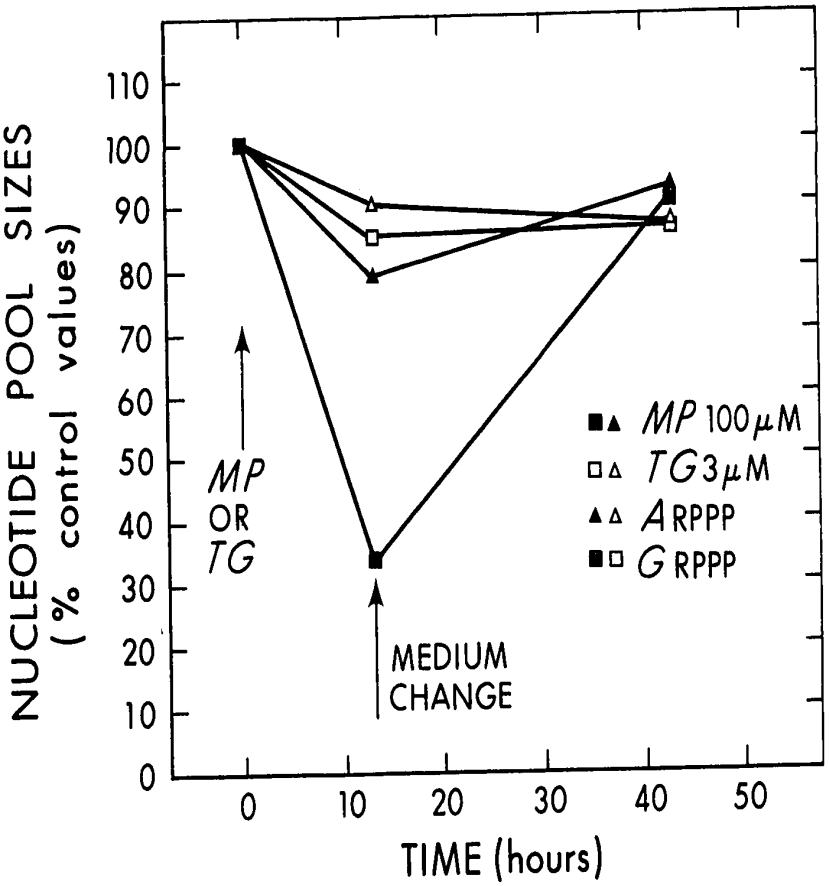

Figure 26. The effects of 13 h exposure to MP and TG on the intracellular purine ribonucleotide pool sizes of cultured L5178Y cells. At 13 h culture fluids were replaced with drug-free media. These are data from the experiment of Figure 24. Acid extracts of cells in treated and untreated cultures were prepared at 13 h, before medium replacement, and at 43 h. Intracellular nucleotide pool sizes were determined by chromatographic analysis of these extracts; nucleotide pool sizes in treated cells are presented as percentages of the values for untreated control cells. Concentration of drugs during exposure: 100 μM MP, filled symbols; 3 μM TG, open symbols. Nucleotides: adenosine triphosphate (▲△); guanosine triphosphate (■□).

reaction (see Figure 24), MP-treated cells had regained nearly normal pools of adenosine triphosphate and guanosine triphosphate. An exposure of 13 h to 0.5 μM 6-methylthioinosine reduced both the adenosine triphosphate and guanosine triphosphate pools by 70%. Coordinate changes in the amounts of adenosine triphosphate and guanosine triphosphate are consistent with a drug-induced inhibition of purine ribonucleotide synthesis de novo at an enzyme step prior to formation of inosine monophosphate. Reductions in the intracellular concentrations of adenine and guanine ribonucleotides were probably responsible for the reduced rates of cell proliferation observed during exposure to 6-methylthioinosine (see Figure 24); concentrations of these nucleotides increased after 6-methylthioinosine exposure was terminated (Figure 25) and the cell proliferation rate rapidly returned to that of untreated control cultures (Figure 24). No delayed cytotoxic reaction was observed in 6-methylthioinosine-treated cultures and it was evident that cells were able to sustain limited periods of purine starvation without apparent effects on viability. Figure 26 compares the effects on cellular adenosine triphosphate and guanosine triphosphate pools of 13 h exposures with 100 μM MP and 3 μM TG.

In contrast to MP, 3 μM TG had only slight effect on intracellular purine ribonucleotide pools, although both agents elicited delayed cytotoxic reactions (Figure 24). The data of Figure 13, Chapter II, suggest that 3 μM TG is ultimately more toxic to cultured L5178Y cells than 100 μM MP.

Cultured L5178Y cells were protected or partially protected against the delayed cytotoxic action of 3 μM TG by simultaneous exposure (13 h) to TG and 20 μM concentrations of any of the following physiological purine derivatives: hypoxanthine, inosine, adenosine and guanosine (K. Horakova and A. R. P. Paterson, unpublished observations). Adenine was not tested. It is evident from the above findings that these purine derivatives did not protect cells against TG by alleviating drug-induced inhibitions of purine nucleotide synthesis since such inhibitions were only minor. Inhibition of TG anabolism would seem to offer a more likely explanation for the protective effects of physiological purine derivatives. These purine derivatives also protected or partially protected cells against the delayed cytotoxic reaction of MP. Protection against MP by purine derivatives is discussed above in terms of possible inhibitions of MP anabolism. Competitive effects at the level of hypoxanthine-guanine phosphoribosyltransferase would also be applicable to TG, since this enzyme is responsible for

conversion of both MP and TG to nucleotides (see Chapter I). Formation of 6-thioguanylate would appear to be a rate-limiting step in the cytotoxic action of TG, and PRPP availability may be the rate-limiting factor involved (130).

D. Discussion

Inhibition of inosinate dehydrogenase by 6-thioinosinate (see Chapter I) may be partially responsible for the 60% reduction in the cellular guanine ribonucleotide pool, which occurred during exposure to MP. Reductions of 20 - 30% in adenine ribonucleotides could reasonably result from inhibitions of adenylosuccinate synthetase, adenylosuccinate lyase and purine ribonucleotide synthesis de novo (see Chapter I). Inhibition of purine ribonucleotide synthesis de novo may also contribute to the reduction in guanine ribonucleotides. It is apparent that the temporary reductions in pool sizes are not lethal; however, sustained reductions might well be fatal. Cells with reduced pools of purine ribonucleotides continued to proliferate and regained normal or near normal pool sizes after MP was removed. The delayed cytotoxic reaction of MP was observed in cells containing normal amounts of adenine and guanine ribonucleotides.

The effects of brief exposure to 6-methylthioinosine on cellular purine ribonucleotide pools provide further evidence that the degree of purine starvation was insufficient to elicit cytotoxic effects. It is evident that cultured L5178Y cells are able to withstand short periods of purine starvation without apparent loss of viability.

The delayed cytotoxic reaction of MP was apparently unrelated to inhibitory effects of drug anabolites at the level of purine ribonucleotide synthesis. A high concentration of thymidine present during exposure to MP did not affect MP-induced reductions in cellular purine ribonucleotide pools, but did afford protection against the delayed cytotoxic reaction. The effects of TG exposure established that inhibitions of purine ribonucleotide synthesis are not necessarily involved in the mechanism of a delayed cytotoxic reaction. Exposure to TG elicited a delayed cytotoxic reaction, but had only slight effect on cellular purine ribonucleotide pools.

MP-induced reductions in cellular purine ribonucleotide concentrations may be responsible for increased cell doubling times observed in cultures during the period of drug-exposure. Adenine or hypoxanthine did not reverse the delayed cytotoxic reaction of prior exposure to MP. However, cells were protected against delayed thiopurine cytotoxicity when any of a number of physiological purine

derivatives were present in culture media during the period of drug exposure. Since inhibitions of purine ribonucleotide synthesis do not appear to be involved in the mechanisms of the delayed cytotoxic reactions of MP and TG, it is likely that the physiological purine derivatives or their metabolites protect cells either directly or indirectly by inhibiting the anabolism of MP and TG.

Incorporation of TG into DNA appears to be centrally involved in the cytotoxic activity of this agent (see Chapter I). The similarity between the delayed cytotoxic reaction of TG and that of MP, and the nature of the delayed cytotoxic reaction itself, suggested that effects at the macromolecular level were responsible for the cytotoxicity of MP. It appeared likely that TG derivatives, formed metabolically from MP (see Chapter I), are responsible for the cytotoxic activity of MP. If this were the case, the mechanisms of the delayed cytotoxic reactions of MP and TG would be identical. Consistent with this possibility was the observation that TG and β-2'-deoxythioguanosine elicited delayed cytotoxic reactions at 0.01 times the concentration of MP required for a comparable effect (see Chapter II).

E. Summary

Intracellular purine ribonucleotide pools were lowered in cultured L5178Y cells during exposure to MP. However, there was no relation between effects on purine ribonucleotide pool sizes and the delayed cytotoxic activity of MP. Thymidine present in culture medium during exposure to MP had no effect on MP-induced reductions in purine ribonucleotide pools, but partially protected cells against the delayed cytotoxic reaction of MP.

Exposure to TG elicited a delayed cytotoxic reaction in cultured L5178Y cells, but had only slight effects on cellular purine ribonucleotide pools.

Adenine and guanine ribonucleotide pools were reduced during brief exposure to 6-methylthioinosine. 6-Methylthioinosine inhibited cell division but apparently did not affect cell viability and the rate of cell proliferation rapidly returned to that of untreated control cultures after 6-methylthioinosine was removed.

MP-induced reductions in cellular purine ribonucleotide pools may be responsible for acute growth inhibitory effects observed in L5178Y cell cultures during exposure to MP.

Adenine or hypoxanthine did not reverse the

delayed cytotoxic reaction of prior exposure to MP. However, physiological purine derivatives present in culture media during drug exposure, did protect or partially protect cells against the delayed cytotoxic activity of MP and TG. These purine derivatives and their metabolites may protect cells by inhibiting thiopurine anabolism.

Intracellular pools of acid-soluble thiopurine derivatives readily declined after MP exposure was terminated.

IV. INCORPORATION OF 6-MERCAPTOPURINE INTO NUCLEIC ACIDS

A. Introduction

Inhibitory effects of MP anabolites at the level of purine nucleotide synthesis are apparently unrelated to the delayed cytotoxic activity of MP (see Chapter III). Similarities between the delayed cytotoxic reactions of MP and of agents that are known to affect DNA, and the very nature of the delayed cytotoxic reaction itself, suggested that effects at the macromolecular level were responsible for MP cytotoxicity (see Chapter II). In addition, the similarity between the delayed effect of MP and that of a 100-fold lower concentration of TG suggested that the cytotoxic activity of MP was mediated by TG anabolites, through their incorporation into DNA.

The experiments described below established that MP is incorporated as TG nucleosides into internucleotide linkage of DNA and RNA of cultured L5178Y cells. A correlation was observed between the extent of incorporation of MP into nucleic acids and the delayed cytotoxic reaction of MP.

B. Materials and Methods

1. Chemicals and Enzymes

MP-S^{35} (20 - 35 mCi/mmole) was purchased from Amersham/Searle Corp., Toronto, Ont. Solutions of MP-S^{35} (3 mM, 1.5 - 2.6 x 10^7 cpm/μmole) were prepared 6 - 8 h before use in sterile twice-concentrated Fischer's medium, pH 8.4 - 8.9 (without horse serum or antibiotic supplements) and were stored at 4° until required. Mycophenolic acid was a gift from T. J. Franklin, Imperial Chemical Industries, Ltd., Maccelesfield, Cheshire, England. Solutions of mycophenolic acid (6mM) were prepared in Fischer's medium by addition of one equivalent of sodium bicarbonate. 6-Thioinosine and 6-thioguanosine were supplied by Drug Research and Development, National Cancer Institute, Bethesda Md.; 6-thioinosinate and 6-thioxanthine were purchased from Sigma Chemical Co., St. Louis, Mo. Yeast RNA (Calbiochem, La Jolla, Calif.) and calf thymus DNA (Worthington Biochemical Corp., Freehold, N.J.) solutions were used as standards for determination of RNA and DNA by absorbancy measurements at 260 nm.

Deoxyribonuclease I (DNase I) from bovine pancreas, crude Crotalus adamanteus venom, 5'-nucleotidase and phosphodiesterase from Crotalus adamanteus venom, crystalline purine nucleoside phosphorylase from

calf spleen and 3'-nucleotidase from rye grass were purchased from Sigma Chemical Co. Micrococcal nuclease from Staphylococcus aureus and phosphodiesterase from bovine spleen were purchased from Worthington Biochemical Corp.

2. Mass Cultures of L5178Y Cells and their Exposure to 6-Mercaptopurine-S^{35}

Five - ten 250 ml cultures of L5178Y cells in stoppered, 700 ml serum bottles were incubated at 37.5° in a water bath placed in a laminar flow clean work station (Edgegard Hood, The Baker Company Inc., Biddeford, Maine) which produced a horizontal flow of sterile air over the work surface. When the cell density in these cultures was 200,000 cells/ml, the stoppers were removed to allow carbon dioxide to diffuse out of the culture medium. This procedure prevented the culture medium from becoming acidic. Cells proliferated exponentially at their usual rate up to densities of about 700,000 cells/ml. The culture procedure was capable of providing 1.75×10^9 exponentially proliferating cells or approximately 1.75 ml of packed cells.

Exposures (13 h) to 100 μM MP-S^{35} began at cell densities of $2 - 2.5 \times 10^5$ cells/ml. Acute inhibitory effects of MP resulted in small increases in cell doubling times during the MP exposure period and the ten 250 ml

cultures contained a total of approximately 10^9 cells at the end of the 13 h treatment interval. After 13 h exposure, cells from 10 ml culture samples were collected by centrifugation, washed and resuspended in 40 ml portions of drug-free medium for demonstration of the delayed cytotoxic reaction to MP. The remainder of the MP-treated cultures was dispensed into pre-cooled centrifuge bottles and chilled in ice. Cells were collected by centrifugation, pooled and washed three times with 100 ml 0.9% NaCl at 4^0. The final cell pellets were stored at -20^0 for subsequent isolation of nucleic acids.

3. Isolation of DNA and RNA

Total DNA and RNA were isolated from cultured L5178Y cells by a modification of the procedure of Caldwell and Henderson (32).

(a). RNA. After the second precipitation of RNA with cetyltrimethylammonium bromide (CTMA bromide), the CTMA-RNA precipitates were washed with ethanolic sodium acetate and the final pellets, which consisted of RNA free of soluble nucleotides, were dissolved in 1.0 ml of water.

