(1968).
6. Reichert, L. E. and Midgley, A. R., Proc. Soc. Exptl. Biol. Med. 128, 1001 (1968).
7. Reece, R. P. and Turner, C. W., Missouri Univ. Ag. Expt. Sta. Bull. 266, (1937).
8. Niswender, G. D., Reichert, L. E., Midgley, A. R., and Nalbandov, A. V., Endocrinology, (in press).
9. Niswender, G. D., Midgley, A. R., Monroe, S. E., and Reichert, L. E., Proc. Soc. Exptl. Biol. Med. 128, 807 (1968).
10. Kragt, C. L. and Meites, J., Proc. Soc. Exptl. Biol. Med. 121, 805 (1966).
11. Kwa, H. G. and Verhofstad, F., J. Endocrinol. 39, 455 (1967).
12. Clemens, G. A. and Meites, J., Endocrinology 82, 878 (1968).
13. Meites, J. and Nicoll, C. S., Ann. Rev. Physiol. 28, 57 (1966).

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Studies on the Mode of Action of *N*-Isopropyl-α-(2-methylhydrazino)-*p*-toluamide (MIH) (33658)

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(Introduced by F. Bernheim)

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N-Isopropyl-α-(2-methylhydrazino)-*p*-toluamide (NIH) is one of a group of hydrazine derivatives that have been shown to be effective in the chemotherapy of experimental animal tumors (1,2). In man, MIH has been used specifically in the treatment of Hodgkin's Disease (3). Its exact mechanism of action is unknown, but the unaltered form is not thought to be biologically active (4) whereas, several of its possible metabolites may affect cellular metabolic processes. For example, in common with other hydrazine derivatives, MIH autoxidizes to produce hydrogen peroxide (5). It is possible that the intracellular generation of peroxide contributes to its cytotoxicity. We have previously described some of the metabolic consequences of the autoxidation of chemotherapeutic agents (6–8). Subsequent to autoxidation, isomerization and cleavage of the azo derivative of MIH may yield a variety of potentially toxic intermediates. These include aldehydes, other hydrazines, and methane. The formation of methane from the *N*-methyl group of MIH was demonstrated in rodents (9), and it is of special interest that this group also appears to be essential for biological activity (10). For these reasons, it was suggested that the transitory formation of methyl free-radicals may be intimately associated with the chemotherapeutic action of MIH (9).

Cytological studies revealed that MIH also exerts a marked influence on the mitotic cycle. In Ehrlich ascites carcinoma cells a suppression of mitosis brought about by a prolongation of interphase has been demonstrated (11). An outstanding feature of the cytological effects of MIH is the appearance of numerous chromatid breaks (11), and the treatment of cells with the drug over many transplant generations resulted in the formation of resistant lines (1). The present experiments were undertaken to explore the possible biochemical relationships between the chemotherapeutic actions of MIH and the development of resistance.

Materials and Methods. Ehrlich ascites cells were grown in male albino Swiss-Webster mice weighing from 21 to 25 g. Transplantation was carried out aseptically by collecting ascites fluid from mice after 8–10 days. The fluid was drawn into heparinized syringes and 0.2 ml (containing approximately 4×10^6 cells) was inoculated intraperitoneally into each receptor animal.

For *in vivo* experiments MIH was dissolved in isotonic saline just before use and injected subcutaneously into mice 6–8 days after inoculation of the ascites tumor. Cells were harvested from the peritoneal cavity by sacrificing the animals at appropriate time intervals after treatment with the drug. If

erythrocytes were present they were lysed by exposing the cells to ice-cold distilled water for 30 sec. The cells were then washed three times with isotonic saline and finally resuspended to the appropriate volume with Krebs–Ringer medium containing 100 mg/100 ml of glucose. The suspensions were standardized after counting with a Coulter (model A) particle counter.

