1954

Frank L. Horsfall, Jr., 1952

The Rockefeller University

EXPERIMENTS ON CHEMICAL ALTERATION OF VIRUS INFECTIONS*

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A SURVEY of the Harvey Lectures of the past 20 years reveals that no fewer than 11 of them considered viruses or the natural history of infections induced by these agents, but that none dwelt on artificial alteration of virus infections. This is readily understood for there was not much that could have been told about modification of such infections until recently. Although it has been known for some time that a variety of substances affect viruses in the test tube and that some may affect the course of infectious processes induced by these agents, it is only within the last few years that systematic studies have been undertaken on the effects of chemical substances on virus infections. But now substances are available which cause alteration of one or more of the infections induced by viruses in animals, plants, or bacteria.

Some hundreds of papers on the effects of various substances on viral agents or infections induced by them have appeared. References to many of these communications may be found in recent reviews of this field.[1,2] As is often the case, most information has been acquired in those areas in which it has been feasible to obtain the most quantitative data. It would be impossible in the brief time before us to undertake an appraisal of available data or even to summarize adequately the more important communications.

Many thousands of substances, either of biological origin or produced in chemical laboratories, have been tested for inhibitory activity against various viruses. The vast majority have been found to be devoid of such activity. But a number of substances have been discovered to possess some inhibitive capacity in certain viral

* Lecture delivered November 20, 1952.

infections. The heterogeneity of these materials is bewildering; some are highly complex structures such as polysaccharides[3-5] or proteins;[6] others are relatively simple structures such as acridines[7-9] or analogs of amino acids.[10,11] In most instances little attention has been directed to the mechanism of action of the inhibitory substance. In only a few cases has the site or mode of action been intensively studied.

For purposes of this lecture, it seems desirable to select certain illustrative examples which have been subjected to extended study and of which we have some personal knowledge. The major portion of the investigations to be considered this evening were carried out by Dr. Harold S. Ginsberg and by Dr. Igor Tamm, with whom it has been my good fortune to be associated during a number of years. Both workers have been kind enough to permit me to use certain of their unpublished data.

The studies to be described have dealt with medium- and small-sized viruses: pneumonia virus of mice, mumps, influenza, and Newcastle disease, each of which leads to infection of some animal species. Each of the agents utilized reacts with certain erythrocytes and causes them to agglutinate. In every case with which we have dealt, the virus particle itself is responsible for this useful reaction, and the concentration of the agent can be determined with reasonable precision by means of hemagglutination *in vitro*. The advantages of using such viruses are obvious, especially when it is necessary to carry out numerous measurements in many different experiments. The chemical substances used, polysaccharides and derivatives of benzimidazole, have been restricted to those which do not demonstrably react with the viruses themselves but do alter infections caused by these agents. One of the major objectives of the work has been to define the mechanisms through which chemical substances alter infectious processes induced by animal viruses.

Our interest in this problem was aroused by an unexpected finding made in 1946.[3] When certain bacteria were given to mice infected with a virus which leads to fatal pneumonia, there occurred an alteration in the course of the virus infection. Instead of dying from pneumonia as was anticipated, animals treated in this odd manner recovered.

Figure 1 shows in summary the results obtained in the initial experiments.[3] Mice were inoculated with various dilutions of pneumonia virus of mice, termed PVM.[12] Two days later they were given one intranasal instillation of a nonhemolytic streptococcus, termed MG.[13] The experiment was terminated on the 11th day when the lungs of all surviving animals were examined. From the data shown, it is clear that not only was there a marked reduc-

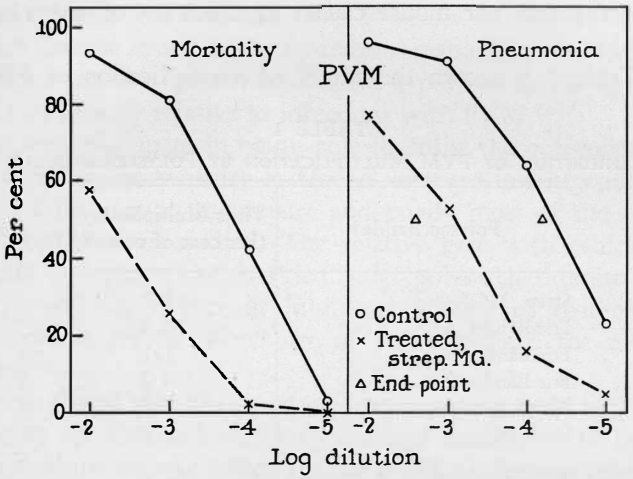


FIG. 1. Effect of one intranasal instillation of streptococcus MG on the mortality and extent of pneumonia in mice infected 2 days previously with various amounts of pneumonia virus of mice (PVM). (From Horsfall and McCarty.[3])

tion in the mortality of treated animals, but also that there occurred a comparable reduction in the extent of pneumonia. In animals treated with the bacterium, the virus titration end-point, indicated by the triangles, corresponded to a dilution of 2.6 log units as compared to 4.2 in controls. Thus, in treated animals, an inoculum containing 40 times more virus was required to produce effects comparable to those found in controls.

Streptococcus MG is an encapsulated bacterium which is entirely nonpathogenic in the mouse. It does not multiply in the mouse lung nor does it persist there for longer than 14 hours. Heat-killed microorganisms are as active against infections caused

by the virus as are living bacteria. One instillation of the bacterium is effective if given as early as 2 weeks before, or as late as 4 days after, inoculation with the virus.[3]

Cell-free extracts of streptococcus MG are as effective as the bacterial bodies themselves. The results of enzyme studies and physicochemical observations indicated that the active component in such extracts might be a polysaccharide. Experiments showed that the purified capsular polysaccharide is active and that only a few micrograms per mouse causes modification of the virus infection.[3]

In Table 1 is shown the extent of multiplication of PVM in

TABLE 1

INHIBITION OF PVM MULTIPLICATION BY POLYSACCHARIDES*

Polysaccharide†	Virus Multiplication (Per cent of control)
Strep. MG‡	3.7
Friedländer A‡	10.0
Friedländer B‡	1.1
Friedländer C‡	6.2
Blood group A	1.6
Dextran	2.3
Strep. saliv. II‡	99.0
Pneumoc. I‡	25.0
Pneumoc. II‡	100.0
Pneumoc. III‡	67.0
Pneumoc. C	55.0
Heparin	50.0

* From Horsfall and McCarty;[3] Ginsberg *et al.*;[5] Ginsberg.[17]
† 0.1 mg. per mouse, intranasally.
‡ Capsular polysaccharide.

mice after one intranasal instillation of various polysaccharides.[3,5] The concentration of virus in the lung was measured 6 days after inoculation of 100 fifty per cent maximum score (MS_{50}) doses.[14] It will be noted that the polysaccharides which restrict multiplication of the virus to 10 per cent or less of the control value appear to have little else in common. So, too, with other polysaccharides which fail to give definite evidence of inhibitory capacity. The

dextran utilized merits comment, for it was synthesized from sucrose *in vitro* by means of a cell-free enzyme.[5] The finding that such a dextran possesses striking inhibitory activity removes any doubt that the active substance is actually polysaccharide in nature.

