RELATIONSHIP BETWEEN STRUCTURE OF BENZIMIDAZOLE DERIVATIVES AND SELECTIVE VIRUS INHIBITORY ACTIVITY

Inhibition of Poliovirus Multiplication and Cytopathic Effects by 2-(α-Hydroxybenzyl)-Benzimidazole, and Its 5-Chloro Derivative*, ‡

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Plates 70 to 72

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Through studies of structure-activity relationships with benzimidazole derivatives and various viruses, compounds with interesting biological activities (1–19) have been obtained. In some instances, new biologically active derivatives were developed in the course of systematic attempts to increase, through structural alteration, the virus inhibitory activity or selectivity of action of existing compounds (3, 5, 8). In other instances, striking biological activities of derivatives were discovered unexpectedly (10, 11, 19). It should be emphasized that no direct inactivating effects on virus infectivity were found (2, 6, 7, 13). Most of the derivatives inhibited (1–9, 12, 15), and some of them enhanced (10, 11, 18) virus multiplication; others had no effect.

The purpose of the present investigation was to explore additional structural approaches to the problem of obtaining benzimidazole derivatives with further increased virus inhibitory activity and selectivity of action. Discovery of highly selective inhibitors of virus multiplication may aid greatly in studies of the chemical basis of virus specificity—a subject about which little is known today.

The present report concerns the virus inhibitory activity and selectivity of a series of benzimidazole and benzotriazole derivatives which have been studied with influenza B virus in the chorioallantoic membrane *in vitro* and with poliovirus type 2 in monkey kidney cell cultures. The results obtained confirm the earlier conclusion (1–3, 5, 8) that extensive substitution in either the benzenoid or the imidazole ring frequently gives compounds of very high virus inhibi-

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tory activity. The selective action of 2-(α-hydroxybenzyl)-benzimidazole (HBB) (19) as an inhibitor of poliovirus multiplication and cytopathic effects is described. Evidence is presented concerning structural features of HBB which are of importance for its selective virus inhibitory action.

Materials and Methods

Viruses.—Stock suspensions of the Lee (4) and "1760" (9) strains of influenza B virus and of the MEF1 (7) strain of type 2 poliovirus were prepared and handled in the manner described previously (4, 7, 9).

Tissue Cultures.—Cultures of chorioallantoic membrane and of monkey kidney cells were similar to those used previously (4, 7). A medium designated buffered glucosol (9) was used with the chorioallantoic membrane, and protein-free Eagle's medium (20) with monkey kidney cells. Three membrane cultures or six monkey kidney cell cultures were used per variable. The inoculum of Lee virus was 2×10^5 EID_{50} (50 per cent egg infective doses) per $cm.^2$ of freely suspended chorioallantoic membrane, and that of MEF1 virus was 5×10^2 $TCID_{50}$ (50 per cent tissue culture infective doses) per monolayer culture consisting of approximately 2.5×10^5 cells. Infected chorioallantoic membrane cultures were incubated at 35°C. for 41 hours and influenza virus was measured in the supernatant by the hemagglutination technique. Infected monkey kidney cell cultures were incubated at 36°C. for 48 hours and poliovirus was measured in the supernatant by infectivity titrations. With each compound two or more experiments were carried out.

Inhibitory Activity of Compounds.—The molar concentration of compound required to cause 75 per cent reduction in yield of influenza or poliovirus was determined in the manner previously described (4, 7).

Toxicity of Compounds.—The molar concentration of compound required to cause 2+ macroscopic damage to chorioallantoic membrane (4) or 3+ microscopic damage to monkey kidney cells (7) was determined in the manner described previously.

Selectivity Ratio.—To obtain an estimate of the selectivity of virus inhibitory action of compounds, the ratio of the toxic to the virus inhibitory concentration was computed (4, 7).

Protective Activity of Compounds against Virus-Induced Cell Damage.—Observations on protection were not possible with influenza B virus in the chorioallantoic membrane because this virus does not cause definite macroscopic or microscopic changes in cells of the chorioallantoic membrane (4).

Such observations were carried out with poliomyelitis and influenza viruses in monkey kidney cell cultures. The amount of virus inoculated was 500 $TCID_{50}$ per culture. The cultures were incubated in the presence or absence of a wide range of concentrations of various compounds and examined 48 hours after infection. In some instances cultures were examined daily for several days. The degree of virus-induced cell damage was expressed in terms of per cent cells affected. The extent of viral cytopathic effects in treated cultures was compared to that in untreated cultures. With many compounds no evidence of protection of cells against virus-induced damage was observed. In the presence of a number of compounds, virus-induced cell damage was slightly and transiently decreased. In such instances there was regularly evidence of compound-induced damage in treated uninfected cultures which were observed in parallel with the treated or untreated infected cultures. With only a few compounds marked reduction in virus-induced cell damage was observed over the course of several days, and no, or only very slight, cell changes were observed in treated uninfected cultures.

On the basis of accumulated experience a criterion of protection was defined. A compound was considered capable of protecting cells against the cytopathic effects of a virus if on any day after infection of cultures the extent of cell damage in the presence of some amount of

compound in treated infected cultures was 25 per cent or less of that in untreated infected cultures. On this basis only two compounds among the twenty-five derivatives examined showed cell-protective activity against viral damage.

EXPERIMENTAL

I. Relationship between Structure of Benzimidazole and Benzotriazole Derivatives and Inhibitory Activity on Influenza B and Poliovirus Type 2 Multiplication

In chemical inhibition studies with influenza B virus in the chorioallantoic membrane and poliovirus type 2 in monkey kidney cells, summarized in Tables I, II, and III, the data were expressed as follows: The 75 per cent virus inhibitory concentration was designated the virus inhibitory concentration or VIC. Virus inhibitory activity relative to benzimidazole was designated relative inhibitory activity or RIA. The concentrations causing 2+ macroscopic changes in the chorioallantoic membrane or 3+ microscopic changes in monkey kidney cells were designated toxic concentrations or TC. Toxicity relative to benzimidazole was designated relative toxicity or RT. The ratio of the toxic to virus inhibitory concentration was designated selectivity ratio or SR. Selectivity ratio relative to benzimidazole was designated relative selectivity or RS. The abbreviated designations, such as VIC for virus inhibitory concentration, are used only in tables and figures.

With a few derivatives significant protection of cells against virus-induced damage was observed. Protective activity may be expressed as the molar concentration of compound which restricts, under specified conditions, the extent of virus-induced cell damage in treated cultures to 25 per cent of that in untreated control cultures.

The derivatives studied have been grouped on the basis of the position and nature of substituents. Within each group compounds are listed in the order of their virus inhibitory activity. Most of the compounds were not completely dissolved at inhibitory or toxic concentrations. All compounds which dissolved poorly were shaken for 1 to 2 hours in protein-free Eagle's medium (20) at 35°C. in a mechanical shaker.

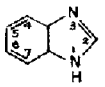
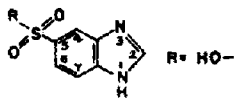
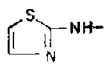
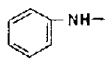
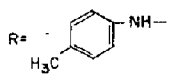
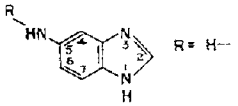
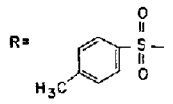
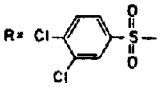
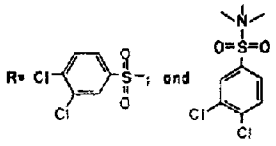
Studies with Influenza B Virus.—Results of studies of influenza virus inhibitory activity and selectivity of action of selected benzimidazole, naphthimidazole, and benzotriazole derivatives are summarized in Tables I and II.

Group A.—Compounds in Group A may be considered as 5-sulfo derivatives of benzimidazole. It is of interest that 5-sulfobenzimidazole itself lacked virus inhibitory activity whereas 5-sulfonamidobenzimidazole was slightly more active, though not significantly more selective, than benzimidazole. Introduction of a methyl group at position 2 reduced the inhibitory activity of the 5-sulfonamido derivative. The 5-sulfonanilide and 5-sulfon-(4-toluidide) derivatives were considerably more active, but at the same time less selective than benzimidazole. Inactivity of 2-(5'-benzimidazolesulfonamido)-thiazole may have been due to very low solubility.

These results indicate that presence of larger substituent groupings at position 5 is frequently associated with increased inhibitory activity. The dependence of the effect of introduction of a methyl group at position 2 on the nature of substituents present in the benzenoid ring is illustrated by the finding that the 5-sulfonamido-2-methyl derivative was less active

TABLE I

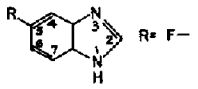
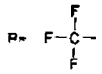
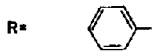
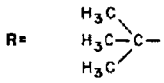
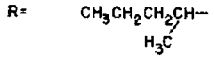
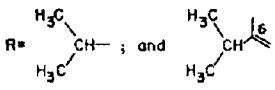
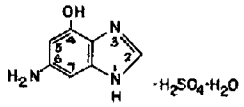
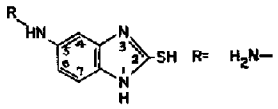
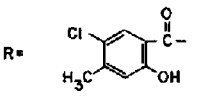
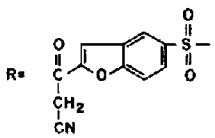
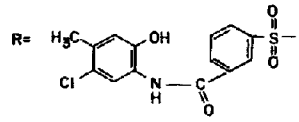
Influenza B Virus Inhibitory Activity and Selectivity of Selected Benzimidazole, Naphthimidazole, and Benzotriazole Derivatives in the Chorioallantoic Membrane in Vitro

Compound					75 per cent virus inhibitory concentration (VIC)	Relative inhibitory activity (RIA)	2+ toxic concentration (TC)	Relative toxicity (RT)	Selectivity ratio (SR)	Relative selectivity (RS)
Group	No.	Benzimidazole derivative	Mol. wt.	Structure						
					μM		μM			
	1	Benzimidazole*	118		3,500	1.0	12,000	1.0	3.4	1.0
A	2	5-Sulfo	198		>10,000	<0.35	>10,000	<1.2	—	—
	3	5-Sulfonamido	197	R= H_2N—	2,700	1.3	10,400	1.2	3.9	1.1
	4	5-Sulfonamido-2-methyl	211	R= H_2N—; and 	5,800	0.60	>6,800	<1.8	>1.2	>0.35
	5	2-(5'-Benzimidazolesulfonamido)-thiazole	280	R= 	>2,000	<1.8	>2,000	<6.0	—	—
	6	5-Benzimidazolesulfonanilide	273	R= 	150	23	290	41	1.9	0.56
	7	5-Benzimidazolesulfon-(4'-toluidide)	287	R= 	180	19	400	30	2.2	0.65
B	8	5-Amino‡	133		>3,500	<1.0	—	—	—	—
	9	5-(4'-Toluenesulfonamido)-	287	R= 	14	250	28	430	2.0	0.59
	10	5-(3',4'-Dichlorobenzenesulfonamido)-	342	R= 	10	350	80	150	8.0	2.4
	11	5-(3',4'-Dichlorobenzenesulfonamido)-1-(3'',4''-dichlorobenzenesulfonyl)-	551	R= 	6.3	560	45	270	7.1	2.1

* *J. Exp. Med.*, 1953, **98**, 245; *J. Bact.*, 1956, **72**, 41; *Virology*, 1957, **4**, 483.

‡ *J. Bact.*, 1956, **72**, 59.

TABLE I—*Continued*

Group	No.	Benzimidazole derivative	Mol. wt.	Structure	75 per cent virus inhibitory concentration (VIC)	Relative inhibitory activity (RIA)	2+ Toxic concentration (TC)	Relative toxicity (RT)	Selectivity ratio (SR)	Relative selectivity (RS)
					μM		μM			
C	12	5-Fluoro	136		2,500	1.4	6,400	1.9	2.6	0.76
	13	5-Trifluoromethyl	186		540	6.5	1,400	8.6	2.6	0.76
	14	5-Phenyl	194		300	12	330	36	1.1	0.32
	15	5-*tert*-butyl	174		260	13	320	38	1.2	0.35
	16	5-(α-Methylbutyl)-	188		78	45	170	71	2.2	0.65
	17	5,6-Diisopropyl	202		310	11	400	30	1.3	0.38
	18	6-Amino-4-hydroxybenzimidazole sulfate§	265		850	4.1	960	13	1.1	0.32
D	19	5-Hydrazino-2-mercapto	180		1,500	2.3	2,200	5.5	1.5	0.44
	20	5-(5′-Chloro-2′-hydroxy-4′-methylbenzamido)-2-mercapto	334		1,300	2.7	1,500	8.0	1.2	0.35
	21	5-(2′-Cyanoacetyl-5′-coumaronesulfonamido)-2-mercapto	412		1,100	3.2	1,300	9.2	1.2	0.35
	22	5-[3′-(5″-Chloro-2″-hydroxy-4″-methylphenylcarbamyl)-benzenesulfonamido]-2-mercapto	489		1,100	3.2	1,700	7.1	1.5	0.44

§ Obtained from Dr. M. Engelman, Columbia University, New York.