The effects of MIH on DNA, RNA, and protein synthesis were determined by measuring the uptake of thymidine-^{3}H, uridine-^{3}H, and arginine-^{3}H, respectively. Isotopes were obtained from the New England Nuclear Corporation. Cells previously exposed to MIH *in vivo* for appropriate time intervals were incubated in duplicate in 25-ml Ehrlenmeyer flasks in a Dubnoff shaker at 37°. The incubation time was 30 min. In addition to 2.5 ml of suspended cells, the flasks contained either 2 μCi of thymidine-^{3}H (6.7 Ci/mmole) or 2 μCi of uridine-^{3}H (12.1 Ci/mmole). Duplicate samples (0.1 ml) of the incubation mixture were removed and placed on filter paper discs. The discs were rapidly dried and washed three times with cold trichloroacetic acid (5%). They were held in Hokin's solution for 30 min at 37° and then in Hokin's solution and ether (1:1) at 37° for 30 min. Finally, they were rinsed twice with ether, dried, and placed in Bray's solution. Radioactivity was determined with a Beckman scintillation counter. The incorporation of arginine into protein was carried out as outlined above except that unlabeled arginine (1.0 mmole) was added to the flasks along with 4 μCi of arginine-^{3}H (1.1 Ci/mmole). Duplicate samples (0.1 ml) were removed; rapidly dried on filter paper discs; and washed three times with cold trichloroacetic acid (5%; washed in a mixture of chloroform, ethanol, and ether (2:2:1); dried; and placed in Bray's solution for counting. The results presented represent the average of duplicate determinations repeated with at least six animals at each time period.

After the cells were stained with Wright's stain, the percentage of mitosis in 1000 counted cells was recorded. The cells had been previously incubated with colchicine (0.2 μg/ml) for 2 hr to arrest them in metaphase.

Results. The growth of Ehrlich ascites cells is initially quite sensitive to MIH. Treatment of tumor bearing animals with a single subcutaneous dose of the drug (200 mg/kg) results in a noticeable prolongation of survival time. In order to examine some of the biochemical changes produced by the drug, ascites cells from animals treated with a single dose of 200 mg/kg were collected at appropriate time intervals, and the incorporation of thymidine-^{3}H, uridine-^{3}H, and arginine-^{3}H into DNA, RNA, and protein, respectively, was measured (Fig. 1). Exposure of cells to MIH caused an inhibition of thymidine incorporation into DNA thymine. The inhibition reached a maximum level (35–40%) when the cells were pulsed *in vitro* with thymidine-^{3}H from 4 to 8 hr after *in vivo* exposure to MIH. After this time thymidine utilization gradually returned to its normal level. Although not illustrated in Fig. 1, at 72 hr no residual effect of the drug on DNA formation was observed.

Inhibition of the fixation of arginine into protein also occurred. The onset of this effect, however, was delayed more than the effect on DNA. As illustrated in Fig. 1, no inhibition of protein synthesis was observed up to 8 hr after the administration of MIH. At 16 hr the incorporation of arginine-^{3}H was maximally depressed, and by 24 hr this pathway appeared to recover somewhat from inhibition. No inhibition was observed at 48 hr.

In contrast to its effects on the synthesis of DNA and protein, MIH consistently produced a marked but transitory enhancement of the incorporation of uridine-^{3}H into RNA. This is also illustrated in Fig. 1. The stimulation of this pathway became maximal between 8 and 16 hr after MIH treatment. At 42 hr uridine utilization returned to its normal level.

The addition of freshly prepared solutions of MIH had no effect on the incorporation of thymidine-^{3}H, uridine-^{3}H, and arginine-^{3}H in Ehrlich cells treated *in vitro*.

Although the synthesis of DNA was only inhibited about 40% after a single exposure of cells to MIH (200 mg/kg), mitosis in the

