Toxicity to Protozoa of Thalidomide Breakdown Products; Counteraction By Nicotinic Acid and Glutamine.* (28665)

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When we reported that nicotinic acid reversed the toxicity of thalidomide toward several protozoa(1), we knew we were dealing with breakdown products of thalidomide. These might be more important than thalidomide itself in producing toxicity. In neutral and aqueous solutions thalidomide is highly unstable, the phthalimide and glutarimide rings opening, with further hydrolysis of the amide groups of glutamine and isoglutamine (2). Here we survey toxicity to the protozoan *Ochromonas danica* of some spontaneous breakdown products of thalidomide and several related compounds. Such information was desirable since it is unknown whether teratogenicity comes from thalidomide *per se* or from its breakdown products.

Materials and methods. Toxicity of the compounds tested (Table I) was surveyed with 4 different protozoa as described(1). Since results among the protozoa were consistent for the compounds listed only the effects on *Ochromonas danica* are given because it was the most sensitive. L-Glutamine and *N*-α-phthaloyl-DL-glutamine were filter-sterilized and added aseptically to minimize breakdown. Phthalic acid, phthalamic acid, hexahydrophthalic acid, Δ^4-hexene-1, 2-*cis*-dicarboxylic acid, phthaloyl-DL-glutamic acid, L-glutamic acid and L-pyroglutamic acid were dissolved and brought to pH 7.0 with KOH. *N*-Methylphthalimide, *N*-hydroxymethylphthalimide, and *N*-chlorophthalimide were dissolved in acetone; *N*-phthalolyl-DL-α-alanine in ethyl alcohol; and α-ethyl-α-phenylglutarimide (Glutethimide: Doriden-Ciba) in ethyl acetate. The remainder of the compounds tested—phthalimide, α-phthalimido-*N*-methyl-DL-glutarimide, α-phthalimido-DL-succinimide, *N*-α-phthaloyl-DL-glutamine, and 3-hydroxy-3-(4-chloro-3-sulfamylphenyl)-phthalimidine (Chlorthalidone: Hygroton-Geigy)—like thalidomide (α-*N*-phthalimido-glutarimide) were dissolved in dilute KOH and neutralized with dilute HCl as described(1). All compounds were prepared as 1.0% stock solutions; they were diluted further with distilled water before addition to the medium.

Results. Inspection of the Table reveals that nicotinic acid predominantly opposes toxicity attributable to the phthalimide derivatives and for the open-chain derivatives of phthalic acid (Groups I and III). Glutamine helps oppose toxicity of compounds having the glutarimide ring and reduced derivatives of phthalic acid (Group II), and the *N*-methyl-, hydroxymethyl- or chloro-derivatives of phthalimide (Group III). The clearest action of glutamine was as a synergist with nicotinic acid in reversing the toxicity of these compounds (Groups I and III), *e.g.* thalidomide toxicity at 2.0 mg/ml is completely nullified by glutamine 2.0 mg/ml plus nicotinic acid 1.0 μg/ml. The toxicity of some of the more soluble test compounds, when added to saturation, could not be reversed by nicotinic acid, glutamine, or both. Doriden, like the other derivatives tested, was toxic at saturation, *e.g.* 0.03 mg/ml, Hygroton, a sedative with a phthalimidine moiety, was not toxic at saturation, *e.g.* 1.0 mg/ml.

Discussion. Since thalidomide may be hydrolyzed at the amide linkages and its aromatic ring metabolically hydroxylated, over 100 derivatives acting *in vivo* are possible (2). It is at present impossible to delineate those features which render a decomposition product teratogenic or those which engender antagonism. A further complication is that glutamine, an antagonist for some phthalimide derivatives, decomposes readily(3); two of its hydrolytic products, glutamic acid and pyroglutamic acid, were inert in

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TABLE I. Reversal of Toxicity of Thalidomide and Related Compounds for *Ochromonas danica*. Growth values expressed as optical densities(1).

Antagonists	Conc (mg/ml)	Reversal agents (mg/ml) None	Nicotinic acid .001	Glutamine 2.0	Nicotinic acid .001 + glutamine 2.0
None	—	1.2	1.2	1.2	1.2
Group I					
Thalidomide	2	.1	.8	.1	1.2
Phthalic acid	1	.1	.8	.1	1.2
Phthalamic acid	1	0	.6	.1	.8
Phthalimide	3	0	.5	.1	.9
N-Phthaloyl-DL-α-alanine	1	.4	1.2	.4	1.2
Phthaloyl-DL-glutamic acid	1	0	.7	0	—
N-α-Phthaloyl-DL-glutamine	1	.4	.9	.6	—
α-Phthalimido-*N*-methyl-DL-glutarimide	1	0	.7	0	—
α-Phthalimido-DL-succinimide	1	0	.4	0	—
Group II					
Hexahydrophthalic acid	3	.4	.4	1.1	—
Δ^4-Hexene-1,2-*cis*-dicarboxylic acid	3	.7	.7	1.2	—
Doriden	.03	.4	.6	1.1	—
Group III					
N-Methylphthalimide	1	0	.5	.7	1.0
N-Hydroxymethylphthalimide	.1	0	.6	.9	1.1
N-Chlorophthalimide	.1	.6	1.0	1.0	1.2

opposing toxicity. The phthalimide ring itself has a certain stability *in vivo;* rabbits fed phthalamic acid excreted phthalic anhydride and phthalic acid(4). One limitation in this work is that ring-hydroxylated derivatives of thalidomide are unavailable; these may be among the more important metabolic products(5). Injection of several insoluble particulates, such as alumina and clay, induce severe teratogenesis(6); thus it is necessary to solubilize thalidomide in order to eliminate such non-specific effects.

