INFLUENZA VIRUS MULTIPLICATION IN THE CHORIOALLANTOIC MEMBRANE IN VITRO: KINETIC ASPECTS OF INHIBITION BY 5, 6-DICHLORO-1-β-D-RIBOFURANOSYL-BENZIMIDAZOLE

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Previous studies on the kinetics of inhibition of influenza virus multiplication by 2,5-dimethylbenzimidazole[1] (designated MB) revealed (1) that this compound prolongs the latent or plateau period and reduces the yield of virus. Furthermore, it appeared (1) that MB interferes with the process of virus multiplication at two stages—early in the latent period and just prior to the emergence of infective virus particles. Studies of glycosides of benzimidazoles have led to the synthesis (2) of a derivative, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazole[1] (designated DRB), which is 35 times more active (3) than the 2,5-dimethyl compound (1). Comparative studies with these compounds with respect to inhibition of influenza virus multiplication, oxygen uptake by the chorioallantoic membrane, and tissue proliferation *in vitro* indicated (3) that DRB is more selective in its inhibitory action on virus multiplication than is MB. In the comparative kinetic studies, which are reported below, independent evidence was obtained supporting this conclusion. In these experiments, the effect of DRB and of MB on the production of virus and of soluble complement-fixing antigen in the chorioallantoic membrane was studied *in vitro*. Release of virus from the membrane was examined also. These studies were facilitated by the adoption of experimental procedures which permitted measurement of virus concentration by the hemagglutination technique during early phases of multiplication. To secure a better understanding of virus-host tissue interaction in the system employed, adsorption of virus onto and yield from the two surfaces of the freely suspended chorioallantoic membrane were studied. The recoverability of the inoculated virus and the production of infective and non-infective hemagglutinating virus were also examined.

Materials and Methods

Culture Medium.—The medium employed has been described previously (4, 5) and contained only inorganic salts and glucose.

[1] The benzimidazole derivatives were obtained through the kindness of Dr. Karl Folkers and Dr. Clifford H. Shunk of the Research Laboratories, Chemical Division, Merck & Company, Inc., Rahway, New Jersey.

Membranes.—The modified procedure (3) for obtaining pieces of chorioallantoic membranes was used.

Membrane Surface Area and Weight.—The mean surface area of the portions of chorioallantoic membrane used was 2.9 $cm.^2$, and the mean wet weight was 20.2 mg.

Viruses.—The Lee virus seed employed differed from that described in an earlier communication (5) in that it was prepared by inoculation at a dilution of 10^{-4} and incubating the embryonated eggs for 30 hours. The seed virus preparation contained $10^{9.43}$ EID_{50} per ml. as determined by the procedure for infectivity titrations described earlier (3). It has been found recently (6) that with Lee virus, incubation of eggs for 72 hours rather than 48 hours gives infectivity titers which are 0.56 log unit higher, and therefore it was computed that the seed virus employed contained $10^{9.99}$ EID_{50} per ml.

Culture Procedure.—The method used has been described (3). To eliminate unadsorbed virus, membranes were washed thoroughly and transferred into fresh medium 1 hour after inoculation. After incubation, the membranes were removed from the medium, pooled in groups, homogenized by grinding with alundum, and taken up in 1.2 ml. of 0.85 per cent NaCl per group of 6 membrane cultures. The suspensions were treated with an equal volume of *Vibrio cholerae* filtrate for 2 hours at 37°C. and centrifuged at 2,000 *g* for 10 minutes. Hemagglutination titrations were carried out on supernatants. For soluble complement-fixing antigen determination, the suspensions were not treated with *V. cholerae* filtrate and were centrifuged at 76,000 *g* for 30 minutes. Such supernatants contained less than 1 per cent of the virus and all of the soluble complement-fixing antigen originally present in the homogenate. Culture media also were pooled in groups, and hemagglutination titrations were performed. Soluble complement-fixing antigen was not measured in the media because early experiments showed that this antigen was not present in detectable amount in the media.

Hemagglutination Titrations.—Test tubes with exactly hemispherical bottoms and an internal diameter of 10 mm. were used. In most titrations serial dilutions of membrane extract or culture medium were made in 2.5 per cent sodium citrate in 0.5 ml. volumes. To each dilution, 0.5 ml. of 0.09 per cent chicken RBC suspension was added. The tubes were held at 21°C. for 2.5 hours. In some titrations, the diluent was 0.85 per cent sodium chloride buffered with 0.01 M phosphate, the concentration of RBC was higher, and the time allowed for settling of RBC was shorter. A strong partial agglutination pattern was taken as the end point. All titrations were carried out in duplicate. It was considered that at the end point one hemagglutinating unit was present in the reaction volume of 1 ml. The volume of pooled media per group was 6 ml. and that of the membrane homogenate was 1.4 ml. The total amount of virus present was computed on this basis.

Soluble Complement-Fixing Antigen Titrations.—Tests were carried out in veronal buffered saline with optimal concentrations of Ca^{++} and Mg^{++} ions (7). Serial dilutions of antigen were titrated against constant amounts (2 to 4 times more than optimal titer) of pooled human influenza B convalescent serum. The final volume was 0.5 ml. 0.1 ml. of antigen, 0.1 ml. of complement (2½ full units), and 0.1 ml. of serum were incubated at 37°C. for 1 hour. 0.2 ml. of 1 per cent suspension of sensitized sheep RBC was added, and incubation was continued for 30 minutes. Tests were read after standing overnight at 4°C. The end point was taken as that dilution, as judged by inspection, at which 50 per cent hemolysis occurred. The usual controls were included.

Infectivity Titrations.—The procedure used was described in an earlier report (3). The infectivity end points were multiplied by a correction factor of $10^{0.56}$ (6). In those titrations in which a 72 hour period of incubation was employed no correction factor was used.

EXPERIMENTAL

Use of the Hemagglutination Technique in Kinetic Experiments.—In earlier studies on kinetic aspects of inhibition of Lee virus multiplication by 2,5-

dimethylbenzimidazole (1) virus concentration was measured by infectivity titrations. The usefulness of this highly sensitive method is impaired by the instability of the infective property and the laboriousness of the procedure. Therefore, the possibility of employing the hemagglutination technique for the measurement of virus concentration in such experiments was explored. It was found that by increasing the amount of virus in the inoculum and by reducing the concentration of RBC in hemagglutination tests it was possible to measure virus during the early phases of multiplication. Experiments showed that a final concentration of 0.045 per cent RBC, though not 0.023 per cent, permitted highly reproducible titrations of virus.

