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Studies on the Haemagglutinating and Complement Fixing Activities, and Infectivity of Murray Valley Encephalitis Virus

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Introduction

Since haemagglutination (HA) by a number of arboviruses was first demonstrated[2,3,4,25], the methods for conducting HA and haemagglutination inhibition (HI) tests with arboviruses have undergone a number of modifications. The most generally useful source of HA antigens for arboviruses is the brain of suckling mice.

Clarke and Casals[7] established the HI and HA test with acetone extracted and protamine sulphate treated arbovirus antigens (mouse brain). Tween-ether treated sindbis virus antigen was recommended by Mussgay and Rott[16]

Complement-fixing (CF) antigen of Murray Valley Encephalitis (MVE) virus was first prepared from infected chorioallantoic membrane extracts of chicken embryos[9] and later from infected mouse brain[1,6,15].

Animal viruses have been extensively studied by zonal celntrifugation techniques. Poliovirus was sufficiently purified by zonal sedimentation to crystallize the virus particles.[23] Foot-and-mouth disease virus has two CF particles which can be separated by zonal centrifugation.[25] Density gradient centrifugation has also been used to study murine leukemia virus,[20] influenza virus[10] adenovirus[19] and Semliki Forest virus[6]

Infectivity, HA, and CF activite materials of various viruses were separated by zonal centrifugation; these are Sindbis virus,[16,17] Dengue virus,[24] and Japanese B Encephalitis (JBE) virus.[13]

In these experiments, the difference in the activity and heat stability of HA, CF and infective components of MVE virus after treatment with acetone, Tween-ether and Tween-ether-protamine sulphate, and a possibility of the separation of these components by zonal centrifugation were studied.

Materials and Methods

Virus[8]: Murray Valley Encephalitis virus (MVE, MRM 66) isolated originally from *C. annulirostris* at Mitchell river in 1960 and had undergone seven mouse brain passages was used.

Virus Infectious Materials : Two to six days old mice were inoculated 10^2 diluted 0.03ml stock infectious suckling mouse brain by the cerebral route. When the mice were begun to die and the rest appeared sick from the infection, the brains were removed and transferred to small, weighed glass bottles held in a bath of dry ice. The frozen mouse brains were homogenized in a homogenizer.

Tween 80 and ether Treatment; The Tween-ether treatment method of Mussgay and Horzineck[17] was used. To one volume of frozen and infected suckling mouse brain with MVE virus nine volumes of borate buffer of pH 9.0, ten volumes of peroxide-free ether, and to this Tween 80 were added in one percent (v/v). The mixture was homogenized, centrifuged in the cold at 2,100 G. for five minutes and the turbid water phase was collected and residual ether was removed by nitrogen bubbling method[16]

Tween-ether-protamine sulphate Treatment; An adaption of the methods of Mussgay and Horzineck[17] and Clarke and Casals[7] were used. A 50mg/ml solution of protamine sulphate was prepared in 0.9 percent NaCl and used immediately after preparation.

The Tween-ether treated virus was chilled in an iced water bath and 0.1 volume of protamine solution was added. The mixture was allowed to stand at 4°C for 30 minutes with occassional shaking and then centrifuged in the cold at 700 G for 15 minutes. The clear supernatant fuid was used as the Tween-ether-protamine sulphate treated virus.

Acetone Treatment : Acetone treated virus was obtained by the procedure of Clarke and Casals[7].

Determination of Infectivity: Infectivity was assayed by $\log_{10}$ $TCID_{50}$ method of Reed and Muench[22] in PK2a (PS) cells using tris Hank's solution in amount of 0.2ml of inocula per tube and five tubes per dilution. The inoculated cells were examined daily over eight days incubation at 37°C. Infectivity was judged by cytopathic effect (CPE).

Determination of HA and CF Activities : The technique of HA test was adapted from the method of Clark and Casals[7]. The method of CF test was adapted from the methods of Fulton and Dumbell[11], Jeon[12], Kwapinsky[14] and Mussgay and Horzinek[17].

Zonal Centrifugation: The untreated(crude virus), Tween-ether treated, and acetone treated viruses were subjected to zonal centrifugation in a linear ten to 60 percent sucrose gradient. The total volume of gradient was 4.6 ml. After centrifugation each sample was possible to collect 270 drops which were divided into 15 fractions (18 drops in each fractions)[23].

