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INHIBITION OF INFLUENZA VIRUS MULTIPLICATION BY N-GLYCOSIDES OF BENZIMIDAZOLES

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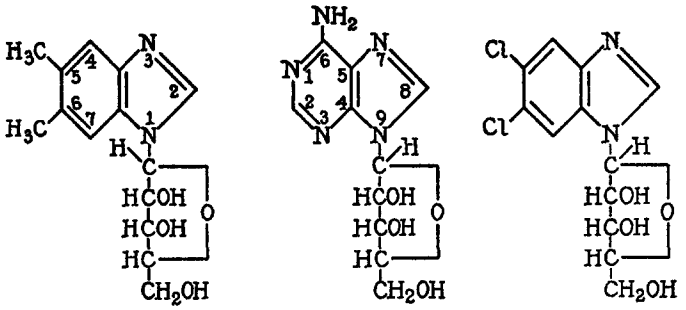
Studies of the effects of benzimidazole derivatives on influenza virus multiplication were undertaken (1) because it appeared likely that such compounds would exhibit inhibitory activity which might be interpreted in terms of present knowledge of vitamin B_{12} and purine metabolism. It was thought that approaches to the striking biological specificity of viruses and to selective inhibition of virus multiplication might best be sought in the area of nucleic acid metabolism (1). As was reported in previous communications (2–4), alkyl derivatives of benzimidazole possess in varying degree the ability to inhibit influenza virus multiplication. Quantitative studies showed that the extent of inhibitory activity depends markedly on the nature and position of substituent groups in the molecule and that certain regularities are observed (4).

In view of the fact that both the 5,6-dimethylbenzimidazole moiety in vitamin B_{12} (5–8) and the adenine and guanine moieties in nucleic acids are linked to pentoses, it appeared fruitful to explore a series of *N*-glycosides of



I

5,6-Dimethyl-1-α-D-ribofuranosyl-benzimidazole or α-ribazole

II

9-β-D-Ribofuranosyladenine or adenosine

III

5,6-Dichloro-1-β-D-ribofuranosyl-benzimidazole (DRB)

FIG. 1

benzimidazoles. It seemed that certain *N*-glycosides might be more active than the corresponding benzimidazoles. A number of ribose derivatives were synthesized (9) and utilized in this investigation. Because of the biochemical importance of ribose-containing compounds, it appeared probable that ribosides would have a striking effect on virus multiplication. As shown in Fig. 1, the pentose linked to the 5,6-dimethylbenzimidazole moiety in vitamin B_{12} is ribose (I). The nucleoside adenosine, which also contains ribose (II) is present not only in pseudovitamin B_{12} (10) and in ribose nucleic acids but also in ADP and ATP and in several coenzymes.

The present studies on inhibition of influenza virus multiplication were carried out with alkyl-, chloro-, or alkyl-chlorobenzimidazoles; the benzimidazole glycosides were derived from ribose and other pentoses, or certain hexoses. It will be shown that the ribofuranose moiety is an important factor relative to inhibitory activity. The striking effect of substituent groups in the benzene ring, with a ribofuranosyl group at position 1, will be demonstrated. In addition, it will be shown that virus multiplication, tissue respiration, and proliferation are not affected in identical degree by certain inhibitory benzimidazole derivatives. It will be demonstrated that 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (*cf.* Fig. 1, III), the derivative which shows the highest inhibitory activity on virus multiplication in membrane culture, also has an inhibitory effect on Lee virus multiplication in mice and embryonated eggs.

Materials and Methods

Culture Medium.—The medium employed for washing and suspension of pieces of chorioallantoic membrane consisted of a sterile buffered salt and dextrose solution (1, 4).

Membrane.—The earlier procedure (4) for obtaining pieces of chorioallantoic membrane from 10- or 11-day embryonated chicken eggs was modified. That part of the membrane which lines the air sac end of the shell was exposed and rinsed with medium. A triangular segment of the shell supporting the area of attachment of the allantoic vein was cut out and discarded. Then the shell, and with it the adherent membrane, was cut into 4 pieces along the long axis of the egg. Cutting the membrane while it was attached to the shell permitted pieces of approximately equal size to be obtained with fewer manipulations. The pieces of membrane were peeled from the shell sections, rinsed in the medium, and transferred into large test tubes containing medium. Statistical analysis of variation in influenza virus cultures with pieces of membrane from individual eggs, *i.e.* among 4 pieces obtained from each egg, showed that, with the modified procedure, the standard deviation of the titers was reduced to a value of 0.69 of that found previously (3).

Pieces of membrane from different eggs were randomized in setting up groups of cultures and not fewer than 6 membrane cultures were included in an experimental group. This was done to avoid systematic errors attributable to variation in membranes from different eggs (3). In some experiments, randomization was accomplished in the following way: 6 eggs were selected at random and, from each egg, 4 pieces of membrane were obtained. Each piece of membrane was assigned to a different group of cultures. Thus, 4 groups of cultures were formed and each group contained a piece from each of the same 6 eggs. This procedure minimized variation due to differences between eggs, and permitted determinations of inhibitory activity with relatively few membrane cultures.

Membrane Surface Area.—With the modified procedure, the mean surface area of membrane pieces was somewhat greater than that reported previously (3). For 16 pieces from 4 eggs, the mean area was 6.6 cm.^{2} with a standard deviation of 1.07. Accordingly, each piece represented about 5.4 per cent of the entire allantoic membrane. On the basis of previous estimates (3, 11), the number of allantoic cells in 6.6 cm.^{2} of membrane is about 10^7.

Viruses.—The Lee virus seed employed was described in an earlier communication (3) and contained $10^{9.3}$ EID_{50} per ml. PR8, FM1, and MB virus seeds were prepared as follows: PR8 was passed serially in the allantoic sac of 10- or 11-day old embryonated eggs with 10^{-2} dilution of infected allantoic fluid as inoculum and 24 hours of incubation. For seed virus, eggs were inoculated with a 10^{-5} dilution and allantoic fluid was harvested after 24 hours. The concentration of infective virus was $10^{8.7}$ EID_{50} per ml. FM1 also was passed serially under identical conditions. For seed virus, eggs were inoculated with a 10^{-4} dilution and allantoic fluid was harvested after 28 hours. The concentration of infective virus was $10^{9.1}$ EID_{50} per ml. MB strain of influenza B virus, isolated in 1950 (12), was passed serially employing a 10^{-4} diluted allantoic fluid as incoulum and a 30 hour period of incubation. Seed virus was prepared under the same conditions. The concentration of infective virus was $10^{9.0}$ EID_{50} per ml. In all cases, the eggs were chilled at −28°C. for 30 minutes prior to harvest. Then allantoic fluids were collected, pooled, and promptly stored at −60°C. in individual tubes which were thawed and used but once.

