THE MODIFYING EFFECTS OF CERTAIN SUBSTANCES OF BACTERIAL ORIGIN ON THE COURSE OF INFECTION WITH PNEUMONIA VIRUS OF MICE (PVM)

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In the course of investigations concerned with problems relative to the pathogenesis of primary atypical pneumonia, a study was undertaken on the effects of inoculating mice with both a virus and a bacterium. The virus employed in these experiments is known as pneumonia virus of mice, and will hereafter be designated PVM. The bacterium used is a non-hemolytic streptococcus, designated streptococcus MG.

When the first experiments were carried out, it was considered that either of two possible results might develop; first, that streptococcus MG would have no discernible influence on the course of an infection induced by PVM; or second, that it might, by contributing to the establishment of a complex infection, cause the results to be more severe than those of infections induced by PVM alone. Surprisingly, neither possibility evolved; instead, the inoculation of streptococcus MG in mice which previously had been inoculated with PVM resulted in a distinctly less severe infection.

This unexpected finding was investigated in detail. It is the purpose of this paper to present the results of studies on the modifying effect of certain bacterial species upon the course of infection with PVM. It will be shown that a number of bacterial species are capable of causing such an effect and that this modification, in striking degree, is obtained when suitable bacteria are given either some days before or after inoculation with this virus. Evidence is presented which indicates that this modification phenomenon is the result of inhibition of multiplication of PVM in lung tissue infected with the virus. In addition, data are presented which suggest that the substance responsible for modification with respect to this virus may be polysaccharide in nature.

Methods

Virus.—Pneumonia virus of mice (PVM), strain 15 (1), was used exclusively. It was maintained by occasional lung passage in albino Swiss mice and stored at -70°C. in a manner identical with that described previously (2).

Virus Titrations.—Titrations of PVM were carried out in groups of albino Swiss mice. Serial decimal dilutions were employed; each dilution was tested in a group of six mice and in each titration at least thirty mice were used. The technique of titrations, as well as the method of calculation of the 50 per cent maximum score end point (M.S.50), was identical with

those described previously (2). Virus titration end points are expressed as the logarithm of the final dilution of infected mouse lungs.

Hemagglutination Titrations.—The technique of hemagglutination titrations with mouse RBC, utilizing serial twofold dilutions, as well as the method of estimating end points, was identical with those previously employed (3). As routine, a 10 per cent suspension in saline of the lungs of a group of six mice was prepared, heated in a water bath at 70°C. for 30 minutes, then centrifuged at 8,000 R.P.M. for 10 minutes, and the supernate tested for hemagglutination. Hemagglutination titration end points are expressed as the logarithm of the final dilution of such supernates.

Reproducibility of Titration End Points.—The results of previous studies (6) showed that virus titration end points with PVM, when carried out under the conditions used in these experiments, are reproducible to ± 0.15 logarithmic unit. They also showed that hemagglutination titration end points with PVM are reproducible to ± 0.10 logarithmic unit.

Bacteria.—The following bacterial species were employed: streptococcus MG (4); *Streptococcus salivarius*, type II (4); Pneumococcus, R36A; Group A hemolytic streptococcus, type 6 (strain S43, glossy); *H. influenzae* type B; and *E. coli*. In each instance appropriate broth cultures were incubated at 37°C. for 18 hours.

Polysaccharides.—The following preparations containing polysaccharides of bacterial origin were used: capsular polysaccharide derived from streptococcus MG by means of an alkali extraction method described previously (4); capsular polysaccharide derived from *Streptococcus salivarius*, type II, by means of a water extraction method described previously (4); specific somatic antigen[1] of *Shigella paradysenteriae*, type V (5); a hapten[1] derived from the latter preparation by a procedure previously described (5); capsular polysaccharide of Pneumococcus, type I; capsular polysaccharide of Pneumococcus, type III; somatic C polysaccharide of Pneumococcus; and capsular polysaccharide[1] of Friedländer bacillus, type B. In addition, the following polysaccharide preparations were employed: blood group A specific substance;[1] agar-agar;[1] gum acacia;[1] Lintner's soluble starch;[1] and heparin. Unless otherwise stated, polysaccharide preparations were tested at a concentration of 2 mg. per cc. in saline and had been heated at 56°C. for 30 minutes.

EXPERIMENTAL

I. Effect of Bacterial Inoculation on the Results of Infection with PVM

Effect of Streptococcus MG.—The effect of intranasal inoculation of streptococcus MG on virus titration end points obtained with PVM in mice was tested.

Groups of mice were inoculated intranasally with dilutions of PVM from 10^{-1} to 10^{-6} in broth containing 10 per cent normal horse serum. Two days and 4 days, respectively, after the virus dilutions had been given, the animals received a second intranasal inoculation *i.e.* 0.05 cc., which consisted either of sterile broth or of an 18 hour broth culture of streptococcus MG. Both the encapsulated bacterium (S), as well as a non-encapsulated variant (R), was used. Mice which died were autopsied and their lungs examined for the presence and amount of pulmonary consolidation. Those which survived the observation period of 12 days were killed and the extent of pulmonary consolidation present in their lungs was recorded. From these data the 50 per cent maximum score end point (7) was determined. It is of importance to point out that the 50 per cent maximum score end point takes into account not only the

[1] These preparations were kindly provided by Dr. Walther F. Goebel, the Rockefeller Institute.

frequency of fatal infections but also the frequency and the degree of pulmonary consolidation in mice which die, as well as in those which survive until killed As is true of titrations of influenza virus in mice (8), this end point is more reproducible with PVM than any other end point so far tested (2). For convenience, this titration end point will be referred to hereafter as M.S.50 end point.

TABLE I

Results of Titrations of PVM in Mice Given Streptococcus MG Intranasally

First inoculation 0.05 cc. I.N.	Interval	Second inoculation 0.05 cc. I.N.	Time of death and extent of pulmonary consolidation in individual mice	Lung lesion score*	Virus titration end point M.S. 50 Log
	days				
PVM dilution					
10^{-1}	2	Sterile broth	D7‡,D7,D7,D8,D8,D10,S++§	32/35	
10^{-2}	"	" "	D7,D7,D7,D7,D7,D8,S++	32/35	
10^{-3}	"	" "	D7,D7,D8,D8,D8,D8,D11	35/35	$10^{-3.85}$
10^{-4}	"	" "	S++,S++,S+,S+,S±	6/25	
10^{-5}	"	" "	S++,S++,S+,S+,S+,S+,S0	7/35	
10^{-1}	2	Strep. MG (S) broth culture	D7,D8,D8,D9,S+++,S+++, S+++	29/35	
10^{-2}	"	" "	D8,D8,D9,S++,S++,S++, S+	22/35	
10^{-3}	"	" "	D9,S+++,S++,S++,S++, S++,S+	17/35	$10^{-2.59}$
10^{-4}	"	" "	D11,S++,S++,S±,S0,S0	9/30	
10^{-5}	"	" "	S±,S0,S0,S0,S0,S0,S0	0/30	
Sterile broth	—	None	S0,S0,S0,S0,S0,S0,S0	0/35	
Strep. MG (S) broth culture	—	"	S0,S0,S0,S0,S0,S0,S0	0/35	

I.N. = intranasal.

