of isozyme patterns was specific for the 3'-isomer.

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1. Bailey, J. M., Clough, J., Stearman, M., Proc. Soc. Exp. Biol. and Med., 1964, v117, 350.
2. Mahy, B. W. J., Rowson, K. E. K., Salaman, M. H., Parr, C. W., Virology, 1964, v23, 528.
3. Notkins, A. L., Scheele, C., J. Nat. Cancer Inst., 1964, v33, 741.
4. Riley, V., Loveless, J. D., Fitzmaurice, M. A., Siler, W. M., Life Sci., 1965, v4, 487.
5. Appella, E., Markert, C. L., Biochem. Biophys. Res. Commun., 1961, v6, 171.
6. Cahn, R. K., Kaplan, N. O., Levine, L., Zwilling, E., Science, 1962, v136, 962.
7. Markert, C. L., ibid., 1963, v140, 1329.
8. Shaw, C. R., Barto, E., Proc. Nat. Acad. Sci., 1963, v50, 211.
9. Markert, C. L., Moller, F., ibid., 1959, v45, 753.
10. Vesell, E. S., Philip, J., Bearn, A. G., J. Exp. Med., 1962, v116, 797.
11. Dawson, D. M., Goodfriend, T. L., Kaplan, N. O., Science, 1964, v143, 929.
12. Cohen, L., Djordjevich, J., Ormiste, V., J. Lab. Clin. Med., 1964, v64, 355.
13. Wieme, R. J., Studies on Agar Gel Electrophoresis; Techniques, Applications, Arscia Uitgaven N. V., Brussels, 1959, 457.
14. Wieme, R. J., Van Maercke, Y., Ann. N. Y. Acad. Sci., 1961, v94, 898.
15. Kline, E. S., Clayton, C. C., Proc. Soc. Exp. Biol. and Med., 1964, v117, 891.
16. McCoy, T. A., Maxwell, M., J. Nat. Cancer Inst., 1959, v23, 385.
17. Smithies, O., Biochem. J., 1959, v71, 585.
18. Dewey, M. M., Conklin, J. L., Proc. Soc. Exp. Biol. and Med., 1960, v105, 492.
19. Fine, I. H., Costello, L. A. in Methods in Enzymology (S. P. Colowick and N. O. Kaplan, eds.), Academic Press, Inc., New York, 1963, v6, 958.
20. Green, H. N., Ghose, T., Nature, 1964, v201, 308.
21. Greenstein, J. P., Cancer Res., 1956, v16, 641.
22. Weber, G., Adv. Cancer Res., 1961, v6, 403.
23. Shrivastava, G. C., Quastel, J. H., Nature, 1962, v196, 876.
24. Van Wijhe, M., Blanchaer, M. C., St. George-Stubbs, S., J. Histochem. Cytochem., 1964, v12, 608.
25. Vesell, E. S., Brody, I. A., Ann. N. Y. Acad. Sci., 1964, v121, 544.
26. Burgess, E. A., Sylvén, B., Cancer Res., 1963, v23, 714.

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Monoamine Oxidase Inhibition by a New Carcinostatic Agent, N-Isopropyl-α-(2-Methylhydrazino)-p-Toluamide (MIH). (30590)

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In 1963, a new class of carcinostatic agents was introduced by Bollag and Grunberg(1). One compound in this class, N-isopropyl-α-(2-methylhydrazino)-p-toluamide hydrochloride (MIH; RO-4-6467/1, NSC-77213), has been subjected to extensive clinical trials in Europe and has been shown to possess considerable activity against Hodgkin's disease (2,3). In addition to an overall 60% response rate, including 30% complete remissions, there appears to be no cross resistance between this drug and others used in the treatment of this disease. The major toxicity, like most other antitumor agents, has been hematopoietic. In addition, troublesome central nervous system toxicity in the form of hyperirritability, euphoria, ataxia and nystagmus has been noted, and the drug also potentiates the effects of chloropromazine derivatives, barbiturates and alcohol.