Culture Procedure.—Intact pieces of membranes, measuring on the average 6.6 cm.^{2}, were suspended in 0.9 ml. of culture medium or a solution of the benzimidazole derivative in the same medium. Immediately thereafter, 0.1 ml. of infected allantoic fluid diluted in cold medium, was introduced. The final concentration of virus was $10^{5.5}$ EID_{50} per ml.; a ratio of approximately 1 EID_{50} to 32 allantoic cells. The culture tubes were closed with rubber stoppers and incubated at 35°C. with continuous horizontal shaking as in earlier studies (1–4). After incubation, the medium was withdrawn and the concentration of virus was measured by the hemagglutination technique (3). In some experiments, the virus was measured by infectivity titrations. For this purpose, pooled membranes were ground with mortar and pestle and then suspended in the culture medium from the same group of cultures. This procedure measures the total amount of infective virus present both in the membrane and in the medium. To eliminate unadsorbed virus in these experiments, membranes were washed and transferred into fresh medium 1 hour after inoculation.

Hemagglutination Titrations.—The procedure used was described in an earlier report (3).

Inhibitory Activity of Compounds.—The method of determining the inhibitory activity of compounds was described in previous communications (3, 4).

Infectivity Titrations.—Serial 0.5 log (*i.e.*, 3.16-fold) dilutions were prepared in chilled broth containing penicillin, 50 units, and streptomycin, 250 μg. per ml. Ten- or 11-day embryonated eggs in groups of 3 or 4 were inoculated allantoically with each dilution, 0.2 ml. per egg. The eggs were incubated at 35°C. for 42 to 46 hours. They were then chilled at −28°C. for 30 minutes, and the 50 per cent infectivity endpoint was determined by the hemagglutination procedure with allantoic fluids.

EXPERIMENTAL

Inhibitory Activity of Certain Benzimidazole Derivatives.—The extent of Lee virus multiplication in cultures was determined by the hemagglutination technique with the culture medium after a 36 hour period of incubation (3). The geometric mean titer of each group of 6 cultures was computed. The degree of inhibition of multiplication was expressed as the percentage value of the control titer and was plotted against the molar concentration of the benzi-

midazole derivative used. Compounds were compared in terms of the molar concentration which caused 75 per cent inhibition (3, 4).

Chloro- and Chloro-alkylbenzimidazoles.—Selected chloro or chloro and alkyl derivatives of benzimidazole were tested for inhibitory activity against Lee virus multiplication and the results were compared with those obtained previously with alkyl benzimidazoles (4). The results of these experiments are summarized in Table I. The low solubility of compounds other than the 5-chloro- and 5-chloro-2-methyl derivatives made it impossible to determine activity precisely. However, the results obtained with the two compounds

TABLE I

Inhibition of Influenza B Virus Multiplication by Chloro and Chloro-Alkyl Derivatives of Benzimidazole

Benzimidazole derivative	No. of experiments	No. of determinations	Inhibitory concentration*	Standard deviation		Inhibitory activity relative to benzimidazole
			$M \times 10^{-4}$	$M \times 10^{-4}$	per cent	
5-Chloro (9)	3	6	7.5	1.8	24	4.7
4,6-Dichloro (9)	2	2	>5.0 <7.5‡			>4.7 <7
5,6-Dichloro (9)	5	8	~2.5§			~14
5-Chloro-2-methyl (9)	2	4	4.7	1.1	23	7.5
5-Chloro-6-methyl (9)	1	2	>5.0 <10‡			>3.5 <4.7
5-Chloro-2,6-dimethyl (9)	1	2	>3.2‖			
5,6-Dichloro-2-methyl (9)	1	2	>0.84‖			
5,6-Dichloro-2-ethyl (9)	2	2	>1.0 <2.5‡			>14 <35

* Concentration giving 75 per cent inhibition of multiplication.

‡ At a concentration slightly higher than the 75 per cent inhibitory concentration, the compound was insoluble.

§ Based on results with concentrations giving 30 to 60 per cent inhibition. Saturated solution gave less than 75 per cent inhibition.

‖ No inhibition at the concentration indicated. Not soluble at higher concentrations.

mentioned indicated that chloro derivatives were 2 to 3 times more active than corresponding methyl compounds. As was reported earlier (4), the 5-methyl and 2,5-dimethyl compounds had inhibitory concentrations of 19 and 13 $\times$ 10^{-4} M, respectively. It should be pointed out that the results obtained with four other compounds (*cf.* Table I) were in accord with those obtained with the more soluble derivatives.

Chloro-N-glycosylbenzimidazoles.—Various *N*-glycosides of 5,6-dichlorobenzimidazole were prepared in order to test the effect of certain pentoses and hexoses on the inhibitory activity. The compound of greatest theoretical interest was the ribofuranosyl derivative of 5,6-dichlorobenzimidazole (*cf.* Fig. 1) because in this case substitution of the particular pentose used was expected to yield a derivative of considerable biochemical significance. As

shown in Table II, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (hereafter referred to as DRB) showed inhibitory activity of a high degree and caused 75 per cent inhibition of Lee virus multiplication at a concentration of 0.38×10^{-4} M; *i.e.*, 12 μg. per ml. Thus, DRB was 92 times more active than the reference compound, benzimidazole. The profound effect of the nature of the glycosyl group on inhibitory activity is apparent from the results given in Table II. When the glycosidic ring was changed from the 5-membered ribo-

TABLE II

Inhibition of Influenza B Virus Multiplication by Chloro-Glycosyl and Chloro-Methyl-Glycosyl Derivatives of Benzimidazole

Benzimidazole derivative	No. of experiments	No. of determinations	Inhibitory concentration*	Standard deviation		Inhibitory activity relative to benzimidazole
			$M \times 10^{-4}$	$M \times 10^{-4}$	*per cent*	
5,6-Dichloro-1-β-D-ribofuranosyl (9)	4	9	0.38	0.031	8.1	92
5,6-Dichloro-1-β-D-ribopyranosyl (9)	2	6	2.3	0.071	3.0	15
5,6-Dichloro-1-β-D-arabinopyranosyl (9)	2	4	11	1.7	15	3.1
5,6-Dichloro-1-β-D-galactopyranosyl (9)	2	2	>10 <20			>1.8 <3.5
5,6-Dichloro-1-β-D-glucopyranosyl (9)	2	3	~11‡			~3.2
5- (or 6-) Chloro-1-β-D-ribofuranosyl (9)	1	2	2.8			13
5,6-Dichloro-2-methyl-1-β-D-ribofuranosyl (9)	3	8	3.6	0.28	7.8	9.7
5,6-Dichloro-2-methyl-1-β-D-glucopyranosyl (9)	1	1	13			2.7

* Concentration giving 75 per cent inhibition of multiplication.

‡ Based on results with concentrations giving 30 to 60 per cent inhibition.

Not soluble at 10×10^{-4} M.

furanoside (*cf.* Fig. 1, III) to the 6-membered ribopyranoside, the inhibitory activity was lowered to 1/6th of that found for DRB. Furthermore, substitution of an arabinopyranosyl moiety for the ribofuranosyl moiety gave a compound which had 1/29th the activity of DRB. A similar low activity was observed with the galactopyranosyl and glucopyranosyl derivatives although the low solubility of these compounds prevented precise measurement of inhibitory activity.