It is well known that inoculation into embryonated eggs of large amounts of PR8 virus results in a yield containing a low proportion of infective to non-infective hemagglutinating particles (8–11). Furthermore, with very large inocula the rate of increase and the total yield of virus particles may be reduced (11). The available evidence indicates that Lee virus behaves similarly (8, 10, 12).

Experiments were carried out to determine whether such effects were operative in the membrane culture system using $10^{7.66}$ EID_{50} of Lee virus per 2.9 cm.^{2} of chorioallantoic membrane. The amount of virus determined by the hemagglutination technique (0.045 per cent RBC) was $10^{1.8}$ units. On the basis of adsorption studies reported below 27 per cent of virus was adsorbed onto the membrane under these conditions, *i.e.* 1.2×10^7 EID_{50}, and the amounts adsorbed onto the allantoic and chorionic surfaces were similar. In earlier communications (1, 5) the number of allantoic cells in the portions of membrane was computed on the basis of previous estimates (13) of the number of such cells in the entire membrane. Work reported recently (11) suggested that previous estimates may have been too large. Direct counts under the phase contrast microscope (14) substantiated this view. On the basis that there are 1.66×10^5 allantoic cells per cm.^{2} of the chorioallantoic membrane (14), the number of allantoic cells used in the present study was estimated to be 4.82×10^5 per culture. Thus, there were 18 EID_{50} of Lee virus per allantoic cell. The number of chorionic cells was not determined in the study (14) referred to above because an extensive capillary network interfered with the visualization of such cells in the membrane from 10 day eggs. However, the chorionic cells were clearly visualized in the membrane from 7 day eggs and the surface area of these cells resembled that of allantoic cells from 10 day eggs. Although the ratio of EID_{50} of virus per chorionic cell cannot be computed on the basis of this evidence, it appears highly probable that it was considerably in excess of 1.

After virus was inoculated, the membranes were washed 1 hour later to remove unadsorbed virus and transferred into fresh medium. Groups of cultures were harvested 1 to 15.5 hours after inoculation. The membranes were ground and resuspended in the media from which they came, except for the 1st hour membranes which were suspended in fresh medium. Aliquots were centrifuged at 2,000 *g* for 10 minutes and stored at −60°C. for infectivity titrations. The remainder was treated with *V. cholerae* filtrate at 37°C. for 2 hours, centrifuged,

and the concentration of virus was measured by the hemagglutination technique employing 0.045 per cent final concentration of RBC.

In Fig. 1 are summarized the results of a representative experiment. The total amount of virus in EID_{50} or hemagglutinating units per group of 6 cultures is plotted against time. The ordinates were adjusted on the basis of the ratio of infectivity to hemagglutination titer (EID_{50}/HA) in the inoculum, *i.e.* $10^{5.86}$. The assumption is not made that in the inoculum the ratio of infective

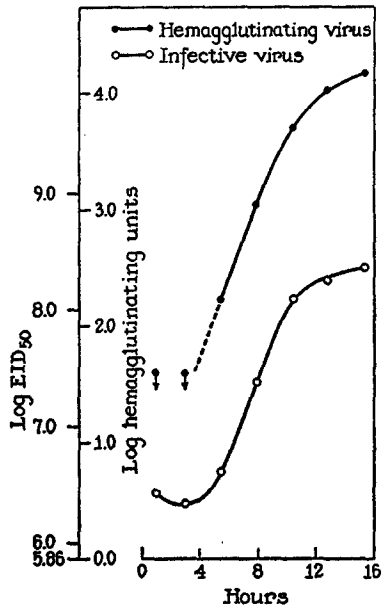

FIG. 1. Multiplication of Lee virus in the chorioallantoic membrane *in vitro*. The total amount of virus inoculated per group of 6 cultures was $10^{8.42}$ EID_{50} or $10^{2.56}$ hemagglutinating units. Final concentration of erythrocytes in hemagglutination titrations was 0.045 per cent. Total amount of virus per group of 6 cultures (membranes + media) is plotted against time. Arrows indicate that the amount of virus present was less than is shown.

to hemagglutinating particles was 1. During the plateau period the mean amount of infective virus was $10^{6.89}$ EID_{50} per group. Since $10^{8.44}$ EID_{50} of Lee virus was inoculated per group at 0 hour, it follows that the amount of virus recovered on or in the membranes during the latent period represented but 0.9 per cent of virus inoculated. No virus could be detected by the hemagglutination technique at 1 and 3 hours. As can be seen, first evidence of a rise in concentration of virus was observed at 5.5 hours, as determined by either infectivity or hemagglutination measurements. Between 5.5 and 10.5 hours the concentration of virus increased logarithmically, and the slopes of the infectivity and hemagglutination curves were identical. However, the EID_{50}/HA ratio was

less than that of the inoculum during the period of logarithmic rise and subsequently tended to become even lower. This finding probably indicates that not all of the hemagglutinating virus which emerged was infective. Comparison of the rate of appearance of infective or hemagglutinating virus during the logarithmic phase after this large inoculum with the rate of appearance of infective virus when the inoculum was only 1/20 as large (1) showed that the two rates also were identical. It appears that in the present system, within the first 10.5 hours, the rate of production of virus particles was not diminished despite the large inoculum but that, among the hemagglutinating particles produced, not all were infective.

Adsorption of Lee Virus by the Chorioallantoic Membrane in Vitro.—Although some information is available with regard to adsorption of influenza virus by the allantoic cells of the chorioallantoic membrane *in vivo* (9, 11, 15), data on adsorption of virus by the membrane *in vitro* are lacking. It was of interest to determine first how much virus was adsorbed when the amounts of virus and membrane were varied, and second the degree of adsorption under the conditions employed in the experiments on the kinetics of multiplication reported below.

Weighed quantities of chorioallantoic membrane from 10 day old embryonated eggs were suspended in nutrient medium (4, 5) and suitably diluted Lee virus was added. The reaction volume was constant at 2 ml. and adsorption was permitted to continue for 1 hour at 35°C. with continuous shaking (5). Then the mixtures were centrifuged at 1,000 *g* for 10 minutes to sediment the membranes. The supernatants were collected, and the concentration of virus was determined by the hemagglutination technique.

