PROPERTIES OF PNEUMONIA VIRUS OF MICE (PVM) IN RELATION TO ITS STATE*

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In a previous communication (1) evidence was presented which indicated that the hemagglutination observed with heated suspensions of lungs infected with pneumonia virus of mice (PVM) was caused by the virus itself, even though the virus had been rendered non-infectious in the course of heating. From the available data it was concluded that the virus has the capacity to combine firmly not only with mouse or hamster erythrocytes but also with lung tissue particles from certain host species, and that appropriate heating serves to release the virus from combination. The occurrence of such combination with PVM appeared to account for its capacity to cause hemagglutination and for other unusual attributes of this virus.

It is the purpose of this paper to present additional evidence in support of the conclusion (1) that combination between PVM and tissue particles does occur, and to elucidate further the mechanism and significance of this phenomenon. It will be shown that the virus can be released from combination by treatment with alkali. Further evidence will be presented to demonstrate that PVM, as it exists *in vivo*, is not combined and that combination occurs in the process of grinding tissues infected with it. Moreover, it will be shown that free infectious virus recovered by a technique described in the preceding paper (2) is not combined and that estimates of its titer can be made directly both *in vivo* and *in vitro*. It will be shown that in demonstrating the presence of PVM or in estimating its titer the results obtained by *in vitro* techniques are more accurate than corresponding results obtained *in vivo*. Finally, evidence will be presented indicating that the virus may occur in several different states and that it possesses certain distinguishing properties in each.

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Methods

Virus.—Pneumonia virus of mice (PVM), strain 15, (3) was used exclusively. It was maintained by occasional passage in albino Swiss mice and stored at −70°C. in a manner identical to that previously employed (4). Suspensions of PVM were prepared according to the several techniques outlined in the preceding communication (2). These preparations were designated, respectively, as (*a*) combined PVM, (*b*) heat-released PVM , (*c*) free PVM, (*d*) heated free PVM, and (*e*) artificially combined PVM and, where required for clarity, the same designations have been used in this paper.

Infectivity Tests.—Titrations of infectivity were carried out in mice utilizing serial decimal dilutions, and end points were calculated by the 50 per cent maximum score end point method as described previously (4).

Hemagglutination Tests.—The technique of hemagglutination tests with mouse RBC, utilizing serial twofold dilutions, and the method of estimating end points were identical with those previously employed (1).

Presentation of Data.—Estimations of virus activity by either *in vivo* or *in vitro* techniques are presented in a manner identical with that used in the preceding paper (2) except where otherwise indicated. It will be noted that in certain tables titers are expressed as reciprocals of the dilution of the actual suspension tested instead of a value representing the final dilution of infected mouse lung included in the suspension.

EXPERIMENTAL

Because of the fact that heat sufficient to release PVM from combination destroys its infectiousness, it was necessary to obtain the earlier evidence which has been cited (1) by comparing the results of tests for infectivity and hemagglutination upon unheated and heated aliquots, respectively, of each suspension of infected lungs. The evidence that hemagglutination is actually caused by the virus and that the virus itself combines with lung tissue particles would obviously be more direct and convincing if the properties of infectiousness and hemagglutination could both be measured directly with each preparation without heating prior to hemagglutination tests.

From theoretical considerations there appeared to be two possible approaches to the objective of obtaining free or uncombined virus capable of causing both infection and hemagglutination. First, it might be possible to effect dissociation of the combination between virus and tissue particles without heating by employing less drastic procedures which would not impair the property of infectiousness. Second, if the virus in infected mouse lungs were only partially or not at all combined *in vivo* and combination occurred during the process of grinding the infected tissue, then it might be possible to recover free virus before it had an opportunity to combine with tissue particles.

Various procedures were utilized in attempts to dissociate virus from combination with tissue particles in suspensions of ground infected mouse lungs. From the results obtained only one procedure appeared promising. When suspensions containing combined PVM were mixed with NaOH or strong alkaline buffers, dissociation became demonstrable by the hemagglutination technique at pH 10.5 or above. An illustrative experiment is presented in

Table I which shows the results of hemagglutination tests with mixtures of mouse lung suspensions and alkaline phosphate buffers held at room temperature for 45 minutes.