Modification of Chloro-N-gylcosylbenzimidazoles.—The effect of substitution in the benzene or imidazole ring on the inhibitory activity of pentose-substituted chloro derivatives of benzimidazole was determined. Previous studies

Published March 1, 1954

(4) with alkyl derivatives had shown that certain substitution in either ring tended to increase inhibitory activity. As shown in Table II, DRB was 6.6 times more active than 5- (or 6-) chloro-β-D-ribofuranosylbenzimidazole, indicating that substitution of two chlorine atoms in the benzene ring was more effective than substitution of one. However, substitution of a methyl group at position 2 in DRB caused a considerable reduction in activity. Thus, substitution at position 2 in this compound had a very different effect from that observed with alkyl benzimidazoles (4). With the latter compounds, substitution of a methyl group at position 2 increased inhibitory activity when one or more methyl groups were present in the benzene ring.

Comparison of the activities of the 5,6-dichloro-2-methyl-1-β-D-ribofuranosylbenzimidazole and the corresponding glucopyranosyl compound (Table II) indicates that, as was observed above, departure from the ribo-

TABLE III

Lack of Inhibitory Activity of Methyl-Glycosyl Derivatives of Benzimidazole

Benzimidazole derivative	Concentration employed	Inhibition of Lee virus multiplication
	$M \times 10^{-4}$	*per cent*
5,6-Dimethyl	19	75
5,6-Dimethyl-1-α-D-ribofuranosyl (13)	35	0
5,6-Dimethyl-1-D-lyxopyranosyl (14)	35	0
5,6-Dimethyl-1-D-arabinopyranosyl (14)	2.2*	0
2,5- (or 2,6-) Dimethyl-1-β-D-ribofuranosyl (9)	35	0

* Not soluble at higher concentrations.

furanose structure in carbohydrate substituents results in decreased inhibitory activity.

Methyl-N-glycosylbenzimidazoles.—In view of the presence of 5,6-dimethyl-1-α-D-ribofuranosylbenzimidazole (also designated α-ribazole) in vitamin B_{12} (5–8), it was important to determine whether or not this compound had inhibitory activity. As shown in Table III, no evidence of such activity was found when the compound was tested at a concentration at which benzimidazole itself caused 75 per cent inhibition of multiplication (4). As was shown above, benzimidazole was only 0.011 as active as DRB. Thus, it appears that for a ribofuranoside, the change from methyl to chloro groups at positions 5 and 6 gives an inhibitory compound of high activity. It was shown previously (4) that 5,6-dimethylbenzimidazole was inhibitory at a concentration of 19×10^{-4} M. Therefore, the present results indicate that the conversion of this compound to a ribofuranoside serves actually to eliminate inhibitory activity. Similarities between these results and those which have been obtained with *Lactobacillus leichmannii* 313 (15) are discussed below. The data presented in

Table III also indicate that glycosides of 5,6-dimethylbenzimidazole which are derived from a pentose other than ribose did not show significant inhibitory activity; the lyxopyranosyl derivative failed to inhibit Lee virus multiplication. In addition, substitution of a methyl group at position 2 in the 5- (or 6-) methyl-1-β-D-ribofuranosyl compound yielded a substance without demonstrable inhibitory activity.

Glycitylbenzimidazoles.—Since riboflavin contains an *N*-ribityl group, it was of interest to test the inhibitory activity of several *N*-glycitylbenzimidazoles. As shown in Table IV, none of the four compounds of this type that were tested showed any inhibitory activity at the concentrations at which they could be tested.

Aspects of the Activity of 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole.—In view of the close structural similarities between α-ribazole, adenosine, and

TABLE IV

Inhibitory Activity of Methyl-glycityl- and Chloro-glycitylbenzimidazoles

Benzimidazole derivative	Concentration employed*	Inhibition of Lee virus multiplication
	M × 10^{-4}	*per cent*
5,6-Dimethyl-1-D-ribityl (16)	18	0
5,6-Dichloro-1-D-arabityl (16)	8.7	0
5,6-Dichloro-1-D-xylityl (16)	3.9	0
5,6-Dichloro-1-D-sorbityl (16)	3.9	0

* The compounds were not soluble at higher concentrations. At the concentrations indicated, the chloro compounds were only partially soluble.

5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, and the high inhibitory activity of the latter compound, detailed studies were undertaken on various aspects of DRB action.

1. Attempts to Block the Inhibitory Effect of DRB.—In a previous communication (4) the failure of a number of compounds to block the inhibitory effect of benzimidazole or 2,5-dimethylbenzimidazole was reported. In the earlier experiments, compounds were used which, it was thought, might serve as metabolites in virus multiplication. When the high inhibitory activity of DRB was discovered, attempts also were made to block its effect. No success was attained and the following compounds failed to block the inhibitory effect of 0.44×10^{-4} M DRB: vitamin B_{12} (0.12 mg./ml.); desoxyribonucleic acid (0.5 mg./ml.); a mixture of adenosine, adenylic acid, guanosine, and guanylic acid (4.4×10^{-4} M of each); and 5,6-dimethyl-1-α-D-ribofuranosylbenzimidazole ($4.4 \times 10^{-4} - 15.1 \times 10^{-4}$ M).

2. Effect of Size of Inoculum on Degree of Inhibition with DRB.—It has been reported that the degree of inhibition of multiplication caused by various

compounds may depend on the amount of virus inoculated (1, 17, 18). This would be expected in multiple cycle experiments if prolongation of the latent period, reduced yield per cycle, or both, were caused by the compound employed. Information is lacking whether or not the degree of inhibition varies with the size of inoculum in single cycle experiments.

Experiments were carried out with pieces of membrane *in vitro* as described above. Varying amounts of Lee virus were used in the inoculum and multiplication occurred in the presence or absence of 0.72×10^{-4} M DRB. Following inoculation, membranes were incubated for 1 hour and then were washed in 3 changes of control medium or DRB-containing medium and transferred into fresh media. This was done in order to remove unadsorbed virus. After 15.5 hours of incubation, membranes and media were harvested, pooled, and 10 per cent suspensions were prepared. Virus concentration was measured by infectivity titrations on the suspensions.

TABLE V

Relation between Size of Inoculum and Degree of Inhibition

	Yield of infective virus*		
Lee virus inoculum	Control	DRB‡	Ratio, Control/DRB
log EID_{50} per ml.	*log*	*log*	*log*
4.5	5.26	~1.62	3.64
5.5	6.12	3.04	3.08
6.5	7.20	4.26	2.94
7.5	6.94	4.79	2.15

* EI_{50} dose contained in 1.0 ml., determined on pooled membranes and media which were collected 15.5 hours after inoculation of virus.

‡ 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole, 0.72×10^{-4} M.