Heating of the Virus : In the study of the HA and CF activities, the virus preparations treated with acetone, Tween-ether and Tween-ether-protamine sulphate, and the untreated virus were heated at 37°C, 56°C and 65°C, for 10, 20, 30, 40 and 60 minutes in a water bath.

Results

Treatment of the Virus with Acetone, Tween-ether and Tween-ether-protamine sulphate : In HA activity, all of these treatments led to eight or 16 fold increase. HA activities after treatment with acetone and with Tween-ether-protamine sulphate were not appreciably different. In CF activity, Tween-ether and acetone led to a two-fold increase. While treatment with Tween-ether-protamine sulphate decreased the activity. The crude virus showed a complete loss of infectivity after treatment with Tween-ether. However, acetone treatment resulted in a three log units decrease (table 1).

Table 1. Haemagglutinating and Complement Fixing Activities, and Infectivity with or without Treatment of Acetone, Tween-ether and Tween-ether-protamine sulphate on Murray Valley Encephalitis Virus (MRM66)

Treatment	Titre of virus material		
	HA@	CF	$\log_{10}TCID_{50}$
Acetone	2,560	160	3.5
Tween-ether	1,280	160	0
Tween-ether-protamine-sulphate	2,560	20	0
Control	160	80	6.5

@ Reciprocal dilution of virus.

Heat Stability of Haemagglutinating and Complement-fixing Activities : The HA activity of crude and acetone treated virus was more heat stable than

Table 2. The Effects of Incubation at 37°C, 56°C and 65°C on HA Activity of Acetone, Tween-ether and Tween-ether-protamine sulphate Treated and Crude Murray Valley Encephalitis Virus (MRM66) Materials

Treatment	Haemagglutinating titre, Incubation at									
	37°C						56°C		65°C	
	0@	10	20	30	40	60	10 to 60		10 to 60	
Acetone	5,120	5,120	5,120	5,120	5,120	160	10	10	10	10
Tween-ether	640	640	640	640	40	10	10	10	10	10
Tween-ether-protamine sulphate	1,280	1,280	1,280	320	40	10	10	10	10	10
Control	160	160	160	160	160	40	10	10	10	10

@ Treating time in minutes.

that of Tween-ether treated and Tween-ether-protamine sulphate treated virus. The former preparations showed its activity at 37°C for 40 minutes exposure without loss of titre.

The CF activity of crude and acetone treated virus was also more heat stable than that of Tween-ether treated and Tween-ether-protamine sulphate treated virus (Table 3).

Usually no HA activity was observed with treated and crude virus after heating at 37°C for 60 minutes while CF activities were increased by the same treatment (Table 2 and 3).

Table 3. The Effects of Incubation at 37°C, 56°C and 65°C on CF Activity of Acetone, Tween-ether and Tween-ether-protamine sulphate Treated and Crude Murray Valley Encephalitis Virus (MRM 66)

Treatments	Complement fixing titre															
	37°C						56°C					65°C				
	0@	10	20	30	40	60	10	20	30	40	60	10	20	30	40	60
Acetone	160	160	320	320	320	320	640	640	640	640	320	640	640	320	320	160
Twee-ether	160	160	160	320	160	160	160	160	160	80	80	80	80	40	40	10
Tween-ether-protamine sulphate	40	40	40	40	40	40	20	20	20	20	10	10	10	10	10	10
Control	80	80	160	160	160	160	320	320	160	160	160	160	160	160	160	80

@ Incubation time in minutes.

Stability of Haemagglutinating Activity at 9°C, and Haemagglutination Inhibition Activity of Tween-ether Treated and Acetone Treated Virus: The HA activity of Tween-ether treated virus was not appreciably changed by the storage at 9°C for 14 days when the HA test was carried out at pH 6.0 and pH 6.4, but at pH 6.7 the titre was decreased two-fold. After storage of Tween-ether treated virus at 9°C for 21 days, HA titres were decreased two-fold at pH 6.0 and pH6.4, and four-fold at pH 6.7 respectively. On the other hand, the HA activity of acetone treated virus was not changed after storage at 9°C for 28 days. The optimal storage pH was 6.0 for Tween-ether treated virus and 6.7 for acetone treated virus (Table 4).