(b). DNA. Phenol layers containing nucleoprotein were mixed with 1.0 ml of sodium citrate solution (0.2 M, in

5 M NaCl) and 4.0 ml of 0.7 M sodium dodecyl sulfate; the mixtures were shaken mechanically for 10 minutes and then centrifuged. Aqueous layers plus interphase layers were removed and mixed with 1.0 ml of sodium citrate solution, 4.0 ml of 8.7 mM sodium dodecyl sulfate, and 4.5 ml of fresh 90% phenol solution. The mixtures were shaken mechanically for 10 minutes and then centrifuged. The viscous aqueous layers were removed and lower layers were re-extracted with 1.0 ml of sodium citrate solution plus 4.0 ml of 8.7 mM sodium dodecyl sulfate. Aqueous layers were combined and extracted four times with ether. Ether was removed with a stream of air; the solutions were mixed with equal volumes of ethanol and centrifuged immediately to collect the fibrous DNA precipitates. Precipitates were washed three times with ethanolic sodium acetate solution and dissolved in 0.5 ml of water.

Samples of DNA and RNA solutions were diluted 300-fold for absorbancy measurements at 260 nm. Yields were calculated on the basis of absorbancy values for standard yeast RNA and calf thymus DNA solutions. Specific activities of S^{35}-labeled nucleic acids were measured by liquid scintillation counting. DNA solutions contained approximately 12 mg DNA/ml and RNA solutions contained approximately 10 mg RNA/ml. These solutions were stored at -20° for subsequent enzymatic degradation.

4. Enzymatic Digestion of DNA and RNA

(a). Digestion to Nucleosides. RNA was hydrolyzed to ribonucleosides and inorganic phosphate by digestion with crude rattlesnake venom, and DNA was hydrolyzed to deoxyribonucleosides and inorganic phosphate by digestion with pancreatic deoxyribonuclease (DNase I) plus crude rattlesnake venom (39).

(i). RNA. Snake venom solution (0.125 ml, 10 mg dried venom/ml) in 0.2 M borate buffer, pH 8.5, containing 0.02 M magnesium chloride and 0.02% v/v mercaptoethanol, was added to 0.125 ml portions of RNA solutions (approximately 10 mg RNA/ml). The mixtures were incubated at 37^{0} for 20 h to effect complete digestion of RNA.

(ii). DNA. DNase I solution (0.025 ml, 2,000 Kunitz units DNase I/ml) in 0.5 M Tris buffer, pH 7, was added to 0.1 ml portions of DNA solutions (approximately 12 mg DNA/ml) and the mixtures were incubated at 37^{0} for 1 h. Snake venom solution (0.125 ml, 4 mg dried venom/ml) containing 0.01% v/v mercaptoethanol was added and the mixtures were incubated at 37^{0} for a further 17 h.

(iii). Protection against oxidation of thiopurine-S^{35}. The concentrations of mercaptoethanol present in digestion mixtures were insufficient to prevent oxidation of S^{35}-labeled TG nucleosides released from

nucleic acids during the periods of incubation at 37°. (Doerr et al., (47), have discussed the oxidation of thiopurines in dilute solution). Oxidation occurred to the extent of 30 - 40% during digestion of DNA and to 50 - 60% during digestion of RNA. This difficulty could not be overcome by increasing the concentration of mercaptoethanol since higher concentrations were inhibitory to the phosphodiesterase present in snake venom. However, S^{35}-labeled TG nucleosides released during enzyme degradation of DNA and RNA were largely protected against oxidation when digestion mixtures contained 1.5 mM β-2'-deoxythioguanosine and 6-thioguanosine, respectively.

(b). Selective Acid Hydrolysis of Purine Deoxyribonucleosides. Purine deoxyribonucleosides are considerably more sensitive to acid hydrolytic cleavage of the glycosidic bond than are the corresponding ribonucleosides. This property was used as a criterion for characterization of S^{35}-labeled TG nucleosides in hydrolyzates of DNA and RNA.

Venom digestions of RNA and DNA were conducted in the presence of 1.5 mM 6-thioguanosine and 1.5 mM β-2'-deoxythioguanosine, respectively; when complete, 0.04 ml of 6 N hydrochloric acid was added to 0.1 ml samples of the hydrolyzates and the acidified mixtures were incubated at 25° for 40 min and then neutralized. This

treatment resulted in the quantitative hydrolysis of purine deoxyribonucleosides in DNA digests to the respective bases, but had no effect on purine ribonucleosides in RNA digests.

(c). Cleavage with Purine Nucleoside Phosphorylase. Guanosine, 6-thioguanosine, deoxyguanosine and β-2'-deoxythioguanosine present in enzyme hydrolyzates of RNA and DNA were converted to their respective bases with crystalline purine nucleoside phosphorylase by a modification of the procedure of LePage (107); 0.025 ml of 0.5 M sodium arsenate-1.0 M sodium acetate buffer solution, pH 6.5, and 1.4 units of purine nucleoside phosphorylase in 3.2 M ammonium sulfate (2 mg protein/ml, 28 units/mg protein) were added to 0.05 ml samples of venom digests of RNA and DNA and the mixtures were incubated at 37^{0} for 60 min.

(d). Deamination by Nitrous Acid. Treatment with nitrous acid at 25^{0} results in the replacement of some primary aromatic amino groups by hydroxyl groups. This process was used as a diagnostic procedure for identification of primary amino substituents. Nucleic acids were degraded with venom and purine nucleoside phosphorylase and 0.05 ml samples of the digests were treated with 0.01 ml of 12 N hydrochloric acid. The protein precipitates were removed by centrifugation and 0.01 ml of 0.2 M

sodium nitrite was added to the supernatant solutions. The mixtures were incubated at 25° for 5 min and then neutralized. This procedure converted authentic TG to 6-thioxanthine.

(e). Digestion of RNA and DNA to 3'-Nucleotides. RNA and DNA were hydrolyzed to 3'-nucleotides by digestion with micrococcal nuclease plus spleen phosphodiesterase (95).

(i). RNA and DNA. Micrococcal nuclease solution (0.025 ml, 2,000 units (Worthington Biochemical Corp.) /ml) in 20 mM Tris buffer, pH 8.6, containing 10 mM calcium chloride was added to 0.1 ml samples of RNA or DNA solutions (approximately 10 mg RNA/ml or 12 mg DNA/ml). The mixtures were incubated at 37° for 2 h and 0.02 ml of 0.5 M sodium succinate-hydrochloric acid buffer, pH 6.5, and 0.025 ml of bovine spleen phosphodiesterase solution (29 units/ml) in water were added. The mixtures were incubated at 37° for 1 h and then a further 0.025 ml of the phosphodiesterase solution was added. Incubation at 37° was continued for a further 1 h and a final 0.025 ml of the phosphodiesterase solution was added. The digestion mixtures were incubated for a final 1 h at 37° to give a total phosphodiesterase incubation time of 3 h. This procedure resulted in complete digestion of DNA or RNA to 3'-nucleotides.

(ii). Oxidation of thiopurine-S^{35}. TG-S^{35} nucleotides released from nucleic acids by digestion with spleen phosphodiesterase were apparently much less sensitive to oxidation during incubation at 37° without carrier, than were the corresponding nucleosides in crude venom digestion mixtures. Nucleic acid hydrolyzates were stored at -20° for subsequent characterization of TG nucleotides by their susceptibility to hydrolysis catalyzed by 3'- or 5'-nucleotidase.

(f). Digestion of RNA and DNA to 5'-Nucleotides. RNA was hydrolyzed to 5'-ribonucleotides by incubation with phosphodiesterase from Crotalus adamanteus venom and DNA was hydrolyzed to 5'-deoxyribonucleotides by incubation with DNase I plus venom phosphodiesterase.

(i). RNA. Venom phosphodiesterase solution (0.125 ml, 1.4 units/ml) in 0.2 M borate buffer, pH 8.5, containing 0.02 M magnesium chloride, was added to 0.125 ml of RNA solutions (approximately 10 mg RNA/ml). The mixtures were incubated at 37° for 20 h to effect complete digestion of RNA.

(ii). DNA. DNase I solution (0.025 ml, 2,000 Kunitz units DNase I/ml) in 0.5 M Tris buffer, pH 7, was added to 0.1 ml of DNA solutions (approximately 12 mg DNA/ml) and the mixtures were incubated at 37° for 1 h. Venom phosphodiesterase solution (0.125 ml, 0.54 units/ml) was added and the mixtures were incubated at 37° for

a further 5 h.

(iii). Oxidation of thiopurine-S^{35}. TG-S^{35} nucleotides released from nucleic acids by digestion with venom phosphodiesterase were apparently much less sensitive to oxidation during incubation at 37^{0} without carrier, than were the corresponding nucleosides in crude venom digestion mixtures. Nucleic acid hydrolyzates were stored at -20^{0} for subsequent characterization of TG nucleotides by their susceptibility to hydrolysis catalyzed by 3'- or 5'-nucleotidase.

(g). Selective Hydrolysis of 3'-Nucleotides with 3'-Nucleotidase. Phosphodiesterase hydrolyzates of RNA were treated with 3'-nucleotidase from rye grass in order to characterize the S^{35}-labeled TG nucleotides. Rye grass 3'-nucleotidase solution (0.1 ml, 2 units/ml) in 0.3 M Tris buffer, pH 7.5, containing 0.1% v/v mercaptoethanol, was added to 0.1 ml samples of phosphodiesterase hydrolyzates of RNA. Mixtures were incubated at 37^{0} for a maximum period of 1 h. Samples were transferred to ice-cooled tubes at various intervals during the incubations and 40 µl portions of these were immediately applied to paper for chromatography, being placed directly upon 6-thioguanosine carrier spots. The kinetics of hydrolysis of TG ribonucleotides were determined from S^{35} radioactivity which co-chromatographed with the 6-thioguanosine

spots. Nucleotides in spleen phosphodiesterase hydrolyzates of RNA were readily hydrolyzed in the presence of 3'-nucleotidase and the reaction was essentially complete within 1 h. Nucleotides in venom phosphodiesterase digests of RNA were unaffected during this interval. 3'-Nucleotidase from rye grass specifically catalyzes hydrolysis of 3'-ribonucleotides, and 3'-deoxyribonucleotides are not substrates for this enzyme (41).

(h). Selective Hydrolysis of 5'-Nucleotides with 5'-Nucleotidase. Phosphodiesterase hydrolyzates of DNA and RNA were treated with 5'-nucleotidase from venom in order to characterize the S^{35}-labeled TG nucleotides. Venom 5'-nucleotidase solution (0.1 ml, 5 units/ml) in 0.2 M borate buffer, pH 8.5, containing 0.02 M magnesium chloride and 0.1% v/v mercaptoethanol, was added to 0.1 ml samples of phosphodiesterase digests of RNA and DNA. Digests were incubated with the enzyme at 37° for a maximum period of 1 h. Samples were transferred to ice-cooled tubes at various intervals during the incubations and 40 µl portions of these were immediately applied to paper for chromatography, being placed upon 6-thioguanosine and β-2'-deoxythioguanosine carrier spots, as appropriate. The kinetics of hydrolysis of TG nucleotides were determined from S^{35} radioactivity which co-chromatographed with the TG nucleoside spots.

Nucleotides in venom phosphodiesterase digests of DNA and RNA were readily hydrolyzed in the presence of 5'-nucleotidase. Nucleotides in spleen phosphodiesterase hydrolyzates of DNA and RNA were unaffected by the 1 h incubation period with 5'-nucleotidase.

5. Paper Chromatographic Systems

Degradation products of nucleic acids were separated by paper chromatography. The presence of venom in hydrolyzates distorted nucleoside spots in some chromatographic systems and, therefore, digest samples were deproteinated by rapid acidification and reneutralization at 4° before chromatography in these systems. Seven lanes of approximately 2.5 x 42.3 cm were cut in sheets (21.5 x 55.4 cm) of Whatman No. 1 paper and each sample (20 - 40 µl) was applied to the paper in a separate lane. Separated components were visualized under ultraviolet light; thiopurine carrier spots were recognized by fluorescence. For assay of radioactivity, the lanes were then cut into 2 cm segments and each was immersed in 18 ml of a toluene-based scintillation fluid and counted with a liquid scintillation counter.

Chromatographic solvents were supplemented with 0.1% v/v mercaptoethanol; the following solvents were used:

Solvent No. 1: 5% w/v disodium hydrogen phosphate and isoamyl alcohol, mutually saturated.(33)

Solvent No. 2: 1 M ammonium acetate, pH 9.0, containing 0.01 M versene and saturated with sodium tetraborate/90% ethanol, 60/140 v/v.

Solvent No. 3: 5% w/v sodium dihydrogen phosphate and isoamyl alcohol, mutually saturated. (40)

Solvent No. 4: 5% v/v isopropanol, 5% w/v ammonium sulfate.

Solvent No. 5: 5% w/v disodium hydrogen phosphate.

R_f values of purine bases, nucleosides and nucleotides in these solvents are recorded in Table 1. All R_f values in Table 1 are those of components and carriers present in samples of enzyme digests of DNA and RNA. Chemical species were identified by comparison with R_f values of authentic compounds or by further enzyme digestion to products of known R_f values.

