Inhibition of Influenza and Mumps Virus Multiplication by 4,5,6- (or 5,6,7-) Trichloro-1-β-D-Ribofuranosyl-benzimidazole

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Results reported earlier (*1*) indicated that the marked influenza virus inhibitory activity of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) was related to (i) the presence of the ribofuranose moiety at position 1, and (ii) the presence of unnatural (Cl) substituents in the benzenoid ring, two such substituents showing a more marked effect than one. It appeared that further increase in activity might be achieved by additional substitution in the benzenoid ring while retaining the ribofuranosyl group at position 1.

4,5,6- (or 5,6,7-) Trichloro-1-β-D-ribofuranosylbenzimidazole (TRB; see Fig. 1) (*2*) was synthesized (*3*) and tested for its inhibitory action on Lee-virus multiplication in chorioallantoic membranes *in vitro* in a manner described previously (*1, 4*). The inhibitory activity of TRB was compared with that of other derivatives in terms of the molar concentration that caused 75-percent inhibition of multiplication. For the purposes of this study, unsubstituted benzimidazole was considered as the reference compound. This substance causes 75-percent inhibition at a concentration of 0.0035*M* or 410 μg/ml and has been assigned a relative inhibitory activity of 1 (*1, 4*). It was found that TRB caused this degree of inhibition of Lee-virus multiplication at a concentration of 0.0000046*M* or 1.6 μg/ml. As can be seen in Table 1, under identical conditions 0.000038*M* or 12 μg/ml of DRB is required to produce the same effect (*1*). On a molar basis, the trichloro compound has 8 times greater activity than the dichloro derivative. It should be emphasized that TRB is 760 times more active, whereas DRB is 92 times more active, than unsubstituted benzimidazole. In Table 1 attention is also directed to the structure-activity relationships (*1*) that establish the importance of the ribofuranose moiety.

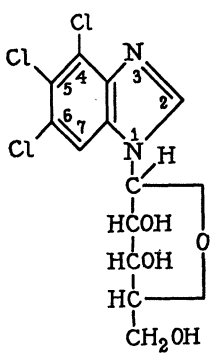

Fig. 1. 4,5,6- (or 5,6,7-) Trichloro-1-β-D-ribofuranosyl-benzimidazole (TRB).

The medium used in these experiments consisted of a buffered salt and sugar solution containing Na_2HPO_4, KH_2PO_4, NaCl, $CaCl_2$, $MgCl_2 \cdot 6H_2O$, and dextrose. In order to determine whether, in the presence of a more complex medium, the degree of inhibition would be similar, a medium (*5*) that included bicarbonate, vitamins, amino acids, and purine and pyrimidine bases was employed in separate experiments. Before incubation was started, the *p*H of the complex medium was adjusted to 7.2 by passing a mixture of CO_2 and O_2 through culture tubes. It was found that the yield of virus at 46 hr in the absence of TRB was no greater in the complex than in the control medium. In the presence of TRB (0.0000065*M*) the degree of inhibition was similar (90 percent) in the two mediums.

Previous attempts to block the inhibitory action of DRB with selected compounds that might serve as metabolites were unsuccessful (*1*). Attempts were made to block the inhibitory action of TRB with a mixture of adenosine (0.001*M*), vitamin B_{12} (25

Table 1. Activities of benzimidazole derivatives as inhibitors of influenza-B virus (Lee) multiplication in the chorioallantoic membrane *in vitro*.*

Benzimidazole derivative	Inhibitory concentration (*M*) †	Inhibitory activity relative to benzimidazole
Benzimidazole‡	0.0035	1.0
5- (or 6-) Chloro-1-β-D-ribofuranosyl	.00028	13
4,6- (or 5,7-) Dichloro-1-β-D-ribofuranosyl	.00010	35
5,6-Dichloro-1-β-D-ribofuranosyl (DRB)	.000038	92
4,5,6- (or 5,6,7-) Trichloro-1-β-D-ribofuranosyl (TRB)	.0000046	760
5,6-Dichloro-1-β-D-ribopyranosyl	.00023	15
5,6-Dichloro-1-β-D-arabinopyranosyl	.0011	3.1
5,6-Dichloro-1-β-D-glucopyranosyl	.0011	3.1

* Each culture consisted of 6.6 cm^2 of chorioallantoic membrane suspended in 1 ml of medium. Inoculum: Lee virus, $10^{6 \cdot 1}$ EID_{50}. Six cultures were used per group and several groups were employed with various concentrations of each compound. Cultures were incubated at 35°C for 36 hr with shaking. Virus was measured by the hemagglutination technique in the medium (*1, 4*).

† Concentration giving 75 percent inhibition of multiplication.

