BENZIMIDAZOLE DERIVATIVES: NEW ENHANCERS OF INFLUENZA VIRUS MULTIPLICATION*

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5-Methyl-2-D-ribobenzimidazole (MRB)[1] restores the capacity of the chorioallantoic membranes from older chicken embryos to produce a large amount of influenza B (Lee) virus after a small inoculum (1, 2). The yield of both cell-associated and released virus is increased, and quantitation by infectivity and hemagglutination titrations gives similar results. MRB requires several hours to act. Once an effect has been established, the compound may be removed and an increase in virus yield still obtained many hours later, although there is a decay in the effect with time. Maximal enhancement is obtained when treatment of membrane cultures is begun before infection and continued for a prolonged period after infection. The available evidence suggests that MRB does not act through the interferon mechanism, but affects another restrictive mechanism that becomes operative in the course of embryological development (2). This mechanism is operative in normal cells and, unlike the interferon mechanism, does not require induction.

It appeared desirable to determine whether derivatives with greater specific and maximal enhancing activity could be found through an analysis of structure-activity relationships. If found and demonstrated to possess an action similar to that of MRB, such compounds would be highly useful in further studies of the mechanism of restriction of influenza virus multiplication.

We report in the present communication that polyhydroxyalkyl-benzimidazoles, varying in the side chain at position 2 in the imidazole ring or in the number of methyl group substitutions in the benzenoid ring, do not show marked differences in enhancing activity. However, a new group of derivatives with increased virus-enhancing activity has been discovered and defined. These derivatives are characterized by the presence of a hydroxyl or methoxyl grouping at position 5 and a methyl grouping at position 1 in the benzimidazolyl nucleus. The action of 5-methoxy-1-methylbenzimidazole on influenza virus multiplication has been examined in some detail and found to be similar to that of MRB.

Materials and Methods

Virus.—Influenza B virus, Lee strain, was prepared and stored as described previously (1).

Medium.—A balanced and buffered salts and glucose medium was used for maintenance of chorioallantoic membranes in vitro (1).

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[1] *Abbreviation used in this paper:* MRB, 5-methyl-2-D-ribobenzimidazole.

Benzimidazole Derivatives.—All benzimidazole derivatives were obtained through the courtesy of Merck, Sharp and Dohme Research Laboratories, Rahway, N. J. The compounds were dissolved in the medium used for chorioallantoic membrane culture; shaking at 37°C facilitated solution.

Chorioallantoic Membrane Cultures.—In some experiments the virus-enhancing activity of benzimidazole derivatives was determined using the procedure described in 1956 (1). In most experiments the more recently described procedure for obtaining chorioallantoic membranes and for setting up cultures was used (2). According to either procedure, each culture that is set up contains membranes from a group of embryos, and the results thus reflect the mean response of a randomly selected group of embryos. In addition, experiments were done in which cultures were set up with membranes from single embryos. These experiments reveal the responses of individual embryos.

Measurement of Virus Yield.—The yield of virus in the culture medium was measured by hemagglutination using a fractional dilution procedure (1, 2). In some experiments yield was measured by a 50% infectivity end point titration procedure (3); the embryonated eggs were incubated for 72 h after inoculation.

Determination of Toxicity of Compounds.—The extent of macroscopic tissue damage caused by certain derivatives was estimated as described previously (4). This could be done in infected chorioallantoic membranes as Lee virus itself does not cause detectable morphological changes during incubation of membranes in vitro.

RESULTS

Polyhydroxyalkyl-benzimidazoles.—The enhancing activity of nine polyhydroxyalkyl-benzimidazoles was compared in chorioallantoic membranes from 10-day old embryonated chicken eggs using the procedure described in 1956 (1). Membrane portions for four equivalent groups were obtained from six eggs selected at random. In each experiment, two such independent sets of four cultures were set up. The effect of each compound on influenza B (Lee) virus yield was determined at several concentrations in two or three experiments, and the concentration giving 150% increase in virus yield was determined graphically (1). The mean results obtained with seven derivatives are summarized in Table I. As can be seen, no marked differences in enhancing activity were ob-

TABLE I

Enhancing Activity of Polyhydroxyalkyl-benzimidazoles on Influenza B (Lee) Virus Multiplication

Benzimidazole derivative	Concentration giving 150% increase in virus yield*
	mM
5-Methyl-2-D-ribo	1.5
5,6-Dimethyl-2-D-ribo	1.5
2-D-Gluco	1.9
5-Methyl-2-D-gluco	1.6
5,6-Dimethyl-2-D-gluco	1.8
5-Methyl-2-D-galacto	1.6
5-Methyl-2-L-rhamno	1.5

* Membranes from 10-day old embryonated eggs were infected with Lee virus, 3×10^4 EID_{50}/ml, and incubated for 41 h. Each value is based on two or three determinations.

served. No signs of morphological damage to the membranes (4) were noted at 3.5 mM concentration of the derivatives listed in Table I. Two other derivatives were examined, i.e., 5-methyl-2-D-arabobenzimidazole and 5-methyl-2-D-mannobenzimidazole. The solubility of these compounds was very low, but enhancement of the order of 100% was observed at 1.75 mM concentration of each, with considerable amounts of undissolved compound present. No damage to the membranes was observed at this concentration.

