Studies on the Biochemical Mode of Action of a Cytotoxic Methylhydrazine Derivative, *N*-Isopropyl-α-(2-methylhydrazino)-*p*-toluamide

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SUMMARY

N-Isopropyl-α-(2-methylhydrazino)-*p*-toluamide inhibited the incorporation of thymidine-^{3}H, deoxycytidine-^{3}H, formate-^{14}C, adenine-8-^{14}C, and 4-amino-5-imidazolecarboxamide-2-^{14}C into DNA, and the utilization of orotic acid-6-^{14}C and leucine-1-^{14}C for the synthesis of RNA and protein, respectively, in L5178Y lymphoma cells. The site of the drug-induced blockade of the formation of DNA did not appear to reside at the level of thymidine kinase (ATP:thymidine 5′-phosphotransferase, EC 2.7.1.21), thymidine monophosphate kinase (ATP:thymidine monophosphate phosphotransferase, EC 2.7.4.9), or DNA nucleotidyltransferase (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase, EC 2.7.7.7). Furthermore, treatment of cells with *N*-isopropyl-α-(2-methylhydrazino)-*p*-toluamide did not influence the rate of loss of thymine-^{3}H from prelabeled DNA. Possible metabolic alterations which might explain the observed results are discussed. The inhibitions of the syntheses of nucleic acids and proteins that were measured were followed by a period during which most of the cell death occurred; this period corresponded with the development of a metabolic imbalance that was characterized by an accumulation of RNA and protein and an increase in cell volume.

INTRODUCTION

Certain derivatives of methylhydrazine have shown considerable antineoplastic activity both in transplanted rodent neoplasms (1–4) and in the treatment of Hodgkin's disease in man (5, 6). The biochemical basis for this growth-inhibitory activity is unknown, although the findings of Rutishauser and Bollag (7) that 1-methyl-2-benzylhydrazine phosphate markedly prolongs the duration of the interphase in Ehrlich ascites carcinoma cells, and that this phenomenon is accompanied by chromatid breaks, which occur apparently either during or after the synthesis of DNA, suggest that nucleic acid metabolism is involved. This possibility gains support from the observation that several cytotoxic derivatives of methylhydrazine possess the ability to degrade DNA *in vitro;* the mechanism of this degradation appears to involve the autoxidation of the methylhydrazine derivative to hydrogen peroxide (8). That such autoxidation can occur in intact cells is consistent with the observations of several laboratories engaged in the study of the metabolism of *N*-isopropyl-α-(2-methylhydrazino)-*p*-toluamide (MIH) in rodents (*3, 9, 10*).

On the basis of results *in vitro,* however, Weitzel *et al.* (11) suggest that cytostatic methylhydrazine derivatives may liberate *in vivo,* in addition to hydrogen peroxide, formaldehyde, azomethine, and *N*-hydroxylmethyl derivatives; thus, growth-inhibitory effects conceivably could result from both oxidation and alkylation of cellu-

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lar constituents. That *N*-demethylation of this class of compounds does indeed occur *in vivo* was shown independently by Kreis and Yen (12) and by Baggiolini *et al.* (13); the metabolic form of the drug that is demethylated to yield the one-carbon fragment has not been identified.

The present study was designed both to elucidate some of the metabolic effects produced by the cytotoxic methylhydrazine derivative MIH and to determine the role of such biochemical alterations in the process of drug-induced cell death.

MATERIALS AND METHODS

L5178Y ascites cells were grown in adult male C57BL × DBA F_1 mice (Cumberland View Farms, Cumberland, Tennessee) weighing 20–25 g. Transplantation was carried out by collecting ascites fluid from donor mice bearing a 7-day growth of lymphoma L5178Y; the fluid was centrifuged for 2 min in a clinical centrifuge (1600 *g*), supernatant peritoneal fluid was decanted, a 10-fold dilution with isotonic saline was made, and 0.1 ml (approximately 1.2×10^6 cells) of the cell suspension was inoculated intraperitoneally into each animal. MIH was dissolved in isotonic saline just prior to use, and was injected intraperitoneally into mice bearing 5–6-day implants of the neoplastic cells. The toxicity of MIH to L5178Y cells was evaluated by injecting mice bearing 5-day implants of lymphomatous cells with a single intraperitoneal dose of MIH. Cells were removed quantitatively from the peritoneal cavities at selected intervals after exposure to the drug and the numbers were determined with a Coulter model A particle counter.

