THE EFFECT OF SULFHYDRYL GROUPS ON PNEUMONIA VIRUS OF MICE (PVM)

By MOGENS VOLKERT, M.D., and FRANK L. HORSFALL, Jr., M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

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The results of previous studies (1, 2) have shown that pneumonia virus of mice (PVM) is very unstable as regards the property of infectiousness but is remarkably stable with respect to its capacity to cause hemagglutination. In the course of studies described in the accompanying paper (3), on the virus-combining component present in the lungs of animal species susceptible to infection with PVM, evidence was obtained which indicated that the titer of the virus, as measured by hemagglutination, is influenced strikingly by the concentration of the lung tissue suspension containing it. Because the virus appeared to be affected deleteriously in suspensions of high concentration, an explanation for this unexpected finding was sought.

It is the purpose of this paper to present the results of experiments concerning the effect of the concentration of lung tissue suspensions on PVM. It will be shown that the virus is adversely affected in concentrated suspensions as well as in the presence of glutathione; that the effect is prevented in both instances by the addition of iodoacetamide; and that, in all probability, the decreased stability of the virus in concentrated lung suspensions or in the presence of glutathione is caused by the presence of sulfhydryl groups.

Methods

Virus.—Pneumonia virus of mice (PVM), strain 15 (4), was employed. Suspensions of infected mouse lungs in saline were prepared as described in the accompanying paper (3). Such suspensions have been shown to contain combined infectious virus (2). Heat-released virus was obtained by heating suspensions at 70°C. for 30 minutes as described previously (2). Free infectious virus was obtained from intact infected lungs by the centrifugation technique described previously (3, 5). Infectivity tests in mice were carried out exactly as in earlier studies (1).

Hemagglutination Tests.—The technique of hemagglutination tests with mouse RBC and the method of estimating end points were identical with those previously employed (2).

Glutathione.—The glutathione used was obtained from a commercial laboratory.[1] A fresh solution adjusted to pH 7.0 in saline was prepared for each experiment and used promptly.

Iodoacetamide.—Dr. Rollin D. Hotchkiss of the Rockefeller Institute prepared and kindly supplied the iodoacetamide used. Solutions were prepared in saline.

EXPERIMENTAL

Stability of PVM in Mouse Lung Suspensions.—Experiments were carried out to determine the effect of the concentration of mouse lung suspensions

[1] Schwarz Laboratories, Inc., New York.

on the PVM contained in them as evidenced by the capacity of the virus to induce infection and to cause hemagglutination following appropriate heating (2).

Suspensions of mouse lungs infected with PVM were prepared in saline. The concentration of the suspensions, in terms of the wet weight of the lungs, ranged from 50 to 10 per cent. Suspensions were held at various temperatures and at intervals aliquots were removed. Hemagglutination titers were determined after aliquots had been heated at 70°C. for 30 minutes. Infectivity titers were determined on unheated aliquots in mice.

The results of typical experiments are shown in Table I. It will be seen that, when suspensions were heated a short time after preparation, the hemagglutination titers of concentrated suspensions were not proportionately higher

TABLE I

The Effect of the Concentration of Mouse Lung Suspensions on the PVM Contained in Them

PVM mouse lung suspenson	Suspension held		Infectivity titer M. S. 50	Hemagglutination titer following 70°C. for 30 min.
	Temperature	Time		
per cent	*°C.*	*hrs.*	*log*	
10	24	1	−4.21	512*
25	24	1	−4.14	256
50	24	1	—	256
10	4	24	−4.21	768
25	4	24	−4.00	0
10	37	10	—	512
50	37	10	—	32

* Reciprocal of titer.

but actually were lower than those of 10 per cent suspensions. With increasing time even more strikingly discrepant results were obtained with concentrated suspensions. The marked decrease in hemagglutination titer which occurred with concentrated lung suspensions varied somewhat from one experiment to another, but it was found regularly that, after 10 hours at 37°C. or 24 hours at 4°C., only very little of the virus originally present in concentrated suspensions could be demonstrated by the hemagglutination technique.

It should be noted, however, that the capacity to induce infection was undiminished in concentrated lung suspensions which had been held at 4°C. for 24 hours, despite the fact that no virus was demonstrable in the same suspensions by the hemagglutination technique after appropriate heating. It appeared, therefore, that PVM was not actually destroyed in concentrated lung suspensions held at 4°C. even though it was rendered no longer demonstrable by the hemagglutination procedure.

