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Concentration and Purification of Japanese Encephalitis Virus with Aqueous Polymer Two Phase System

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ABSTRACT: To obtain the concentrated and purified Japanese encephalitis (JE) virus preparation, the polyethylen glycol (PEG) precipitation method and the two phase system of aqueous polymer solution of sodium dextran sulfate (DS) and PEG were applied. The concentrated and partial purified JE virus precipitated with 5% or 8% PEG still contained cellular fragments. It was required further purification of that preparation. The concentrated JE virus with high purity was obtained by two step method of phase separation using DS-PEG system without loss of infectivity and biological properties. The suitable system of phase separation was given by the application of DS 0.5-PEG 8.0 (0.3 M NaCl) in initial step and DS 0.5-PEG 8.0 (1 M NaCl) in second concentration. JE virus preparation obtained with this system indicated 200 times more over of concentration effect, 455 fold of the concentration factor and 2.27 of infectivity index. This method was recommended for the purification and the concentration of JE virus from the large volume of starting material.

The distribution of some animal viruses in aqueous polymer two-phase system was introduced by Wesslen *et al.* (1959), Norrby *et al.* (1960), Albertsson *et al.* (1960) and Philipson *et al.* (1960). It was found that the virus particles were collected in a small volume phase and the phase system was able to use for the concentration of virus suspension without high speed centrifugation. The advantage procedure of the phase separation was reported by Philipson *et al.* (1960). The tow-step method of phase separation was excellent technique to obtain the concentrated virus preparation with high degree of purity. Furthermore, these methods were also suitable to deal with a large volume of starting material. However, little knowledge about the concentration and purification of arboviruses applying with this method are still remained. The present paper deals with the partition of Japanese encephalitis virus in aqueous two phase system of sodium dextran sulfate and polyethylen glycol.

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MATERIALS AND METHODS

Virus and cells: Nakayama-NIH strain of Japanese encephalitis (JE) virus was passaged 41 times in suckling mouse brain and adapted five times in vero cells in this laboratory. Vero cells grown in Eagles MEM supplemented with 2% calf serum, 0.1% yeast extract and 0.1 % polyvinylpyrolidone in 500 ml Rowx bottles were washed and infected with JE virus. The culture was maintained with Eagles MEM supplemeted with 0.5% bovine serum albumine. After 1, 2 and 3 day at 37°C incubation, the fluids were collected and stored at -75°C before use.

Infectivity assay: The plaque titration was performed with chick embyonated cells and the titers were presented as plaque forming units (PFU) per ml.

Hemagglutination (HA) test: HA titration was carried out by the method of Clarke and Casals (1958) . The adequate pH for the test was 6.4.

Preparation of polyethylen glycol 6,000 (PEG) precipitation: To make a final concentration of PEG required, appropriate amount of stock solution of PEG was added to the virus suspension with stirring in ice bath. The mixture was kept at 4°C for 1 hour and centrifuged at 8,000rpm (Refrigerator centrifuge, Tomy Seiko Co. Model RS 18P-2, Roter No.2N) for 15 minutes. The precipitate was resuspended with 0.01 M phosphate buffer saline (PBS) supplemented 0.3% bovine serum albumine at the proportion of 1/50 volumes of original matrial.

Preparation of phase system: Twenty percent (w/w) sodium dextran sulfate (DS) (μ/w 500,000 17% sulfate was contained) in 0.01 M PBS and 30% (w/w) PEG in 0.01 M PBS were made as stock solutions and kept at 4°C. Phase separation was obtained by the mixture of stock solutions of DS, PEG and freshly prepared NaCl solution. The final concentration of each reagents was presented in a formula of DSa-PEGb (n M NaCl). The sign of a and b mean the final percent (w/w) of DS and PEG and n means the final molarity of NaCl. The concentrated virus preparation was obtained in DS layer in this system. Consequently, DS in the virus preparation was removed by the method of Philipson *et al.* (1960). The virus preparation was added 3 M KCl at the propotion of 0.67 ml per 1 ml of DS and the sedimentation was removed by centrifugation at 3,000 rpm for 30 minutes. The supernatant was the purified and concetrated preparation and it was stored at -75°C.

Electronmicroscopic observation: Negative stain of the concetrated virus was carried out by the procedure of Hayashi *et al.* (1972). The preparations were observed with JEM 100B electronmicroscope.