The average cellular content of DNA and RNA was determined by pentose analyses (14), using deoxyadenosine and adenosine, respectively, as the standards. The protein content of the residue after extraction of nucleic acids was determined by the biuret reaction (15) with crystalline bovine serum albumin serving as the standard. The average volume of the cells was estimated in hematocrit tubes by measuring the volume occupied by a known number of cells.

Drug-induced metabolic effects were measured by administering a single intraperitoneal dose of MIH to mice bearing 6-day growths of L5178Y ascites cells. At selected time intervals thereafter, either 200 μg of thymidine-^{3}H (6.6×10^3 cpm/μg), 100 μg of orotic acid-6-^{14}C hydrate (1.6×10^4 cpm/μg), 125 μg of DL-leucine-1-^{14}C (1.7×10^4 cpm/μg), 660 μg of deoxycytidine-^{3}H (1.1×10^3 cpm/μg), 90 μg of formate-^{14}C (11.8×10^4 cpm/μg), 50 μg of adenine-8-^{14}C (2.7×10^4 cpm/μg), or 90 μg of 4-amino-5-imidazolecarboxamide-2-^{14}C (1.5×10^4 cpm/μg) were administered by intraperitoneal injection to each mouse and were allowed 1 hr for metabolic utilization. Sodium nucleates were isolated by the method of Tyner *et al.* (16). In experiments involving the incorporation of thymidine, orotic acid, deoxycytidine, and formate into nucleic acids, the sodium nucleates were hydrolyzed with 70% perchloric acid for 1.5 hr (17), desalted on charcoal columns and the desired purine and pyrimidine components of the nucleic acids were purified and analyzed as described by Danneberg *et al.* (18). When adenine and 4-amino-5-imidazolecarboxamide were employed as the isotopic substrates, the sodium nucleates were hydrolyzed with 0.4 *N* KOH at 37° for 1 hr to separate the RNA from DNA. The DNA was precipitated subsequently by acidification and isolated by centrifugation. Purines were liberated by hydrolyzing with 10% trichloroacetic acid for 0.5 hr at 90°, and were purified and analyzed as described previously (19, 20), except that radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer. After exposure of the cells to leucine-^{14}C, residual protein was isolated and analyzed as previously described (21).

Enzyme extracts were prepared and the activities of thymidine kinase (ATP: thymidine 5′-phosphotransferase, EC 2.7.1.21), thymidine monophosphate kinase (ATP:thymidine monophosphate phosphotransferase, EC 2.7.4.9), and thymidine monophosphate nucleotidase (5′-ribonucleotide phosphohydrolase, EC 3.1.3.5) were assessed by methods previously described (22). The activity of DNA nucleotidyltransferase (deoxynucleosidetriphosphate:

DNA deoxynucleotidyltransferase, EC 2.7.7.7) was assayed by the method of Mantsavinos and Canellakis (23), except that radioactivity was determined by scintillation spectrometry.

RESULTS

The growth-inhibitory effects of MIH on L5178Y lymphoma cells were measured by quantitatively determining the number of ascites cells in the peritoneal cavities of mice bearing 5-day implants of the neoplasm at selected intervals of time after a single intraperitoneal injection (300 mg/kg) of this agent (Fig. 1). That such an exposure to MIH is capable of causing cell death is shown by the marked decrease in the number of cells present in the abdominal cavities; tumor-bearing mice given this quantity of MIH survived approximately 3 days longer than did similar untreated tumor-bearing animals.