As shown in Table V, the yield of virus in the control cultures inoculated with $10^{4.5}$ to $10^{6.5}$ EID_{50} per ml. was directly proportional to the amount of virus inoculated. The fact that an inoculum of $10^{7.5}$ did not yield more virus than one of $10^{6.5}$ EID_{50} per ml. probably indicates that the number of infective doses of virus introduced was greater than that of susceptible host cells. As indicated above, the number of allantoic cells per piece of membrane is about 10^7. The results presented in Table V indicate that the degree of inhibition at the end of the first cycle of multiplication (2) was not greatly influenced by the quantity of virus inoculated. This was most evident with inocula of $10^{5.5}$ and $10^{6.5}$ EID_{50}. In both cases, multiplication in DRB-treated cultures was restricted to approximately 0.1 per cent of that in control cultures. With an inoculum of $10^{4.5}$ EID_{50}, the titer of virus in the treated cultures was too low to be reliable. With the largest inoculum employed, the ratio between infective doses and allantoic cells was sufficiently high to introduce some uncertainties in the results (19).

3. Lack of Direct Inactivating Effect of DRB on Lee Virus.—The effect of DRB on the infectivity of Lee virus *in vitro* was determined. It has been shown (20) that the rate of inactivation of influenza viruses is markedly affected by environmental factors. Throughout the present investigation, pieces of membrane were suspended in a chemically defined medium and inoculated by adding virus to the medium. During multiplication in membrane culture, virus may be present at intervals in the membrane, the medium, or both. To simulate conditions in the culture system, an extract of membrane was added to the medium in experiments on the direct effect of DRB on virus infectivity.

Membranes were harvested (3) and a 5 per cent suspension by volume was prepared in culture medium and homogenized in a modified Waring blendor. The homogenate was centrifuged at 2,000 *g* for 10 minutes and the supernatant was removed and diluted with an equal volume of medium. Membranes contain about 93 per cent of water (3), and the final concentration of solids derived from the membranes in the enriched medium was less than 0.2 per cent. To give $10^{6.5}$ EID_{50} of Lee virus per ml., infected fluid was diluted $10^{-2.5}$ in the enriched medium or in a solution of DRB, 0.98×10^{-4} M in this medium. Infectivity titrations were carried out on the control specimen immediately and on both the control and DRB-containing specimens after incubation at 35°C. for 2 hours.

TABLE VI

Lack of Direct Effect of DRB on Infectivity of Lee Virus

Hrs. at 35°C.	Infectivity titers* *in ovo*	
	Control medium	Medium plus DRB‡
	log	*log*
0	−8.87	—
2	−8.50	−8.66

* EI_{50} computed to 1.0 ml.

‡ 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole.

Table VI gives the results of such an experiment. There was no notable difference in the titers of the control and DRB-containing specimens after incubation. Thus, DRB appeared to have no direct inactivating effect on the infectious property of Lee virus.

4. Lack of Effect of DRB on Virus Hemagglutination.—In the present study, hemagglutination titrations were frequently employed for the measurement of virus concentration. To determine if DRB had any effect on virus-erythrocyte interaction, the following experiments were done: when a suspension of chicken erythrocytes, 0.36 per cent, in buffered saline was mixed with an equal volume of culture medium containing DRB, 3.4×10^{-4} M, no visible agglutination of the erythrocytes occurred. When serial dilutions of DRB in buffered saline,

a constant amount of Lee virus, *i.e.* 4 hemagglutinating doses in the final mixture, and chicken erythrocytes, 0.36 per cent, were mixed, no inhibition of hemagglutination was demonstrable at the lowest dilution of DRB tested, *i.e.* 0.83×10^{-4} M. Thus, no evidence was obtained that the compound combined with the virus or with receptor areas on the surface of erythrocytes.

5. Lack of Effect of DRB on Adsorption of Virus by Membrane.—Experiments were carried out to determine if DRB affected adsorption of Lee virus by membrane *in vitro*.

Pieces of membrane from 10-day embryonated eggs were suspended in 1.8 ml. of culture medium or in a 1.3×10^{-4} M solution of DRB in this medium. The wet weight of pieces of membrane was 0.4 gm. per sample. Then 0.2 ml. of Lee virus, diluted 10^{-1} in culture medium was added to give $10^{7.8}$ EID_{50} per ml. in the final mixture. Controls were similar in all respects except that pieces of membrane were omitted.

TABLE VII

Lack of Effect of DRB on Adsorption of Lee Virus by Membrane in Vitro

Membrane	Incubation		Centrifugation	Hemagglutination titer*	
				Control medium	DRB‡
gm.	*°C.*	*hrs.*			
None	4	2	None	32	—
"	35	2	Supernatant	32	32
0.4	35	2	"	3	3

* Expressed as the reciprocal of dilution at the endpoint.

‡ 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole, 1.3×10^{-4} M.

As shown in Table VII, DRB did not affect the amount of Lee virus adsorbed by the membrane, nor the hemagglutination titer of virus incubated in the absence of membrane.

6. Persistence of DRB Inhibition.—It was shown previously (1, 3) that, following withdrawal of 2,5-dimethylbenzimidazole from the culture medium, the inhibitory effect of the compound on Lee virus multiplication in membranes *in vitro* disappeared. Experiments were conducted to determine if inhibition caused by DRB was similarly reversible.

Pieces of membrane were suspended in culture medium or in 0.54×10^{-4} DRB in the same medium and inoculated with sufficient Lee virus to give $10^{5.5}$ EID per ml. After incubation for 36 hours, hemagglutination titers were determined on the medium of certain groups of cultures and the membranes were then transferred into fresh medium with or without added DRB. After a total of 69 hours of incubation, the media from all groups were collected and the concentration of virus was measured by the hemagglutination technique.

The results of a representative experiment are given in Table VIII. The multiplication of Lee virus at 36 hours in the DRB-treated cultures was re-

stricted to about 4 per cent of the control value. At 69 hours, similar degrees of inhibition were found in groups 3 and 4, indicating that the process of multiplication remained inhibited even when membranes were transferred at 36 hours to fresh media not containing DRB. Thorough washing of DRB-treated membranes with fresh media at the time of transfer had no effect on persistence of inhibition. In similar experiments with 2,5-dimethylbenzimidazole, 19 $\times 10^{-4}$ M, the concentration of virus was low in the treated group at the earlier time period and reached a maximal level comparable to that of controls after further incubation in the absence of the compound. This confirms the results obtained previously with the 2,5-dimethyl compound (1, 3).

Persistence of inhibition after withdrawal of DRB was investigated further. The effect of pretreatment of membranes with the compound followed by steps

TABLE VIII

Persistence of Inhibition of Lee Virus Multiplication by DRB upon Withdrawal of the Compound

Culture group	DRB* in culture medium		Membranes transferred to fresh medium at 36 hrs.	Hemagglutination titer‡ of medium		
				36 hrs.		69 hrs.
	0–36 hrs.	37–69 hrs.			Per cent of control	
1	0	0	−	—	—	79
2	0	0	+	75	100	21
3	+	+	+	3.2	4.2	3.2
4	+	0	+	3.4	4.5	5.0

* 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole, 0.54 $\times 10^{-4}$ M.