Each of the capsular polysaccharides derived from the three types of Friedländer bacilli is active, though none is related to any other serologically. In contrast, capsular polysaccharide of pneumococcus type II is wholly inactive, though it is related serologically to that obtained from Friedländer type B.[15] Thus, it appears that the structural configurations responsible for serological specificity are not those which cause a polysaccharide to possess inhibitory activity relative to infections with PVM.[3,5]

We were fortunate in being able to enlist the enthusiastic collaboration of Dr. Walther F. Goebel in these investigations, and he was kind enough to prepare and purify most of the polysaccharides that were studied. The relative ease with which large amounts of highly purified Friedländer polysaccharide can be obtained[15] and the degree of inhibitory activity of that obtained from type B led to the choice of this substance for extended studies.

It was found that the route of administration markedly affects inhibitory activity, and only on intranasal instillation of the substance could a definite effect against PVM be demonstrated.[3] Although quantities of the polysaccharide which inhibit multiplication of the virus cause no lung lesions in mice, larger quantities may do so.[16] This raised the possibility that the decrease in viral multiplication was due simply to some nonspecific local damage in the lung. If this were the explanation, it seemed probable that other viruses which multiply in the same tissue should be inhibited by the substance in a similar manner.

In Table 2 it is seen that the inhibitory effect is specific for PVM and that little or no effect is obtained against influenza A or B virus multiplying in the lung of the same host species.[3,5,17] Moreover, oxidation with periodic acid almost completely abolishes the pneumotoxic activity of the polysaccharide but has no effect on its inhibitory activity.[3,5,16] These results make it doubtful that nonspecific tissue damage is responsible for the observed effects. The fact that the inhibitory activity of a single instillation

TABLE 2

EFFECT OF FRIEDLÄNDER B POLYSACCHARIDE ON MULTIPLICATION OF DIFFERENT VIRUSES*

Virus†	Host	Virus Multiplication (Per cent of control)
PVM	Mouse‡	1.1
IAV	Mouse‡	63.0
IBV	Mouse‡	100.0

* From Horsfall and McCarty;[3] Ginsberg *et al.*;[5] Ginsberg.[17]
† IAV = influenza A virus; IBV = influenza B virus.
‡ 0.1 mg. polysaccharide per mouse, intranasally.

of the substance persists for 2 or more weeks[3] is correlated with the fact that the polysaccharide itself persists for very long periods in the mouse lung.[17]

That the polysaccharide does not react with the virus itself seems clear from a number of findings. The substance does not diminish infectivity when held with PVM *in vitro*[3] nor does it sediment with the virus in high gravitational fields.[17] It does not inhibit hemagglutination nor diminish adsorption of the virus by either RBC or mouse lung tissue.[3,18] That the polysaccharide does not block cell receptors is deduced from the fact that the substance does not diminish adsorption of the virus by the intact mouse lung.[3,17]

These findings suggest that the substance is active during the multiplication process itself but do not indicate whether the effect is on (*a*) penetration of the cell by the virus, (*b*) processes related to proliferation of the agent in the cell, or (*c*) release of newly formed virus particles from the infected cell.

That Friedländer polysaccharide is a potent inhibitor of multiplication of PVM is evident from quantitative studies. Figure 2 shows the degree of inhibition obtained 6 days after infection with 100 MS_{50} doses of the agent as a function of the quantity of polysaccharide given.[3] As little as 2 μg. given once before inoculation causes over 90 per cent inhibition of multiplication. More than 99 per cent inhibition is obtained with any quantity greater than 12 μg. The degree of inhibition obtained is not a function of the technique used to measure viral concentration, and meas-

urements made by infectivity titrations yield results almost identical with those secured by hemagglutination *in vitro*.[3,16]

In attempting to unravel the mechanism of the inhibitory effect, it seemed desirable to fragment the infectious process and to examine each step as closely as feasible. The ideal approach would have been to reach down to the cellular level and work with individual host cells and virus particles, as can be done with bac-

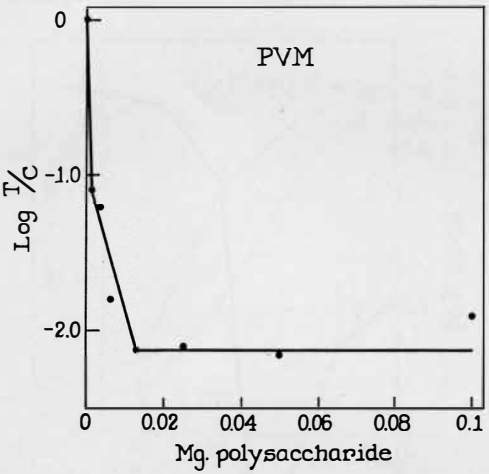


FIG. 2. Extent of inhibition of multiplication of PVM relative to the amount of Friedländer type B capsular polysaccharide given. Concentration of virus in mouse lungs was measured 6 days after inoculation with 100 MS_{50} doses; T = concentration in treated mice; C = concentration in control mice. (From Horsfall and McCarty.[3])

terial viruses. Because this has not as yet been accomplished with an animal virus, it was necessary to be content with one-step growth experiments.[19,20]

Like bacterial viruses[19,21] and influenza viruses,[20,22] PVM gives evidence of multiplying in discrete cycles.[23] One of the major tenets of modern virology is that a cycle of multiplication is attributable to the series of events which develops as a result of infection of individual host cells by individual virus particles. Each cycle has four chief parts which evolve in fixed sequence: the first is adsorption of virus by host cells; the second is the

eclipse or latent period during which the virus disappears; the third is the incremental period in which new virus appears in the cells and increases in amount; and the fourth is release of newly formed virus particles from the infected cells. In each successive cycle of multiplication, this sequence of events recurs.

Thus, the concentration of virus in infected tissue tends to increase in stepwise fashion, and each step represents one cycle of multiplication of the agent within susceptible cells. The

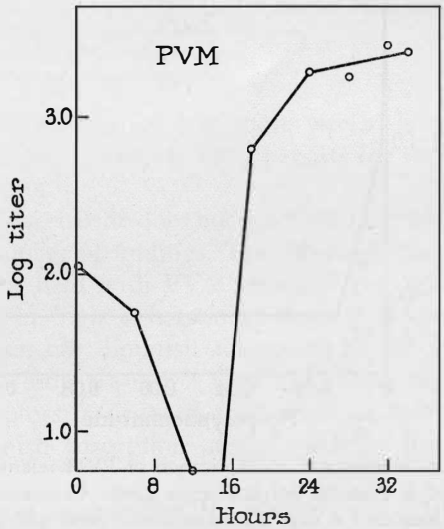


FIG. 3. Single cycle of multiplication of PVM in the mouse lung. (From Ginsberg and Horsfall.[23])

metabolic processes concerned with the development of new virus particles operate during the second and third parts of each cycle.[24-26] Virus multiplication, as such, is confined to the latent period and the incremental period.