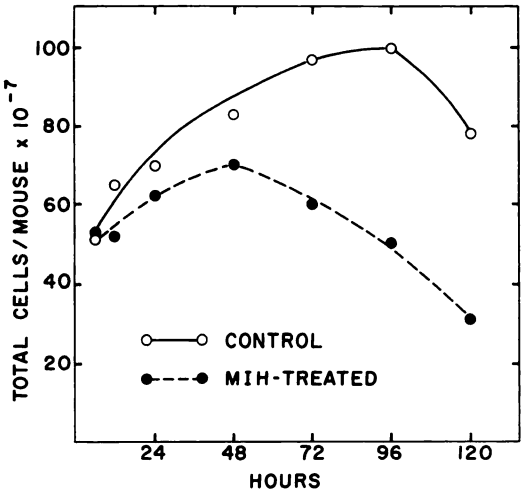

FIG. 1. *The effects of MIH on the growth of lymphoma L5178Y ascites cells*

Mice bearing 5-day implants of tumor were given a single intraperitoneal dose of 300 mg of MIH per kilogram. At selected time intervals thereafter, ascites cells were harvested quantitatively from the abdominal cavities and the numbers were determined with a Coulter model A particle counter. Each point represents the mean value of results from 3–4 determinations, each representing the pooled cells from 2 animals.

Some biochemical effects produced by MIH in these neoplastic ascites cells were estimated by measuring the rate of incorporation of thymidine-^{3}H, orotic acid-6-^{14}C and leucine-1-^{14}C into DNA, RNA, and protein, respectively (Table 1); exposure of the cells to MIH (300 mg/kg) caused a pronounced inhibition of thymidine incorporation into DNA thymine, which reached a maximum of about 70% when the thymidine-^{3}H was administered from 1 to 3 hr after MIH. By 24 hr after the drug, inhibition of this pathway appeared to be relieved completely. The degree of inhibition of thymidine utilization for DNA formation induced by MIH increased in a linear fashion with respect to the quantity of drug to which the cells were exposed (Fig. 2). The rates of incorporation of orotic acid into RNA uracil and of adenine and 4-amino-5-imidazolecarboxamide into RNA purines also were depressed by MIH (Tables 1 and 5); however, the synthesis of RNA appeared to recover from the inhibition within 12 hr after the drug (Table 1). Retardation of the fixation of leucine into residual protein also

TABLE 1

Incorporation of thymidine-^{3}H, orotic acid-6-^{14}C and DL-*leucine-1-^{14}C into DNA, RNA, and protein, respectively, of MIH-treated L5178Y ascites cells*

Mice bearing 6-day implants of L5178Y lymphoma cells received a single intraperitoneal dose of 300 mg of MIH per kilogram of body weight. At selected time intervals after dosage with the drug, either thymidine-^{3}H (6.6×10^3 cpm/μg) at a level of 200 μg per animal, orotic acid-6-^{14}C hydrate (1.6×10^4 cpm/μg) at a level of 100 μg per animal or DL-leucine-1-^{14}C (1.7×10^4 cpm/μg) at a level of 125 μg per animal was administered and 1 hr was allowed for metabolic utilization. Each figure represents the mean value (± the standard error) obtained with 3–28 mice.

Time after MIH (hr)	cpm/μmole $\times 10^{-2}$		cpm/mg
	DNA thymine	RNA uracil	Residual protein
0	125.5 ± 6.9	18.0 ± 1.0	5470 ± 260
1	46.2 ± 4.9	9.8 ± 1.4	5580 ± 460
3	44.8 ± 4.0	7.6 ± 1.3	3870 ± 600
12	86.2 ± 6.3	16.6 ± 0.9	2830 ± 620
24	161.0 ± 9.6	23.0 ± 5.7	5750 ± 850