It will be noted that hemagglutination occurred with the untreated mixture held at pH 10.5, but that when readjustment was made to pH 7.5 hemagglutination was no longer demonstrable. Hemagglutination also occurred with the supernate obtained by centrifuging at high speed a portion of this untreated mixture, but after adjustment to pH 7.5 the titer was reduced. With the mixture held at pH 10.9 the corresponding hemagglutination titers were much higher than at the lower pH but, following readjustment to pH 7.5, the titers were reduced. Maximal titers were attained with the mixture held at pH 11.1 and readjustment of pH caused no diminution in titer. These

TABLE I

Effect of pH upon Combined PVM. Release of Virus by Alkali

Mixtures of mouse lung suspension and buffer		Hemagglutination titer*			
		Uncentrifuged		15,000 R.P.M. supernate	
Mouse lung	pH	Untreated	Adjusted to pH 7.5	Untreated	Adjusted to pH 7.5
PVM	10.5	64	0	256	64
PVM	10.9	1024	256	2048	512
PVM	11.1	2048	1536	2048	4096
IAV‡	11.1	0	0	0	0
Normal	11.3	0	0	0	0

* Titer = reciprocal of dilution of mouse lung suspension.

‡ IAV = influenza A virus, PR8 strain.

findings indicate that at pH 10.5 partial but reversible dissociation occurred, whereas at pH 11.1 dissociation was relatively complete and apparently irreversible. It would appear that at this latter pH the tissue particles which combined with PVM were so altered as to be incapable of recombining with the virus. It will be noted that suspensions of mouse lungs infected with influenza A virus (PR8) and suspensions of normal mouse lung held at pH 11.1 caused no reaction when similarly tested, indicating that the hemagglutination observed with preparations of PVM is attributable to the presence of this virus and not to an effect produced in common with other viruses nor to nonspecific factors capable of causing hemagglutination. This was further borne out by serological evidence.

Suspensions of mouse lungs infected with PVM which, following treatment with alkali, caused agglutination of mouse RBC were tested for hemagglutination inhibition in the presence of serum. The suspensions used for this purpose

had been readjusted to pH 7.5 and the method of testing was the same as that previously described (1). It was found that hemagglutination caused by alkali-treated suspensions was inhibited by serum containing neutralizing antibodies against PVM but not by serum which did not contain such antibodies. Serum inhibition of hemagglutination by alkali-treated suspensions of PVM appeared to be similar in specificity and in degree to that obtained with heated PVM suspensions.

Experiments were also carried out to determine whether the capacity of alkali-treated PVM suspensions to produce hemagglutination was heat-stable. It was found that when such suspensions were heated at 70°C. for 30 minutes prior to neutralization, the hemagglutination property was destroyed, whereas, when they were heated following readjustment to pH 7.5, tests for hemagglutination showed no appreciable alteration of titer.

Although from these and other similar data it seemed clear that PVM could be dissociated from combination, not only be heating but also by treatment with alkali, the desired objective of obtaining free infectious virus was not achieved. It was found that at pH values high enough to effect dissociation (*i.e.*, above pH 10.5) the property of infectiousness was either very markedly reduced or completely destroyed.

The possibility of recovering free virus from infected lung tissue before combination occurred was also explored. It will be recalled that fluid exuding from the cut surfaces of mouse lungs infected with PVM causes agglutination of the mouse erythrocytes but that suspensions prepared from the same lungs fail to cause hemagglutination (5). These observations suggested that some of the virus present in infected mouse lungs is free and combines with erythrocytes or tissue particles when the infected lungs are ground (1). It will also be recalled that combination with mouse RBC occurs more slowly and less completely at 4°C. than at 37°C., or at room temperature (1). With these points in mind a procedure was devised for recovering PVM from infected lungs under conditions unfavorable for combination and with as little exposure as possible to tissue constituents. This procedure is described in detail in the preceding paper (2). By means of this technique suspensions of PVM are obtained which are not only infectious but also capable of causing hemagglutination directly. It will be recalled that the essential features of the technique which is used to obtain free infectious PVM are the following: (*a*) the infected lungs are perfused *in situ* to remove RBC; (*b*) the lungs are not ground, but instead are cut up into small pieces; (*c*) the lung pieces are gently shaken with cold, buffered saline and this mixture is then centrifuged in the cold at high speed; (*d*) the clear supernate is employed in subsequent tests.

In Table II the results of tests for infectivity and hemagglutination with suspensions of PVM prepared by the technique outlined above are compared

with the results of similar tests with suspensions of ground infected mouse lungs. For both types of preparation lungs containing blood and lungs perfused *in situ*, respectively, were used.