RESULTS

Growth curve of JE virus in chick embryonated (CE) cells and vero cells and the preparation of JE virus

It was found that JE virus was released in the culture fluid after infection of 12 hours and reached at maximum titer of infectivity after infection of 16 hours or 20 hours both in CE cells infected with moi 1 and vero cells infected with moi 5 respectively. Consequently, the

fluid virus from vero cell cultures infected with the virus was combined and harvested at 24, 48 and 72 hours after infection by changing the maintenance medium respectively (Fig.1).

Effect of polyethylen glycol (PEG) and dextran sulfate (DS) on the infectivity of JE virus

Although it seemed to be preserved the infectivity of the virus with PEG, the infectivity of the virus kept at 4°C with or without PEG and DS have decreased gradually after 12 or 14 hours. According to this results, it was considered that the procedure for the JE virus concentration and purification with PEG and DS should be done within 18 or 24 hours in each step (Fig. 2).

Partial purification and concetration of JE virus by precipitation with PEG

It was attempted to precipitate JE virus by adding various final concentration of PEG in 0.25 M NaCl and 0.5 M NaCl solutions. The most highest value of the concentration factor and the virus recovery were indicated 44 or 39 fold and 88.1% or 78.9% in the systems of 5% or 8% PEG with 0.25 M NaCl respectively (Table 1). The precipitate suspended with 0.01 M PBS was usually associated with PEG. The difficulties of the removal of PEG in the concentrated and partially purified preparation of JE virus in this method were remained. The highly purified preparation of the virus could not be obtained without the additional procedure of column chromatography or density gradient sedimentation.

Phase separation with DS-PEG in 0.3 M NaCl

Phase diagram was obtained as follows: Certain concentrations of DS dissolved in PBS containing 0.3 M NaCl produced the white turbidity by adding with certain concentrations of PEG. At this time, the concentrations of DS and PEG in PBS were registered. The

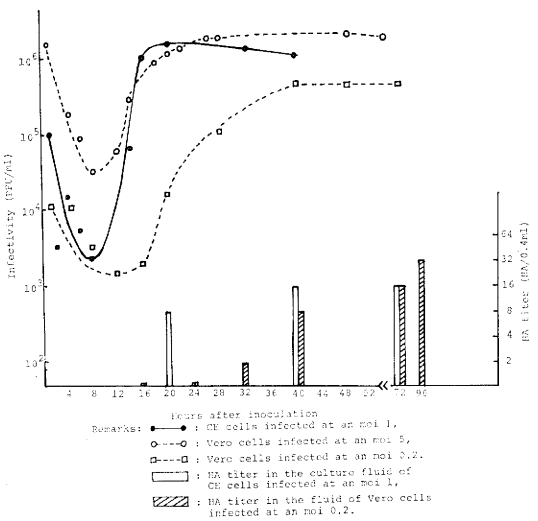

Fig. 1. Growth curve of JE virus in Vero and CE cells.

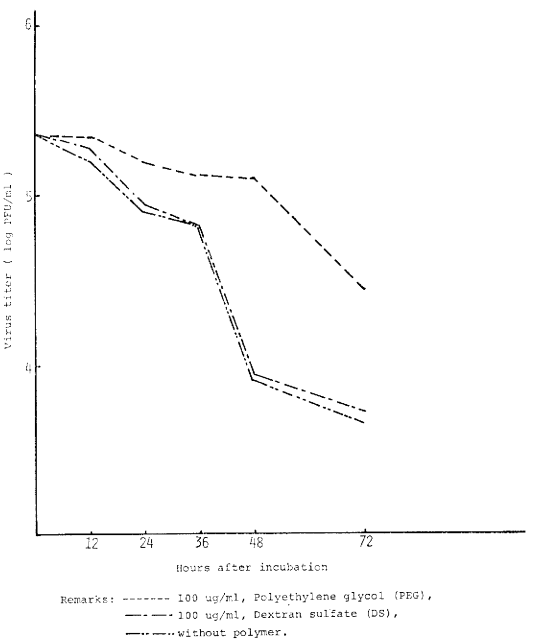

Fig. 2. Effect of PEG and DS on the infectivity of JE virus.

white turbidity was escaped by adding with the distilled water. At this time, the concentrations of DS and PEG were calculated and registered again. The production and disappearance of the turbidity due to variety of the concentrations of DS and PEG were given in the phase diagram as shown in Fig. 3. It was necessary to be selected the concentration of DS and PEG, particularly, as possible as small volume of DS for the production of turbidity, because JE virus was concentrated in DS phase.