5-Methoxy- and 5-Ethoxybenzimidazoles.—In preliminary experiments using membranes from 10-day old embryonated eggs (1), the 5-methoxy and 5-ethoxy derivatives of benzimidazole increased Lee virus yield at 1.0 mM concentration, but decreased the yield at 3.5 mM concentration. Variable results were obtained in the intervening range. Of the two compounds, 5-ethoxybenzimidazole was considerably more active, both as an enhancer and an inhibitor. Inspection of the membranes (4) revealed toxic changes at concentrations above 1.0 mM. The degree of toxic changes varied directly with the concentration. 5-Methoxybenzimidazole, 7.0 mM, and 5-ethoxybenzimidazole, 3.5 mM, caused marked damage (3+) to chorioallantoic membranes. For comparison, unsubstituted benzimidazole, 7.0 mM, causes only minimal (±) macroscopic damage (4).

Earlier studies have shown that substitution of a methyl group at position 1 in the benzimidazolyl nucleus results in a loss of inhibitory activity for influenza virus multiplication (5). While unsubstituted benzimidazole causes 75% inhibition of virus yield at 3.5 mM concentration, 1-methylbenzimidazole causes such an effect at 7.2 mM concentration.

It was of great interest, therefore, to determine whether substitution of a methyl group at position 1 in the benzimidazolyl nucleus in derivatives such as 5-methoxybenzimidazole might reduce the potential for virus-inhibitory activity and toxicity, and thereby yield a better enhancing compound. It was found that 3.5 mM 5-methoxy-1-methylbenzimidazole is nontoxic, while 3.5 mM 5-methoxybenzimidazole causes moderate damage. Furthermore, the 5-methoxy-1-methyl and related derivatives proved highly active as enhancers.

5-Methoxy- and 5-Hydroxy-1-methylbenzimidazoles.—Comparative experiments were carried out with 5-methoxy-1-methylbenzimidazole and MRB to determine the maximal enhancement obtainable with each and the concentrations required, and also the concentrations giving equivalent enhancement. These experiments were carried out in membranes from 13-day old embryonated eggs using the procedure in which membrane portions for nine equivalent groups are obtained from 12 eggs selected at random for each experiment (2).

Fig. 1 shows that, on the basis of the molar concentration required for maximal enhancement, 5-methoxy-1-methylbenzimidazole is approximately twice as active as MRB. The data recorded in Fig. 1 and results from other experiments indicate that 5-methoxy-1-methylbenzimidazole causes maximal enhancement in the concentration range from 1.75 to 3.5 mM, with 2.5 mM as

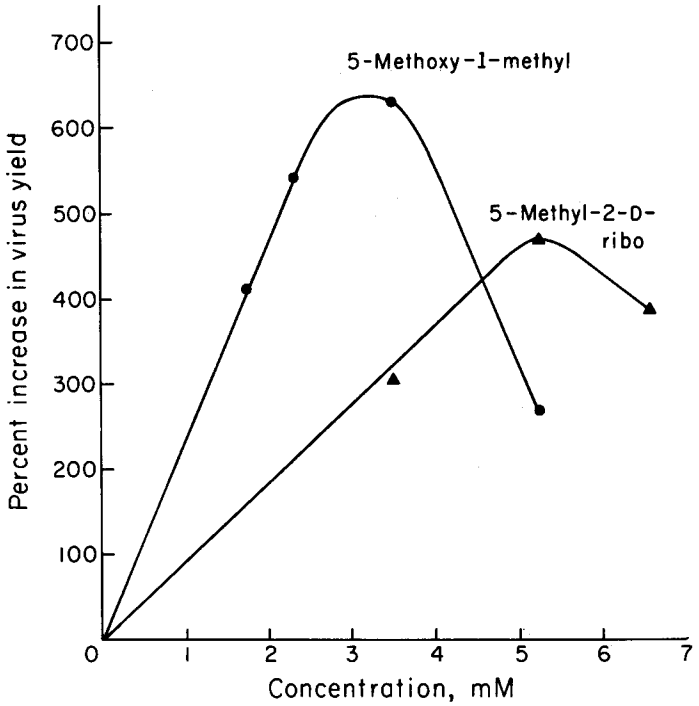

FIG. 1. Relationship between concentration of 5-methoxy-1-methylbenzimidazole and 5-methyl-2-D-ribobenzimidazole and degree of enhancement of Lee virus multiplication. Cultures of chorioallantoic membrane from 13-day old eggs were inoculated with Lee virus, 3×10^4 EID_{50}/ml, and incubated for 41 h. Each culture contained 12 1.8 cm^2 pieces of membrane in 6 ml of medium. The inoculum was equivalent to 0.03 EID_{50} of virus per allantoic or chorionic cell on membrane surfaces. Yield was measured in the medium by hemagglutination titration. The effects of the two compounds were compared in the same experiment. Mean results of two experiments.

the estimated peak concentration. The peak concentration fluctuates somewhat from experiment to experiment, which reflects biological variation in the chorioallantoic membranes. MRB causes maximal enhancement at a concentration of 5.2 mM (Fig. 1 and ref. 2). The maximal enhancing effect obtainable with the 5-methoxy-1-methyl derivative is 1.4-fold greater than that obtainable with MRB. Fig. 1 also shows that, on the basis of equivalent molar virus-enhancing concentrations, 5-methoxy-1-methylbenzimidazole is 2.6 times more active than MRB.

The effects of 5-methoxy-1-methylbenzimidazole (1.75 mM) and MRB (3.5 mM) on the yield of infective virus were also compared. Table II shows that both compounds increased the yield of infective virus and that the degree of enhancement is similar as measured by infectivity and hemagglutination titrations.

