SPECTRUM AND CHARACTERISTICS OF THE VIRUS INHIBITORY ACTION OF 2-(α-HYDROXYBENZYL)-BENZIMIDAZOLE*, ‡

By HANS J. EGGERS, M.D., and IGOR TAMM, M.D.

(From The Rockefeller Institute)

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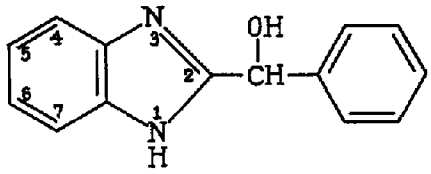
Recently, selective inhibition of poliovirus by 2-(α-hydroxybenzyl)-benzimidazole was described (1–3).

Following the original report (1) concerning inhibition of poliovirus by 2-(α-hydroxybenzyl)-benzimidazole (HBB), it was shown in quantitative studies (2, 3) that HBB inhibits the multiplication and the cytopathic effects of poliovirus type 2 in monkey kidney cells, but does not inhibit influenza B virus in the same cell system or in the chorioallantoic membrane from embryonated chicken eggs. At concentrations sufficient to cause marked inhibition of poliovirus, the compound had no effect, or only a slight effect, on the morphologic appearance of monkey kidney cells. Thus, HBB showed significant biological selectivity in its virus inhibitory action. Inhibition of viral cytopathic effects appeared to be due to inhibition of virus multiplication. It should be emphasized that HBB was virostatic, but not virocidal, for poliovirus.



2-(α-Hydroxybenzyl)-benzimidazole

Text-Fig. 1. Structure of 2-(α-hydroxybenzyl)-benzimidazole (HBB).

Studies of structure-activity relationships with HBB and related compounds suggested (3) that the hydroxybenzyl grouping at position 2 in the imidazole ring was of fundamental importance for the selective virus inhibitory action of HBB (*cf.* Text-fig. 1). HBB could not readily be considered a close structural analog of any known metabolite. Furthermore, the fact that the compound did not damage cells at virus inhibitory concentrations, made it seem unlikely that HBB was acting as an antagonist of a metabolite required by both the host cell and the virus.

These findings suggested a new approach to the study of mechanism of virus multiplication: it appeared that with the aid of HBB and related com-

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pounds, information might be obtained concerning some of the specific biological and biochemical features of poliomyelitis and related viruses.

In the present communication the spectrum of virus inhibitory activity of HBB is described. It is shown that the compound acts by inhibiting some intracellular step in the reproductive cycle of HBB-susceptible enteroviruses, and that it does not inhibit any of the cellular metabolic processes which have been studied; *i.e.*, respiration, glycolysis, and turnover of RNA and proteins. Undisturbed growth of HeLa and ERK cells in the presence of HBB is reported. It is shown that on passage of HBB-susceptible viruses in the presence of the compound, insusceptible variants emerge.

Materials and Methods

Viruses.—Poliovirus types 1 (Mahoney), 2 (MEF1), and 3 (Saukett) were obtained from the Connaught Laboratories through Dr. Francis L. Black. Dr. M. Theiler supplied the arbor B, and Dr. S. M. Buckley the arbor C viruses. Adenovirus types 2, 3, and 4 were obtained from Dr. I. W. McLean, Jr.; para-influenza 2 and 3 from Dr. R. M. Chanock; and mumps virus from Dr. J. P. Utz. Vaccinia virus was obtained as a calf lymph preparation from the New York City Department of Health (4). When used, it had been through nine passages in the chorioallantoic membrane of embryonated chicken eggs in this laboratory. The Lederle live poliovirus vaccine strains were obtained from Dr. H. R. Cox. All other viruses were supplied by Dr. A. B. Sabin. The strain designations of most of the viruses used are given in Table I; the designations of some are indicated in the text.

Most of the viruses were propagated in primary cultures of trypsinized kidney tissue from rhesus monkeys. The culture tubes were held stationary at 36°C. ECHO 28 was grown in rhesus monkey kidney cells by the procedure described by Pelon (5), except that incubation was carried out at 35°C. Cultures to be inoculated with reoviruses (formerly ECHO 10) were carefully washed to remove residual calf serum which is inhibitory to the viruses (6); after inoculation, the tubes were kept in a roller drum.

Coxsackie A types 11, 13, and 18 were propagated in ERK cells (7). In some experiments, ECHO 9 (Hill) was also grown in this cell line.

Herpes simplex virus was propagated in HeLa cells. Poliovirus types 1 (V. Richardson) and 2 (P 712-Ch-2ab) were also grown in this cell line in some experiments. The P 712-Ch-2ab strain used in HeLa cells had been passed 41 times in ERK cells by Dr. A. B. Sabin, and several times in HeLa cells in this laboratoy.

Seed virus was obtained by collecting supernatant fluids from infected cultures when cytopathic effects were complete. In the following instances seed virus was not prepared in the tissue culture system in which it was ultimately used: arbor B virus was propagated in the brain of adult white mice, arbor C viruses in HeLa cells, and vaccinia virus in the chorioallantoic membrane.

Most virus seeds were stored at −25°C. The following viruses were stored at −55°C.: ECHO types 16, 19, 23, and 28; arbor B and C; the myxoviruses; herpes simplex (with 50 per cent rabbit serum); and vaccinia.

Compound.—2-(α-Hydroxybenzyl)-benzimidazole (HBB)[1] was suspended in protein-free Eagle's medium and the suspension shaken in a mechanical shaker at 35°C. for 1 hour. 98

[1] HBB, 2-(α-hydroxybenzyl)-benzimidazole was obtained through Dr. Karl Folkers of the Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey.

μM HBB dissolved almost completely. When serum-containing medium was used, as for ERK and HeLa cells, the serum was added after shaking.

Monkey Kidney Cell Cultures.—Rhesus monkey kidney cell cultures in screw cap tubes were secured from Microbiological Associates, Inc. When used, the monolayer cultures were 8 to 10 days old and contained approximately 2.5×10^5 cells per tube. In all experiments protein-free Eagle's medium was used. In approximately one-half of the experiments Eagle's basal medium (8) was used, whereas in the other half, Eagle's minimum essential medium was employed (9).

Monkey kidney cell cultures in 60 mm. plastic culture dishes[2] were prepared as follows: kidneys removed from rhesus monkeys were trypsinized and the culture dishes were seeded with 5 ml. of a 1:200 suspension of cells in growth medium per dish. The growth medium consisted of Hanks' solution (10) with 0.5 per cent lactalbumin hydrolysate and 2 per cent calf serum which had been inactivated at 56°C. for 30 minutes. The cultures were incubated at 36°C. in a humidified atmosphere of 5 per cent CO_2 in air. The cell sheets were confluent 5 to 7 days after seeding of the dishes. The number of cells per dish was approximately 2×10^6.

ERK Cell Cultures.—ERK cells were propagated as described previously (11), with the exception that the maintenance medium contained glucose in the final concentration of 1 gm., rather than 4 gm., per liter. In experiments with ERK cells in screw cap tubes the medium was changed every 2nd day.

HeLa Cell Cultures.—A HeLa cell strain adapted to calf serum was obtained through Dr. A. B. Sabin. The growth medium consisted of Eagle's minimum essential medium with 10 per cent calf serum which had been inactivated at 56°C. for 30 minutes. The maintenance medium consisted of Eagle's minimum essential medium with 5 per cent chicken serum which had been inactivated at 56°C. for 30 minutes. In most experiments with HeLa cells in screw cap tubes the medium was changed every 2nd or 3rd day.

Measurement of Infective Virus.—With most viruses infectivity titrations were performed in monkey kidney cells. HeLa cells were used to titer herpes simplex virus, and ERK cells for titration of Coxsackie A types 11, 13, and 18. With poliovirus types 1 (V. Richardson) and 2 (P 712-Ch-2ab), and ECHO 9 (Hill) virus, both the inhibition experiments and the simultaneous infectivity titrations were carried out in more than one cell type, as indicated below. Tube cultures of monkey kidney, HeLa, and ERK cells were used in virus infectivity titrations employing a 50 per cent end point procedure. Dish cultures of monkey kidney cells were used for plaque assay of infective virus.

Virus titrations in tube cultures: Serial 10-fold dilutions of virus were made in Eagle's medium, and 0.1 ml. aliquots of each dilution were inoculated into groups of three to six tubes, containing 0.9 ml. of an appropriate maintenance medium. The cultures were incubated at 36°C. in a stationary position. They were observed every 2nd day, and the final readings were made 7 or 8 days after inoculation. Concentration of infective virus was expressed in terms of 50 per cent tissue culture infective doses ($TCID_{50}$) per ml.

Plaque assay: Plastic dishes with confluent cell sheets were washed twice with 5 ml. of phosphate-buffered saline (PBS) (12), and inoculated with 0.2 ml. of serial 5-fold dilutions of virus in PBS. Two to three dishes were used per dilution. After an adsorption period of 60 minutes at 36°C., the cultures were washed twice with PBS, and 7.5 ml. of an overlay was added. It consisted of equal volumes of 1.9 per cent ionagar and of two times concentrated Hanks' solution containing 0.2 per cent bovine albumin and 0.2 per cent yeast extract. After addition of the overlay the cultures were inverted and incubated for 3 days at 36°C. in a humidified atmosphere of 5 per cent CO_2 in air. Two ml. of a second overlay containing 0.0025

[2] TCPD 60 15, Falcon Plastics Company, Los Angeles.

per cent neutral red was then added to each culture. Plaques were counted 12 to 18 hours later. Concentration of infective virus was expressed as the number of plaque-forming units (PFU) of virus per milliliter.