TABLE I—*Continued*

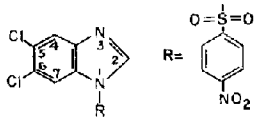
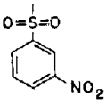
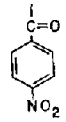
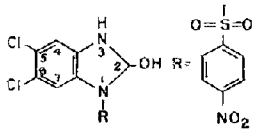
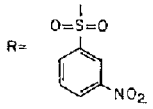
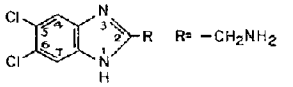
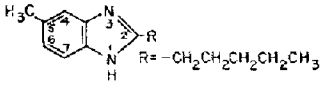
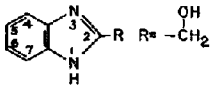
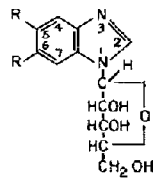
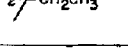
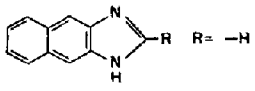
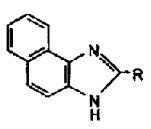
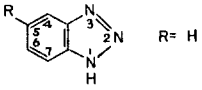
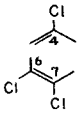
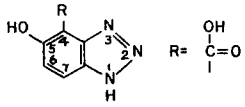
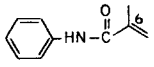
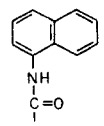
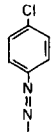
Group	No.	Benzimidazole derivative	Mol. wt.	Structure	75 per cent virus inhibitory concentration (VIC)	Relative inhibitory activity (RIA)	2+ toxic concentration (TC)	Relative toxicity (RT)	Selectivity ratio (SR)	Relative selectivity (RS)
					μM		μM			
E	23	1-(*p*-Nitrobenzenesulfonyl)-5,6-dichloro	372		>5,300	<0.66	>5,300	<2.3	—	—
	24	1-(*m*-Nitrobenzenesulfonyl)-5,6-dichloro	372		>5,300	<0.66	>5,300	<2.3	—	—
	25	1-(*p*-Nitrobenzoyl)-5,6-dichloro	336		>5,300	<0.66	>5,300	<2.3	—	—
	26	1-(*p*-Nitrobenzenesulfonyl)-2-hydroxy-5,6-dichlorobenzimidazoline	390		820	4.3	>1,900	<6.3	>2.3	>0.58
	27	1-(*m*-Nitrobenzenesulfonyl)-2-hydroxy-5,6-dichlorobenzimidazoline	390		570	6.1	3,000	4.0	5.3	1.6
F	28	2-Aminomethyl-5,6-dichloro	216		860	4.1	1,800	6.7	2.1	0.62
	29	2-(β-Aminoethyl)-5,6-dichloro	230	R= $-CH_2CH_2NH_2$	270	13	600	20	2.2	0.65
	30	2-(γ-Aminopropyl)-5,6-dichloro	244	R= $-CH_2CH_2CH_2NH_2$	270	13	670	18	2.5	0.74
	31	2-Amyl-5-methyl	202		73	48	210	57	2.9	0.85
	32	2-Heptyl-5-methyl	230	R= $-CH_2CH_2CH_2CH_2CH_2CH_2CH_3$	42	83	72	170	1.7	0.50

TABLE 1—*Continued*

Group	No.	Benzimidazole derivative	Mol. wt.	Structure	75 per cent virus inhibitory concentration (VIC)	Relative inhibitory activity (RIA)	2 + toxic concentration (TC)	Relative toxicity (RT)	Selectivity ratio (SR)	Relative selectivity (RS)
					μM		μM			
G	33	2-Hydroxymethyl	148	 R= $-CH_2OH$	>5,200	<0.67	>5,200	<2.3	—	—
	34	2-(α-Hydroxyethyl)-	162	R= $-CHOHCH_3$	>5,300	<0.66	>5,300	<2.3	—	—
	35	2-(α-Hydroxybenzyl)-	224	R= $-CHOH-C_6H_5$	>3,800	<0.92	>3,800	<3.2	—	—
	36	2-(α-Hydroxybenzyl)-5-chloro	259	R= $-CHOH-C_6H_5$; and 	>4,900	<0.71	>4,900	<2.4	—	—
	37	2-(α-Hydroxybenzyl)-5,6-dichloro	293	R= $-CHOH-C_6H_5$; and 	>2,900	<1.2	>2,900	<4.1	—	—
	38	2-(α-Hydroxybenzyl)-5,6-dimethyl	252	R= $-CHOH-C_6H_5$; and 	>5,300	<0.66	>5,300	<2.3	—	—
	39	2-Benzoyl	222	R= $-CO-C_6H_5$	>8,500	<0.41	>8,500	<1.4	—	—
	40	2-Benzyl	208	R= $-CH_2-C_6H_5$	>4,800	<0.73	>4,800	<2.5	—	—
	41	2-(p-Aminobenzenesulfonamido)-	288	R= $-NH-SO_2-C_6H_4-NH_2$	>2,700	<1.3	>2,700	<4.4	—	—
H	42	5,6-Dimethyl-1-(β-D-ribofuranosyl)-	278	 R= H_3C-	2,600	1.3	>2,600	<4.6	>1.0	>0.29
	43	2-Ethyl-5,6-dichloro-1-(β-D-ribofuranosyl)-	347	R= Cl— ; and 	890	3.9	1,700	7.1	1.9	0.56
		Naphthimidazole derivative								
I	44	Naphth-2,3-imidazole‖	168	 R= —H	900	3.9	1,300	9.2	1.4	0.41
	45	2-Ethylnaphth-2,3-imidazole‖	196	R= $-CH_2CH_3$	>3,500	<1.0	>3,500	<3.4	—	—
	46	2-Hydroxynaphth-2,3-imidazole‖	184	R= —OH	>3,500	<1.0	>3,500	<3.4	—	—
	47	Naphth-1,2-imidazole‖	168	 R= —H	370	9.5	790	15	2.1	0.62

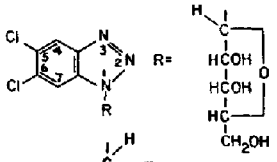
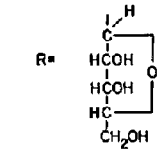
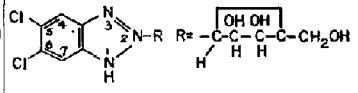
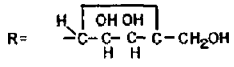
‖ Obtained from Dr. D. J. Brown, The Australian National University, Canberra.

TABLE I—*Continued*

Compound					75 per cent virus inhibitory concentration (VIC)	Relative inhibitory activity (RIA)	2+ toxic concentration (TC)	Relative toxicity (RT)	Selectivity ratio (SR)	Relative selectivity (RS)
Group	No.	Benzotriazole derivative	Mol. wt.	Structure						
					μM		μM			
	48	Benzotriazole	119	 R= H	7,500	0.47	11,000	1.1	1.5	0.44
	49	5-Chloro	154	R= Cl—	610	5.7	860	14	1.4	0.41
J	50	5,6-Dichloro	188	R= Cl—; and 	54	65	100	120	1.9	0.56
	51	4,5,6-Trichloro	223	R= Cl—; and 	12	290	44	270	3.7	1.1
	52	4,5,6,7-Tetrachloro	257	R= Cl—; and 	3	1,200	9	1,300	3.0	0.88
	53	5-Fluoro	137	R= F—	2,600	1.3	5,100	2.4	2.0	0.59
	54	5-Trifluoromethyl	187		180	19	400	30	2.2	0.65
K	55	5,6-Dimethyl	147	R= H_3C—; and 	>5,300	<0.66	>5,300	<2.3	—	—
	56	6-Amino-4-hydroxybenzotriazole dihydrochloride§	223	R= H— ; and  ·2 HCl	1,400	2.5	2,500	4.8	1.8	0.53
	57	5-Hydroxybenzotriazole-4-carboxylic acid¶	179		7,200	0.49	9,200	1.3	1.3	0.38
	58	5-Hydroxybenzotriazole-6-carboxylic acid¶	188	R= H— ; and  ·½ H_2O	6,700	0.52	9,000	1.3	1.3	0.38
	59	5-Hydroxybenzotriazole-6-carboxanilide¶	254	R= H—; and 	1,200	2.9	1,600	7.5	1.3	0.38
L	60	5-Hydroxybenzotriazole-4-carboxy-α-naphthylamide¶	304	R= 	13	270	40	300	3.1	0.91
	61	4-(*p*-Chlorophenylazo)-5-hydroxybenzotriazole¶	274	R= 	4.3	810	19	630	4.4	1.3

¶ Obtained from Dr. F. H. Adams, American Cyanamid Company, Bound Brook, New Jersey.

TABLE—I *Continued*

Group	No.	Benzotriazole derivative	Mol. wt.	Structure	75 per cent virus inhibitory concentration (VIC)	Relative inhibitory activity (RIA)	2+ toxic concentration (TC)	Relative toxicity (RT)	Selectivity ratio (SR)	Relative selectivity (RS)
					μM		μM			
M	62	1-(α-D-Ribofuranosyl)-5,6-dichloro	320		1,500	2.3	2,100	5.7	1.4	0.41
	63	1-(β-D-Ribofuranosyl)-5,6-dichloro	320		750	4.7	1,400	8.6	1.9	0.56
	64	2-(α-D-Ribofuranosyl)-5,6-dichloro	320		550	6.4	1,100	11	2.0	0.59
	65	2-(β-D-Ribofuranosyl)-5,6-dichloro	320		830	4.2	800	15	0.96	0.28

than 5-sulfonamidobenzimidazole. It was found earlier that whereas such introduction decreased the inhibitory activity of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (2), it increased the activity of 5-methyl, 4,5-dimethyl, 5,6-dimethyl, and 5-chlorobenzimidazole (1, 2).

Group B.—Compounds in this group may be considered to be 5-amino derivatives of benzimidazole. 5-Aminobenzimidazole itself was inactive (21); in contrast, the toluenesulfonamido and dichlorobenzenesulfonamido derivatives were highly active. The toluenesulfonamido derivative was less selective whereas the dichlorobenzenesulfonamido derivative was more selective than unsubstituted benzimidazole. The inhibitory activity of the dichlorobenzenesulfonamido derivative was further increased by introduction of a second dichlorobenzenesulfonyl group at position 1.

These results lend further support to the contention that the presence of large substituents at position 5 is associated with high inhibitory activity. Comparison of the structures and activities of 5-benzimidazolesulfon-(4′-toluidide) and 5-(4′-toluenesulfonamido)-benzimidazole reveals a 13-fold difference in activity in favor of the latter. As can be seen in Table I, the only structural difference between these compounds involves the sulfonamido link between the benzimidazole and toluene ring structures.

Group C.—This group includes five compounds with different substituents attached to position 5, and two compounds with two substituents in the benzenoid ring. It is of interest that the 5-trifluoromethyl derivative was more active than the 5-fluoro compound. The

5-phenyl, 5-*tert*-butyl, and 5-(α-methylbutyl) derivatives were compounds of moderate to high virus inhibitory activity. The 5,6-diisopropyl and 6-amino-4-hydroxy derivatives were also considerably more active than benzimidazole. All compounds in this group were less selective than benzimidazole.

These results, too, support the contention that substitution of larger groups at position 5 causes a marked increase in virus inhibitory activity.

Group D.—Each of the four derivatives of 2-mercaptobenzimidazole included in this group has a different substituent at position 5. These substituents range from the relatively simple hydrazino group to the complex 3-(5′-chloro-2′-hydroxy-4′-methylphenylcarbamyl)-benzenesulfonamido grouping. All four derivatives showed similar low virus inhibitory activity, which may be due to the presence of the mercapto group at position 2 or to the nature of the substituents at position 5. The selectivity of these derivatives was also low.

Group E.—In this group are included three derivatives of 5,6-dichlorobenzimidazole and two derivatives of 2-hydroxy-5,6-dichlorobenzimidazoline. The first three compounds were inactive. Since 5,6-dichlorobenzimidazole itself is approximately fourteen times more active than benzimidazole (2), the failure of the 1-nitrobenzenesulfonyl and 1-nitrobenzoyl derivatives to inhibit influenza virus multiplication may be due to a possible inactivating effect of these substitutions at position 1. Similar observations with other derivatives have been reported previously (2). The 1-(*p*-nitrobenzenesulfonyl) and 1-(*m*-nitrobenzenesulfonyl) derivatives of 2-hydroxy-5,6-dichlorobenzimidazoline were of low activity, but the meta compound was 1.6 times more selective than benzimidazole.