* Numerator = sum of arithmetic values 1, 2, 3, 4, and 5 assigned to lung lesions corresponding to +, ++, +++, ++++, and D++++, respectively.

Denominator = number of mice multiplied by 5 (*i.e.*, maximum possible score).

‡ D7 = mouse died with ++++ pulmonary consolidation on 7th day.

§ S++ = mouse survived 12 days but at autopsy showed ++ pulmonary consolidation.

The results which were obtained in one part of the experiment are presented in detail in Table I. It will be observed that there is a striking difference in the frequency with which fatal infection and extensive pulmonary consolidation occurred in mice which received streptococcus MG 2 days after inoculation with PVM as compared with those which received broth. An amount of virus (*i.e.*, a dilution of 10^{-3}), which caused complete consolidation of the lungs, and death of all mice given sterile broth, led only to the development of approxi-

mately 50 per cent pulmonary consolidation and the death of but a single mouse among those given a broth culture of streptococcus MG. Similarly, among mice inoculated with a 10^{-5} dilution of PVM, those which subsequently received broth showed, on the average, approximately 25 per cent pulmonary consolidation, whereas those which received streptococcus MG showed, with one questionable exception, no evidence of pneumonia. The virus titration end point (M.S.50) obtained in mice which were given sterile broth was $10^{-3.85}$; in mice which received broth culture of streptococcus MG it was $10^{-2.59}$. The difference between these two virus titration end points (*i.e.*, $-3.85-(-2.59)=$ -1.26 logarithmic units) indicates that at least 18 times (*i.e.*, log 18 = 1.26) more virus was required to cause pneumonia and death in mice given streptococcus MG as compared with control animals given sterile broth.

TABLE II

Effect of Intranasal Instillation of Streptococcus MG on the Results of PVM Titrations in Mice

First inoculation 0.05 cc. I.N.	Interval	Second inoculation 0.05 cc. I.N.	Virus titration end point M.S. 50 Log	Difference from broth control Log
	days			
PVM dilutions	—	None	−4.45	—
" "	2	Sterile broth	−3.85	—
" "	"	Strep. MG (S)	−2.59	−1.26
" "	"	" " (R)	−2.79	−1.06
" "	4	Sterile broth	−5.00	—
" "	"	Strep. MG (S)	−3.82	−1.18
" "	"	" " (R)	−3.39	−1.61
Geometric mean				−1.27

S = encapsulated type-specific bacterium.
R = non-encapsulated variant.

The results which were obtained in the complete experiment are presented in Table II. In this table, as in those which follow, the detailed experimental results are condensed as much as possible in the interest of both conciseness and precision, and a single value, the M.S.50 virus titration end point, is used to give quantitative expression to all of the results which were obtained in individual mice in each virus titration. As was indicated above, at least thirty mice were used in each titration. From the data shown in Table II it can be seen that, when streptococcus MG was given intranasally, the virus titration

end point was significantly lower in each instance than it was in mice which received sterile broth. The geometric mean difference (*i.e.*, the sum of the differences expressed as logarithms divided by the number of differences) of the titration end points from the corresponding control end points was −1.27 logarithmic units which represents an end point lower by 20-fold. This surprising result indicates that the effect of streptococcus MG was to lessen the consequences of infection with PVM. Because of the regularity with which the virus titration end point was lowered, even though the bacterium was given as long as 2 or 4 days after the virus had been inoculated, the modifying effect was investigated in more detail.

Streptococcus MG in Mouse Lungs.—Although, as was shown previously (4), streptococcus MG is not pathogenic for mice, it was not known for what period the bacteria would survive in the lungs of mice following intranasal inoculation.

Groups of mice either untreated, or those which had received 1,000 M.S.50 doses of PVM 24 hours previously, were given undiluted culture of streptococcus MG intranasally. Animals were killed at 2 hour intervals thereafter and cultures were made from the cut surfaces of their lungs.

It was found that the streptococcus could be recovered from the lungs of almost all inoculated mice during the first 10 hours after instillation of the bacteria but that in no instance could it be recovered later than the 14th hour and did not later reappear. It appears, therefore, that this microorganism is incapable of surviving for more than a relatively short time in the mouse lung and that the period of survival is not increased if the mouse has been infected previously by PVM. The intranasal inoculation of mice with streptococcus MG did not lead to the development of pulmonary lesions discernible either in the gross or on microscopic examination.

Reproducibility of Modification.—Further experiments were carried out to determine the reproducibility of the modifying effect of streptococcus MG on infection induced with PVM.

Groups of mice, which had been given appropriate dilutions of PVM intranasally, were inoculated with one of the following: sterile broth, streptococcus MG culture, the supernatant fluid from such a culture following centrifugation at 10,000 R.P.M., or sedimented bacterial cells resuspended to original volume in saline.

The results of this experiment are shown in Table III. It will be seen that in mice, which received either whole culture or resuspended bacterial cells 3 days after inoculation with PVM, the virus titration end point was significantly lower than in mice which received broth or culture supernate. Moreover, when the order of the inoculations was reversed, and virus was given 2 days after intranasal inoculation of bacteria, similar results were obtained. It will be evident also that mixing resuspended bacterial cells with either normal serum or antistreptococcus MG serum had no effect upon modifying activity. This immune serum had a titer of 1:5,000 in agglutination tests against streptococcus MG. Both the normal and the immune serum were shown to possess

no neutralizing antibodies against PVM. The geometric mean difference in virus titration end points between the broth controls and the various groups of mice which received preparations containing streptococcus MG was −1.79 logarithmic units; a 60-fold lower end point. To state this in another way, the results obtained indicate that it required 60 times more virus to produce comparable pulmonary disease in mice given streptococcus MG as compared with control animals.