Metabolic Activities of Monkey Kidney Cells.—The procedures used for measurement of oxygen uptake, and for determinations of incorporation of adenosine-8-C^{14} into RNA, and of C^{14}-L-alanine into proteins, were described previously (13). Glucose utilization was measured by the procedure of Park and Johnson (14), and lactic acid production by the procedure of Hullin and Noble (15). The number of cells used was varied in accordance with the requirements of the various procedures. The cell counts were made in a hemocytometer after staining with crystal violet. In all experiments Eagle's basal medium without serum was used (8).

EXPERIMENTAL

I. Susceptibility of Viruses to the Inhibitory Action of 2-(α-Hydroxybenzyl)-benzimidazole (HBB)

Spectrum of Virus Inhibitory Activity of HBB.—The ability of HBB to inhibit viral cytopathic effects was investigated with prototype strains of all types of polio and ECHO viruses; with six types each of Coxsackie A and Coxsackie B virus; and with arbor B and C, reo, adeno, myxo, herpes simplex, and vaccinia virus strains.

With most viruses, tube cultures of monkey kidney cells and protein-free Eagle's medium were used. Herpes simplex virus was studied in HeLa cells, and Coxsackie A types 11, 13, and 18 in ERK cells. The media used with HeLa and ERK cells are described above. In most experiments 30 to 500 $TCID_{50}$ of virus was inoculated per culture, but in some, larger inocula were used, as indicated in Text-figs. 2 to 7. Infected cultures were incubated in the presence or absence of HBB. The concentrations of HBB originally used were 98 and 493 μM. In later experiments the higher concentration was replaced with 219 μM HBB. Three to six cultures were used per variable.

The procedure was as follows: medium was removed from cultures by suction, and to each was added 0.9 ml. of fresh medium with or without HBB. The cultures were gassed with 5 per cent CO_2 in air, and each received 0.1 ml. of medium containing virus. In each experiment uninfected control cultures, with or without compound, were included, and titrations of the virus seeds were performed. The cultures were incubated at 36°C., and they were examined daily or every other day. The final reading was made 7 or 8 days after inoculation. Virus-induced cell damage was expressed as per cent cells affected. At least two experiments were carried out with each virus.

A virus was considered to be inhibited by HBB if in the presence of 219 μM HBB the extent of virus-induced cell damage was less than 25 per cent of that in untreated control cultures on any day after infection. With most, but not all of the sensitive viruses studied, 75 per cent or greater inhibition of viral cytopathic effects was also observed at 98 μM HBB. In contrast, none of the insensitive viruses was significantly inhibited at 219 or 493 μM HBB. The foregoing definition of susceptibility to inhibition by HBB has been found useful in that it provides an objective measure on the basis of which viruses may be classified as sensitive or insensitive to HBB. Clearly, the characteristics of inhibition can be determined only on the basis of time curves.

Typical results of such inhibition experiments with HBB and 54 viruses are depicted in Text-figs. 2 to 7. A summary of the virus inhibitory spectrum of

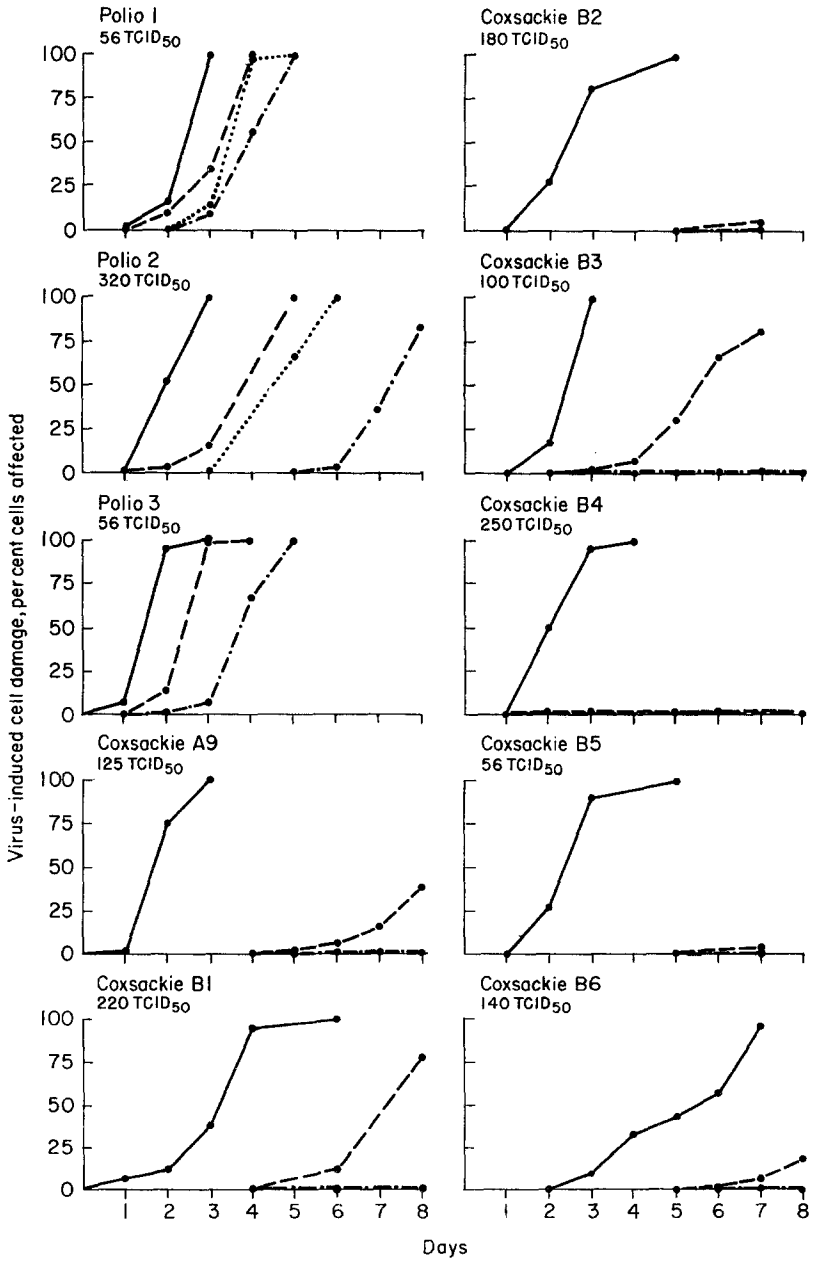
HBB is presented in Table I. The table also includes influenza B virus which was studied previously (2, 3), and arbor C strain Oriboca and Coxsackie A18 strain D 52112. Of the total of 57 viruses examined, 34 were inhibited by HBB. As can be seen, only enteroviruses were inhibited by this compound. However,

TABLE I

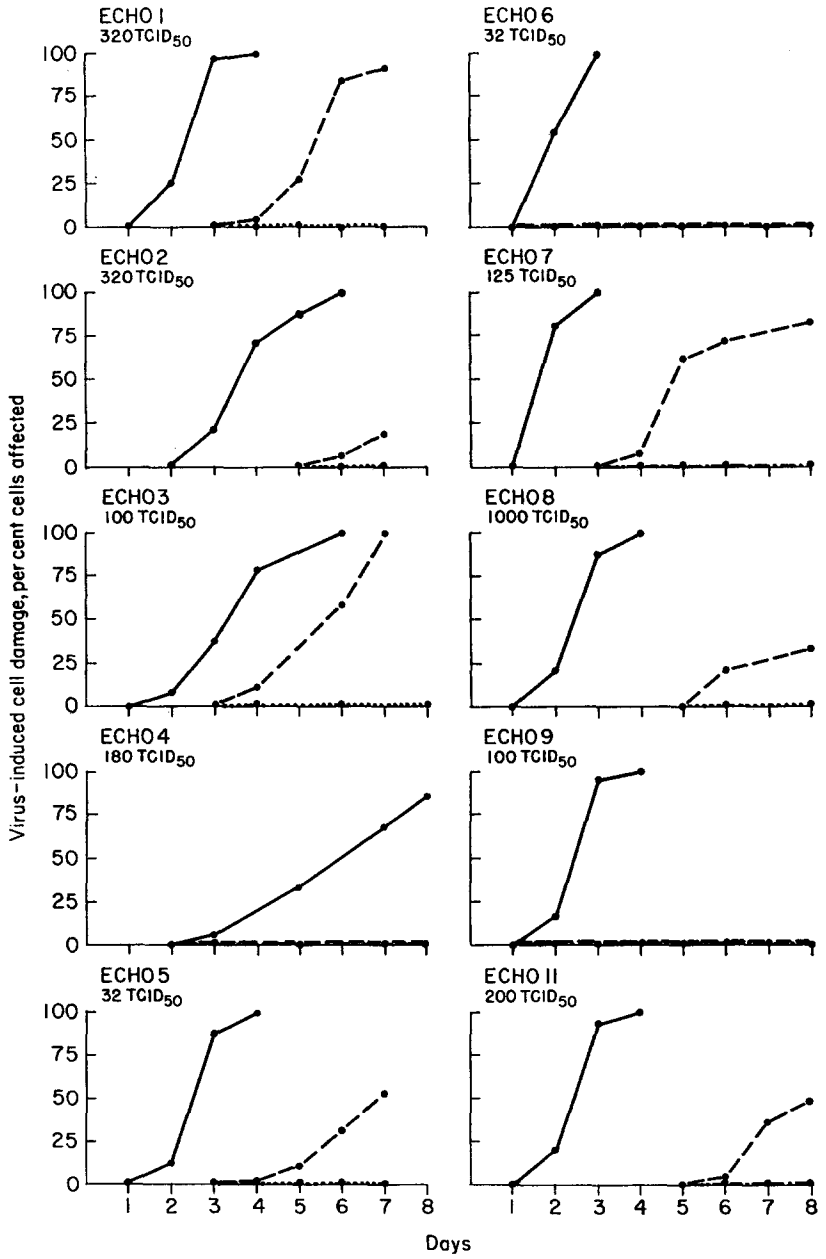
Spectrum of Virus Inhibitory Activity of 2-(α-Hydroxybenzyl)-benzimidazole (HBB)