Because of its hydrazine structure and the presence of the above side effects, we considered the possibility that some of these symptoms might be due to inhibition of the enzyme, monoamine oxidase (MAO). The latter was determined by measurement of the

inhibition of guinea pig liver monoamine oxidase and potentiation of the central nervous system effects of L-tryptophan. The results of these experiments form the substance of this report.

Methods. MAO activity of guinea pig liver was determined using a modification of the method of Weissbach *et al*(4). Male NIH all-purpose guinea pigs, weighing 250 to 500 g, were injected intraperitoneally (IP) with a single dose of 175 mg/kg of MIH and sacrificed by a blow on the head at a predetermined time. The liver was quickly excised, weighed and homogenized in cold distilled water with a Potter-Elvehjem homogenizer. After straining through 2 layers of cheesecloth, the homogenate volume was adjusted with cold distilled water so that each ml contained 200 mg of liver. The homogenate was then centrifuged at 1000 *g* for 30 minutes at —4°C in an International PR centrifuge. The supernatant fluid was removed and used as the source of enzyme for the assay.

To each 4 ml quartz cuvette were added 0.5 ml of 0.5 molar phosphate buffer pH 7.4, 0.1 ml of the liver homogenate, and distilled water to a volume of 3.1 ml. Immediately after addition of 0.4 ml of kynuramine (0.5 μmol), the contents of the cuvette were mixed, and the reaction followed at an ultraviolet absorption wave length maxima of 360 mμ from 1 to 30 minutes at 5-minute intervals using a Beckman Model DB Recording Spectrophotometer. Distilled water replaced kynuramine in the sample which served as a blank. Livers of animals treated IP with 20 mg/kg of pheniprazine (JB 516), a known potent *in vivo* and *in vitro* MAO inhibitor, were simultaneously run at various intervals, and the results compared to MIH-treated animals. Untreated animals were used as controls with each determination, and all samples were run in duplicate. All drugs were administered in 0.85% saline solution. MAO activity was expressed in terms of percent of the control activity for that particular determination.

Estimation of MAO inhibitory activity of MIH, monomethylhydrazine, JB 516, and N-isopropyl terepthalamic acid (a metabolite of MIH) was determined *in vitro*. These compounds, in molar concentrations equivalent to an *in vivo* dose of MIH (assuming equal distribution of the drug in body water), were each incubated at 37°C in a Dubnoff shaker with 2 g of guinea pig liver mince and 0.4 ml of 0.5 M phosphate buffer pH 7.4. Following an incubation period of 30 to 90 minutes, the supernatant fluid was decanted, and the liver prepared for MAO assay as described above.

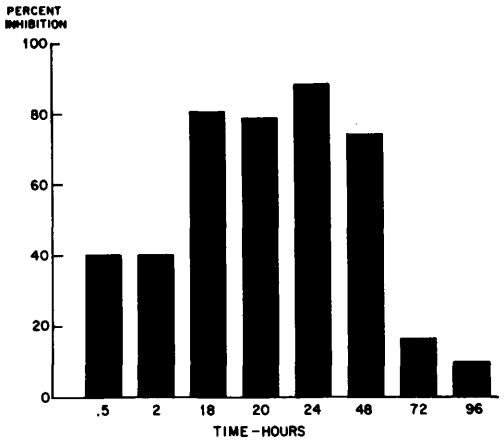

FIG. 1. Guinea pig liver monoamine oxidase inhibition by a single intraperitoneal dose of 175 mg/kg of MIH. Each bar represents the average value of 5 guinea pigs.