The results of two such experiments are summarized in Fig. 2. As can be seen, there is a linear relationship between the logarithm of unadsorbed virus and the amount of membrane used. It is also evident that the slope of the line was steeper with decreasing amounts of virus, indicating that with less virus more complete adsorption occurred. However, the absolute amount of virus adsorbed was highest with the largest amount of virus added. Appropriate controls showed that, under the experimental conditions employed, no demonstrable inactivation of the hemagglutinating ability of virus particles occurred, that no significant amounts of virus were eliminated in centrifugation, and that no demonstrable inhibition of virus hemagglutination occurred due to the presence of components such as serum released from the membrane during incubation. Thus the observation that with decreasing amounts of virus in the inoculum an increasing proportion was removed from the medium was probably solely due to adsorption of virus by the membrane. Since the data show that the absolute and relative amounts of virus adsorbed by the membrane vary in this way both with membrane mass and virus concentration, it seems that the reaction behaves like an equilibrium reaction. However, it was impossible to fit all the data exactly to simple mass action equations including those derived for adsorption of bacteriophage (16).

Amount of Virus Adsorbed in Kinetic Experiments.—In kinetic experiments reported below $10^{7.66}$ EID_{50} of Lee virus per culture was added, and the average amount of membrane in each was 20.2 mg. On the basis of the results given in Fig. 2, it was expected that 20.2 mg. of membrane would adsorb less than 50 per cent of the inoculum and that, therefore, the reduction in concentration of virus in the medium could not be satisfactorily determined by conventional techniques.To circumvent this difficulty the amount of membrane was varied from

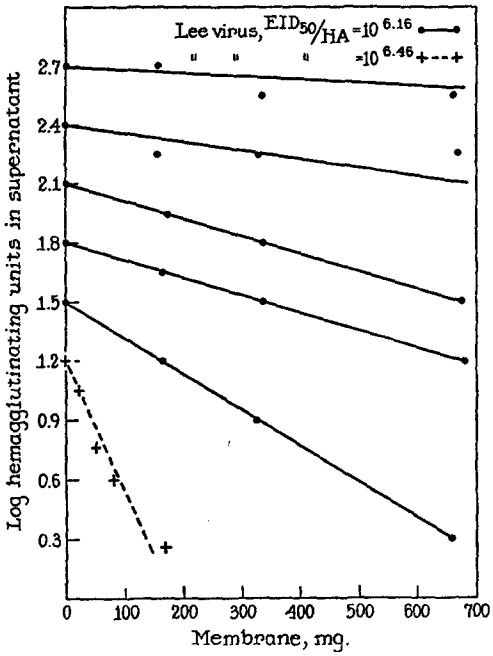

FIG. 2. Adsorption of Lee virus by the chorioallantoic membrane *in vitro* as a function of the amount of virus and membrane used. Time of interaction was 1 hour. Final concentration of erythrocytes in hemagglutination titrations was 0.18 per cent, except in titrations of samples for the lowest curve; in this case the erythrocyte concentration was 0.045 per cent and the titers obtained were divided by 4.

20 to 170 mg. and a curve was obtained relating the amount of inoculated virus adsorbed to the amount of membrane present. The results of one such experiment are given in the lower part of Fig. 2. As can be seen, there is again a linear relationship between the logarithm of unadsorbed virus and the amount of membrane. Computations show that 20.2 mg. of membrane adsorbed 27 per cent of the virus in the inoculum. It should be emphasized that these relationships apply to the total virus present, *i.e.* infective-hemagglutinating + non-infective-hemagglutinating (11), as measured by the hemagglutination technique. Thus, if in this system two virus preparations were employed con-

taining the same number of infective-hemagglutinating particles but different numbers of non-infective-hemagglutinating particles, it may be expected that the proportion of virus particles adsorbed would depend on the total number present.The lower two curves depicted in Fig. 2 were obtained with two different virus preparations characterized as follows: virus used for the upper curve: $EID_{50}/HA = 10^{9.16}/10^{3.0} = 10^{6.16}$; virus used for the lower curve: $EID_{50}/HA = 10^{9.99}/10^{3.53} = 10^{6.46}$. In hemagglutination titrations the concentration of RBC was 0.18 per cent final. In both cases $10^{7.66}$ EID_{50} was used as the inoculum, and therefore the total number of virus particles differed by a factor of two. As can be seen, the proportion of hemagglutinating virus adsorbed was greater in the second case because a smaller total number of virus particles, though not a smaller number of infective particles, was used. If adsorption of infective and non-infective hemagglutinating particles is random, then it would be expected that the proportion of infective particles adsorbed was also greater in the second case.

Adsorption of Lee Virus by the Two Sides of the Chorioallantoic Membrane.—Experiments were carried out to determine the amount of Lee virus adsorbed by each of the two sides of the chorioallantoic membrane, since in cultures used virus had equal access to both sides.

Ten day embryonated eggs were used. The eggs were preselected for uniform weight and for regularity of the chorioallantoic border near the albumin. Entire chorioallantoic membranes were secured from two eggs by a technique developed recently (14) and suspended in buffered saline. The membrane from one egg was turned inside out, whereas the membrane from the other egg was used with the chorionic layer facing outward. Each membrane was folded along its longitudinal axis and the open end was closed tightly with thread. The sacs prepared in this manner contained approximately 1 ml. of saline and were checked for leaks. The sacs were then suspended in nutrient medium in test tubes measuring 25 × 150 mm. 2 ml. (final volume) was used per tube. Lee virus added to the medium had access only to the outside surface of the sacs. The preparations were incubated for 1 hour with continuous shaking. Control fluid was incubated similarly. The concentration of virus in the medium was measured by the hemagglutination technique.

The results of a representative experiment are summarized in Table I. It is apparent that the two surfaces adsorbed virus to a closely similar extent. As can be seen, the allantoic surface adsorbed 75 per cent and the chorionic surface 82 per cent of the virus present in the medium in 1 hour.

Multiplication of Lee Virus in the Allantoic and Chorionic Layers.—The extent of multiplication of Lee virus in the allantoic and chorionic layers was determined. Membranes were prepared and incubated in the manner described above. To eliminate unadsorbed virus, membranes were washed and transferred into fresh medium 1 hour after inoculation. After a 24 hour period of incubation the concentration of virus was determined in the medium by the hemagglutination technique.