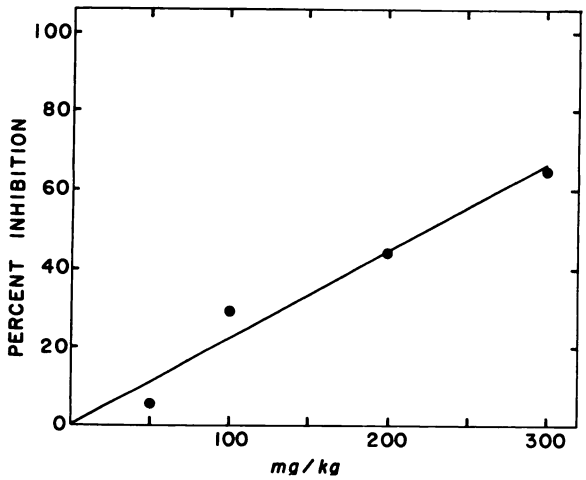

FIG. 2. *The effect of varying concentrations of MIH on the incorporation of thymidine-^{3}H into DNA*

Mice bearing 6-day implants of L5178Y lymphoma cells received a single intraperitoneal dose of MIH. Three hours after dosage with the drug, thymidine-^{3}H (6.6×10^3 cpm/μg), at a level of 200 μg per animal was administered and 1 hr was allowed for metabolic utilization. Each point represents the mean value, expressed as percentage inhibition, obtained with 4 mice.

occurred; however, the onset of inhibition of protein synthesis was more delayed than that of the nucleic acids, and only slight inhibition of leucine incorporation was produced by MIH at periods when DNA and RNA formation were inhibited markedly. The maximum decrease in the utilization of leucine-^{14}C for the synthesis of proteins occurred when the isotope was administered 12 hr after the MIH.

To determine whether the inhibition of the synthesis of DNA induced by MIH is manifest at the level of the enzymes that phosphorylate thymidine or on the polymerization process, thymidine kinase, thymidine monophosphate kinase, thymidine monophosphate nucleotidase, and DNA nucleotidyltransferase activities were assayed in extracts prepared from cells exposed to the methylhydrazine derivative and in extracts to which MIH was added; the results are presented in Table 2. Neither the addition of MIH (either 1×10^{-4}M or 1×10^{-3}M) to cell-free ex-

TABLE 2

Thymidine kinase, thymidylate kinase, thymidylate nucleotidase and DNA nucleotidyltransferase activities of MIH-treated L5178Y ascites cells

Six-day growths of L5178Y lymphoma cells from untreated mice or from those treated intraperitoneally 3 hr previously with 300 mg of MIH per kilogram of body weight were used as the enzyme source. Each value represents the mean value of 2 determinations.

	mμmoles/hr/mg			
Cell-free extract	Thymidine kinase	Thymidine monophosphate kinase	Thymidine mono-phosphate nucleotidase	DNA nucleotidyl-transferase
Untreated	19.1	97.2	6.3	0.12
Untreated + 1×10^{-3} M MIH	18.0	92.6	7.5	0.10[a]
Pretreated (300 mg of MIH/kg)	23.7	106.7	7.2	0.12

[a] The concentration of MIH in this assay was 1×10^{-4} M.

tracts nor the preparation of these extracts from cells treated *in vivo* 3 hr previously with 300 mg of MIH per kilogram, caused a decrease in the activities of these enzymes. Thus, blockade of the incorporation of thymidine-^{3}H into DNA cannot be accounted for by MIH-induced inhibition of these enzymes.

TABLE 3

Retention of thymine-^{3}H residues in DNA of MIH-treated L5178Y ascites cells

Mice bearing 6-day implants of L5178Y lymphoma cells received thymidine-^{3}H (6.6×10^3 cpm/μg) at a level of 32 μg per animal. Two hours later a single intraperitoneal dose of 300 mg of MIH per kilogram of body weight was given, and cells were collected at various time intervals thereafter. The total radioactivity incorporated into the cold 0.4 M perchloric acid insoluble fraction of the cell population was determined. Each figure represents the mean value obtained with 3 mice.