Table 4. Stability of Haemagglutinating Activity of Tween-ether Treated Murray Valley Encephalitis Virus (MRM66)

Treatment	Storage at 9°C	HA titre		
		6.0@	6.4	6.7
Acetone	0(day)	128	128	256
Tween-ether	0	256	128	128
Acetone	14	128	128	256
Tween-ether	14	256	128	64
Acetone	21	128	128	256
Tween-ether	21	128	64	16
Acetone	28	128	128	256
Tween-ether	28	32	8	0

@ 6.0: pH6.0

Haemagglutinating and Complement-fixing Activities, and Infectivity of the Treated and Crude Virus by Centrifugation Technique: Infectivity and haemagglutinating activity of crude virus material were closely corelated. The peak of infectivity, most of the HA activity and one of the peak of CF activity were recovered in the middle part of the gradient. However, the second peak of CF activity showed a tendency of extension toward thinner fractions. These results may suggest that the crude virus particles responsible to the infectivity, haemagglutinating activity and a part of complement-fixing activity (Fig. 1a).

No difference could be found in HI titre, using MVE virus immune serum, between acetone and Tween-ether treated viruses. It was also shown that the haemagglutinins of Tween-ether preparations are possible to use in the HI test as those of the acetone treated virus (Table 4 and 5).

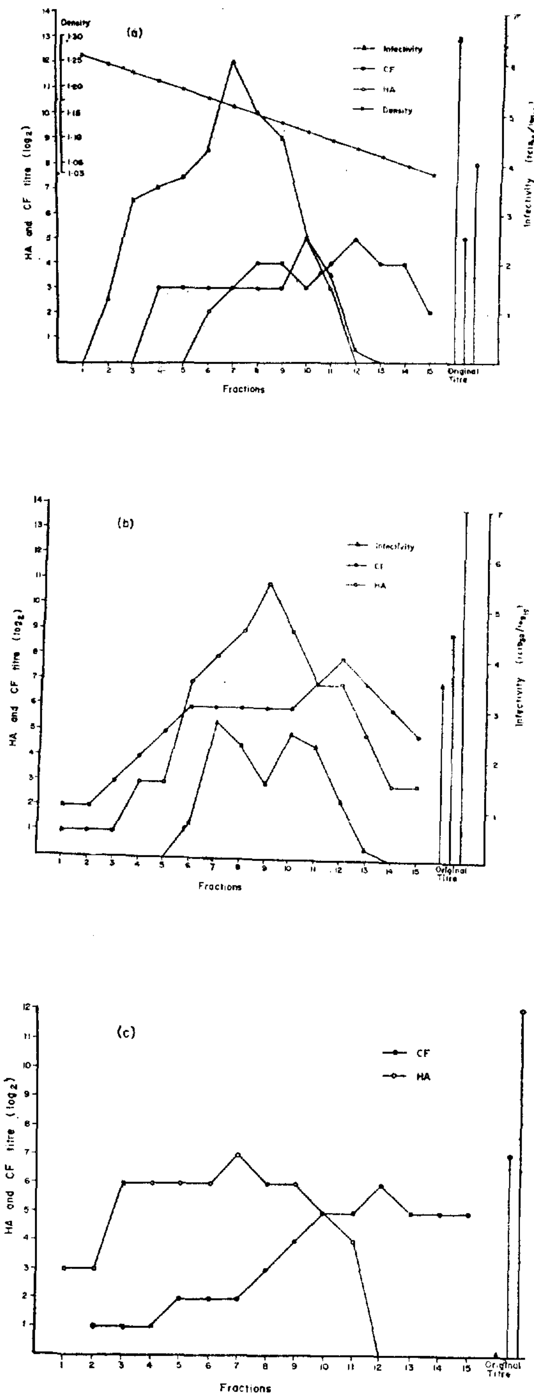

Fig. 1. Distribution of infectivity, haemagglutinating and complement-fixing activities of the fractions collected after zonal centrifugation in a linear 10 to 60 percent sucrose gradient of MVE virus (a) crude virus, (b) acetone treated and (c) Tween-ether treated virus.

Table 5. Haemagglutination Inhibition of Tween-ether Treated and Acetone Treated Virus by Anti-Murray Valley Encephalitis Rabbit Serum

Antigen	No. of test	HI titre
Acetone treated	1	2,560
Control	2	1,280
Tween-ether treated	1	2,560
Control	2	1,280

In the isolation of HA active component from CF active component by treating the virus with Tween-ether, the former showed its predominant activity in the lower half of the gradient, but with a peak in the middle part of the gradient. This may due to the haemagglutinin released from virus particles by Tween-ether treatment[16] The peak of CF activity occurred in the upper half of gradient, and the CF active fraction exhibited neither HA activity nor the infectivity (Fig. 1c).