C. Results

1. Identification of 6-Thioguanine in DNA and RNA Isolated from Cultured L5178Y Cells Exposed to 6-Mercaptopurine.

(a). Mycophenolic Acid Protection. The similarity between the delayed cytotoxic reaction of MP and that of a 100-fold lower concentration of TG suggested that cytotoxic effects of MP might be mediated by TG anabolites,

Table 1. Paper chromatography of enzyme digests of DNA and RNA isolated from L5178Y cell cultures; average R_f values

Compound	R_f values				
	Solvent 1	Solvent 2	Solvent 3	Solvent 4	Solvent 5
MP	0.36			0.32	
TG	0.27			0.22	0.24
TX	0.36				
MPR	0.62				
TGR	0.52	0.20	0.50	0.44	0.45
TGdR	0.54	0.64	0.51	0.46	0.49
5'-MPRP	0.81	0.03	0.72		
A	0.36				0.33
G	0.40				0.37
AR	0.48		0.56	0.46	0.40
GR	0.59	0.31	0.62	0.56	0.52
AdR	0.50		0.58	0.50	0.45
GdR	0.62	0.73	0.64	0.58	0.58
5'-ARP	0.69	0.06			
5'-GRP	0.76	0.04			
5'-AdRP	0.69	0.30			
5'-GdRP	0.78	0.20			
3'-ARP	0.63	0.29			
3'-GRP	0.76	0.20			
3'-AdRP	0.63	0.31			
3'-GdRP	0.75	0.23			

R_f values are those of components and carriers present in samples of enzyme digests of DNA and RNA. Chemical species were identified by comparison with R_f values of authentic compounds or by further enzyme digestion to products of known R_f values.

through incorporation into DNA. It is likely that the enzymes responsible for conversion of inosinate to guanylate are also responsible for the conversion of 6-thioinosinate to 6-thioguanylate (see Chapter I). The suggestion[3] was made that if conversion of 6-thioinosinate to 6-thioguanylate is essential to the cytotoxic activity of MP, then mycophenolic acid should protect cells against cytotoxic effects of MP exposure. Mycophenolic acid is an antibiotic which inhibits both enzymes involved in conversion of inosinate to guanylate (34,61,172). Figure 27 illustrates the effects on L5178Y cell proliferation of simultaneous exposure (13 h) to 0.5 μM mycophenolic acid and 100 μM MP. The concentration of mycophenolic acid was chosen on the basis of dose,response data; these demonstrated that inhibition of cell proliferation was reversed after 13 h exposure by replacing culture fluids with drug-free media (for example, see Figure 33, mycophenolic acid treatment alone). Mycophenolic acid protected cells against the delayed cytotoxic reaction of MP (Figure 27). This result is compatible with the hypothesis that conversion of 6-thioinosinate to 6-thioguanylate is essential to the delayed cytotoxic activity of MP. In addition, this experiment supports the earlier conclusion that inhibitions of purine nucleotide synthesis are not centrally involved in the lethal effects of MP. Simultaneous exposure (17 h) to 0.3 μM mycophenolic acid and

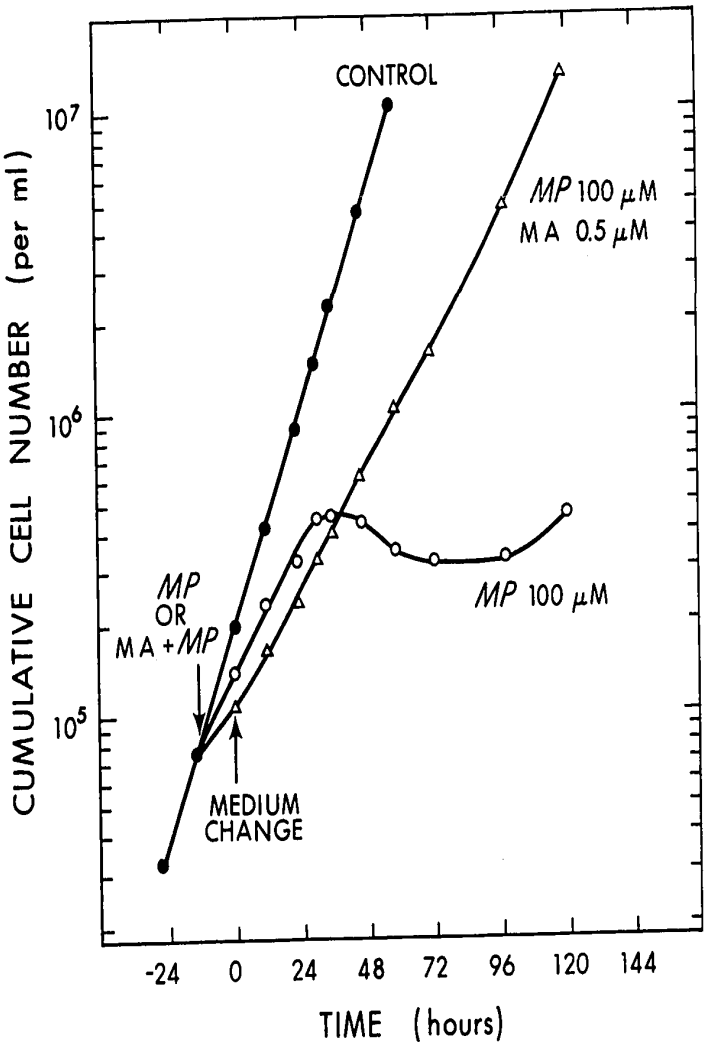

Figure 27. The effect of 13 h exposure to MP and MP plus mycophenolic acid (MA) on the proliferation of cultured L5178Y cells. Culture fluids were replaced with drug-free media at 0 h. Concentrations of agents during exposure are indicated in the figure.

100 μM MP also protected cultured L5178Y cells against delayed cytotoxic effects (Figure 28), however, under the same conditions 0.3 μM mycophenolic acid did not protect against the delayed cytotoxic reaction of 0.5 μM β-2'-deoxythioguanosine (Figure 29). This result would be expected if mycophenolic acid protected cells against MP by inhibiting conversion of 6-thioinosinate to 6-thioguanylate since anabolism of β-2'-deoxythioguanosine would not involve the enzymatic steps known to be affected by mycophenolic acid. Over the 17 h drug exposure period, cell proliferation was inhibited in these cultures; inhibitions were: 11% for MP alone; 2% for β-2'-deoxythioguanosine alone; 32% for mycophenolic acid alone; 43% for MP plus mycophenolic acid; 34% for β-2'-deoxythioguanosine plus mycophenolic acid.

(b). Treatment with MP-S^{35}. On the basis of the above results, incorporation of MP into nucleic acids was investigated in exponentially proliferating cultures of L5178Y cells. DNA and RNA were isolated from mass cultures at the end of 13 h exposure to 100 μM MP-S^{35}. At this time, cells in 10 ml culture samples were washed and resuspended in drug-free media to ascertain effects of the MP treatment on cell proliferation. These cells continued to divide for approximately 24 h after MP was removed and the delayed cytotoxic reaction to MP exposure was observed after

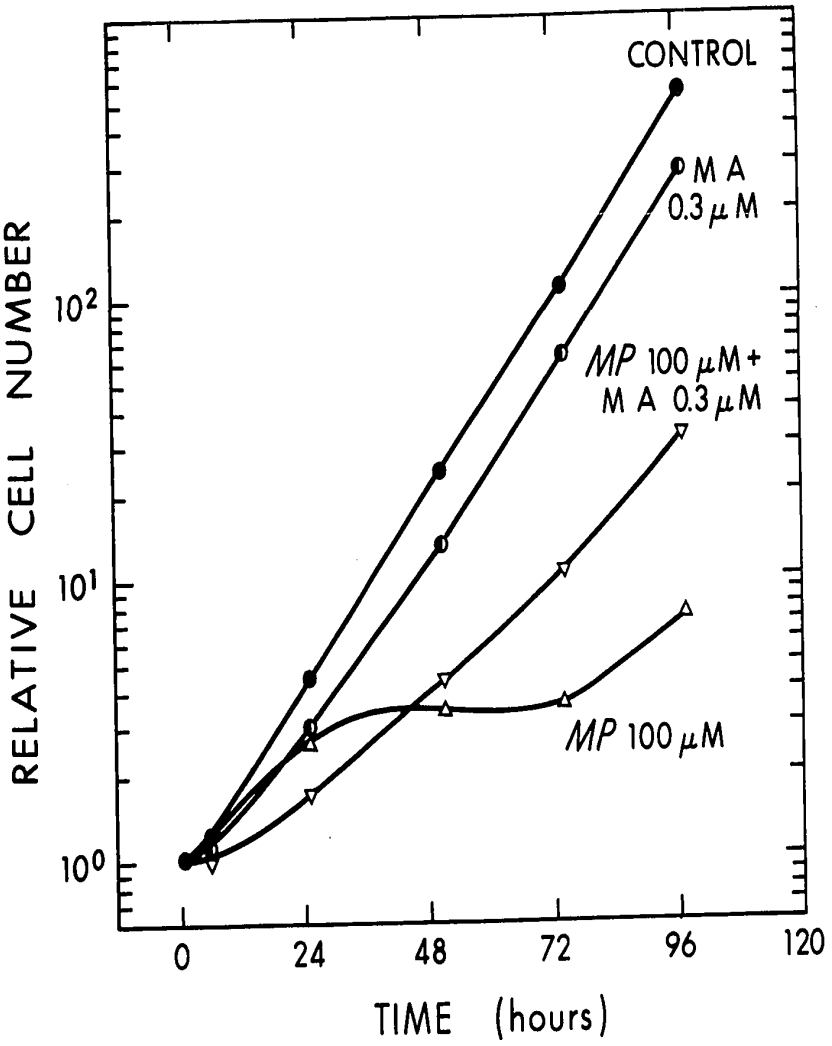

Figure 28. Cell proliferation in L5178Y cultures after 17 h exposure to MP, mycophenolic acid (MA), and MP plus mycophenolic acid. Culture fluids were replaced with drug-free media containing 10 μM guanosine at 0 h and cell numbers are relative to those at 0.75 h. Concentrations of drugs during exposure are indicated in the figure. Guanosine was not present in culture media during the drug exposure period. The data of this figure and Figure 29 were part of the same experiment; all cultures were prepared from the same parent culture 7 h before drug treatments were initiated.

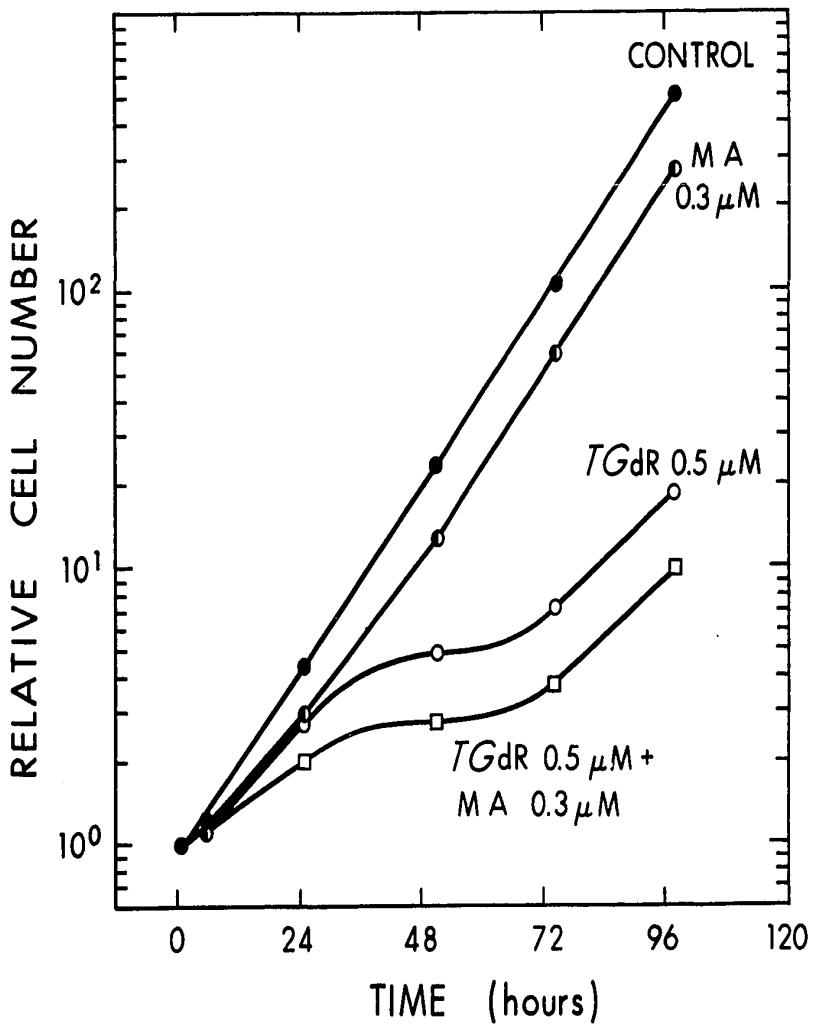

Figure 29. Cell proliferation in L5178Y cultures after 17 h exposure to β-2'-deoxythioguanosine (TGdR), mycophenolic acid (MA), and β-2'-deoxythioguanosine plus mycophenolic acid. Culture fluids were replaced with drug-free media containing 10 μM guanosine at 0 h and cell numbers are relative to those at 0.75 h. Concentrations of drugs during exposure are indicated in the figure. Guanosine was not present in culture media during the drug exposure period. The data of this figure and Figure 28 were part of the same experiment; all cultures were prepared from the same parent culture 7 h before drug treatments were initiated.

1.25 - 1.50 doublings (for example, see Figure 33, MP-S^{35} treatment alone). It was concluded that L5178Y cells treated with MP under mass culture conditions behaved similarly to conventionally cultured cells.

(c). Degradation of DNA with DNase I plus Venom. The isolated DNA (total yield 5.1 mg DNA, specific activity 80,500 cpm/mg DNA) was hydrolyzed with DNase I plus rattlesnake venom in the absence of thiopurine carrier. When digestion was complete, β-2'-deoxythioguanosine was added to samples of the digest, and components of these mixtures were separated by paper chromatography with solvents 1, 4 and 5. Results of paper chromatography are summarized in Table 2. Samples of the DNA digest were acidified and rapidly reneutralized at 4° to remove protein before chromatography. Two major peaks of radioactivity were observed in each of the developed chromatograms. One peak, accounting for 40 - 50% of the total activity was associated with the β-2'-deoxythioguanosine spot in each solvent (Table 2). A lesser peak, accounting for 30 - 40% of the total activity, had high R_f values in these solvent systems and presumably represented a charged S^{35}-derivative. The activity associated with TG reflects the sensitivity of β-2'-deoxythioguanosine

Table 2. Paper chromatography of venom digests of DNA and RNA isolated from MP-S^{35}-treated L5178Y cells.