It will be noted that hemagglutination was not demonstrable with any of the suspensions of ground lungs until they had been heated appropriately and also that filtration through a Coors No. 3 candle removed all of the virus from such suspensions as judged by the results of both infectivity and hemagglutination tests. It will be seen that each of the supernates obtained from

TABLE II

Results of Virus Titrations with Free and Combined PVM

Infected mouse lungs	Preparation	Virus titer*		
		Infectivity M.S. 50	Hemagglutination	
			Unheated	70°C. 30 min.
Not perfused	Ground; 1,000 R.P.M.			
	Supernate	132	0	128
	Filtrate‡ of above	0	0	0
	Cut; 12,000 R.P.M.			
	Supernate	47	32	64
	Filtrate‡ of above	8	32	64
Perfused	Ground; 1,000 R.P.M.			
	Supernate	332	0	64
	Filtrate‡ of above	0	0	0
	Cut; 12,000 R.P.M.			
	Supernate	57	96	64
	Filtrate‡ of above	96	64	96

* Titer = reciprocal of dilution of mouse lung suspension.

‡ Coors No. 3 filter.

lungs which had not been ground caused hemagglutination on direct test and that the titers obtained were closely similar to those observed following heating as well as to the corresponding infectivity titers. Moreover, it is evident that with these preparations filtration did not remove significant amounts of the virus since following this procedure both hemagglutination and infectivity titers remained essentially unchanged.

The results indicate that PVM in supernates obtained from unground lungs is infectious and not combined. When prepared from lungs containing blood, free virus is obtained but the yield is generally somewhat lower than that obtained from perfused lungs. When prepared from perfused lungs, the free virus is capable of combining with and agglutinating mouse RBC at

dilutions practically identical with those of corresponding heated aliquots. Present evidence indicates that approximately 10 per cent of the virus demonstrable in infected lungs can be recovered in the free state by this technique. Although this is not a very high yield, it has sufficed for additional investigations on the properties of PVM.

In Table II the difference in filterability between free infectious PVM and combined PVM is strikingly apparent. With free PVM the titers obtained before and after filtration were closely similar, whereas with combined PVM no virus was demonstrable following filtration by either infectivity or hemagglutination tests. These findings are not surprising from either a theoretical or an experimental standpoint. It would be expected that free virus particles should be relatively uniform and smaller in size than the particles containing virus and tissue constituents in combination. Experimental evidence that the apparent discrepancy in size between free PVM and combined PVM is not only real but even measurable was presented in the preceding paper (2). From the results of quantitative studies carried out in the high speed angle centrifuge, it appears that free PVM or PVM released from combination by appropriate heating is relatively small with dimensions of the order of 40 millimicrons, whereas combined PVM appears to be relatively large with minimal dimensions of the order of 140 millimicrons (2).

It was found that free infectious PVM not only was indistinguishable in size from heat-released PVM but possessed other properties in common with it. Agglutination of mouse RBC in direct tests with free infectious PVM was found to be specifically inhibited by serum containing neutralizing antibodies against this virus but not by serum which did not contain such antibodies. The results of tests with free infectious PVM for neutralization in mice and for inhibition of hemagglutination *in vitro* were closely similar by both methods and of the same order of magnitude and specificity as those obtained indirectly in the earlier studies (1) with unheated combined PVM and heat-released aliquots, respectively. Heated aliquots of free PVM, which agglutinated mouse RBC in essentially the same titer as the corresponding preparations from which they were derived, were also used in tests for agglutination inhibition. It was found that under standard conditions hemagglutination by unheated or heated free PVM was inhibited by specific immune serum to the same degree.

It will be recalled that in a previous communication (1) it was found that PVM, both unheated (*i.e.*, combined) and heated (*i.e.*, heat-released), were apparently destroyed by the action of proteolytic enzymes (*e.g.*, trypsin and chymotrypsin) but not by nucleases (*e.g.*, ribonuclease and desoxyribonuclease). Similar experiments carried out with free PVM indicated that both infectiousness and the capacity to cause hemagglutination were destroyed by the same two proteolytic enzymes and were unaffected by the nucleases.

Free PVM, like combined PVM and heat-released PVM, appeared not to be dialyzable. Infectiousness was lost in the course of this procedure, but hemagglutinating activity was retained and was confined exclusively to the water-insoluble sediment.