The 5-ethoxy-1-methyl derivative was not available, however examination of 5-hydroxy-1-methylbenzimidazole showed that this compound is even more active than the 5-methoxy-1-methyl derivative. As can be seen in Fig. 2, the maximal enhancing effect of 5-hydroxy-1-methylbenzimidazole (3.3 mM) is 2.4

TABLE II

Enhancing Effect of 5-Methoxy-1-methylbenzimidazole and 5-Methyl-2-D-ribobenzimidazole (MRB) on the Production of Infective Lee Virus

Treatment of chorioallantoic membrane	Virus yield*	
	EID_{50}/ml $\times 10^6$	HAU/ml
None	19	50
5-Methoxy-1-methylbenzimidazole, 1.75 mM	280	400
5-Methyl-2-D-ribobenzimidazole (MRB), 3.5 mM	220	360

* Membranes from 13-day old embryonated eggs were infected with Lee virus, 3×10^4 EID_{50}/ml, and incubated for 41 h.

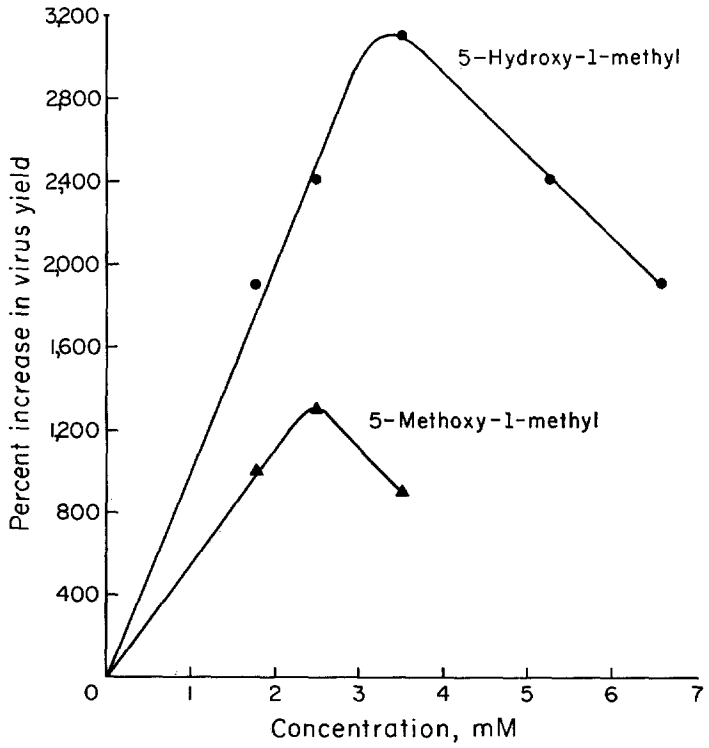

FIG. 2. Relationship between concentration of 5-hydroxy-1-methylbenzimidazole and 5-methoxy-1-methylbenzimidazole and degree of enhancement of Lee virus multiplication. For procedure see Fig. 1. Mean results of two experiments.

times greater than that of 5-methoxy-1-methylbenzimidazole (2.5 mM). On the basis of estimated equivalent virus-enhancing concentrations, the 5-hydroxy-1-methyl derivative was 1.8-fold more active than the 5-methoxy-1-methyl compound in these experiments. Fig. 3 shows the results of separate experiments in which the effects of these compounds were compared in a lower concentration range. The percent enhancement figures at the 1.75 mM concentration were lower in these experiments than in experiments summarized in Fig. 2, again reflecting biological variation in the chicken embryos. In the experiments summarized in Fig. 3, 5-hydroxy-1-methylbenzimidazole was 1.5 times more active

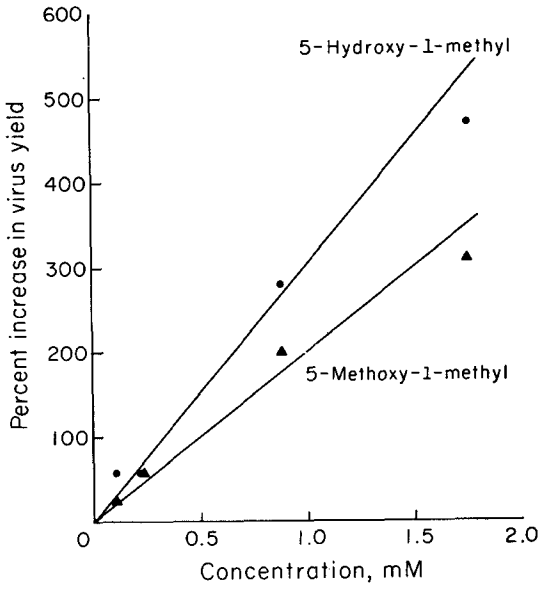

FIG. 3. Relationship between concentration of 5-hydroxy-1-methylbenzimidazole and 5-methoxy-1-methylbenzimidazole and degree of enhancement of Lee virus multiplication. For procedure see Fig. 1. Mean results of two experiments.

than 5-methoxy-1-methylbenzimidazole on the basis of equivalent virus-enhancing concentrations.

6-Methoxy- and 6-Hydroxy-1-methylbenzimidazoles.—A comparison of position 5- vs. 6-substituted derivatives in Fig. 4 shows that while 6-hydroxy-1-methylbenzimidazole at 0.44 mM concentration has greater enhancing activity than the 5-hydroxy-1-methyl compound, maximal enhancement obtained with the 6-hydroxy-1-methyl derivative at higher concentrations is much less than that obtainable with the 5-hydroxy-1-methyl compound (Fig. 2). The results in Fig. 5 provide confirmatory evidence that, at low concentrations 6-hydroxy-1-methylbenzimidazole shows greater enhancing activity than the 5-hydroxy-1-methyl compound. The 6-methoxy-1-methyl derivative produces maximal

enhancement of a low order at 0.11 mM concentration and it inhibits virus multiplication at 0.22 mM concentration.