‡ Expressed as the reciprocal of dilution at endpoint.

to facilitate removal of the substance from the membranes prior to inoculation of virus was studied.

Pieces of membrane were incubated for varying periods of time in the presence of DRB. Control membranes were similarly incubated in the absence of the compound. Thereafter, the membranes were washed, transferred into fresh medium not containing DRB, and incubated for 4 hours to allow the compound to diffuse into the medium. The membranes were again washed and transferred into the final media. All cultures then were inoculated with Lee virus, $10^{5.5}$ EID_{50} per ml. After 36 hours of incubation, the virus content of the medium was determined by the hemagglutination technique.

Table IX gives the results of two such experiments. Membranes pretreated with DRB for 3 hours subsequently supported virus multiplication only about 29 per cent as well as control membranes. Membranes pretreated with the substance for 32 hours yielded only about 8 per cent as much virus as controls. In both experiments, membranes which were not pretreated with the compound but were incubated with DRB after inoculation showed inhibition of

greater degree than did pretreated membranes. However, it appears clear that pretreatment of membranes and procedures designed to remove the compound left the membranes in a state in which they were unable to support maximal virus multiplication.

In other experiments, membranes were pretreated with DRB in a similar manner and then, after thorough washing, were incubated and shaken in fresh medium in the absence of the compound for as long as 12 hours. When inoculated with Lee virus and incubated in fresh medium, such membranes still showed definite restriction in their capacity to support multiplication of the agent.

TABLE IX

Effect of Pretreatment of Membranes and Removal of DRB on Multiplication of Lee Virus

Experiment	Culture group	Pretreatment with DRB*		Postinoculation DRB		Hemagglutination titer of medium‡	
		Concentration	Hrs.	Concentration	Hrs.		Per cent of control
		$M \times 10^{-4}$		$M \times 10^{-4}$			
I	1	None	0–3	None	7–43	84	100
	2	0.73	0–3	"	7–43	24	29
	3	None	0–3	0.73	7–43	<2	<3
II	1	None	0–32	None	36–72	141	100
	2	0.99	0–32	"	30–72	11	8
	3	None	0–32	0.99	36–72	<2	<2

* After this period all membranes were washed, transferred to fresh medium containing no DRB, and incubated for 4 hours. Then they were again washed and transferred to fresh media before inoculation.

‡ Determined 36 hours after inoculation with Lee virus.

7. *Effect of DRB on Oxygen Uptake of Membranes.*—The effects of DRB and 2,5-dimethylbenzimidazole on oxygen consumption of the membrane were determined.

Oxygen uptake was measured by the direct method in the Warburg apparatus. Each flask contained a weighed quantity, ranging from 70 to 130 mg., of membrane from 10- or 11-day old embryonated eggs and 2 ml. of culture medium. In the center well, 0.2 ml. of 10 per cent KOH and a rolled piece of filter paper were present. The flasks were shaken 125 times per minute employing a stroke length of 2 cm. Two flasks were employed per variable in each experiment. The temperature was 35°C. Oxygen consumption was expressed in μl. of oxygen taken up in 1 hour per 100 mg. of tissue.

At a concentration of 0.72×10^{-4} M, DRB caused 99.9 per cent inhibition of Lee virus multiplication as measured by infectivity titrations at 15.5 hours, *i.e.*, at the end of the first multiplication cycle (3). It was shown previously

(3) that, in the presence of 26×10^{-4} M 2,5-dimethylbenzimidazole, multiplication was restricted to 1.5 per cent of the control value as measured at 15.5 hours. That 2,5-dimethylbenzimidazole had no effect on oxygen consumption of the membranes when used at this concentration (1) was confirmed (*cf.* Table X). However, in the presence of 44×10^{-4} M, oxygen uptake was markedly reduced. This concentration is 3.3 times the concentration which causes 75 per cent inhibition as measured by hemagglutination titrations on the culture medium after 36 hours of incubation (4). When DRB was employed at 2.2×10^{-4} M, *i.e.* 5.8 times the inhibitory concentration, no effect on oxygen

TABLE X

Effect of Benzimidazole Derivatives on Oxygen Consumption of Chorioallantoic Membranes in Vitro

Experiment No.	Benzimidazole derivative		Oxygen consumption*		
			Hrs.		
		Concentration	0–1	4–5	20–21
		M × 10^{-4}			
1	None	—	27.2	22.7	14.4
	2,5-Dimethyl	26	26.1	25.2	14.4
2	None	—	27.4	24.6	17.0
	2,5-Dimethyl	44	7.7	2.5	0.0
3	None	—	21.2	17.8	16.6
	DRB	2.2	28.2	19.7	15.5
4	None	—	28.6	26.2	16.8
	DRB	3.3	26.2	18.6	7.6

* μl. oxygen per 100 mg. of membrane in 1 hour. Each figure represents the mean of two determinations.

consumption was observed. With 3.3×10^{-4} M, *i.e.* 8.7 times the inhibitory concentration, a moderate reduction in oxygen uptake was demonstrable. Thus, in terms of molar concentration, much less DRB than 2,5-dimethylbenzimidazole is needed to cause a reduction in oxygen uptake, but, in terms of inhibition activity, a considerably larger amount of DRB is required before oxygen uptake is reduced. It should be emphasized again that, at appropriate concentration, both compounds cause marked inhibition of virus multiplication without affecting tissue respiration.

It is known (21, 22) that influenza virus multiplication requires oxygen. That the compounds used are capable of causing marked inhibition of virus multiplication without affecting oxygen uptake indicates that such inhibition is not brought about through interference with oxidative energy-yielding re-

actions. The results obtained suggest that DRB has a relatively greater specificity of action than 2,5-dimethylbenzimidazole because of the greater differential between concentrations which inhibit virus multiplication and oxygen uptake, respectively.

8. Effect of DRB on Membrane Proliferation.—In this and previous studies (1–4) on the inhibitory activity of benzimidazole derivatives on influenza virus multiplication, pieces of membrane were suspended in culture medium and shaken in an incubator. Under these conditions, almost no proliferation of the cells of the membrane can be demonstrated microscopically. Although not growing, membranes under such conditions metabolize actively as is shown by their oxygen uptake. To learn whether membrane proliferation was inhibited by benzimidazole derivatives, roller tube cultures were employed.

Small pieces of chorioallantoic membrane, 1 to 1.5 mm. in diameter, were placed along the inner walls of clean test tubes, 15 x 150 mm. Ten pieces were used per tube. The pieces were placed in position with a platinum spatula and a minimal amount of culture medium. The tubes were held at room temperature for 15 to 30 minutes at an angle of 45°, so that the tissue pieces would adhere to the glass. Only the chemically defined culture fluid, used for membrane cultures (1, 3), was employed and each tube received 1.5 ml. The tubes were tightly stoppered, placed in a roller tube apparatus maintained at 37°C., and rotated at 8 to 10 revolutions per hour. The growth of the tissue pieces was followed by repeated observation.