Time after MIH (hr)	cpm/10^6 cells $\times 10^{-3}$		Total cpm $\times 10^{-5}$	
	untreated	MIH-treated	untreated	MIH-treated
3	1.27	1.28	8.60	6.84
24	0.92	0.98	6.57	6.65
48	0.84	0.87	6.48	6.86
72	0.45	0.72	5.10	6.62

Since Berneis *et al.* (8) reported that MIH caused degradation of DNA *in vitro*, through the intermediate production of hydrogen peroxide, the possibility existed that the drug induces a depolymerization of DNA *in vivo* that interferes with synthesis by reducing the ability of DNA to function effectively as a primer. To determine whether extensive breakdown of DNA occurs in drug-treated cells, the DNA of intact L5178Y cells was prelabeled with thymidine-^{3}H; the retention of this label in the DNA of untreated and MIH-treated cells was then compared. The results in Table 3 show that no preferential loss of thymine-^{3}H occurred from the DNA of MIH-treated cells. That the more rapid decrease in the specific radioactivity of DNA from the untreated population is attributable to dilution by newly synthesized cells is consistent with the relative stability of the total quantity of radioactivity present in the entire neoplastic population.

To probe further for the site of the metabolic lesion induced by MIH on the DNA biosynthetic pathway in L5178Y cells, other isotopic substrates that can trace the formation of DNA through both the purine and pyrimidine nucleotide synthetic routes were employed (Tables 4 and 5). The results indicated that a similar degree of inhibition of the synthesis of DNA was induced by MIH regardless of the metabolic pathway monitored, suggesting that a site of blockade common to all these isotopic tracers may be involved in the

TABLE 4

Incorporation of formate-^{14}C and deoxycytidine-^{3}H into the nucleic acids of MIH-treated L5178Y ascites cells

Mice bearing 6-day implants of L5178Y lymphoma cells received a single intraperitoneal dose of 300 mg of MIH per kilogram of body weight. Three hours after dosage with the drug, either formate-^{14}C (11.8×10^4 cpm/μg) at a level of 90 μg per animal or deoxycytidine-^{3}H (1.1×10^3 cpm/μg) at a level of 660 μg per animal was administered, and 1 hr was allowed for metabolic utilization. Each figure represents the mean value (± the standard error), obtained with 3–4 mice.

Isotopic substrate	MIH	cpm/μmole $\times 10^{-2}$			
		DNA thymine	N.A.[a] cytosine	N.A. adenine	N.A. guanine
Formate-^{14}C	−	76.7 ± 2.7		124.7 ± 10.8	11.9 ± 1.0
	+	31.5 ± 5.1		37.6 ± 5.7	4.0 ± 0.3
Deoxycytidine-^{3}H	−	6.0 ± 0.4	3.3 ± 0.3		
	+	3.1 ± 0.4	1.9 ± 0.3		

[a] Nucleic acid (mixed DNA and RNA).

TABLE 5

Incorporation of adenine-8-^{14}C and 4-amino-5-imidazolecarboxamide-2-^{14}C into the DNA and RNA purines of MIH-treated L5178Y ascites cells

Mice bearing 6-day implants of L5178Y lymphoma cells received a single intraperitoneal dose of 300 mg of MIH per kilogram of body weight. Three hours after dosage with the drug, either adenine-8-^{14}C (2.7×10^4 cpm/μg) at a level of 50 μg per animal or 4-amino-5-imidazolecarboxamide-2-^{14}C (1.5×10^4 cpm/μg) at a level of 90 μg per animal was administered, and 1 hr was allowed for metabolic utilization. Each figure represents the mean value (± the standard error), obtained with 3–12 mice.

