INHIBITION OF INFLUENZA VIRUS MULTIPLICATION BY ALKYL DERIVATIVES OF BENZIMIDAZOLE

I. Kinetic Aspects of Inhibition by 2,5-Dimethylbenzimidazole as Measured by Infectivity Titrations

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That 2,5-dimethylbenzimidazole inhibits the multiplication of influenza A or B virus in chorioallantoic membrane cultures *in vitro* was reported in an earlier paper (1). The evidence obtained indicated that the compound exerted an inhibitory effect when added after the host tissue had been infected by the virus. Moreover, the inhibitory effect was reversible: after a period during which multiplication was suppressed, reduction in the concentration of the substance resulted in undiminished multiplication of the virus in treated membranes. In addition, the finding that the compound had, at inhibitory levels, no effect on the oxygen consumption of membranes suggested that metabolic pathways other than those concerned with oxidative reactions were affected.

One approach to an understanding of the mechanism of the inhibitory action of 2,5-dimethylbenzimidazole is to obtain answers to the following questions: (*a*) How does the compound affect the curve of increase in concentration of the virus? (*b*) What phases of the multiplication cycle of the virus are affected by the compound? The concept that multiplication of influenza virus takes place in discrete cycles, each of which can be subdivided into (*a*) an adsorptive period, (*b*) a plateau or latent period, and (*c*) an incremental period, is supported by considerable experimental evidence (2–7). The results reported in this paper are in accord with this conception.

In this communication, experimental results are presented which provide a basis for an analysis of the kinetics of inhibition of influenza B virus multiplication by 2,5-dimethylbenzimidazole. Infectivity titrations were used throughout this study so that events occurring in the first cycle of multiplication could be examined. In the accompanying papers (8, 9), results obtained by hemagglutination titrations are presented and the inhibitory effects of a number of alkyl derivatives of benzimidazole are described.

Materials and Methods

The accompanying communication (8) contains detailed descriptions and analyses of the procedures used. The techniques employed in the present study are described briefly below:—

Virus.—The Lee strain of influenza B virus was passed serially in the allantoic sac of 10 or 11 day old chicken embryos. The inoculum was 0.1 ml. of a 10^{-3} dilution of infected allantoic fluid. Incubation at 35°C. was carried out for 24 hours. To prepare seed virus for experiments *in vitro*, Lee virus, diluted similarly, was inoculated into 11 day old embryos which were incubated for 28 hours and then chilled at −28°C. for 1 hour. Allantoic fluid was harvested, pooled, and stored in a number of tubes at −60°C. Each tube was thawed and used but once. The infected allantoic fluid used as seed virus contained $10^{9.5}$ EID_{50} per ml.

Cultivation of Lee Virus in Vitro.—Intact pieces of chorioallantoic membrane obtained from 10 or 11 day old embryos were suspended in a nutrient fluid medium, designated N.F.2 in a previous report (1). The average surface area of each piece of membrane was 11.5 cm.^{2} and the wet weight was 74.1 mg. per culture tube. The volume of nutrient fluid was 2.0 ml. and contained $10^{5.5}$ EID_{50} of Lee virus per ml. 2,5-Dimethylbenzimidazole was used at a concentration of 0.0026 M. The membrane cultures were incubated at 35°C. with continuous horizontal shaking (8). At various times, groups of 6 membranes were collected, washed in three changes of 0.85 per cent NaCl, and either promptly frozen and stored overnight at −28°C. or immediately ground with mortar and pestle and a small amount of alundum. A 10 per cent suspension in broth containing 10 per cent normal horse serum was prepared. The suspension was centrifuged at 2,000 *g* for 10 minutes and the supernatant was frozen and stored at −60°C. Infectivity titrations were carried out *in ovo* on supernatants prepared in this manner.

Infectivity Titrations.—Serial tenfold dilutions were prepared in chilled 0.85 per cent NaCl solution, buffered at pH 7.2 with phosphate, containing penicillin, 50 units, and streptomycin, 250 μg. per ml. 10 or 11 day embryonated eggs in groups of 6 were inoculated allantoically with each dilution, 0.1 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 end point was determined by the hemagglutination procedure with the allantoic fluids.