Under the conditions described above, proliferating cell areas surrounding the pieces of membrane reached maximal size in approximately 3 days. Two types of proliferation were observed: (1) a monocellular layer consisting of what appeared to be flat polygonal cells, and (2) cyst-like structures surrounded by flat cells and often traversed by spindle-shaped cells. The latter type of proliferation appeared earlier than the former and frequently the cyst-like structures grew out from areas of membrane containing relatively large blood vessels. Cyst-like structures occurred less frequently than monocellular layers and pieces of membrane which showed multiple cyst-like structures exhibited reduced proliferation of the monocellular layer type.

The effects of DRB and 2,5-dimethylbenzimidazole on membrane proliferation were determined. In the presence of 0.54×10^{-4} M DRB which causes approximately 99 per cent inhibition of Lee virus multiplication, proliferation of cyst-like structures was unaffected, but the rate of monocellular layer proliferation was somewhat reduced. However, in the presence of 19×10^{-4} M 2,5-dimethylbenzimidazole, which also causes approximately 99 per cent inhibition of Lee virus multiplication, proliferation by cyst-like formation was considerably restricted and only a slight degree of outgrowth of cells in a monocellular layer took place. Thus, when employed at equivalent virus inhibitory concentration, DRB showed only a slight effect on membrane proliferation, whereas 2,5-dimethylbenzimidazole caused marked restriction of growth.

In view of the results obtained in experiments on persistence of the inhibitory effect of DRB and 2,5-dimethylbenzimidazole on Lee virus reproduction, it was important to determine whether the effect of these compounds on tissue proliferation was reversible.

Tissue culture experiments were carried out employing 0.73×10^{-4} M DRB and 26×10^{-4} M 2,5-dimethylbenzimidazole as supplements. At these concentrations the inhibitory effects of the compounds on Lee virus multiplication are closely similar. After a 48 hour period of incubation, the media containing supplements were replaced with fresh media free of the compounds. Control cultures were incubated with or without supplement during the entire experimental period. At 96 hours, the fluids present were exchanged for fresh fluids of the same kind. Two tissue culture tubes were used per variable.

TABLE XI

Reversibility of Inhibition of Tissue Proliferation by DRB or 2,5-Dimethylbenzimidazole upon Withdrawal of the Compound

Culture group	Supplement in culture medium		Tissue proliferation*				
	Hrs.		Hrs. of incubation				
	0–48	49–96	24	48	72	96	120
1	None	None	+	++	+++	++++	++++
2	DRB‡	DRB‡	0	±	+	++	+++
3	DRB‡	None	0	±	+++	++++	++++
4	2,5-Dimethyl§	2,5-Dimethyl§	0	0	0	0	0
5	2,5-Dimethyl§	None	0	0	+	++	+++

* Degree of proliferation in monocellular layer estimated from 0 to ++++.

‡ 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole, 0.73×10^{-4} M.

§ 2,5-Dimethylbenzimidazole, 26×10^{-4} M.

Table XI shows that proliferation of tissues in the presence of 0.73×10^{-4} M DRB was retarded. However, upon removal of the compound, proliferation rapidly reached the degree shown by cultures incubated in the absence of the compound. 2,5-Dimethylbenzimidazole, 26×10^{-4} M, suppressed tissue proliferation throughout the period of observation. Subsequent to removal of the compound at 48 hours, proliferation of tissue cells began and continued for 3 days. Thus, both compounds were capable of causing reversible restriction of tissue proliferation when used at appropriate concentrations. Of the two, DRB showed a smaller effect on tissue proliferation in relation to virus inhibitory activity than did 2,5-dimethylbenzimidazole. On the other hand, it should be emphasized that inhibition of virus multiplication disappeared promptly on withdrawal of the 2,5-dimethyl compound but not on withdrawal of DRB.

Inhibition of PR8, MB, and FM1 Virus Multiplication by DRB.—Most of the experiments on the inhibitory activity of benzimidazole derivatives, both earlier (1–4) and present, were carried out with the Lee strain of influenza B

virus. To ascertain whether the multiplication of other influenza viruses was inhibited in a closely comparable manner by such compounds, experiments were conducted with the PR8 and FM1 strains of influenza A virus and with the MB strain of influenza B virus.

Suitable seeds for each strain were prepared as described above. With $10^{5.5}$ EID_{50} of virus per ml. in the inoculum, hemagglutination titer–time curves were determined in membrane cultures with the three strains of influenza virus. As shown in Fig. 2, the yield of PR8 virus appeared to be approximately two-fold higher than that of the other viruses. However, there was no indication of a significant difference in the time when maximal titers were reached in the

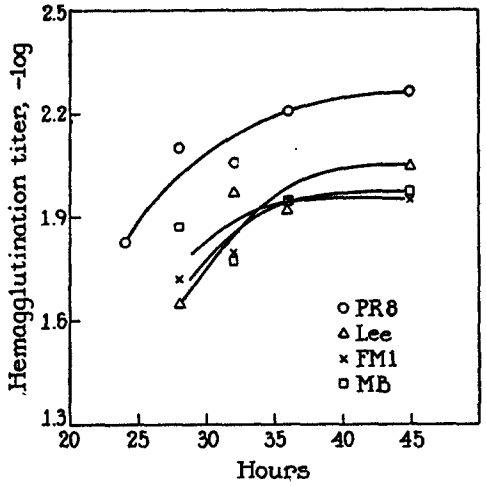


FIG. 2. Hemagglutination titer–time curves of Lee, PR8, FM1, and MB viruses in the media of chorioallantoic membrane cultures *in vitro*. Initial EI_{50} titer of each virus in the medium, computed to 1 ml., was $10^{-5.5}$.

medium. In order to assure adequate time for multiplication and release when inhibitory compounds were present, the 45 rather than 36 hour time interval was chosen for collection of media. Detailed information regarding the kinetics of multiplication in the presence or absence of inhibitory compounds was available only for Lee virus (2).

The inhibitory effect of 20×10^{-4} M 2,5-dimethylbenzimidazole and of 0.54×10^{-4} M DRB was studied in groups of six membrane cultures. As can be seen in Table XII, there do not appear to be significant differences in the degree to which multiplication of the several influenza viruses was inhibited by the same compound or in the inhibition ratios obtained with the two compounds in respect to the various viruses. Thus, no evidence was obtained that multiplication of representative strains of influenza A or B virus was affected in different ways by these two inhibitory compounds.

Inhibition of Lee Virus Multiplication in Chicken Embryos and Mice.—The fact that a chemical substance shows certain biological activities in *in vitro* cultures does not provide evidence that it will exert similar effects in the living animal. Conditions having to do with transport to the site of action, chemical alteration, and rate of excretion may radically alter the effects of a compound *in vivo*. In view of the high inhibitory activity of DRB on influenza virus multiplication *in vitro*, this compound was used in experiments with chicken embryos and mice. Because of the low solubility of DRB in aqueous media, *i.e.* of the order of 3.5×10^{-4} M, the compound was employed in suspension.