TABLE III

Effect of Streptococcus MG on the Results of PVM Titrations in Mice

First inoculation 0.05 cc. I.N.	Interval	Second inoculation 0.05 cc. I.N.	Virus titration end point M.S. 50 Log	Difference from broth control Log
	days			
PVM dilutions	—	None	−4.43	—
" "	3	Sterile broth	−4.59	—
" "	"	Strep. MG (S)	−2.35	−2.24
" "	"	" supernate	−4.27	−0.32
" "	"	" cells	−2.46	−2.13
Sterile broth	2	PVM dilutions	−3.14	—
Strep. MG (S)	"	" "	−1.19	−1.95
" supernate	"	" "	−3.67	+0.53
" cells	"	" "	−1.78	−1.36
" " + Norm. serum	"	" "	−1.77	−1.37
" " + Anti-MG "	"	" "	−1.41	−1.73
Geometric mean (culture supernates)				+0.10
" " (preparations containing strep. MG)				−1.79

Effect of Time Interval.—Experiments were carried out to determine at what time intervals before, as well as after, inoculation of the virus a significant modifying effect could be obtained with cultures of streptococcus MG. The results of these experiments are presented graphically in Fig. 1 in which the logarithm of the virus titer is plotted against the time at which the test materials were given in relation to inoculation with the virus. It is evident that the intranasal inoculation of mice with sterile broth alone prior to infection by PVM is not wholly without effect upon the results of virus infection, as manifest in the titration end point. This was most evident when broth was given either 1 or 2 days before the virus. Much more striking, however, is the effect upon the virus titration end point of the intranasal inoculation of streptococcus MG culture. With the exception of the tests carried out 14 days before the virus

was given, each of the differences in titration end point between broth control groups and streptococcus MG groups is significant. The difference was most striking on the 2nd or 3rd day before the virus was given but was of almost equal extent from the 1st through the 4th day after the virus was inoculated.

In the total of eighteen experiments with streptococcus MG carried out at various intervals before or after inoculation with the virus, the geometric mean difference between the observed virus titration end points has a value of −1.62 logarithmic units which corresponds to a 42-fold lower end point, on the

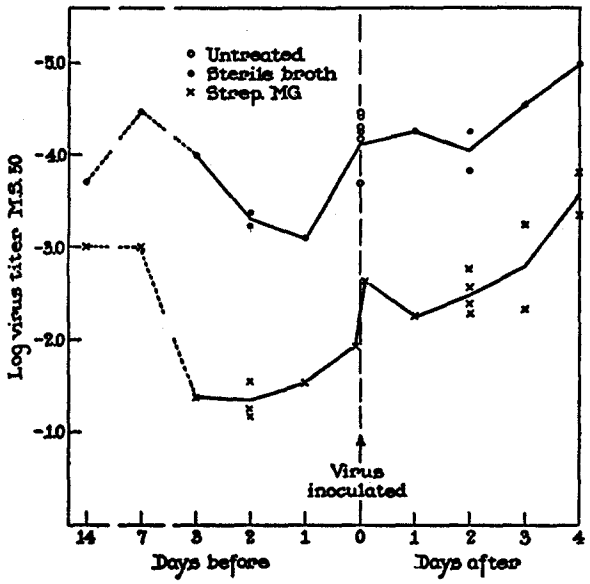

FIG. 1. Effect of intranasal instillation of streptococcus MG, relative to time, on virus titration end points with PVM in mice. Each end point was determined by the results obtained in at least thirty mice.

average, in mice given the bacterial culture as compared with those which received sterile broth.

Effect of Intraperitoneal Injection.—In all of the experiments described so far, only intranasal inoculation of streptococcus MG was employed. It seemed of importance to determine whether a similar effect could be obtained if the bacteria were given by another route. In Table IV are shown the results of tests analogous to those already described but with the difference that the bacterial culture was given intraperitoneally. It is obvious that in no instance was there a significant reduction in the virus titration end point even though widely different time intervals were employed and large amounts of bacteria were injected.

Various other tests have been done also and, so far, it has not been possible

to obtain any modifying effect against PVM unless active material is given intranasally.

Effect on Influenza Virus.—It seemed of considerable interest to determine whether or not a similar modifying effect could be obtained with a different virus. For this purpose influenza A virus was selected because it, like PVM, is considered to be strictly pneumotropic for mice. The results of a number of different experiments similar to those already described with PVM are shown in Table V. It is obvious that the intranasal inoculation of streptococcus MG, either at various intervals before inoculation with influenza A virus or at inter-

TABLE IV

Effect of Intraperitoneal Injection of Streptococcus MG on the Results of PVM Titrations in Mice

First inoculation 0.5 cc. I.P.*	Interval	Second inoculation 0.05 cc.I.N.	Virus titration end point M.S. 50 Log	Difference from control Log
	days			
Sterile broth	14	PVM dilutions	−4.25	—
Strep. MG (0.05 cc.)‡	"	" "	−3.98	−0.27
Sterile broth	7	" "	−4.39	—
Strep. MG (0.05 cc.)	"	" "	−4.38	−0.01
Saline	2	" "	−3.24	—
Strep. MG (0.05 cc.)	"	" "	−4.13	+0.78
" (5.0 cc.)	"	" "	−3.65	+0.22
Geometric mean				+0.18

* I.P. = intraperitoneal.

‡ Bacterial cells from indicated quantity of broth culture.

vals after such inoculation, had in no instance a significant effect upon the virus titration end point. Even when 10 times concentrated bacterial cells were given 2 days before infection with this virus, there was no discernible reduction in titration end point.

Effect of Various Bacterial Species.—The effect of bacterial species other than streptococcus MG was tested. In these experiments various Gram-positive cocci, both encapsulated and non-encapsulated, as well as certain Gram-negative bacilli, were included. In each instance fresh, live cultures were employed. The results of a number of such experiments are presented in summary form in Table VI. It will be noted that in some instances bacterial preparations were given 3 days after inoculation with PVM; in other instances the bacterial preparations were given first, and 2 days later dilutions of PVM were inoculated.

It will be apparent from the results obtained that each of the Gram-positive cocci tested exerted a very striking modifying effect. Similarly, both of the Gram-negative bacilli tested caused a marked reduction in the virus titration end point as compared to appropriate controls. It should also be pointed out that the cell-free supernate obtained from a culture of *Streptococcus salivarius*, type II, was without effect as, it will be recalled, were similar supernates derived from streptococcus MG cultures. The geometric mean difference from

TABLE V

Effect of Intranasal Instillation of Streptococcus MG on the Results of Influenza A Virus Titrations in Mice

First inoculation 0.05 cc. I.N.	Interval	Second inoculation 0.05 cc. I.N.	Virus titration end point M.S. 50 Log	Difference from control Log
	days			
Sterile broth	14	IAV* dilutions	−6.81	—
Strep. MG	"	" "	−6.71	−0.10
Sterile broth	7	" "	−6.36	—
Strep. MG	"	" "	−6.03	−0.33
Saline	2	" "	−6.63	—
Strep. MG	"	" "	−6.18	−0.45
" " (10 × concentration)	"	" "	−6.30	−0.33
IAV dilutions	2	Sterile broth	−6.98	—
" "	"	Strep. MG	−6.57	−0.41
" "	1	Sterile broth	−6.29	—
" "	"	Strep. MG	−6.52	+0.23
Geometric mean				−0.23

* IAV = influenza A virus (PR8 strain).

the controls of the virus titration end points, obtained when these bacterial preparations had been inoculated, was −2.07 logarithmic units, which corresponds to an end point lower by approximately 120-fold.