The results of two experiments are summarized in Table II. Production of

virus occurred when either the allantoic or the chorionic surface was infected, and no significant difference was found in the mean amount of virus produced in 4 cultures of each kind. The differences observed between pairs of cultures may have been due to variation in the ability of membranes from different eggs to support virus multiplication (5). That the layer facing inward did not liberate virus was established by hemagglutination titrations on the fluid en-

TABLE I

Adsorption of Lee Virus by the Two Surfaces of the Chorioallantoic Membrane

Membrane surface	Virus in medium, hemagglutinating units*	Per cent of control
None	32	100
Allantoic	8	25
Chorionic	5.6	18

* Hemagglutinating units determined with 0.045 per cent erythrocytes in the final mixture. The virus preparation used contained $10^{9.15}$ EID_{50} of Lee virus per ml. of infected allantoic fluid; in the reaction mixture $10^{7.05}$ EID_{50} was present in 2 ml. Interaction proceeded at 35°C. for 1 hour.

TABLE II

Multiplication of Lee Virus in the Allantoic or the Chorionic Layer

Membrane cultures	Yield of virus, hemagglutinating units*	
	Allantoic	Chorionic
1 and 2	256	354
3 and 4	128	512
5 and 6	128	256
7 and 8	256	64
Geometric mean	178	229

* Inoculum: $10^{7.05}$ EID_{50} of Lee virus. Incubation: 24 hours. Virus was measured in the medium employing 0.18 per cent erythrocytes in the final mixture.

closed in each sac. No detectable amount of virus was found in such fluid from any membrane sac. The finding that the chorionic layer produced and liberated large amounts of Lee virus is at variance with results reported for the BAR strain of influenza A virus (17).

Recovery of Adsorbed Virus.—It was of interest to determine how much of the virus which had been adsorbed by the chorioallantoic membrane could be recovered. The membranes from the experiments on adsorption of virus by both sides of the membrane (*cf.* Fig. 2) were collected, homogenized, suspended in saline, treated with *V. cholerae* filtrate, and the amount of virus was measured

by the hemagglutination technique. The amount recovered was related to the amount adsorbed. The results of one such experiment are summarized in Table III. Only a small fraction of the virus adsorbed by membranes could be recovered from the membranes. Extrapolation to the 20.2 mg. amount of membrane showed that, under conditions which were used in the experiments reported below, only 2.5 per cent of the adsorbed virus could be recovered. This value is in agreement with that found in the experiment represented in Fig. 1, and reinforces the conclusion that, of the virus inoculated, only a small fraction is recoverable in or on the membranes during the latent period. This result is in agreement with findings reported earlier (15) on the disappearance of PR8 virus inoculated into intact embryonated eggs.

TABLE III

Recovery of Adsorbed Lee Virus from Membrane

Membrane	Hemagglutinating units of virus*					
	In supernatant, not adsorbed	Adsorbed		Recovered from membrane		
mg.	*units*	*units*	*per cent*	*units*	*per cent of adsorbed*	*per cent of total*
—	70	—	—	—	—	—
48	26	44	63	1.6	3.6	2.3
83	18	52	74	2.8	5.4	4.0
169	8	62	89	5.8	9.4	8.3

* Hemagglutinating units determined with 0.045 per cent erythrocytes in the final mixture. The virus preparation used contained $10^{9.99}$ EID_{50} per ml. of infected allantoic fluid; in the reaction mixture $10^{7.66}$ EID_{50} was present. Interaction proceeded at 35°C. for 1 hour.

Effect of Specific Immune Serum on Residual Adsorbed Virus.—Experiments were carried out to determine whether the residual adsorbed virus could be removed from the membranes with specific immune serum.

Groups of 6 membrane cultures were prepared. Each piece weighed approximately 20.2 mg. and $10^{7.66}$ EID_{50} of Lee virus was used per culture. After inoculation the membranes were incubated at 35°C. for 1 hour with continuous shaking. They were then washed in 3 changes of medium and transferred into fresh medium or into normal or immune serum diluted with medium. After an additional period of incubation, all membranes were washed again and transferred into medium and incubation was continued. Groups of membranes were collected at time intervals; they were homogenized, suspended in 6 ml. of medium per group, and the concentration of virus was measured by infectivity titrations.

The results of two such experiments are summarized in Table IV. The mean amount of virus per group in control and normal serum cultures during the latent period was $10^{6.61}$ EID_{50}. On the basis that 27 per cent of the virus inoculated was adsorbed and that 2.5 per cent of the adsorbed virus should be re-

coverable, the expected amount in the membranes was computed to be 1.86×10^6 EID_{50}. This agrees with the mean determined value of 0.47×10^6. At 5.5 hours there was evidence of an increase in virus; the mean amount was $10^{7.19}$ EID_{50}. Following exposure of the membranes to 5 per cent immune serum for 0.5 hour, the residual baseline titer was reduced 90 per cent. On subsequent incubation in the absence of immune serum, the titer in the treated membranes rose to the same level as in controls. When 20 per cent immune serum and a 1 hour period of treatment were used, the reduction in the baseline titer was of the same order of magnitude. In this case, however, the titer at 5.5 hours was

TABLE IV

Effect of Specific Immune Serum on Infectivity Titer Baseline of Membranes during the Latent Period

Experiment No.	Treatment of membranes			Lee virus in membranes, log EID_{50} per group*			
	Serum‡	Concentration	Time	Time, *hrs.*			
		per cent	*hrs.*	1	1.5	3	5.5
1	—	—	—	7.05	6.38	—	—
	Normal	5	0.5	—	6.88	6.85	7.92
	Immune	5	0.5	—	5.88	5.75	7.88
					2.5	4	5.5
2	—	—	—		6.63	—	—
	Normal	20	1.0		6.69	6.19	6.90
	Immune	20	1.0		5.42	5.36	6.00

* Amount present initially: $10^{8.42}$ EID_{50} per group.

‡ Heated at 56°C. for 30 minutes.

significantly lower than the control titer. These results show that 90 per cent of the residual virus, which itself constitutes only 2.5 per cent of the virus adsorbed, was eliminated by specific immune serum. It appears likely that infection is initiated by the adsorbed virus which cannot be recovered (15).

Effect of DRB on the Increase of Virus and Soluble Complement-Fixing Antigen.—The multiplication of Lee virus in chorioallantoic membranes was studied in the presence or absence of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB). Membrane cultures were prepared in the manner described above, and groups of 6 cultures were collected at successive intervals. Virus concentration was measured by the hemagglutination technique in the membranes and in the media. The concentration of the soluble complement-fixing antigen was determined in the membranes because the bulk of this antigen was not released into the medium.