As shown in Figure 3, inoculation of mice with a large amount of PVM leads to a series of events which has the aspects of a single cycle of multiplication.[23] Measurements by both infectivity and hemagglutination show that about 10 per cent of the virus reaching the lung succeeds in initiating infection. After inoculation there is a latent period of about 15 hours' duration which is

followed by a rise or incremental period of approximately the same length. The complete multiplication cycle requires about 30 hours, and the yield of virus or step size corresponds to an increment of approximately 16-fold.

When the polysaccharide is given during the latent period following inoculation with PVM, results such as those shown in Figure 4 are obtained.[16] No evidence of multiplication appears if the substance be given at 4 hours and only very little virus

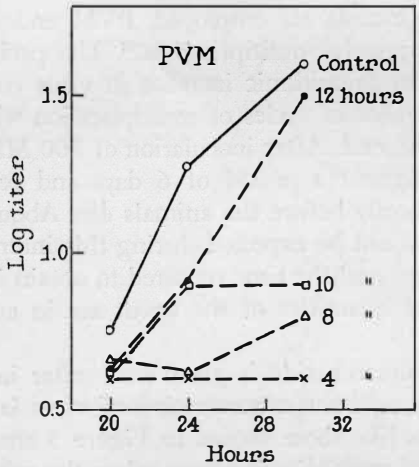


FIG. 4. Inhibition of multiplication of PVM in the mouse lung when Friedländer type B capsular polysaccharide (0.1 mg. per mouse) was given at various times during the latent period of a single cycle. The abscissa indicates time after inoculation with the virus. (From Ginsberg and Horsfall.[16])

proliferation occurs when the substance is given at 8 or 10 hours after inoculation. However, at 12 hours there is almost no effect upon the first cycle of multiplication.

Thus, the polysaccharide is inhibitory when given during the first two-thirds of the latent period. This finding affords evidence that the substance acts during intracellular multiplication and that the inhibitory effect is not attributable to prevention of penetration of the susceptible cell. In addition, the fact that at 12 hours the compound does not diminish the yield of virus from the first cycle is indicative that it does not prevent release of virus particles from

infected cells. Thus, it appears that the inhibitory effect is due to interruption of a process which leads to the development of new virus particles.

When the polysaccharide is given after the latent period, say at 18 hours, it has no effect upon the yield of virus from the first cycle of multiplication but prevents proliferation during the next cycle.[16] The available evidence indicates that the process leading to the inhibitory effect on the second cycle is identical with that on the first.

When small inocula are employed, PVM undergoes what appears to be progressive multiplication.[27] The probability is high that the apparent logarithmic increase in virus concentration results from a number of cycles of multiplication which get out of phase after the second. After inoculation of 100 MS_{50} doses, PVM multiplies throughout a period of 6 days and reaches maximal concentration shortly before the animals die. About five cycles of multiplication would be expected during this interval. The maximal yield of virus and the time required to obtain it after inoculation of different quantities of the agent are in accord with this view.

When the polysaccharide is given once after inoculation with 100 MS_{50} doses, and the concentration of virus is determined at intervals, results like those shown in Figure 5 emerge.[3] There is little evidence of multiplication even when the substance is given as late as 4 days after inoculation. Such findings indicate that the polysaccharide can inhibit proliferation of the virus during any cycle of multiplication from the first through the fourth or fifth.

To this point we have been concerned chiefly with evidence indicating that Friedländer polysaccharide acts by interrupting intracellular multiplication of PVM. To inhibit virus proliferation is one thing; to restrict the progress of a viral disease may be quite another. It will be recalled that in the initial experiments with streptococcus MG, treated mice recovered from an infection which killed control animals.[3] One may ask: What is the relation between the extent of multiplication of a virus and the extent of the disease induced? A corollary would be: Does inhibition of virus multiplication result in predictable modification of the disease process?

PVM possesses properties well suited to this problem. The extent of the lung lesions the virus induces is a function of the size of the inoculum.[27] With large inocula, complete pulmonary consolidation develops and mice then die. With small inocula, only partial consolidation occurs and animals recover. The amount of pneumonia can be estimated with considerable precision and under given conditions is closely reproducible.[27,28]

In a system such as that postulated for the multiplication of

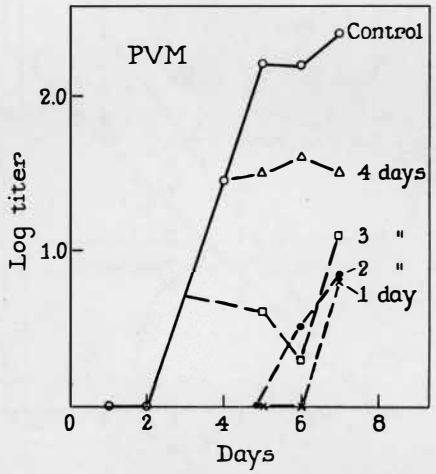


FIG. 5. Inhibition of multiplication of PVM in the mouse lung when Friedländer type B capsular polysaccharide (0.1 mg. per mouse) was given at various times after inoculation of the virus. (From Horsfall and McCarty.[3])

PVM, the virus concentration at the end of a given cycle depends upon the amount of virus inoculated. The increment of virus from each cycle is constant, but the total yield is a function of the number of host cells infected. Without going into the mathematical basis for this conclusion, let us look at the data which support it.[27] As shown in Figure 6, a straight line is obtained when the logarithm of the virus concentration divided by the amount of virus inoculated is plotted against time. The upper line depicts both the rate and extent of virus multiplication during the first 8 days of the disease. Another straight line is obtained when the logarithm of the amount of pneumonia divided by the

quantity of virus inoculated is plotted similarly. The lower line depicts both the rate of increase and the extent of lung lesions during the first 10 days of the disease.

This evidence indicates that there is a relationship between virus concentration and the extent of the disease induced.[27,29] Because the two lines shown have the same intercept at zero time and only their slopes are different, one can equate virus con-

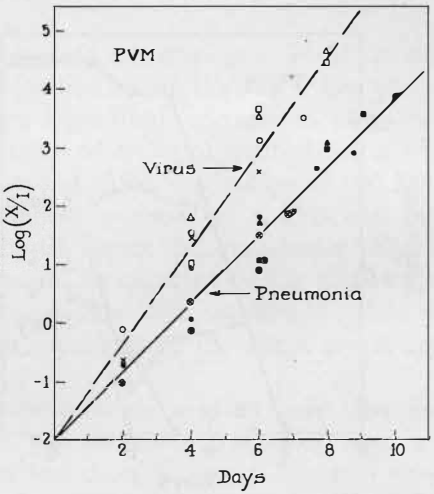


FIG. 6. Relationship between the concentration of PVM in the mouse lung and the extent of pneumonia relative to time after inoculation of the virus. X = amount of virus or pneumonia per lung; I = amount of virus inoculated. (From Horsfall and Ginsberg.[27])

centration and extent of lung lesions during the proliferative period and in fact compute one from the other. Tests show that computations of the amount of pneumonia expected as a function of virus concentration agree with observed values within ±10 per cent.[27,29]

Because the extent of pneumonia is directly related to the virus concentration, it follows that inhibition of multiplication resulting in diminished virus concentration should cause restriction in the progress of the disease.