TABLE XII

Extent of Inhibition of Lee, PR8, MB, and FM1 Virus Multiplication by Benzimidazole Derivatives

Benzimidazole derivative	Concentration	Virus: Lee		PR8		MB		FM1	
		Hemagglutination titer of culture medium*							
			Per cent of control		Per cent of control		Per cent of control		Per cent of control
	$M \times 10^{-4}$								
None	—	205	100	183	100	166	100	82	100
2,5-Dimethyl	20	19	9	10	5	32	19	<2	<2
None	—	132	100	193	100	161	100	85	100
DRB	0.54	32	24	58	30	16	10	15	17
Inhibition ratio, 2,5-dimethyl / DRB			0.37		0.17		1.9		<0.12

* Expressed as the reciprocal of dilution. Mean of two experiments, except for MB in which case results of one experiment are recorded.

1. Effect of DRB in Chicken Embryos.—The effect of DRB on Lee virus multiplication in embryonated eggs was determined as follows:—

Groups of 6 eggs, 10 or 11 days old, were injected allantoically with 0.5 ml. of buffered saline or with an homogenized suspension of DRB in buffered saline. The saline contained penicillin, 50 units, and streptomycin, 250 μg. per ml. Then $10^{2.5}$ EID_{50} of Lee virus was inoculated by the same route and the eggs were incubated at 35°C. for 32 hours. The allantoic fluids were harvested and the concentration of virus in each fluid was measured by the hemagglutination technique. The geometric mean titer was computed for each group.

The results of a representative experiment are given in Table XIII. Marked inhibition of Lee virus multiplication was obtained with DRB. However, it is difficult to relate the degree of inhibition obtained *in vivo* to that found in

membrane culture because nothing yet is known about the distribution of the inhibitory compound in the embryonated egg.

2. Effect of DRB in Mice.—The effect of DRB on Lee virus multiplication in mice was determined as follows:—

Swiss mice, 3 to 4 weeks old, were inoculated intranasally under light ether anesthesia with $10^{2.5}$ MS_{50} of Lee virus. Two and 8 hours later, each mouse received by intraperitoneal injection 0.5 ml. of either buffered saline or a suspension of 2 mg. of DRB in buffered saline. Thereafter, similar injections were repeated twice a day. At 48 hours, groups of 6 mice were killed and their lungs were removed. Ten per cent lung suspensions were prepared in saline The concentration of virus was determined by hemagglutination titration after treatment with *Vibrio cholerae* filtrate according to the technique described previously (23).

TABLE XIII

Inhibition of Lee Virus Multiplication by DRB in Intact Embryonated Eggs

DRB	Hemagglutination titer* of allantoic fluid	
		Per cent of control
mg.		
0	565	100
1	183	32
4‡	<16	<2.8

Inoculum: Lee virus, $10^{2.5}$ EI_{50}.

* Expressed as the reciprocal of dilution at endpoint.

‡ Undissolved 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole was present in the allantoic cavity at the end of the experiment.

TABLE XIV

Inhibition of Lee Virus Multiplication by DRB in the Lungs of Mice

DRB	Hemagglutination titer* of lung suspension	
		Per cent of control
mg./mouse/day		
0	1770	100
4	160	9

Inoculum: Lee virus, $10^{2.5}$ MS_{50}.

* Expressed as the reciprocal of dilution at endpoint.

Table XIV gives the results of a representative experiment. DRB caused a definite inhibition of Lee virus multiplication in the mouse lung. As in the case of experiments in the intact egg, a quantitative evaluation of the inhibitory activity of DRB in the mouse was not feasible.

Toxicity of DRB for Chicken Embryos and Mice.—Neither chicken embryos nor mice showed ill effects from injections of as much as 4 mg. of DRB except

for transient drowsiness and roughing of fur exhibited by mice. No deaths were observed among chicken embryos at 44 hours after they had been injected allantoically with 4 mg. of the compound at the beginning of the experiment and again at 20 hours. No deaths were observed among mice injected intraperitoneally twice daily for 8 days with 2 mg. of the compound, *i.e.*, a total of 32 mg. When examined 2.5 months later, the mice appeared perfectly healthy. Among 12 such mice, two pregnancies occurred subsequently and the offspring, 7 and 5 in number respectively, appeared normal.

DISCUSSION

The fact that 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) is the most active inhibitor of influenza virus multiplication among the benzimidazole derivatives studied invites an analysis of structure-activity relationships in the light of present biochemical knowledge. It appears clear that the high inhibitory activity of DRB is dependent both on the chloro and ribofuranosyl substituents present in the molecule. Chloro derivatives not containing pentose moieties are considerably more active than their methyl counterparts. In this connection, the growth inhibitory activity of 5,6-substituted benzimidazoles against *Lactobacillus leichmannii* 313 has been reported (15) to depend on the nature of the substituent groups in the following manner: $CH_3 < Br < Cl$. With a ribofuranosyl group at position 1, the presence of two chlorine atoms rather than one in the benzene ring increases inhibitory activity against influenza virus markedly. The role of chloro substituents is further underlined by the finding that for the ribofuranoside, the corresponding 5,6-dimethyl derivative is inactive as an inhibitor. Unless the latter compound is altered in membrane cultures and rendered inactive, this finding raises the possibility that 5,6-dimethyl-1-α-D-ribofuranosylbenzimidazole, the moiety in vitamin B_{12}, may be concerned in the process of influenza virus multiplication.

Thus, relative to inhibitory activity, the importance of an unnatural benzimidazole nucleus, particularly in respect to the benzene ring, seems clear. On the other hand, it should be emphasized that the high activity of DRB owes much to the presence of a ribofuranose moiety as the chloro-ribofuranosylbenzimidazoles are considerably more active than the corresponding chloro benzimidazoles. Furthermore, with two chlorine atoms present in the benzene ring, the presence of glycosyl groups other than ribofuranosyl results in reduced inhibitory activity. The inhibitory activity of DRB on influenza virus multiplication places it among the most active chemical compounds found to exhibit such activity (24).

It has been reported (15) that the growth inhibiting activity of benzimidazole, or of the 5,6-substituted methyl, bromo, or chloro derivatives against *Lactobacillus leichmannii* 313 is not found for the D-glucoside, and that the activity of the 5,6-dimethyl compound is absent in the D-xyloside. Thus, there appears

to be a similar effect on inhibitory activity against both *Lactobacillus leichmannii* and influenza virus when 5,6-dimethylbenzimidazole is converted into a glycoside. It should be emphasized that conversion of 5,6-dichlorobenzimidazole into a glycoside is also accompanied by lowered inhibitory activity against influenza virus multiplication, except for the ribopyranoside or the ribofuranoside. For the ribopyranoside, there is no change in inhibitory activity, whereas, for ribofuranoside, there is a considerable increase.