Effect of the concentration of DS in the single step of aqueous two phase system on the concentration of JE virus

It was necessary to examine the concentration of DS for the production of turbidity in the single step method of phase separation under the condition of the fixed concentration of PEG and NaCl. The variety of precipitation due to the concentration of DS in the 8% PEG and 0.3 M NaCl solution was shown in Table 2. JE virus was highly concentrated in cases of DS 0.2-PEG 8.0 (0.3 M NaCl) or DS 0.5-PEG 8.0 (0.3 M NaCl) respectively. It was noted that, when DS was applied at higher concentrations than 0.8% in this system, the DS precipitate become increase in volume than that in case

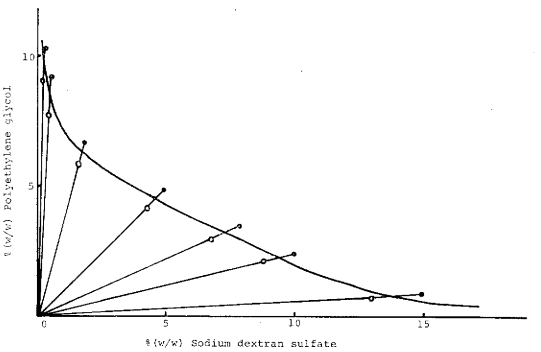

Fig. 3. Phase diagrams of DS-PEG with 0.3 M NaCl.

Table 1. Partial purification of JE virus by precipitation with various concentration of PEG and NaCl

Preparation	Virus titer (PFU/ml)	Concent. factor	Virus recovery (%)
original material (50ml)	1.9×10^5	1	100
3% PEG (0.25M NaCl)	4.8×10^6	25	50.5
5% PEG (0.25M NaCl)	8.4×10^6	44	88.1
8% PEG (0.25M NaCl)	7.5×10^6	39	78.9
10% PEG (0.25M NaCl)	2.3×10^6	12	24.2
15% PEG (0.25M NaCl)	2.5×10^6	13	32.9
18% PEG (0.25M NaCl)	2.6×10^6	14	34.2
3% PEG (0.5M NaCl)	1.9×10^5	1	19.7
5% PEG (0.5M NaCl)	3.0×10^6	16	31.6
8% PEG (0.5M NaCl)	3.0×10^6	16	31.6
10% PEG (0.5M NaCl)	3.1×10^6	16	32.6
15% PEG (0.5M NaCl)	2.0×10^6	11	21.1
18% PEG (0.5M NaCl)	1.2×10^6	6	12.6

1) The precipitate was resuspended with one fiftyth volume of the original volume.
2) Concentration factor means the value of PFU/ml of precipitate dividing by original one.

of DS 0.5-PEG 8.0 (0.3 M NaCl). Consequently, the concentration of the virus might be performed effectively (Table 2) in these cases. It was indicated that the adequate concentration of DS should be selected between 0.2% and 0.5%.

Effect of pH on JE virus concentration in the phase separation system

The phase system was prepared by 0.5% DS and 8% PEG adjusted pH with 1 N HCl or 1 N KOH respectively. Concentration factor was presented by dividing the hemagglutination (HA) titer in DS phase with that of the starting material. So far as the HA titer rising was concerned, the virus was concentrated sufficiently at pH 8.0 in DS 0.5-PEG 8.0 (0.3 M NaCl) system.

Concentration of JE virus with two step method of the phase separation

It was found that the preparation of JE virus concentrated with one step method of

Table 2. The effect of DS concentration in aqueous two phase system in 8% PEG on JE virus purification

Preparation	Volume (ml)	HA titration		Virus titration	
		HA/ml	Conc. factor	Virus titer (PFU/ml)	Conc. factor
1. Original material	200	20	1	1.6×10^6	1
2. DS 0.2-PEG 8.0 (0.3M NaCl)	1.0[1)]	320	16	2.5×10^7	15.6
3. DS 0.5-PEG 8.0 (0.3M NaCl)	1.5	320	16	2.0×10^7	12.5
4. DS 0.8-PEG 8.0 (0.3M NaCl)	5.5	160	8	7.5×10^6	4.7
5. DS 1.0-PEG 8.0 (0.3M NaCl)	6.2	80	4	2.9×10^6	1.8
6. DS 3.0-PEG 8.0 (0.3M NaCl)	50.0	20	1	2.6×10^6	1.6

1) The volumes obtained in the bottom phase of DS by single step of the aqueous two phase system were indicated.