In toxicity determinations (4) both the 6-methoxy-1-methyl and the 6-hydroxy-1-methyl derivative caused macroscopic morphological changes in the

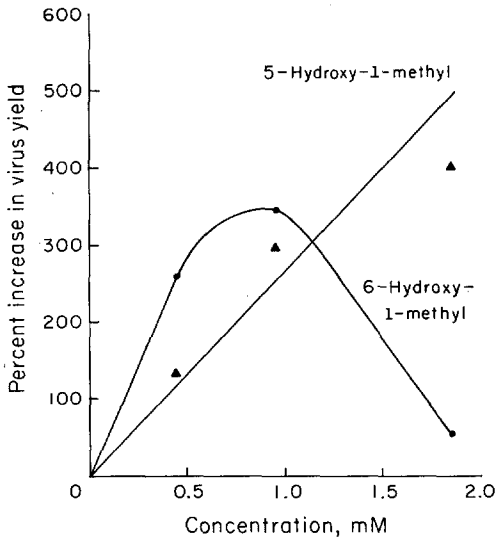

FIG. 4. Relationship between concentration of 5-hydroxy-1-methylbenzimidazole and 6-hydroxy-1-methylbenzimidazole and degree of enhancement of Lee virus multiplication. For procedure see Fig. 1. Mean results of two experiments.

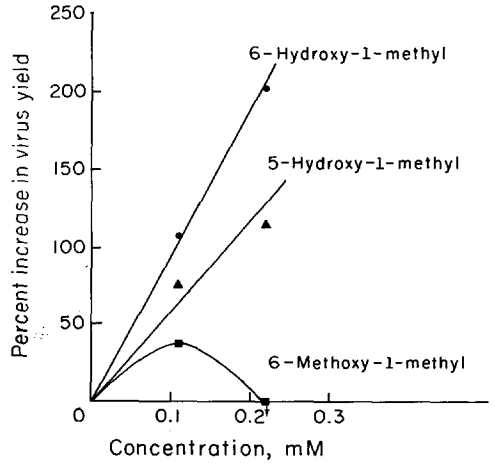

FIG. 5. Relationship between concentration of 6-hydroxy-1-methylbenzimidazole, 5-hydroxy-1-methylbenzimidazole, and 6-methoxy-1-methylbenzimidazole and degree of enhancement of Lee virus multiplication. For procedure see Fig. 1. Mean results of three experiments.

membranes at a concentration of 3.5 mM (cf. Table III). 6-Methoxy-1-methylbenzimidazole was somewhat more toxic than the 6-hydroxy-1-methyl derivative. In contrast, 5-methoxy-1-methyl and 5-hydroxy-1-methyl derivatives showed no toxicity.

Additional Derivatives.—Three additional derivatives have been examined: 5-methoxy-1,2-dimethylbenzimidazole; 5-methoxy-1-β-D-ribofuranosylbenzimidazole; and 6-methoxy-1-β-D-ribofuranosylbenzimidazole. In two experiments with 5-methoxy-1,2-dimethylbenzimidazole, the mean control yield in membranes from 10-day old embryonated eggs was 32 hemagglutination units (HAU)/ml; at 3.5 mM concentration the compound caused 63% inhibition of yield although no gross morphological evidence of damage to the membrane was noted; at 1.75 mM concentration the yield was increased 210%. Although the intrinsic enhancing activity of the 5-methoxy-1,2-dimethyl derivative is

TABLE III

Toxicity of Enhancers of Lee Virus Multiplication in the Chorioallantoic Membrane

Concentration	Toxicity of benzimidazole derivatives*			
	5-Methoxy-1-methyl	5-Hydroxy-1-methyl	6-Methoxy-1-methyl	6-Hydroxy-1-methyl
mM				
3.5	0	0	++ to +++	++
1.75			+ to ++	±
0.9			± to +	

* Membranes from 13-day old embryonated eggs were infected with Lee virus, 3×10^4 EID_{50}/ml, and incubated for 41 h. Untreated membranes showed no macroscopic damage. Macroscopic damage was estimated as described previously (4).

comparable to that of the 5-methoxy-1-methyl compound, the maximal tolerated concentration is lower, and therefore maximal obtainable enhancement is also lower.

In experiments carried out in membranes from 11- or 13-day old embryonated eggs, the 1-β-D-ribofuranoside of 5-methoxybenzimidazole (1.75 and 3.5 mM) showed lower enhancing activity than 5-methoxy-1-methylbenzimidazole. The 1-β-D-ribofuranoside of 6-methoxybenzimidazole (0.875 and 1.75 mM) showed less inhibitory activity than 6-methoxy-1-methylbenzimidazole. No gross damage to membranes was noted.

Enhancing Action of 5-Methoxy-1-methylbenzimidazole.—The action of 5-methoxy-1-methylbenzimidazole as a representative of a new group of enhancers of Lee virus multiplication was investigated in detail and compared with that of 5-methyl-1-D-ribobenzimidazole (MRB) (1, 2).

Dependence of enhancement on age of membranes: Table IV shows that 1.75 mM 5-methoxy-1-methylbenzimidazole caused only a small percentage increase in the very high yield of Lee virus in chorioallantoic membranes from 8-day old

TABLE IV

Dependence of Enhancement of Lee Virus Multiplication by 5-Methoxy-1-methylbenzimidazole on Age of Chorioallantoic Membrane

Age of chorioallantoic membrane	Control	Yield of virus in chorioallantoic membrane in vitro, HAU/ml*	
		5-Methoxy-1-methyl-benzimidazole, 1.75 mM	Increase in treated cultures
days			%
8	323	422	31
10	75	172	129
13	28	157	406

* Cultures were infected with Lee virus, 3×10^4 EID_{50}/ml, and incubated for 41 h with or without 5-methoxy-1-methylbenzimidazole. Mean of two experiments.

embryonated chicken eggs. The increase was greater in 10-day old membranes, and greatest (406%) in 13-day old membranes, in which the control yield after a small virus inoculum was approximately 1/10th of that in membranes from 8-day old embryonated eggs. In absolute terms, 1.75 mM 5-methoxy-1-methylbenzimidazole made possible the production of an additional amount of 97–129 HAU of virus in the chorioallantoic membranes of varying age.