The structural formulae of α-ribazole, adenosine, and DRB shown in Fig. 1 show the close chemical relationship between these compounds. Evidence is accumulating that there is a close metabolic relationship between vitamin B_{12} and nucleosides; however, the details of the role these compounds play with respect to each other are not yet clear. The results presented in this report are in agreement with the hypothesis that DRB causes inhibition of influenza virus reproduction through interfering with metabolic processes involving ribofuranosides; indeed, it seems not improbable that nucleic acid metabolism is affected.

The role of nucleic acids in the process of reproduction of the several virus categories may not be identical in every instance. Thus, plant viruses appear to contain RNA but no DNA, animal viruses apparently may contain either DNA, RNA, or both, while bacterial and insect viruses are thought to contain DNA but no RNA. Such diversity in nucleic acid composition of these agents suggests that the relative roles of host cell and virus nucleic acids in the process of virus multiplication may be widely different in dissimilar host categories. It seems probable that the susceptibility of a host to a particular virus is ultimately determined by DNA. However, the possibility exists that, in the biosynthetic processes concerned with virus multiplication, RNA also may play an important, even a specific role. With animal viruses, it may prove more fruitful at this stage to attempt the elucidation of the role of RNA rather than DNA. Consideration of the available information concerning virus reproduction and RNA provides support for this idea.

Earlier work (25–27), including microscopic observation combined with spectrophotometric and cytochemical measurements, revealed that after infection of cells by certain animal viruses nucleoli may show increased size and increased RNA concentration, and that RNA in the cytoplasm may also increase. Studies on uninfected animal tissues have shown that RNA is present both in the nucleus and cytoplasm of cells (28); in the nucleus, the highest concentration is found in the nucleolus (29); in the cytoplasm, the concentration is high in microsomes and in mitochondria (30). RNA from nucleoli are chemically different from those in cytoplasm and their turnover is higher in the nucleus than in the cytoplasm (31–40). Chemical differences exist among RNA from different cytoplasmic fractions (33). It may be that RNA show specific features both as to location in the cell and chemical structure. That animal and plant viruses show preferences with respect to their sites of intracellular multiplication is well known. Furthermore, RNA from dissimilar plant viruses are chemically different, whereas those from closely related virus strains are similar (41, 42).

Such data provide some support for the hypothesis that RNA may play an im-

portant role in virus multiplication. If this idea has any validity, then it seems possible that in some host-virus systems the RNA of the host might play a role not less important than the RNA of the virus particle. Unfortunately, little is known about the synthesis of RNA or the intermediary metabolism of ribofuranosides, and present knowledge of the role of RNA in cellular metabolism is limited. It has been found (43, 44) that a constant correlation exists between a high content of RNA and rapid protein formation. Results of turnover studies with N^{15} are in agreement with the concept that RNA play some role in protein metabolism (45).

Several features of the effects of DRB on influenza virus multiplication require comment. Because the compound does not inactivate extracellular virus, does not interfere with adsorption of the virus by host tissue, and does not prevent release of newly formed virus from host cells, it seems probable that the site of action is intracellular. The fact that the process of virus multiplication is considerably more sensitive to the inhibitory effect of the compound than is host tissue respiration indicates that oxidative energy-yielding reactions are not involved in the mechanism of inhibition by DRB. This suggests that the compound possesses some specificity of action with respect to the process of influenza virus reproduction. Comparison of the results obtained with DRB and 2,5-dimethylbenzimidazole indicates that the degree of such specificity of action is greater with the former compound. With the latter substance, the differential between the concentrations at which the compound inhibits virus multiplication and tissue respiration was narrower. Similar relationships were observed with respect to tissue proliferation, except that the effects of the two compounds on tissue proliferation and virus multiplication were more nearly related.

The fact that, at sufficient concentration, DRB has a retarding effect on proliferation of membrane cells *in vitro* may reflect a reduction in the rate of processes involved in tissue growth rather than a damaging effect resulting from irreversible changes in the biosynthetic machinery of the cells. This view is strengthened by the demonstration that the cells promptly start multiplying when the compound is withdrawn and continue to multiply to a normal extent. That the action of 2,5-dimethyl-benzimidazole is of a similar type appears likely.

Although the responses of the host tissue relative to respiration and reversibility of inhibition of virus multiplication are characterized by features which suggest differences in the mechanism of action of DRB and 2,5-dimethylbenzimidazole, the quantitative effect of the two compounds on the multiplication of several influenza A and B virus strains was rather uniform. In each case, DRB was approximately 35 times more active as an inhibitor. Furthermore, at constant concentration, each of the compounds inhibited the multiplication of the various strains to a similar extent.

That DRB inhibits influenza virus multiplication in the embryonated egg

and the mouse is of interest mainly because only a few chemical compounds have been reported to produce this result without causing serious effects in the living host.

SUMMARY

Chloro derivatives of benzimidazole were found to be 2 to 3 times more active than corresponding methyl derivatives in causing inhibition of Lee virus multiplication in chorioallantoic membrane cultures *in vitro*. The most active benzimidazole derivative thus far tested is 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB); it caused 75 per cent inhibition of Lee virus multiplication in membrane cultures at a concentration of 0.38×10^{-4} M. On the other hand, 5,6-dimethyl-1-α-D-ribofuranosylbenzimidazole, the moiety present in vitamin B_{12}, failed to inhibit Lee virus multiplication at a concentration of 35×10^{-4} M. Other *N*-glycosides of 5,6-dichlorobenzimidazole were considerably less active than DRB.

In single cycle experiments, the degree of inhibition of Lee virus multiplication by DRB in membrane cultures was not dependent on the amount of virus in the inoculum. This compound did not inactivate the infectivity of extracellular Lee virus, had no effect on virus-erythrocyte interaction, did not interfere with the adsorption of the virus by the host tissue, nor affect the release of newly formed virus from the membrane. The inhibitory effect of DRB on Lee virus multiplication, in contrast to that of 2,5-dimethylbenzimidazole, persisted after transfer of infected membranes into fresh culture medium not containing the compound. Both DRB and the 2,5-dimethyl compound caused 99 per cent inhibition of Lee virus multiplication without affecting oxygen uptake of the membrane. Tissue proliferation of membrane pieces in roller tube culture was not significantly affected by DRB at inhibitory concentration, whereas at equivalent concentration the 2,5-dimethyl compound did restrict cellular growth. At higher concentrations, both compounds caused retardation of cell proliferation. This effect was reversible on removal of either compound from the medium. The multiplication of several strains of influenza A and B viruses, *i.e.* Lee, MB, PR8, and FM1, was inhibited to the same degree by each of the two compounds; DRB was 35 times more active than the 2,5-dimethyl compound relative to each of the strains.

DRB caused inhibition of Lee virus multiplication in intact embryonated chicken eggs and in mice without causing significant signs of toxicity in either host. Some of the implications of these findings are discussed in relation to the mechanism of the inhibition of influenza virus multiplication.

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