The results of tests of such a therapeutic hypothesis are shown

in Figure 7.[16] Infected mice were treated once with 20 μg. of Friedländer polysaccharide either on the 2nd or 3rd day after inoculation with 100 MS_{50} doses of PVM. In animals treated on the 2nd day, virus multiplication was restricted to 4 per cent and pneumonia to 43 per cent of that in controls. All treated animals

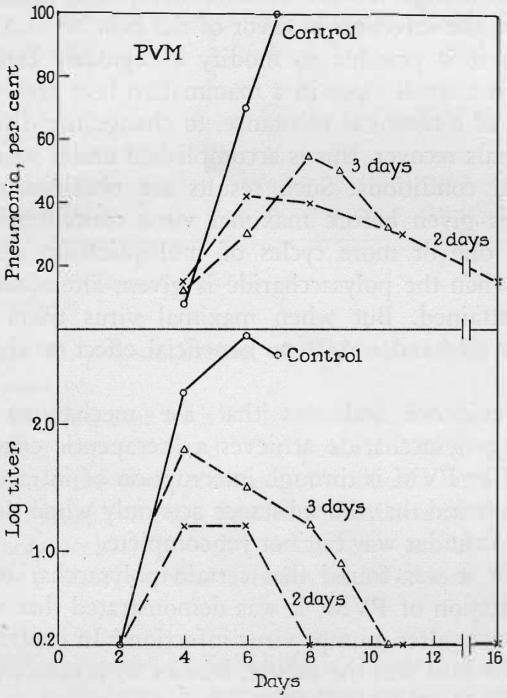


FIG. 7. Results of treatment of mice infected with PVM by Friedländer type B capsular polysaccharide (0.02 mg. per mouse) either on the 2nd or the 3rd day after inoculation of the virus. (From Ginsberg and Horsfall.[16])

recovered, but all controls died by the 7th day. On treatment at the 3rd day, virus proliferation was held to 12 per cent and lung lesions were restricted to 55 per cent of that found in controls. Two-thirds of the animals treated at this late period recovered. The extent to which the development of pneumonia was restricted corresponded, within ± 15 per cent, to what was computed from the virus concentration found in treated animals.

Thus, with PVM, reduction in virus multiplication appears to be directly correlated with reduction in the severity of the disease induced. When the polysaccharide is given so long after inoculation that virus multiplication has gone through two or three cycles[23] and the development of pneumonia has begun, the substance acts as though it were a chemotherapeutic agent and alters the course of the infection in favor of the host.[3,16]

Although it is possible to modify a regularly fatal infection induced with a small virus in a mammalian host and, with a few micrograms of a chemical substance, to change the disease so that treated animals recover, this is accomplished under strictly defined experimental conditions. Such results are obtained only if the compound is given before maximal virus concentration has developed. If one or more cycles of multiplication remain to be completed when the polysaccharide is given, alteration of the infection is attained. But when maximal virus titers have been reached, the substance exerts no beneficial effect on the infectious process.[3,16]

Present evidence indicates that the mechanism by which Friedländer polysaccharide achieves a therapeutic effect in infections caused by PVM is through interruption of intracellular virus multiplication and that the substance acts only when the proliferative process is under way but not yet complete.

Soon after it was found that certain polysaccharides interrupt the multiplication of PVM, it was demonstrated that some of the same substances alter mumps virus infection.[5] In contrast to PVM, for which the host was the mouse, studies with mumps virus were carried out in the chick embryo.

In Table 3 is shown the extent of multiplication of mumps virus in the allantoic sac after injection of various polysaccharides.[5] The concentration of virus in the allantoic fluid was measured 6 days after inoculation of 100 fifty per cent embryo infective (EI_{50}) doses. Among the substances examined, only capsular polysaccharides from Friedländer bacilli restrict multiplication of the agent. It appears clear that serological relationship between polysaccharides is not correlated with inhibitory activity relative to the mumps agent. As in the case of PVM, the serologically unrelated Friedländer polysaccharides are active, while pneu-

TABLE 3

INHIBITION OF MUMPS VIRUS MULTIPLICATION BY POLYSACCHARIDES*

Polysaccharide†	Inhibition with PVM	Virus Multiplication (Per cent of control)
Friedländer A‡	+	6.2
Friedländer B‡	+	0.8
Friedländer C‡	+	0.8
Strep. MG‡	+	100.0
Blood group A	+	100.0
Dextran	+	72.0
Pneumoc. II‡	0	59.0
Pneumoc. III‡	0	18.0

* From Ginsberg *et al.*[5]
† 1.0 mg. per chick embryo, intra-allantoically.
‡ Capsular polysaccharide.

mococcus type II polysaccharide, though related to Friedländer type B,[15] is inactive.

It will be recalled that with PVM evidence for specificity of the inhibitory effect was obtained: Compounds inhibitory for PVM had no effect on the multiplication of influenza A or B virus.[3,5] With mumps virus, there are further indications of specificity for, among 6 polysaccharides with inhibitory activity relative to PVM,[3,5] only 3 show similar activity in infections with mumps virus.[5]

TABLE 4

EFFECT OF FRIEDLÄNDER B POLYSACCHARIDE ON MULTIPLICATION OF DIFFERENT VIRUSES*

Virus†	Host	Virus Multiplication (Per cent of control)
Mumps	Ch. embryo‡	0.8
IAV	Ch. embryo‡	100.0
IBV	Ch. embryo‡	71.0
NDV	Ch. embryo‡	100.0

* From Ginsberg *et al.*[5]
† IAV = influenza A virus; IBV = influenza B virus; NDV = Newcastle disease virus.
‡ 0.6 to 1.0 mg. per chick embryo, intra-allantoically.

The data in Table 4 provide additional evidence on this point.[5] Friedländer B polysaccharide, one of the most active in infections with either mumps virus or PVM, has in the chick embryo no discernible effect on the multiplication of influenza A, influenza B, or Newcastle disease virus. Even when the smallest possible inoculum of the latter agents is employed, and polysaccharide is given some hours before the virus, multiplication is not inhibited.

Because Friedländer polysaccharide was used in detailed studies on the mechanism of the inhibitory effect with PVM in the mouse, the same substance was employed in extended investigations with mumps virus in the chick embryo. With the mumps agent, the route of administration of the compound proved to be almost as crucial as with PVM. Inhibition of virus multiplication is obtained readily if the polysaccharide is injected into the allantoic cavity. Similar inhibition occurs also on injection of the substance into the yolk sac, but only if the compound is transported to the allantoic sac, a transport which occurs in about 50 per cent of embryos.[5]

The polysaccharide causes no evidence of toxicity in the chick embryo; as much as 10 mg. injected into the allantoic sac does not affect the rate of growth or development and does not produce demonstrable microscopic lesions in the allantoic membrane or the tissues of the embryo.[5] These findings, in conjunction with those indicative of a specific effect on mumps virus proliferation, support the idea that the inhibitory effect is not the result of some nonspecific damage to the allantoic membrane.