Hemagglutination Titrations.—Serial twofold dilutions were made in buffered saline. To each dilution an equal volume of a 0.36 per cent chicken RBC suspension which had been standardized photometrically was added. A strong partial agglutination was taken as the end point. The titer was expressed as the dilution at the end point.

EXPERIMENTAL

Effect of 2,5-Dimethylbenzimidazole on Infectivity Titer-Time Curve.—The multiplication of Lee virus in chorioallantoic membrane cultures was studied in the presence or absence of 2,5-dimethylbenzimidazole. In all experiments, the concentration of the virus in the membrane *per se* was measured at various periods by infectivity *in ovo*. This procedure made it possible to examine the earliest phases of the multiplication process. Because it was feasible to study the first cycle of multiplication in the membrane, the length of the infectivity latent period and of the incremental period could be determined. In addition, the rate of increase in infective virus during the incremental period could be estimated.

Fig. 1 shows the composite results of four experiments on the infectivity titer-time curve with Lee virus in membrane cultures. Each point represents the

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geometric mean of at least two titrations. The infectivity titer of the control membranes showed no definite change between 0.5 and 5.5 hours and the duration of the latent period was approximately 8 hours. In view of the differences in the experimental conditions employed, it is noteworthy that the latent period was similar to that found with Lee virus in the membrane of intact embryos (2, 10). At 8 hours, the beginning of a rapid rise in the titer of infective virus was demonstrable and a logarithmic increase in titer continued until 15.5 hours. The duration of the first incremental period was approxi-

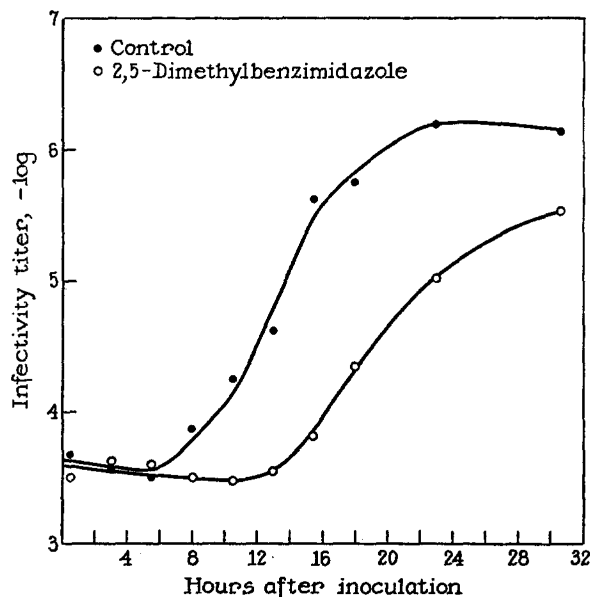

FIG. 1. Effect of 2,5-dimethylbenzimidazole, 0.0026 M, on infectivity-time curve of Lee virus in the chorioallantoic membrane *in vitro*. Initial titer (EI_{50}) of virus in the medium was $10^{-4.5}$.

mately 7.5 hours. The titer at the end of the incremental period, *i.e.* 15.5 hours, was 2.12 log units higher than at the beginning. From 16 to 23 hours, the titer continued to rise, but at a much slower rate, and after 23 hours there was no further increase. On the basis of the estimate (8) that 3.5 per cent of the available host cells were infected initially and the probability that each cell yields approximately 35 infective Lee virus particles (2), the supply of susceptible cells would be exhausted in two cycles of multiplication if the major portion of the virus particles from the first cycle was capable of initiating infection in the second cycle.

As is shown also in Fig. 1, the presence of 2,5-dimethylbenzimidazole did not affect the titer level during the plateau period. It did, however, cause an in-

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crease of 6 to 7 hours in the duration of the latent period. The rate of increase in titer during the incremental period was lower than that obtained with control membranes. Comparison of the rates is difficult because of differences in the shape of the two curves. The curve obtained in the presence of the compound showed no definite break indicative of the end of the first cycle. Instead, the rate of increase in titer gradually decreased. The yield of virus measured at 15.5 hours was but 1.5 per cent of the control value. At 30.5 hours, the yield as determined in the membrane was 25 per cent of the control value.