The relationship between age of chorioallantoic membrane and degree of enhancement of Lee virus yield by 5-methoxy-1-methylbenzimidazole is closely similar to that previously described for MRB (1, 2).

Relationship between yields in control and treated cultures: Fig. 6 shows the relationships between yield in controls and percent enhancement in cultures treated with 1.75 mM 5-methoxy-1-methylbenzimidazole. The data are derived from 20 experiments carried out with membranes from 13-day old embryonated eggs between August 22, 1970, and October 17, 1972. Each culture contained portions of chorioallantoic membrane from 12 embryonated eggs (2), and thus each point records the mean result for chorioallantoic membranes from a group of 12 embryos. It is apparent that percent increase in Lee virus yield in the presence of 5-methoxy-1-methylbenzimidazole is inversely related to the control yield, although biological variation is considerable. The grand mean yields were as follows: control, 28 HAU/ml; treated, 166 HAU/ml. Thus 1.75 mM 5-methoxy-1-methylbenzimidazole caused, on the average, a 490% increase in virus yield. This compares with a 650% increase in a large series of separate experiments with 3.5 mM 5-methyl-2-D-ribobenzimidazole (2). With the latter compound, too, percent increase in yield in treated cultures is inversely related to control yield (1).

The relationship between control yield and degree of enhancement by 5-methoxy-1-methylbenzimidazole was also investigated in chorioallantoic membranes from individual chicken embryos.

Six 13-day old embryonated eggs were selected at random and from each membrane nine 1.9 cm^2 portions were punched out and distributed among three cultures, as follows: control, 5-

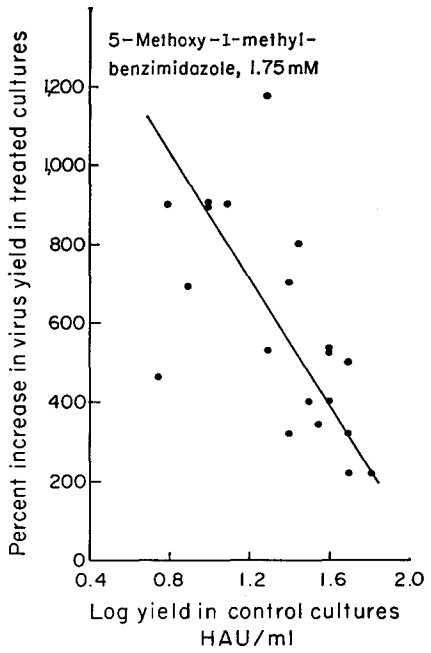

FIG. 6. Relationship between yield of Lee virus in control cultures and percent increase in yield in the presence of 1.75 mM 5-methoxy-1-methylbenzimidazole. For procedure see Fig. 1. Results of 20 experiments.

methoxy-1-methylbenzimidazole (1.75 mM), and MRB (3.5 mM). Each culture contained 5.7 cm^2 of membrane in 3.0 ml of medium and 9×10^4 EID_{50} of Lee virus as the inoculum. The cultures were incubated for 41 h and the yield of virus was determined by hemagglutination titration of the medium. Three such experiments were carried out, on these dates: July 20 and 27, and August 10, 1971. In the fourth experiment on September 7, 1971, the procedure was varied to include two control cultures, as well as one culture with each of the two compounds. For this experiment eight 1.9 cm^2 portions of membrane were obtained from each of six embryonated eggs, and each culture contained 3.9 cm^2 of membrane in 2 ml of medium and 6×10^4 EID_{50} of Lee virus as the inoculum.

Fig. 7 shows that similar results were obtained with the 5-methoxy-1-methylbenzimidazole and MRB used at nearly equivalent enhancing concentrations. With both compounds the degree of enhancement is inversely related to the control yields in the membranes from individual chicken embryos. There are, however, some low yielders, whose capacity to make virus is not markedly enhanced by the benzimidazole derivatives, and a few fairly high yielders, which are able to make even more virus in response to the compounds. The grand mean yields from the four experiments were: control, 41 HAU/ml; 5-methoxy-1-methylbenzimidazole (1.75 mM), 111 HAU/ml; and MRB (3.5 mM), 105 HAU/ml. The mean yield values for treated cultures from the four