Viruses inhibited by HBB*	Viruses not inhibited by HBB
Polio 1 (Mahoney)	Arbor B (West Nile)
Polio 2 (MEF1)	Arbor C (Marituba; Oriboca)
Polio 3 (Saukett)	
	Reo 1 (Lang) }
Coxsackie A9 (Woods)	Reo 2 (Jones) } Formerly ECHO 10
	Reo 3 (Dearing) }
Coxsackie B1 (P.O. Dalldorf)	
Coxsackie B2 (Ohio 1)	Adeno 2 (Ind-2)
Coxsackie B3 (Nancy)	Adeno 3 (JF)
Coxsackie B4 (Powers)	Adeno 4 (RN)
Coxsackie B5 (Faulkner)	
Coxsackie B6 (1-51-21)	Influenza B (1760)
	Para-influenza 2, croup-associated (Greer)
ECHO 1 (Farouk)	Para-influenza 3, hemadsorption 1 (C-243)
ECHO 2 (Cornelis)	Mumps (Utz)
ECHO 3 (Morrisey)	
ECHO 4 (Pesascek)	Herpes simplex (RE)
ECHO 5 (Noyce)	
ECHO 6 (D'Amori)	Vaccinia
ECHO 7 (Wallace)	
ECHO 8 (Bryson)	Coxsackie A7 (AB IV Habel)
ECHO 9 (Hill)	Coxsackie A11 (D 52148)
ECHO 11 (Gregory)	Coxsackie A13 (D 5359)
ECHO 12 (Travis 2-85)	Coxsackie A16 (D 52109)
ECHO 13 (11-4-1D)	Coxsackie A18 (D 52112)
ECHO 14 (Tow)	
ECHO 15 (Charleston 96-51)	ECHO 22 (Harris)
ECHO 16 (Harrington)	ECHO 23 (Williamson)
ECHO 17 (CHHE-29)	ECHO 28 (2060)
ECHO 18 (Metcalf)	
ECHO 19 (Burke)	
ECHO 20 (JV-1)	
ECHO 21 (Farina)	
ECHO 24 (de Camp)	
ECHO 25 (JV-4)	
ECHO 26 (11-3-6)	
ECHO 27 (1-36-4)	

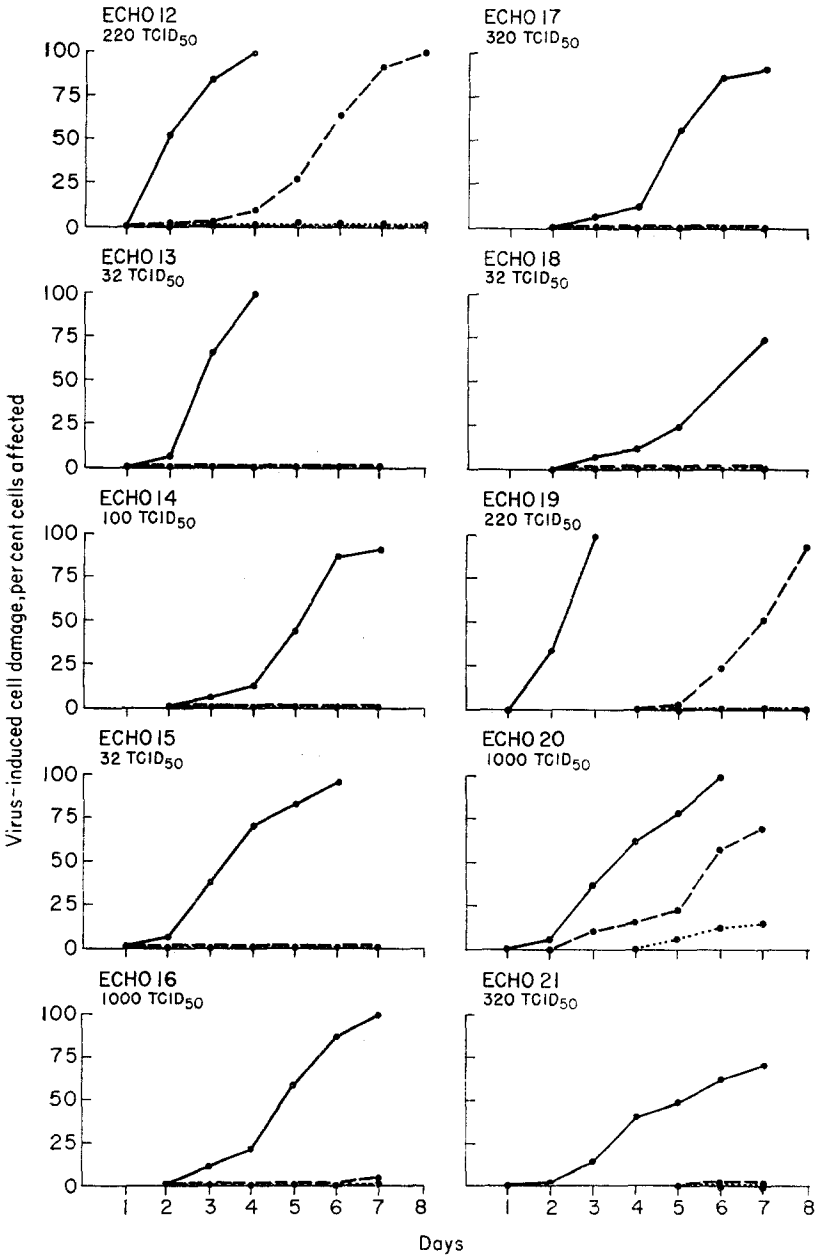
* More than 75 per cent reduction in virus-induced cell damage in the presence of 219 μM HBB.



TEXT-FIG. 2. Inhibition by 2-(α-hydroxybenzyl)-benzimidazole (HBB) of the cytopathic effects of polio 1 to 3; Coxsackie A9; and Coxsackie B 1 to 6 in monkey kidney cells. Concentration of HBB: —— none; - - - 98 μM; ···· 219 μM; -·-·- 493 μM.



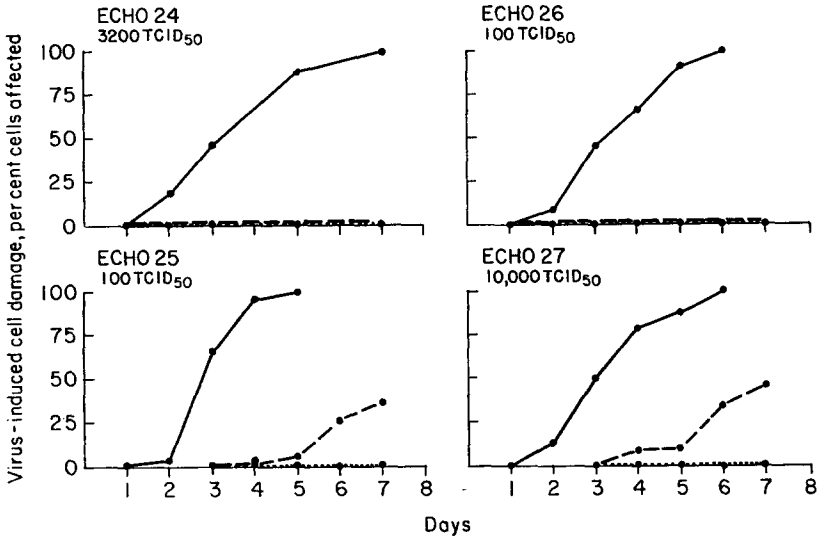
TEXT-FIG. 3. Inhibition by 2-(α-hydroxybenzyl)-benzimidazole (HBB) of the cytopathic effects of ECHO 1 to 9 and ECHO 11 in monkey kidney cells. Concentration of HBB: —— none; – – – 98 μM; ···· 219 μM; –·–·– 493 μM.



TEXT-FIG. 4. Inhibition by 2-(α-hydroxybenzyl)-benzimidazole (HBB) of the cytopathic effects of ECHO 12 to 21. Concentration of HBB: —— none; - - - 98 μM; · · · · 219 μM; -·-·- 493 μM.

not all viruses which are classified as enteroviruses were susceptible to inhibition by HBB.