The demonstration of hemagglutination directly with preparations of free infectious PVM made it possible to show that combination of the virus with mouse RBC occurs in a manner similar to that observed with preparations of the virus following heating at 70°C. (1). It was found that combination between mouse RBC and unheated free virus occurred rapidly, was stable over a period of at least 48 hours, and did not dissociate spontaneously. The

TABLE III

Artificial Combination of Free Infectious PVM with Normal Tissue
Results of Tests for Infectivity and Hemagglutination

Mixture of free virus and suspension of	Virus titer*				
	Uncentrifuged	Centrifuged			
		Supernate			Resuspended sediment
	Hemagglutination 70°C.	Infectivity M.S. 50	Hemagglutination		Hemagglutination 70°C.
			Unheated	70°C.	
Mouse RBC	128	2	0	4	128
" lung	128	4	0	4	64
Cotton rat lung	128	8	0	8	64
Cotton rat RBC	64	40	128	256	0
Chick embryo tissues	128	36	128	128	0

* Titer = reciprocal of dilution of mouse lung suspension.

virus could be released from combination, however, by heating at 70°C. and was then capable of recombining with fresh RBC.

It was found, moreover, that free infectious PVM combined not only with mouse RBC but also with particles obtained from perfused and washed normal mouse or cotton rat lungs. Similar combination did not occur with suspensions of cotton rat RBC or of chick embryo tissues. The results of an experiment demonstrating these findings are shown in Table III.

Mixtures of free virus and 10 per cent suspensions of RBC or tissue in equal volumes were shaken gently at room temperature for 2 hours. Following centrifugation at 15,000 R.P.M. for 15 minutes, the supernates were removed and the sediments resuspended to initial volume. The supernates were tested for infectivity, and also for hemagglutination, both unheated and after being heated at 70°C. for 30 minutes. Aliquots of each mixture prior to

centrifugation and of the resuspended sediments were likewise heated at 70°C. and tested for hemagglutination.

It will be noted that when mixed with mouse RBC, or with mouse or cotton rat lung particles, all of the virus was bound as indicated by the failure of the unheated supernates to cause hemagglutination. The low infectivity titers of these supernates corresponded closely to the hemagglutination titers obtained with heated aliquots, indicating that small amounts of combined infectious virus remained in the supernates. That most of the virus in these mixtures did combine and was sedimented under these conditions is shown by the relatively high titers obtained when the sediments were resuspended, heated at 70°C., and tested for hemagglutination. It will be seen that these titers

TABLE IV

Demonstration of the Instability of Infectiousness with PVM

Preparation	Virus titer*		
	Infectivity M.S. 50	Hemagglutination	
		Unheated	70°C.
Free PVM, tested immediately.....	83	64	96
" " " after 24 hrs. at 25°C..	3	64	96
Combined PVM, tested immediately...	1320	0	512
" " " after 24 hrs. at 25°C.....	2	0	1024

* Titer = reciprocal of dilution of mouse lung suspension.

compared closely with those obtained similarly with the same mixtures prior to centrifugation.

On the other hand, the virus did not combine with cotton rat RBC or chick embryo tissue as indicated by the finding with each method of testing that the virus was demonstrable in relatively high and similar titers in the supernates and was not demonstrable in the resuspended sediments. The somewhat lower titers obtained in tests for infectivity than in the corresponding tests for hemagglutination are not surprising in view of the fact that with PVM the capacity to cause hemagglutination is stable (1), whereas the property of infectiousness is relatively unstable (1–4).

Direct experimental evidence for this important difference in the stability of these two properties is shown in Table IV. It will be seen that with preparations of both free and combined virus the infectivity titers fell practically to zero during 24 hours at room temperature whereas the hemagglutination titers remained unchanged.

In interpreting and evaluating the results of *in vivo* and *in vitro* tests carried

out with a single preparation of virus or in comparing the results obtained with different preparations, differences in stability between various properties of PVM need to be taken into consideration. It is now apparent that with PVM the property of infectiousness is affected adversely by a variety of environmental factors which do not appreciably affect the capacity to produce hemagglutination or certain other relatively stable properties; *e.g.*, specific antigenicity.

In a previous communication (4) the results of multiple titrations of PVM in mice were analyzed to determine the experimental error of such titrations and to evaluate the significance of differences in titration end points with this agent. In that study suspensions of ground mouse lungs infected with PVM were used and, in the light of present knowledge, it appears evident that the virus was in a combined state. It was found that the standard deviation of the distribution of end points for the whole series was 0.383 log unit and it was pointed out how, with this value known, the significance of differences between end points can be estimated with reasonable accuracy.