Group F.—Five compounds are included and each has a different substituent at position 2. The first three are derivatives of 5,6-dichlorobenzimidazole. Introduction of the aminomethyl group reduced the inhibitory activity of 5,6-dichlorobenzimidazole (2) to ⅛ whereas introduction of either the β-aminoethyl or γ-aminopropyl group had no effect on inhibitory activity, but increased the solubility of the compounds.

Both the 2-heptyl-5-methyl and the 2-amyl-5-methyl derivatives were highly active, with the 2-heptyl-5-methyl compound being two times more active than the 2-amyl-5-methyl derivative. This is of interest because in earlier studies it was found that although the 2-ethyl-5-methyl derivative was considerably more active than the 2,5-dimethyl compound, further lengthening of the alkyl side chain at carbon 2 by one or two alkyl residues (giving propyl and butyl derivatives) did not increase inhibitory activity (1).

All five new derivatives were less selective than unsubstituted benzimidazole.

Group G.—This group includes an additional series of nine derivatives substituted at position 2. None of these compounds showed inhibitory activity on influenza virus multiplication. As is reported below, two of these compounds, *i.e.* 2-(α-hydroxybenzyl)-benzimidazole and its 5-chloro and 5,6-dichloro derivatives, are active as inhibitors of poliovirus multiplication and cytopathogenicity.

It was found earlier that 5-methyl-2-D-ribobenzimidazole and certain other polyhydroxyalkyl derivatives of benzimidazole increase the yield of influenza virus in the chorioallantoic membrane (10, 11, 18). It appeared on the basis of these studies that the presence of hydroxyl groupings was the essential structural feature of position 2 substituted compounds with enhancing activity. The present finding that the 2-hydroxymethyl and 2-(α-hydroxyethyl) derivatives possess no enhancing activity indicates that a single hydroxyl group is not sufficient to impart such activity.

Group H.—Two 1-β-D-ribofuranosyl derivatives are included: the 5,6-dimethyl and the 2-ethyl-5,6-dichloro compounds. Both showed low inhibitory activity and selectivity.

5,6-Dimethyl-1-(β-D-ribofuranosyl)-benzimidazole differs from the benzimidazole riboside moiety in vitamin B_{12} only in that the ribose moiety is in the β rather than α linkage. It was shown earlier that the α-linked ribofuranoside of 5,6-dimethylbenzimidazole possesses no

influenza virus inhibitory activity (2). In contrast, the 5,6-dichloro derivative of β-D-ribofuranosylbenzimidazole is 92 times more active and 2.4 times more selective than benzimidazole (2). Thus, for high activity the presence of chlorine rather than methyl substituents in the benzenoid ring appears essential. Certain other halogen derivatives of β-linked ribofuranosylbenzimidazole have also been examined and found to be highly active (5).

The fact that introduction of an ethyl group at position 2 of the 5,6-dichloro-1-β-D-ribofuranosyl compound lowered its inhibitory activity to $\frac{1}{23}$ is in line with the earlier observation that introduction of a methyl group reduced the activity to $\frac{1}{9.5}$ (2). These findings provide a contrast to the observation that such substitution increases the inhibitory activity of derivatives containing alkyl substituents in the benzenoid ring (1).

Group I.—Four naphthimidazole compounds are included. The parent compound, naphth-2,3-imidazole was of low activity and selectivity. Substitution of an ethyl or hydroxy group at position 2 inactivated the compound. Naphth-1,2-imidazole was 2.4 times more active than naphth-2,3-imidazole, but it, too, showed low selectivity. These findings parallel some of the observations with benzimidazole derivatives.

Group J.—This group includes benzotriazole and four derivatives with one to four chlorines in the benzenoid ring. Benzotriazole was only one-half as active as benzimidazole. However, with each chlorine introduced the activity increased markedly. 4,5,6,7-Tetrachlorobenzotriazole was the most active compound among the sixty-five included in Table I. It caused 75 per cent inhibition of influenza virus multiplication at a concentration of 3.0×10^{-6} M or 0.8 μg./ml. The selectivity of the tri- and tetrachloro derivatives was similar to that of benzimidazole. Benzotriazole, and the mono- and dichloro derivatives were less selective.

The series of four chloro derivatives of benzotriazole provides a striking example of dependence of high virus inhibitory activity on multiple substitution in the benzenoid ring. Benzotriazole itself was less active than benzimidazole and the monochloro derivative of benzotriazole was only 1.2 times more active than the corresponging benzimidazole, but the di-, tri-, and tetrachloro derivatives of benzotriazole were approximately five times more active than the corresponding benzimidazoles. Furthermore, the benzotriazole derivatives were considerably more soluble at pH 7.2 in the medium used.

Group K.—This group includes two benzotriazole derivatives substituted at position 5, and two others which have two substituents in the benzenoid ring. The 5-trifluoromethyl compound was much more active than the 5-fluoro derivative, but both were less selective than benzimidazole. 5,6-Dimethylbenzotriazole was inactive and the 6-amino-4-hydroxy compound showed low activity and low selectivity.

The potentiating effect on inhibitory activity of introduction of larger groups is strikingly illustrated by the 15-fold difference in activity between the 5-trifluoromethyl and 5-fluoro derivatives. Failure of 5,6-dimethylbenzotriazole to inhibit may be due to low solubility or intrinsic inactivity of the compounds.

Group L.—All of the benzotriazole derivatives in this group possess an hydroxyl group at position 5. In addition, each compound has a second substituent at either position 4 or 6. The carboxylic acid derivatives of 5-hydroxybenzotriazole showed very low activity and selectivity. The 6-carboxanilide derivative was 2.9 times more active than benzimidazole, but it, too, was non-selective. In contrast, the 4-carboxy-α-naphthylamide was very highly active, and in selectivity of action it was similar to benzimidazole. The 4-(*p*-chlorophenylazo) compound was even more active, and it was slightly more selective than the reference compound.

The 4-carboxy-α-naphthylamide and 4-(*p*-chlorophenylazo) derivatives provide striking examples of correlation between presence of large substituents in the benzenoid ring and high virus inhibitory activity. The low activity of the 6-carboxanilide derivative is noteworthy, because it suggests that the position of the substituent in the benzenoid ring may be impor-

tant. Earlier work showed (1) that introduction of methyl groups at positions 4 and 5 of the benzenoid ring cumulatively increased the inhibitory activity of benzimidazole derivatives; in contrast, introduction of a methyl group at position 6 did not cause an increase in activity.

Group M.—This group includes four D-ribofuranosyl derivatives of 5,6-dichlorobenzotriazole. All four compounds showed low virus inhibitory activity and selectivity. The fact that substitution of the D-ribofuranose moiety in β-linkage at position 1 resulted in marked inactivation rather than in potentiation of the inhibitory activity of 5,6-dichlorobenzotriazole is surprising because the β-D-ribofuranosides of six halogenated benzimidazoles were all more active than the corresponding parent compounds without the ribofuranosyl moiety (2, 5). This provides another example of the dependence of the effect of a substituent not only on its own nature and position, but also on the structural characteristics of other parts of the molecule.

Summary of Influenza Virus Inhibitory Activity and Selectivity of Certain Benzimidazole, Naphthimidazole, and Benzotriazole Derivatives. In Table II the compounds studied are listed in order of their influenza virus inhibitory activity. As can be seen, a considerable number, *i.e.* eighteen, showed no detectable inhibitory activity and four additional compounds were less active than benzimidazole. Forty-three derivatives were more active than benzimidazole. The most active derivative, 4,5,6,7-tetrachlorobenzotriazole, was 1200 times more active than the reference compound; however, it was slightly less selective than benzimidazole.

Three compounds, 5-sulfonamidobenzimidazole, 5-hydroxybenzotriazole-4-carboxy-α-naphthylamide, and 4,5,6-trichlorobenzotriazole were closely similar to benzimidazole in selectivity of action. The relative virus inhibitory activities of these compounds were 1.3, 270, and 290 respectively.

Only four compounds showed somewhat greater selectivity than benzimidazole; 1-(*m*-nitrobenzenesulfonyl)-2-hydroxy-5,6-dichlorobenzimidazoline, 5-(3′,4′- dichlorobenzenesulfonamido) - benzimidazole, 5 - (3′,4′ - dichlorobenzenesulfonamido)-1-(3″,4″-dichlorobenzenesulfonyl)-benzimidazole, and 4-(*p*-chlorophenylazo)-5-hydroxybenzotriazole. The relative virus inhibitory activities of these compounds were 6.1, 350, 560, and 810, respectively. Thus, most of the compounds studied were less selective than benzimidazole, and none was highly selective.

Studies with Poliovirus Type 2.—Of the sixty-five benzimidazole, naphthimidazole, and benzotriazole derivatives studied with influenza virus in the chorioallantoic membrane, twenty-five were investigated with poliovirus in monkey kidney cells. The results of studies of the relationship between structure of these selected compounds and their poliovirus inhibitory activity and selectivity are summarized in Table III. To facilitate cross-reference, compounds listed in Table III are referred to by the same numbers as were used in Tables I and II.

Group A.—The poliovirus inhibitory activity of 5-sulfonamidobenzimidazole was similar to that of the reference compound, but the sulfonamido derivative was approximately twice

TABLE II

Activities and Selectivities of Benzimidazole, Naphthimidazole, and Benzotriazole Derivatives as Inhibitors of Influenza B Virus Multiplication

No.	Derivative	Inhibitory activity relative to benzimidazole	Selectivity relative to benzimidazole
1	Benzimidazole	1.0	1.0
2	5-Sulfobenzimidazole	<0.35	—
5	2-(5′-Benzimidazolesulfonamido)-thiazole	<1.8	—
8	5-Aminobenzimidazole	<1.0	—
23	1-(*p*-Nitrobenzenesulfonyl)-5,6-dichlorobenzimidazole	<0.66	—
24	1-(*m*-Nitrobenzenesulfonyl)-5,6-dichlorobenzimidazole	<0.66	—
25	1-(*p*-Nitrobenzoyl)-5,6-dichlorobenzimidazole	<0.66	—
33	2-Hydroxymethylbenzimidazole	<0.67	—
34	2-(α-Hydroxyethyl)-benzimidazole	<0.66	—
35	2-(α-Hydroxybenzyl)-benzimidazole	<0.92	—
36	2-(α-Hydroxybenzyl)-5-chlorobenzimidazole	<0.71	—
37	2-(α-Hydroxybenzyl)-5,6-dichlorobenzimidazole	<1.2	—
38	2-(α-Hydroxybenzyl)-5,6-dimethylbenzimidazole	<0.66	—
39	2-Benzoylbenzimidazole	<0.41	—
40	2-Benzylbenzimidazole	<0.73	—
41	2-(*p*-Aminobenzenesulfonamido)-benzimidazole	<1.3	—
45	2-Ethylnaphth-2,3-imidazole	<1.0	—
46	2-Hydroxynaphth-2,3-imidazole	<1.0	—
55	5,6-Dimethylbenzotriazole	<0.66	—
48	Benzotriazole	0.47	0.44
57	5-Hydroxybenzotriazole-4-carboxylic acid	0.49	0.38
58	5-Hydroxybenzotriazole-6-carboxylic acid	0.52	0.38
4	5-Sulfonamido-2-methylbenzimidazole	0.60	>0.35
3	5-Sulfonamidobenzimidazole	1.3	1.1
42	5,6-Dimethyl-1-(β-D-ribofuranosyl)-benzimidazole	1.3	>0.29
53	5-Fluorobenzotriazole	1.3	0.59
12	5-Fluorobenzimidazole	1.4	0.76
19	5-Hydrazino-2-mercaptobenzimidazole	2.3	0.44
62	1-(α-D-Ribofuranosyl)-5,6-dichlorobenzotriazole	2.3	0.41
56	6-Amino-4-hydroxybenzotriazole dihydrochloride	2.5	0.53
20	5-(5′-Chloro-2′-hydroxy-4′-methylbenzamido)-2-mercaptobenzimidazole	2.7	0.35
59	5-Hydroxybenzotriazole-6-carboxanilide	2.9	0.38
21	5-(2′-Cyanoacetyl-5′-coumaronesulfonamido)-2-mercaptobenzimidazole	3.2	0.35
22	5-[3′-(5″-Chloro-2″-hydroxy-4″-methylphenylcarbamyl)-benzenesulfonamido]-2-mercaptobenzimidazole	3.2	0.44
43	2-Ethyl-5,6-dichloro-1-(β-D-ribofuranosyl)-benzimidazole	3.9	0.56
44	Naphth-2,3-imidazole	3.9	0.41
18	6-Amino-4-hydroxybenzimidazole sulfate	4.1	0.32
28	2-Aminomethyl-5,6-dichlorobenzimidazole	4.1	0.62
65	2-(β-D-Ribofuranosyl)-5,6-dichlorobenzotriazole	4.2	0.28
26	1-(*p*-Nitrobenzenesulfonyl)-2-hydroxy-5,6-dichlorobenzimidazoline	4.3	>0.68
63	1-(β-D-Ribofuranosyl)-5,6-dichlorobenzotriazole	4.7	0.56
49	5-Chlorobenzotriazole	5.7	0.41
27	1-(*m*-Nitrobenzenesulfonyl)-2-hydroxy-5,6-dichlorobenzimidazoline	6.1	1.6
64	2-(α-D-Ribofuranosyl)-5,6-dichlorobenzotriazole	6.4	0.59
13	5-Trifluoromethylbenzimidazole	6.5	0.76
47	Naphth-1,2-imidazole	9.5	0.62
17	5,6-Diisopropylbenzimidazole	11	0.38
14	5-Phenylbenzimidazole	12	0.32
15	5-*tert*-butylbenzimidazole	13	0.35
29	2-(β-Aminoethyl)-5,6-dichlorobenzimidazole	13	0.65
30	2-(γ-Aminopropyl)-5,6-dichlorobenzimidazole	13	0.74
7	5-Benzimidazolesulfon-(4′-toluidide)	19	0.65
54	5-Trifluoromethylbenzotriazole	19	0.65
6	5-Benzimidazolesulfonanilide	23	0.56
16	5-(α-Methylbutyl)-benzimidazole	45	0.65
31	2-Amyl-5-methylbenzimidazole	48	0.85
50	5,6-Dichlorobenzotriazole	65	0.56
32	2-Heptyl-5-methylbenzimidazole	83	0.50
9	5-(4′-Toluenesulfonamido)-benzimidazole	250	0.59
60	5-Hydroxybenzotriazole-4-carboxy-α-naphthylamide	270	0.91
51	4,5,6-Trichlorobenzotriazole	290	1.1
10	5-(3′,4′-Dichlorobenzenesulfonamido)-benzimidazole	350	2.4
11	5-(3′,4′-Dichlorobenzenesulfonamido)-1-(3″,4″-dichlorobenzenesulfonyl)-benzimidazole	560	2.1
61	4-(*p*-Chlorophenylazo)-5-hydroxybenzotriazole	810	1.3
52	4,5,6,7-Tetrachlorobenzotriazole	1200	0.88