The following viruses were inhibited: three types of poliovirus; Coxsackie A9; Coxsackie B types 1 to 6; and most of the ECHO virus types. Not inhibited were the following enteroviruses: Coxsackie A types 7, 11, 13, 16, and 18; and ECHO types 22, 23, and 28. Other insusceptible viruses were arbor B and C, reo 1 to 3, adeno 2 to 4, influenza B, para-influenza 2 and 3, mumps, herpes simplex, and vaccinia. As reported elsewhere (16), Salisbury strain H.G.P., a



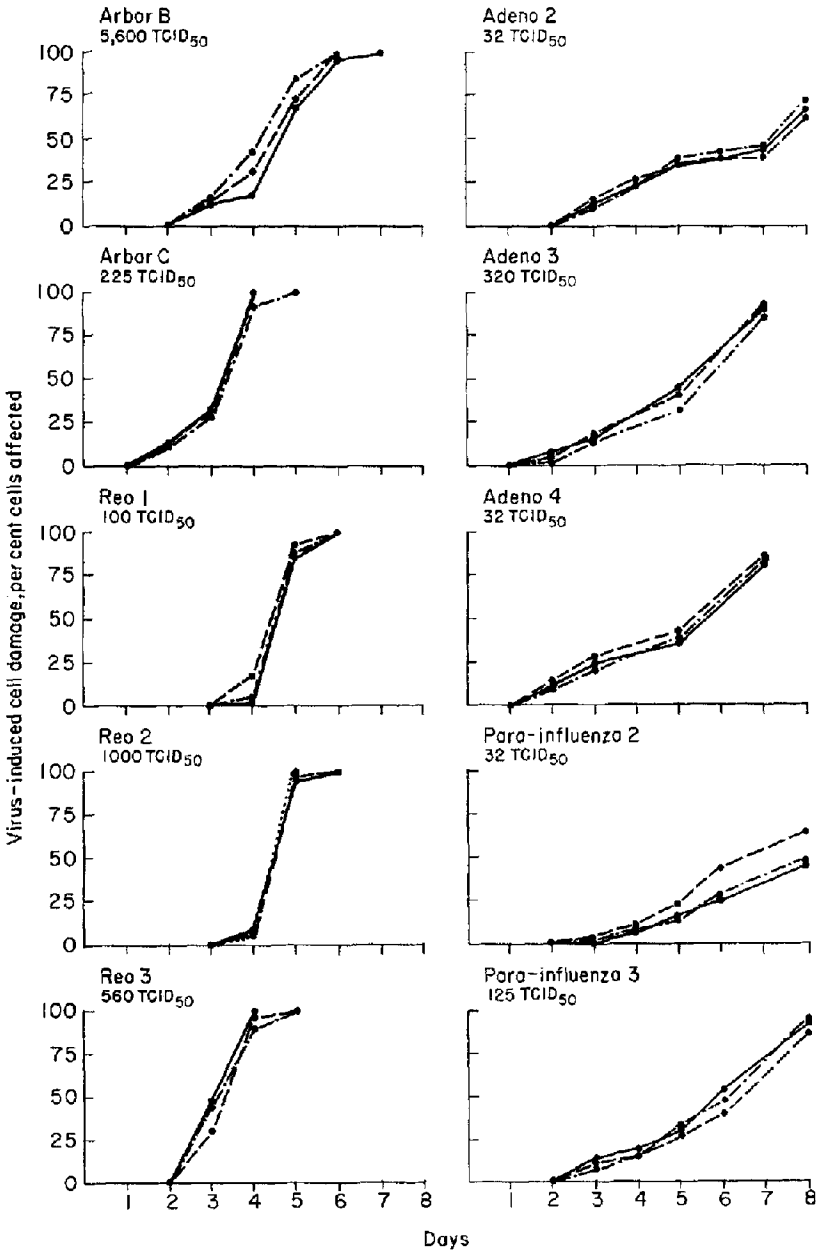
TEXT-FIG. 5. Inhibition by 2-(α-hydroxybenzyl)-benzimidazole (HBB) of the cytopathic effects of ECHO 24 to 27. Concentration of HBB: —— none; - - - 98 μM; ···· 219 μM.

hitherto unclassified virus which causes mild upper respiratory disease (17), was also not inhibited by HBB.

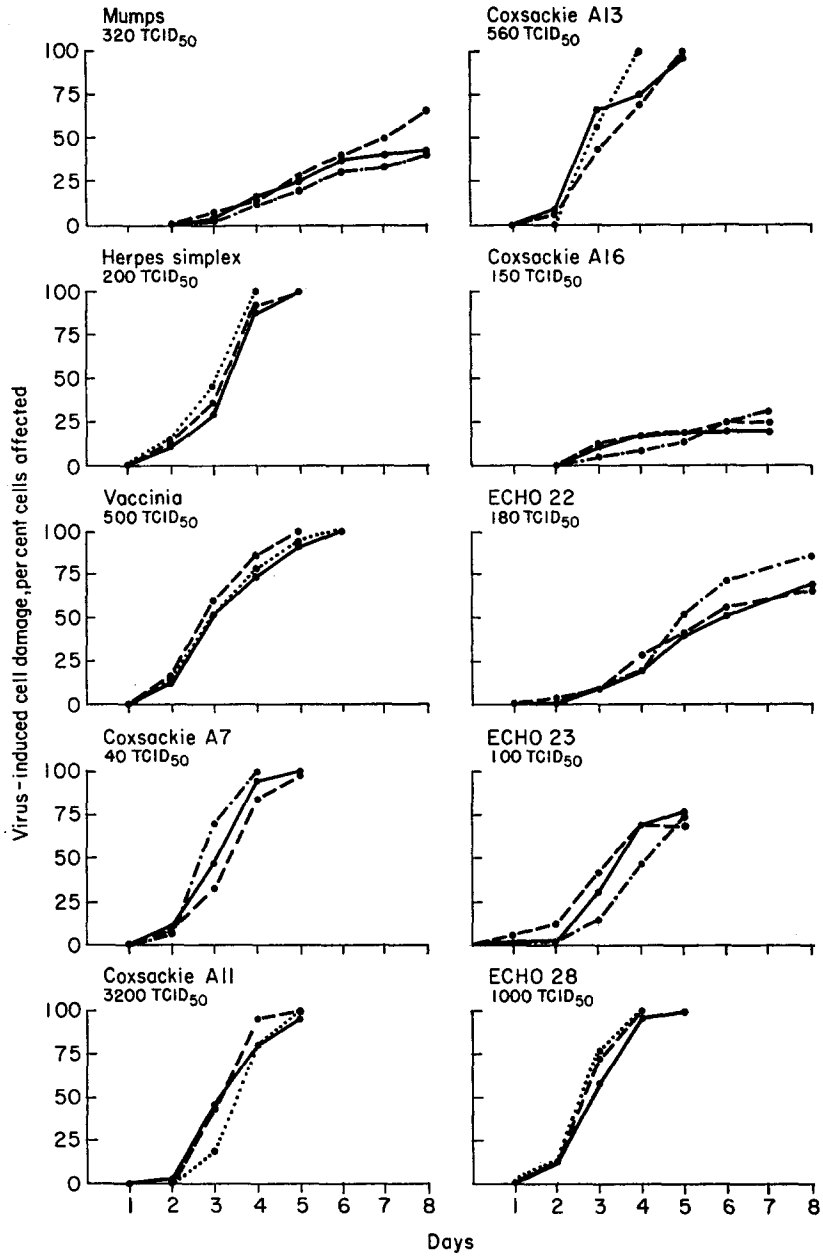
Clearly, HBB as a virus inhibitor has a highly specific range of action. The specificity of the virus inhibitory action of this compound has interesting implications with respect to virus classification. These are discussed below and elsewhere (16).

The inhibitory effect of HBB on the cytopathogenicity of Coxsackie B4 and ECHO 6 viruses in monkey kidney cells is illustrated in Figs. 1 to 6.

Viruses which were inhibited by HBB showed considerable quantitative differences in the degree of susceptibility to inhibition by this compound, as demonstrated in Text-figs. 2 to 5. Coxsackie B4, ECHO 4, and ECHO 24 virus prototypes were highly susceptible in that in all experiments 98 μM HBB completely prevented the development of virus-induced cell damage in cultures



TEXT-FIG. 6. Lack of inhibitory effect of 2-(α-hydroxybenzyl)-benzimidazole (HBB) on the cytopathogenicity of arbor B and C; reo 1 to 3; adeno 2 to 4; and para-influenza 2 and 3 in monkey kidney cells. Concentration of HBB: —— none, – – – 98 μM, ···· 219 μM, –·–·– 493 μM.



TEXT-FIG. 7. Lack of inhibitory effect of 2-(α-hydroxybenzyl)-benzimidazole (HBB) on the cytopathogenicity of mumps; herpes simplex; vaccinia; Coxsackie A types 7, 11, 13, 16; and ECHO 22, 23, and 28 in monkey kidney, HeLa, or ERK cells (see text). Concentration of HBB: —— none, – – – 98 μM, ···· 219 μM, -·-·- 493 μM.

infected with these viruses. On the other hand, polio 1, polio 3, and ECHO 20 were only slightly inhibited; the development of viral cytopathic changes was delayed only 1 to 2 days at 98 μM HBB. Polio 1 was least sensitive. With these viruses, and polio 2 (2, 3), higher concentrations of HBB also failed to prevent the ultimate development of virus-induced cell damage. The other virus types exhibited various intermediate degrees of susceptibility.