That the polysaccharide does not act on mumps virus itself seemed clear from the fact that the compound does not reduce infectivity of the virus when held with it *in vitro*,[5] nor does it inhibit hemagglutination by the agent.[18] That the polysaccharide does not block receptors of the allantoic membrane is evident from the fact that mumps virus is absorbed in a normal manner from the allantoic fluid even when the allantoic membrane of the living embryo is exposed to 40 mg. of the substance for some hours before injection of the agent.[18]

The extent of inhibition of mumps virus multiplication obtained with various quantities of the polysaccharide is shown in Figure 8.[5] Virus concentration was measured 6 days after inocula-

tion with 100 EI_{50} doses. As little as 5 μg. given 3 hours after the virus causes more than 90 per cent inhibition of multiplication. More than 95 per cent inhibition is obtained with quantities ranging from 50 to 200 μg. As with PVM,[16] the degree of inhibition is not dependent upon the procedure used to measure virus concentration, and infectivity titrations yield results which correspond closely with those found by hemagglutination *in vitro*.[5]

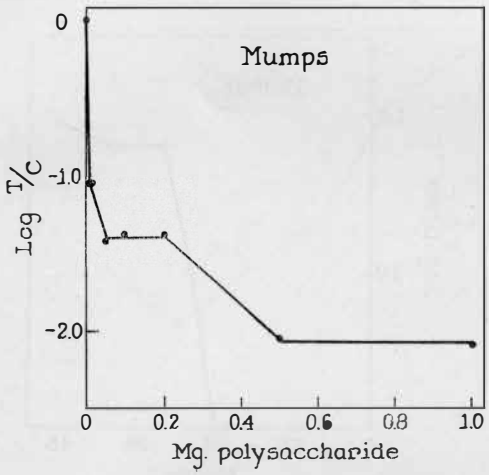


FIG. 8. Extent of inhibition of multiplication of mumps virus relative to the amount of Friedländer type B capsular polysaccharide injected. Concentration of virus in allantoic fluids was measured 6 days after inoculation with 100 EI_{50} doses; T = concentration in treated chick embryos; C = concentration in controls. (From Ginsberg *et al.*[5])

Like PVM,[23] mumps virus also gives evidence of multiplication in discrete cycles.[30] As shown in Figure 9, inoculation of very large amounts of the agent into the allantoic sac results in a series of developments which has the features of a single cycle of multiplication. There appears to be a latent period of about 24 hours' duration which is followed by a rise or incremental period of 8 to 12 hours. One complete multiplication cycle may require about 36 hours, and the yield of virus or step size corresponds to an increment of approximately 64-fold.

When the polysaccharide is given during the latent period, say

at 3 hours after inoculation with a large amount of mumps virus, results different from those obtained with PVM are found.[30] The yield of virus after the 2nd multiplication cycle at 3 days is only 10 per cent of that in controls. But after the 4th cycle at 6 days the virus concentration is nearly identical with that in controls.

As shown in Figure 10, the degree of inhibition is affected by the quantity of mumps virus injected.[5,30] When 1.0 mg. of the polysaccharide is given and virus concentration is measured at 6

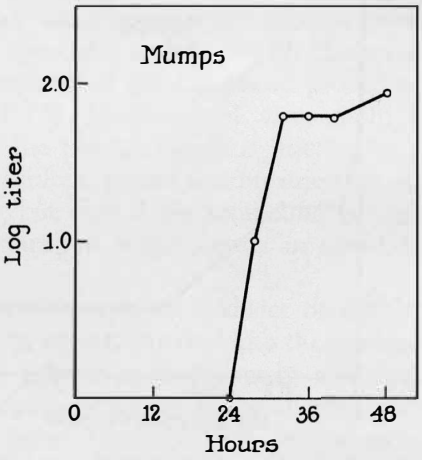


FIG. 9. Single cycle of multiplication of mumps virus in the allantoic sac. (From Ginsberg and Horsfall.[30])

days, more than 97 per cent inhibition is obtained after inocula of 10 to 1,000 EI_{50} doses, but a progressive decrease in inhibition develops as more and more virus is given, and after 10^6 EI_{50} doses there is none.

In seeking an explanation, the possibility of a variant virus resistant to the effects of the polysaccharide arose. A number of facts pointed in this direction. With small inocula, the polysaccharide restricts multiplication markedly but does not completely interrupt proliferation of the mumps agent. Even relatively large amounts of polysaccharide, for example 1.0 mg. per embryo, given after small inocula of virus do not entirely prevent multiplication.[5] With large inocula, although multiplication is re-

stricted by the substance during 2 cycles, maximal proliferation of the agent eventually occurs.[30]

The occurrence of a resistant variant of mumps virus is readily demonstrable. Serial passage of the agent along with small amounts of the polysaccharide, say 50 μg., results regularly in the emergence of such a variant. Moreover, a single passage with a large inoculum in chick embryos given 1.0 mg. of the substance yields a resistant strain by the 6th day. Available techniques do

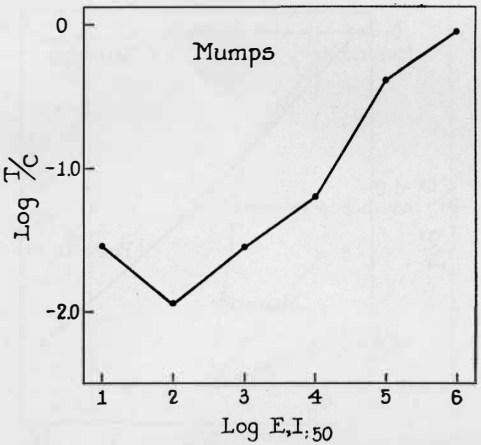


FIG. 10. Extent of inhibition of multiplication of mumps virus by Friedländer type B capsular polysaccharide (1.0 mg. per chick embryo) relative to the amount of virus inoculated. T = concentration of virus in treated chick embryos; C = concentration in controls. (From Ginsberg *et al.*;[5] Ginsberg and Horsfall.[30])

not permit a reliable determination of the proportion of the resistant variant in the virus population, but a ratio of 1 variant to 10,000 typical particles would conform with the data.[30]

The extent to which the variant is resistant to inhibition by the polysaccharide is illustrated in Figure 11.[30] The concentration of the parent or variant virus was measured 6 days after inoculation of 100 EI_{50} doses in embryos given various quantities of polysaccharide. Although multiplication of the parent virus is markedly inhibited by as little as 50 μg., that of the variant is unaffected by as much as 1.0 mg. Resistant strains could not be maintained in-

definitely on passage in the absence of the polysaccharide. After numerous serial passages along with the substance, full sensitivity to the inhibitory effect was regained on 5 passages without the polysaccharide in normal embryos.[30]

Except for resistance to the effects of the polysaccharide, the variant virus possesses no other properties which serve clearly to distinguish it from the parent agent. Infectivity, hemagglutinating, enzymatic, and immunological features are indistinguishable