To evaluate the degree of inhibition of monoamine oxidase in the brain of guinea pigs, the following procedure was employed: Two groups of 6 animals were given a single IP injection of MIH (175 mg/kg) and 3 groups of 6 animals were injected IP with single doses of JB 516 (20 mg/kg). Another group of 9 animals was chronically treated with single daily IP doses of 100 mg/kg of MIH for 5 days. Each group of animals was then given a single IP injection of 1000 mg/kg of L-tryptophan at various intervals. The L-tryptophan was prepared by suspending the finely ground powder in 0.5% saline solution of sodium carboxy methyl cellulose to assist dispersion. The effects were noted at ½- to 1-hour intervals up to 24 hours from the time of the tryptophan injection.

Results. MAO inhibition—in vivo. The results of the MAO determination are illustrated in Fig. 1. At a dose of 175 mg/kg, which was determined to be well under lethal dose for guinea pigs (one death in 100 ani-

mals), 40% inhibition was seen at 30 minutes and at 2 hours. The peak inhibition for this dose was 88% and occurred at 24 hours after MIH treatment. As has been noted for other MAO inhibitors(5), the effects after a single dose persisted to a significant degree through 48 hours and thereafter trailed off.

Results with pheniprazine were similar to those reported by Horita(6) with complete inhibition at 30 minutes persisting for at least 24 hours. Control animals were analyzed with each experiment, and the results were remarkably consistent from day to day with 0.1 ml of the homogenate, as prepared above, metabolizing about 0.5 μmol of substrate in 30 minutes at room temperature.

In vitro *incubations*. Incubation of guinea pig liver alone, in 0.5 M phosphate buffer pH 7.4 at 37°C for periods of one hour or more, resulted in an average loss of 40% of the available enzyme. Samples from the same animals without incubation consistently metabolized about 0.5 μmol of kynuramine, while incubated samples metabolized only 0.3 μmol. When compared to controls, liver incubated in the presence of MIH for periods up to 90 minutes did not inhibit monoamine oxidase. The major metabolic product of MIH, N-isopropyl terepthalamic acid(7), also produced no inhibition. On the other hand, monomethylhydrazine and pheniprazine produced complete inhibition when incubated with liver mince for one hour.

Accentuation of effects of L-tryptophan on the central nervous system. It has been demonstrated that the effects of L-tryptophan on the brain can be markedly potentiated in guinea pigs by pre-treatment with a monoamine oxidase inhibitor(8). A typical syndrome is produced within one-half hour consisting of hyperpyrexia, jerking movements of the head, paralysis of the hind legs, arching of the back, prostration, cyanosis and death when a dose of 1000 mg/kg is administered IP. These effects are produced by accumulation in the brain of pharmacologically active amines resulting from the metabolism of tryptophan. The effects can be prevented by co-administration of a centrally active decarboxylase inhibitor, which prevents the accumulation of these amines by inhibiting their decarboxylation to the active form. Administration of a single dose of L-tryptophan alone at 1000 mg/kg did not produce this syndrome in 10 control animals. There were no deaths in either group of 6 guinea pigs pre-treated with 175 mg/kg of MIH and then given L-tryptophan at 2 and at 24 hours. Animals treated with L-tryptophan at 2 hours appeared irritable but demonstrated no other CNS symptoms, while the animals treated at 24 hours appeared normal. When a single dose of pheniprazine was followed by L-tryptophan at 30 minutes, 18 hours and 24 hours, all the animals had the typical syndrome within 30 minutes, and all 6 animals in each of the 3 groups died between 2 and 3 hours. Chronic treatment of guinea pigs with MIH followed by tryptophan at 2 hours after last dose, however, resulted in death of all animals preceded by CNS symptoms similar to those observed in the pheniprazine-treated animals. No deaths occurred in a control group of 5 guinea pigs given 100 mg/kg of MIH IP daily for as long as 8 days. To rule out the possibility that MIH was also inhibiting brain decarboxylase, thereby preventing expression of brain MAO inhibition, a group of 6 animals was given both single doses of MIH (175 mg/kg) and of JB 516 (20 mg/kg) followed in 24 hours by a single dose of L-tryptophan (1000 mg/kg). All of these animals exhibited the characteristic syndrome and expired within 3 hours.