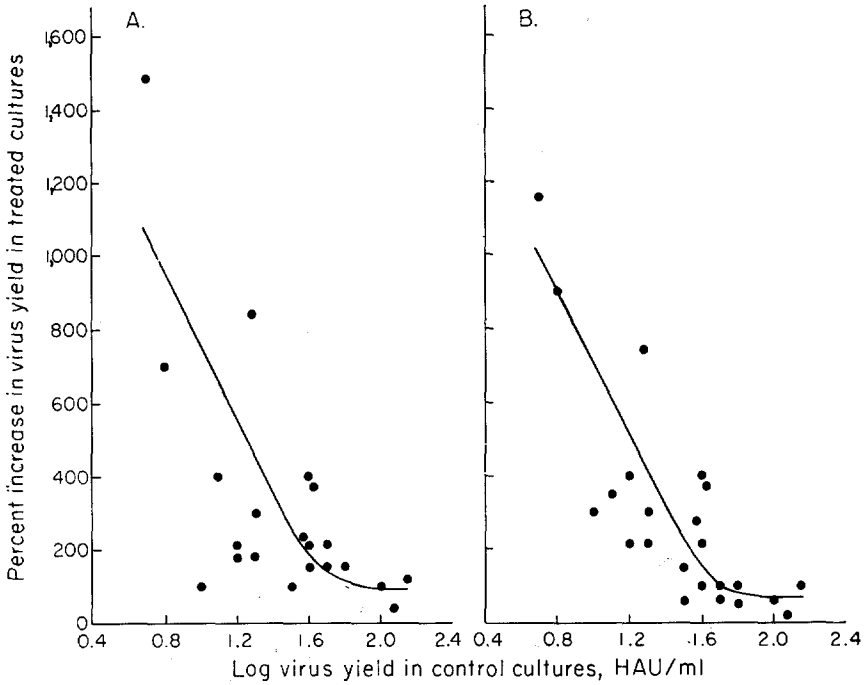

FIG. 7. Relationship between yield in control cultures and percent increase in yield in the presence of (*A*) 1.75 mM 5-methoxy-1-methylbenzimidazole or (*B*) 3.5 mM 5-methyl-2-D-ribobenzimidazole. For procedure see text. Results of determinations with chorioallantoic membranes from 24 individual eggs in four experiments.

experiments gave the following virus yield ratios,

$$\frac{\text{5-methoxy-1-methylbenzimidazole}}{\text{5-methyl-1-D-ribobenzimidazole}} : 1.15;\ 1.13;\ 1.08;\ \text{and}\ 0.96.$$

Among the 24 individual embryonated eggs studied, close correspondence in responses to the two derivatives was observed in membranes from 17 eggs. With these 17 embryos, the yields in cultures derived from the same membrane and treated with one or the other compound were within a 1.4-fold range of each other, and variation appeared to be random. In two membranes, the virus yield ratios, 5-methoxy-l-methylbenzimidazole/5-methyl-2-D-ribobenzimidazole, were 1.69 and 1.78 and in remaining five, the yield ratios were 0.37, 0.34, 0.50, 0.44, and 0.52. In these comparisons, allowance was made for the differences between computed mean yields in cultures treated with the two compounds.

These results suggest that there is no systematic difference in the responses of membranes from individual chicken embryos to the enhancing effects of 5-methoxy-1-methylbenzimidazole and MRB on Lee virus multiplication. Control experiments described below substantiate this view.

Three control experiments were carried out in which triplicate untreated cultures were set up from individual eggs. Six embryonated eggs were used for each experiment. Each of the

three cultures prepared from the same chorioallantoic membrane contained three 1.8 cm^2 portions of membrane in 3 ml of medium. The dates were: August 3, October 26, and December 28, 1971. In addition, results in duplicate controls were available from the experiment of September 7, 1971. With the chorioallantoic membranes from the 24 embryonated eggs examined, the yields among replicate groups were within a 1.4-fold range of each other in 20 of the eggs. In the remaining four, the yields in triplicate groups from membranes of the individual eggs were as follows: 20, 16, 25; 16, 40, 40; 40, 80, 100; and 40, 32, 28 HAU/ml. The greatest difference between yields in any two groups from the same membrane was 2.5-fold. The greatest difference between yields from different membranes was 74-fold. The yields in triplicate control cultures from the membrane that produced the least virus were 2.5, 2, and 2 HAU/ml; the yields from the highest producer were 160, 160, and 160 HAU/ml.

The relationship between virus yield in controls and degree of enhancement was also investigated with 3.5 mM 5-methoxy-1-methylbenzimidazole and the results were similar to those obtained with the compound at 1.75 mM concentration.

Effects of pre- and posttreatment: Results of experiments summarized in Table V show that cultures pretreated with 5-methoxy-1-methylbenzimidazole for 17 h, and then washed thoroughly to remove the compound, produced considerably more virus than cultures not treated at any time. However, treatment with 5-methoxy-1-methylbenzimidazole both before and after infection gave a greater increase in Lee virus yield than treatment before or after infection only. To obtain maximal enhancement, prolonged treatment with 5-methoxy-1-methylbenzimidazole, as with MRB (2), is necessary.

Effect of combined treatment: Similarities in the virus-enhancing effects of 5-methoxy-1-methylbenzimidazole and MRB suggested that the action of the two compounds may be additive in combined treatment. Results in Table VI

TABLE V

Enhancing Effects of Pre- and Posttreatment with 5-Methoxy-1-methylbenzimidazole and 5-Methyl-2-D-ribobenzimidazole on Virus Yield

Period of treatment from beginning of experiment*		Yield of virus in chorioallantoic membrane in vitro‡	
Before infection	After infection	HAU/ml	Increase in treated cultures
h	*h*		%
0–17	17–64		
Control	Control	22	—
5-Methoxy-1-methyl	Control	61	177
5-Methyl-2-D-ribo	Control	79	259
Control	5-Methoxy-1-methyl	135	514
Control	5-Methyl-2-D-ribo	101	359
5-Methoxy-1-methyl	5-Methoxy-1-methyl	192	773
5-Methyl-2-D-ribo	5-Methyl-2-D-ribo	180	818

* Membranes from 13-day old embryonated eggs were infected with Lee virus, 3×10^4 EID_{50}/ml. Control refers to medium without 5-methoxy-1-methylbenzimidazole (1.75 mM) or 5-methyl-2-D-ribobenzimidazole (3.5 mM).

‡ Mean of three experiments.