Effect of Bacterial Concentration.—A series of tests was carried out to determine what effect the concentration of the bacterial suspension would have on modification with PVM. Four species of Gram-positive cocci and one species of Gram-negative bacilli were tested in a concentration equivalent to that of the undiluted culture and also in a concentration 10 times that of the culture. In each of these experiments the bacterial preparations were inoculated intranasally 2 days before the virus dilutions.

In Fig. 2 are presented graphically the results which were obtained. The difference in virus titration end point obtained in control mice as compared with treated mice is plotted against the concentration of the bacterial suspensions used. It will be observed that in each instance, as the bacterial concentration was increased, the modifying effect also increased. The geometric mean difference in titration end point with the one time concentrated suspensions was −1.46 logarithmic units; with the 10 times concentrated suspensions this difference was greater than −2.20 logarithmic units. This rep-

TABLE VI

Effect of Intranasal Instillation of Various Bacterial Species on the Results of PVM Titrations in Mice

First inoculation 0.05 cc. I.N.	Interval	Second inoculation 0.05 cc. I.N.	Virus titration end point M.S. 50 Log	Difference from control Log
	days			
PVM dilutions	3	Sterile broth	−4.59	—
" "	"	*Streptococcus salivarius* type II	−2.48	−2.11
" "	"	" " supernate	−4.36	−0.23
" "	"	" " cells	−2.75	−1.84
Sterile broth	2	PVM dilutions	−3.14	—
Pneumococcus R36A	"	" "	−0.81	−2.33
Group A hemolytic streptococci, type 6	"	" "	−0.83	−2.31
Saline	2	" "	−3.00	—
H. influenzae, type B	"	" "	−1.35	−1.65
E. coli	"	" "	−0.79	−2.21
Geometric mean (preparations containing bacteria)				−2.07

resents, on the average, more than a sixfold increase in the modifying effect with a tenfold increase in bacterial concentration.

Effect of Heat-Killed Bacteria.—Inasmuch as the experiments so far described were carried out with live bacteria, it was of interest to determine whether heat-killed bacteria would be capable of exerting a similar modifying effect. A considerable series of experiments to test this point was carried out and the results of some of the more pertinent are summarized in Table VII. Heat-killed suspensions of streptococcus MG were given intranasally either 2 days before or after inoculation with dilutions of PVM. It will be seen that heating bacteria for 30 minutes at 56, 70, or 80°C. caused no significant change in their modifying activity with respect to the virus. The geometric mean difference in virus

titration end points from the controls was −1.63 logarithmic units; a 42-fold lower end point. The results of these experiments led us to hope that it might be possible to separate from the bacterial cells the substance responsible for this phenomenon. One of the chief objectives of this investigation was to learn the chemical nature of the substance.

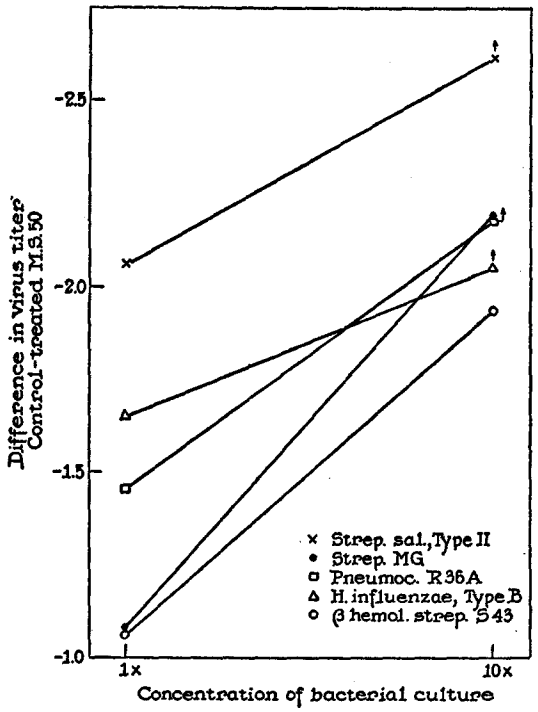

FIG. 2. Effect of intranasal instillation of different concentrations of various bacterial species on virus titration end points with PVM in mice. The bacteria were given 2 days before the virus. Each end point was determined by the results obtained in at least thirty mice. Arrows indicate that the difference in titer was greater than that shown.

II. Effect of Polysaccharide Preparations on the Multiplication of PVM in the Mouse Lung

Up to this point all experiments had been carried out *in vivo* and they required very large numbers of animals, a minimum of thirty mice for each titration. They also required considerable time, a minimum of 12 days. Consequently, an effort was made to obtain results more rapidly and with fewer animals.

It will be recalled that PVM possesses the capacity to cause agglutination of mouse erythrocytes (3). It has been shown that hemagglutination is caused by the virus particles themselves and that the hemagglutination technique can be used to measure the concentration

of virus present in infected lungs (3). Efforts were directed toward devising a technique whereby the modifying effect could be studied by means of the hemagglutination phenomenon. Although it had been shown previously (3) that in the infected mouse lung the increase in virus and in hemagglutination titers parallel each other very closely during the first 7 days after intranasal inoculation with PVM, it was not known whether the size of the virus inoculum would significantly affect the shape of the curve of virus multiplication.

Rate of Multiplication of PVM.—Experiments were carried out to determine this and also to determine more accurately on what day after inoculation with the virus it could be expected that maximum hemagglutination titers would be

TABLE VII

Effect of Intranasal Instillation of Heat-Killed Streptococcus MG on the Results of PVM Titrations in Mice

First inoculation 0.05 cc. I.N.	Interval	Second inoculation 0.05 cc. I.N.	Virus titration end point M.S. 50 Log	Difference from control Log
	days			
PVM dilutions	2	None	−4.26	—
" "	"	Strep. MG	−2.31	−1.95
" "	"	" " (56°C.)*	−3.17	−1.09
Sterile broth	2	PVM dilutions	−3.14	—
Strep. MG	"	" "	−1.19	−1.95
" " (70°C.)*	"	" "	−1.93	−1.21
Saline	2	" "	−3.23	—
Strep. MG (10 × concentrated)	"	" "	−1.24	−2.19
" " " (70°C.)*	"	" "	−1.81	−1.42
Strep. MG (10 × concentrated) (80°C.)*	"	" "	−1.63	−1.60
Geometric mean				−1.63

* Heated for 30 minutes at indicated temperature.

present in the lungs of infected animals. The results of these experiments are presented graphically in Fig. 3. The logarithm of the hemagglutination titer is plotted against time after inoculation with PVM. Three different amounts of virus were used; 10, 100, and 1,000 M.S.50 doses. It will be noted that there is very little difference between the two curves obtained when either 100 or 1,000 doses of virus was given and that when no more than 10 doses was inoculated the curve was not greatly different from the others. With 100 or 1,000 doses of virus maximum titers were obtained on the 6th day, and were maintained through the 7th day after inoculation.