In addition to the infectivity titrations on the membranes, hemagglutination titrations were carried out on the culture media. Positive hemagglutination reactions were obtained at 15.5 hours with medium from control cultures but not until 30.5 hours with that from cultures containing 2,5-dimethylbenzimidazole. As determined by the hemagglutination procedure, the titer of the medium at 30.5 hours was 1:64 and 1:2, respectively, in control and treated groups. On this basis, multiplication in the treated groups was restricted to 3.1 per cent of control. The relation between the amount of virus in the membrane and that in the medium at various periods is considered below.

Amount of Infective Virus in Membrane and Medium.—The infectivity titers of both the membranes and the culture media were determined at various intervals after inoculation so that changes in the concentration and in the total amount of virus in both phases of the membrane cultures could be estimated.

Groups of membrane cultures were prepared and treated exactly as described above. At various intervals after inoculation, both the media and the membranes were collected simultaneously from a group of cultures. The membranes were washed and suspensions were prepared, frozen, and stored as described above. The media were pooled, frozen, and stored similarly. Infectivity titers were determined on each group of membranes and the corresponding pooled culture media.

Table I shows the results of such an experiment. In the cultures employed in this study, the ratio of the volume of the membrane to that of the medium was approximately 1:27. Therefore, at equivalent titers, the total amount of virus in the membrane *per se* was about 27 times less than that in the medium. At 0.5 hour, the titers were closely similar, but the membrane contained about 20 times less virus than the medium. Both at 3.0 and 5.5 hours, the titer of the membrane was about 5 times higher than that of the medium, yet the total amount of virus in the membrane was about 6 times less than in the medium. At 15.5 hours, the titer of the membrane was about 36 times higher than that of the medium, but the total amounts of virus in the two phases were nearly equal. At 36 hours, the titers were identical and therefore the medium contained about 27 times more virus than the membrane. Similar relationships between the amounts of virus in the two phases were found when 2,5-dimethylbenzimidazole was present in inhibitory amount in the medium.

From these results it appears evident that during the plateau or latent period

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the concentration of infective virus in the membrane was higher than that in the culture medium. The marked increase in the concentration of virus in the membrane at 15.5 hours was reflected by a less marked increase in the concentration of virus in the medium. This indicates that virus formed in the membrane was released into the medium but that there was a lag in the equalization of the concentrations. At 36 hours, the concentrations of infective virus in the two phases of the culture were equal. This may be taken as an indication that the release of newly formed virus into the medium was completed. On the basis of the amounts present in the two phases at 36 hours, it appears that approximately 96 per cent of virus formed in membrane was released into the medium.

Effect of 2,5-Dimethylbenzimidazole on Various Phases of the Multiplication Cycle.—Experiments were carried out in which the time of addition of 2,5-

TABLE I

Infectivity Titers of Lee Virus in the Membranes and Culture Media

Time after inoculation	Membranes		Media		Ratio, $\frac{\text{Membranes}}{\text{Media}}$	
	Infectivity titer*	Amount of virus‡	Infectivity titer*	Amount of virus§	Concentration	Amount
hrs.	*log*	*log*	*log*	*log*		
0.5	3.67	3.54	3.52	4.82	1.4	0.053
3.0	3.56	3.43	2.85	4.15	5.1	0.192
5.5	3.50	3.37	2.85	4.15	4.5	0.166
15.5	5.79	5.66	4.23	5.53	36.0	1.35
36.0	6.00	5.87	6.00	7.30	1.0	0.037

* Expressed as the reciprocal of dilution.

‡ On the basis of 0.074 gm. of membrane, expressed as EID_{50}.

§ On the basis of 2.0 ml. of medium, expressed as EID_{50}.

dimethylbenzimidazole to the membrane cultures was varied over a wide range: from 3 hours prior to inoculation of the virus to 13 hours after inoculation. This was done in order to determine how late after inoculation of virus 2,5-dimethylbenzimidazole could be added and an inhibitory effect obtained.