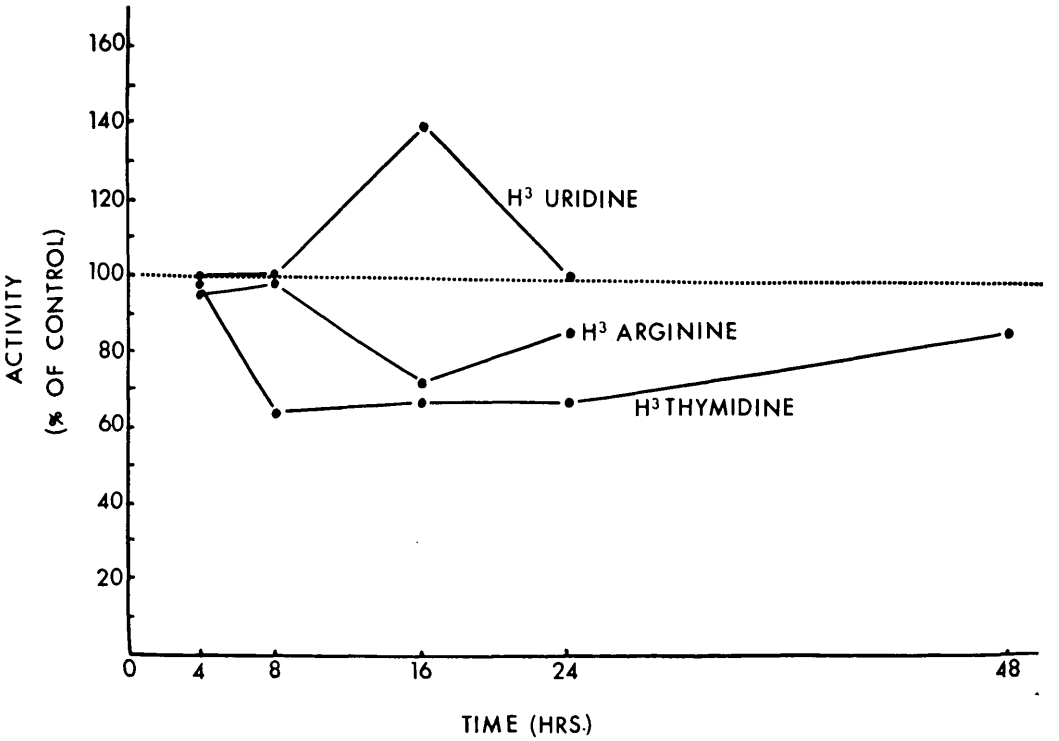

FIG. 1. The effect of MIH (200 mg/kg) on the incorporation of thymidine-^{3}H, uridine-^{3}H, and arginine-^{3}H in Ehrlich ascites cells.

cell population was completely blocked. This is illustrated in Table I. It may be noted that maximal inhibition of cell division was observed 8 hr after the drug was administered, and that it remained suppressed beyond the period in which the synthesis of DNA was restored to its original value.

The prolonged suppression of mitosis accompanied by the shorter retardation of DNA and protein synthesis suggested that these latter effects were coincidental rather than the cause of mitotic arrest. We therefore attempted to determine whether the pronounced effect on cell division could be attributed to some more primary effect of MIH on other metabolic processes. Cells which had been exposed to MIH (200 mg/kg) 8 hr previously were harvested and incubated *in vitro* to study the rates of respiration, aerobic glycolysis, and glucose oxidation via the hexose monophosphate shunt. Despite the fact that cell division had ceased and DNA and protein synthesis were maximally inhibited, there was no difference between MIH-treated and control cells in the oxygen uptake and in the production of $^{14}CO_2$ from either glucose-1-^{14}C or glucose-6-^{14}C. However, the aerobic glycolysis of MIH-treated cells was depressed about 25% when compared to untreated cells.

TABLE I. Mitotic Index and Thymidine-^{3}H Incorporation into DNA by Ehrlich Ascites Cells.

After injection (hr)	Mitotic index for 1000 cells (%)		Thymidine-^{3}H incorporation after MIH (% of control)
	Control (saline)	MIH (200 mg/kg)	
4	5.5	5.0	100
8	3.8	0.5	62
24	3.5	0.2	65
48	3.5	0.8	87
72	3.5	1.5	100

As shown in Table II when Ehrlich ascites cells were exposed to MIH (200 mg/kg) three times at 72-hr intervals (total dose = 600 mg/kg) survival time of the tumor-bearing animals was nearly doubled. In addi-

TABLE II. Development of MIH Resistant Sublines in Ehrlich Ascites Cells.

Transfer generation	Av survival (days) treated/untreated	Karyotype changes
Treatment: MIH, 200 mg/kg every third day × 3 doses		
Passage 1	23/12	2 Translocation in 80% of the cells in mitosis
Passage 2	15/12	
Treatment: MIH, 200 mg/kg × 2 days, 100 mg/kg × 2 days		
Passage 1	22/12	Some polyploidy; giant cells
Passage 2	23/12	Occasional translocation
Passage 3	18/12	2 Translocations/cell in about 30% of cells undergoing mitosis
Passage 4	15/12	2 Translocations in every cell in mitosis

tion, two new metacentric chromosomes were observed in 80% of the cells undergoing mitosis. These cells became resistant to the drug after the first passage, and the survival time, in days, of the treated animals compared to untreated animals, decreased from 23/13 in the first passage to 15/12 in the second passage. Instead of administering MIH at intervals to coincide with the resumption of DNA synthesis, the drug also was administered so that the same total dose (600 mg/kg) was received in a single 72-hr period during which DNA synthesis remained suppressed. In these experiments the development of resistance was delayed through four passages of the cells (Table II). The resistant cell line developed in this slow manner was also characterized by the appearance of two new metacentric chromosomes in every cell in mitosis.