Extracts of Streptococcus MG.—

Cell-free extracts of streptococcus MG were prepared in the following manner: Extract B- concentrated bacterial cells were extracted at 37°C. in the presence of purified lysozyme preparation from egg white at a concentration of 0.5 mg. per cc. Extract D- packed bacterial cells were ground with powdered glass and extracted with saline. Both extracts were rendered essentially cell-free by centrifugation at 10,000 R.P.M. The modifying effect of the extracts was measured in the following way: Each of a group of six mice was given 0.05 cc. of extract intranasally and 2 or 3 days later each was given 100 M.S.50 doses of PVM intranasally. On the 5th, 6th, 7th, and 8th days, respectively, after inoculation of the virus groups of mice were killed and suspensions of their lungs, following appropriate treatment, were tested for hemagglutination.

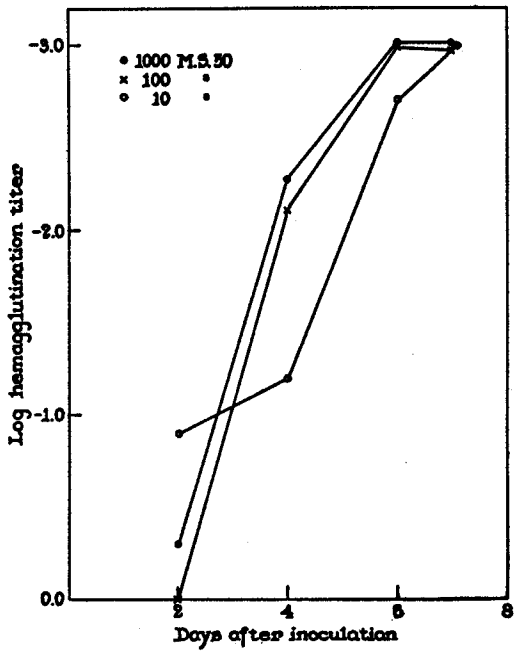

FIG. 3. Relation between hemagglutination titer of lung suspensions, time after intranasal inoculation, and quantity of PVM given mice. Each end point was determined by the results obtained in a group of at least six mice.

The results of one such experiment are shown in Table VIII. It will be seen that, when saline was given before the virus, the hemagglutination titers reached maximal levels between the 6th and 8th days after inoculation as was to be expected from the control curves shown in Fig. 3. It will be noted also that with mice, which were given a cell-free extract of streptococcus MG either 2 or 3 days before the virus, hemagglutination was either not demonstrable or the titers found were much lower than in comparable controls. The geometric mean difference in the hemagglutination titers for the treated animals as compared to the controls was −1.83 logarithmic units; a reduction in titer of 68-fold.

It was of some importance to determine with what reproducibility the modifying effect could be demonstrated under the conditions just described. In Table IX are shown the results of three tests carried out with one cell-free extract of streptococcus MG and six tests carried out with another. In each instance the extracts were given intranasally 2 days before the mice received

TABLE VIII

Effect of Intranasal Instillation of Cell-Free Extracts of Streptococcus MG on Multiplication of PVM in Mouse Lungs

First inoculation 0.05 cc. I.N.	Interval	Second inoculation 0.05 cc. I.N.	Interval	Hemagglutination titer* Log	Difference from comparable control Log
	days		*days*		
Saline	2	PVM (100 M.S. 50)	5	−1.81	—
"	"	" "	6	−2.11	—
"	"	" "	7	−2.41	—
"	"	" "	8	−2.71	—
Extract D	2	" "	5	0.00	−1.81
" "	"	" "	6	0.00	−2.11
" "	"	" "	7	−0.60	−1.81
" "	"	" "	8	−0.90	−1.81
Saline	3	" "	5	−1.81	—
"	"	" "	6	−1.98	—
"	"	" "	7	−2.58	—
"	"	" "	8	−2.71	—
Extract D	3	" "	5	0.00	−1.81
" "	"	" "	6	−0.60	−1.38
" "	"	" "	7	−1.38	−1.20
" "	"	" "	8	0.00	−2.71
Geometric mean					−1.83

* Titer of mouse lung suspensions.

100 doses of virus and the lungs were removed from each group of animals 6 days after inoculation with PVM. It will be apparent that the irregularities in the results obtained were not very striking. The differences observed in the hemagglutination titers with the control and treated groups, respectively, had a geometric mean value of −1.39 logarithmic units with a mean deviation of ±0.28 logarithmic unit, corresponding to a 25-fold reduction in titer with a mean deviation of ±twofold.

Stability of Active Component.—On the basis of these results it appeared fea-

sible to undertake a study of the effects of various procedures upon the modifying activity of cell-free extracts of streptococcus MG. The results obtained with some of the more pertinent procedures are shown in Table X. In this series of experiments, as in those just described, the test materials were given 2 days before the virus and mouse lungs were removed 6 days after inoculation with it.

