Lethal Effect of Certain Benzimidazoles and Benzenes on Early Chick Embryo.*† (24305)

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Little is known of the biochemical mechanism(s) interrupted by the benzimidazoles. Unsubstituted benzimidazole inhibition in yeast is reversed by adenine and guanine(1), but the 2, 5-alkylbenzimidazoles are only partially reversed by Vit. B_{12}(2,3). A characteristic order of inhibitory activity has been found in several biological systems(2,4-8) in which 2-ethyl-5-methylbenzimidazole is considerably more active than either the 2, 5-dimethyl or 5, 6-dimethyl analogues and all alkyl analogues are more active than unsubstituted benzimidazole. However, increasing the alkyl chain past 2 carbons in the 2 position does not further increase inhibition of influenza virus multiplication(4). Dimethyldiaminobenzene (a degradation product of 5, 6-dimethylbenzimidazole and B_{12}) and its analogue, dichlorodiaminobenzene, also have characteristic orders of inhibition depending upon the Vit. B_{12} requirement of the system being inhibited. Dichlorodiamine inhibits synthesis of Vit. B_{12} and riboflavin in microorganisms(9) and inhibits the growth of non-requiring-B_{12} and/or riboflavin microorganisms but has no effect on organisms (including mice) which require these vitamins(10). The opposite is true of dimethyldiamine; it reverses the dichlorodiamine or dibromodiamine in microorganisms able to synthesze the vitamins(3,10) and inhibits those organisms which require an exogenous source of Vit. B_{12}(11). However, in virus inhibition the dichlorodiamine is only slightly more effective than the dimethyldiamine and the 2 are equally toxic to the chorioallantoic membrane(12).

In our study the order of lethality of the benzimidazoles to the early chick embryo is identical to that found in virus inhibition and the order of the benzenes is the same as that found in organisms able to synthesize Vit. B_{12}.

Materials and methods. In addition to previously mentioned benzenes, the benzimidazole analogues; 5, 6-dimethyl, 2, 5-dimethyl, 2-ethyl-5-methyl and 2-hepta-5-methyl; and methylamide (an inhibitor of Vit. B_{12} in microorganisms)(13,14) were studied as lethal agents in early chick embryo. The effect of Vit. B_{12} on 3 of the compounds was tested.

The benzimidazoles and benzenes were completely dissolved in dilute HCl (0.06-0.13 N), 10 mg per ml, then diluted with double distilled water (used throughout) to the concentration injected into the egg (Table I). Methylamide and Vit. B_{12} were dissolved in water. The benzimidazoles and Vit. B_{12} were autoclaved for 10-15 min. at 15 lb pressure before injection, but the benzenes, because of their unstable nature, were autoclaved dry and dissolved in sterile HCl.

Eggs from 2 strains of White Leghorns, Mt. Hope and random bred, were used. The eggs were never stored for more than 3 days previous to injections. Groups of 10 to 25 eggs were used per treatment. Each egg was dabbed with tincture of iodine, wiped with 70% ethanol, punctured with a mechanical egg shell puncher, and injected through the air cell into the albumen with a sterile #27 needle mounted on a 1 ml syringe with marked plunger. Each egg received 0.1 ml solution. The eggs were immediately incubated in Jamesway forcedraft incubators at 99-100°F. They were candled the fifth day of incubation and daily thereafter until the 18th day. Each infertile or dead embryonated egg was removed and opened for examination. The percent dead of fertile eggs per experimental

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TABLE I. Lethal Effect of Several Inhibitors on Chick Embryos following Injection into Eggs Prior to Incubation.