Nucleic acid	Treatment	Chromatographic solvent	Identity of activity peaks	R_f of activity peaks	CPM in activity peaks	% of total activity on chromatogram	
DNA	DNase I + venom, acidified-reneutralized	1	TG	0.26	267	5)	90
			TGdR	0.52	2,410	50)	
			"sulfite"	0.93	1,704	35)	
	DNase I + venom, stored -20° for 24 h	1	TGdR	0.54	3,189	37)	90
			"TGsulfinatedR"	0.74	4,483	53)	
	DNase I + venom + 0.22 mM TGdR, acidified-reneutralized	1	TG	0.27	364	8)	93
			TGdR	0.52	3,383	73)	
			"sulfite"	0.94	535	12)	
	DNase I + venom + 0.22 mM TGdR, stored at -20° for 24 h	1	TGdR	0.54	6,500	83)	95
			"TGsulfinatedR"	0.74	909	12)	
	DNase I + venom + 1.5 mM TGdR	1	TGdR	0.55	8,078	90)	97
			"TGsulfinatedR"	0.79	656	7)	
RNA	venom, acidified-reneutralized	1	TGR	0.52	583	37)	91
			"sulfite"	0.93	857	54)	
	venom, stored -20° for 120 h	1	TGR	0.52	587	25)	92
			"TGsulfinateR"	0.75	1,570	67)	
	venom + 0.22 mM TGR, acidified-reneutralized	1	TGR	0.51	921	66)	93
			"sulfite"	0.93	373	27)	

Table 2 (continued):

RNA	venom + 0.22 mM TGR, stored -20° for 120 h	1	TGR	0.54	1,311	61)	87
			"TGsulfinateR"	0.74	567	26)	
	venom + 1.5 mM TGR	1	TGR	0.50	2,531	87)	94
			"TGsulfinateR"	0.76	202	7)	
DNA	DNase I + venom, acidified-reneutralized	4	TG	0.22	161	4)	88
			TGdR	0.46	1,624	42)	
			"sulfite"	0.84	1,596	42)	
	DNase I + venom + 0.22 mM TGdR, acidified-reneutralized	4	TG	0.22	211	6)	87
			TGdR	0.46	2,206	65)	
			"sulfite"	0.86	556	16)	
RNA	venom, acidified-reneutralized	4	TGR	0.44	424	29)	86
			"sulfite"	0.82	830	57)	
	venom + 0.22 mM TGR, acidified-reneutralized	4	TGR	0.45	661	59)	90
			"sulfite"	0.82	350	31)	
DNA	DNase I + venom, acidified-reneutralized	5	TG	0.24	239	5)	89
			TGdR	0.48	2,350	51)	
			"sulfite"	0.88	1,529	33)	
	DNase I + venom + 0.22 mM TGdR, acidified-reneutralized	5	TG	0.25	336	7)	89
			TGdR	0.50	3,221	71)	
			"sulfite"	0.93	518	11)	
RNA	venom, acidified-reneutralized	5	TGR	0.45	559	37)	89
			"sulfite"	0.82	787	52)	
	venom + 0.22 mM TGR, acidified-reneutralized	5	TGR	0.45	899	68)	92
			"sulfite"	0.86	319	24)	

to acid hydrolysis.

(d). Oxidation of β-2'-Deoxythioguanosine-S^{35} Released from DNA. Chromatography with solvent 1 suggested that the lesser peak was an oxidation product of β-2'-deoxythioguanosine-S^{35}. This unidentified S^{35}-labeled derivative was probably inorganic sulfite. When samples were chromatographed with solvent 1 without prior acidification and reneutralization the unidentified component had lower R_f value (Table 2) and was located in the purine nucleoside monophosphate region of the chromatogram (see Table 1). This derivative was probably the deoxyribonucleoside of 2-aminopurine-6-sulfinate, an oxidation product of TG (47). Purine-6-sulfinates are very sensitive to acid catalyzed hydrolysis of the carbon-sulfur bond and inorganic sulfite is a product of acid treatment (47). The "sulfinate" derivative was apparently an artifact derived by oxidation of β-2'-deoxythioguanosine-S^{35} released from DNA during digestion at 37°. When carrier β-2'-deoxythioguanosine was present throughout the period of digestion of DNA, there was a decrease in the proportion of the total radioactivity associated with the "sulfinate" derivative and a corresponding increase in the proportion associated with β-2'-deoxythioguanosine (Table 2). The "sulfinate" derivative accounted for only 7% of the total activity

when digestion was conducted in the presence of 1.5 mM β-2'-deoxythioguanosine. In subsequent experiments, digestion of DNA with DNase I plus venom was conducted in the presence of 1.5 mM β-2'-deoxythioguanosine; 90 - 95% of the total activity in hydrolyzates of S^{35}-labeled DNA co-chromatographed with β-2'-deoxythioguanosine in both solvents 1 and 3.

(e). Degradation of RNA with Venom. RNA (total yield 12 mg RNA, specific activity 19,800 cpm/mg RNA) was degraded with rattlesnake venom in the absence of carrier thiopurine. When digestion was complete, 6-thioguanosine was added to samples of the hydrolyzate and components of these mixtures were separated by paper chromatography in solvents 1, 4 and 5. Results of paper chromatography are summarized in Table 2. Samples were acidified and re-neutralized to remove protein before chromatography and the result was entirely analogous to that described above for DNA. Two peaks of radioactivity were observed in each of the developed chromatograms; one peak of activity was associated with 6-thioguanosine in each solvent, whilst the other peak was found at high R_f values in these solvent systems and probably represented sulfite-S^{35}.

(f). Oxidation of 6-Thioguanosine-S^{35} Released from RNA. When RNA hydrolyzates were chromatographed in solvent 1 without prior acidification and reneutralization, the unidentified peak of radioactivity was found at lower R_f values (Table 2). This component was probably the ribonucleoside of 2-aminopurine-6-sulfinate, formed by oxidation of 6-thioguanine-S^{35} during the period of incubation at 37°. When 6-thioguanosine was present in digestion mixtures throughout the period of digestion of RNA, there was a decrease in the proportion of the total radioactivity associated with the "sulfinate" derivative and a corresponding increase in the proportion associated with carrier 6-thioguanosine (Table 2). The presumed sulfinate derivative accounted for only 7% of the total activity when digestion was conducted in the presence of 1.5 mM 6-thioguanosine. In subsequent experiments digestion of RNA with venom was conducted in the presence of 1.5 mM 6-thioguanosine; 80 - 90% of the total activity in hydrolyzates of S^{35}-labeled RNA co-chromatographed with 6-thioguanosine. No detectable activity accompanied 6-thioinosine in solvent 1 (see Table 1 for R_f values). The radioactivity released by venom also co-chromatographed with 6-thioguanosine in solvent 3.

(g). Chromatography of Nucleic Acid Hydrolyzates in Saturated Borate Solution. The chromatographic systems described above did not separate 6-thioguanosine and β-2'-deoxythioguanosine. In a separate experiment, DNA and RNA were isolated from L5178Y cells at the end of 13 h exposure to 100 μM MP-S^{35} and the nucleic acids were degraded to nucleosides in the presence of 1.5 mM concentrations of β-2'-deoxythioguanosine and 6-thioguanosine, respectively. Samples of these hydrolyzates were subjected to paper chromatography with solvent 2, which contains a saturating concentration of sodium borate. Ribonucleosides are separated from corresponding deoxyribonucleosides by chromatography in this solvent; radioactivity released by digestion of RNA co-chromatographed with 6-thioguanosine and was separated from radioactivity released by digestion of DNA which co-chromatographed with β-2'-deoxythioguanosine (see Table 1 for R_f values). When a sample of the RNA digest containing both 6-thioguanosine and β-2'-deoxythioguanosine was chromatographed in solvent 2, the activity was associated with the 6-thioguanosine spot and no detectable activity was associated with the separated β-2'-deoxythioguanosine spot.

(h). Selective Acid Hydrolysis of Purine Deoxyribonucleosides. Purine deoxyribonucleosides are considerably more sensitive to acid-catalyzed hydrolysis of the glycosidic bond than are the corresponding ribonucleosides. This property was used as a further criterion for characterization of the S^{35}-labeled TG nucleosides present in hydrolyzates of DNA and RNA. DNA and RNA were degraded to nucleosides in the presence of 1.5 mM concentrations of β-2'-deoxythioguanosine and 6-thioguanosine, respectively. Digests were incubated with 1.7 N hydrochloric acid at 25^{0} for 40 min and then neutralized. Samples of the acid-treated digests were chromatographed on paper with solvent 1. Effects of this treatment are summarized in Table 3. The procedure resulted in quantitative conversion of deoxyadenosine to adenine, deoxyguanosine to guanine and quantitative conversion of β-2'-deoxythioguanosine to TG. Ninety five percent (2710 cpm) of the total activity in samples of the acid-treated DNA hydrolyzate was associated with TG. Acid treatment had no apparent effect on adenosine, guanosine or 6-thioguanosine and 85% (760 cpm) of the total activity in samples of the acid-treated RNA hydrolyzate co-chromatographed with 6-thioguanosine. Venom digests of RNA were treated with acid in the presence of both 6-thioguanosine and β-2'-deoxythioguanosine. Acid treatment resulted in conversion of

Table 3. Characterization of the S^{35}-labeled derivatives in venom hydrolyzates of DNA and RNA isolated from MP-S^{35}-treated L5178Y cells.

Nucleic acid hydrolyzate	Treatment	Identity of activity peaks	R_f of activity peaks, solvent 1	CPM in activity peaks	% of total activity on chromatogram
DNA	None	TGdR	0.54	5,130	95
	1.7 N Hydrochloric acid, 25°, 40 min	TG	0.27	2,710	95
	None	TGdR	0.55	8,100	90
	Purine nucleoside phosphorylase, 37°, 1 h	TG	0.26	3,600	81
		TGdR	0.50	340	8
	Purine nucleoside phosphorylase, 37°, 1 h; nitrous acid, 25°, 5 min	TX	0.36	1,600	71
		"sulfite"	0.90	410	19
RNA	None	TGR	0.51	1,570	86
	1.7 N Hydrochloric acid, 25°, 40 min	TGR	0.51	760	85
	None	TGR	0.50	2,500	87

Table 3. (Continued):

RNA	Purine nucleoside phosphorylase, 37°, 1 h	TG	0.26	970	69
		TGR	0.50	150	10
	Purine nucleoside phosphorylase, 37°, 1 h; nitrous acid, 25°, 5 min	TX	0.36	350	50
		"sulfite"	0.90	160	23

β-2'-deoxythioguanosine to TG, whilst 6-thioguanosine was unaffected. Radioactivity in the acid-treated digests was associated with 6-thioguanosine and no detectable activity was associated with the separated TG spot. These results established that the S^{35}-labeled TG nucleoside in RNA digests was a ribonucleoside, whilst the S^{35}-labeled TG nucleoside in DNA digests was a deoxyribonucleoside.

Venom digests of S^{35}-labeled DNA and RNA were treated further, first with purine nucleoside phosphorylase and then with nitrous acid in order to confirm that the radioactive thiopurine base associated with the nucleic acids was TG.

(i). Cleavage with Purine Nucleoside Phosphorylase. DNA and RNA were digested in the presence of 1.5 mM concentrations of β-2'-deoxythioguanosine and 6-thioguanosine, respectively. Ninety percent (8100 cpm) of the total activity in samples of the DNA hydrolyzate co-chromatographed with β-2'-deoxythioguanosine in solvent 1. Eighty-seven percent (2500 cpm) of the total activity in samples of the RNA hydrolyzate co-chromatographed with 6-thioguanosine in solvent 1. The DNA and RNA digests were incubated at 37^{0} for 1 h with purine nucleoside phosphorylase in the presence of arsenate as ribosyl and deoxyribosyl group acceptor. Components of these mixtures were separated by paper chromatography with solvent 1.

Effects of incubations with phosphorylase are summarized in Table 3. Treatment with phosphorylase converted β-2'-deoxythioguanosine and 6-thioguanosine to TG. Eighty-one percent (3600 cpm) of the total activity in samples of the DNA digest and 69% (970 cpm) of the total activity in samples of the RNA digest co-chromatographed with TG. Small amounts of unreacted carrier and S^{35}-labeled TG nucleosides remained in digestion mixtures. These may have been present as disulfides during phosphorylase digestion and as such would probably be resistant to phosphorolytic cleavage of the glycosidic bond. Upon chromatography disulfides would be reduced by mercaptoethanol present in the chromatographic solvent. Unreacted β-2'-deoxythioguanosine accounted for 8% (340 cpm) of the total activity in samples of the phosphorylase-treated DNA hydrolyzate; unreacted 6-thioguanosine accounted for 10% (150 cpm) of the total activity in samples of the phosphorylase-treated RNA hydrolyzate. Similar proportions of unreacted TG nucleoside were found after only 30 min phosphorylase digestion. No detectable activity accompanied MP in chromatograms of the phosphorylase-treated digests of both DNA and RNA.

Susceptibility to phosphorylase-catalyzed cleavage of the glycosidic bond demonstrated that the S^{35}-labeled TG nucleosides, released by digestion of RNA and DNA, had the β-configuration about the anomeric carbon

of the sugar moiety (107).

(j). Deamination by Nitrous Acid. The action of nitrous acid on phosphorylase-treated hydrolyzates of DNA and RNA provided further confirmation that the S^{35}-labeled thiopurine was TG. The effects of nitrous acid are summarized in Table 3. Treatment with nitrous acid resulted in the deamination of TG to 6-thioxanthine in these digests. Seventy-one percent (1600 cpm) of the total activity in samples of the nitrous acid-treated DNA digest and 50% (350 cpm) of the total activity in samples of the nitrous acid-treated RNA digest co-chromatographed with 6-thioxanthine in solvent 1. Nitrous acid is an oxidizing agent and a certain amount of oxidation of S^{35}-labeled TG occurred during nitrous acid treatment. A minor peak of activity (R_f 0.90) was observed in chromatograms of nitrous acid-treated DNA and RNA digests (solvent 1). Oxidation of the thiopurines is discussed above; the minor peak of activity was probably inorganic sulfite-S^{35}. "Sulfite" accounted for 19% (410 cpm) of the total activity in samples of the nitrous acid-treated DNA digest and 23% (160 cpm) of the total activity in samples of the nitrous acid-treated RNA digest.

2. Incorporation of 6-Mercaptopurine into Internucleotide Linkage of DNA and RNA as 6-Thioguanine

The experiments described above demonstrated that TG was associated with DNA and RNA in MP-treated L5178Y cell cultures. These results implied that MP is incorporated as TG nucleosides into 3',5'-phosphodiester linkages of RNA and DNA in cultured L5178Y cells. However, it was conceivable that TG nucleotides (which would be 5'-phosphoryl derivatives) might be "bound" to polynucleotides; hence the need to demonstrate that 3',5'-phosphates of 6-thioguanosine and β-2'-deoxythioguanosine are present in RNA and DNA. In order to establish the nature of the association between TG nucleosides and the nucleic acids, DNA and RNA were hydrolyzed to 3'- and 5'-monophosphates with purified phosphodiesterases. Phosphodiesterase hydrolyzates were then subjected to further digestion with 3'- and 5'-nucleotidases to determine the position of the phosphate substituent in the TG nucleotides.

(a). Degradation of Nucleic Acids with Phosphodiesterases. DNA and RNA were both degraded to 3'-monophosphates with micrococcal nuclease and spleen phosphodiesterase; RNA was hydrolyzed to 5'-monophosphates with venom phosphodiesterase and DNA was hydrolyzed to 5'-monophosphates with DNase I plus venom phosphodiesterase. Phosphodiesterase degradations and chromatography of

hydrolyzates were conducted in the absence of carrier TG nucleotides. Nucleotides of TG appeared to be considerably more resistant to oxidation in these digestion mixtures than did the corresponding nucleosides in crude venom hydrolyzates. Samples of the phosphodiesterase hydrolyzates were chromatographed on paper with solvent 1. Results of paper chromatography are summarized in Table 4. In solvent 1, a single peak (R_f 0.73 - 0.74) of radioactivity was observed in the nucleotide region of the chromatograms for DNA- and RNA-derived 5'-monophosphates and for RNA-derived 3'-monophosphates (Table 4).