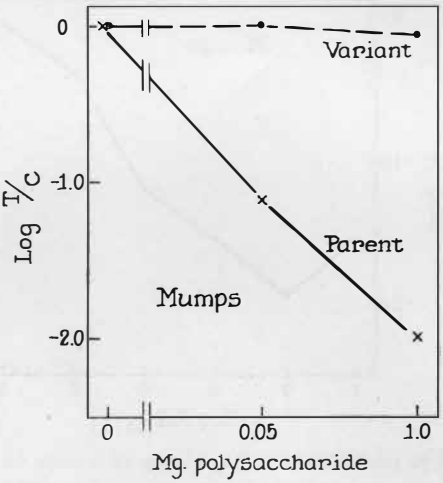


FIG. 11. Resistance of a variant of mumps virus to inhibition by Friedländer type B capsular polysaccharide. T = concentration of virus in treated chick embryos; C = concentration in controls. (From Ginsberg and Horsfall.[30])

from those of typical mumps virus. There is, however, evidence indicating that the rate of multiplication is slower than that of the parent.[30]

Segregation of a resistant variant from a virus population by an inhibitory chemical substance is formally analogous to selection of resistant mutants from bacterial populations by chemotherapeutic agents.[31] That the variant can be obtained at will and from various strains of the agent is indicative that mumps virus populations are not homogeneous and that natural variants in such populations possess distinctive properties. Procedures available

for the study of animal viruses do not permit rigorous proof that the variant is in fact a mutant, but it seems obvious that the findings are not at odds with such a view.

When small inocula of mumps virus are employed, the multiplication of the agent appears to be progressive and, as with PVM, discrete cycles after the first tend to disappear in a curve of logarithmic increase.[30] After 100 EI_{50} doses are given, the virus

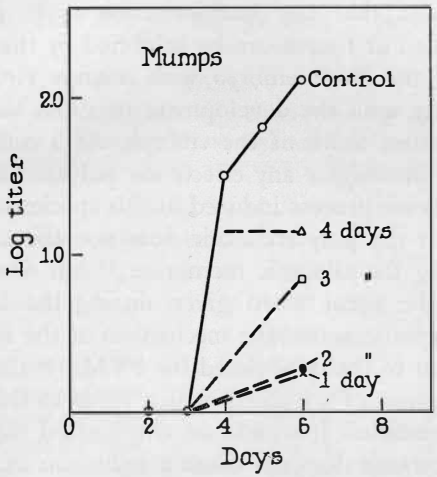


FIG. 12. Inhibition of multiplication of mumps virus in the allantoic sac when Friedländer type B capsular polysaccharide (1.0 mg. per chick embryo) was given at various times after inoculation of the virus. (From Ginsberg *et al.*[5])

multiplies in the allantoic sac during a period of 6 days. About four cycles of multiplication can be expected in this interval. Both the maximum yield of virus and the time required to obtain it after inoculation of various quantities of the agent are in accord with this concept.

When 1.0 mg. of the polysaccharide is given after inoculation of 100 EI_{50} doses and the concentration of virus is measured at the 6th day, results such as shown in Figure 12 are obtained.[5] It will be noted that there is only slight evidence of proliferation when the substance is given either on the 1st or the 2nd day after

inoculation. Little multiplication of the agent occurs when the substance is given on the 3rd day, and, even when it is not given until the 4th day, there is definite limitation in the degree of multiplication.

These findings indicate that, despite the potential occurrence of a resistant variant, the polysaccharide markedly impedes multiplication of mumps virus at any time through the 4th day after inoculation, as is the case also with PVM.[3] In addition, they support the idea that any multiplication cycle from the first through the third or fourth can be inhibited by the substance.

Infection of the chick embryo with mumps virus is not associated constantly with the development of gross lesions and does not regularly cause death of the embryo. As a consequence, it is not feasible to investigate any effects the polysaccharide may have on the mild disease process induced in this species by the agent.

The fact that the polysaccharide does not affect adsorption of mumps virus by the allantoic membrane,[18] but does inhibit multiplication of the agent when given during the latent period,[30] supports the hypothesis that the mechanism of the inhibitory effect is closely similar to that postulated for PVM:[3,16] that inhibition is due to interruption of a process which leads to the development of new virus particles. It should be emphasized that, as too with PVM,[3] the substance does not cause a reduction in the concentration of preformed virus and an inhibitory effect is demonstrable only if the substance is given before maximal virus concentration has developed.[5]

With both PVM and mumps, attention has been drawn to the specificity of the inhibitory action of the polysaccharide. In the mouse, the substance has no effect on the multiplication of influenza A or B virus.[5,5,17] In the chick embryo, the compound does not alter the proliferation of either of these agents or of Newcastle disease virus.[5] If, as the evidence suggests, the inhibitory action of the polysaccharide is attributable to blocking of cellular metabolic systems necessary for virus multiplication, then it appears that influenza and Newcastle disease viruses do not demand the same metabolic systems that PVM and mumps virus need for proliferation.

One approach to this hypothesis is by means of interference

experiments. There is much evidence pointing to the probability that interference is dependent on competition between two viruses for cellular systems essential to multiplication of the agents.[32-34] If viral interference and chemical inhibition of virus multiplication are dependent upon similar metabolic mechanisms, then there should be a close correlation between the results of the two types of experiments with the same agents.

A summary of evidence indicating that there is such a correlation is shown in Table 5. Interference experiments with two

TABLE 5

CORRELATION BETWEEN CHEMICAL INHIBITION OF MULTIPLICATION AND INTERFERENCE*

Effect of Polysaccharide† on Multiplication	Viruses	Interference	
		Between Viruses	In Host Species
Inhibition of both	PVM-Mu	+	Mouse
Inhibition of one, not the other	PVM-IA	0	Mouse
Inhibition of one, not the other	PVM-IB	0	Mouse
Inhibition of one, not the other	Mu-IA	0	Mouse
Inhibition of one, not the other	Mu-IB	0	Ch. embryo
Inhibition of one, not the other	Mu-IA	0	Ch. embryo
No effect on either	IA-IB	+	Mouse
No effect on either	ND-IA	+	Mouse
No effect on either	IA-IB	+	Ch. embryo
No effect on either	IA-ND	+	Ch. embryo
No effect on either	ND-IB	+	Ch. embryo

* From Ginsberg and Horsfall;[35,37] Ginsberg.[36]

† Friedländer B polysaccharide.

viruses are of necessity carried out in one host species. Although mumps and Newcastle disease viruses do not multiply, in the classical sense, in the mouse, it has been demonstrated that both agents cause reactions in this species which make it feasible to employ them in interference experiments.[35,36] Friedländer polysaccharide markedly inhibits proliferation of both PVM[3,16] and mumps virus.[5] Presumably, therefore, these two viruses require some of the same metabolic systems for multiplication. From this

one may predict that they should show interference, as has been demonstrated in fact, in the mouse.[35] Although the polysaccharide inhibits multiplication of either PVM or mumps virus, it has no effect on proliferation of influenza A or B virus either in the mouse or the chick embryo.[3,5,17] As a consequence, it may be assumed that influenza viruses do not require the metabolic systems blocked by polysaccharide. If the systems needed by PVM and mumps virus are different from those required by influenza viruses, it would be expected that PVM and influenza viruses, as well as mumps and influenza viruses, should not show interference. This expectation is borne out by the results of experiments in either the mouse or chick embryo.[37] Influenza and Newcastle disease viruses show reciprocal interference in both host species.[36,38-40] Therefore, these three agents are assumed to utilize similar metabolic systems during multiplication. If the requirements of these agents are in fact similar, one may anticipate that a substance which has no effect on the multiplication of one should not affect multiplication of the others. As is indicated, this is indeed the case.[3,5,17]

This correlation between the results of dissimilar experiments with five viruses in two host species does not of itself provide direct evidence as to the nature of the mechanisms of virus interference or chemical inhibition of virus multiplication, but it does afford support for the idea that the mechanisms are similar.