In the distribution of HA and CF activities, and infectivity of acetone treated virus, both activities were recovered in all of the fractions collected. One peak of CF and HA activities, and one of the peak of infectivity were recovered in the upper half of the gradient. The three different peaks are likely located in a similar region of the gradient, however, the CF active peak and HA active peak showed such a tendencies as toward a lesser and a denser density, respectively. Two peaks of infectivity were observed, one in the middle part of the gradient, and the other in the upper half of the gradient. These three activities were not separated from each other by zonal centrifugation (Fig. 1b).

Discussion

Arbovirus particles break with a resultant loss of infectivity when subjected to the action of ether and detergents. The haemagglutinating and conplement-fixing antigens obtained after these treatments retain certain characters of the virions from which they are derived[16,18].

To eliminate normal arbovirus inhibitors and to purify arbovirus antigens derived from mouse brain, acetone, Tween-ether or Tween-ether-protamine sulphate were used. These caused a significant increase in HA activity, but not in CF activity. Tween-ether-protamine sulphate treatment rather caused a decrease in CF activity (Table 1). These results are in accordance with those of Cheng[6]. with Semliki Forest virus.

From these results it appears that Tween-ether treatment of MVE virus grown in mouse brain may be useful in the preparation of a high-titred, non-infectious, and stable antigen for use in the HI test. Additionally, Tween-ether treatment may possible with a small amount of virus material, and gives a high titred noninfectious haemagglutinin. The optimum pH for HA activity was found to differ after acetone treatment and Tween-ether treatment, that is, in Tween-ether treated virus was displaced to the acid side (Table 4). This result is similar to that of Mussgay and Rott [16] with Sindbis virus. The use of Sindbis virus, prepared with Tween-ether, at a pH of 5.8 not caused an appreciable change in HA titre, whereas in the case of untreated Sindbis virus concentrate 32 fold of HA titre was decreased at this pH.

The HA activity of Tween-ether treated virus was not appreciably changed by storage at pH 6.0, 9°C for 14 to 21 days (Table 4), and treatment with Tween-ether not affected to the HI activity of virus in immune serum (Table 5). A similar result was also found by Mussgay and Rott[16] with Sindbis virus.

The HA activity of Tween-ether-protamine sulphate treated virus was not suitable for use in the HI test because at 37°C over 30 minutes resulted in an unstable activity.

Some variation occurred in the CF activity of acetone, Tween-ether and Tween-ether-protamine sulphate treated MVE virus on exposure to heat, but usually the CF activity was much less affected by heat than the HA activity. HA and CF activities of crude virus (complete virus particles) were more heat stable than those of the Tween-ether treated virus (broken virus particles) (Table 2 and 3). A similar increase in CF activity after heating was also found by Planterose et al.[21] with foot-and-mouth disease virus.

By density gradient centrifugation of crude MVE virus two peaks of CF activity were demonstrated,

one accompanied by HA activity and infectivity in the dense part of gradient, and the other in the upper half of the gradient (low density). No CF particles of crude virus in the low density was observed, and acetone treatment increased HA activity and decreased infectivity. Two peaks of infectivity after acetone treatment occurred in the same part of gradient as with crude virus. These infectivity peaks represent complete virus particles which may not completely destroyed by acetone treatment. The CF and HI active peaks were difficult to distinguish from the infectivity (Fig. 1a and 1 b). While Tween-ether treatment produced a complete release of the noninfectious HA and CF antigens from the virion, acetone treatment produced only a partial release of these antigens and some were still associated with the infective virus particle. These results implied that the HA and CF activities were associated with different parts of the complete virion. A similar effect of Tween-ether treatment to MVE virus was found as was found in Sindbis virus[16]: that was a loss of infectivity and an appearance of virus specific sub-units having HI and CF activity. The CF components having a buoyant density less than the complete virus[17] appeared to be protein in nature, and were probably split products of the virus core and envelope. Protamine sulphate treatment of Dengue virus also resulted in CF and HA activity associated with particles other than the complete virus[6]. Stevens and Schlesinger[24] found that the non-infectious HA sub-units released from Dengue virus with lipid solvents were of greater density than the infectious virus, as was the case with MVE virus. Noninfectious HA and CF antigens have also been demonstrated by density gradient centrifugation with JBE virus after treatment with protamine sulphate and sonication by Kitaoka and Nishimura[13].