		cpm/μmole $\times 10^{-2}$			
Isotopic substrate	MIH	DNA adenine	DNA guanine	RNA adenine	RNA guanine
Adenine-8-^{14}C	−	50.6 ± 4.0	7.4 ± 0.7	150.7 ± 9.6	13.7 ± 1.0
	+	18.4 ± 2.2	3.1 ± 0.4	50.2 ± 5.6	7.2 ± 0.9
4-Amino-5-imidazole-carboxamide-2-^{14}C	−	18.9 ± 2.8	15.7 ± 2.4	59.3 ± 7.1	28.1 ± 4.0
	+	5.0 ± 0.7	5.1 ± 1.1	14.2 ± 1.2	10.9 ± 1.0

inhibition of the formation of DNA caused by this agent. The decrease in the rate of synthesis of DNA would not appear to be a result of the unavailability of a supply of one or more of the deoxyribonucleotides required for the formation of DNA, since a mixture of deoxycytidine, deoxyadenosine, and deoxyguanosine did not reverse the MIH-induced inhibition of the incorporation of thymidine-^{3}H into the DNA of

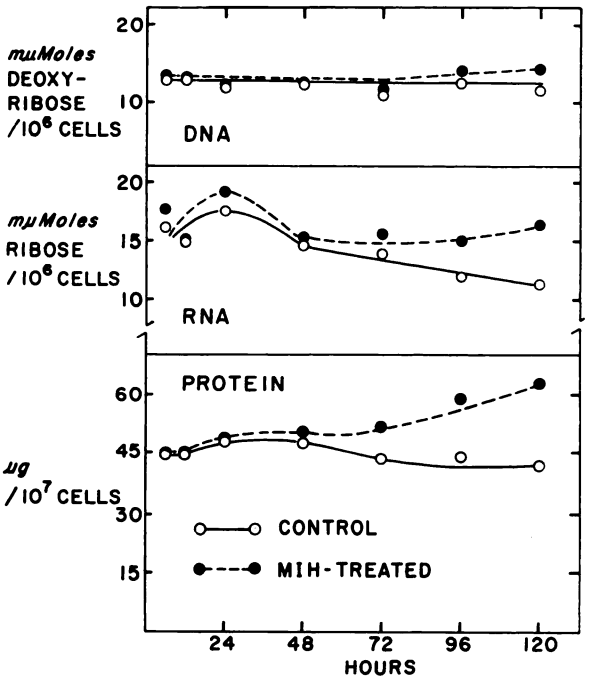

FIG. 3. *The effects of MIH on the average cellular content of DNA, RNA, and protein*

Mice bearing 5-day implants of tumor were given a single intraperitoneal dose of 300 mg of MIH per kilogram. At selected time intervals thereafter, ascites cells were collected from the abdominal cavities; the average cell contents of DNA and RNA were determined by pentose analyses and of residual protein by the biuret reaction. Each point represents the mean value of results from 3–4 determinations, each representing pooled cells from 2 animals.

L5178Y ascites cells; however, no direct evidence to support this concept is available.

The relatively transient effects on nucleic acid and protein metabolism that have been described largely precede the major loss in lymphomatous cells from the abdominal cavities of methylhydrazine-treated animals (Fig. 1). To determine changes in metabolic processes that accompanied cell death, the average DNA, RNA, and protein contents of cells exposed to the drug were measured. The results in Fig. 3

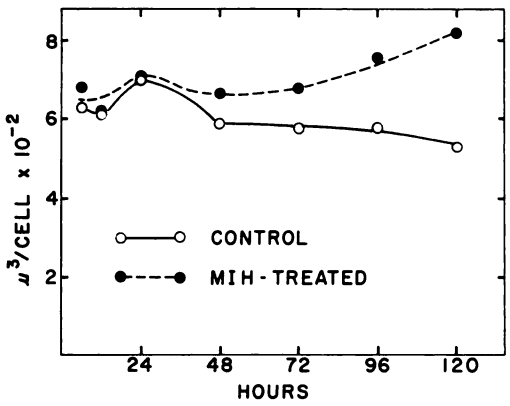

FIG. 4. *The effect of MIH on the average volume of lymphoma L5178Y ascites cells*