It should be emphasized that in spite of these wide quantitative differences among HBB-sensitive viruses in susceptibility to inhibition by HBB, no difficulties were encountered in distinguishing between HBB-susceptible and insusceptible viruses. This is evident when Text-figs. 2 to 5 are compared with Text-figs. 6 and 7.

As mentioned above, a few of the viruses used were studied in HeLa or ERK rather than in monkey kidney cells. Since these viruses, *i.e.* herpes simplex, and Coxsackie A types 11, 13, and 18, were not inhibited by HBB, it was important to determine whether viruses, which are susceptible to HBB in monkey kidney cells, would also be inhibited in HeLa or ERK cells. Poliovirus types 1 (V. Richardson) and 2 (P 712-Ch-2ab) were examined in HeLa cells, and ECHO 9 (Hill) virus, in ERK cells. HBB inhibited each virus in the cells used. In these inhibition experiments both growth and maintenance media were employed, with similar results.

Lack of Relationship between Attenuation and Susceptibility to HBB.—Early in the course of the present studies, after only the three types of poliovirus and a few Coxsackie and ECHO virus types had been examined, it appeared possible that there might be a relationship between attenuation of a virus and degree of susceptibility to HBB. To explore this possibility two highly attenuated, and four virulent strains of poliovirus type 1 were examined as to their susceptibility to HBB. The attenuated strains used were Sabin's and Cox's live poliovirus vaccine strains. The virulent strains were the Mahoney strain and three strains isolated by Dr. A. B. Sabin and Dr. G. Berg from stools of paralytic cases in Cincinnati in 1955. The three "field strains" were in the first monkey kidney tissue culture passage, and gave similar yields when infected cultures were incubated at 36° or 40°C. (18). The vaccine strains gave much lower yields at the higher temperature (19, 20).

No relationship was found between attenuation of the strains and the degree of susceptibility to HBB; the attenuated strains were only slightly susceptible whereas among the wild strains slightly or markedly HBB-susceptible strains were encountered. Similar results were obtained with poliovirus type 2 strains.

Emergence of HBB-insusceptible Variants on Passage of Susceptible Strains in the Presence of HBB.—It was stated above that with most of the HBB-susceptible viruses a delay in the development of cytopathic changes rather than complete prevention of such changes was observed in the presence of 98 μM HBB. This observation raised the possibility that the populations of virus particles which grew out in the presence of HBB might be insusceptible

to HBB, or at least less susceptible than the parent virus populations. Experiments were performed in which virus populations, which had grown out in the presence of HBB, were examined for susceptibility to HBB.

Eight tissue culture tubes containing approximately 2.5×10^5 monkey kidney cells per tube were inoculated with approximately 2×10^5 $TCID_{50}$ of Coxsackie A9 virus contained in 1 ml. of tris-buffered saline (21). After adsorption for 30 minutes at 36°C., the residual unadsorbed virus was removed, and the cells were washed twice with 10 ml. of tris-buffered saline. Half of the tubes then received 1 ml. of Eagle's medium; the other half received 1 ml of Eagle's medium containing 98 μM HBB. After 2 days of incubation at 36°C., most of the cells in infected untreated cultures showed cytopathic changes. The culture tubes were frozen and thawed rapidly two times, and their contents pooled, and stored at −20°C. Cells in the infected treated cultures showed an almost complete cytopathic change in 4 days. These cultures were then handled in the same manner as untreated controls.

The pooled materials from each group were diluted 1:100, and the procedure described above was repeated: virus grown once with HBB was grown again in the presence of HBB; control virus was grown again without HBB. The tubes were collected two days after inoculation, and the contents pooled within each group after two cycles of rapid freezing and thawing. At the time of harvest, all cells in the untreated control cultures showed virus-induced damage, whereas in treated cultures approximately 85 per cent of cells were so affected.

The susceptibility to HBB of the parent Coxsackie A9 strain and of the virus populations derived from it after two passages either in the presence or absence of HBB was determined. As can be seen in Text-fig. 8, the parent strain was highly susceptible to HBB. However, virus which had been grown in the presence of HBB for two passages was no longer susceptible to inhibition by this compound. HBB in concentrations up to 219 μM did not even delay the development of the cytopathic effects due to the HBB-insusceptible variant. In an experiment not depicted in Text-fig. 8, 493 μM HBB did delay, but did not prevent, the development of the viral cytopathic effects of the variant.

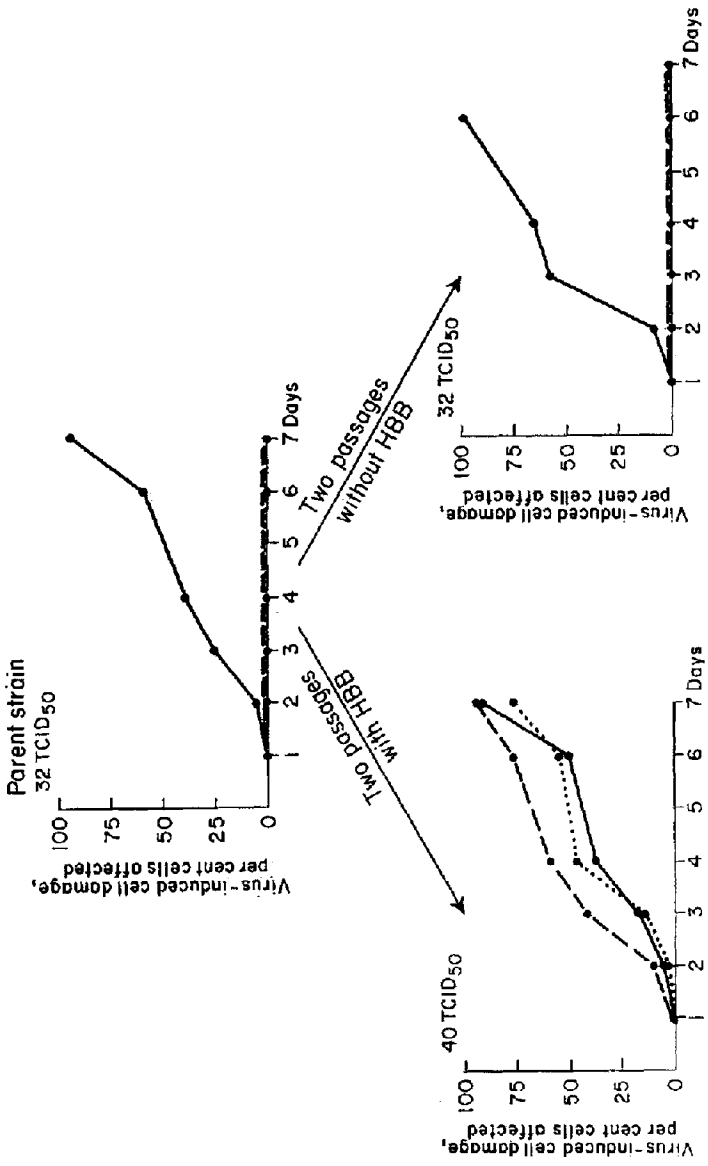
In contrast, the Coxsackie A9 virus strain which had been passaged twice in the absence of HBB was similar to the parent population in being highly susceptible to inhibition by HBB. In a further experiment with lower concentrations of HBB it was found that the parent virus and the virus passed twice in the absence of HBB were quantitatively closely similar in their susceptibility to HBB.

The HBB-insusceptible variant was neutralized by specific rabbit immune serum against the parent Coxsackie A9 strain. Also, the cytopathic changes due to the insusceptible variant were indistinguishable from those caused by the parent virus.

These results indicate that in the presence of HBB, an HBB-insusceptible variant of the HBB-susceptible Coxsackie A9 virus was obtained. Whether the emergence of the variant was due to selection alone or to mutation accompanied by selection cannot be decided at this time.

Similar findings have also been obtained with ECHO 7 virus.

Microscopic Cell Changes Due to HBB.—The earlier results (3) concerning



TEXT-FIG. 8. Emergence of an HBB-insusceptible variant of Coxsackie A9 after two passages in the presence of 98 μM HBB. Concentration of HBB: —— none, - - - 98 μM, ... 219 μM.

the effects of HBB on the microscopic appearance of monkey kidney cells in culture were amply confirmed in the present studies. Monkey kidney cells exposed to 493 μM HBB regularly showed slight changes after 3 to 5 days of incubation. The degree of these changes varied with the batch of cultures; the changes were minimal in cultures of excellent quality, but in occasional batches of cultures in which untreated cells showed some granular changes, the changes due to 493 μM HBB became moderate to marked on prolonged incubation. No morphologic changes attributable to the compound were observed in cells incubated in the presence of 98 μM HBB, except in a few instances. At 219 μM, slight changes were occasionally observed after 5 to 7 days of incubation. In "blind" tests it was not possible to distinguish consistently between untreated control cultures and those incubated in the presence of 219 μM HBB. Similar results were obtained with ERK cell cultures. HeLa cells seemed to be more prone to degenerative changes on prolonged incubation in the presence of 219 μM HBB than in its absence. It is also of interest that HeLa cells, maintained under non-optimal conditions (medium not changed frequently enough), degenerated earlier and more extensively in the presence of 219 μM than in untreated control cultures.

II. Nature of Virus Inhibitory Action of 2-(α-Hydroxybenzyl)-benzimidazole (HBB)

To explain the inhibitory effect of HBB on the cytopathogenicity of certain viruses, experiments were carried out to determine whether HBB inactivates the infectivity of susceptible viruses or inhibits their multiplication.