Summary

Throughout the studies the following experimental results were obtained and summarized.

1. Treatment of MVE virus with acetone, Tween-ether and Tween-ether-protamine sulphate caused an eight to 16 fold increase in HA activity.
2. Treatment with acetone and Tween-ether resulted in a four fold increase in CF activity. Treatment with Tween-ether-protamine sulphate decreased the activity.
3. The crude virus showed a complete loss of infectivity after treatment with Tween-ether, but three log unit was decreased with acetone treatment.
4. The HA activity of treated and crude virus was disappeared after heating at 37°C for 60 minutes but CF activity was increased.
5. Tween-ether or acetone treatment equally applicable to the preparation of haemagglutinin for HI test.
6. Zonal centrifugation of crude virus in a linear ten to 60 percent sucrose gradient showed two peaks of CF activity, and one of high buoy ant density part accompanied by HA activity and infectivity and the other of lower density part. Acetone treatment brought a decrease of the high density CF activity but not affected the second peak of low density found with crude virus, and resulted in increased HA activity and decreased infectivity. The peaks of HA, CF and infectivity after acetone treatment were not clearly separated. Tween-ether treatment caused a loss of the peak of CF activity found in the area of high density with crude virus, but the peak in the area of low density was not affected. This peak of CF activity was separated from noninfectious HA activity. The HA and CF activities were considered to be contributed by different parts of the varion.

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腦炎바이러스의 赤血球凝集力價와 補體結合力價 및 感染力에 관한 研究

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이 實驗은 腦炎바이러스(MVE virus)들 acetone, Tween-ether 그리고 Tween-ether-protamine-sulphate로 처리한 후 그 바이러스가 지니고 있는 赤血球凝集力價, 補體結合力價 그리고 感染力價의 변화 여부를 관찰함과 아울러 超遠心分離法에 의하여 이 바이러스가 지니고 있는 위의 세 가지 活性物質의 分劃 可能性을 검토한 결과 다음과 같은 결과를 얻었다.

1) Acetone, Tween-ether 그리고 Tween-ether protamine-sulphate 처리에 의하여 MVE virus가 지니는 赤血球凝集力價는 8~16 倍로 증가 하였다.

2) Acetone 및 Tween-ether 처리로 補體結合力價는 4倍로 증가 되었으나 Tween-ether protamine-sulphate 처리로는 저하되는 경향이 있었다.

3) Tween-ether로 처리하면 感染力價는 완전히 상실 되나 acetone으로 처리하면 TCID50/$\log_{10}$ 3 이 감퇴 되었다.

4) 위의 화학제의 처리와 관계없이 바이러스의 赤血球凝集力價는 37°C에서 10분간 加熱됨으로써 완전 소실 되었으나 補體結合力價는 상승 하였으며 이 活性度는 65°C에서 20분간 까지도 계속 유지 되었다.

5) 처리되지 않은 바이러스와 acetone 처리된 바이러스의 補體結合力價나 赤血球凝集力價는 Tween-ether 나 Tween-ether-protamine-sulphate로 처리한 바이러스의 그것보다 熱에 대해서 더 安定하였다.

6) 赤血球凝集抑制反應用 抗原製造에 있어서 Tween-ether로 처리하거나 acetone으로 처리하여 만든 두가지 抗原은 모두 同一한 力價를 보였다.

7) 10~60%의 sucrose gradient centrifugation에서 처리되지 않은 바이러스는 두개의 補體結合피크가 低濃渡와 高濃渡에서 각각 나타났으며 低濃渡에서 얻은 재료는 赤血球凝集力과 感染力을 동반하고 있었다.

8) Acetone으로 처리된 바이러스의 超遠心分離에서는 위에 말한 두 補體結合피크 중 高濃渡部의 것은 감소 되었으나 다른 하나는 영향을 받지 않았다. 그리고 赤血球凝集最高力價가 증가된 반면 感染最高力價는 감퇴 되었으며 이 세 가지 活性은 acetone 처리 후에는 명확히 分劃되지 않았다.

Tween-ether로 처리된 바이러스는 超遠心分離에 의하여 高濃渡部에 있는 補體結合피크는 완전 소실된 반면 低濃渡部에 있는 補體結合最高力價는 영향을 받지 아니 하였으며 이 피크는 感染力을 동반하지 않는 赤血球凝集素와 分劃이 가능 하였다.