as selective. Both the sulfonanilide and sulfon-(4-toluidide) derivatives were moderately active, and the latter also showed increased selectivity. Except for some minor differences,

TABLE III

Poliovirus Type 2 Inhibitory Activity and Selectivity of Benzimidazole and Benzotriazole Derivatives in Monkey Kidney Cells in Vitro

Compound			75 per cent virus inhibitory concentration (VIC)	Relative inhibitory activity (RIA)	3+ toxic concentration (TC)	Relative toxicity (RT)	Selectivity ratio (SR)	Relative selectivity (RS)
Group	No.	Benzimidazole derivative						
			μM		μM			
	1	Benzimidazole	2800	1	5200	1	1.9	1
A	3	5-Sulfonamido	2400	1.2	>8800	<0.59	>3.7	>1.9
	6	5-Benzimidazolesulfonanilide	130	22	>270	<19	>2.1	>1.1
	7	5-Benzimidazolesulfon-(4′-toluidide)	96	29	>400	>13	>4.2	>2.2
B	9	5-(4′-Toluenesulfonamido)-	21	130	>60	<87	>2.9	>1.5
	10	5-(3′,4′-Dichlorobenzenesulfonamido)-	12	230	>100	<52	>8.3	>4.4
	11	5-(3′,4′-Dichlorobenzenesulfonamido)-1-(3″,4″-dichlorobenzenesulfonyl)-	10	280	>100	<52	>10	>5.3
C	13	5-Trifluoromethyl	350	8.0	>450	<12	>1.3	>0.68
F	29	2-Aminomethyl-5,6-dichloro	130	22	950	55	7.3	3.8
	30	2-(β-Aminoethyl)-5,6-dichloro	~60	~47	~60	~87	~1	~0.53
	31	2-(γ-Aminopropyl)-5,6-dichloro	~60	~47	~60	~87	~1	~0.53
G	33	2-Hydroxymethyl	>3800	<0.74	>3800	<1.4	—	—
	34	2-(α-Hydroxyethyl)-	>3500	<0.80	>3500	<1.5	—	—
	35	2-(α-Hydroxybenzyl)-	36	78	>1100	<4.7	>31	>16
	36	2-(α-Hydroyxbenzyl)-5-chloro	22	130	>640	<8.1	>29	>15
	37	2-(α-Hydroxybenzyl)-5,6-dichloro	140	20	>1300	<4.1	>9.1	>4.8
	38	2-(α-Hydroxybenzyl)-5,6-dimethyl	740	3.8	>1000	<5.2	>1.4	>0.74
	39	2-Benzoyl	740	3.8	>1100	<4.7	>1.5	>0.79
	40	2-Benzyl	95	29	>360	<14	>3.8	>2.0
		Benzotriazole derivative						
J	48	Benzotriazole	2800	1	11000	0.47	3.9	2.1
	49	5-Chloro	500	5.6	1600	3.3	3.2	1.7
	50	5,6-Dichloro	73	38	260	20	3.6	1.9
	51	4,5,6-Trichloro	23	120	50	100	2.2	1.2
	52	4,5,6,7-Tetrachloro	8.1	350	12	430	1.5	0.79
K	54	5-Trifluoromethyl	240	12	>450	12	1.9	1.0

the inhibitory activity and selectivity of action of compounds in this group were similar with influenza and poliovirus.

Group B.—The toluenesulfonamido and dichlorobenzenesulfonamido derivatives were highly active, and showed increased selectivity. The activity and selectivity of compounds in this group were similar with influenza and poliovirus.

Group C.—5-Trifluoromethylbenzimidazole was eight times more active, but not more

selective than the reference compound. Similar results were also obtained with influenza virus.

Group F.—The 2-aminomethyl-5,6-dichloro derivative was one-half as active, but several times more selective than the corresponding 2-β-aminoethyl and 2-γ-aminopropyl compounds, which appeared to be considerably more toxic for monkey kidney cells than for the chorioallantoic membrane. All three derivatives were more active against poliovirus in monkey kidney cells than against influenza virus in the chorioallantoic membrane.

Group G.—From the viewpoint of selective inhibition of poliovirus multiplication this group contains derivatives of greatest interest. Of the six compounds which possessed inhibitory activity on poliovirus multiplication, three were of sufficiently low toxicity so that at virus inhibitory concentrations only slight or no microscopic cell changes due to compound were seen at 48 hours. Two of the three, *i.e.* 2-(α-hydroxybenzyl)-benzimidazole (HBB) and the 5-chloro derivative of HBB, were also capable of causing marked inhibition of the cytopathic effects of poliovirus, as is shown below. The third compound, *i.e.* the 5,6-dichloro derivative of HBB, was much less active as an inhibitor of poliovirus multiplication and cytopathic effects than either the parent molecule or the 5-chloro derivative.

The data on structure-activity relationships summarized in Table III indicate that the hydroxybenzyl grouping at position 2 is of fundamental importance for the selective virus inhibitory action of HBB. As can be seen in Table III, the 2-hydroxymethyl and 2-(α-hydroxyethyl) derivatives of benzimidazole were inactive, and the 2-benzoyl and 2-benzyl derivatives showed low selectivity although they were slightly to moderately active.

Compared to HBB or its 5-chloro derivative, the low activity and selectivity of the 5,6-dimethyl derivative are striking. This observation and the finding that the activity of the 5,6-dichloro derivative was also relatively low, indicate that introduction of chloro or methyl substituents at both the 5 and 6 positions is undesirable from the viewpoint of virus inhibitory potency and selectivity.

It should be emphasized that none of the compounds in group G showed any inhibitory activity with influenza B virus in the chorioallantoic membrane. HBB was also studied with influenza virus in monkey kidney cells, and, as is reported below, no inhibition of virus yield or virus-induced cell damage was observed.

Group J.—Benzotriazole was equal in activity to benzimidazole. The chloro derivatives which contained one to four chlorine atoms in the benzenoid ring were progressively more active as the number of substituents was increased. However, the highly active tri- and tetrachloro derivatives were less selective than benzotriazole or the mono- and dichloro derivatives.

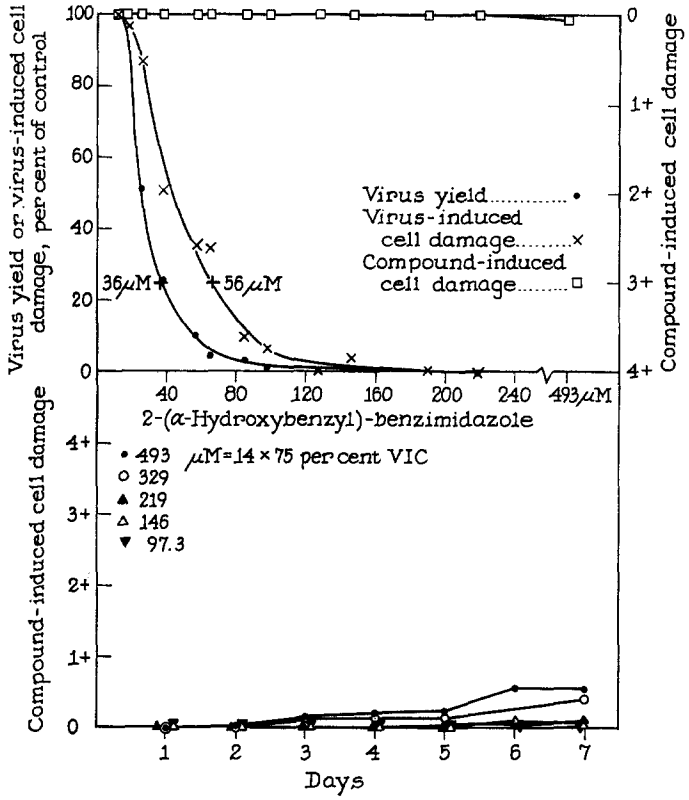
Benzotriazole showed greater activity with poliomyelitis than influenza virus, whereas with the tri- and tetrachloro derivatives the reverse was true. The mono- and dichloro derivatives showed similar activity with the two viruses. With respect to selectivity, benzotriazole and the mono- and dichloro derivatives were all somewhat more selective in inhibiting poliomyelitis than influenza virus multiplication. No striking differences were observed in this respect with the two viruses and tri- and tetrachloro derivatives.

Group K.—The 5-trifluoromethyl compound was twelve times more active, but not more selective than benzimidazole. Similar results were obtained with influenza virus.

Summary of Poliovirus Inhibitory Activity and Selectivity of Certain Benzimidazole and Benzotriazole Derivatives.—Of the twenty-five compounds studied, two lacked poliovirus inhibitory activity and the activity of another was similar to that of benzimidazole. Twenty-one derivatives were more active than the reference compound. The most active derivative, 4,5,6,7-tetrachlorobenzotriazole, was 350 times more active than the reference compound; however, it was slightly less selective than benzimidazole. Several compounds

were similar to benzimidazole in selectivity of action, or showed somewhat greater selectivity.

Two compounds stood out in that they were both much more active and selective than benzimidazole. 2-(α-Hydroxybenzyl)-benzimidazole (HBB) and 2-(α-hydroxybenzyl)-5-chlorobenzimidazole showed 78 and 130 times greater

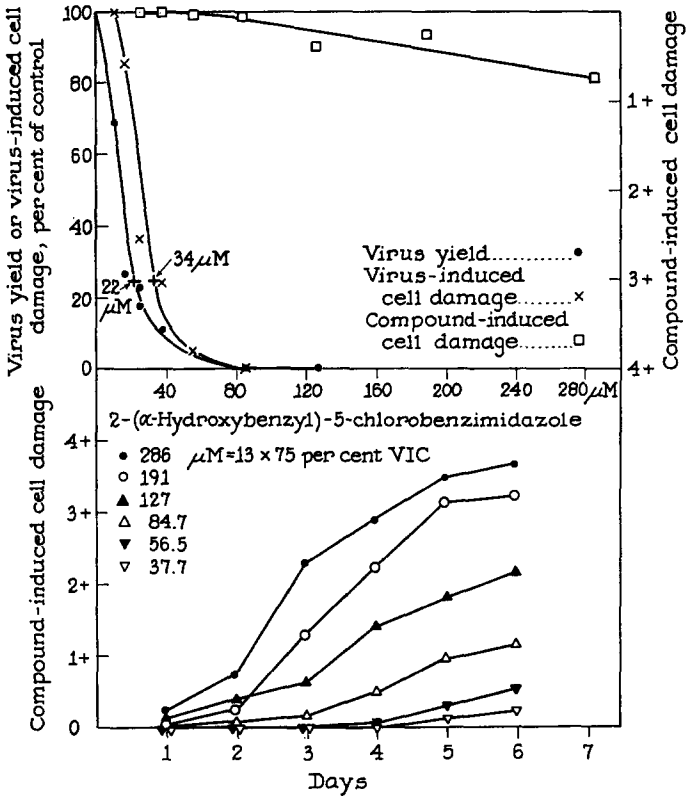


TEXT-FIG. 1. Inhibition of multiplication and cytopathic effects of poliovirus type 2 by 2-(α-hydroxybenzyl)-benzimidazole (HBB), and toxicity of the compound in monkey kidney cells.

activity, respectively, and more than 15-fold greater selectivity than benzimidazole. These compounds, and also the 5,6-dichloro derivative of HBB are discussed below.