The results of one such experiment are given in Fig. 3. An increase in the amount of hemagglutinating virus in the membrane was demonstrable at 5.5 hours; the latent period was probably shorter than 4 hours. Between the 4th and 8th hours the amount of virus in the membranes increased rapidly; from the 8th to the 23rd hour the amount of virus increased at a much slower rate. Accumulation of virus in the media between the 4th and 8th hour took place at

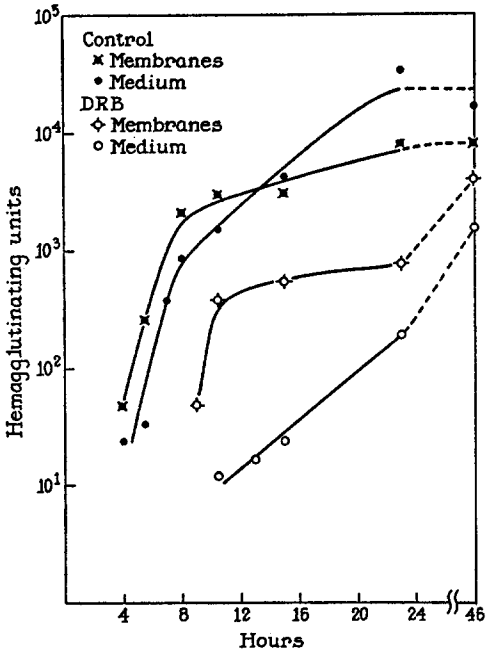

FIG. 3. Effect of DRB, 0.000055 M, on hemagglutination-time curve of Lee virus in chorioallantoic membrane cultures *in vitro*. The compound was added immediately before virus. The total amount of virus inoculated per group of 6 cultures was $10^{2.56}$ hemagglutinating units. In hemagglutination titrations the final concentration of erythrocytes was 0.045 per cent. The amounts of virus in membranes and media were determined separately and they are plotted separately in hemagglutinating units per group of 6 cultures.

a rate identical to that of increase in virus in membranes; however, the curve was displaced to the right to the extent of 1 hour indicating that there was a lag between virus production and release (18-20). The rate of increase between the 8th and 23rd hour was faster in the media than in the membranes. As a result, after 14 hours, the amount in the media exceeded that present in the membranes. These results indicate that virus was continually being liberated into the medium. On the basis that 0.69×10^2 hemagglutinating units of virus was adsorbed per group of 6 membranes, it can be computed that the yield at 23 hours was 580 times the amount of virus adsorbed.

In the presence of 0.000055 M DRB the rate of increase in the membranes

during the period of rapid rise was not significantly different from that observed in the absence of DRB. However, the incremental phase was delayed by 5 hours. The rate of increase between 10.5 and 23 hours was similar to that seen in the control. Virus continued to increase in the membranes to the end of the period of observation, *i.e.* 46 hours. In the presence of DRB the curve for the media showed two striking characteristics: the lag between appearance of virus in the membranes and in the media was longer than in the control, and the slopes of the curves between 10.5 and 23 hours were closely similar. This suggests, that, although because of the lower concentration of virus in the cells in the presence of DRB, release may have begun later than in the absence of this compound, the rate of release was similar in both cases. Thus, it appears that DRB had no effect on the process of release and that the delay in onset of release may have been due to low concentrations of virus in the membranes in the treated groups. As a corollary, it should be emphasized that the degree of inhibition, as determined by relating the amount of virus in treated cultures to that in controls, was greater for the media than for the membranes.

To obtain a representative view of total multiplication and the degree of inhibition, the amounts of virus present in the membranes and in the media were added. In the construction of the upper two curves in Fig. 4 the summated values for hemagglutinating virus in the membranes and the media were used. These curves resemble closely the curves of virus in membranes. In the lower part of Fig. 4, the curves describing the rise in soluble complement-fixing antigen in the membranes in the presence or absence of DRB indicate that the compound prolonged the period when no soluble complement-fixing antigen could be detected and decreased the amount of antigen produced. The extent of inhibition of complement-fixing antigen production, computed on the basis of data shown for 10.5, 15, 23, and 46 hours, was 3.2 times less than that of inhibition of virus formation. This difference was not statistically significant ($0.05 < p < 0.1$).

Effect of DRB on Various Phases of Lee Virus Multiplication.—To determine the exact time when processes inhibitable by DRB take place, experiments were carried out in which the time of addition of the compound to membrane cultures was varied over a wide range—from 3 hours prior to inoculation of the virus to 5 hours after inoculation.

Groups of membrane cultures were prepared and treated as described above. The procedure differed slightly in that each piece of membrane was suspended in 0.4 ml. of medium. Lee virus, 0.1 ml., then was added. At various periods before or after introduction of the virus, 0.5 ml. of medium containing DRB was added. To control membranes, 0.5 ml. of medium without the compound was added at various periods. The membranes and media, in groups of 6, were collected 10.5 hours after inoculation of virus. Suspensions of the membranes were prepared and the concentration of virus was determined in membranes and media by the hemagglutination technique.

The sum of the amounts of virus present in the two phases was expressed in hemagglutinating units per group. Soluble complement-fixing antigen was measured only in the membranes. Fig. 5 summarizes the results of 3 such experiments. Each point represents the geometric mean of 2 or 3 determinations. The amount of virus in control cultures fell within a narrow range of variation indicating that the addition of medium alone at various intervals did not cause

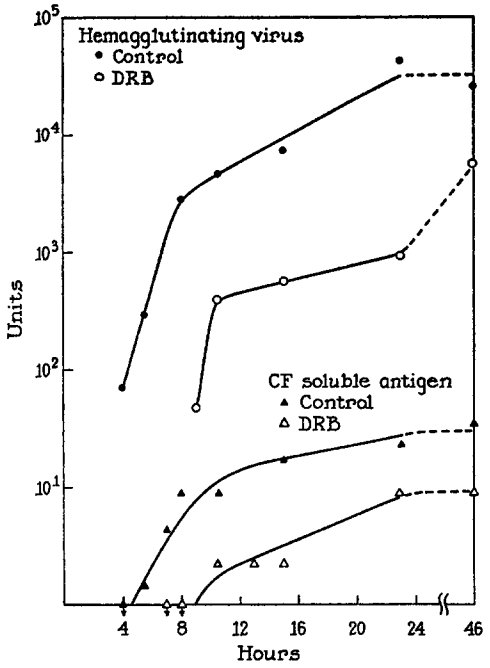

FIG. 4. Effect of DRB, 0.000055 M, on hemagglutination-time curve and on soluble complement-fixing antigen–time curve in chorioallantoic membrane cultures infected with Lee virus. The sums of the values of hemagglutinating virus in membranes and media shown in Fig. 3 are plotted. Soluble complement-fixing antigen was measured in the membranes, and the values plotted represent amount per group of 6 cultures.