In a subsequent report (1) the infectivity and hemagglutination titers of various PVM mouse lung suspensions were compared and found in most instances to be closely similar. For this comparison infectivity titers in mice were estimated as in the analysis cited above (4) and hemagglutination titers were determined by using an appropriately heated aliquot of each suspension. Although the standard deviation of titration end points by the hemagglutination technique was not determined, the results obtained indicated that it did not exceed and was probably less than the standard deviation obtained with the *in vivo* method. This is not surprising when it is recalled that end points are determined by the *in vivo* method from the results obtained following the intranasal inoculation of mice with serial tenfold dilutions, whereas with the *in vitro* technique serial twofold dilutions are employed, and the uncontrollable factor of virus multiplication is eliminated.

In the present study an analysis was made to estimate the experimental error in titrations of free infectious PVM by each of 3 different procedures. These included: (*a*) a determination of infectiousness in mice, (*b*) a direct test for hemagglutination with each unheated suspension of free virus prepared as outlined above, and (*c*) a hemagglutination test with an appropriately heated aliquot of each suspension. 16 individual preparations were included in this analysis; each was titered by all 3 procedures, with the exception of 1 preparation which was not tested *in vitro* after heating. Multiple titrations were carried out on all but 3 of the preparations by the *in vivo* technique and on all but 2 by the *in vitro* tests. Deviations, variances, and standard deviations were computed according to the usual methods as described previously (4) and the results are presented in Table V.

It will be seen that the standard deviation of end points obtained by the

in vivo method of titration with the preparations of free virus in this series was 0.155 log unit or approximately half the value obtained in similar titrations of combined PVM in the previous experiments (4). The standard deviations of end points in hemagglutination tests with unheated and heated aliquots, respectively, were practically identical and had a value of approximately 0.10 log unit. The number of preparations included in this series is not large and the number of titrations carried out is not optimal for statistical analysis. The results obtained, however, indicate that the experimental error in titrations of free PVM by any of the techniques employed does not exceed and probably

TABLE V

Deviations, Variances, and Standard Deviations of Titration End Points with Free Infectious PVM

Titration of	PVM mouse lung suspension	No. of preparations	No. of titrations	Geometric mean titer Log	Geometric mean deviation from mean titer Log	Variance	Standard deviation
Infectiousness for mice	Unheated	16	36	−2.80	±0.13	0.024	0.155
		13*	**28**	**−2.98**			
Hemagglutination *vs.* mouse RBC	Unheated	16	47	−3.05	±0.06	0.009	0.095
		13*	**39**	**−3.04**			
Hemagglutination *vs.* mouse RBC	70°C. 30 min.	15	45	−3.09	±0.09	0.011	0.105
		12*	**36**	**−3.07**			

* Three preparations which had infectivity titers significantly lower than hemagglutination titers were excluded.

is less than the experimental error previously found in titrations of combined PVM *in vivo* (4).

In Fig. 1 is shown graphically the degree of correlation obtained between infectivity and hemagglutination titers on the same 16 preparations of free infectious PVM used in the above analysis. The position of each point was determined by plotting the logarithm of the infectivity titer against the logarithm of the direct hemagglutination titer obtained with each unheated preparation. The connected cross points indicate the corresponding hemagglutination titers obtained after heating each preparation at 70°C. for 30 minutes. Most of the points represent the geometric mean value of several titrations by each technique with each preparation. It will be seen that 13 of the points are distributed along a straight line with a slope of 1 which happens

to intercept both axes at zero; they are within or close to an area enclosed by the broken lines which indicate the limits of probable significant deviations from the line. It should be noted that 3 points deviate significantly from the line and that each does so because the virus titer was considerably less than the corresponding hemagglutination titers. In view of the recognized instability of the property of infectiousness with this virus, these discrepancies can be attributed to partial loss of infectiousness prior to testing. It is of importance to point out that in no instance was the infectivity titer significantly higher than the corresponding hemagglutination titers. It is also of importance