Although much has been learned in these studies on the mechanism by which a polysaccharide inhibits the multiplication of two animals viruses, there seems to be small likelihood that the investigations can be carried to a point where the mechanism can be described in precise biochemical terms. The great difficulties of polysaccharide structural chemistry, the uncertainty regarding cell systems which might be blocked by these complex substances, and the lack of effective antagonists of known constitution have led us to undertake studies with other inhibitory substances of precisely defined structure.

For studies on the mechanism of chemical alteration of virus infections, influenza viruses possess certain advantages over PVM and mumps virus. Unlike the latter agents, influenza viruses are pathogenic for a number of small animal species. In addition, the

influenza viruses have relatively short cycles of multiplication,[20,22] they readily and rapidly proliferate in tissue culture systems, and their concentration can be measured with considerable precision *in vitro*.[41]

In the hope that host cell metabolism might be affected in a selective manner and the proliferation of influenza virus restricted in consequence, Dr. Igor Tamm initiated and carried forward a series of studies with analogs of certain of the B vitamins. When it was discovered that some of these compounds did in fact inhibit the multiplication of influenza A and B viruses, it became evident that an extended investigation was warranted. We were fortunate in being able to interest Dr. Karl Folkers, Research Laboratories

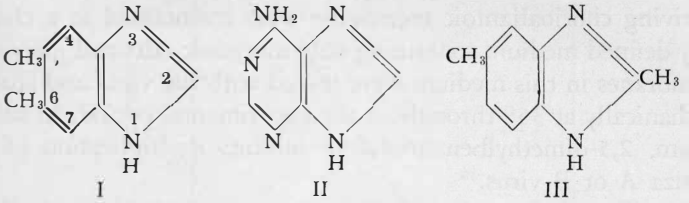


FIG. 13. Structure of 5,6-dimethylbenzimidazole (I), adenine (II), and 2,5-dimethylbenzimidazole (III). (Courtesy, *Yale J. Biol. & Med.*, 24, 559, 1952.)

of Merck & Company, in this problem. He became a close collaborator and kindly undertook the preparation of a variety of compounds.

Because it seemed probable that an alteration in nucleoprotein metabolism would be reflected in an effect upon virus multiplication, it was decided to concentrate efforts on compounds bearing some relation to vitamin B_{12}. This vitamin is known to play an important role in nucleoprotein metabolism. That there is a close metabolic relationship between vitamin B_{12} and desoxyribonucleic acid is apparent from considerable evidence involving microbial growth.[42] The vitamin contains a benzimidazole moiety[43] and, when it was found that certain substituted benzimidazoles possess inhibitory activity relative to influenza virus multiplication,[44] a detailed study with such compounds was undertaken.

In Figure 13,[45] are shown the structure of 5,6-dimethylbenzi-

midazole (I), the moiety present in vitamin B_{12}; that of the purine adenine (II); as well as that of 2,5-dimethylbenzimidazole (III), an analog of the B_{12} moiety. It will be noted that the purine and the two benzimidazole derivatives all possess a bicyclic skeleton of the same size and have a common imidazole ring. From this it appears that there is a relationship between the structural configuration of adenine, a constituent of nucleic acid, and that of the benzimidazole moiety of vitamin B_{12}, which regulates nucleic acid metabolism. As will be demonstrated, both the 5,6-dimethyl and the 2,5-dimethyl benzimidazole derivatives are active as inhibitors of influenza virus multiplication.

In order to simplify the virus-host cell system as much as possible and to make results as reproducible as feasible, portions of surviving chorioallantoic membrane were maintained in a chemically defined medium containing only inorganic salts and glucose.[46] Membranes in this medium were mixed with the virus and shaken mechanically at 35° throughout the experimental period. In such a system, 2,5-dimethylbenzimidazole inhibits multiplication of influenza A or B virus.[45]

As illustrated in Figure 14, at a concentration of about 0.0015 *M*, the substance causes a definite diminution in the yield of influenza B virus.[45] The degree of inhibition increases as the concentration of the substance is increased under the fixed experimental conditions. Such a relationship between the concentration of benzimidazole derivative and the extent of restriction in virus multiplication obtains with all the compounds studied thus far.[47-50] This makes it possible to determine with considerable precision the quantity of a compound capable of causing inhibition of fixed extent. For purposes of comparison, the activity of various derivatives is expressed in terms of the molar concentration capable of causing 75 per cent inhibition of multiplication.

That the inhibitory effect is not due to direct action of the substance on the virus is evident from the following findings: The compound does not inactivate the infectivity of influenza virus when held with it *in vitro*. Moreover, it does not inhibit hemagglutination by the agent and does not diminish adsorption of the virus by susceptible host cells. That the inhibitory activity of the compound is not attributable to either nonspecific or

irreversible effects is indicated by the facts that it does not affect the oxygen consumption of the host tissue and that, after prolonged exposure to the substance, membranes still retain in undiminished degree the capacity to support influenza virus multiplication when the concentration of the compound is sufficiently reduced.[45]

These findings led to an analysis of the effects of various other alkyl derivatives of benzimidazole on the multiplication of in-

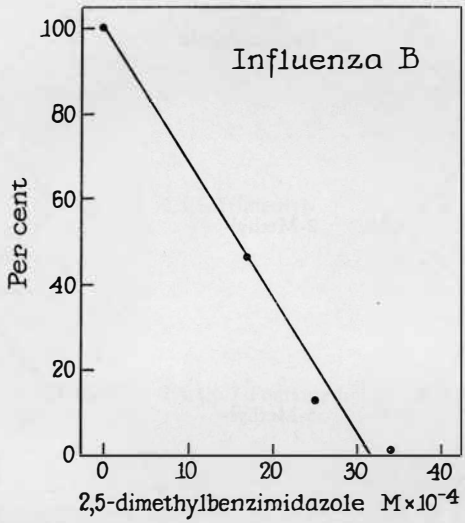
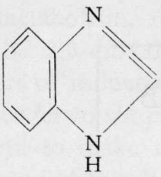
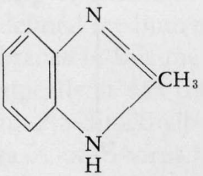
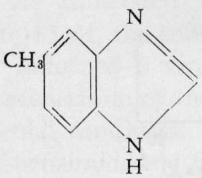
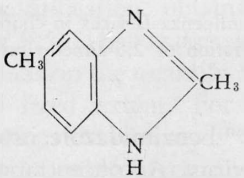