In other experiments a fresh 50 per cent suspension of normal mouse lungs in saline was added to a 10 per cent suspension of infected lungs. Aliquots of the mixture were held at 37°C. or 4°C. and at intervals samples were removed and tested as before. The results obtained were similar to those described above. The addition of normal lung tissue to suspensions of infected lungs caused a prompt reduction in the amount of virus which could be released by heating and, with increasing time, the amount of virus demonstrable by the hemagglutination technique progressively decreased.

Effect of Glutathione on Stability of PVM.—Because tissue suspensions provide reducing conditions, experiments were carried out to determine whether the addition of a reducing substance, *e.g.* glutathione, to infected mouse lung suspensions would exert an effect upon PVM similar to that observed with concentrated suspensions.

To 10 per cent suspensions of mouse lungs infected with PVM (combined infectious virus[2]) a fresh solution of glutathione was added to give a concentration of 0.005 M. The mixtures were held at 24°C. and at intervals samples were removed. Infectivity titers were determined in the usual manner in mice. Hemagglutination titers were determined following heating at 70°C. for 30 minutes.

The results of representative experiments are shown in Table II. It was found that the addition of 0.005 M glutathione to PVM mouse lung suspensions had no effect upon the infectivity titer of the suspensions but, following heating of the mixtures, no virus could be demonstrated by the hemagglutination technique. These results indicate that in the presence of glutathione PVM is incapable of withstanding the degree of heating employed as routine to release the virus from combination with the lung tissue component (2, 3).

If the effect of glutathione on the heat stability of PVM were due to the sulfhydryl groups of the compound, it would be expected that the addition of iodoacetamide, which combines with sulfhydryl groups (6), would prevent the effect of glutathione on the virus.

To 10 per cent suspensions of infected mouse lungs was added either iodoacetamide solution to give a concentration of 0.01 M or a fresh solution of glutathione to give a concentration of 0.005 M. The mixtures were held at 24°C. and at intervals samples were removed. To aliquots of those mixtures which contained glutathione, iodoacetamide was then added. Hemagglutination titers were determined following heating in the usual manner.

The results of typical experiments are shown in Table II. It is evident that the addition of iodoacetamide to a lung suspension containing glutathione completely prevented the effect of glutathione on the heat stability of PVM. Moreover, when 0.005 M glutathione was employed, iodoacetamide (0.01 M)

[2] As indicated in the accompanying paper, combined infectious virus refers to PVM present in ground lung tissue suspensions; although fully infectious the virus does not cause hemagglutination because it is in combination with a lung tissue component.

could be added 1 hour after the addition of glutathione and elimination of the effect of glutathione on the virus was obtained.

Similar experiments were carried out with free PVM. Both heat-released (2) and free infectious virus (5) were employed. It will be recalled that free PVM possesses the advantage that it causes hemagglutination directly and, unlike combined PVM, does not require further treatment to unmask this property.

TABLE II

The Effect of Low Concentrations of Glutathione and Iodoacetamide on PVM

PVM	Added before incubation		Incubation at 24°C. for	Added after incubation	Infectivity titer M. S. 50	Heating	Hemagglutination titer
	Glutathione	Iodoacetamide		Iodoacetamide			
					log		
Combined infectious virus	0	0	1 hr.	0	−4.21	70 C. for 30 min.	512*
" "	0.005 M	0	1 "	0	−4.11	" "	0
" "	0	0.01 M	1 "	0	—	" "	512
" "	0.005 M	0	1 "	0.01 M	—	" "	512
Heat-released virus	0	0	2 hrs.	0	—	None	512
" "	0.005 M	0	2 "	0	—	"	512
" "	0.005 M	0	1 min.	0	—	70 C. for 30 min.	0
" "	0.005 M	0	2 hrs.	0	—	" "	0
" "	0.005 M	0	24 "	0	—	" "	256
" "	0	0.01 M	1 hr.	0	—	" "	512
" "	0.005 M	0	1 "	0.01 M	—	" "	512
" "	0.005 M	0.01 M	1 "	0	—	" "	512
Free infectious	0.005 M	0	1 "	0	—	None	512

* Reciprocal of titer.

The results of these experiments also are presented in Table II. It will be seen that the presence of glutathione (0.005 M) caused no reduction in the hemagglutination titer of either heat-released or free infectious virus, but that, when the mixtures were heated within 2 hours of their preparation, the capacity to cause hemagglutination was lost. It was found that the effect of glutathione on the virus was produced promptly; 1 minute after the addition of glutathione no virus could be demonstrated by means of hemagglutination if the mixture was heated at 70°C. With increasing time the effect of glutathione progressively decreased and when mixtures were heated 6 to 24 hours after preparation maximal hemagglutination titers were again obtained. It is well known that

glutathione is unstable in solution and that on standing it gradually becomes oxidized to the disulfide form. It appears probable that elimination of the effect of glutathione on PVM with increasing time of incubation of a mixture is similar to the effect which was obtained when iodoacetamide (0.01 M) was added 1 hour after the mixture had been prepared.