No.	Compound	μM/egg	No. of groups	Total fertile eggs	% dead 18 days	Lethal index*
	None	—	4	132	5.4 (3–10)	—
	HCl (.06-.13 N)	.1 ml	11	142	7.9 (0–22)	—
1.	Methylamide	13.6	1	17	0.0	.0
	"	16.9	1	19	26.	1.5
	"	169.5	1	15	13.	.1
2.	1,2-Dimethyl-4,5-diaminobenzene	3.7	1	13	8.	2.1
	"	5.5	1	15	20.	3.6
	"	7.3	1	15	20.	2.7
3.	1,2-Dichloro-4,5-diaminobenzene	1.99	3	48	14.3 (14–15)	7.2
	"	2.8	4	60	33.7 (13–67)	11.9
4.	5,6-Dimethylbenzimidazole	3.4	1	13	15.	4.4
	"	4.8	1	13	23.	4.8
	"	6.8	1	14	14.	2.0
5.	2,5-Dimethylbenzimidazole	1.7 (1.4–2)	3	50	23.3 (20–25)	14.0
	"	3.1 (2.7–3.4)	5	60	24.0 (6–40)	7.7
	"	5.6 (4.8–6.8)	3	36	13.3 (0–30)	2.3
6.	2-Hepta-5-methylbenzimidazole	1.3	2	33	20.5 (12–29)	15.8
	"	2.2	3	51	29.3 (26–31)	13.4
	"	3.0	3	46	51.0 (25–69)	16.8
	Oct.-Nov.; random bred, white leghorn					
7.	2-Ethyl-5-methylbenzimidazole	2.19	2	34	29.0 (25–33)	13.2
	"	3.12	2	38	37.5 (31–44)	12.0
	Feb.-March; Mt. Hope, white leghorn					
	"	.62	2	26	31.0 (20–22)	35.5
	"	1.25	2	23	40.0 (30–50)	32.0
	"	2.19	2	20	65.0 (60–70)	29.7
	"	3.12	3	37	59.0 (55–64)	19.0

* Lethal index expresses % dead/μMol inhibitor. ( ) = Range of individual group results.

group was, in each case, averaged. For comparison of inhibitors a lethal index (indicating dose effectiveness) was devised by dividing percent dead embryos by the number of micromoles of inhibitor per egg. Vit. B_{12} and various normalities of HCl required to dissolve the highest concentration of inhibitors served for control injections.

Results. Table I shows that injection of dilute HCl produced only slightly greater mortality than found in noninjected controls. Thus the HCl in which inhibitors were dissolved cannot be considered to be a significant factor in producing the mortality found in inhibitor groups. When dose size of compounds, particularly 3, 6, and 7, was increased, mortality also increased but the lethal index decreased or increased depending on the in-

hibitors (Table I). The relationship of lethal index to change in dose size also varied with other inhibitors (Table I), thus making it difficult to compare the compounds as lethal agents. However, by averaging the lethal indices the compounds are rated in the following order given with their respective averaged values: methylamide, 0.5; 1, 2-dimethyl-4, 5-diaminobenzene, 2.8; 5, 6-dimethylbenzimidazole, 3.7; 2, 5-dimethylbenzimidazole, 8.0; 1, 2-dichloro-4, 5-diaminobenzene, 9.5; 2-ethyl-5-methylbenzimidazole, 12.6 (Oct.-Nov., random bred); 2-hepta-5-methylbenzimidazole, 15.5; and 2-ethyl-5-methylbenzimidazole, 29.0 (Feb.-Mar., Mt. Hope).

The lethal effect of 2-ethyl-5-methylbenzimidazole, but that of no other inhibitor, could be correlated with seasonal or genetic differences or both. This inhibitor had considerably greater effect when injected into Mt. Hope eggs during Feb. and Mar. than when injected into random bred eggs during Oct. and Nov. (Table I).

Vit. B_{12} (100-500 μg) injected alone produced an average of 3.3% mortality with a range of 0-10%; these values do not differ from those found in noninjected controls (Table I). B_{12} is, therefore, nonlethal at these levels. B_{12} at high levels had no effect on mortality produced by 2-hepta-5-methylbenzimidazole and 1, 2-dichloro-4, 5-diaminobenzene, but it decreased mortality produced by the 2-ethyl-5-methyl analogue under conditions resulting in greater lethality of this compound; *i.e.*, when 100 μg B_{12} (but not 50 μg) with 2.19 mcMol inhibitor were injected during Feb. and Mar. into Mt. Hope eggs, the mortality decreased from 65 to 40%; also, 250 μg B_{12} with 3.12 mcMol inhibitor reduced mortality from 56 to 33%. However, B_{12} had no effect when the same combinations of B_{12} and inhibitor were injected into random bred eggs during Oct. and Nov. Unpublished data on the 1½-day-incubated egg gave essentially the same results.