In our experiments, hydrolysis on autoclaving yielded the more toxic breakdown products; when filter-sterilized solutions of phthalic acid, phthalamic acid, and phthalimide were added to the medium, they were less toxic.

Our results agree with those of others who studied the effect of thalidomide and some derivatives (including some of the compounds studied by us) on the development of leucocytes(7); these results and those on other systems(8) point out that glutamine is a likely antagonist of thalidomide. Indeed, addition of glutamine in developing chick embryos corrected a postulated block in purine biosynthesis induced by "3- and 4-hydroxy metabolites" of thalidomide(5). Simple substituted phthalimides can be teratogenic(9): *e.g., n*-butyl-phthalimide was teratogenic in rats, inducing, among other things, malformed limbs.

Our results may add interest to the findings that antimetabolites of nicotinic acid may cause congenital abnormalities in animals. Pregnant rats fed the nicotinic acid antagonist, 6-aminonicotinamide, developed fetal abnormalities(10). Similar results have been obtained in chick embryos with the nicotinic acid antagonists, 6-aminonicotinamide and 3-acetylpyridine(11). The activity of glutamine along with nicotinic acid in overcoming the toxicity of thalidomide and its derivatives may reflect a simple biosynthetic relationship: Nicotinamide adenine dinucleotide (NAD) synthesis in tissue culture was shown to be optimal in the presence of glutamine(12); NAD also overcomes thalidomide toxicity like nicotinic acid(1).

Bacteria are insensitive to many antimetazoal factors as we observed for thalidomide(1), and which was confirmed by others (13). Inasmuch as ". . . ordinary reasonable

toxicity studies at an experimental level would not have been likely to reveal potential intrauterine developmental defects in embryos to whose mothers the drug may have been given"(14), a recourse to protozoa for studying toxicity was in order. Protozoa have been used for studying antimetabolic actions of drugs; with some drugs they mimic man and other animals in their response(15,16, 17), despite their lack of metazoan metabolic detoxification mechanisms.

Summary. The toxicity of thalidomide and some of its breakdown products were tested on protozoa, mainly the flagellate *Ochromonas danica*. Toxicity attributed to the phthalimide ring or phthalic acid was reversed by nicotinic acid. Glutamine contributed to this reversal, especially with compounds having a phthalimide group or a partly hydrogenated phthalic acid; glutamine was especially effective with Doriden which has a glutarimide group, but lacks a phthalimide group. These results with protozoa are in accord with the reported teratogenicity of nicotinamide antagonists.

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1. Frank, O., Baker, H., Ziffer, H., Aaronson, S., Hutner, S. H., Leevy, C. M., *Science,* 1963, v139, 110.

2. Williams, R. T., *Lancet,* 1963, vI, 723.

3. Tritsch, G. L., Moore, G. E., *Exp. Cell Res.,* 1962, v28, 360.

4. Bray, H. G., Hybs, Z., Thorpe, W. V., *Biochem. J.,* 1951, v48, 192.

5. Boylen, J. B., Horne, H. H., Johnson, W. J., *Lancet,* 1963, vI, 552.

6. Williamson, A. P., Blattner, R. J., Lutz, H. R., PROC. SOC. EXP. BIOL. AND MED., 1963, v112, 1022.

7. Roath, S., Elves, M. W., Israels, M. C. G., *Lancet,* 1963, vI, 249.

8. Bassil, G. T., Faigle, J. W., Keberle, H., Riess, W., Schmid, K., *Brit. Med. J.,* 1962, vII, 672.

9. Bignami, G., Bovet, D., Bovet-Nitti, F., Rosnati, V., *Lancet,* 1962, vII, 1333.

10. Chamberlain, J. G., Nelson, M. M., PROC. SOC. EXP. BIOL. AND MED., 1963, v112, 836.

11. Landauer, W., Clark, E. M., *J. Exp. Zool.,* 1962, v151, 253.

12. Friedland, I. M., Fuller, L., Dietrich, L. S., *J. Biol. Chem.,* 1962, v237, 3829.

13. Nyström, C., *Scand. J. Clin. Lab. Invest.,* 1963, v151, 102.

14. Leake, C. D., *Ann. Rev. Pharmacol.,* 1963, v3, 429.

15. Baker, H., Frank, O., Pasher, I., Ziffer, H., Hutner, S. H., Sobotka, H., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 965.

16. Aaronson, S., Bensky, B., Shifrine, M., Baker, H., *ibid.,* 1962, v109, 130.

17. Baker, H., Frank, O., Hutner, S. H., Aaronson, S., Ziffer, H., Sobotka, H., *Experientia,* 1962, v18, 224.

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Distribution of an Antihistamine Substance(s) in Extracts of Human Tumor and "Normal" Tissues.* (28666)

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The existence of a naturally occurring antihistamine substance(s) in extracts of human and animal tissues has been amply confirmed (1,9). Recently, we reported the presence and distribution of this active principle(s) in different kinds of human tissues and described a standard method for its extraction and

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