Lack of Inactivating Effect of HBB on Virus Infectivity.—From among the 34 viruses whose cytopathogenicity was inhibited by HBB, five were chosen for investigation of the possible effects of HBB on virus infectivity.

Virus seeds, diluted 1:100 in protein-free Eagle's medium with or without HBB, were incubated at 36°C. for 24 or 48 hours in culture tubes which did not contain cells, and the amount of remaining infective virus was determined. In the virus titrations, HBB was diluted out to ineffective levels.

Table II shows that HBB had no effect on the infectivity of Coxsackie B4, or ECHO types 6, 9, 11, or 12. It was shown previously that the compound does not inactivate the infectivity of poliovirus type 2 (2, 3).

Inhibition of Virus Multiplication by HBB.—Ten viruses were chosen for study of the effects of HBB on virus multiplication. Four were inhibited, and six were not inhibited by HBB in experiments on viral cytopathogenicity. The yields of these viruses in monkey kidney cells were determined in the presence or absence of HBB.

The procedure was described above. With most viruses, 25-320 $TCID_{50}$ of virus was inoculated per culture. In some instances larger inocula were used, as indicated in Table III. Three

to six cultures were used per variable. The concentration of HBB was 219 μM. The length of incubation was varied depending on the speed of development of virus-induced cell damage in infected untreated controls. The cultures were collected when the cytopathic changes in untreated cultures became marked. Before harvesting, the extent of virus-induced damage

TABLE II

Lack of Inactivating Effect of 2-(α-Hydroxybenzyl)-benzimidazole (HBB) on Infectivity of Enteroviruses

Virus	Strain	Time at 36°C.	Infective virus per ml.	
			Control	HBB, 493 μM
		hrs.		
Coxsackie B4	Powers	24	2.4×10^5 PFU	3.5×10^5 PFU
ECHO 6	D'Amori	24	3.2×10^8 $TCID_{50}$	2.5×10^8 $TCID_{50}$
ECHO 9	Hill	24	3.2×10^7 $TCID_{50}$	3.2×10^7 $TCID_{50}$
ECHO 11	Gregory	48	1.6×10^7 $TCID_{50}$	2.0×10^7 $TCID_{50}$
ECHO 12	Travis	24	1.35×10^8 PFU	1.9×10^8 PFU

TABLE III

Inhibition of Virus Multiplication and Cytopathogenicity by 2-(α-Hydroxybenzyl)-benzimidazole (HBB)

Virus	Inoculum	Incubation	Virus yield*		Cell damage†	
			Control	HBB, 219 μM	Control	HBB, 219 μM
	$TCID_{50}$	*days*				
Coxsackie B4 (Powers)	320	3	⩾8.2	⩽1.8	100	0
ECHO 6 (D'Amori)	320	4	8.7	⩽1.5	94	0
ECHO 7 (Wallace)	25	3	8.6	⩽1.5	100	0
ECHO 9 (Hill)	100	7	7.3	⩽1.5	63	0
Croup-associated (Greer)	56	6	6.4	6.3	78	47
Hemadsorption 1 (C-243)	56	6	7.2	6.9	88	81
Mumps (Utz)	200	7	5.8	5.1	41	41
Vaccinia	56	6	6.9	6.6	89	94
ECHO 22 (Morrison)	10,000	5	6.9	6.6	71	83
ECHO 22 (R. Host)	3,200	4	5.3	5.8	25	31

* Expressed as log $TCID_{50}$ per ml.

† Expressed as per cent cells affected.

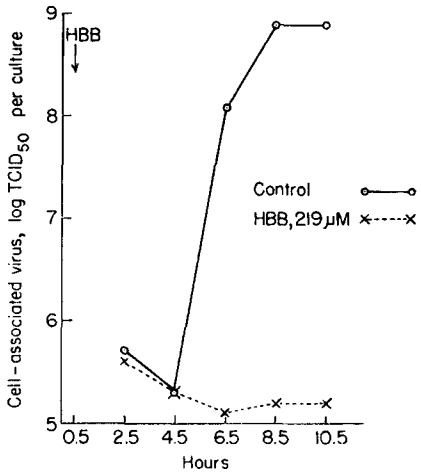
in both treated and control cultures was determined by microscopic examination. The cultures were then subjected to three cycles of rapid freezing and thawing, and assayed for infective virus. This procedure measured the sum total of cell-associated and released virus.

The results of these experiments are summarized in Table III. As can be seen, 219 μM HBB caused apparently complete inhibition of multiplication of

those viruses, *i.e.* Coxsackie B4, and ECHO types 6, 7, and 9, whose cytopathic effects it had prevented in experiments described above. The inhibitory effect of the compound on the cytopathogenicity of these viruses was confirmed (*cf.* Table III).

In contrast, HBB had no effect, or, in some instances, only a questionable effect on the yield of those viruses, *i.e.* croup-associated, hemadsorption 1, mumps, vaccinia, and ECHO 22, whose cytopathogenicity it did not inhibit. Similar results have been obtained with the HBB-susceptible polio type 2, and the HBB-insusceptible influenza B virus (2, 3).

These results suggest that HBB inhibits the cytopathogenicity of certain viruses by inhibiting their multiplication.



TEXT-FIG. 9. Single cycle growth curve experiment with ECHO 6 (D'Amori) virus in the presence or absence of 219 μM HBB.

Inhibition of ECHO 6 Virus Multiplication by HBB in Single Cycle Experiments.—It was of interest to determine whether HBB inhibits some intracellular steps in the viral reproductive sequence. Using one of the HBB-susceptible viruses (ECHO 6), single cycle experiments were carried out in which the compound was introduced after adsorption of virus to cells, and the yield of cell-associated virus was determined.

Cultures of monkey kidney cells in small Petri dishes were inoculated with 6.3×10^6 $TCID_{50}$ of virus per culture contained in 1 ml. volumes of Eagle's medium. The amount of virus inoculated per culture was considered equivalent to 4.3×10^6 infective units. Since the number of cells per Petri dish was, on the average, 1.9×10^6, the virus-cell multiplicity was approximately 2. The cultures were incubated at 36°C. for 30 minutes, whereupon they were washed twice to remove unadsorbed virus. The cultures then received 5 ml. of Eagle's medium with or without HBB (219 μM), and incubation at 36°C. was continued. Two cultures were used per variable. At intervals, groups of cultures were collected, the medium removed

and discarded, and the cell monolayers washed three times with Hanks' solution. Each culture then received 3 ml. of Eagle's medium, and the cultures were subjected to three cycles of rapid freezing and thawing to liberate the cell-associated virus, which was assayed by a 50 per cent infective end point titration procedure.

As can be seen in Text-fig. 9, the amount of cell-associated virus present during the latent period was closely similar in control and HBB-treated cultures. In the interval between 4.5 and 8.5 hours after inoculation, virus in control cultures increased rapidly, and reached maximal levels, whereas no increase was observed in treated cultures.

These results indicate that HBB inhibits some intracellular step in the reproductive sequence of ECHO 6 virus. Similar results have been obtained with Coxsackie B4, ECHO 7, and ECHO 12 viruses.

III. Lack of Effect of 2-(α-Hydroxybenzyl)-benzimidazole (HBB) on Metabolic Activities and Multiplication of Cells

The lack of toxic effects on the morphology of cells as well as the virtually undisturbed reproduction of insensitive viruses in the presence of 219 μM HBB suggested that host cells continue to perform vital metabolic processes at virus inhibitory concentrations of the compound. To obtain direct evidence on this point, various metabolic activities and the rate of multiplication of cells were investigated in the presence or absence of HBB.

Oxygen Uptake.—Oxygen uptake by monkey kidney cells incubated in the presence or absence of HBB was determined by the direct method in the Warburg apparatus at 35°C. The procedure used was described previously (13).

The results summarized in Table IV show that HBB, at concentrations which are markedly inhibitory for many viruses, had no effect on oxygen uptake by monkey kidney cells.

Glucose Utilization and Lactic Acid Production.—The amounts of glucose utilized and lactic acid produced by monkey kidney cells in the presence or absence of HBB were determined. In these experiments monkey kidney cells were used in suspension or as a monolayer.

Experiments with cells in suspension were carried out in 25 x 150 mm. test tubes. To 1 ml. volumes of a cell suspension containing 8 x 10^6 cells per ml., equal volumes of protein-free Eagle's medium with or without HBB were added. One or two tubes were used per variable. They were gassed with 5 per cent CO_2 in air, stoppered tightly, and incubated with shaking for 3 hours at 35°C. The cell suspensions were then centrifuged at 1,500 R.P.M. for 10 minutes. The supernatants were collected and diluted 1:100 for glucose determinations (14) and 1:20 for lactic acid measurements (15).

Experiments with cells in monolayers were carried out in 32 ounce prescription bottles. The growth medium in bottles containing complete monolayers was replaced with protein-free Eagle's medium with or without HBB. The volume of medium used, *i.e.* 40 ml. per bottle, was chosen to provide an amount of medium per cell comparable to that used in tube cultures. Three bottles were used per variable. They were gassed with 5 per cent CO_2 in air and incubated for 24 hours at 37°C. During incubation they remained stationary. The supernatants

were then collected and used for determinations of glucose and lactic acid. The cells were removed with 1:5000 versene, suspended in protein-free Eagle's medium, pooled within groups, and counted.