Discussion. The parent compound of MIH, 1-methyl-2-benzyl hydrazine, was initially synthesized in search of compounds with monoamine oxidase inhibitory properties and was found to be a cytostatic agent(1). A series of hydrazine derivatives was then synthesized in search of a compound with a higher therapeutic index, thus from this group evolved MIH. MIH appears to be a "hit and run" inhibitor of monoamine oxidase, a phenomenon described by Brodie(9). Peak inhibition occurred at 24 hours. Information obtained in our laboratory using radioisotopically labeled MIH has shown that greater than 90% of the drug is excreted in 24 hours. Persistent inhibition at 48 and 72 hours after a single dose is probably related to the time required to synthesize new enzyme.

When compared to pheniprazine, MIH does not appear to be a potent inhibitor of liver MAO, and indeed, was less effective as an inhibitor of brain MAO. The reason for the decreased effect on the central nervous system is not readily apparent since pharmacological studies have shown that the drug readily passes the blood brain barrier(7). Inhibition of brain decarboxylase might explain these results(8), but co-administration of MIH and pheniprazine followed by L-tryptophan produced the effects expected of monoamine oxidase inhibition alone. MIH has been shown to auto-oxidize in aqueous media and physiologic fluid to N-isopropyl terepthalamic acid(7). This degradation suggests the release of monomethylhydrazine, a compound which has not yet been identified in animals or patients treated with MIH. It is not known whether the parent compound or free methylhydrazine acts as the inhibitor of MAO *in vivo*. However, *in vitro*, the parent compound, MIH, had no inhibitory properties, while free monomethylhydrazine is a potent inhibitor at equimolar doses. The metabolite of MIH, N-isopropyl terepthalamic acid, was inactive. Both the delay in onset of peak inhibition and the *in vitro* experiments may indicate that the parent compound must be altered metabolically before it is active as a MAO inhibitor.

The dose used in these experiments in guinea pigs caused death in one out of 100 animals treated. In our own clinical study, the drug is given orally at a dose of 100-150 mg/m^2 body surface area (2.5-3.5 mg/kg) daily in divided doses for periods up to several months. Since the effect of a single dose in animals is delayed and prolonged, one would expect the daily administration of MIH for extended periods to deplete gradually the available supply of the enzyme and the effects to be cumulative.

Several adverse side effects of MAO inhibition have received considerable attention in the literature. Of particular interest are the severe hypertensive reactions associated with headaches, cerebrovascular accidents and pulmonary edema, associated with the ingestion of foods rich in tyramine, particularly ripe cheeses but also beers, wines and yogurt(10). In addition, orthostatic hypotension, usually a by-product of treatment of hypertension with MAO inhibitors has been noted when these drugs are used for depressive reactions.

It is of interest that a patient has been observed with hypertension and Hodgkin's disease whose blood pressure returned to normal following treatment with MIH.*

Although we were unable to demonstrate potentiation of the effects of L-tryptophan on the central nervous system after a single dose of MIH, chronic administration of MIH did result in significant inhibition of brain MAO. In addition, severe side effects associated with administration of MAO inhibitors, hypertensive reactions and orthostatic hypotension, are associated with inhibition of monoamine oxidase outside the brain.

MIH is now being marketed in Europe under the name of Natulan. In this country, it is being widely used in studies designed by the Lymphoma Task Force. Physicians using this drug in the future should be cognizant of the possibility of unwanted side effects associated with inhibition of monoamine oxidase.

Summary. N-isopropyl-α-(2-methylhydrazino)-p-toluamide (MIH), a methylhydrazine derivative, is a member of a new class of carcinostatic agents with significant therapeutic effect in the treatment of Hodgkin's disease. It was found to inhibit guinea pig liver monoamine oxidase to a significant degree with a prolonged effect after a single dose. The compound was not as potent as a known MAO inhibitor, pheniprazine (JB 516). The CNS effects of a tryptophan load following a single dose of MIH were minimal; however, following chronic treatment with MIH, such a tryptophan load produced lethal convulsions indicating significant inhibition of brain MAO. The clinical implications are discussed. Physicians using this compound in the treatment of malignancies should beware of unwanted side effects associated with monoamine oxidase inhibitors.