Two peaks of activity were observed in chromatograms of DNA-derived 3'-monophosphates (see Table 4). The major peak of activity was found in the nucleotide region of the chromatogram (R_f 0.75). The minor component (R_f 0.53) was β-2'-deoxythioguanosine-S^{35}, apparently derived from the major component because, when degradation of DNA to 3'-monophosphates was complete, further incubation at 37^0 led to a progressive increase in the proportion of β-2'-deoxythioguanosine-S^{35} in the hydrolyzate.

Samples of phosphodiesterase digests were also chromatographed with solvent 2 (see Table 4). This solvent contains a saturating concentration of sodium borate. Deoxyribosyl 3'-monophosphates, deoxyribosyl 5'-monophosphates and ribosyl 3'-monophosphates behave similarly, but are

Table 4. Paper chromatography of phosphodiesterase digests of DNA and RNA isolated from MP-S^{35}-treated L5178Y cells

Nucleic acid	Digestion enzymes	Identity of activity peaks	CPM in activity peaks (solvent I)	% of total activity on chromatogram (solvent I)	R_f of activity peaks in solvent I	R_f of activity peaks in solvent 2
DNA	DNase I + venom phosphodiesterase	5'-TGdRP	2150	93	0.74	0.18
	Micrococcal nuclease + spleen phosphodiesterase	3'-TGdRP	2320	90	0.75	0.18
		TGdR	230	9	0.53	0.65
RNA	Venom phosphodiesterase	5'-TGRP	450	76	0.74	0.05
	Micrococcal nuclease + spleen phosphodiesterase	3'-TGRP	420	84	0.73	0.18

separated in this solvent from the corresponding ribosyl 5'-monophosphates (see Table 1). Borate complexes with ribose derivatives when the cis-glycol grouping of the sugar is unsubstituted. Two peaks of activity were observed in chromatograms (solvent 2) of spleen phosphodiesterase hydrolyzates of DNA. The major peak (R_f 0.18) was located in the deoxyribonucleotide region of the chromatogram. The origin of the minor component, β-2'-deoxythioguanosine-S^{35} (R_f 0.65), is discussed above. Venom phosphodiesterase hydrolyzates of DNA and RNA and spleen phosphodiesterase hydrolyzates of RNA gave single peaks of radioactivity in solvent 2. The peak of activity was located in the deoxyribonucleotide region (R_f 0.18) in chromatograms of DNA-derived 5'-monophosphates. The same R_f value was observed for the peak of activity in chromatograms of RNA-derived 3'-monophosphates. This, together with the preceeding results, demonstrated that the radioactive component in spleen phosphodiesterase hydrolyzates of RNA was probably the 3'-monophosphate of 6-thioguanosine. In contrast, when venom phosphodiesterase hydrolyzates of RNA were chromatographed in Solvent 2, the peak of activity was located in the 5'-ribonucleotide region, close to the origin (R_f 0.05). The radioactive component in these hydrolyzates was probably the 5'-monophosphate of 6-thioguanosine.

(b). Selective Hydrolysis of 5'-Nucleotides with 5'-Nucleotidase. Phosphodiesterase digests of DNA and RNA were treated further with 5'-nucleotidase to establish the position of the phosphate substituent on the sugar moiety of the S^{35}-labeled TG nucleotides. Rates of hydrolysis of TG nucleotides were determined by measuring the rate of appearance of the corresponding S^{35}-labeled TG nucleosides in reaction mixtures. Figures 30 and 31 present the rates of 5-nucleotidase-catalyzed hydrolysis of TG nucleotides to TG nucleosides. Hydrolysis proceeded at a constant rate over the 1 h incubation period in venom phosphodiesterase and DNase I plus venom phosphodiesterase digests of S^{35}-labeled RNA and DNA, respectively. No reaction was observed over the 1 h incubation period in micrococcal nuclease plus spleen phosphodiesterase hydrolyzates of RNA and DNA. These results indicated that phosphate was on the 5'-hydroxyl group in TG nucleotides released by venom phosphodiesterase. Similarly, resistance to hydrolysis demonstrated that the phosphate substituent was on the 3'-hydroxyl group in the TG nucleotides released by micrococcal nuclease plus spleen phosphodiesterase.

(c). Selective Hydrolysis of 3'-Nucleotides with 3'-Nucleotidase. Phosphodiesterase digests of RNA were treated further with 3'-nucleotidase and rates of hydrolysis of S^{35}-labeled TG nucleotides were determined

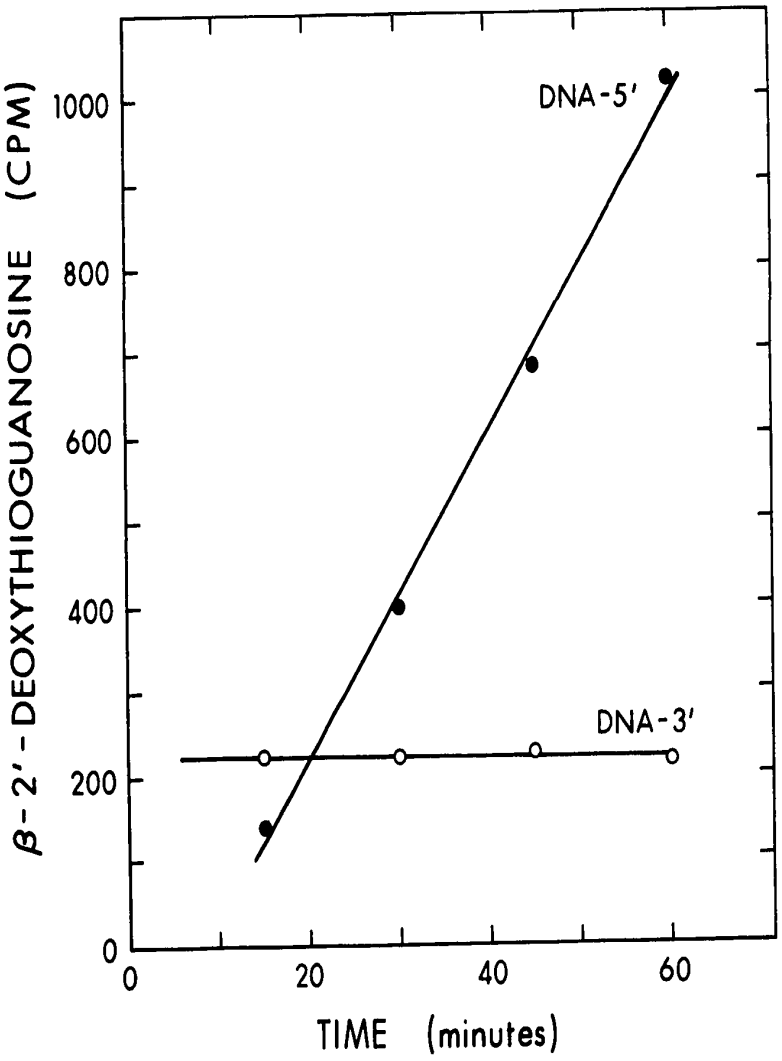

Figure 30. 5'-Nucleotidase-catalyzed hydrolysis of TG-S^{35} nucleotides in phosphodiesterase digests of DNA isolated from MP-S^{35}-treated L5178Y cell cultures. DNA-5', DNase I + venom phosphodiesterase digest of DNA (●); DNA-3', micrococcal nuclease + spleen phosphodiesterase digest of DNA (o).

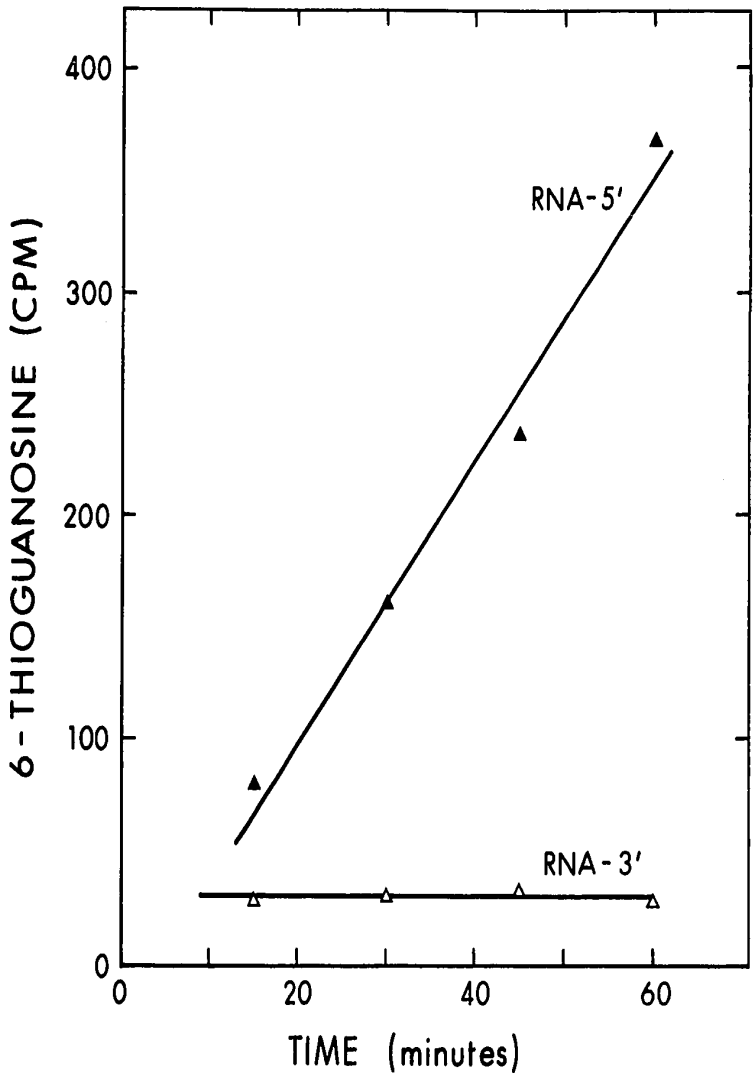

Figure 31. 5'-Nucleotidase-catalyzed hydrolysis of TG-S^{35} nucleotides in phosphodiesterase digests of RNA isolated from MP-S^{35}-treated L5178Y cell cultures. RNA-5', venom phosphodiesterase digest of RNA (▲); RNA-3', micrococcal nuclease + spleen phosphodiesterase digest of RNA (△).

(Figure 32). Hydrolysis proceeded rapidly in the micrococcal nuclease plus spleen phosphodiesterase digest of RNA and the reaction was essentially complete within the 1 h incubation period. No reaction was observed over the 1 h incubation period in the venom phosphodiesterase hydrolyzate of RNA. This result confirmed that phosphate was on the 3'-hydroxyl group in the TG ribonucleotide released from RNA by micrococcal nuclease plus spleen phosphodiesterase.

These data demonstrate that MP is incorporated as TG nucleosides in 3',5'-phosphodiester linkage of DNA and RNA chains.

3. Incorporation of 6-Mercaptopurine into Nucleic Acids and the Delayed Cytotoxic Reaction

The experiments described in the preceeding section demonstrated that MP is incorporated as TG into nucleic acids of cultured L5178Y cells. Levels of incorporation expressed as nmoles TG/mg nucleic acid are presented in Table 5. The data of Table 5 were derived from experiments which investigated the relevance of such incorporation to the delayed cytotoxic reaction of MP. A relation appears to exist between incorporation of MP as TG into nucleic acids and the cytotoxicity of MP.

DNA and RNA were isolated from MP-sensitive

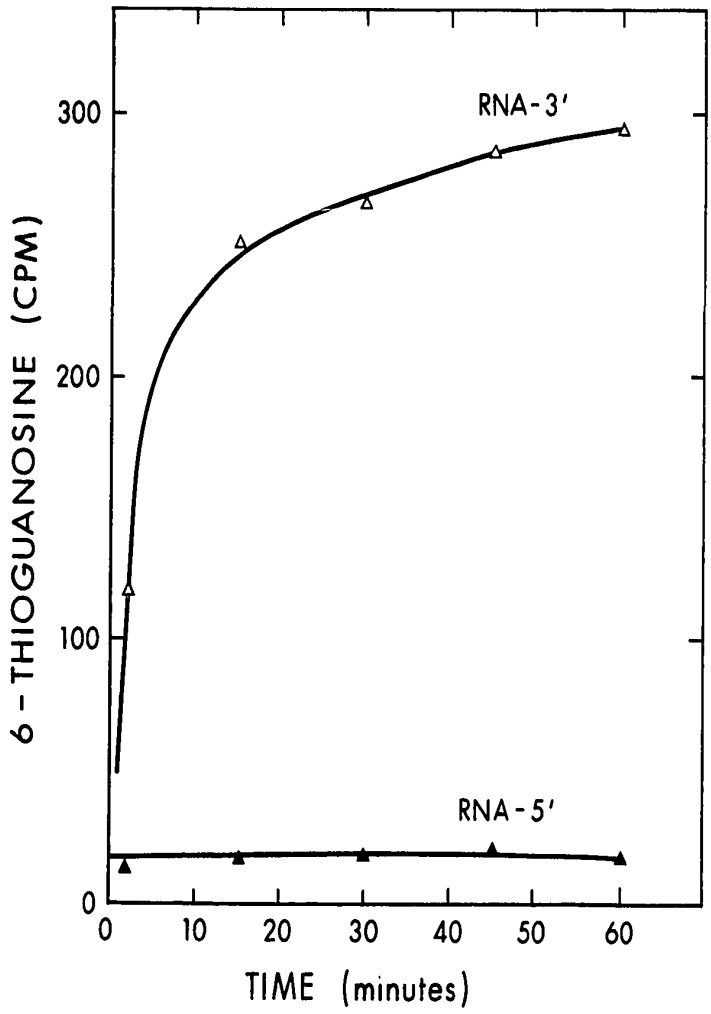

Figure 32. 3'-Nucleotidase-catalyzed hydrolysis of TG-S^{35} nucleotides in phosphodiesterase digests of RNA isolated from MP-S^{35}-treated L5178Y cell cultures. RNA-5', venom phosphodiesterase digest of RNA (▲); RNA-3', micrococcal nuclease + spleen phosphodiesterase digest of RNA (△).

Table 5. Incorporation of MP as TG into nucleic acids of cultured L5178Y cells.