TABLE IV

Lack of Inhibitory Effect of 2-(α-Hydroxybenzyl)-benzimidazole (HBB) on Oxygen Uptake by Monkey Kidney Cells in Vitro

Cumulative O_2 uptake, μl. per 3 hrs. per 1 × 10^6 cells*			
Control	HBB		
	98 μM	219 μM	493 μM
6.38	6.75	6.01	5.95

* Number of cells per vessel: 16.3 × 10^6.

TABLE V

Lack of Inhibitory Effect of 2-(α-Hydroxybenzyl)-benzimidazole (HBB) on Glucose Utilization and Lactic Acid Production in Monkey Kidney Cells in Vitro

A. Cells in suspension

Glucose utilized				Lactic acid produced			
μM per 3 hrs. per 1 × 10^6 cells*							
Control	HBB			Control	HBB		
	98 μM	219 μM	493 μM		98 μM	219 μM	493 μM
0.94	0.98	0.98	0.99	1.76	1.78	1.88	1.88

* Number of cells per vessel: 8.0 × 10^6.

B. Cells in monolayer

Glucose utilized				Lactic acid produced			
μM per 3 hrs. per 1 × 10^6 cells*							
Control	HBB			Control	HBB		
	98 μM	219 μM	493 μM		98 μM	219 μM	493 μM
1.16	1.32	1.35	1.23	2.91	3.27	3.27	3.09

* Number of cells per bottle: 11.7 to 15.0 × 10^6. The experiment lasted 24 hours.

As can be seen in Table V, HBB had no effect on glucose utilization and lactic acid production in monkey kidney cells in suspension or in monolayer. As would be expected, more lactic acid was produced per mole of glucose utilized in the stationary monolayers than in the agitated suspensions of cells.

Incorporation of Adenosine into RNA and of Alanine into Proteins.—Uptake of adenosine-8-C^{14} into RNA and of C^{14}-L-alanine into proteins of monkey kidney cells was studied in the presence or absence of HBB. The procedures used were described previously (13).

In a 3 hour period, HBB had no effect on incorporation of adenosine-8-C^{14} into RNA, or of C^{14}-L-alanine into proteins of monkey kidney cells (*cf.* Table VI). In another experiment, incubation was extended to 9 hours and the concentration of HBB was 98 μM. Under these conditions also, HBB showed no effect on uptake of precursors into RNA or proteins.

TABLE VI

Lack of Inhibitory Effect of 2-(α-Hydroxybenzyl)-benzimidazole (HBB) on Uptake of Adenosine-8-C^{14} into RNA and of C^{14}-L-Alanine into Proteins of Monkey Kidney Cells in Vitro

Uptake per 3 hrs.							
Adenosine-8-C^{14}*				C^{14}-L-Alanine‡			
c.p.m. per 4 × 10^6 cells				c.p.m. per mg. of protein§			
Control	HBB			Control	HBB		
	19.5 μM	98 μM	493 μM		19.5 μM	98 μM	493 μM
111	98	123	128	32	29	—	28

* 0.2 μc./ml.

‡ 0.03 μc./ml.

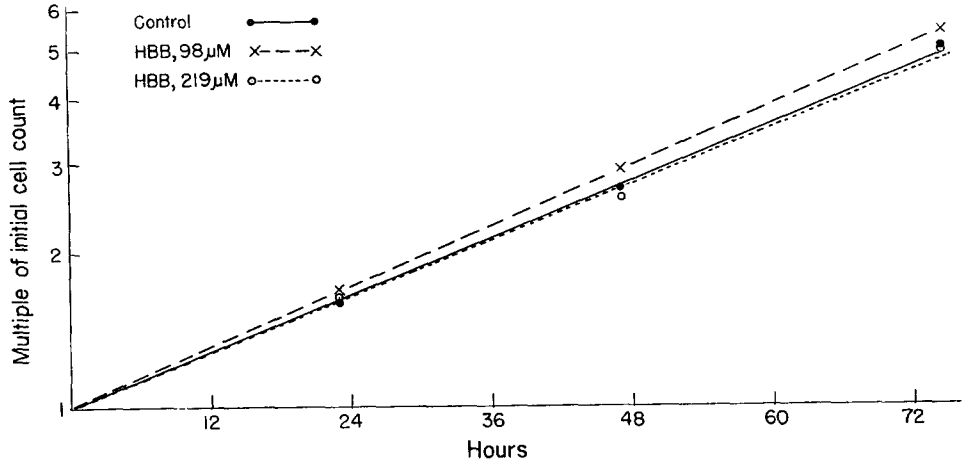
§ The protein fraction was obtained from 20 × 10^6 cells.

Multiplication of Cells.—The rates of multiplication of HeLa and ERK cells were studied in the presence or absence of HBB. The compound was used at concentrations sufficient to cause marked inhibition of susceptible viruses.

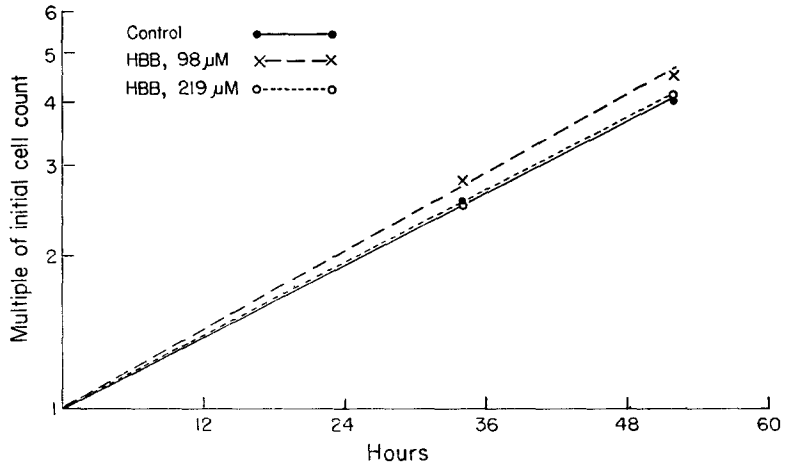
Plastic Petri dishes measuring 6 cm. in diameter were planted with approximately 10^5 cells per dish. The cells were introduced in 5 ml. volumes of growth medium with or without compound. Three dishes were used per variable. The cultures were incubated at 36°C. in a CO_2 incubator. Cells were enumerated within the area covered by three or four small fields which had been marked on the bottom of each dish prior to introduction of cells. The initial cell count was carried out 20 to 24 hours after seeding, and all subsequent counts were related to the initial count. In this manner normalized multiplication curves were obtained.

No obvious differences were noted in the attachment of cells to the plates in the presence or absence of HBB. Multiplication curves of HeLa and ERK cells, shown in Text-figs. 10 and 11, indicate that HBB at concentrations of 98 and 219 μM did not significantly influence the rate of multiplication of these cells. The latest cell counts were performed 3 or 4 days after planting. At subse-

quent times accurate counting was not possible because of the high density of cell populations, but inspection revealed no apparent differences between treated and untreated cultures.



TEXT-FIG. 10. Multiplication of HeLa cells in the presence or absence of 2-(α-hydroxybenzyl)-benzimidazole (HBB).



TEXT-FIG. 11. Multiplication of ERK cells in the presence or absence of 2-(α-hydroxybenzyl)-benzimidazole (HBB).

DISCUSSION

The virus inhibitory action of 2-(α-hydroxybenzyl)-benzimidazole is characterized by two important features: (*a*) HBB does not inhibit the multiplica-

tion and cytopathic effects of all viruses, but rather has a specific range of action; (*b*) HBB, at virus inhibitory concentrations, does not interfere with vital metabolic processes of host cells.

The spectrum of virus inhibitory activity of HBB is generally in line with the present classification of viruses (16). All viruses which are inhibited by HBB belong to one group, the enteroviruses and include the three types of poliovirus: Coxsackie A9; Coxsackie B types 1 to 6; and ECHO virus types 1 to 9, 11 to 21, and 24 to 27. None of the arbor, reo, adeno, myxo, herpes, or pox viruses examined were inhibited by HBB. Some enteroviruses were also insusceptible to HBB; these exceptions underscore the existing heterogeneity among enteroviruses.

The HBB-insusceptible enteroviruses include Coxsackie A types 7, 11, 13, 16, and 18; and ECHO types 22, 23, and 28. The following comments are pertinent:

1. It appears that Coxsackie A viruses, as a group, are insusceptible. This includes Coxsackie A7 (AB IV strain) (22, 23), which represents the so-called Russian "poliovirus type 4" (22). Among the six Coxsackie A viruses examined, the only HBB-susceptible virus was type 9—a virus which has been considered to be related to the ECHO group (24).
2. ECHO 22 and 23 viruses cause characteristic cytopathic effects in monkey kidney cells (25) which also set them apart from other enteroviruses.
3. The ECHO 28 strain examined represents the 2060 virus (25)—an upper respiratory disease agent, which possesses some of the characteristics of enteroviruses, but which in certain respects differs from most of them.

In summary, the HBB-insusceptible enteroviruses exhibit additional properties which, along with insusceptibility to HBB, serve to distinguish them from most enteroviruses.

The insusceptibility of reoviruses to HBB is also of special interest, because they were originally known as ECHO 10 viruses, but were reclassified when it became evident that they differed from enteroviruses in a number of properties (26).