Mice bearing 5-day implants of tumor were given a single intraperitoneal dose of 300 mg of MIH per kilogram. At selected time intervals thereafter, ascites cells were collected from the abdominal cavities, and their average volume was estimated in hematocrit tubes by measuring the volume occupied by a known number of cells. Each point represents the mean value of results from 3–4 determinations, each representing the pooled cells from 2 animals.

show that an imbalancing of metabolic processes occurred after exposure of cells to MIH. The average cellular content of DNA deoxyribose remained constant in cells isolated from untreated animals, whereas a slight but not statistically significant increase in DNA, 96–120 hr after the drug, occurred in those cells treated with MIH. The average cellular content of RNA ribose of untreated lymphoma L5178Y cells varied with growth as previously described (24); thus, a peak cellular content of RNA occurred 6 days after tumor cell implantation. Subsequently, a progressive decrease in the cellular RNA content occurred; however, exposure of these cells to MIH prevented the decrease in RNA content. The cellular content of residual protein, which decreased only slightly with increasing cell age, also was sensitive to MIH, and a pronounced accumulation occurred. This drug-induced imbalancing of metabolic processes was accompanied by an increase in the average volume of the neoplastic cells (Fig. 4).

DISCUSSION

Exposure of L5178Y lymphoma cells to the methylhydrazine derivative MIH caused pronounced inhibition of the synthesis of DNA. The inhibition could be detected by utilizing isotopic tracers that monitored the formation of DNA through both the purine and the pyrimidine deoxyribonucleotide biosynthetic pathways, a finding which suggested that either the site of blockade by MIH was common to each of the isotopic tracers employed, or the intracellular concentration of some essential metabolite was depressed to a level that limited the formation of DNA. The activities of thymidine kinase, thymidine monophosphate kinase, thymidine monophosphate nucleotidase, and DNA nucleotidyltransferase were not depressed in cell-free extracts prepared from MIH-treated cells, nor were these enzymic activities sensitive to the addition of MIH. These results appear to eliminate these enzymes as possible sites of MIH-induced blockade; however, it is conceivable that some metabolite of MIH is an active inhibitor of one or more of these enzymes. Although enzymic activities of cell-free extracts prepared from MIH-treated cells were not lower than those of normal cells, the presence of an inhibitory metabolite may have been masked by dilution during preparation of the cell-free extracts.

The precise site of the metabolic lesion which yields inhibition of the synthesis of DNA, RNA and protein is unknown, and depression of the rate of formation of these polymers may well be due to blockade of some event which leads to inhibition of cell

division. The observed alterations would thus be considered secondary events. An equally plausible hypothesis, however, is that the drug interferes with the primer function of DNA in the DNA nucleotidyltransferase reaction by a drug-induced modification of the structure of preformed DNA molecules. The evidence that this class of agents causes chromatid breaks in Ehrlich ascites carcinoma cells might be indicative of this kind of lesion; in contrast to the alkylating agents, however, no complete breaks in the structure of the chromosomes are observed in the drug-treated cells (7). These cytological findings are not inconsistent with the present results which indicate that MIH did not influence the rate of loss of thymine-^{3}H from prelabeled DNA. Thus, minor alterations in the structure of the DNA may be produced by the methylhydrazine derivatives, and these changes could be expressed as a few lesions in the DNA chain; however, no extensive depolymerization of DNA occurs in drug-treated cells. Such structural alterations, if they occur, can be envisioned to interfere with the primer functions of DNA; thus, this concept not only explains the decrease in the rate of formation of DNA, but it also accounts for the inhibition of the synthesis of RNA in MIH-treated L5178Y cells, a process which essentially parallels in duration the inhibition of the biosynthesis of DNA. It is of interest that the time course for drug-mediated inhibition of the formation of proteins does not correspond to that of the nucleic acids; there is a lag in the appearance of inhibition by MIH of the fixation of isotopic leucine into residual protein, and this inhibition reaches a maximum at a time when the synthesis of the nucleic acids is recovering from the effects of the drug. No data are available to decide whether these effects are attributable to either distinct metabolic lesions in these biosynthetic pathways or a single alteration of a metabolic event.