Groups of membrane cultures were prepared and treated as described above. The procedure differed slightly in that each piece of membrane was suspended in 0.9 ml. of medium. Diluted Lee virus, 0.2 ml., then was added. At various periods before or after introduction of the virus, 0.9 ml. of medium containing 2,5-dimethylbenzimidazole was added. To control membranes, 0.9 ml. of medium without the compound was added simultaneously. The membranes, in groups of 6, were collected 15.5 hours after inoculation of virus. Suspensions were prepared and infectivity titrations were carried out as described above.

Fig. 2 shows the results obtained in three separate experiments. Each point represents the geometric mean of at least two infectivity titrations. The titers of the control membranes all fell within a narrow range (standard deviation =

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0.14 log unit), indicating that the addition of medium alone at various intervals before or after introduction of the virus did not cause any demonstrable effect on the extent of multiplication of the agent. The titers of membranes in cultures containing 2,5-dimethylbenzimidazole were considerably lower than those of controls, even when the compound was not added until 8 hours after introduction of the virus. Exposure of membranes to the substance for 3 hours before inoculation did not cause greater inhibition of multiplication, *i.e.* 99 per cent, than occurred when virus and compound were added simultaneously. As the interval between inoculation and addition of the compound was increased up to 3 hours, the degree of inhibition progressively

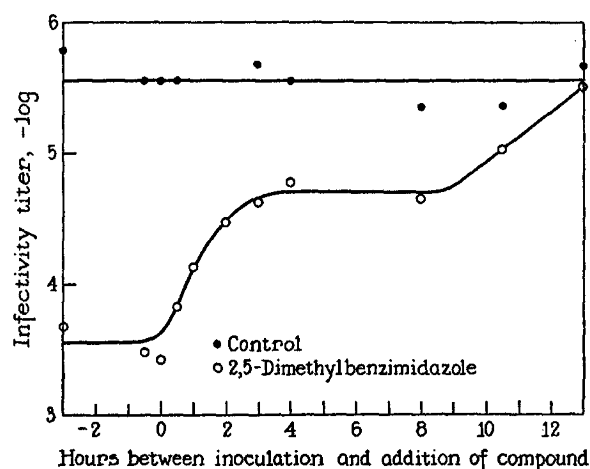

FIG. 2. Inhibition of Lee virus multiplication in the chorioallantoic membrane *in vitro* relative to time of addition of 2,5-dimethylbenzimidazole, 0.0026 M, as measured by infectivity titrations. Initial titer (EI_{50}) of virus in the medium was $10^{-4.5}$.

decreased from 99 to 87 per cent. When the substance was given at either 4 or 8 hours after the virus, inhibition equal in extent to that secured at 3 hours occurred. But when the compound was introduced either 10.5 or 13 hours after inoculation, a significant degree of inhibition of multiplication was not demonstrable.

These results show that 2,5-dimethylbenzimidazole, in the concentration employed: (*a*) restricts the multiplication of Lee virus to about 1 per cent of the control value when given before or along with the agent, (*b*) inhibits multiplication to a degree inversely proportional to the time after inoculation when added during the first third of the latent period, and (*c*) inhibits multiplication to a moderate degree even when added during the last two-thirds of the latent period.

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DISCUSSION

That the inhibitory activity of 2,5-dimethylbenzimidazole is not attributable to a direct effect upon influenza virus *per se,* while the agent is in an extracellular position, may be inferred from the results of this study and that which preceded it (1). In addition, it is clear that the compound does not affect the adsorptive process; in its presence, the virus is adsorbed by susceptible cells in the usual manner (1). On the basis of the alterations caused by 2,5-dimethylbenzimidazole in the kinetics of the first cycle of multiplication of influenza virus, it is adduced that the substance diminishes the rate of biosynthetic processes necessary for reproduction of the virus.

Several phases of the multiplication cycle are altered by the presence of the compound: The duration of the plateau or latent period is extended by approximately 80 per cent. The rate at which the concentration of virus increases during the incremental period appears to be diminished. The concentration of virus at the end of the cycle is only a small fraction of the control value. These findings provide strong support for the concept that the compound reduces the efficiency of the multiplication process *per se*. However, they do not shed any light on the precise nature of this process.