It was found that the activity of cell-free extracts was not altered by heating at 100°C. for 30 minutes; that the active substance in such extracts was not

TABLE IX

Effect of Intranasal Instillation of Streptococcus MG Extracts on Multiplication of PVM in Mouse Lungs

First inoculation 0.05 cc. I.N.	Interval	Second inoculation 0.05 cc. I.N.	Interval	Hemagglutination titer*		Difference from control Log	Deviation from mean
				Control mice Log	Treated mice Log		
	days		*days*				
Extract B	2	PVM (100 M.S. 50)	6	−2.88	−1.10	−1.78	+0.38
" "	"	" "	"	"	−1.23	−1.65	+0.26
" "	"	" "	"	−2.72	−1.08	−1.64	+0.25
Extract D	2	" "	6	"	−1.71	−1.17	−0.22
" "	"	" "	"	−2.78	−1.60	−1.18	−0.21
" "	"	" "	"	−2.72	−1.20	−1.52	+0.13
" "	"	" "	"	"	−1.51	−1.21	−0.18
" "	"	" "	"	−2.88	−1.66	−1.22	−0.17
" "	"	" "	"	−2.11	0.00	−2.11	+0.72
Geometric mean						−1.39	±0.28

* Titer of mouse lung suspensions.

dialyzable; that it was precipitable by half-saturated ammonium sulfate, and also by 80 per cent ethyl alcohol, and that it was not significantly altered by brief treatment with N/10 NaOH or HCl. Of more interest was the finding that the activity of the extracts was unaffected by incubation with; (1) a mixture of crystalline trypsin and chymotrypsin; (2) crystalline ribonuclease; and (3) highly purified desoxyribonuclease. Each enzyme was used in a concentration of 0.1 mg. per cc. It is apparent that none of the procedures enumerated caused any striking reduction in the activity of the extracts. The geometric mean difference of the hemagglutination titers from the controls was —1.75 logarithmic units; a reduction in titer of 56-fold. These results indicate that the substance responsible for the modifying effect is remarkably stable and is relatively large with respect to molecular dimensions. In casting about

among the various classes of substances which might be expected to withstand all of the procedures employed, it seemed probable that substances of polysaccharide nature should be considered. By means of precipitation tests with antistreptococcus MG serum, it was shown that both extracts B and D contained approximately 2 mg. per cc. of specific capsular polysaccharide (4).

Effect of Polysaccharide Preparations.—A number of different polysaccharide preparations and certain related substances were tested as a consequence. In Table XI are shown in summary form the results of certain of these experiments.

TABLE X

Effect of Various Procedures on Activity of Cell-Free Extracts of Streptococcus MG

First inoculation 0.05 cc. I.N.		Interval	Second inoculation 0.05 cc. I.N.	Interval	Hemagglutination titer*		Difference from control Log
Extract	Procedure				Control mice Log	Treated mice Log	
		days		*days*			
B	None	2	PVM (100 M.S. 50)	6	−2.88	−1.10	−1.78
"	100° C. 30 min.	"	" "	"	"	−1.35	−1.53
"	Dialysis *vs.* saline	"	" "	"	"	−1.10	−1.78
"	Ppt. ½ sat. $(NH_4)_2SO_4$	"	" "	"	"	−0.60	−2.28
"	Ppt. 80 per cent alcohol	"	" "	"	"	−1.61	−1.27
"	N/10 NaOH	"	" "	"	"	−1.89	−0.99
"	N/10 HCl	"	" "	"	"	−1.14	−1.74
D	None	"	" "	"	−2.72	−1.20	−1.52
"	Trypsin + chymotrypsin	"	" "	"	"	−0.90	−1.82
"	Ribonuclease	"	" "	"	"	−0.84	−1.88
"	Desoxyribonuclease	"	" "	"	"	0.00	−2.72
Geometric mean							−1.75

* Titer of mouse lung suspensions.

A standard procedure was followed. In each instance 0.1 mg. of the test substance was given intranasally to each mouse and 2 days later the mice received, by the same route, 100 M.S.50 doses of PVM. The lungs of these animals as well as those of control mice were removed 6 days after inoculation with the virus and the hemagglutination titers determined. Each preparation was tested in two or more separate experiments.

It was found that preparations of the capsular polysaccharide of streptococcus MG, the capsular polysaccharide of Friedländer bacillus type B, the specific somatic antigen of *Shigella paradysenteriae* type V, as well as a hapten derived from the same preparation, blood group A specific substance, and ordinary agar-agar, each exerted a very striking modifying effect under the conditions of these experiments. The geometric mean difference of the hemag-

glutination titers obtained in all of the experiments carried out with these preparations was −1.89 logarithmic units; a reduction in titer of 78-fold.

TABLE XI

Effect of Intranasal Instillation of Various Polysaccharide Preparations on Multiplication of PVM in Mouse Lungs

First inoculation 0.05 cc. I.N. 0.1 mg.	Interval	Second inoculation 0.05 cc. I.N.	Interval	Geometric mean difference of hemagglutination titers* from controls Log
	days		*days*	
Streptococcus MG, capsular polysaccharide	2	PVM (100 M.S. 50)	6	−1.43
Friedländer type B, capsular polysaccharide	"	" "	"	−1.97
Shigella paradysenteriae, type V, antigen	"	" "	"	−1.74
" " " " hapten	"	" "	"	−2.13
Blood group A specific substance	"	" "	"	−1.80
Agar	"	" "	"	−2.26
Geometric mean				−1.89
Pneumococcus type I, capsular polysaccharide	2	PVM (100 M.S. 50)	6	−0.60
Pneumococcus type III, capsular polysaccharide	"	" "	"	−0.17
Pneumococcus C polysaccharide	"	" "	"	−0.25
Streptococcus salivarius, type II, capsular polysaccharide	"	" "	"	−0.01
Gum acacia	"	" "	"	−0.06
Starch, soluble	"	" "	"	−0.66
Heparin	"	" "	"	−0.30
Geometric mean				−0.29

* Titer of mouse lung suspensions.

Surprisingly, it also was found that the capsular polysaccharides of Pneumococcus, type I and type III, as well as of *Streptococcus salivarius* type II, the somatic C polysaccharide of Pneumococcus, as well as gum acacia, soluble starch and heparin, were almost without modifying effect and that none of them caused a significant reduction in hemagglutination titer. The geometric mean difference in titer in all of the tests carried out with these latter preparations was −0.29 logarithmic unit; a twofold reduction in titer.

These results appear to lend support to the idea that the substance respon-

sible for the modification observed with PVM is a large, non-diffusible molecule which may be polysaccharide in nature. Unfortunately, however, those substances which were found to possess distinct modifying activity with respect to this virus are those, among the preparations tested, about which least is known as to molecular structure. So far, it has not been possible to discover any clue regarding a possible molecular configuration possessed in common by the substances which are active as modifiers against PVM.

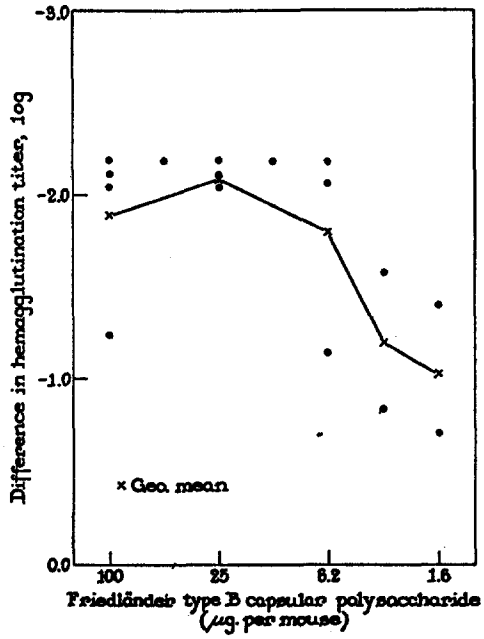

FIG. 4. Effect of intranasal instillation of different quantities of Friedländer polysaccharide on hemagglutination titer of suspensions of mouse lungs infected with PVM. The polysaccharide preparation was given 2 days before the virus. Each end point was determined by the results obtained in a group of at least six mice.