any demonstrable effect on the multiplication of the agent. The amount of virus present in cultures to which DRB had been added along with the virus was 4 per cent of the mean amount in controls. Exposure of membranes to the substance for 3 hours before inoculation did not cause greater inhibition of multiplication than occurred when virus and compound were added simultaneously. This indicates that the speed of penetration of the compound is probably not an important variable and that duration of the contact of host cells with the compound was not *per se* of appreciable importance. As the interval between inoculation and addition of the compound was increased up to 3

hours, the degree of inhibition progressively decreased and, at later time intervals, no significant inhibition was observed. The inhibitory effect of DRB on the production of soluble complement-fixing antigen was even more strikingly dependent on time of administration; the effect was considerably less marked when the compound was given 0.5 hours after inoculation rather than along with the virus. When DRB was given 1 hour after inoculation or later, no

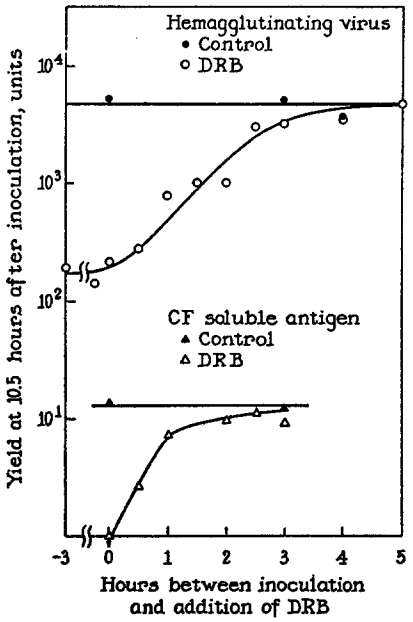

FIG. 5. Inhibition of Lee virus multiplication and of soluble complement fixing antigen formation in the chorioallantoic membrane *in vitro* relative to time of addition of DRB, 0.000055 M. The total amount of virus inoculated per group of 6 cultures was $10^{2.56}$ hemagglutinating units. Final concentration of erythrocytes in hemagglutination titrations was 0.045 per cent. Yield was measured at constant time, *i.e.* 10.5 hours after inoculation. Total amount of hemagglutinating virus per group of 6 cultures (membranes + media) was measured. Soluble complement-fixing antigen was measured in membranes.

significant effect was observed on production of soluble complement-fixing antigen. Thus, it appeared that the processes inhibitable by DRB were of shorter duration in the case of the soluble complement-fixing antigen than in the case of virus particles.

Effect of MB on Various Phases of Lee Virus Multiplication.—In an earlier report (1), the relation between time of addition and inhibitory effect was described for 2,5-dimethylbenzimidazole (MB). In the earlier experiments (1) $10^{6.36}$ EID_{50} (corrected value) of Lee virus was used as the inoculum per culture, samples were collected at 15.5 hours, and virus was measured in the membranes

by infectivity titrations. To compare more closely the inhibitory effects of DRB and MB, experiments with the latter compound were repeated employing the same conditions under which DRB inhibition was studied. The inoculum consisted of $10^{7.66}$ EID_{50} of Lee virus per culture. MB was given simultaneously with the virus or up to 6 hours later, and all samples were harvested 10.5 hours after inoculation. Virus was measured by the hemagglutination technique, both

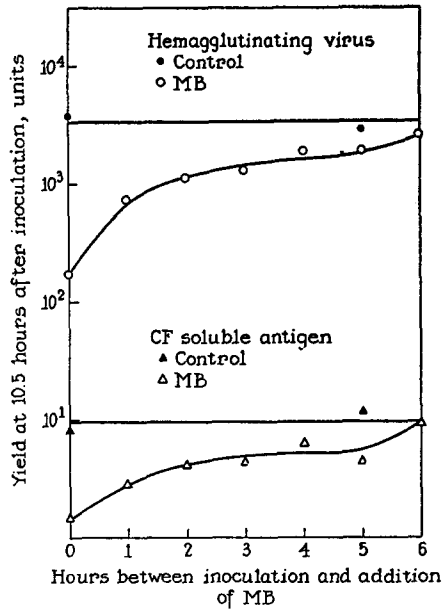

FIG. 6. Inhibition of Lee virus multiplication and of soluble complement-fixing antigen formation in the chorioallantoic membrane *in vitro* relative to time of addition of MB, 0.0021 M. The total amount of virus inoculated per group of 6 cultures was $10^{2.56}$ hemagglutinating units. Final concentration of erythrocytes in hemagglutination titrations was 0.045 per cent. Yield was measured at constant time, *i.e.* 10.5 hours after inoculation. Total amount of hemagglutinating virus per group of 6 cultures (membranes + media) was measured. Soluble complement-fixing antigen was measured in membranes.

in the membranes and in the media. The amount of soluble complement-fixing antigen was determined in the membranes.

In Fig. 6, the results of 3 experiments are summarized. As can be seen, when MB was given 2 hours after inoculation, the degree of inhibition of virus multiplication was considerably less than when the compound was administered along with the virus. However, even when the compound was given as late as 4 to 5 hours after inoculation, an appreciable degree of inhibition was obtained. No inhibition was observed when the interval between inoculation and introduction of MB was prolonged to 6 hours. These results agree with those re-

ported earlier (1) in that the process of virus multiplication was affected by the compound at two intervals, early and late. These results are in contrast to those obtained with DRB and indicate that the mechanism of action of the two compounds is different. It can also be seen in Fig. 6 that with MB the relationship between the time of addition and the inhibitory effect was similar both for virus and soluble complement-fixing antigen.

Effect of DRB and of MB at Higher Concentrations.—To obtain additional evidence that 5 hours after inoculation virus synthesis could no longer be

TABLE V

Comparison of the Effect of DRB and MB at Higher Concentration When Added 5 Hours after Inoculation

Benzimidazole derivative*		Virus‡	
		Hemagglutinating units§	Per cent of control
	M		
None	—	6160	100
DRB	0.000055	3396	53
"	0.000096	3299	55
"	0.00017	4935	80
None	—	7180	100
DRB	0.00096	5130	71
MB	0.0021	2020	28
"	0.0038	453	6.3

* Added 5 hours after inoculation.

‡ Lee virus, $10^{7.66}$ EID_{50} per culture, was used in the inoculum, and the cultures were incubated for 10.5 hours.