L5178Y Cell Lines	Treatment	Incorporation into DNA nmoles/mg DNA	Incorporation into RNA nmoles/mg RNA
MP sensitive	13 h, 100 µM MP-S^{35}	2.96	0.73
MP sensitive	18.5 h, 100 µM MP-S^{35}. Nucleic acids extracted at time of delayed cytotoxic reaction, 27 h after MP exposure terminated.	0.96	0.18
MP sensitive	13 h, 100 µM MP-S^{35} plus 0.5 µM mycophenolic acid	0.19	0.09
T1 cells, partially tolerant to MP	13 h, 100 µM MP-S^{35}	1.18	0.35
T1 cells, partially tolerant to MP	13 h, 100 µM MP-S^{35}. 0.2 µM MMPR was present 3 h before and during MP exposure	2.83	0.78
H1 cells, partially tolerant to MP	13 h, 100 µM MP-S^{35}	0.78	0.20

L5178Y cell cultures at the end of 13 h exposure to 100 μM MP-S^{35}. The levels of incorporation of MP into these nucleic acids were: 2.96 nmoles TG/mg DNA and 0.73 nmoles TG/mg RNA (Table 5). These levels of incorporation would be roughly equivalent to 0.4% replacement of guanine by TG in DNA and 0.1% replacement of guanine by TG in RNA, assuming that the nucleic acids contained equal proportions of the 4 bases. DNA and RNA were also isolated from L5178Y cell cultures at the time of the delayed cytotoxic reaction. Cells in a single 250 ml culture were exposed to 100 μM MP-S^{35} for 18.5 h and were then washed and resuspended in drug-free medium and harvested for isolation of nucleic acids 27 h later, when the cytotoxic effects of MP exposure were observed. The MP-S^{35}-treated cell population underwent 1.2 doublings during the 27 h period between removal of the labeled drug and onset of the delayed cytotoxic reaction. Therefore S^{35}-labeled nucleic acids were diluted by unlabeled nucleic acids which were synthesized during the period of abortive proliferation after MP-S^{35} was removed. Appreciable amounts of nucleic acid-incorporated TG-S^{35} remained in these cells at the time of the delayed effect (Table 5). DNA contained 0.96 nmoles TG/mg and RNA contained 0.18 nmoles TG/mg. TG present in nucleic acids was the major thiopurine remnant of MP exposure in these dying cells. The total intracellular pool of acid soluble

thiopurine derivatives at this time is only 1 - 2% (1 - 2 nmoles/ml tissue) of that found in cells at the end of the MP exposure period (see Chapter III). This result was confirmed in the present experiment during isolation of the S^{35}-labeled nucleic acids. After precipitation of RNA, the aqueous supernatant containing soluble tissue nucleotides (32) had only 0.5% of the activity found for an equivalent number of cells that were extracted at the end of the MP-S^{35} exposure period.

The S^{35}-labeled RNA and DNA were enzymatically degraded in order to verify that the incorporated thiopurine in nucleic acids was still TG at the time of the delayed cytotoxic reaction. Samples of the digests were chromatographed in solvent 1. Eighty-three percent (1600 cpm) of the total activity in samples of DNA hydrolyzate co-chromatographed with β-2'-deoxythioguanosine. Seventy-two percent (320 cpm) of the total activity in samples of RNA hydrolyzate co-chromatographed with 6-thioguanosine. It was apparent that little if any chemical modification of TG present in nucleic acids occurred during the period of abortive proliferation after MP exposure was terminated.

Mycophenolic acid present in culture medium during MP exposure protected cells against the delayed cytotoxic reaction of MP (see above). Mycophenolic acid did not protect cells against the delayed cytotoxic

reaction of β-2'-deoxythioguanosine. These results suggested that cytotoxic effects of MP were mediated by TG anabolites since, by analogy with effects on guanylate synthesis, mycophenolic acid might be expected to inhibit conversion of 6-thioinosinate to 6-thioguanylate. The effect of mycophenolic acid on incorporation of MP-S^{35} into nucleic acids was investigated. DNA and RNA were isolated from L5178Y cell cultures at the end of 13 h exposure to 100 μM MP-S^{35} plus 0.5 μM mycophenolic acid. Samples of the cells were also resuspended in drug-free media to determine the effects of this treatment on cell proliferation. Simultaneous exposure to mycophenolic acid and MP protected cells against the delayed cytotoxic reaction of MP (Figure 33), whilst incorporation of MP-S^{35} into nucleic acids was considerably reduced (Table 5). Addition of mycophenolic acid reduced incorporation of MP into DNA by 94% and reduced incorporation into RNA by 88%. This experiment suggested that conversion of MP to TG anabolites and their incorporation into nucleic acids are essential to the cytotoxic activity of MP.

L5178Y cells that were maintained in culture for extended periods (longer than 6 weeks) often developed partial tolerance to 13 h exposure with 100 μM MP. This characteristic developed in cell cultures which had never been exposed to MP. One such cell line (designated H1)

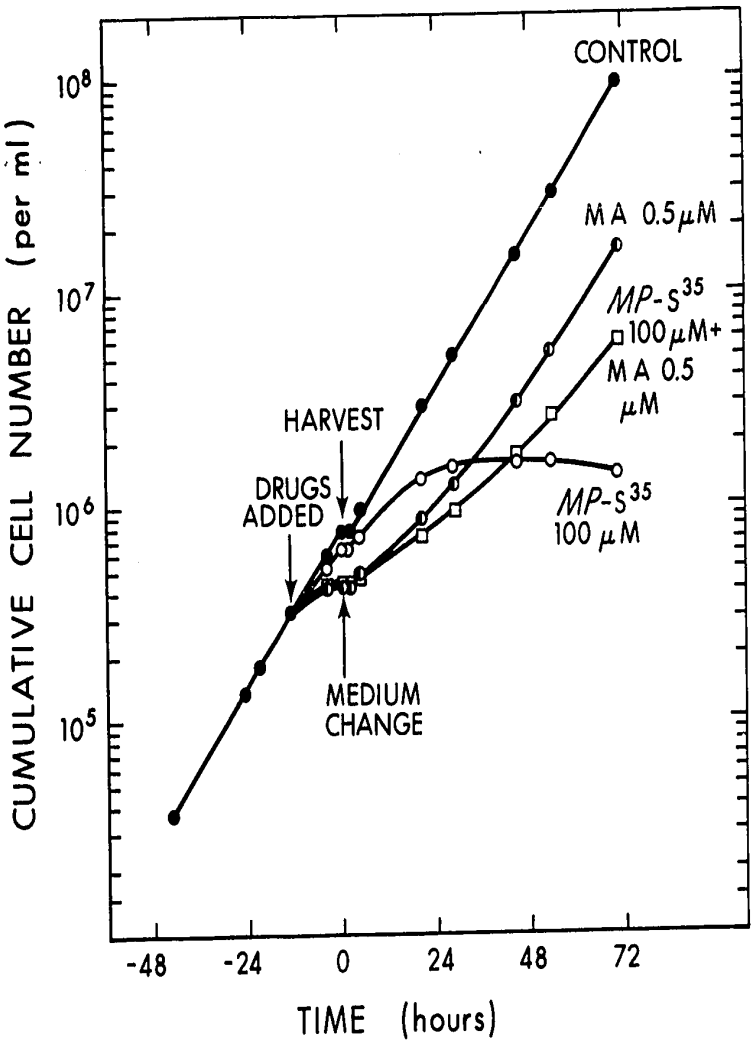

Figure 33. Cell proliferation in L5178Y cultures exposed for 13 h at high cell density to MP-S^{35}, mycophenolic acid (MA), and MP-S^{35} plus mycophenolic acid. Cell numbers plotted at negative times are actual cell numbers per ml in these cultures; at 0 h, 10 ml samples were removed and culture fluids in these samples were replaced with drug-free media. The remainder of the MP-S^{35} plus mycophenolic acid-treated cells were collected at 0 h for determination of the incorporated MP-S^{35} in the isolated nucleic acids of these cells. Concentrations of drugs during exposure are indicated in the figure.

was shown to anabolize hypoxanthine at the same rate as MP-sensitive cells (F. F. Snyder, Ph.D. Thesis, Department of Biochemistry, University of Alberta, 1973) and H1 cells proliferated at their normal rate in culture medium containing methotrexate (0.03 μM), hypoxanthine (40 μM) and thymidine (5 μM). H1 cells did not proliferate in medium containing methotrexate (0.03 μM) and thymidine (5 μM) without hypoxanthine. Therefore, it was apparent that H1 cells had hypoxanthine-guanine phosphoribosyltransferase activity. H1 cells were tolerant to 13 h exposure with both 100 μM MP and 1 μM β-2'-deoxythioguanosine (Figure 34). The effects of these treatments on H1 cells may be compared with the effects of the same treatments on cells which had been maintained in culture for less than six weeks (Figure 35).

Incorporation of MP into nucleic acids was investigated in H1 cells and also in another L5178Y cell culture line (designated T1) which spontaneously developed partial tolerance to 13 h exposure with 100 μM MP. DNA and RNA were isolated from H1 and T1 cell cultures at the end of 13 h exposure to 100 μM MP-S^{35}. Samples of the cells were also resuspended in drug-free media to determine the effects of this treatment on cell proliferation. The effects were similar to those shown in Figure 34. A slight inflexion was observed on the growth curve of these MP-S^{35}

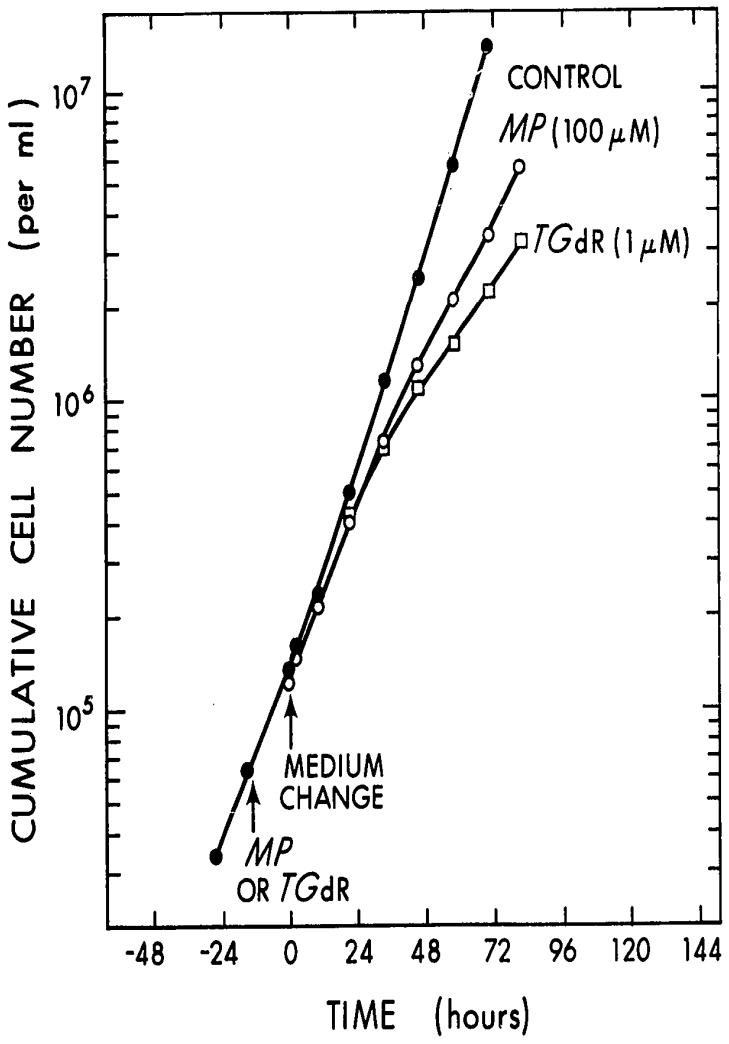

Figure 34. The effects of 13 h exposure to MP and β-2'-deoxythioguanosine (TGdR) on the proliferation of a spontaneously tolerant line (H1) of cultured L5178Y cells. Cells were previously maintained in culture for an extended period (longer than 6 weeks) during which time they developed partial tolerance to 13 h exposure with MP and β-2'-deoxythioguanosine. Concentrations of drugs during exposure are indicated in the figure.

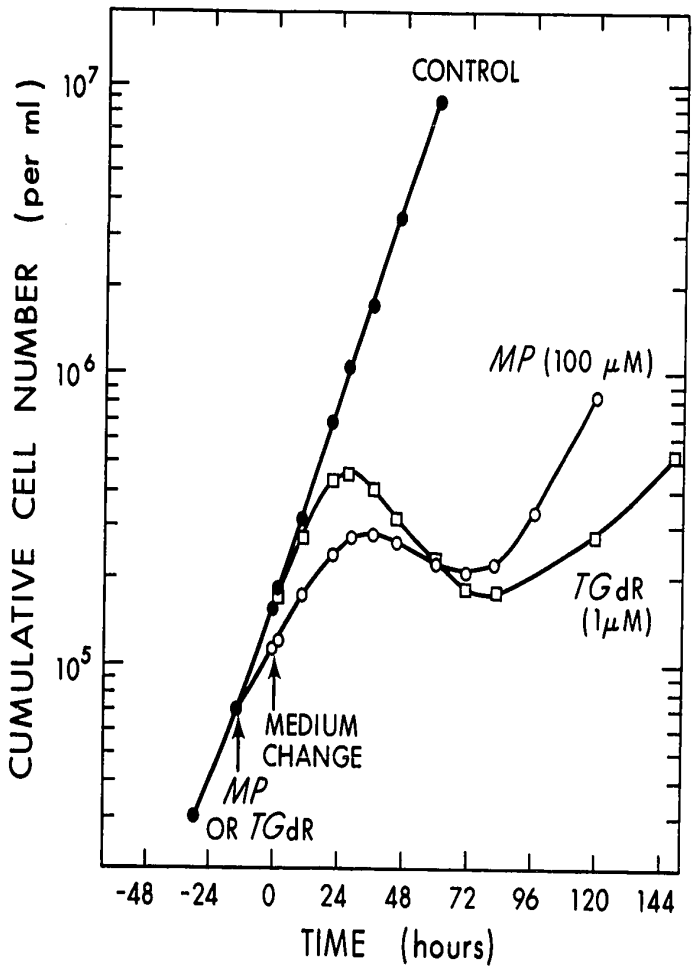

Figure 35. The effects of 13 h exposure to MP and β-2'-deoxythioguanosine (TGdR) on the proliferation of briefly cultured L5178Y cells. These data were obtained with cells which had been maintained in culture for less than six weeks. Concentrations of drugs during exposure are indicated in the figure.

treated cells; this occurred at the time that the delayed cytotoxic effects of MP-S^{35} exposure were observed in cultures of MP-sensitive cells. H1 and T1 cells were larger than normal at 22 h after resuspension but the median cell volumes (1410 cu μm, 1330 cu μm) were lower than the median volume (1630 cu μm) of MP-S^{35}-treated sensitive cells (median volume of untreated MP-sensitive cells, 1110 cu μm). Cellular damage induced by MP-S^{35} exposure was sub-lethal in H1 and T1 cells and large cells apparently continued to divide beyond the critical period of post-exposure proliferation. Cell volumes and proliferation rates subsequently returned to normal in the MP-S^{35}-treated cultures of H1 and T1 cells.