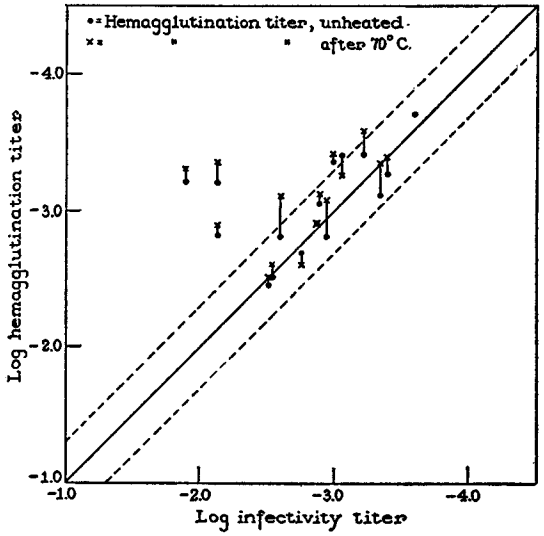

FIG. 1. Relation of infectivity and hemagglutination titers with various preparations of free infectious PVM.

that in no instance was there a definitely significant deviation between hemagglutination titers obtained with unheated and heated aliquots.

In Table V the geometric mean titers obtained for all 16 preparations by each method of testing are given. It will be seen that the values obtained by the *in vitro* techniques were almost identical and that the mean titer determined by the *in vivo* technique, although somewhat lower, was of the same order of magnitude. Recalculations of these values, after omitting the 3 preparations which had significantly lower infectivity than hemagglutination titers, did not appreciably alter the mean hemagglutination titers but obviously resulted in a higher value for the *in vivo* titer. With this correction the mean values obtained by each method were practically the same, and there was no significant difference between any 2 of them.

These findings indicate that in each preparation the greater part of the virus demonstrable by any of the methods employed was capable of combining with and agglutinating mouse RBC, and suggest that most of the virus in each of these preparations was in a free state and was not combined with tissue particles.

DISCUSSION

Present evidence appears to justify the conclusion that PVM may occur in a free state or in combination with particles obtained from certain tissues although not from other tissues. Moreover, the virus, whether in the free or combined state, may lose the property of infectiousness either as a result of some experimental procedure such as heating, or spontaneously due to unfavorable conditions sometimes difficult to identify or control. As indicated

TABLE VI

Observed States of PVM

State of virus	Results of tests for		
	Infectivity in mice	Hemagglutination	
		Unheated	70°C.
1. Free, infectious	+	+	+
2. " non-infectious	0	+	+
3. Combined infectious	+	0	+
4. " non-infectious	0	0	+

in Table VI, PVM may occur in any one of 4 different states and in each state gives a different pattern of reactions with the 3 most convenient methods of testing. It has been shown that free infectious virus gives positive reactions in tests for infectivity and in tests for hemagglutination before and after heating, and that the titers obtained are comparable. With free virus which has lost its infectiousness the results of hemagglutination tests remain unchanged even though the virus cannot be detected by tests for infectivity. With combined virus hemagglutination occurs only after the combination has been dissociated, and the virus released as by heating at 70°C. or by treatment with alkali. With a fully infectious preparation the titers of infectivity obtained with an unheated aliquot, and of hemagglutination with a heated aliquot, closely correspond. When combined virus has lost its infectiousness, its presence and titer are indicated by results obtained with only one of the 3 convenient procedures for testing, namely, hemagglutination following heat dissociation of the combination.

In Table VII various properties of PVM are tabulated in relation to each

of the 4 different states in which it appears that this virus may occur. It is seen that free virus, whether infectious or non-infectious, has properties identical with those of heat-released virus. Combined virus, whether infectious or non-infectious, differs from free virus in that it fails to cause hemagglutination or to combine with added tissue particles; it cannot be tested by the agglutination-inhibition technique and does not react in complement fixation tests with specific immune serum; it is easily sedimentable and not readily filterable.

TABLE VII

Properties of PVM in Relation to Each Observed State

Property	State			
	Free		Combined	
	Infectious	Non-infectious	Infectious	Non-infectious
Infectivity	+	0	+	0
Hemagglutination	+	+	0	0
Combination with added lung tissue particles	+	+	0	0
Easily sedimentable	0	0	+	+
Readily filterable	+	+	0	0
Antigenicity	−	+	+	−
Neutralizable by immune serum	+		+	
Agglutination inhibition by immune serum	+	+		
Complement fixation	−	+	0	0
Destruction by proteolytic enzymes	+	+	+	+
Water insolubility (dialysis)	+	+	+	+

− = not tested.