TABLE VI

Effect of Combined Treatment with 5-Methoxy-1-methylbenzimidazole and 5-Methyl-2-D-ribobenzimidazole on Virus Yield

Treatment	Yield of virus in chorioallantoic membrane in vitro*	
	HAU/ml	Increase in treated cultures
		%
Control	74	—
5-Methoxy-1-methyl, 0.88 mM	357	382
5-Methyl-2-D-ribo, 1.75 mM	226	205
5-Methoxy-1-methyl, 0.88 mM + 5-methyl-2-D-ribo, 1.75 mM	501	577

* Membranes from 13-day old embryonated eggs were infected with Lee virus, $10^{4.5}$ EID_{50}/ml, and incubated for 41 h. Mean of two experiments.

show that the combined effect of 0.88 mM 5-methoxy-1-methylbenzimidazole plus 1.75 mM MRB is equivalent to the sum of the effects of either compound alone.

Enhancing effect in chorioallantoic membranes from COFAL-negative embryos: Chorioallantoic membranes from 13-day old COFAL-negative chick embryos, obtained from Spafas, Inc., Norwich, Conn., produce smaller amounts of Lee virus than membranes from eggs produced by the random-bred flock at Shamrock Farms, North Brunswick, N. J. (2). Although both flocks consist of White Leghorn hens, the flocks are genetically different. In an experiment with membranes from 18 individual COFAL-negative eggs, the mean yield in controls was <2 HAU/ml, while the yield in membranes treated with 1.75 mM 5-methoxy-1-methylbenzimidazole was 48. Similar results have been obtained with MRB (2).

DISCUSSION

We have found no marked differences in the enhancing activity of a group of polyhydroxyalkyl-benzimidazoles on influenza B (Lee) virus multiplication. Examined in chorioallantoic membranes from 10-day old chicken embryos, the derivatives caused a 150% increase in virus yield at concentrations in the range from 1.5 to 1.9 mM. It appears that neither the overall molecular configuration of polyhydroxyalkyl-benzimidazoles, nor all of its details are critically important for enhancing activity. However, the presence of the hydroxyl groupings in the side chain attached at position 2 in the benzimidazolyl nucleus is critically important for enhancing activity, as 5-methyl-2-butylbenzimidazole is a highly active inhibitor (5). This derivative, which has a four-membered alkyl side chain at position 2, causes 75% reduction in influenza virus yield at a concentration of 0.17 mM.

One compound in this group, i.e. 5-methyl-2-D-ribobenzimidazole (MRB), has been studied extensively in previous work (1, 2). In membranes from 13-day

old embryos, 3.5 mM 5-methyl-2-D-ribobenzimidazole increases virus yield by 650%, on the average. At 5.2 mM concentrations, which is the concentration causing peak enhancement, the increase in virus yield is 1.4 times greater than at 3.5 mM. This gives an estimated average peak enhancement of 910% for this compound.

Members of a new group of enhancers show marked dependence of the activity on molecular structure, and the enhancing activity of several members of the new group exceeds that of MRB. 5-Hydroxy-1-methylbenzimidazole (Fig. 8) is the derivative that produces the greatest increase in Lee virus yield after a small inoculum in chorioallantoic membranes from 13-day old embryonated eggs. We estimate that the maximal effect obtainable with this compound at the optimal concentration of 3.3 mM is 3.4 times greater than that obtained with MRB, and is equivalent to a 30-fold increase, on the average, over the control yield in untreated cultures.

A review of Figs. 1–5 and Table III suggests that two factors determine the

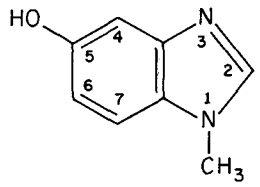

FIG. 8. The structure of 5-hydroxy-1-methylbenzimidazole—a highly active enhancer of influenza virus multiplication (mol wt 148.16).

dose-effect curves observed: (*a*) the specific enhancing activity of the compounds, and (*b*) toxicity. According to specific activity, the rank order of the derivatives is as follows: 6-hydroxy-1-methyl > 5-hydroxy-1-methyl > 5-methoxy-1-methyl > 5-methyl-2-D-ribo; no exact rank assignment is possible for 6-methoxy-1-methylbenzimidazole. The rank order based on maximal enhancement at the highest tolerated concentration is different: 5-hydroxy-1-methyl > 5-methoxy-1-methyl > 5-methyl-2-D-ribo > 6-hydroxy-1-methyl > 6-methoxy-1-methyl. The hydroxyl grouping either at position 5 or 6 in the benzimidazolyl nucleus is clearly superior to the methoxyl grouping in that the hydroxy derivatives display both higher specific activities and higher tolerated dose levels than the corresponding methoxy compounds. Substitution at position 5 is much superior to substitution at position 6 insofar as the tolerated dose, and therefore the maximal possible enhancing effect is concerned, but the highest specific enhancing activity was displayed by the 6-hydroxy-1-methyl derivative.

To summarize the central findings: the high maximal enhancement obtainable with 5-hydroxy-1-methylbenzimidazole can be ascribed to two factors: (*a*) the hydroxyl grouping at position 5 in the benzimidazolyl nucleus imparts enhancing activity in high degree; (*b*) the methyl grouping at position 1 reduces

the toxicity of the benzimidazole moiety. The hydroxyl grouping imparts enhancing activity of a high order also when substituted at position 6, however such substitution is associated with toxicity that vitiates the enhancing effect. All these considerations apply also to 5-methoxy-1-methylbenzimidazole, with the qualification that the methoxyl grouping is inferior to the hydroxyl grouping in imparting enhancing capability to the molecule. The relative enhancing activities of the derivatives are given in Table VII.