Levels of incorporation of MP-S^{35} as TG-S^{35} in DNA and RNA of T1 and H1 cells were lower than those found in nucleic acids of MP-sensitive cells (Table 5). DNA and RNA isolated from T1 cells contained respectively 40% and 48% of the amount of TG found in nucleic acids of MP-sensitive cells. The corresponding values for DNA and RNA of H1 cells were 26% and 27%. It was apparent that the development of partial tolerance to MP exposure was accompanied by a reduced capacity for incorporation of MP as TG into nucleic acids of these cells.

6-Methylthioinosine potentiates the cytotoxic action of MP apparently by enhancing anabolism of the thiopurine base (138). The effect of 6-methylthioinosine was tested in relation to the cytotoxic action of MP and incorporation of MP into nucleic acids in one of the cell lines which had developed partial tolerance to MP exposure. 6-Methylthioinosine (0.2 μM) was added to T1 cell cultures 3 h before MP-S^{35} and was present in culture medium during 13 h exposure with 100 μM MP-S^{35}. DNA and RNA were isolated from the T1 cell cultures at the end of 13 h exposure with 100 μM MP-S^{35} in the presence of 0.2 μM 6-methylthioinosine. 6-Methylthioinosine stimulated incorporation of MP-S^{35} as TG-S^{35} into nucleic acids (Table 5). The levels of incorporation were similar to those found in nucleic acids isolated from MP-sensitive cells at the end of 13 h exposure with 100 μM MP-S^{35} alone. In a separate experiment with these cells, 6-methylthioinosine was shown to potentiate the delayed cytotoxic reaction of MP exposure (Figure 36).

6-Thioxanthylate has been shown to accumulate in MP-treated cells (122,138) suggesting that amination of this derivative may be a rate-limiting step in conversion of 6-thioinosinate to 6-thioguanylate. In the present investigation, it became evident that the medium concentration of glutamine was not a limiting factor for

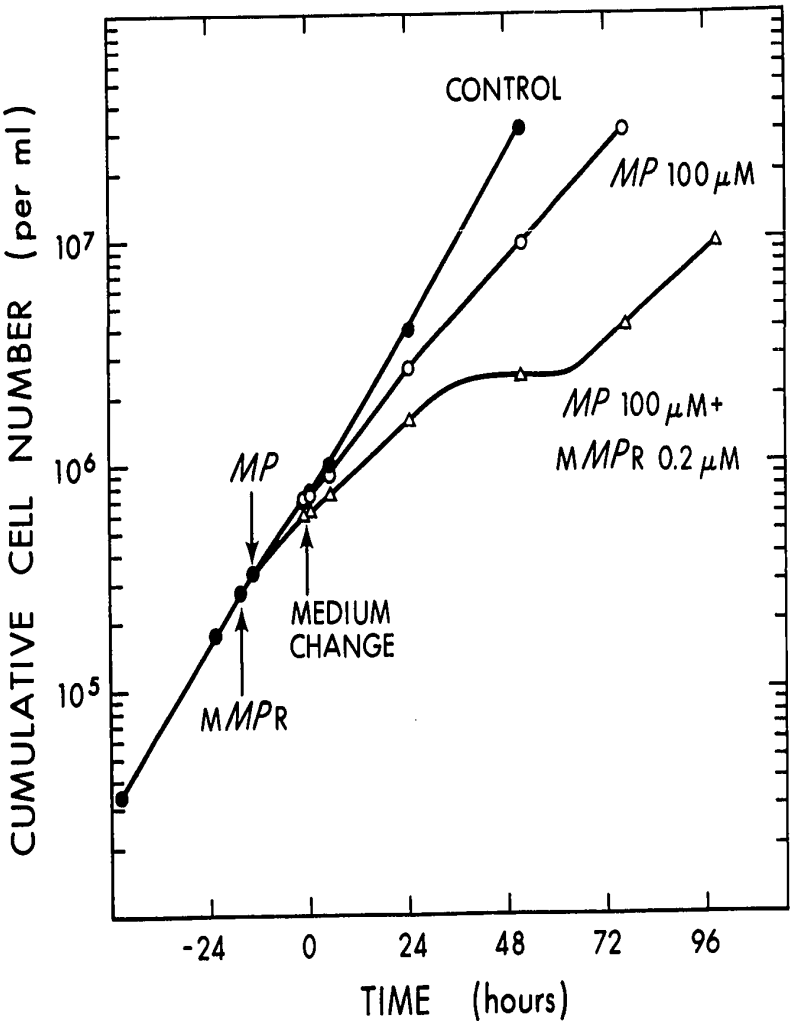

Figure 36. Cell proliferation in spontaneously MP-tolerant L5178Y cultures exposed at high cell density to MP and MP plus 6-methylthioinosine (MMPR). Cells of this culture line (designated T1) were previously maintained in culture for an extended period (longer than 6 weeks) during which time they developed partial tolerance to 13 h exposure with MP. Cell numbers plotted at negative times are actual cell numbers per ml in these cultures. 6-Methylthioinosine was added 3 h before MP and was present in culture media during 13 h exposure to MP; at 0 h, culture fluids were replaced with drug-free media. Concentrations of drugs during exposure are indicated in the figure.

conversion of 6-thioxanthylate to 6-thioguanylate in L5178Y cell cultures; increasing the concentration of glutamine by as much as 8 times (8 x 1.40 mM) during MP exposure did not potentiate the delayed cytotoxic reaction of MP.

D. Discussion

The similarity between the delayed cytotoxic reaction of MP and those of TG and β-2'-deoxythioguanosine suggested that lethal effects of MP were mediated by TG anabolites of MP. Consistent with this hypothesis was the observation that TG and β-2'-deoxythioguanosine were toxic to L5178Y cells at approximately 0.01 times the concentration of MP required for a comparable effect (see Chapter II). In the experiments of this chapter, MP was shown to be incorporated as TG nucleosides in 3',5'-phosphodiester linkages of DNA and RNA of cultured L5178Y cells. Therefore, it seems likely that cytotoxic effects of the two thiopurines share a common biochemical mechanism and that the delayed cytotoxic reaction of MP is in reality that of TG.

A body of evidence suggests that incorporation of TG into DNA is involved centrally in the cytotoxic activity of this drug (see Chapter I). However, the

mechanism by which the analog so incorporated exerts its toxic effect is not evident. 5-Bromodeoxyuridine and ethyl methanesulfonate are both agents which affect DNA and both produced delayed cytotoxic effects in L5178Y cell cultures (see Chapter II). Kao and Puck (97) concluded that chromosomal aberration rather than mutational events was the main factor involved in cell death induced by both X-radiation and ethyl methanesulfonate. It is possible that incorporation of TG into DNA may induce cell death by a similar mechanism. Bases (14) has described the similarity between the delayed effects of MP and low doses of X-irradiation on cultured HeLa cells. It would appear that this mode of cell death is not unique to MP and TG and that the ultimate factors responsible for cell kill will represent a general property which TG shares with other agents rather than a specific property of the thiopurine.

Scannell and Hitchings (156) previously demonstrated that deoxythioguanosine was present in DNA isolated from a MP-resistant subline of Adenocarcinoma 755 after treatment with MP. The amount of deoxythioguanosine associated with the DNA of the resistant tumor was twice that found in the sensitive parent line and was of the same order of magnitude as that reported for incorporation of TG into DNA under comparable conditions (106). On the

basis of these results, Scannell and Hitchings (156) proposed that incorporation of TG into DNA is not responsible for cytotoxic effects of MP and TG. However, it is apparent that incorporation per se is not the final step in the action of MP or TG and it is conceivable that mechanisms of resistance may be operative even at this locus. Resistant mutants could possibly arise in which toxic effects of the DNA-incorporated analog are suppressed in some manner.

In the present investigation, a relation was observed between cytotoxic effects of MP and incorporation of MP as TG into nucleic acids in the following instances: (a) mycophenolic acid protected L5178Y cells against the delayed cytotoxic reaction of MP and depressed incorporation of MP as TG into nucleic acids, presumably by inhibiting conversion of 6-thioinosinate to 6-thioguanylate; (b) partial tolerance to MP exposure was associated with a reduced capacity for incorporation of MP as TG into nucleic acids; (c) 6-methylthioinosine potentiated the cytotoxic effect of MP in partially tolerant cells and stimulated incorporation into nucleic acids; in addition, (d) nucleic acid-incorporated TG was found to be the major thiopurine derivative persisting in MP-sensitive cells at the time of the delayed cytotoxic reaction to MP exposure.

These results are compatible with a biochemical mechanism for the delayed cytotoxic reaction of MP, involving incorporation of TG anabolites into nucleic acids. The level of incorporation of MP as TG into DNA was 4 times that into RNA in MP-sensitive cells. The reason for the higher incorporation into DNA is unclear. Factors affecting MP-cytotoxicity did not indicate whether incorporation of TG into either DNA or RNA is responsible for the delayed lethal effect of MP. However, in view of the evidence relating cytotoxic effects of TG to incorporation into DNA, it seems likely that the delayed cytotoxic reaction of MP results from incorporation of TG into DNA.

Electron dense, virus-like particles were observed in electron micrographs of sections of MP-treated L5178Y cells that were fixed at the time of the delayed cytotoxic reaction to MP exposure (D. M. Tidd, D. Scraba and A. R. P. Paterson, unpublished observations). These particles were associated with the outer cell membrane of MP-treated cells and were not seen in electron micrographs of untreated control cells. The origin of the virus-like particles is not known. However, it is noteworthy that MP has been used in mice to induce thymic lymphomas (46,119). These MP-induced lymphomas have been passaged in vivo by cell-free transfer and this would suggest a viral etiology (46,119). It is possible that

incorporation of MP as TG into DNA may induce synthesis of a latent tumor virus. Lowy et al., (117) recently reported induction of murine leukemia virus by 5-bromodeoxyuridine in mouse embryo cell cultures. Similarly, Gerber (64) and Hampar et al. (73) have reported 5-bromodeoxyuridine activation of the Epstein-Barr virus genome in "virus-negative" human cells. 5-Bromodeoxyuridine is incorporated into DNA and this drug elicited a delayed cytotoxic effect in cultures of L5178Y cells (see Chapter II). It is conceivable that incorporation of MP as TG into DNA may activate a viral genome in murine lymphoma L5178Y cells. However, the MP-induced appearance of virus-like particles was not characterized further and it is not known whether this effect is relevant to the delayed cytotoxic activity of MP.

E. Summary

6-Thioguanosine-S^{35} and β-2'-deoxythioguanosine-S^{35} were identified in venom hydrolyzates of RNA and DNA isolated from MP-S^{35}-treated L5178Y cell cultures. Both paper chromatography and selective acid hydrolysis established that the S^{35} released by digestion of S^{35}-labeled DNA was a TG deoxyribonucleoside, whilst the S^{35} released by digestion of RNA was a TG ribonucleoside.

Further treatment of venom hydrolyzates of S^{35}-labeled DNA and RNA confirmed that the S^{35}-labeled thiopurine moiety was TG. Incubation with purine nucleoside phosphorylase resulted in conversion of the labeled derivative in both digests to TG; this also established that the TG nucleosides had the β-configuration about the anomeric carbon of the sugar. Subsequent treatment with nitrous acid converted S^{35}-labeled TG to 6-thioxanthine.

Digestion with purified phosphodiesterases demonstrated that MP was incorporated as TG nucleosides in 3',5'-phosphodiester linkages of DNA and RNA chains. 3'-Nucleotides of TG-S^{35} were released from S^{35}-labeled DNA and RNA by digestion with micrococcal nuclease plus spleen phosphodiesterase. The 5'-ribonucleotide of TG-S^{35} was released from S^{35}-labeled RNA by digestion with venom phosphodiesterase and the 5'-deoxyribonucleotide of TG-S^{35} was released from S^{35}-labeled DNA by digestion with DNase I plus venom phosphodiesterase. The position of the phosphate substituent on the TG nucleotides was established by their susceptibility to hydrolysis catalyzed by 3'- and 5'-nucleotidases.

A relation was observed between incorporation of MP as TG into nucleic acids and delayed cytotoxicity. Mycophenolic acid protected cells against the delayed

cytotoxic effect of MP and suppressed incorporation into nucleic acids. The spontaneous development of partial tolerance to MP exposure was associated with a reduced capacity for incorporation of MP as TG into DNA and RNA. 6-Methylthioinosine potentiated cytotoxic effects of MP in a partially tolerant cell line and stimulated incorporation into DNA and RNA. Nucleic acid-incorporated TG was the major thiopurine derivative persisting in MP-sensitive cells at the time of the delayed cytotoxic reaction to MP exposure.

The delayed cytotoxic activity of MP is probably mediated by TG anabolites through their incorporation into DNA. It is likely that one and the same biochemical mechanism is responsible for delayed cytotoxic effects of both MP and TG.

V. THE CELL CYCLE PHASE SPECIFICITY OF 6-MERCAPTOPURINE

A. Introduction

The experiments described in Chapter IV demonstrated that MP is incorporated as β-2'-deoxythioguanosine in 3',5'-phosphodiester linkages of DNA and RNA chains. A relation was observed between the extent of this incorporation and the delayed cytotoxic activity of MP. The similarity between the delayed cytotoxic reactions of MP and TG suggested that incorporation of TG anabolites into DNA is responsible for the lethal effects of MP. This would imply that cells are only sensitive to MP during the DNA-synthetic period of the cell cycle. In support of this hypothesis were the MP concentration-survival curves (Figures 15 and 16, Chapter II) which exhibited a saturation-kill effect similar to that reported for agents which are known to affect cells only during a particular phase of the cell cycle (27).

The present experiments, performed by S. C. Kim, were designed to test the phase specificity of MP. Synchronized populations of L5178Y cells were exposed to MP during specific portions of their mitotic cycle and effects of these treatments on cell viability were determined.

B. Materials and Methods

1. Cloning Assay for Cell Survival

The cloning assay for cell survival is described in Chapter II, Section B.6.

2. Synchronization of Cells

The conditions of the synchrony method of Doida and Okada (48) were adjusted to produce optimal yields of synchronized cells. Cultures (100 ml) containing 10^7 exponentially proliferating cells were exposed to 1.5 mM thymidine for 5 - 5.5 h, whereupon the medium was changed by an automatic pumping system which removed thymidine medium and introduced colcemid containing medium over a 0.7 h interval; the resultant medium contained 0.1 mM thymidine and colcemid at a concentration of 0.015 - 0.02 µg/ml. After 5 h incubation, the cells (then in metaphase) were washed and resuspended in drug-free media. Cells began to divide about 0.5 h after release from colcemid and cell numbers increased 160 - 170% during the following 1.5 h.