It should be emphasized, however, that these differences between PVM in the free and combined states are not attributable to any intrinsic alteration in the virus itself, for, when the virus is dissociated from combination, it retains all of the properties of free virus excepting only the capacity to produce infection. The virus in either the free or the combined state is destroyed by proteolytic enzymes, is non-dialyzable, and is water-insoluble. With either free or combined PVM the only distinguishing difference between the infectious and non-infectious states lies in the capacity to induce evidence of infection in susceptible animals and, dependent upon this, in the capacity to be demonstrably neutralized by specific immune serum *in vivo*.

It is apparent that Tables VI and VII are oversimplified presentations of the available data. It is impossible by present techniques to make certain

that all of the virus in a given preparation is actually free. It is equally impossible to determine whether all of the virus, either free or combined, is infectious. By differences in titers obtained with the 3 techniques employed and by more elaborate procedures described earlier, it is possible, however, to estimate with some approximation of accuracy which states of the virus are present in a given preparation and in what relative proportions.

Of greater importance, however, is recognition of the fact that PVM may occur in 4 different states, and that in each state the demonstrable properties of the virus are distinctive. The test for infectivity, that most generally used in the study of viruses, obviously fails to detect non-infectious free or combined virus in a suspension even when present in high titer and capable of specific activity in other respects. Employing methods of differential separation such as filtration or centrifugation, most of the virus might be recovered or lost in one or another fraction depending upon the state of the virus in the preparation used. It is quite obvious that in attempts to determine the physical or chemical properties of PVM, or to estimate the size of virus particles, the results obtained are greatly influenced by the state of the virus in the preparations used.

It is interesting to speculate upon the significance of the combination which occurs between PVM and the substance present in certain tissues. The substance occurs in the lungs of animals susceptible to infection by this pneumotropic virus but not in the extra-embryonic fluids or tissues of chick embryos which apparently are not readily susceptible to infection by it. Does the occurrence of combination in any way determine the apparent strict pneumotropism of this agent? Or does the occurrence of combination reduce the opportunity of the virus to invade susceptible tissue cells?

Present evidence indicates that some, if not all, of the virus as it occurs in infected mouse lungs *in vivo* is free and that combination results when the lungs are removed and ground, thus exposing the virus particles to lung tissue constituents with which they may have no contact *in vivo*. As an hypothetical illustration, if the virus multiplies in the cytoplasm of susceptible pulmonary cells and the substance responsible for combination occurs in the nucleus, combination would not be expected *in vivo* but would result only when the cells were traumatized and the virus and combining substance intimately mixed as in the process of grinding. The technique of obtaining free infectious virus from perfused lungs which are cut in small pieces but not ground, by greatly diminishing the trauma to cells, would be expected to reduce the amount of combining substance liberated from tissues and thereby provide less opportunity for virus particles and this tissue constituent to come together and combine.

Although combined virus when inoculated by the intranasal route is capable of producing pulmonary infection in susceptible animals, it seems improbable

that the virus actually penetrates susceptible tissue cells in this complex state. It appears more likely that in mouse lungs, possibly at the membrane of the susceptible tissue cells, the combination is dissociated and the released virus enabled to enter and multiply within the cell. Evidence in support of this concept might be obtainable by identification of the combining substance, by the demonstration of a cellular mechanism capable of dissociating combined virus, and by evidence that tissues lacking this mechanism could be infected with virus in the free but not in the combined state.

It is possible that relatively stable combination with tissue particles as exhibited by PVM is a property peculiar to this virus alone. It seems more likely, however, that this phenomenon is not unique and that other viruses may also be found to behave in a similar manner. If other viruses are found to be capable of combining firmly with tissue substances, present estimates of their physical and chemical properties may require re-evaluation. The fact that PVM may occur in stable combination affords a basis for re-assessing methods used to prepare suspensions of viruses, particularly agents which are transmissible only with difficulty or which are demonstrable only in low titer and perhaps also those which appear to be transmissible exclusively by cellular transplants.

SUMMARY

Additional evidence is presented that PVM is capable of combining firmly with lung tissue particles or erythrocytes from certain susceptible species. Release of the virus from such combination can be effected by treatment with alkali as well as by heating. Free virus expressed from infected lungs without grinding the tissues is infectious and causes hemagglutination when tested directly. The concentration of virus can be estimated more accurately by means of the hemagglutination technique than from the results of infectivity tests in mice. Present evidence indicates that PVM may occur in 4 different states and that in each state it manifests distinctive properties.

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