The fact that many different viruses multiply to high yields in the presence of HBB implies that treated cells continue to perform important biosynthetic and energy-yielding processes. It was therefore not unexpected that HBB, at concentrations sufficient to cause marked inhibition of susceptible enteroviruses, had no effect on oxygen uptake, glucose utilization, lactic acid production, uptake of labelled adenosine into RNA, or uptake of labelled alanine into proteins of host cells. Further evidence that HBB does not disturb processes of vital importance to cells is provided by the demonstration that cells continue to multiply at the usual rate in presence of HBB. All of these results are in line with the finding reported earlier (2, 3) and confirmed in the present studies that HBB at virus inhibitory concentrations causes no, or only minor, morphologic changes in cells. The combined evidence suggests strongly that HBB does

not act as an antagonist of a metabolite required by both the host cell and the virus.

The fact that HBB delays or prevents the cytopathic effects of only those viruses whose multiplication it inhibits, suggests that inhibition of virus-induced cell damage is due to inhibition of virus multiplication. It has also been reported (2, 3) that the curves describing the relationships between concentration of compound and inhibition of poliovirus yield or reduction in virus-induced cell damage were approximately parallel. This finding also suggested (3) that the reduction in virus-induced cell damage was due to inhibition of the viral reproductive process.

HBB at 219 or 493 μM prevented completely the development of the cytopathic effects of many of the susceptible viruses. However, with numerous susceptible viruses, a delay, rather than complete prevention of the development of viral cytopathic effects was observed in the presence of 98 μM HBB. The possibility was therefore considered that the development on prolonged incubation of viral cytopathic effects in treated cultures may have been due to the presence in virus strains of two or more kinds of virus particles with different susceptibilities to the action of HBB. If this be true, the proportion of HBB-insusceptible virus particles, or of particles with low susceptibility, and their growth rate would determine the time after virus inoculation when virus "break-through" would occur in the presence of HBB at a certain concentration. Also, the virus growing out in the presence of HBB would be expected to be less susceptible to the compound than the original virus population. It is of considerable interest that in experiments with two viruses, *i.e.* Coxsackie A9 and ECHO 7, HBB-insusceptible variants were obtained on passage of the parent strains in the presence of the compound. Further work is required to determine whether the emergence of the HBB-insusceptible particles was due to selection alone or to mutation accompanied by selection.

As to the mechanism of the virus inhibitory action of HBB, there is strong evidence that HBB inhibits some intracellular step in virus multiplication. Furthermore, preliminary experiments have indicated that HBB acts at a late stage in the reproductive cycle of enteroviruses (3, 27), but the possibility that the compound may also have effects during the early intracellular stages has not been excluded. It should be emphasized that HBB has no direct inactivating effect on the infectivity of enteroviruses, and that it does not appear to interfere with the adsorption of enteroviruses to host cells (27). It is noteworthy that the virus inhibitory activity of HBB is not restricted to one cell system, but can be demonstrated in at least three cell systems, *i.e.* in monkey kidney, HeLa, and ERK cells, employing different media.

The results thus far obtained suggest that elucidation of the precise mechanism whereby HBB acts may reveal an important step in the reproduction of enteroviruses, which may be specific for this group of viruses.

SUMMARY

2-(α-Hydroxybenzyl)-benzimidazole (HBB) inhibited the cytopathic effects of the following enteroviruses: polio 1 to 3; Coxsackie A9; Coxsackie B 1 to 6; and ECHO virus types 1 to 9, 11 to 21, and 24 to 27. The following enteroviruses were not inhibited: Coxsackie A types 7, 11, 13, 16, and 18; and ECHO types 22, 23, and 28. Other HBB-insusceptible viruses were: arbor B and C, reo 1 to 3; adeno 2 to 4; influenza B; para-influenza 2 and 3; mumps; herpes simplex, and vaccinia.

HBB had no inactivating effect on viral infectivity, but rather inhibited some intracellular step in the reproductive cycle of susceptible viruses. With all viruses examined, inhibition of viral cytopathic effects appeared to be due to inhibition of virus multiplication. Virus inhibition by HBB was demonstrable in monkey kidney, HeLa, and ERK cells.

HBB-susceptible viruses varied quantitatively in their susceptibility to the compound, and different strains of the same virus also exhibited varying susceptibility. No relationship was found between attenuation of polioviruses and their susceptibility to the compound. After passage of HBB-susceptible enteroviruses in the presence of the compound, virus populations with lowered susceptibility to HBB were obtained.

At virus inhibitory concentrations, HBB did not affect the morphology of cells, nor the following cellular metabolic activities: oxygen uptake; glucose utilization; lactic acid production; and incorporation of adenosine into RNA, and of alanine into proteins. The rates of multiplication of HeLa and ERK cells were not significantly altered by HBB at virus inhibitory concentrations.

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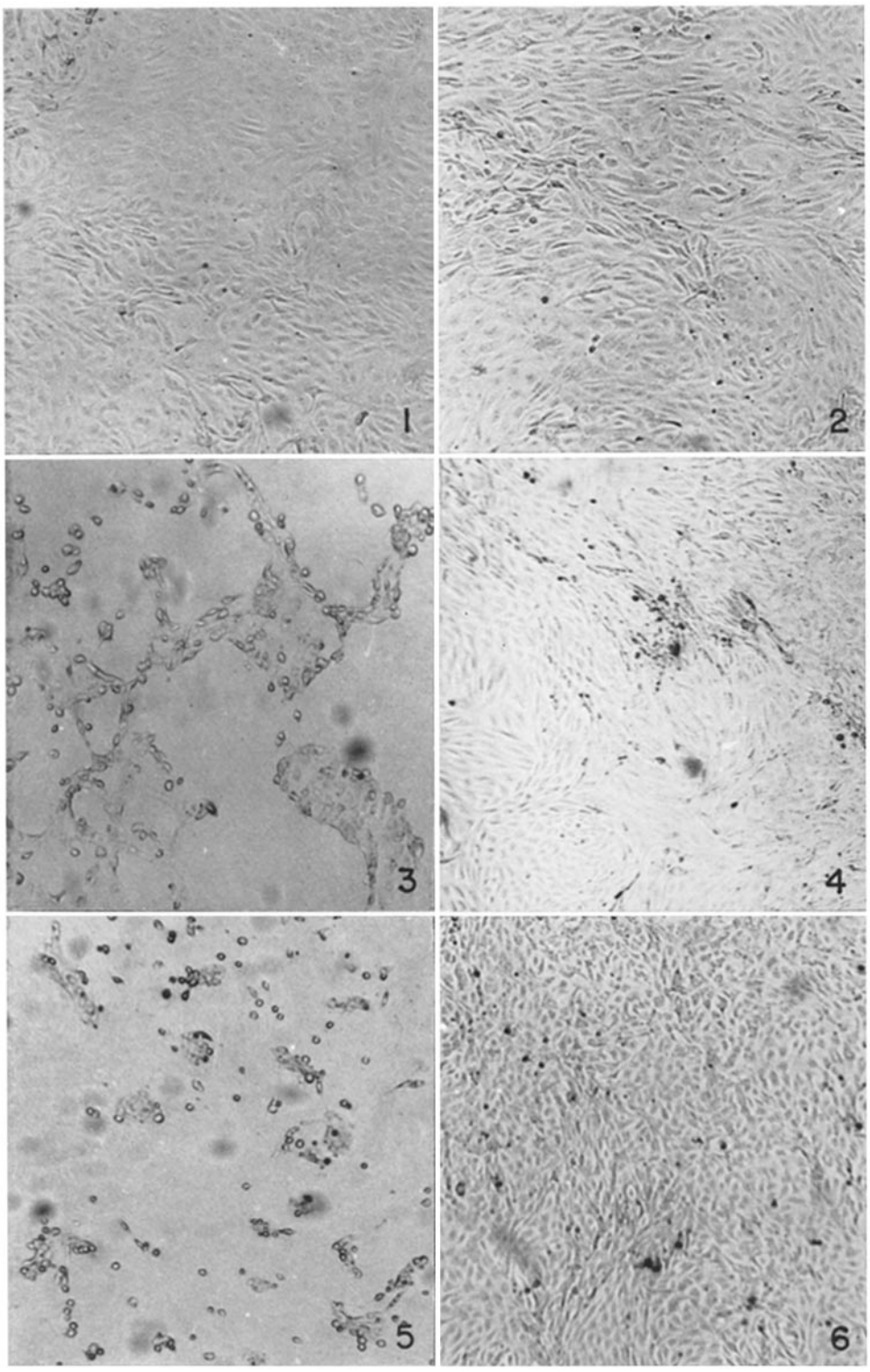
EXPLANATION OF PLATE 73

Black and white photomicrographs of monkey kidney cells demonstrating inhibition by 2-(α-hydroxybenzyl)-benzimidazole (HBB) of the cytopathic effects of Coxsackie B4 (Powers) and ECHO 6 (D'Amori) viruses. Amounts of virus inoculated per culture: Coxsackie B4, 200 $TCID_{50}$; ECHO 6, 50 $TCID_{50}$. The concentration of HBB was 98 μM. Cultures were incubated for 5 days. × 37.

FIG. 1. Uninfected, untreated culture.
FIG. 2. Uninfected, treated culture.
FIG. 3. Coxsackie B4-infected, untreated culture.
FIG. 4. Coxsackie B4-infected, treated culture.
FIG. 5. ECHO 6-infected, untreated culture.
FIG. 6. ECHO 6-infected, treated culture.



(Eggers and Tamm: Virus inhibitory action of HBB)