§ Expressed as the total for 6 cultures. In hemagglutination titrations 0.045 per cent final chicken erythrocytes were used; membranes and media from each group were titrated separately and the amounts of virus were added.

affected by DRB but could be inhibited by MB, experiments were carried out in which the two compounds were used at higher concentrations. The amount of virus was $10^{7.66}$ EID_{50} per culture, the compounds were added 5 hours after inoculation, and all groups were harvested at 10.5 hours. The results of such experiments are summarized in Table V. DRB failed to cause a significant degree of inhibition even when the concentration was increased to 3.1 times the concentration at which the compound caused 96 per cent inhibition (*cf.* Fig. 5) when given along with the virus. As expected, MB caused a moderate degree of inhibition when employed at a concentration of 0.0021 M (*cf.* Fig. 6). As was demonstrated above, at this concentration the compound, like DRB, caused 96 per cent inhibition when given simultaneously with the virus (*cf.* Fig. 6). When the concentration was increased to 0.0038 M, inhibition by MB was

marked. It appears that, 5 hours after inoculation, processes inhibitable by MB were still operating, whereas at this time those inhibitable by DRB had come to an end.

Effect of DRB and of MB in Experiments with Smaller Inocula.—Experiments were carried out to determine how late, after inoculation of relatively small amounts of virus, DRB or MB could be added and an inhibitory effect obtained.

TABLE VI

Comparison of the Effect of DRB and MB When Added 9 or 22 Hours after Small Inocula

Lee virus inoculated	Benzimidazole derivative	Time of addition of compound	Virus*	
			Hemagglutinating units‡	Per cent of control
EID_{50} per culture		*hrs.*		
$10^{5.56}$	None	—	1501	100.0
"	DRB§	0	0	—
"	"	9	57	3.8
"	"	22	790	53.0
$10^{3.56}$	None	—	346	100.0
"	DRB§	22	47	14.0
$10^{5.56}$	None	—	1039	100.0
"	MB‖	0	50	4.8
"	"	9	111	11.0
"	"	22	790	76.0
$10^{3.56}$	None	—	346	100.0
"	MB‖	22	69	20.0

* Membranes were incubated for 36 hours.

‡ Expressed as total for 6 cultures. In hemagglutination titrations 0.18 per cent final chicken erythrocytes were used; membranes and media from each group were titered separately and the amounts of virus were added.

§ 0.000055 M.

‖ 0.0021 M.

In these experiments $10^{5.56}$ or $10^{3.56}$ EID_{50} of Lee virus per culture was used as the inoculum, the compound was given along with the virus or at 9 or 22 hours after inoculation, and membranes and media were collected 36 hours after inoculation. The results of two such experiments are summarized in Table VI. With $10^{5.56}$ as inoculum a marked degree of inhibition was obtained when DRB, 0.000055 M, or MB, 0.0021 M, was added 9 hours after inoculation but not when the compounds were added 22 hours after inoculation. With $10^{3.56}$ EID_{50} of virus as inoculum a considerable degree of inhibition was obtained even when the compounds were added 22 hours after inoculation. This was expected because with small inocula the number of cells infected initially was but a small

fraction of the total and, therefore, several cell cycles of multiplication probably took place before high titers were reached. Under these conditions the inhibitable processes would repeat themselves successively as more cells became infected with virus produced earlier by other cells. It should be emphasized that with an inoculum of $10^{3.56}$ EID_{50} virus was not present in the control at 22 hours in an amount detectable by the hemagglutination technique and, therefore, that more than 99 per cent of the virus present at 36 hours was formed between 22 and 36 hours. In natural infections in animal hosts, in which the inoculum usually is small, this result suggests that virus multiplication may be inhibited by such compounds a considerable time after infection has been initiated.

DISCUSSION

It has been reported that with PR8 virus (9, 11, 15) regardless of the amount inoculated in the allantoic cavity *in vivo* the proportion of virus adsorbed is constant in unit time, except when 3×10^8 or more particles are inoculated (11) in which case the proportion adsorbed is smaller. In the present *in vitro* experiments in which it was possible to vary the amounts of both membrane and virus, the relationship between the amount of membrane and the amount of Lee virus adsorbed in 1 hour was exponential. Furthermore, with decreasing amounts of virus more complete adsorption occurred. In retrospect, it is probable that with an inoculum of $10^{6.36}$ EID_{50} of Lee virus and an area of 11.5 $cm.^2$ of membrane per culture as employed in earlier studies (1), more than 90 per cent of the virus was adsorbed. The computations made in the earlier studies (1, 5) were based on such an assumption. However, when larger inocula and smaller amounts of membrane were used only a small proportion of the virus inoculated was adsorbed, as was the case in kinetic experiments described in the present report.

With $10^{7.66}$ EID_{50} per 2.9 $cm.^2$ of chorioallantoic membrane in free suspension the process of influenza virus multiplication was characterized by a low EID_{50}/HA ratio in the yield. This observation accords with those made by others (8, 11, 13). In spite of the low EID_{50}/HA ratio in the yield, it is probable that measurements of hemagglutinating virus reliably reflected the total amount of virus produced in the presence or absence of inhibitory compounds. Available evidence (11, 21) does not provide good support for the hypothesis that hemagglutinating particles are precursors of infective particles. Furthermore, DRB acts early in the latent period, *i.e.* prior to the emergence of hemagglutinating or infective virus particles. It should be reemphasized that the rate of appearance of virus particles was not diminished despite the large inocula and that the methods chosen had great advantages as to precision and ease of execution. Studies with smaller inocula have demonstrated that 2,5-dimethylbenzimidazole (MB) inhibits the production of infective virus to an extent similar to the inhibition of hemagglutinating virus (5). The kinetic characteristics of inhibition

appear not to depend on the method of measurement of virus particles. That 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) is capable of inhibiting production of infective virus has been demonstrated previously (3).

It is possible to distinguish between the modes of action of DRB and MB on the basis of kinetic evidence of two kinds. Firstly, those processes inhibitable by DRB are of shorter duration than those inhibited by MB. This is true for the synthesis of both hemagglutinating virus and soluble complement-fixing antigen. Secondly, with MB the relationship between the time of addition and the inhibitory effect is similar both for virus and soluble complement-fixing antigen; in contrast, with DRB the inhibitable processes are of shorter duration for the soluble complement-fixing antigen than for virus particles. Thus, of the two compounds DRB is not only 35 times more active on a molar basis but its effect is more restricted in time. This evidence, combined with that reported previously (3) regarding the effect of these two compounds on oxygen uptake by the chorioallantoic membrane and on tissue proliferation *in vitro*, indicates that of the two DRB is considerably more selective in its virus inhibitory action. This is of interest from the points of view of both the mechanism of action of benzimidazole derivatives and their possible preventive or therapeutic usefulness in virus infections.