3. Exposure to 6-Mercaptopurine

Cultures were exposed to MP for specified intervals at various times after release from colcemid. At the

end of each exposure, cells were washed and resuspended in drug-free media and plated in soft agar medium for assay of viability by the cloning method.

4. DNA Synthesis

Rates of DNA synthesis were determined by pulse-labeling with ^{14}C-thymidine; culture samples were incubated with 150 μM ^{14}C-thymidine at 37° for 20 min, cells were collected on Millipore filters (1.2 μm pore size), washed twice with 15 ml of 5% w/v trichloracetic acid, and air dried. Filters were counted in a liquid scintillation system.

C. Results

L5178Y cells that were exposed successively to thymidine (1.5 mM, 6 h) and colcemid (0.015 μg/ml, 5 h) divided synchronously upon release from colcemid (Figure 37); cell numbers began to rise by 0.5 h after release and within the following 1.5 h the cell population increased 1.65-fold. A second, less synchronized wave of mitosis also occurred in which the cell numbers increased 1.66-fold. The time interval between the 2 half-value points on the rising portions of the synchronous growth curve provided a value of 8.6 h for the cell generation time.

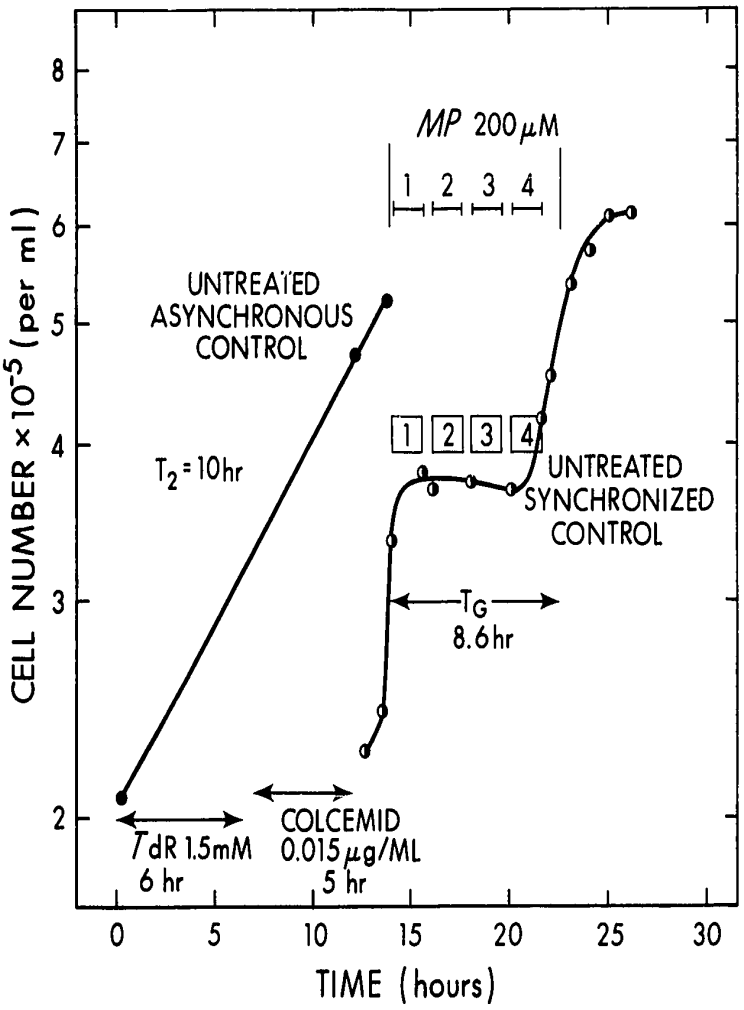

Figure 37. Exposure of synchronized L5178Y cells to MP during specific portions of the intermitotic interval. Following release from the colcemid block, cultures were treated with 200 μM MP during one of the four intervals indicated. These cultures were then assayed for cell viability (Figure 38).

Portions of the synchronized culture were exposed to 200 μM MP for 1.5 h intervals at the indicated times after release from colcemid (Figure 37). Effects of these MP treatments on the ability of cells to form macroscopic colonies in soft agar medium are shown in Figure 38. The plating efficiencies of untreated control cultures, asynchronous and synchronized, were 70% and 34%, respectively. MP exposure during the first, second and fourth quarters of the intermitotic interval had only slight effects on the plating efficiency of the synchronized cells; however, plating efficiency was reduced to less than 10% of that of the untreated, synchronized control cells by MP exposure during the third quarter of the intermitotic period. This period of maximum MP sensitivity corresponded to the time after release from colcemid at which the rate of ^{14}C-thymidine incorporation into acid-insoluble material was greatest (Figure 39).

D. Discussion

It is apparent that L5178Y cells were most sensitive to MP during the third quarter of the intermitotic period; this result was obtained in 4 experiments (including that presently reported) in which MP concentrations during exposure were between 200 and 1000 μM.

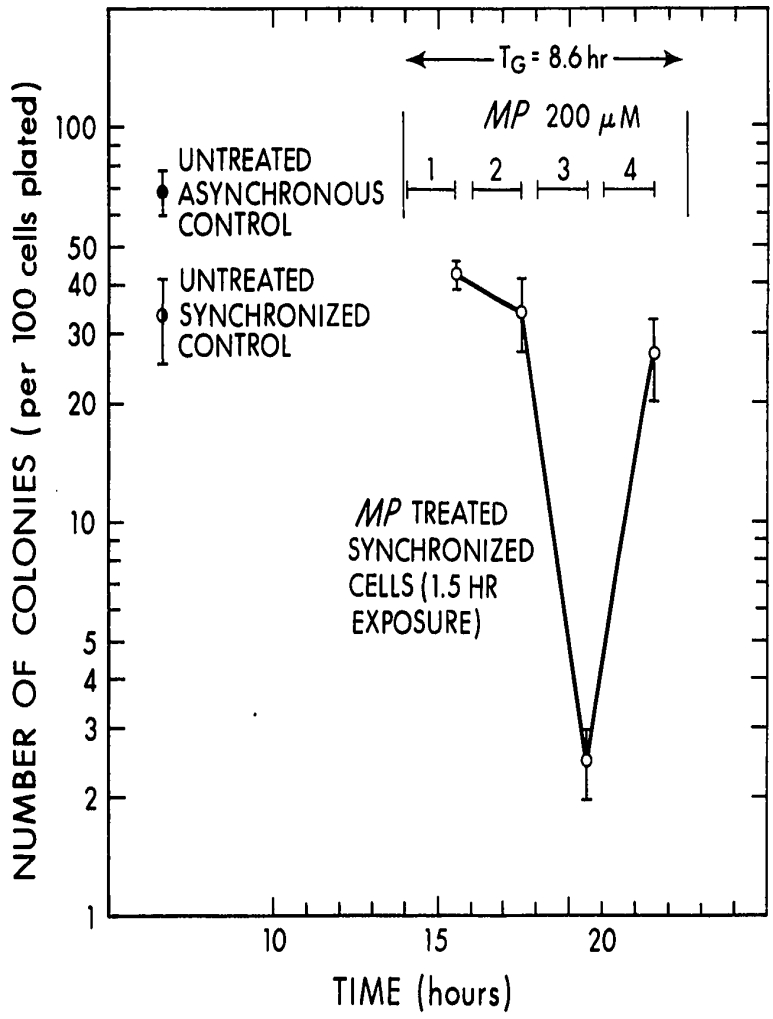

Figure 38. Viability of MP-exposed cells from the experiment of Figure 37.

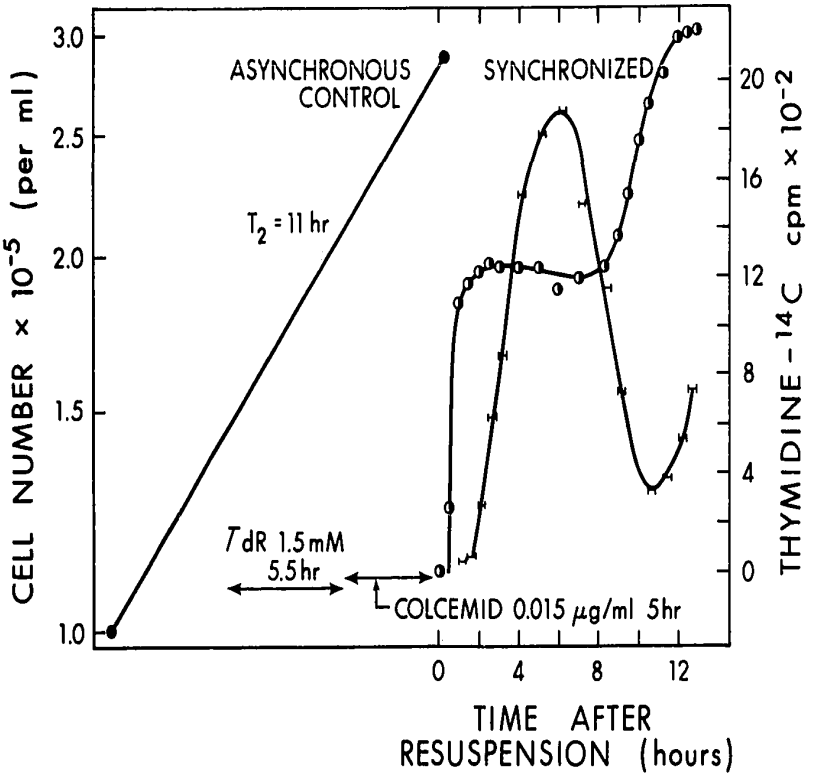

Figure 39. ^{14}C-Thymidine incorporation into acid-insoluble material in synchronized cultures of L5178Y cells. ◐, Cell number in synchronized cultures; ▬ ^{14}C-thymidine (cpm) in acid-insoluble material of synchronized cultures. ●, Cell number in asynchronous control culture.

Treatment with MP did not affect the viability of mitotic cells (data not shown). The period of MP sensitivity corresponded with that at which that rate of DNA synthesis was maximal (Figure 39). It may be concluded that cells were most sensitive to the cytotoxic effects of MP exposure during the mid S-phase of the cell cycle. This result was compatible with the hypothesis that incorporation of TG anabolites in internal nucleotide positions of DNA chains is responsible for the cytotoxic activity of MP. A similar cell cycle phase specificity was reported for β-2'-deoxythioguanosine. Barranco and Humphrey (13) demonstrated that synchronized Chinese hamster ovary cells were sensitive to β-2'-deoxythioguanosine during the early and middle portions of the S-phase of the mitotic cycle. The similarity to the present results provides further evidence for the postulate that cytotoxic effects of MP and TG are mediated by a common mechanism.

E. Summary

Division of cultured L5178Y cells was synchronized by the method of sequential thymidine and colcemid arrest. A synchronized wave of mitosis occurred upon release of the colcemid block and cells, so treated, exhibited maximum sensitivity to cytotoxic effects of MP exposure during

the third quarter or mid S-phase of the subsequent intermitotic interval. This result supports the hypothesis that incorporation of TG anabolites into 3',5'-phosphodiester linkages of DNA chains is responsible for the cytotoxic activity of MP.

VI. GENERAL CONCLUSIONS

A number of carcinolytic agents have been shown to destroy the long term reproductive capacity of cells without producing immediate signs of cytotoxicity. These agents are said to induce mitotic (65) or reproductive (18) death and they may be distinguished from treatments which are "acutely" toxic to cells. It was apparent in the present investigation that MP and TG belong to the former category.

Altered DNA structure may be responsible for delayed lethal effects. 5-Bromodeoxyuridine is incorporated into DNA whilst polynucleotides are alkylated by reaction with ethyl methanesulfonate; both drugs induced a delayed cytotoxicity in cultures of L5178Y cells. Incorporation of TG into DNA is thought to be centrally involved in the cytotoxic activity of this drug and MP was shown to be incorporated as TG in internal nucleotide positions of DNA and RNA chains. A relation was observed between the extent of this incorporation and MP cytotoxicity which would suggest that the delayed cytotoxic reaction of MP is in reality that of TG. In addition, cultured L5178Y cells were shown to be most sensitive to the cytotoxic effects of MP exposure during the mid DNA-synthetic period of the cell cycle. This result

provides further evidence for the suggestion that incorporation of TG anabolites into DNA is responsible for the cytotoxic activity of MP. A similar cell cycle phase specificity was reported for β-2'-deoxythioguanosine (13).

It is evident that the ability to alter the structure of DNA is a common property, shared by the different agents which elicit delayed lethal effects. In attempting to account for the mode of cell death, a situation might be envisaged in which cell processes would continue for a limited period before errors in replication and repair of genetic material accumulated.

Incorporation of TG anabolites into DNA may also be the mechanism responsible for the chemotherapeutic effects of MP against human leukemia. Scheduling of MP treatments would be a critical factor affecting the response to therapy. It might be predicted that an optimum response would be achieved when the concentration of MP in body fluids was maintained at a value sufficient to give maximum incorporation throughout a period in which proliferating elements of the neoplasm would realise at least one complete replication of their DNA. A subsequent interval of no treatments would allow normal proliferating cell populations to recover before the treatment was repeated. The procedure of high dose intermittent therapy (19) may approximate to this ideal. Results of the present

investigation would recommend that MP be administered by continuous infusion over a period that was related to predetermined cell kinetics of the leukemia.

An alternative approach to the optimization of MP therapy might involve in vivo synchronization of malignant cell populations. Acute lymphoblastic leukemic cells are accumulated in the early part of the S-phase by treating the patients with arabinosylcytosine (J. J. Akabutu, personal communication). A synchronized phase of DNA synthesis occurs in such cells after release from the arabinosylcytosine block and therapeutic effects might be enhanced by treatment with MP at this time.

Mycophenolic acid protected L5178Y cells against the delayed cytotoxic reaction of MP. This illustrates the possibility of inducing antagonistic effects when drugs are used in combination. It is likely that arabinosylcytosine would also protect cells against simultaneous exposure to MP by inhibiting the passage of cells into the MP-sensitive phase of the mitotic cycle. Drugs which inhibit ribonucleotide reductase would probably reduce cytotoxic effects of MP by inhibiting both DNA synthesis and the conversion of MP to deoxyribonucleotide derivatives of TG. Care must therefore be taken in selecting drugs for simultaneous combination therapy.

In metabolic terms, MP is far removed from a

site of action involving incorporation of TG anabolites into DNA. β-2'-Deoxythioguanosine might achieve some chemotherapeutic advantage over MP in that fewer metabolic steps would be required for incorporation of the thiopurine into DNA. β-2'-Deoxythioguanosine may well be effective against certain types of tumors which have formerly exhibited poor responses to therapy with MP (127).

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