* Frei, E., III, personal communication.

1. Bollag, W., Grunberg, E., Experientia, 1963, v19, 130.

2. Mathé, G., Schweisguth, O., Schneider, M.,

Amiel, J. L., Berumen, L., Brule, G., Catton, A., Schwartzenberg, L., Lancet, 1963, v2, 1077.

3. Todd, I. D. H., Brit. Med. J., 1965, v1, 628.

4. Weissbach, H., Smith, T. E., Daly, J. W., Witkop, B., Udenfriend, S., J. Biol. Chem., 1960, v235, 1160.

5. Spector, S., Shore, P. A., Brodie, B. B., J. Pharmacol. Exp. Therap., 1960, v128, 15.

6. Horita, A., ibid., 1958, v122, 176.

7. Oliverio, V. T., Denham, C., De Vita, V. T., Kelly, M. G., Cancer Chem. Rep., 1964, v42, 1.

8. Hodge, J. V., Oates, J. A., Sjoerdsma, A., Clin. Pharm. and Ther., 1964, v5, 149.

9. Brodie, B. B., Pharmacologist, 1964, v6, 12.

10. Goldberg, L. I., J.A.M.A., 1964, v190, 456.

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Rapid Changes in Bacteria Following Introduction into Hypertonic Media. (30591)

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When the osmotic environment of Gram-negative bacteria is changed the bacteria respond by losing or gaining water in hypertonic or hypotonic solutions respectively(1). In solutions of non-permeating solutes the bacteria remain in a dehydrated condition and, if the concentration of the solute is great enough, plasmolysis occurs and the protoplast shrinks to a small volume and breaks away from the cell wall. Solutes which can pass freely across the cell membrane allow osmotic equilibrium to be established across the membrane, and the cell volume at equilibrium will be approximately equal to the volume of the cell before it was introduced into the solution of the permeating solute. A convenient technique for study of the changes which bacteria undergo on immersion in various osmotic environments is measurement of the changes in the light scattering ability of suspensions of the bacteria(2,3). However, a limitation to the methods so far used has been the inability to follow the rapid changes in light scattering which occur immediately after bacteria are introduced into a new osmotic environment. From a knowledge of the kinetics of the osmotic dehydration of bacteria obtained from such a technique and rates of entry of permeating substances, estimates of the hydraulic permeability and other pertinent information about the permeability properties of the bacteria might be made. By modifying the rapid mixing stopped-flow apparatus designed by Gibson and Milnes(4) the changes in the intensity of light scattered at 90° to an incident beam can be followed approximately 2.0 to 3.0 milliseconds after a suspension of Gram-negative bacteria is rapidly and efficiently introduced into a new osmotic environment.

Description of apparatus. The modification made to the Gibson-Milnes apparatus is illustrated in Fig. 1; the remainder of the apparatus is exactly as in the original design. The mode of operation is the same as described by Gibson and Milnes(4) except that a suspension of bacteria is mixed with a solution of the solute under investigation by pulling the arm of the hydraulic actuator. A parallel beam of light is directed through the mixed bacterial suspension approximately 5 mm from the second mixer. This distance represents a time of 2.0 to 3.0 milliseconds at the flow rates obtained in the apparatus. A small lens, placed at its focal distance from the illuminated suspension, collects light laterally scattered from the incident beam and directs this light onto the cathode of an end-window photomultiplier. The signal from the photomultiplier is fed into a cathode follower and then into an oscilloscope. The sensitivity of the photomultiplier is varied as required by using different load resistors and the time constant of the circuit is kept at 5 milliseconds by the insertion of an appropriate capacitor in parallel with the load resistor. The oscilloscope trace is triggered by the operation of a microswitch, actuated by the arm

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