It was of interest to determine whether concentrations of glutathione greater than 0.005 M would cause even more marked effects on PVM. Therefore, further experiments, similar to those described above, were performed.

To preparations of combined infectious virus (10 per cent suspensions of infected mouse lungs), heat-released virus, or free infectious virus was added a fresh solution of glutathione to give a concentration of 0.05 M. The mixtures were held at 37°C. and at intervals samples were removed and their hemagglutination titers determined. To aliquots of these mixtures, either before incubation or following it, a solution of iodoacetamide was added to give a concentration of 0.1 M.

The results of typical experiments are presented in Table III. It was found that, when 0.05 M glutathione was present in suspensions containing combined PVM, it was necessary to add iodoacetamide (0.01 M) immediately if the effect of glutathione on the virus was to be inhibited. One hour after the addition of glutathione in this concentration, the addition of iodoacetamide failed to eliminate the effect of glutathione on the hemagglutinating capacity of the virus.

Moreover, with 0.05 M glutathione, striking direct effects were obtained on the hemagglutination titer of free PVM. As is shown in Table III, the hemagglutination titer of either heat-released or free infectious virus rapidly decreased in the presence of 0.05 M glutathione and, after 1 hour at 37°C., little or no virus was demonstrable by the hemagglutination technique even when heating was not employed. With free infectious virus it was found that the capacity of the virus to induce infection also decreased rapidly in the presence of 0.05 M glutathione. When iodoacetamide (0.1 M) was added simultaneously with glutathione, the striking reduction in hemagglutination titer was completely prevented but, if iodoacetamide was not added until 1 hour after the addition of glutathione, the reduction in hemagglutination titer was not reversed. Combined infectious virus, heat-released virus, and free infectious virus appeared to be affected in an identical manner by glutathione.

The results of these experiments indicate clearly that in the presence of glutathione the stability of PVM, either in the combined or the free state, is decreased. In the presence of relatively low concentrations of glutathione (*i.e.*, 0.005 M) the effect can be either prevented or eliminated by the addition of iodoacetamide. However, in the presence of relatively high concentrations of glutathione (*i.e.*, 0.05 M) the effect can be prevented but cannot be eliminated by the addition of iodoacetamide. The capacity of free PVM to cause hemag-

glutination is unaffected by low concentrations of glutathione but rapidly decreases, as also does the capacity to induce infection, when high concentrations are present. The reduction in the hemagglutination titer of free virus under these conditions can be prevented but not completely reversed by the addition of iodoacetamide.

TABLE III

The Effect of High Concentrations of Glutathione and Iodoacetamide on PVM

PVM	Added before incubation		Incubation at 24°C. for	Added after incubation	Infectivity titer M. S. 50	Heating	Hemagglutination titer
	Glutathione	Iodoacetamide		Iodoacetamide			
					log		
Combined infectious virus	0	0	1 hr.	0	—	70 C. for 30 min.	512*
" "	0.05 M	0	1 "	0	—	" "	0
" "	0	0.1 M	1 "	0	—	" "	256
" "	0.05 M	0	1 "	0.1 M	—	" "	8
" "	0.05 M	0.1 M	1 "	0	—	" "	256
Heat-released virus	0	0	1 hr.	0	—	None	512
" "	0.05 M	0	1 min.	0	—	"	256
" "	0.05 M	0	30 "	0	—	"	32
" "	0.05 M	0	1 hr.	0	—	"	0
" "	0	0.1 M	1 "	0	—	"	256
" "	0.05 M	0	1 "	0.1 M	—	"	16
" "	0.05 M	0.1 M	1 "	0	—	"	256
Free infectious virus	0	0	1 "	0	−2.61	"	256
" "	0.05 M	0	1 "	0	−0.72	"	2

* Reciprocal of titer.

Effect of Iodoacetamide on Stability of PVM in Lung Suspensions.—The effect of glutathione on PVM appeared to be analogous to the effect of concentrated lung suspensions on the virus. Because iodoacetamide was capable of preventing the effect of glutathione and of eliminating its action under appropriate conditions, experiments were carried out to determine whether the addition of iodoacetamide to concentrated PVM lung suspensions would prevent the marked reduction in hemagglutination titer which occurs in such suspensions.