It was of interest to determine how small an amount of highly purified material would be capable of exerting a significant modifying effect with respect to this virus. The results of a series of such experiments with Friedländer bacillus type B capsular polysaccharide, a highly purified preparation which had been autoclaved, are shown in Fig. 4. The difference in the hemagglutination titer obtained as compared with that found with appropriate controls is plotted against the micrograms of polysaccharide given intranasally to each mouse. It will be seen that there was no striking difference in the modifying effectiveness of amounts as small as 6 μg. per mouse as compared with the effect obtained with 100 μg. per mouse. In general, the lungs of mice which received these

quantities failed to show any capacity to cause hemagglutination. It will be noted that, when quantities as minute as 1.6 μg. per mouse were given intranasally, significant modification was observed.

Effect of Derivatives of Friedländer Polysaccharide.—The polysaccharide preparation which was used for these experiments seemed especially suitable for further studies. This preparation was oxidized with M/50 periodic acid at pH 5.2.[2] The reaction was continued until specific serological activity, as judged by the precipitin reaction, had been destroyed. Fractions obtained at various time intervals during the reaction were tested for their modifying effect as also

TABLE XII

Effect of Intranasal Instillation of Friedländer Capsular Polysaccharide on Multiplication of PVM in Mouse Lungs

First inoculation I.N.		Interval	Second inoculation I.N.	Interval	Hemagglutination titer,* difference from control Log
Material	Amount				
	mg.	*days*		*days*	
Friedländer, type B, capsular polysaccharide	0.1	2	PVM (100 M.S. 50)	6	−1.90
Friedländer, type B, capsular polysaccharide	0.006	"	" "	"	−1.80
Same, treated with periodic acid	0.1	"	" "	"	−2.10
" " " " "	0.006	"	" "	"	−1.80
Same, aldobionic acid derivative	1.0	"	" "	"	−0.30
" " " "	0.1	"	" "	"	0.00

* Titer of mouse lung suspensions.

was the aldobionic acid[3] derived from the original polysaccharide by acid hydrolysis.

The results of experiments with the untreated polysaccharide, the periodic acid–oxidized material, and the aldobionic acid, are shown in Table XII. It will be seen that the aldobionic acid showed no evidence of significant modifying activity even when amounts as large as 1 mg. were given to each mouse. The material obtained after oxidation of the polysaccharide with periodic acid, however, appeared to be equally as effective as the untreated polysaccharide and in each instance as little as 6 μg. per mouse was sufficient to reduce markedly the

[2] Oxidation with periodic acid was kindly carried out by Dr. Walther F. Goebel, the Rockefeller Institute.

[3] This material was kindly prepared and provided by Dr. Walther F. Goebel, the Rockefeller Institute.

hemagglutination titer as compared to controls. With this quantity a 64-fold reduction in titer was observed in both instances.

It should be pointed out that, following oxidation of the Friedländer carbohydrate with periodic acid, the oxidized polysaccharide may be regarded as a polyaldehyde which has not suffered gross depolymerization as evidenced by the fact that the substance, like the specific polysaccharide from which it is

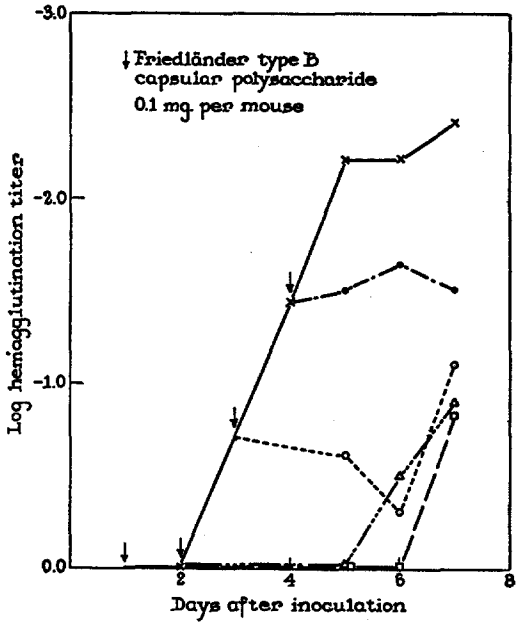

FIG. 5. Effect of intranasal instillation of Friedländer polysaccharide on hemagglutination titer of suspensions of mouse lungs infected with PVM. Groups of mice were given polysaccharide once only, as indicated by the arrow, at various times following inoculation with virus. Lungs were removed and tested at times indicated by the end points. Control end points were obtained simultaneously with lungs of mice which had received either saline or 0.1 mg. of aldobionic acid at a corresponding time after inoculation with virus. Each end point was determined by the results obtained in a group of at least six mice.

derived, does not diffuse through semipermeable membranes. The oxidized carbohydrate, although no longer capable of giving a positive precipitin reaction in the presence of specific antiserum, did, nevertheless, still react with homologous antibody as shown by the results of specific inhibition tests. Aldobionic acid is, of course, a relatively small molecule and is readily dialyzable. If molecular integrity is a criterion of activity, one would not expect this latter substance to show a modifying effect.

Interruption of Multiplication.—All of the experiments, in which the hemagglutination technique was used to measure the modifying activity of various

substances of bacterial origin, were carried out under standardized conditions. In each, the preparations to be tested were given 2 days before 100 M.S.50 doses of virus was inoculated and the lungs of inoculated animals were removed 6 days after the virus was given. It was of obvious importance to determine whether polysaccharide materials would, like intact bacterial cells, be capable of causing a modifying effect if given at various times after the virus had been inoculated.

A series of experiments to test this point was carried out and the results are presented graphically in Fig. 5. The logarithm of the hemagglutination titer obtained is plotted against time after inoculation of the virus. When either saline or the aldobionic acid was given intranasally at various intervals after the virus had been inoculated, there was no significant effect; and the curve of increase in hemagglutination titer, *i.e.* in virus concentration, with time was closely similar to the control curve shown in Fig. 3. However, when 0.1 mg. per mouse of Friedländer polysaccharide was given at various intervals after inoculation with the virus, the hemagglutination titers obtained were significantly lower than in control mice. It should be noted that the reduction in titer from the controls was, on the average, inversely proportional to the length of time between the inoculation of the virus and the subsequent injection of the polysaccharide.