Although some cell death occurred during the period in which inhibition of the synthesis of nucleic acids and proteins was measured, maximum loss of cells occurred after these biosynthetic processes had recovered from the inhibitory action of the drug. Thus, it is difficult to implicate directly these effects in the phenomenon of cell death. Rather, it would appear that some additional secondary event is responsible for the major decrease in the number of cells in the abdominal cavities of MIH-treated mice. The exact site of the lesion responsible for the death of the lymphoma cells is not evident from the available data; however, an imbalancing of metabolic processes accompanied the major decrease in cell number. This imbalance was characterized by an accumulation of RNA and protein and an increase in the average size of the cells. Whether the relatively short-lived inhibition of the synthesis of either DNA, RNA, or protein is directly responsible or whether such blockade triggers a metabolic event responsible for the imbalance remains to be elucidated.

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REFERENCES

1. W. Bollag and E. Grunberg, *Experientia* **19**, 130 (1963).
2. W. Bollag, *Cancer Chemotherapy Rept.* **33**, 1 (1963).
3. V. T. Oliverio, C. Denham, V. T. DeVita and M. G. Kelly, *Cancer Chemotherapy Rept.* **42**, 1 (1964).
4. A. C. Sartorelli and S. Tsunamura, *Proc. Am. Assoc. Cancer Res.* **6**, 55 (1965).
5. G. Mathe, O. Schweisguth, M. Schneider, J. L. Amiel, L. Berumen, G. Brule, A. Cattan and L. Schwarzenberg, *Lancet* **ii**, 1077 (1963).
6. G. Martz, A. D'Alessandri, H. J. Keel and W. Bollag, *Cancer Chemotherapy Rept.* **33**, 5 (1963).
7. A. Rutishauser and W. Bollag, *Experientia* **19**, 131 (1963).
8. K. Berneis, M. Kofler, W. Bollag, A. Kaiser and A. Langemann, *Experientia* **19**, 132 (1963).

9. K. Berneis, M. Kofler, W. Bollag, P. Zeller, A. Kaiser and A. Langemann, *Helv. Chim. Acta* **46**, 2157 (1963).
10. J. Raaflaub and D. E. Schwartz, *Experientia* **21**, 44 (1965).
11. G. Weitzel, F. Schneider and A.-M. Fretzdorff, *Experientia* **20**, 38 (1964).
12. W. Kreis and W. Yen, *Experientia* **21**, 284 (1965).
13. M. Baggiolini, M. H. Bickel and F. S. Messiha, *Experientia* **21**, 334 (1965).
14. W. C. Schneider, *in* "Methods in Enzymology" (S. P. Colowick and N. D. Kaplan, eds.), Vol. 3, p. 680. Academic Press, New York, 1955.
15. E. Layne, *in* "Methods in Enzymology," (S. P. Colowick and N. D. Kaplan, eds.), Vol. 3, p. 447. Academic Press, New York, 1955.
16. E. P. Tyner, C. Heidelberger and G. A. LePage, *Cancer Res.* **13**, 186 (1953).
17. A. Marshak and J. Vogel, *J. Biol. Chem.* **189**, 597 (1951).
18 P. B. Danneberg, B. J. Montag and C. Heidelberger, *Cancer Res.* **18**, 329 (1958).
19. A. C. Sartorelli, *Methods Med. Res.* **10**, 285 (1964).
20. A. C. Sartorelli and B. A. Booth, *Biochim. Biophys. Acta* **55**, 214 (1962).
21. B. A. Booth and A. C. Sartorelli, *J. Biol. Chem.* **236**, 203 (1961).
22. A. C. Sartorelli and B. A. Booth, *Cancer Res.* **25**, 1393 (1965).
23. R. Mantsavinos and E. S. Canellakis, *Cancer Res.* **19**, 1239 (1959).
24. S. Tsunamura and A. C. Sartorelli, *Cancer Res.* **26**, 75 (1966).