Suspensions of mouse lungs infected with PVM, which in terms of wet weight ranged from 50 to 10 per cent, were prepared in saline. To aliquots of these suspensions a solution of

iodoacetamide was added to give a concentration of 0.01 M. The suspensions and mixtures were held at 37°C. and at intervals samples were removed. To aliquots of the suspensions iodoacetamide (from 0.01 to 0.1 M) was then added, following which all samples were heated at 70°C. for 30 minutes and their hemagglutination titers determined in the usual manner.

The results of typical experiments are shown in Table IV. It will be seen that the addition of iodoacetamide did not alter the hemagglutination titer of 10 per cent suspensions and, moreover, that such suspensions could be held at 37°C. for 20 hours without the addition of iodoacetamide and still show maximal

TABLE IV

The Effect of Concentration of Mouse Lung Suspensions and of Iodoacetamide on PVM

PVM mouse lung suspension	Added before incubation	Incubation at 37°C. for	Added after incubation	Hemagglutination titer following 70°C. for 30 min.
	Iodoacetamide		Iodoacetamide	
per cent		*hrs.*		
10	0	0	0	512*
"	0.01 M	0	0	512
"	0	20	0	512
50	0	0	0	256
"	0	0	0.01 M	2048
"	0	10	0	32
"	0	10	0.01 M	1024
"	0	20	0	0
"	0	20	0.01 M	16
"	0	20	0.1 M	16
"	0.01 M	20	0	1024

* Reciprocal of titer.

hemagglutination titers after appropriate heating. On the other hand, the addition of iodoacetamide to 50 per cent supensions of infected lungs resulted in a marked increase in their hemagglutination titers following heating. In the presence of iodoacetamide the titer of 50 per cent suspensions was found regularly to be five times higher than that obtained with 10 per cent suspensions.

When iodoacetamide was added to 50 per cent suspensions and the mixture then was incubated for 20 hours, no significant decrease in hemagglutination titer occurred. Even when it was added following 10 hours' incubation of 50 per cent suspensions, an almost undiminished hemagglutination titer was obtained following heating, whereas during an identical period in the absence of iodoacetamide a very marked reduction in titer occurred. When the incubation period with such concentrated suspensions was 20 hours, in the absence of iodoacetamide no virus could be demonstrated by the hemagglutination technique.

The results of these experiments indicate clearly that iodoacetamide is capable of preventing the deleterious effect of concentrated lung suspensions upon the heat stability of PVM and even of restoring this. Moreover, they show that during incubation at 37°C. the virus in concentrated lung suspensions becomes progressively less stable to heat. After 10 hours' incubation this effect is almost completely eliminated by the addition of iodoacetamide but after 20 hours' incubation the effect is almost entirely irreversible even though much iodoacetamide is added.

DISCUSSION

The results obtained in this study indicate that the presence of sulfhydryl groups is an important factor which influences the stability of PVM. In the presence of sulfhydryl groups provided by 0.005 M glutathione the capacity of the virus to induce infection is unaltered but it is promptly rendered less stable to heat. The effect of this concentration of glutathione on the heat stability of the virus is eliminated either by the spontaneous oxidation of glutathione which occurs in solution or by the addition of iodoacetamide which combines with sulfhydryl groups. In the presence of sulfhydryl groups provided by 0.05 M glutathione irreversible changes in the virus take place, the capacity to induce infection is lost rapidly, and there is associated with this change a corresponding decrease in hemagglutination titer. Both free and combined virus are similarly affected by high concentrations of glutathione and, although the effect can be prevented completely by the addition of iodoacetamide, it is not eliminated by the addition of this substance, once it has occurred.

In concentrated lung tissue suspensions changes are induced in the virus which are strikingly similar to those which occur in the presence of glutathione. Under appropriate conditions (*e.g.*, 4°C.) in concentrated suspensions the capacity of the virus to induce infection is unaltered but marked instability to heat develops. This latter change, like that which occurs in the presence of glutathione, can be prevented by the addition of iodoacetamide. Similarly, if the reaction has not been allowed to proceed for too long a period (*e.g.*, 10 hours), the decreased heat stability of the virus can be eliminated by the addition of iodoacetamide. After a longer time an irreversible change in heat stability occurs analogous to that obtained with 0.05 M glutathione, and this is not eliminated by iodoacetamide.

The finding that the infectious as well as the hemagglutinating properties of PVM are both similarly affected in either concentrated lung tissue suspensions or in the presence of glutathione, and that these effects are prevented or eliminated in like degree by iodoacetamide, indicates that in both instances the alterations in the properties of the virus are dependent upon the presence of sulfhydyl groups.

SUMMARY

Evidence is presented which indicates that PVM is affected adversely in concentrated lung tissue suspensions or in the presence of glutathione. Because iodoacetamide inhibits or eliminates these effects in a similar manner, it is concluded that sulfhydryl groups are essential to their development.

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