Inhibition of Poliovirus Multiplication and Cytopathic Effects by 2-(α-Hydroxybenzyl)-benzimidazole (HBB), and Its 5-Chloro and 5,6-Dichloro Derivatives.— Of the twenty-three benzimidazole and benzotriazole derivatives which were capable of inhibiting the yield of poliovirus in monkey kidney cells only two, *i.e.* 2-(α-hydroxybenzyl)-benzimidazole (HBB), and the 5-chloro derivative of

HBB, showed significant protective activity against virus-induced cell damage, as illustrated in Text-figs. 1 and 2. The 5,6-dichloro derivative of HBB did not reproducibly cause 75 per cent inhibition of viral cytopathic effects (*cf.* Text-fig. 3). It should be emphasized that neither HBB nor its chloro derivatives were completely dissolved at the higher concentrations employed.

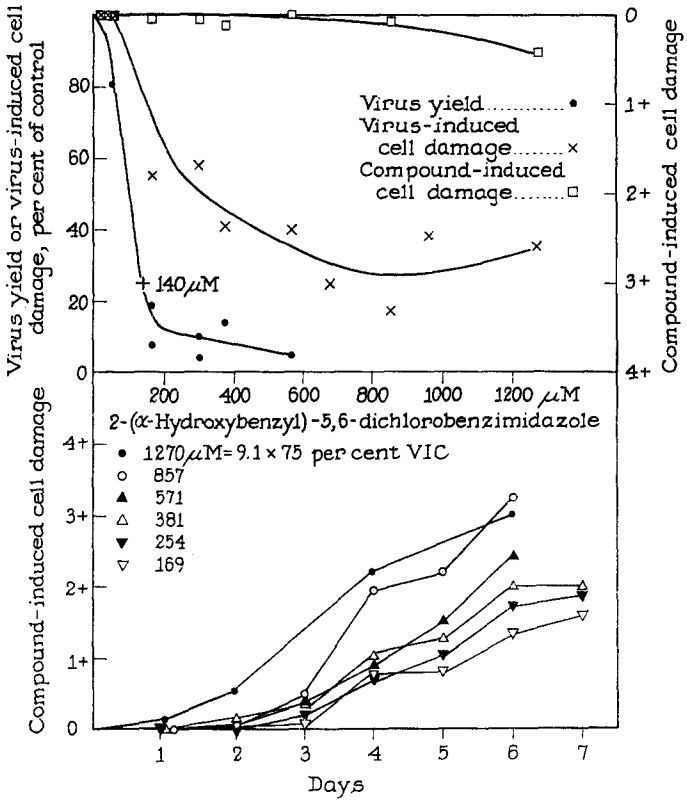


TEXT-FIG. 2. Inhibition of multiplication and cytopathic effects of poliovirus type 2 by 2-(α-hydroxybenzyl)-5-chlorobenzimidazole, and toxicity of the compound in monkey kidney cells.

The upper parts of Text-figs. 1 to 3 show curves relating compound-induced cell damage, and inhibition of virus yield and virus-induced cell damage, to concentration of HBB and its derivatives. All determinations were carried out at 48 hours. The lower parts show time curves of compound-induced cell damage.

With HBB, the curves describing the relationship between concentration of compound and inhibition of virus yield or reduction in virus-induced cell

damage were approximately parallel. At a concentration of 190 μM, all cells appeared protected. Over the greater part of the concentration range studied, no compound-induced cell changes were observed in uninfected cultures. At 493 μM, which was the highest concentration used, slight cell changes due to compound were observed. The time curves of compound-induced cell damage



TEXT-FIG. 3. Inhibition of multiplication and cytopathic effects of poliovirus type 2 by 2-(α-hydroxybenzyl)-5,6-dichlorobenzimidazole, and toxicity of the compound in monkey kidney cells.

show that even at 493 μM the extent of HBB-induced cell changes increased only slightly on prolonged incubation.

With the monochloro derivative, the curves describing inhibition in virus yield and reduction in virus-induced cell damage were also approximately parallel. At a concentration of 85 μM all cells appeared protected, but the monochloro derivative caused definite toxic effects at the higher concentrations. Compound-induced cell changes were observed in 48 hours, and they became marked as incubation was continued.

With both HBB and its 5-chloro derivative the concentrations at which 75 per cent reduction in virus-induced cell damage was observed were 1.5 times higher than those sufficient to cause 75 per cent reduction in virus yield.

With the dichloro derivative, the curve relating reduction in virus-induced cell damage to concentration of compound deviated considerably from that describing the relationship between concentration and inhibition in virus yield, and, regardless of concentration,the highest degree of protection observed was only 60 to 80 per cent. It should be emphasized that this compound was much less active than the other two in inhibiting the yield of virus. As with the monochloro derivative, significant cytotoxic changes were observed at the higher concentrations. These changes became marked when incubation was continued for a period of 6 to 7 days.

The results obtained suggest that reduction in poliovirus-induced cell damage was due to inhibition of the viral reproductive process. This conclusion is supported by results obtained with influenza B virus in monkey kidney cells. HBB not only failed to inhibit the multiplication of influenza virus, but it also had no significant protective effect against influenza virus-induced cell damage.

Progression of Compound-Induced Cell Damage.—The time curves of compound-induced cell damage obtained with 20 benzimidazole and benzotriazole derivatives lacking protective activity against poliovirus-induced cell damage are shown in Text-figs. 4 and 5. Each of these 20 compounds reduced the yield of poliovirus, but showed low selectivity.

With most compounds increase in cell damage was gradual with time, and observations made at 48 hours were representative of the total time course of toxic phenomena, particularly at lower concentrations of the compounds. However, with a few compounds, a rather abrupt increase in microscopic cell damage was observed after the 2nd day, particularly at higher concentrations.

Poliovirus Inhibitory Activity of Certain Benzimidazole Derivatives.—In earlier studies of the effects of selected benzimidazole derivatives on influenza B virus multiplication (4, 5, 7), certain compounds were found to be of particular interest. Three such compounds have been studied with poliovirus type 2, and the results are summarized in Table IV and in Text-fig. 6.

2-Ethyl-5-methylbenzimidazole was the most selective compound among the alkyl derivatives studied with influenza virus (4). Furthermore, this compound was nineteen times more active than benzimidazole. As an inhibitor of poliovirus, the 2-ethyl-5-methyl derivative was not significantly different from the reference compound in selectivity of action, and its relative inhibitory activity was 7.2.

5-(or 6-)Bromo-4,5-(or 5,7-) dichloro-1-β-D-ribofuranosylbenzimidazole was, among the compounds examined, the most active and selective inhibitor of influenza virus multiplication (5). This compound was 1950 times more active

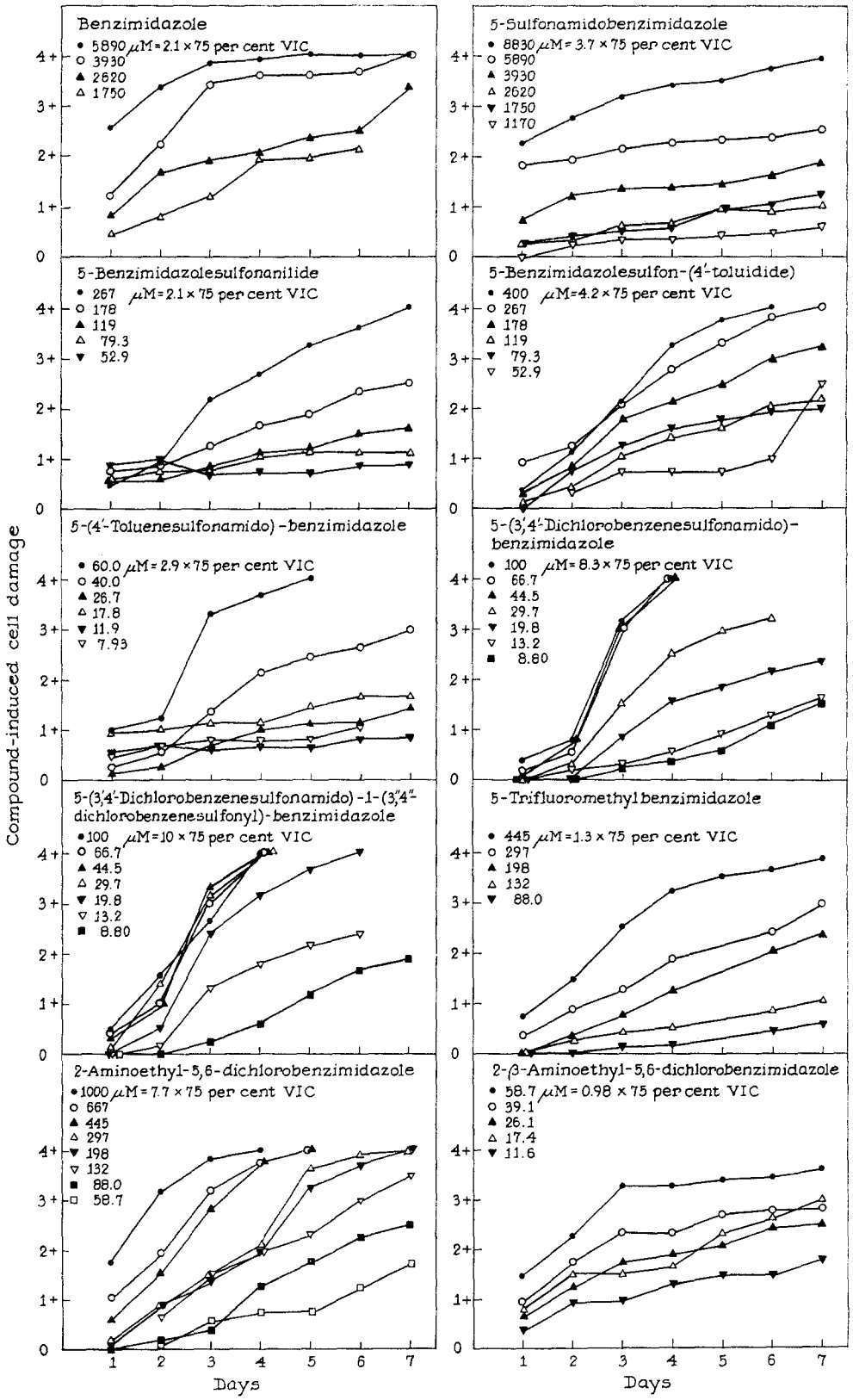


FIG. 4

TEXT-FIGS. 4 and 5. Compound-induced damage-time curves with selected benzimidazole and benzotriazole derivatives in monkey kidney cells.