Discussion. The seasonal and/or genetic variation in lethality of 2-ethyl-5-methylbenzimidazole and the reversal by B_{12} may be due to seasonal and genetic dependent variation in deposition of Vit. B_{12} in the yolk by the hen. A normal seasonal variation in B_{12} deposition has been reported(15) in which there is greater deposition in the fall than in winter and spring. This is in accordance with the seasonal variation found in our investigatons. The large amounts of B_{12} required to affect a response could be due to binding of B_{12} by the egg albumen, but further work is needed to clarify this point. The data presented here are not adequate to determine whether the variation found with 2-ethyl-5-methylbenzimidazole is predominately related to seasonal or genetic differences or both. However, Landauer(16) found lethal and teratogenic action of compounds in chick embryos to be related to both seasonal and genetic differences.

The failure of 2-hepta-5-methylbenzimidazole to show greater lethality than 2-ethyl-5-methylbenzimidazole makes the relative order of activity of benzimidazoles exactly the same as that found with the virus multiplication inhibitory work(4); this would seem to indicate that the same mechanisms are interrupted in the early embryo as in virus multiplication. The relative lethality of the benzenes, however, did not agree with the virus work, nor with the toxicity to the chorioallantoic membrane taken from 10-day embryos(12), nor inhibition of B_{12} requiring microorganisms (11), but did agree with the inhibition of microorganisms which are able to synthesize Vit. B_{12} and riboflavin(9,10). The implication is, thus, that the early embryo synthesizes Vit. B_{12} and/or riboflavin. Work with B_{12} deficient eggs(17) and B_{12} levels in the yolk and embryonic tissue(15) indicate that the chick embryo either does not require B_{12} during the first week of incubation or is able to synthesize it. It should be noted, in this connection, that the 3 compounds which are natural moieties of Vitamin B_{12}, methylamide, 1, 2-dimethyl-4, 5-diaminobenzene, and 5, 6-dimethylbenzimidazole, were the least lethal of the compounds tested.

Summary. (1) The lethality of 7 inhibitors structurally related to Vit. B_{12} was determined in chick embryos by injecting several doses of each of them into the albumen prior to incubation. (2) Vit. B_{12} alone was nonlethal and failed to influence the lethality of 2-hepta-5-methylbenzimidazole and 1, 2-dichloro-4, 5-diaminobenzene. (3) Lethality

of 2-ethyl-5-methylbenzimidazole as well as its partial reversal by high levels of Vit. B_{12} varied with seasonal and/or genetic differences. (4) The relative order of lethality of benzimidazoles was the same as in virus inhibitory work, while that of benzenes was the same as the inhibition of microorganisms which synthesize B_{12} and/or riboflavin. (5) The 3 inhibitors which are natural moieties of Vit. B_{12}, methylamide, 1, 2-dimethyl-4, 5-diaminobenzene, and 5, 6-dimethylbenzimidazole, were the least lethal of the compounds tested. The implications of these findings are discussed.

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Latex-Leptospiral Agglutination Test. (24306)

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The successful use of sensitized latex particles(1) in an agglutination test for rheumatoid arthritis(2) suggested applications in the serodiagnosis of leptospirosis. Latex-leptospiral suspensions were found to form macroscopic specific aggregates with hyperimmune rabbit serum, and the study was extended to the human infection. This report gives details of the technic, and compares results of the latex agglutination test with those obtained in standard agglutination-lysis and microscopic agglutination tests.

Materials and methods. Polystyrene latex particles (diameter, 0.81 μ, in 11% aqueous suspension)* were diluted 1:10 in distilled water, filtered through Whatman #40 paper to remove any aggregates, then adjusted with water until a further 1:100 dilution in glycine-buffered saline solution† had an optical density of 0.25 $\pm$ 0.01 at 650 mμ. The optical density measurements were made in 12 x 75 mm cuvettes, using the Coleman Jr. Spectrophotometer 6A with adapter 6.106. Leptospiral suspensions were prepared from 7-day-old cultures in modified Korthof's medium with yeast extract(3). The organisms were killed by overnight exposure to formalin, which was added to give a final concentration of 0.5%. They were then concentrated by spinning 20 min. at 13,000 G, washed twice with formalinized (0.5%) 0.85% NaCl solution (FS), and resuspended in about 1 ml FS per 100 ml original culture. The concentrate was homogenized, using a tuberculin syringe with 22 gauge needle, and spun 3 min.

* Kindly provided by the Dow Chemical Co., Midland, Mich.

† One liter of aqueous solution contained 10.0 gm NaCl, 7.31 gm NH_2CH_2COOH, and 3.5 ml NaOH (1 N); pH was 8.2 at 25°C.