Although the 5-hydroxy-1-methyl derivative is the compound of choice in studies of influenza virus enhancement, our investigation of the action of the new group of enhancers has so far been carried out with the 5-methoxy-1-methyl derivative. The evidence obtained to date has not revealed any differences in the actions of 5-methoxy-1-methylbenzimidazole and MRB. The enhancing effect of both compounds can be characterized as restoration of the capacity of

TABLE VII

Relative Enhancing Activity of Benzimidazole Derivatives on Influenza B (Lee) Virus Multiplication

Benzimidazole derivative	Peak enhancing concentration	Relative activity based on equivalent enhancing concentration	Relative activity based on maximal enhancing effect
	mM		
5-Methyl-2-D-ribo	5.2	1	1
5-Methoxy-1-methyl	2.5	2.6	1.4
5-Hydroxy-1-methyl	3.3	4.3	3.4
6-Methoxy-1-methyl	0.1	—	<1
6-Hydroxy-1-methyl	0.8	6.7	<1

membranes from older chicken embryos to produce large amounts of virus after a small inoculum of Lee virus. Both compounds are able to increase virus yield when used to pretreat membranes, but their effect is greatest when pretreatment is followed by prolonged treatment after infection. There is marked variation in control yields from membranes of individual embryonated eggs derived from random-bred flocks of White Leghorn hens. No significant differences, exceeding experimental variation, have been found in the responses of individual membranes to 5-methoxy-1-methylbenzimidazole and MRB, which supports the view that the two compounds act by the same mechanism. The fact that combined treatment with the two compounds gives an additive effect also supports this possibility.

The nature of the mechanism by which certain benzimidazole derivatives increase the yield of influenza virus in the chorioallantoic membrane is not yet clear. In early work (1), two possibilities were considered to explain the virus-enhancing action of 5-methyl-2-D-ribobenzimidazole: (*a*) the compound, or a conversion product thereof in cells, may serve as a metabolite; (*b*) the compound

may interfere with certain normal cell functions. In a recent report (2) the action of MRB was viewed as overcoming a virus-restrictive mechanism that is expressed in the course of embryological development. It was suggested that the compound may improve the functional capabilities of some cellular structure required in influenza virus multiplication or that it may block an endogenous virus inhibitor.

It is of interest to note that, while nontoxic for the chorioallantoic membrane, 3.5 mM MRB causes morphological changes in monolayer cultures derived from trypsinized membranes (2) or rhesus monkey kidneys (6) and reduces influenza virus yield. The virus-enhancing action in the chorioallantoic membrane thus clearly depends on the physiological state of the developing chick embryo.

SUMMARY

The enhancing activity of 5-methyl-2-D-ribobenzimidazole on influenza B (Lee) virus yield in chorioallantoic membranes from 10-day old embryonated eggs was compared with that of eight other polyhydroxyalkyl-benzimidazoles. No marked differences in activity were noted with the following six derivatives: 5,6-dimethyl-2-D-ribo; 2-D-gluco; 5-methyl-2-D-gluco; 5,6-dimethyl-2-D-gluco; 5-methyl-2-D-galacto; and 5-methyl-2-L-rhamno. None caused morphological damage to the membranes at a concentration of 3.5 mM. The solubility of the 5-methyl-2-D-arabo and 5-methyl-2-D-manno derivatives was too low to permit quantitative comparisons, but both were active and nontoxic at a concentration of 1.75 mM.

5-Hydroxy-1-methylbenzimidazole and 5-methoxy-1-methylbenzimidazole are more active than 5-methyl-2-D-ribobenzimidazole both with respect to specific activity and maximal enhancement at the highest tolerated dose. The hydroxyl substituent is superior to the methoxyl grouping. Substitution at position 5 is superior to substitution at position 6 with respect to the tolerated dose level and therefore the maximal effect obtainable, but the 6-hydroxy-1-methyl derivative showed the highest specific activity.

5-Methoxy-1-methylbenzimidazole increases the yield to a comparable extent as measured by infectivity and hemagglutination titrations. The responses of membranes from individual chicken embryos to the enhancing action of 5-methoxy-1-methylbenzimidazole and 5-methyl-2-D-ribobenzimidazole are similar. 5-Methoxy-1-methylbenzimidazole restores the capacity of membranes from older chicken embryos to produce a large amount of virus after a small inoculum. This derivative increases the yield of virus in membranes treated before infection only. Maximal enhancement is obtained with prolonged treatment, starting before, and continuing after infection. 5-Methoxy-1-methylbenzimidazole increases the yield of virus from COFAL-negative embryos in which the control yield is very low. Combined treatment with moderate doses

of 5-methoxy-1-methylbenzimidazole and 5-methyl-2-D-ribobenzimidazole gives an additive effect.

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APPENDIX

Additional evidence that substitution of a methyl group at position 1 in the benzimidazole nucleus reduces influenza virus-inhibitory activity and cytotoxicity has been obtained with the 5-chloro and 5-chloro-1-methyl derivatives. The 75% virus-inhibitory concentration of 5-chlorobenzimidazole is 0.75 mM (3, 4), but that of 5-chloro-1-methylbenzimidazole is 3.2 mM. 5-Chlorobenzimidazole causes 4+ macroscopic damage at 3.0 mM, and 1+ at 1 mM (4), whereas 5-chloro-1-methylbenzimidazole causes 1+ damage at 3.5 mM, and 0 damage at 2.3 mM. Quantitative comparison of the virus-inhibitory activity and toxicity of the 5,6-dichloro and 5,6-dichloro-1-methyl derivatives is not possible because of the poor solubility of these compounds, however the 75% virus-inhibitory concentation of 5,6-dichlorobenzimidazole is ~0.25 mM (3), whereas that of 5,6-dichloro-1-methylbenzimidazole is 0.48 mM.

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