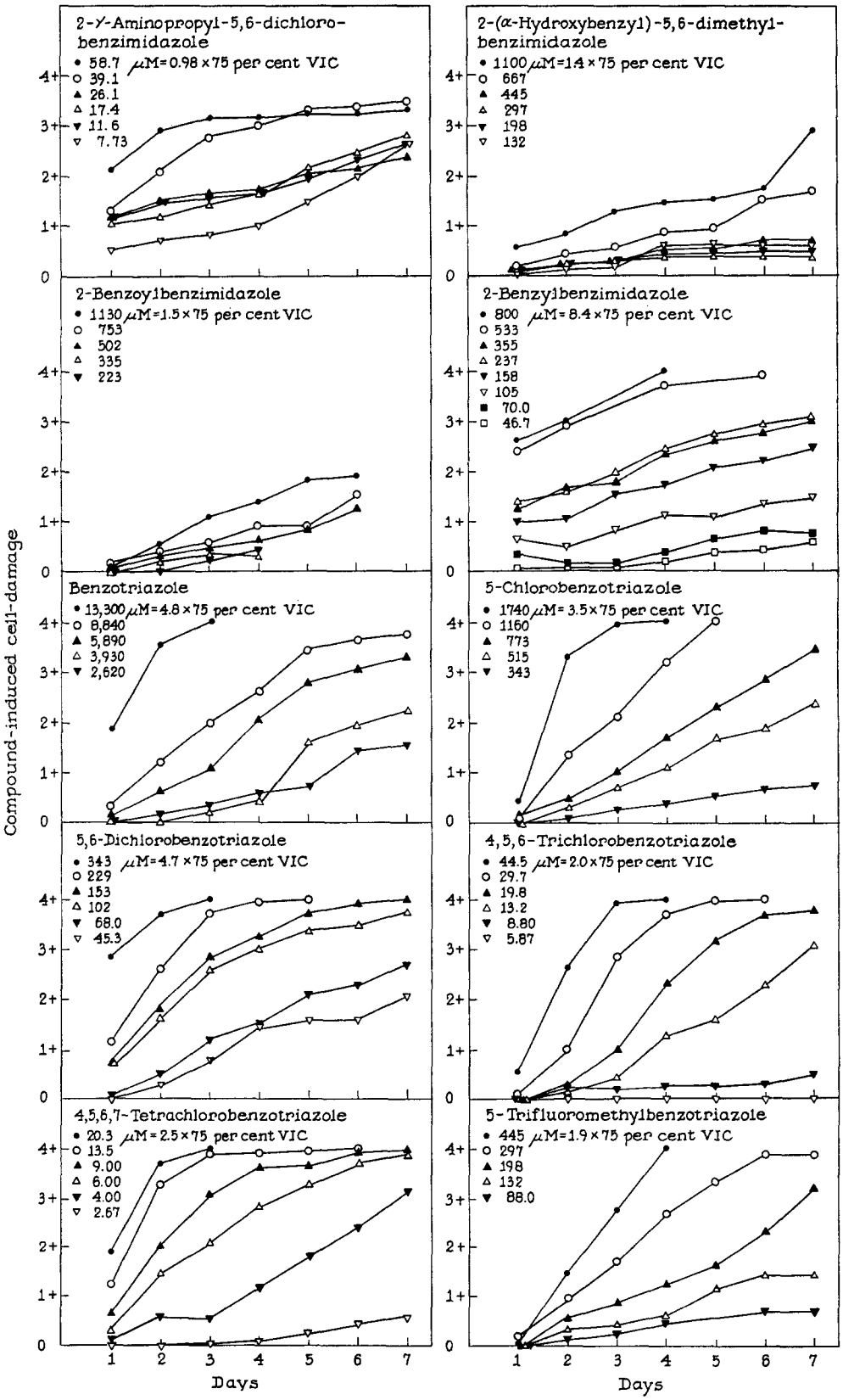

2-γ-Aminopropyl-5,6-dichloro-benzimidazole
• 58.7 μM=0.98×75 per cent VIC
○ 39.1
▲ 26.1
△ 17.4
▼ 11.6
▽ 7.73
2-(α-Hydroxybenzyl)-5,6-dimethyl-benzimidazole
• 1100 μM=14×75 per cent VIC
○ 667
▲ 445
△ 297
▼ 198
▽ 132
2-Benzoylbenzimidazole
• 1130 μM=1.5×75 per cent VIC
○ 753
▲ 502
△ 335
▼ 223
2-Benzylbenzimidazole
• 800 μM=8.4×75 per cent VIC
○ 533
▲ 355
△ 237
▼ 158
▽ 105
■ 70.0
□ 46.7
Benzotriazole
• 13,300 μM=4.8×75 per cent VIC
○ 8,840
▲ 5,890
△ 3,930
▼ 2,620
5-Chlorobenzotriazole
• 1740 μM=3.5×75 per cent VIC
○ 1160
▲ 773
△ 515
▼ 343
5,6-Dichlorobenzotriazole
• 343 μM=4.7×75 per cent VIC
○ 229
▲ 153
△ 102
▼ 68.0
▽ 45.3
4,5,6-Trichlorobenzotriazole
• 44.5 μM=2.0×75 per cent VIC
○ 29.7
▲ 19.8
△ 13.2
▼ 8.80
▽ 5.87
4,5,6,7-Tetrachlorobenzotriazole
• 20.3 μM=2.5×75 per cent VIC
○ 13.5
▲ 9.00
△ 6.00
▼ 4.00
▽ 2.67
5-Trifluoromethylbenzotriazole
• 445 μM=1.9×75 per cent VIC
○ 297
▲ 198
△ 132
▼ 88.0
Compound-induced cell-damage
0
1+
2+
3+
4+
1
2
3
4
5
6
7
Days

FIG. 5

than benzimidazole. With poliovirus, the relative inhibitory activity was 670, and it was not significantly more selective than benzimidazole.

Finally, 5-methyl-2-D-ribobenzimidazole was remarkable in that it increased the yield of influenza B virus (7). With poliovirus this compound showed slight inhibitory activity on virus yield.

None of these derivatives showed protective activity against poliovirus-induced cell damage.

TABLE IV

Poliovirus Type 2 Inhibitory Activity and Selectivity of Certain Benzimidazole Derivatives in Monkey Kidney Cells in Vitro

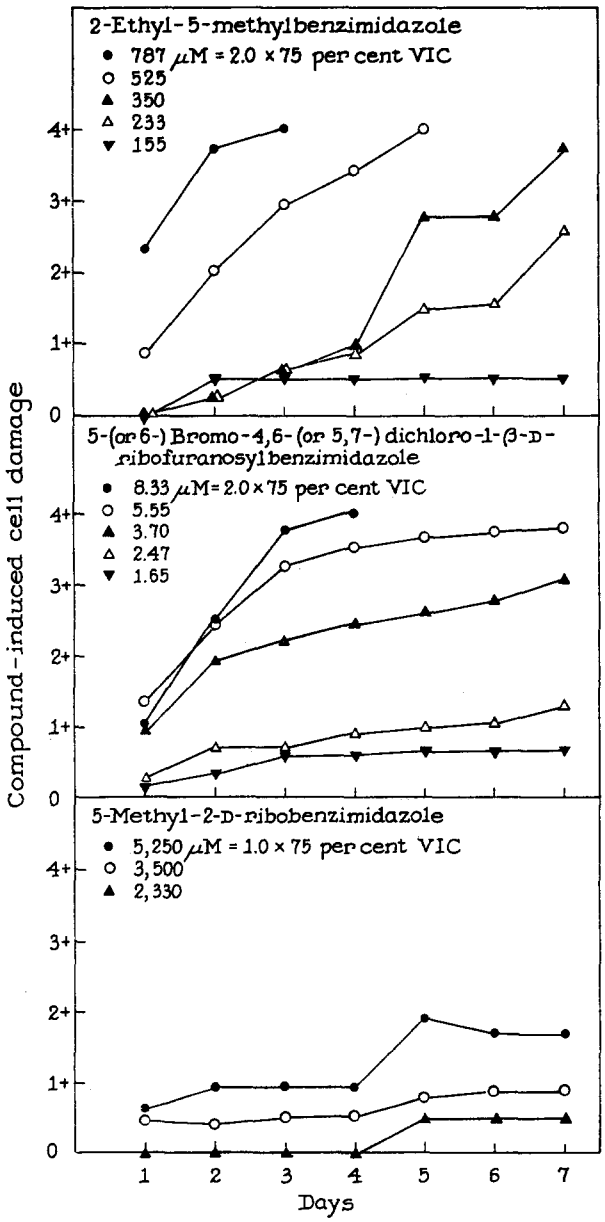
Benzimidazole derivative	75 per cent virus inhibitory concentration	Relative inhibitory activity	3+ toxic concentration	Relative toxicity	Selectivity ratio	Relative selectivity
	μM		μM			
Benzimidazole	2800	1	5200	1	1.9	1
2-Ethyl-5-methyl	390	7.2	~680	~7.6	~1.7	~0.89
5-(or 6-)Bromo-4,6-(or 5,7-)dichloro-1-β-D-ribofuranosyl	~4.2	~670	~8.4	~620	~2.0	~1.1
5-Methyl-2-D-ribo	~5200	~0.54	>5200	<1.0	>1.0	>0.53

II. Characteristics of Poliovirus Type 2 Inhibition by 2-(α-Hydroxybenzyl)-benzimidazole (HBB)

Photomicrographic Demonstration of Inhibition by HBB of Virus-Induced Cell Damage.—Inhibition of poliovirus cytopathic effects by HBB is illustrated in photomicrographs of monkey kidney cells shown in Figs. 1 to 11. In these experiments the amount of poliovirus inoculated was 500 $TCID_{50}$ per culture, and the number of cells per culture was approximately 2.5×10^5. The concentration of HBB was 493 μM.

Cultures shown in Figs. 1 to 4 were photographed 48 hours after the beginning of the experiment. Fig. 1 demonstrates the appearance of normal cells in an uninfected untreated culture. Lack of effect of HBB on the microscopic appearance of uninfected monkey kidney cells is illustrated in Fig. 2. The cytopathic effects of poliovirus type 2 can be seen in Fig. 3. Most of the cells have fallen off the glass and the few remaining cells show marked contraction and rounding up of the cytoplasm. Fig. 4 shows an infected culture incubated in the presence of HBB. It is evident that the compound has prevented the occurrence of viral cytopathic changes at this time.

The findings at 96 hours are illustrated in Figs. 5 to 8. The appearance of cells in uninfected treated cultures (Fig. 6) was similar to that in uninfected untreated cultures (Fig. 5). In the infected untreated cultures (Fig. 7) only a few severely damaged cells remained on glass. In treated infected cultures (Fig. 8) a moderate number of cells were undergoing degenerative changes due to poliovirus, although many still appeared normal.



TEXT-FIG. 6. Compound-induced damage-time curves with certain benzimidazole derivatives in monkey kidney cells.

In view of the appearance at 96 hours of viral lesions in treated cultures, the possibility was investigated that the compound had been inactivated during the interval between 48 and 96 hours. The results are illustrated in Figs. 9 to 11. The cultures shown in Figs. 9 to 11 were photographed at 96 hours. Fig. 9 illustrates the extent of virus-induced damage in infected cultures which were treated with HBB for 96 hours without a change in medium. Fig. 10 shows an infected treated culture whose medium was replaced with a fresh solution of compound at 48 hours. As can be seen, the extent of virus-induced cell damage in this culture is similar to that in the culture (Fig. 9) in which the HBB-containing medium was not changed. Clearly, the development of virus-induced cell damage in treated cultures on prolonged incubation was not due to inactivation of HBB.

Fig. 11 shows a culture whose medium, containing compound, was replaced at 48 hours with control medium without compound. This culture showed more marked viral changes than the cultures which were treated with compound for the entire 96-hour period. Thus, the suppressive effect of HBB on viral activity was reversible, as removal of compound was followed by increased viral activity.

Effects of Size of Virus Inoculum and Concentration of Compound on Inhibition of Virus-Induced Cell Damage by HBB.–

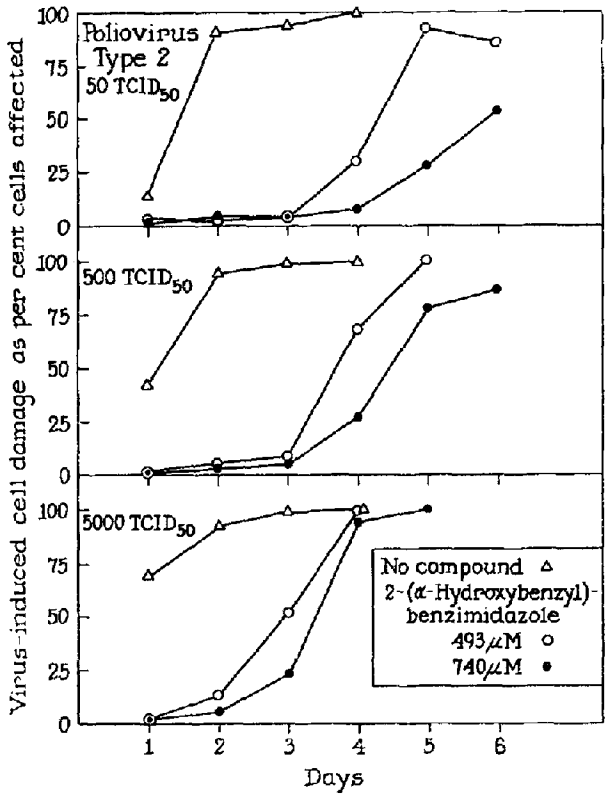
Monkey kidney cell cultures were inoculated with varying amounts of poliovirus and incubated in the presence or absence of HBB which was used at two concentrations. The amount of virus inoculated per culture was 50, 500, or 5000 $TCID_{50}$, and the concentration of HBB was 493 or 740 μM. The cultures were examined daily for 6 days and virus-induced cell damage was recorded as per cent cells affected.

The mean results of two such experiments are shown in Text-fig. 7. At 24 hours the extent of virus-induced damage in untreated cultures was approximately proportional to the amount of virus inoculated; at 2 days viral cytopathic changes in untreated cultures were near maximal regardless of the size of the inoculum. The curves depicting the course of events in treated cultures show that the cell protective effect varied directly with the concentration of compound, and inversely with the size of virus inoculum. With the lowest virus inoculum (50 $TCID_{50}$) and the higher concentration of HBB (740 μM), virus-induced cell damage was delayed 4 days; with the highest virus inoculum (5000 $TCID_{50}$) and the lower concentration of compound (493 μM) the viral cytopathic changes were delayed about 2 days.