‡ Considered as the reference compound.

μg/ml), folic acid (0.00017*M*), and coenzyme 1 (0.0001*M*). It was found that such supplementation of the simpler medium did not result in increased virus production by the chorioallantoic membrane. Furthermore, no significant blocking of the inhibitory action of 0.0000065*M* TRB by the supplements used was noted.

Previous studies (*1*) showed that DRB is capable of inhibiting Lee-virus multiplication in embryonated eggs and in mice without causing significant signs of toxicity in either host. In view of these results and the results described here, extension of the animal studies to other viruses appeared indicated. The effect of DRB and of TRB on the multiplication of mumps virus in 8-day-old embryonated eggs was determined. As can be seen in Table 2, both compounds caused marked inhibition of multiplication of mumps virus in the allantoic sac of embryonated eggs. Precise quantitative comparison of the effects of DRB and TRB *in vivo* is vitiated by the low solubility of these compounds. In the *in vivo* experiments, both DRB and TRB were used in suspension form. Under the experimental conditions employed, neither compound caused obvious slackening of the spontaneous activity of the embryos. No deaths attributable to DRB or TRB occurred.

The results reported here lend further support to the contention (*1*) that an unnatural benzimidazole nucleus, particularly with respect to the benzenoid ring, is of great importance in relation to the virus-inhibitory activity of the ribofuranosides of benzimidazole. Previous observations (*1, 3*) that the inhibitory activity cannot be blocked by certain suspected metabolites have been extended. The finding that DRB and TRB inhibit mumps-virus multiplication *in vivo* without causing apparent damage to the host indicates that these compounds are selective in their action. However, it is doubtful (*1, 6*) that these compounds interfere with chemical reactions that are specific for the viruses and do not occur in the uninfected host. It appears that the explanation for the selective action may concern the relative quantitative importance of certain metabolic processes for the virus as compared with the host and intracellular factors of accessibility.

Table 2. Inhibition of mumps-virus multiplication by benzimidazole derivatives *in ovo*.*

Compound	Incubation (hr)	Hemagglutination titer† of allantoic fluid
None	96	128
DRB	96	< 2
TRB	96	< 2
None	120	512
DRB	120	16
TRB	120	22

* Inoculum : mumps virus, $10^{2 \cdot 9}$ EID_{50}. Thirty minutes after inoculation of virus each egg received, by allantoic injection, 0.25 ml of saline, or compound suspended in saline. One milligram of either DRB or TRB was injected per egg.

† Expressed as the reciprocal of dilution at end-point. Six eggs were used per group, and aliquots of infected allantoic fluid were pooled for titration. Final concentration of chicken RBC was 0.045 percent.

References and Notes

1. I. Tamm *et al., J. Exptl. Med.* **99**, 227 (1954).
2. This and other benzimidazole derivatives were obtained through the kindness of Karl Folkers and Clifford H. Shunk of the Research Laboratories, Chemical Division, Merck & Co., Inc., Rahway, N.J.
3. C. H. Shunk *et al.*, data in preparation.
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On the Nonidentity of Bence-Jones Proteins

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A century ago Bence-Jones first described urinary proteins that now bear his name and are identified by the property of coagulation at low temperature (45° to 55°C) with dissolution on boiling. These proteins occur rarely, always in association with some pathology and are most often, but not invariably, found in the urine of patients with multiple myeloma. Some workers have concluded that Bence-Jones proteins are chemically identical, although it has been shown by immunological and physicochemical analysis that different patients may excrete biologically and molecularly dissimilar proteins. Recently, in a study of Bence-Jones proteins obtained from nine cases none were found to be identical in all the physical properties studied—that is, sedimentation constant (s_{20}), diffusion constant, isoelectric point *pI*, *p*H-mobility curve, and stability in dilute acid or alkali (*1*).

In further characterization of normal plasma proteins and of the pathological proteins in multiple myeloma by means of N-terminal amino acid analysis, Bence-Jones proteins from eight cases have thus far been studied. The fluorodinitrobenzene method of Sanger (*2*) was used to detect and estimate the N-terminal amino acids (that is, terminal residues having a free amino group). Buffered silica gel or celite was employed for chromatographic separation of the dinitrophenyl (DNP) derivatives, and paper chromatography for their identification (*3–5*). As is shown in Table 1, six of the eight proteins were similar in s_{20} (about 3.3 Svedberg units) but were distinguishable by their electrophoretic properties. Proteins B, E, and G migrated electrophoretically with skewed patterns indicative of heterogeneity but unlike Ma did not separate into two components within the *p*H stability range (*p*H 5 to *p*H 9). A, D, F, and Ag con-