FIG. 14. Extent of inhibition of multiplication of influenza B virus in chorioallantoic membranes *in vitro* relative to the concentration of 2,5-dimethylbenzimidazole in the medium. (From Tamm *et al.*[45])

fluenza viruses. As is shown in Table 6,[47-50] benzimidazole itself inhibits the multiplication of influenza B virus. A concentration of no more than 0.0035 *M* causes 75 per cent inhibition in the multiplication of the agent. The addition of a methyl group at the 2-position in the imidazole ring has, as can be seen, no definite effect on the inhibitory activity of the compound. However, the addition of such a group at the 5-position in the benzene ring yields a substance that is twice as active as the unsubstituted compound. The inhibitory activity of the 5,6-dimethyl compound,

TABLE 6

INHIBITION OF INFLUENZA B VIRUS MULTIPLICATION BY BENZIMIDAZOLE DERIVATIVES*

Benzimidazole Derivative		Inhibitory Conc.†	Inhibitory Activity Relative to Benzimidazole
		M × 10^{-4}	
	Benzimidazole	35	1.0
	2-Methyl-	31	1.1
	5-Methyl-	19	1.8
	2,5-Dimethyl-	13	2.7

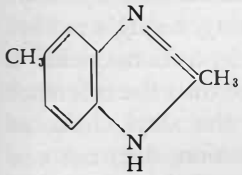
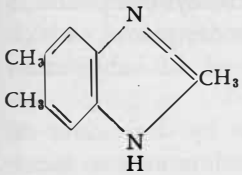
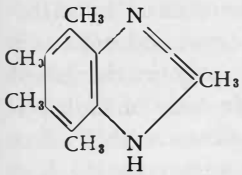
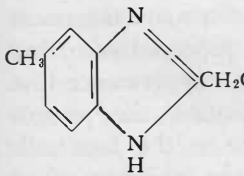
* From Tamm *et al.*[47–50]

† Concentration yielding 75 per cent inhibition of multiplication.

the moiety present in vitamin B_{12}, is almost identical with that of the 5-methyl compound shown in this table. That a substituent group at the 2-position does bear on the activity of these derivatives is evident from the results obtained when methyl groups are placed at both positions 2 and 5. With the 2,5-dimethyl com-

TABLE 7

INHIBITION OF INFLUENZA B VIRUS MULTIPLICATION BY BENZIMIDAZOLE DERIVATIVES*

Benzimidazole Derivative		Inhibitory Conc.†	Inhibitory Activity Relative to Benzimidazole
		$M \times 10^{-4}$	
	2,5-Dimethyl-	13	2.7
	2,5,6-Trimethyl-	8.9	3.9
	2,4,5,6,7-Pentamethyl-	2.2	16
	2-Ethyl-5-methyl-	1.8	19

* From Tamm *et al.*[47–50]

† Concentration yielding 75 per cent inhibition of multiplication.

pound, inhibitory activity is nearly 3 times greater than that of benzimidazole itself.

As is shown in Table 7,[47–50] the addition of further methyl groups at various positions in the benzene ring leads to a progressive increase in inhibitory activity when a methyl group is

also present at position 2 in the imidazole ring. It is seen that the 2,5,6,-trimethyl compound is about 4 times more active than the reference compound, unsubstituted benzimidazole. The 2,4,5,6,7-pentamethyl compound is over 16 times more active as an inhibitor than is benzimidazole. In this case, a concentration of only 0.0002 *M* is sufficient to cause 75 per cent inhibition in the multiplication of influenza B virus. This concentration is equivalent to 40 μg. of the substance per ml. In addition, if an ethyl rather than a methyl group is added at position 2 in the imidazole ring, a single methyl substituent in the benzene ring, as at the 5-position, yields a highly active compound 19 times more effective than the reference substance. To increase further the length of the alkyl chain, as with a propyl or butyl substituent at the 2-position, does not add to activity. It will be noted that the 2-ethyl 5-methyl compound is 7 times more active than the 2,5-dimethyl compound, which provides additional evidence of the importance of substituents at the 2-position in the imidazole ring.

Inhibition of influenza virus multiplication by derivatives of benzimidazole is not dependent upon the procedure used to measure virus concentration and is as readily demonstrated by infectivity titrations as by hemagglutination *in vitro*. Inhibition is obtained when an active compound is added early in the latent period and can be demonstrated during a single cycle of multiplication.[47,48] Because such compounds have no demonstrable effect upon influenza viruses *per se,* nor upon their adsorption by host cells, it seems probable that their inhibitory effects are the result of some mechanism which alters biochemical processes occurring during virus multiplication. In the absence of any evidence that viruses themselves carry out metabolic activities, the present tendency is to ascribe the altered metabolism to the host cell. Whether the metabolic processes involved in the inhibitory effect of benzimidazole derivatives are in fact related to nucleoprotein synthesis remains to be determined.

It is clear that the structural configuration of various alkyl derivatives of benzimidazole markedly affects their capacity to cause inhibition of the multiplication of influenza viruses under the experimental conditions employed.[47–50] Synthetic compounds of precisely known structure may prove to be potent tools of wide

usefulness in attempts to unravel some of the problems of virus proliferation.

As was indicated earlier, a major objective of the investigations described this evening has been to define the mechanisms through which chemical substances alter infectious processes induced by animal viruses. It is obvious that this objective has not as yet been achieved. However, the efforts expended in pursuing it have not been unrewarding, and a considerable amount of new information has been acquired during the elusive chase.

To summarize briefly: From studies on kinetics of inhibition there emerges the concept that both the polysaccharides and benzimidazole derivatives examined impede virus multiplication by affecting processes which occur during the latent period. Such processes appear to be metabolic and this leads to the idea that they are features of the host cell rather than the proliferating virus. Should it be found that a virus in action, that is, a virus multiplying in a living host cell, possesses attributes different from a virus *in vitro*, and actively participates in metabolic transformations, this hypothesis may require drastic revision.

That the affected processes are subtle and not such as are essential to continued life of the host cell is deduced from the lack of demonstrable toxicity of inhibitory substances and the absence of an effect on oxidative metabolism by benzimidazole derivatives. Further evidence for the subtle nature of these processes stems from the specificity of the inhibitory activity of certain substances, particularly the polysaccharides which interrupt the multiplication of certain viruses but do not affect that of others in the same host tissue. This has led to the hypothesis that some viruses require metabolic processes different from those needed by others, a concept which is supported by the results of interference experiments with the same agents.

That multiplication is itself impeded by the effects of inhibitory substances appears from the fact that the yield of newly formed virus particles from infected cells is diminished, and the expected complement of new virus cannot be found no matter how the host cells may be fragmented or extracted.

Finally, in the case of pneumonia virus of mice, an animal virus with dimensions of only 40 mμ, it appears that there is a direct

relationship between concentration of virus in the lung and the objective manifestations of the disease, which is to say the amount of pneumonia. With this agent, inhibition of multiplication, under strictly defined conditions, results in a predictable diminution in the pathological process. This, then, is an instance in which the administration of a chemical substance during the course of a severe disease modifies the virus infection so that the host recovers.

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