Relationship between Time of Addition of HBB and Inhibition of Virus-Induced Cell Damage.—In experiments on the relationship between time of addition of HBB and inhibition of poliovirus cytopathic effects in monkey kidney cells, the amount of virus used in the inoculum was 2×10^6 or 5×10^2 $TCID_{50}$, and HBB was added 30 minutes before virus inoculation, along with the virus, or at varying intervals after virus inoculation.

The experiments with the lower inoculum were carried out in the usual manner. In experiments with the higher inoculum cultures were washed twice with warm Eagle's medium with

or without HBB 30 minutes after virus inoculation to remove unadsorbed virus. The washing was carried out at 24°C., and the time required was subtracted from the total time between virus inoculation and addition of compound. In both kinds of experiments control groups of infected cultures received an equal volume of medium, but no compound. The cultures were examined at intervals and virus-induced cell damage was recorded as per cent cells affected.

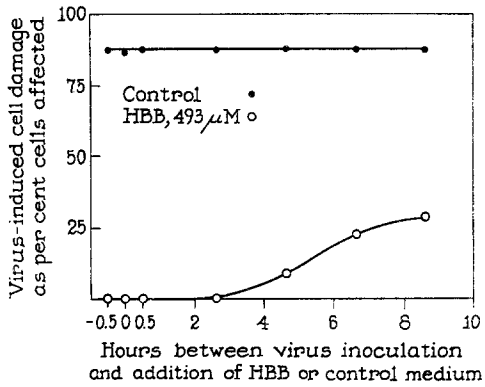


TEXT-FIG. 7. Effects of size of virus inoculum and concentration of compound on inhibition by 2-(α-hydroxybenzyl)-benzimidazole (HBB) of poliovirus type 2-induced cell damage in monkey kidney cells.

Text-fig. 8 depicts the mean results of two experiments in which 2×10^6 $TCID_{50}$ of virus was inoculated per culture. It should be mentioned that under the conditions used, earliest evidence of viral cytopathic effects in the untreated controls was observed 6 to 8 hours after inoculation. As can be seen in Text-fig. 8, HBB was capable of causing marked inhibition of viral cytopathic effects when given as late as 3 to 5 hours after virus inoculation; *i.e.*, during the exponential phase of poliovirus multiplication (22). This suggests that HBB may inhibit a late step in the reproductive sequence of poliovirus.

In two experiments in which the inoculum was 5×10^2 $TCID_{50}$ per culture, the extent of virus-induced damage in untreated controls at 24 hours was equivalent to approximately 3 per cent involvement of the monolayer. At 48 hours, approximately 90 per cent of cells in untreated infected cultures were markedly altered, whereas in cultures which had received HBB 24 hours previously, only 20 per cent of cells showed viral cytopathic effects at this time. These results are in line with those described above.

Lack of Inactivating Effect of HBB on Virus Infectivity.—Stability of poliovirus in the presence of HBB was studied at a final dilution of 1:100 of the seed virus preparation in protein-free Eagle's medium.



TEXT-FIG. 8. Relationship between time of addition of 2-(α-hydroxybenzyl)-benzimidazole (HBB) and inhibitory effect on poliovirus type 2-induced cell damage in monkey kidney cells. Extent of virus-induced cell damage was determined at constant time, i.e. 24 hours after inoculation.

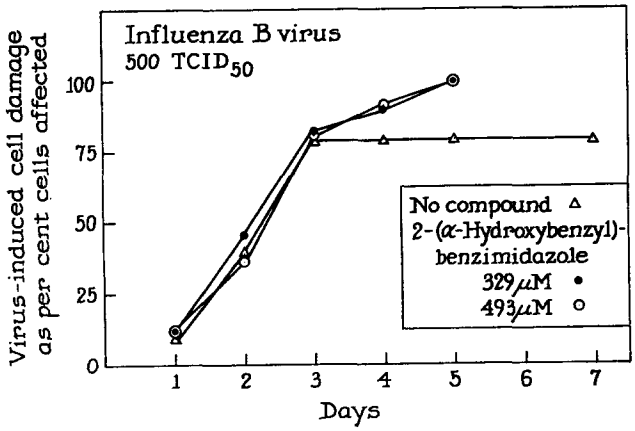
No inactivation of poliovirus by the compound occurred when the virus was incubated with 1,100 μM HBB for 40 hours or with 4,400 μM HBB for 3 hours.

Lack of Inhibitory Effect of HBB on Multiplication and Cytopathogenicity of Influenza B Virus in Monkey Kidney Cells.—It was shown above that HBB had no inhibitory effect on production of influenza B virus in the chorioallantoic membrane *in vitro*. Experiments were carried out to determine the effects of HBB on the yield of influenza B virus in monkey kidney cells and on cytopathic changes caused by this virus. The experimental procedures were similar to those used with poliovirus.

HBB at a concentration of 740 μM had no effect on the yield of influenza B virus in monkey kidney cells, as determined by hemagglutination titrations. In a separate experiment with 493 μM HBB, the yield of virus was measured by both hemagglutination and infectivity titrations. The compound had no

effect on virus yield as measured by either procedure. The results summarized in Text-fig. 9 show that HBB did not protect cells against influenza virus-induced damage.

Experiments were also carried out to determine whether HBB had any effects on the yield of influenza A virus in the chorioallantoic membrane *in vitro*. No inhibition or enhancement of yield was observed in experiments in which 3.2×10^5 EID_{50} of the PR8 strain of influenza A virus was inoculated per culture, the concentration of HBB was 1110 or 3770 μM, the cultures were incubated for 41 hours, and the virus was measured by the hemagglutination procedure.



TEXT-FIG. 9. Lack of inhibitory effect of 2-(α-hydroxybenzyl)-benzimidazole (HBB) on influenza B virus-induced cell damage in monkey kidney cells.

DISCUSSION

The demonstration that 2-(α-hydroxybenzyl)-benzimidazole (HBB) and certain related derivatives possess selective inhibitory activity on poliovirus type 2 multiplication adds another group of compounds to the growing list of benzimidazole derivatives with remarkable effects on virus multiplication. It should be emphasized that each group is characterized by a different set of structure-activity relationships.

HBB is remarkable, because it is among the first synthetic chemical compounds to show significant biological selectivity in its virus inhibitory action. At concentrations which cause marked inhibition of poliovirus type 2 multiplication, this compound has no effect on the multiplication of influenza B virus, and it shows no or only slight effects on the microscopic structure of monkey kidney cells. Finally, HBB, and its 5-chloro derivative, are the only synthetic chemicals which have shown significant inhibitory activity on viral cytopatho-

genicity. The 5-chloro derivative is 1.6 times more active, but also more toxic than HBB.

In the communication which follows, the virus inhibitory spectrum of HBB is described, and the remarkable selectivity of HBB is fully brought out (23). As will be shown, HBB has no inhibitory effects on the several metabolic activities of monkey kidney cells which have been studied. Furthermore, it does not inhibit the growth of cells. The usefulness of HBB as an aid in virus classification has been established (24).

As to the mechanism of action of HBB, it should be emphasized that this compound has no direct inactivating effect on the infectivity of poliovirus. HBB appears to be capable of interfering with a late step in the reproductive sequence of poliovirus. There is evidence that HBB inhibits viral cytopathic effects through inhibiting virus multiplication. The inhibitory effect of HBB is reversible in that after removal of the compound, increased viral activity develops.

Studies of the structure-activity relationships with HBB and related compounds have suggested that the hydroxybenzyl grouping at position 2 in the imidazole ring of the benzimidazole molecule is of fundamental importance for the selective virus inhibitory action of HBB. The structural and metabolic (23) evidence which is available does not suggest that HBB acts as an antagonist of a metabolite required both by the host cell and the virus; indeed, there is strong evidence against such a possibility. The subject of metabolic antagonism and selective virus inhibition is discussed in detail elsewhere (25), and the selective virus inhibitory action of HBB is compared with that of M-8450, helenine, and interferon.

The new, highly active inhibitors of ribonucleic acid (RNA) biosynthesis, described previously (2, 3, 5, 8), are β-linked ribofuranosides of halogenated benzimidazoles, and they have played a significant part in studies on the requirements and mechanism of virus multiplication (8, 9, 12–15), as well as in studies on the role of RNA in protein synthesis (16, 17).

Finally, certain structurally very different derivatives of benzimidazole possess the unique ability of increasing the yield of influenza virus from infected tissue without affecting the metabolic activities of the cells (11, 18). These derivatives are 2-(aldopolyhydroxyalkyl)-benzimidazoles, of which the compound that has been studied most intensively is 5-methyl-2-D-ribobenzimidazole.

Clearly, certain benzimidazole derivatives have opened new approaches to the problems of the mechanism and requirements of virus multiplication and the mechanisms of virus-induced cell damage. It should be mentioned, however, that numerous benzimidazole derivatives which have been examined for virus inhibitory activity have been found to be inactive or have shown virus inhibitory activity of variable degree accompanied by low selectivity of action. In the

present studies, much new evidence was obtained indicating that extensive substitution in either the benzenoid or imidazole ring frequently gives compounds of very high inhibitory activity on virus multiplication. However, no highly selective inhibitors of influenza virus multiplication were found.

Some years ago (8) the view was expressed that there appeared to be numerous as yet untapped possibilities for increasing the virus-inhibitory effectiveness of benzimidazole derivatives through further modification of structure. This prediction has been fulfilled; yet, the same prediction may be made again with even greater justification than before.

SUMMARY

The virus inhibitory activity and selectivity of certain benzimidazole, benzotriazole, and naphthimidazole derivatives were determined with influenza B and polio type 2 viruses.

Among the sixty-five compounds examined, several were highly active inhibitors of influenza B virus multiplication in the chorioallantoic membrane *in vitro*. The following compounds, listed in order of increasing inhibitory activity, were more than 100 times as active as benzimidazole: 5-(4′-toluenesulfonamido)-benzimidazole, 5-hydroxybenzotriazole-4-carboxy-α-naphthylamide, 4,5,6-trichlorobenzotriazole, 5-(3′,4′-dichlorobenzenesulfonamido)-benzimidazole, 5-(3′,4′-dichlorobenzenesulfonamido) - 1 - (3″,4″ - dichlorobenzenesulfonyl)-benzimidazole, 4-(*p*-chlorophenylazo)-5-hydroxybenzotriazole, and 4,5,6,7-tetrachlorobenzotriazole. However, none showed high selectivity.

Of the sixty-five compounds studied with influenza virus, twenty-five were also examined with poliovirus type 2 in monkey kidney cells *in vitro*. Included in this group were five of the seven most active inhibitors of influenza virus, listed above. All five were more than 100 times as active in inhibiting poliovirus multiplication as the reference compound. In addition to these, two other compounds were highly active: 2-(α-hydroxybenzyl)-benzimidazole (HBB), and 2-(α-hydroxybenzyl)-5-chlorobenzimidazole, with relative inhibitory activities of 78 and 130, respectively. These two compounds, and the much less active 5,6-dichloro derivative of HBB, were the only ones which showed no, or only slight, toxic effects on cells at concentrations sufficient to cause considerable inhibition of poliovirus multiplication. Furthermore, HBB and the 5-chloro derivative were the only compounds which caused significant inhibition of the cytopathic effects of poliovirus.

HBB, and its 5-chloro and 5,6-dichloro derivatives had no effect on the multiplication of influenza B virus in the chorioallantoic membrane. In addition, HBB failed to inhibit influenza B virus multiplication and cytopathic effects in monkey kidney cells.

Inhibition of poliovirus-induced cell damage by HBB was characterized by the following features: the curves relating reduction in virus yield or cytopathic

effects to concentration of the compound followed an approximately parallel course; somewhat higher concentrations were required to inhibit virus-induced cell damage than to reduce virus yield. HBB suppressed viral cytopathic effects for a period of time which varied directly with the concentration of compound, and inversely with the size of virus inoculum. The development of virus-induced cell damage in treated cultures on prolonged incubation was not due to inactivation of HBB. The inhibitory effect of HBB on virus-induced cell damage was reversible by removal of the compound. HBB inhibited viral cytopathic effects when given during the exponential increase phase in virus multiplication. Inhibition of virus-induced cell damage by HBB was demonstrated by photomicrographs. HBB did not inactivate the infectivity of poliovirus type 2.