It appears clear that processes inhibitable by DRB cease before soluble complement-fixing antigen or virus particles have been produced in demonstrable amounts in the infected tissue. Thus, it is possible that DRB interferes with the synthesis of precursor substances for both these virus materials. The results secured in this study leave undecided the question of whether or not the soluble complement-fixing antigen may itself be a precursor of influenza virus particles (21). The finding that the processes inhibitable by DRB are of shorter duration for the soluble complement-fixing antigen may mean that in this case they are less extensive or simpler than in the case of virus particles, since the end product, the soluble complement-fixing antigen, is much smaller and immunologically simpler than the virus particle. In terms of the chemical reactions likely to be involved, it seems probable that DRB interferes with nucleic acid metabolism (3). That the mechanism of inhibition caused by MB is probably more complex than reduction in the rate of a single biosynthetic process was recognized in earlier studies (1).

On the basis of available evidence it seems likely that both MB and DRB interfere with processes which are not specific for virus production, but may take place in the uninfected cell as well. The important point seems to be that inhibition of virus multiplication can be obtained under conditions which leave large areas of host tissue metabolism unaffected as indicated, *e.g.*, by lack of effect on oxygen uptake.

Although broad-spectrum antibiotics like chlortetracycline (aureomycin), oxytetracycline (terramycin), and chloramphenicol exert selective inhibitory action not

only on a large number of bacteria but also on intracellular parasites of the psittacosis group and on rickettsiae, viruses of the size of vaccinia or smaller are not thus affected. This immediately raises the question of the mechanism of their selective action because the available evidence suggests that a broad-spectrum agent may cause inhibition of microbial growth through interference with a chemical process common to host and parasite rather than through inhibition of chemical reactions specific for each parasite. It has not been possible to explain the inhibitory action of chlortetracycline, oxytetracycline or chloramphenicol on the growth of bacteria in terms of inhibition of specific enzyme reactions so far studied. Most of such reactions studied have been of the catabolic type. More positive results have been obtained in studies on biosynthetic reactions. Evidence is now accumulating that these three antibiotics inhibit net protein synthesis in bacteria at concentrations which inhibit their growth (22, 23). On the other hand, it has been reported (22) that at the same concentrations these compounds show a stimulating effect on nucleic acid synthesis in *Staphylococcus aureus*. However, more recent work (23) indicates that one of the compounds, chloramphenicol, has no effect on ribose or desoxyribose nucleic acid synthesis in *Escherichia coli*. Of great interest is the finding that chlortetracycline and chloramphenicol are capable, at low concentration, of inhibiting proliferation of cells obtained from the chicken embryo and cultivated *in vitro* (24).

If the process inhibited by a broad-spectrum antibiotic is common to host and parasite, the mechanism of selective inhibition may concern factors such as sites and rates of protein synthesis in the infected cell; intracellular permeability barriers; and the precise nature and degree of dependence of intracellular parasite reproduction on host cell metabolism.

From these studies and considerations certain features emerge by which compounds which inhibit virus reproduction can be assessed. The chemical agents should not only be highly active but also selective in their action; they should interfere only with a narrow segment of the metabolism of the infected cell, and this segment should be one which permits a quantitatively differential response on the part of the parasite and the host; such interference with metabolic processes ought to be reversible. On the last point, it has been shown that the inhibitory effect of DRB on tissue proliferation was reversible on withdrawal of the compound, although the effect on virus multiplication persisted (3), whereas the effect of MB was reversible in both regards (3–5). No explanation was offered in regard to the persistence of the virus inhibitory effect of DRB. It is possible that DRB combines with some cellular component and fails to diffuse out. It should be pointed out that in the membrane experiments on virus multiplication the host cells did not multiply demonstrably, whereas in experiments on tissue proliferation in roller tube cultures cells divided in the presence of DRB although at a slow rate. On withdrawal of the compound and on continued division of cells in roller tube cultures the intracellular concentration of DRB would be expected to fall, and its effect may disappear.

The various evidences reported previously (3) and in the present communication that DRB is a considerably more interesting compound than MB are cor-

roborated by the finding that DRB inhibits Lee virus multiplication in mice and chicken embryos without serious damage to the host (3), whereas MB fails to do so (25).

SUMMARY

A procedure is described for kinetic studies on the multiplication of Lee virus in the chorioallantoic membrane *in vitro* employing the hemagglutination technique for measurement of virus concentration. A linear relationship was found between the logarithm of virus adsorbed and the amount of membrane used. Of the virus adsorbed less than 10 per cent could be recovered from the membrane. Of the recoverable virus 90 per cent was eliminated by specific immune serum. Lee virus was adsorbed by the allantoic and chorionic layers of the membrane to a similar degree. Multiplication occurred in both layers and to a similar extent. When $10^{7.66}$ EID_{50} of Lee virus was inoculated per 2.9 cm.^{2} of chorioallantoic membrane, the ratio of infectivity to hemagglutination titer in the yield was low, although the rate of appearance of virus particles was not diminished despite the large inocula.

Virus produced in membranes was liberated rapidly and continually into the medium. 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), 0.000055 M, prolonged the latent period by more than 100 per cent. The rate of increase during the period of rapid rise was similar in the presence or absence of DRB, but the yield was markedly reduced at the end of this period in the presence of DRB. The amount of the virus in the membranes continued to rise in the presence of DRB and eventually approached the maximal levels reached much earlier in the controls. Measurement of the amount of virus in the media indicated a greater degree of inhibition than did measurement in the membranes. Comparative studies with two benzimidazole derivatives on the dependence of the inhibitory effect on the time of addition of the compound showed that processes which could be inhibited by DRB were of shorter duration than those inhibited by 2,5-dimethylbenzimidazole (MB). With MB the relationship between the time of addition and the inhibitory effect was similar both for virus and for soluble complement-fixing antigen; with DRB the inhibitable processes were of shorter duration for the complement-fixing antigen than for virus particles. DRB was not only 35 times more active on a molar basis but also was more selective in its action than MB. DRB interfered with processes which preceded the emergence of either soluble complement-fixing antigen or virus particles. Some of the implications of these findings are discussed in relation to the mechanism of inhibition of influenza virus multiplication by benzimidazole derivatives.

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