Discussion. Gale *et al.* (4), have reported that the synthesis of DNA, RNA, and protein is markedly inhibited after *in vitro* incubation of Ehrlich ascites cells with "aged solutions" of MIH. Similar effects of MIH on these major metabolic pathways have been described by Sartorelli and Tsunamura (12), (in animals bearing L5178Y lymphoma cells) and by Weitzel *et al.* (13), (in animals bearing Ehrlich ascites cells). We have confirmed the finding (4) that freshly prepared solutions of MIH are devoid of such activity in Ehrlich cells. The clear implication of these experiments is that MIH must be converted to some biologically active form. The nature of the active metabolite(s) of MIH and the mechanism(s) of their effects on macromolecular synthesis in tumor cells is unknown. However, the requirement of the *N*-methyl group for chemotherapeutic activity (10) and the demonstration that this group is converted to methane (9) has strongly implicated the importance of a transitory methyl free-radical in its action.

In contrast to the experiments of Gale *et al.* (4), Sartorelli and Tsunamura (12), and Weitzel *et al.* (13), we have not been able to demonstrate an inhibitory effect of MIH on the incorporation of uridine-^{3}H into RNA. In our experiments, MIH has consistently stimulated this process in a transitory manner. The reasons for this difference are not clear. Since we have not characterized the species of RNA formed, the possible mechanisms of this effect are also not clear. The fact that the stimulation either precedes or coincides with the returns of protein synthesis to normal levels suggests that the two events are related. In any case, it seems that under the conditions of our experiments, inhibition of RNA synthesis is not a prerequisite for the chemotherapeutic action of MIH. In agreement with other workers, we have found that a single dose of 200 mg/kg of MIH will depress the incorporation of thymidine-^{3}H into DNA for periods up to 48 hr. With this amount of drug DNA synthesis was depressed about 40%. As reported by Sartorelli and Tsunamura (12), this effect is dosage related. For example, we have found that DNA synthesis is inhibited about 60% when the amount of MIH is increased to 300 mg/kg. It is of special interest that after 48 hr, when DNA synthesis has almost returned to normal levels, cell division remains completely depressed. At 72 hr, when the synthesis of DNA is identical with that of control cells, mitosis is still markedly lowered. These experiments suggest that MIH has a primary

effect on some process which leads to mitotic arrest and that its "short-lived" action on DNA (and protein) synthesis either follow from this action or are unrelated to it. Our experiments do not help to define the nature of this primary effect. We were not able to demonstrate any action of MIH on cellular energy metabolism other than a small inhibition of aerobic glycolysis. The view that MIH exerts a primary effect on some process other than the synthesis of nucleic acids and protein is in accordance with that of Sartorelli and Tsunamura (12). They demonstrated that cell death occurred after these biosynthetic processes had recovered from the inhibitory action of the drug. The site of the lesion responsible for the death of lymphoma cells in their experiments was not clear. It is anticipated that further experiments with the new cell lines resistant to MIH may help to define the minimum metabolic events associated with cell death.

Although the chemotherapeutic action of MIH on tumor cells may be unrelated to its effect on nucleic acid synthesis, the rapid development of resistance can be induced by administering the drug at selected intervals during which DNA synthesis is not inhibited. Thus a resistant cell line was developed in a single transplant generation by treating the cells with MIH at intervals to coincide with the return of DNA synthesis to control levels. If the same dose of drug was administered to animals in consectutive 24-hr periods; so that the total dose was received during the period of maximum depression of DNA synthesis, then resistance did not develop until the fourth transplant generation. These observations may have clinical relevance in the treatment of Hodgkin's disease. The resistant cell lines produced after MIH treatment were characterized by karyotype analysis and all scorable cells contained two new metacentric chromosomes. A description of these chromosomes has been presented elsewhere (14). Whether these new chromosomes are causally related to the development of resistance is not known. However, similar chromosomal changes in a resistant line of L1210 cells have been previously described after treatment with methotrexate (15).

Summary. The relationship between some of the chemotherapeutic effects of *N*-isopropyl-α-(2-methylhydrazino)-*p*-toluamide (MIH) and the development of resistance in Ehrlich ascites cells was explored. Whereas DNA and protein synthesis is moderately inhibited in these cells after the *in vivo* administration of the drug, the synthesis of RNA is stimulated. Cell division is markedly suppressed by MIH for periods of time beyond its effects on nucleic acid metabolism. Energy metabolism of the cells is not significantly affected by the drug. It is concluded that the primary chemotherapeutic effect of MIH is on some process associated with cell division other than nucleic acid or energy producing metabolism. A line of cells resistant to MIH was developed. Resistance was induced much more rapidly when MIH was given to coincide with periods of normal DNA synthesis. Resistance developed more slowly when the drug was given over a period in which DNA synthesis was depressed. In both cases, the resistant line was characterized by the appearance of two new metacentric chromosomes in each cell in mitosis.