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EXPLANATION OF PLATES

PLATE 70

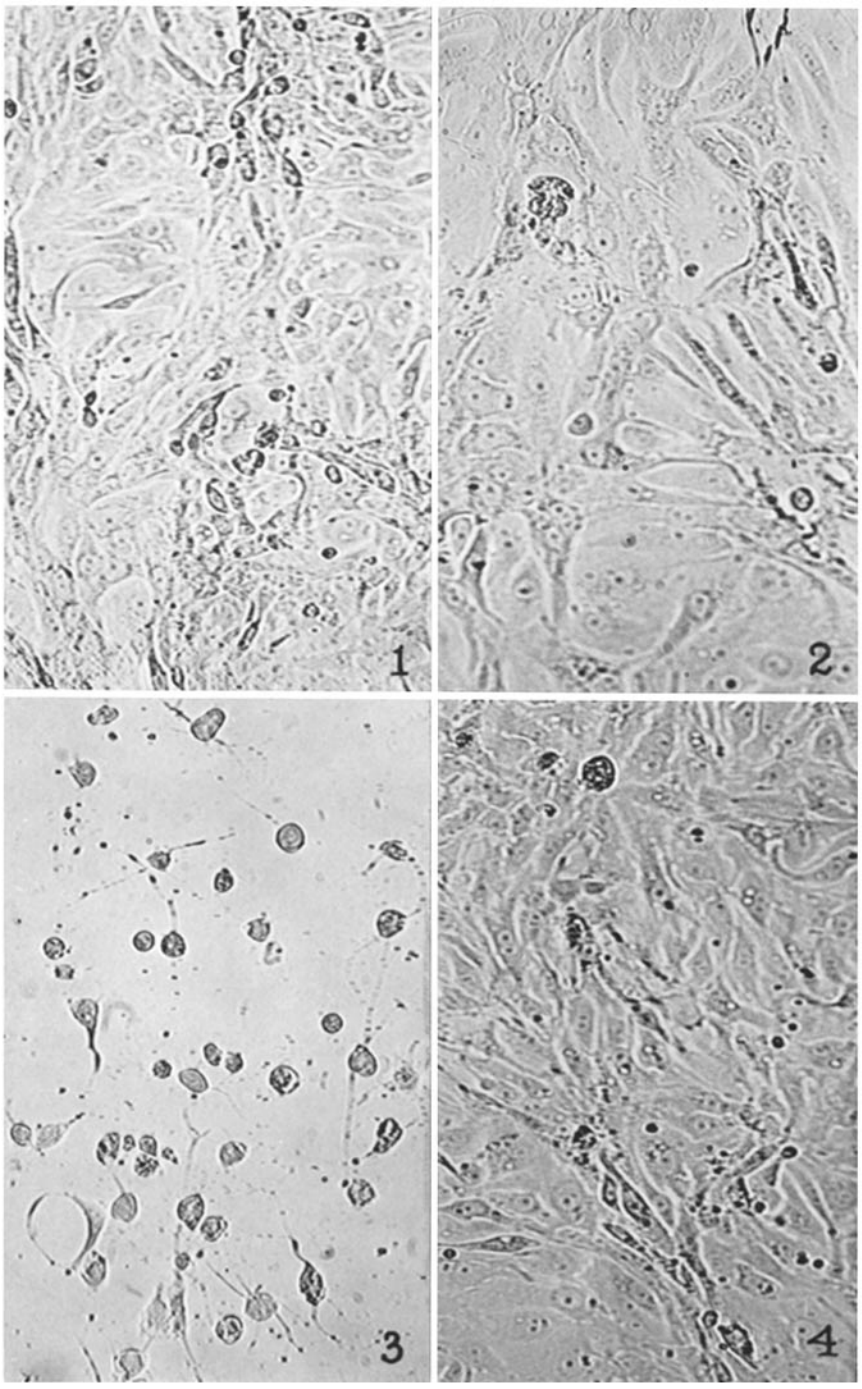
Black and white photomicrographs of monkey kidney cells demonstrating inhibition of poliovirus-induced cell damage by 2-(α-hydroxybenzyl)-benzimidazole (HBB). The amount of poliovirus type 2 inoculated was 500 $TCID_{50}$ per culture and the concentration of HBB was 493 μM. Cultures were incubated for 48 hours. × 160.

FIG. 1. Uninfected, untreated culture.

FIG. 2. Uninfected, treated culture.

FIG. 3. Infected, untreated culture.

FIG. 4. Infected, treated culture.



(Tamm *et al.*: Virus inhibitory activity of benzimidazoles)

PLATE 71

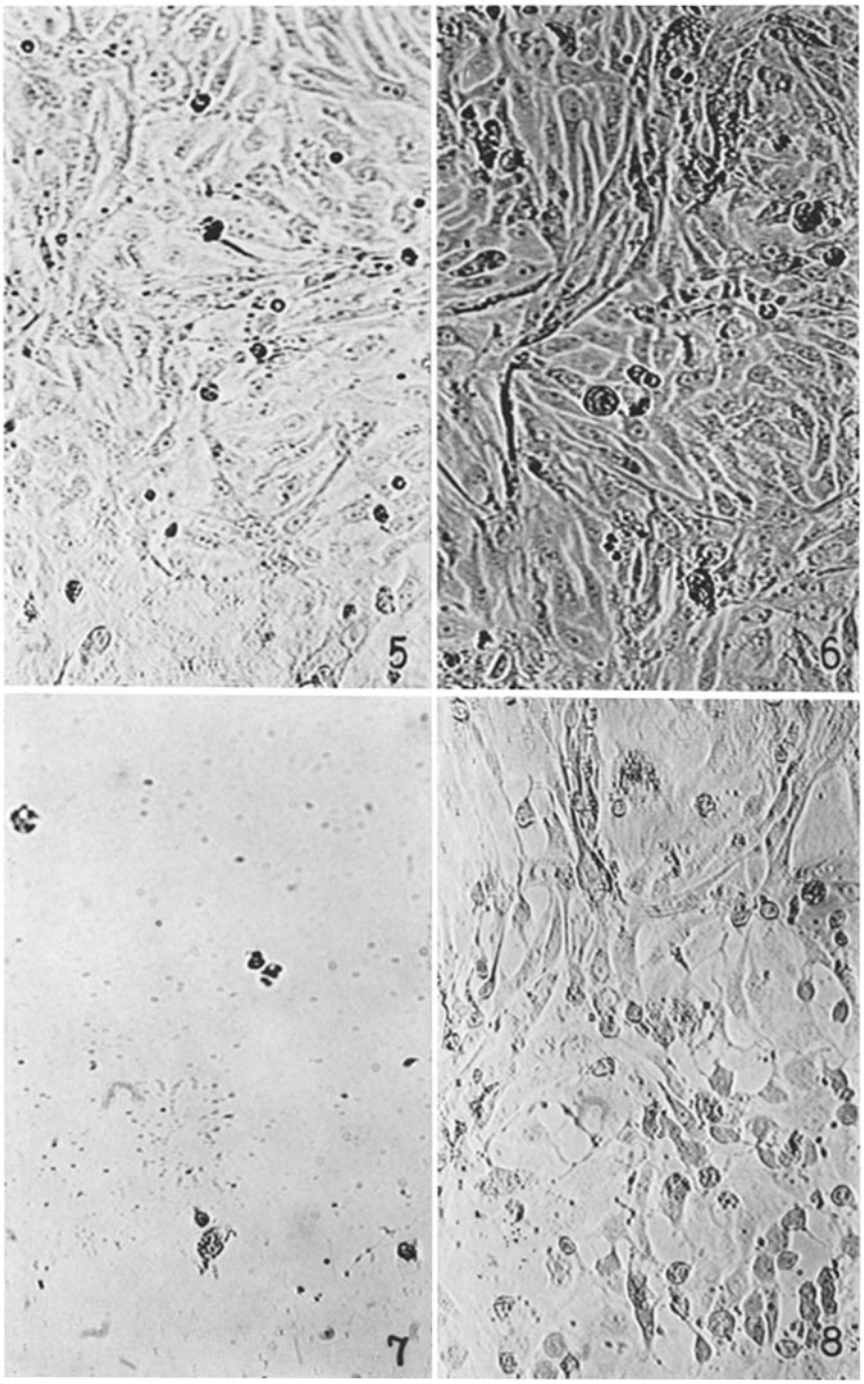
Black and white photomicrographs of monkey kidney cells demonstrating inhibition of poliovirus-induced cell damage by 2-(α-hydroxybenzyl)-benzimidazole (HBB). The amount of poliovirus type 2 inoculated was 500 $TCID_{50}$ per culture and the concentration of HBB was 493 μM. Cultures were incubated for 96 hours. $\times$ 160.

FIG. 5. Uninfected, untreated culture.

FIG. 6. Uninfected, treated culture.

FIG. 7. Infected, untreated culture.

FIG. 8. Infected, treated culture.



(Tamm *et al.*: Virus inhibitory activity of benzimidazoles)

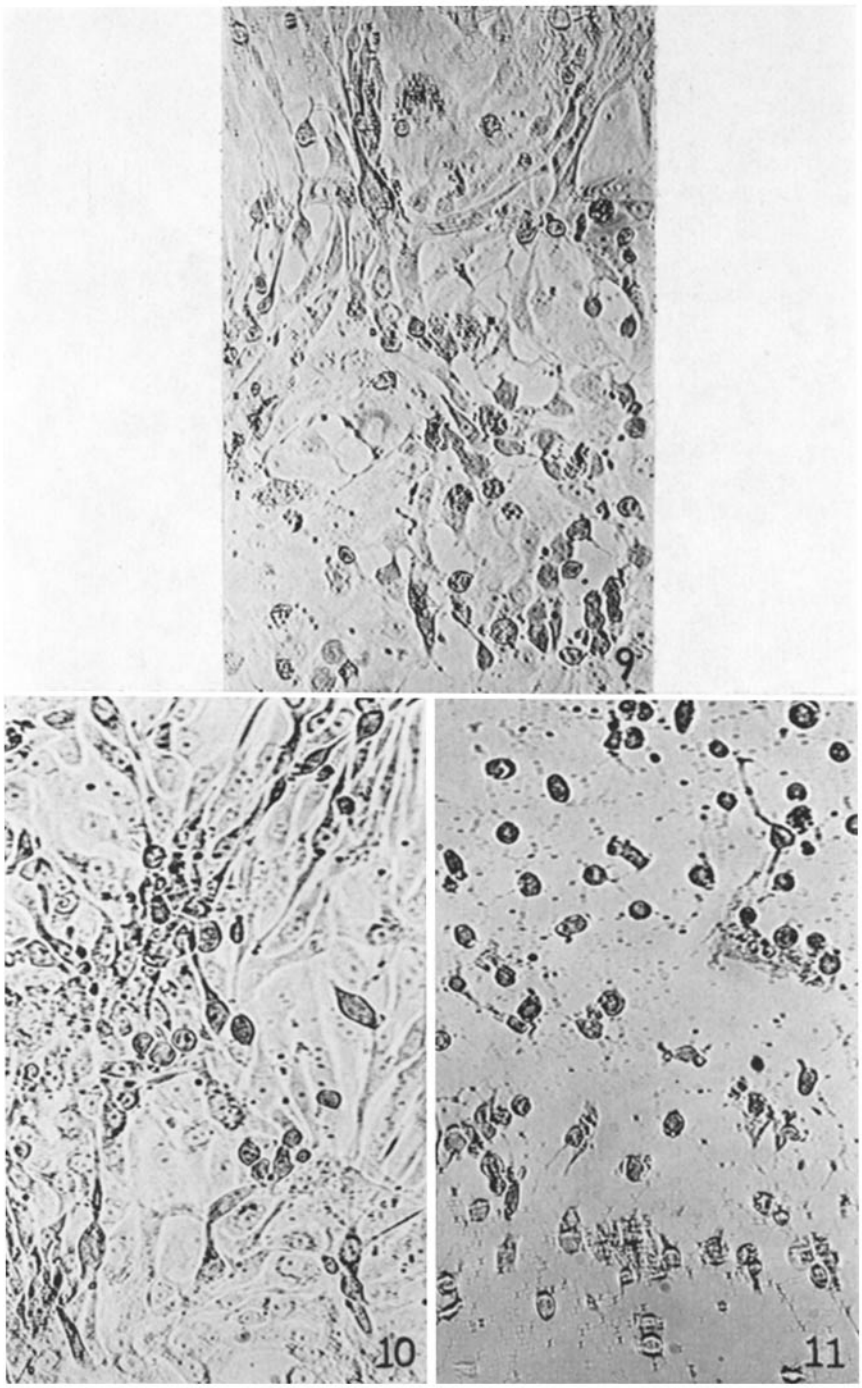
PLATE 72

Black and white photomicrographs of monkey kidney cells demonstrating inhibition of poliovirus-induced cell damage by 2-(α-hydroxybenzyl)-benzimidazole (HBB). The amount of poliovirus type 2 inoculated was 500 $TCID_{50}$ per culture and the concentration of HBB was 493 μM. Cultures were incubated for 96 hours. × 160.

FIG. 9. Infected culture, treated with HBB for 96 hours; medium not changed.

FIG. 10. Infected culture, treated with HBB for 96 hours; at 48 hours medium containing HBB replaced with fresh HBB-containing medium.

FIG. 11. Infected culture, treated with HBB for 48 hours; at 48 hours medium replaced with fresh medium without HBB.



(Tamm *et al.*: Virus inhibitory activity of benzimidazoles)