The curves shown in Fig. 5 suggest that relatively little additional increase in virus titer occurred after the intranasal instillation of the polysaccharide. It appears, therefore, that this polysaccharide preparation is, like intact bacterial cells, capable of inhibiting or interrupting further multiplication of virus even when it is given at a time when much virus multiplication has already occurred. In order to show this effect, it was essential to give the polysaccharide before maximal hemagglutination titers had been reached. If the polysaccharide was given 5 days or more after inoculation with PVM, little or no modifying activity could be demonstrated. By this time, of course, the virus titer had almost, if not actually, reached maximal levels.

DISCUSSION

The evidence obtained in this study indicates that a substance or substances present in certain bacterial species are capable of exerting a striking modifying effect upon the course of infection with PVM in mice. A similar effect is obtained also with some materials which are not of bacterial origin. These active materials show this effect following intranasal instillation but failed to show it following injection by other routes. Not only do the active substances modify the results of infection with PVM, but also, and of more importance, they appear to cause inhibition or interruption of multiplication of the virus. This is most clearly demonstrable if active material is given after infection with PVM has been established, when actual multiplication of the virus is occurring.

Each of the soluble substances which was found to be active contained one polysaccharide or another. The most active substances so far tested are, in fact, polysaccharide preparations; certain of these preparations were highly purified and some had been subjected to rigorous treatment. Yet, in amounts as small as a few micrograms per mouse, such polysaccharide preparations show distinct modifying effects with respect to PVM. Not all polysaccharide preparations tested are active as modifiers against this virus; a number of materials, both of bacterial and of other origin, were without significant effect. Present evidence suggests that the active component is associated with certain highly purified polysaccharides and raises the possibility that it may itself be polysaccharide in nature. As yet it has not been possible to obtain evidence as to the chemical structure of the active component.

It appears that active substances are capable of causing a significant alteration in the phenomena which result ordinarily when PVM is inoculated intranasally in susceptible mice. Under appropriate experimental conditions, it can be shown that mice given such substances intranasally not only survive the inoculation of quantities of virus which are fatal for control animals, but also fail to develop pulmonary consolidation as do controls. In order to show the modifying effect, it is not essential to give active substances prior to inoculation with the virus; closely similar results are obtained when the material is given as late as 4 days after the virus. Present evidence indicates that the normal course of infection with PVM can be modified in favor of the host, by means of active soluble material, even at a time when demonstrable evidences of infection are already present. However, once maximal infection has developed, *i.e.* when either full blown pulmonary consolidation or maximal virus titer has been reached, active materials appear no longer to be capable of exerting an appreciable modifying effect.

Published reports indicate that, in rare instances, one or another substance may exert some influence upon infections induced by certain viruses. In this regard, the following seem pertinent: Infections induced by some strains of the largest of known viruses, *i.e.* those which form elementary bodies and are members of the psittacosis-lymphogranuloma group, appear to be somewhat altered by either sulfonamide drugs or penicillin (10). Certain acridine compounds (11) and an analogue of tryptophane (*i.e.*, 5-methyl tryptophane) (12) appear to inhibit the multiplication of some strains of bacteriophage but do so only at concentrations which also interfere with growth of the bacterial host cells. Evidence has been presented (13) to show that tobacco mosaic virus is inactivated when mixed with a substance derived from yeast and that this inactivator is a polysaccharide. A number of chemical substances, most notably zinc sulfate, have been shown to induce a state of decreased susceptibility to intranasal inoculation of poliomyelitis virus (14). An acridine compound (*i.e.*, nitroakridin 3582) has been shown to have an inhibiting effect on influenza B virus in

embryonated eggs (15). With the exceptions noted, it appears that there is but little evidence which indicates that the course of a virus infection can be altered significantly, once it has been initiated, by any substance other than specific antibodies against the virus itself.

It is apparent that it would be of considerable theoretical importance to have knowledge of the mechanism by which active substances are capable of exerting a modifying effect upon the course of infection induced by PVM. It seems probable that knowledge of the chemical structure of the substance or substances responsible for modification, with respect to this virus, may be necessary before it will be possible to understand the mechanism by which the effect occurs. In the absence of decisive information as to the chemical structure of the substance, it has not yet been feasible to carry out conclusive experiments which would elucidate the underlying mechanism. Nonetheless, there were opportunities to test certain possible mechanisms.

It will be recalled that PVM enters into firm combination with suitable erythrocytes as well as with lung tissue from species susceptible to infection with the virus (3). Neither type of combination undergoes spontaneous dissociation. Following combination of virus with erythrocytes, agglutination of red blood cells occurs. Following combination with lung tissue particles, virus can be sedimented along with such particles in centrifugal fields of relatively small magnitude (9). It seemed possible that modification might result from chemical blockade of virus "receptors" thought to be present on cells of the respiratory tract.

Numerous experiments were carried out to test this hypothesis but no evidence in favor of the theory was obtained. Preparations which are highly active as modifiers failed to cause any hemagglutination of erythrocytes susceptible to agglutination by the virus. The treatment of erythrocytes under various conditions with active preparations failed to yield any evidence of inhibition of hemagglutination by the virus. Furthermore, treatment under various experimental conditions of lung tissue, or particles derived therefrom, with active preparations failed to reduce their capacity to combine with the virus. In the light of these experiments, it seems improbable that modification is to be explained on so simple a basis as blockade of virus "receptors" present on cells of the lining membrane of the respiratory tract.

It seems more probable that active preparations may exert their modifying effect by competing with PVM for some intracellular system essential to the multiplication of this virus. To devise direct tests for this hypothesis has not been possible because at the present time there is no information regarding the nature of such systems. On the other hand, indirect evidence has been obtained during these studies which is perhaps suggestive. Most important is the fact that the modifying effect with respect to the virus is clearly demonstrable even as late as 4 days after infection with the virus has been established,

at a time when much multiplication of virus has occurred. It seems of considerable significance, in this same regard, that the extent of the modifying effect appears to be almost inversely proportional to the titer the virus has reached at the time when active material is given. Moreover, it appears that, following the injection of active polysaccharide preparations in infected animals, very little, if any, further multiplication of PVM occurs.

SUMMARY

Evidence is presented which indicates that certain polysaccharide preparations derived from various bacterial species, as well as similar materials not of bacterial origin, are capable of lessening the severity of infection with pneumonia virus of mice (PVM) and inhibiting multiplication of the virus in mouse lungs infected with this agent. It seems probable that modification with respect to the virus is mediated by a substance which may be polysaccharide in nature.

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