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1. Bollag, W. and Grunberg, E., Experientia **19**, 130 (1963).

2. Zeller, P., Gutman, H., Hegedus, B., Kalser, A., Langemann, A., and Muller, M., Experientia **19**, 129 (1963).

3. Brunner, K. W. and Young, C. W., Ann. Internal Med. **63**, 69 (1965).

4. Gale, G. R., Simpson, J. G., and Smith, A. B., Cancer Res. **27**, 1186 (1967).

5. Berneis, K., Kofler, M., Bollag, W., Kaiser, W., and Langemann, A., Experientia **19**, 132 (1963).

6. Hochstein, P. and Cohen, G., Ann. N. Y. Acad. Sci. **100**, 876 (1963).

7. Cohen, G. and Hochstein, P., Biochemistry **3**, 895 (1964).

8. Miller, D. S., Laszlo, J., McCarty, K. S., Guild, W. E., and Hochstein, P., Cancer Res. 27, 632 (1967).

9. Dost, F. N. and Reed, R. J., Biochem. Pharmacol. 16, 1741 (1967).

10. Bollag, W., *in* "Symposium on Chemotherapy of Cancer," p. 191. Elsevier, Amsterdam, (1964).

11. Rutishauser, A. and Bollag, W., Experientia 19, 131 (1963).

12. Sartorelli, A. C. and Tsunamura, S., Mol. Pharmacol. 2, 275 (1966).

13. Weitzel, V. G., Schneider, F., Hirschmann, W. D., Durst, J., Thauer, R., Ochs, H., and Kummel, D., Z. Physiol. Chem. 348, 443 (1967).

14. Huang, A., Gutterman, J., and Hochstein, P., Experientia, in press (1968).

15. Biedler, June L., Albrecht, A. M., and Hutchison, D. J., Cancer Res. 25, 246 (1965).

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Dietary Cadmium, Iron, and Zinc Interactions in the Growing Rat. (33659)

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The depressing effect of dietary cadmium on blood hemoglobin has been demonstrated in several species (1–3). A mutual antagonism between Cd and Zn has been established (3–7) and a partial amelioration of cadmium toxicity in the chick was noted with Cu fed in conjunction with 100 ppm of Zn (1). With Zn at 25 ppm, both Fe and Cu supplements were shown to ameliorate Cd toxicity.

Cadmium decreases longevity in male mice and possesses innate toxicity (8). These toxic properties of Cd make it desirable to elucidate the factors which will minimize the adverse effects on animals of environmental Cd contamination. The present experiments were undertaken to test the interactions between dietary Cd, Fe, and Zn in the growing rat with respect to effect on body weight gain, blood hemoglobin concentration and tissue levels of trace elements.

To test these interactions, weanling rats were fed diets identical in all respects, within each experiment, except for the level of Cd, Fe, and Zn. These trace elements were added to the basal diet alone and in all possible combinations so that the influence of one on the response to others could be determined. In addition to dietary variables, two experiments involved cage type (galvanized vs stainless steel) as an environmental contribution to Zn ingestion in combination with dietary supplements of Cd and Fe alone and together. Evidence is presented on the protective effect of Fe, in combination with Zn, against Cd toxicity.

Methods. Expt. 1. Forty weanling rats (av wt. 46 ± 7 g) were assigned by weight and sex in a 2 × 2 factorial arrangement of dietary treatments. These were: diet 1, basal (B) (Table I), 100 ppm Fe (as $FeSO_4 \cdot 7H_2O$), O Cd (Table I); diet 2, 100 ppm Cd (as $CdCl_2$); diet 3, 400 ppm Fe, 0 Cd; and diet 4, 400 ppm Fe, 100 ppm Cd. Animals were kept individually in wire cages. Feed was offered *ad libitum* from porcelain feeders and distilled water from glass bottles with stainless steel tubes.

After 4 weeks, rats were weighed, anesthetized with diethyl ether, blood was drawn from the posterior vena cava of each rat for hemoglobin determination and liver was excised from one-half of each group chosen at random. Blood hemoglobin was determined as oxyhemoglobin with 0.1% Na_2CO_3 as a diluent (9). Livers were freeze-dried, ground, ashed, and an aliquot of each was used for spectrographic analysis.[3]

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[3] Analysis was performed in the Spectrographic Laboratory, New York State College of Agriculture, Ithaca, New York 14850.