The fact that 2,5-dimethylbenzimidazole prolongs the latent period serves to focus attention on processes which precede the emergence of infective virus particles. It appears that the longer the addition of the compound is withheld during the latent period, the smaller is the extent of inhibition as measured at a fixed time; *i.e.,* 15.5 hours. During the first 3 hours of the latent period, there is an approximate inverse proportionality between these two variables. This indicates that the earlier in a cycle the rate of processes involved in reproduction of new virus can be reduced, the more marked will be the reduction in the yield of virus. That this relationship is not attributable merely to differences in the duration of contact of the host cells with the compound seems probable because pretreatment of the membrane with the substance does not cause a greater inhibitory effect than is secured when inoculation and addition of the compound are nearly simultaneous.

When the compound is given at 4 or even at 8 hours after inoculation, a small but definite degree of inhibition is demonstrable at 15.5 hours. This raises the possibility that during much of the last half of the latent period, the substance does not exert an inhibitory effect but that just prior to the emergence of infective virus particles some final step in virus reproduction is retarded. It should be emphasized that, if the substance is given after the end of the latent period, no inhibition can be demonstrated during that particular cycle.

In studies on substances which retard bacterial growth, Moore and Boylen (11) were able to distinguish between three types of inhibitory action. They concluded that only one of the inhibitory types could be interpreted as repre-

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senting a simple inhibition of the rate of a single reaction sequence. With this type of inhibition, there was no delay in the attainment of the final decreased growth rate, and the microorganisms multiplied logarithmically. With the other two types of inhibition, the growth curves were more complex. These findings serve to underline the importance of investigating complete growth curves when the mechanism of action of an inhibitory substance is under study. That this applies also to investigations on inhibitors of virus multiplication has been emphasized previously (12).

It is apparent from the data secured in this study that 2,5-dimethylbenzimidazole causes several alterations in the kinetics of the multiplication process of influenza B virus. This finding as well as that bearing on quantitative differences in the inhibitory effect as related to time of addition of the compound suggests that the mechanism of inhibition is more complex than reduction in the rate of a single biosynthetic process.

The finding that, during the incremental period, newly formed virus particles accumulate considerably more rapidly in the membrane than in the medium provides evidence for a lag in the release of such particles from infected cells. Inasmuch as the concentrations of virus in the two phases of the culture do not become equal until late in the second cycle of multiplication, it is apparent that investigations on the first cycle are best carried out by measurements on the membrane *per se*. It should be emphasized that, under the experimental conditions employed in this study, the inoculum and the number of susceptible host cells are limiting factors which restrict the multiplication process to two cycles. By 36 hours, at least 95 per cent of the newly formed virus is released into the medium. As a consequence, the total yield of virus from the infected membrane can be determined satisfactorily by means of measurements on the culture medium provided they are carried out at the time indicated or later. Data which support this conclusion are presented in the accompanying papers (8, 9).

SUMMARY

At a concentration of 0.0026 M, 2,5-dimethylbenzimidazole caused a number of alterations in the first cycle of multiplication of influenza B virus, Lee strain, in chorioallantoic membrane cultures *in vitro*. As determined by infectivity titrations *in ovo* on the membrane *per se*, the following alterations were observed: The duration of the latent period was increased by 80 per cent. The rate of increase in titer during the incremental period was somewhat decreased. The yield of virus was decreased by about 99 per cent.

When the compound was added to membrane cultures at various periods before or after inoculation with the virus, the following findings were obtained: On addition before or along with the virus, the substance caused about 99 per cent inhibition of multiplication. When added during the first 2 hours after inoc-

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ulation, the compound caused inhibition of a degree which was inversely proportional to the time of addition. When added 3 to 8 hours after inoculation, the substance caused about 80 per cent inhibition. When added after the end of the latent period, no definite inhibition was obtained in the first cycle of multiplication.

These results are interpreted as indicating that 2,5-dimethylbenzimidazole acts by reducing the rate of biosynthetic mechanisms necessary for the reproduction of influenza virus particles.

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