Table 3. The effect of pH on JE virus purificaiton in a two phase system of 0.5 percent (w/w) DS and 8 percent (w/w) PEG

pH[1)]	Volume ratio[2)] (top/bottom)	HA titer (HA/ml)	Concentration factor
Original material		40	1
6.5	31.3	20	0.5
6.7	32.3	40	1
7.0	34.7	40	1
7.3	33.5	40	1
7.8	33.5	640	16
8.0	44.5	1,280	32
8.6	51.6	320	8

1) The pH value was adjusted with 1N HCl or 1N KOH.
2) The volume ratio means the value of dividing the volume of top phase with the volume of bottom phase.

DS 0.5-PEG 8.0 (0.3 M NaCl) still contained a lot of cellular fragments by the electronmicroscopic observation. It was necessary to perform further purification of JE virus preparation concentrated with one step method. The two step method of the phase separation was available for this purpose. The procedure of the first step was performed with DS 0.5-PEG 8.0 (0.3 M NaCl) system and that of the second step was made by the phase systems of DS 0.5-PEG 8.0 (0.8 M NaCl) and DS 0.5-PEG 8.0 (1 M NaCl) respectively. The preparation of JE virus in the bottom phase of DS concentrated with the first step of phase separation indicated 306 fold of concentration factor and 6.89 of infectivity index. It was considered that this high infectivity index of partial purified perparation might be due

Table 4. Concentration of JE virus with two step method of aquous two phase system of DS-PEG

Preparation	Volume (ml)	Infectivity (PFU/ml)	Concentration factor	Infectivity index
Step 1				
Starting material	2,000	1.6×10^6	1	1.0
Bottom phase	45	4.9×10^8	306	6.89
Top phase	2,904	8.5×10^4	0.05	0.07
Step 2 (1 M NaCl)				
Bottom phase	23.5	1.0×10^6	6.3	0.14
Interface	1.0	8.8×10^8	550	0.56
Top phase	2.0	8.5×10^8	530	1.06
Step 2 (0.8 M NaCl)				
Bottom phase	21.5	1.8×10^7	113	0.24
Interface	2.0	1.4×10^9	875	1.76
Top phase	0.5	3.0×10^9	813	0.94

Remarks : Infectivity index was presented a value obtained by dividing the virus titer of concentrated specimens with that of the starting material.

Table 5. Concentration of JE virus with two step method of aqueous two phase system of DS-PEG

Preparation	Volume (ml)	HA titration		Virus titration		Infectivity index
		HA/ml	Concentration factor	PFU/ml	Concentration factor	
Step 1						
1. Starting material	2,000	160	1	2.2×10^6	1	1.0
2. Bottom phase	35	640	4	4.6×10^8	209	3.63
3. Top phase	2,875	10	0.06	1.9×10^5	0.09	0.12
Step 2 (1 M NaCl)						
4. Bottom phase	22	ND	ND	8.0×10^6	3.6	0.04
5. Interface	5	80	0.5	9.1×10^7	41.4	0.10
6. Top phase	10	10,240	64	1.0×10^9	455	2.27

Remarks : See Table 4.

to the existence of DS by the adsorption of the virus onto the cells. Such a partial purified preparation of JE virus was divided into two parts for the attempt to perform further purification. The purified virus was obtained successfully in the top phase of both procedure applying DS 0.5-PEG 8.0 (1 M NaCl) and DS 0.5-PEG 8.0 (0.8 M NaCl) systems for the second concentration respectively. The preparation in the interfaces and bottom phases of both systems still contained a lot of cellular particles. As shown in Table 4, the most of JE virus particles was still contained in the bottom phase and interface of DS 0.5-PEG 8.0 (0.8 M NaCl) system applied as the second concentration. It was demonstrated that the purification of JE virus could be accomplished effectively with the second concentration using DS 0.5-PEG 8.0 (1 M NaCl) system rather than DS 0.5-PEG 8.0 (0.8 M NaCl) system.

Further experiment for the practical application of the purification of JE virus

The concentrated JE virus preparation with high purity was obtained in the top phase at the second step of the two step method according to the system of DS 0.5-PEG 8.0 (0.3 M NaCl) and DS 0.5-PEG 8.0 (1 M NaCl). The final purified preparation showed 455 fold of concentration factor and 2.27 of infectivity index. The high index value of the bottom phase in the initial step and of the top phase in the second step might be due to

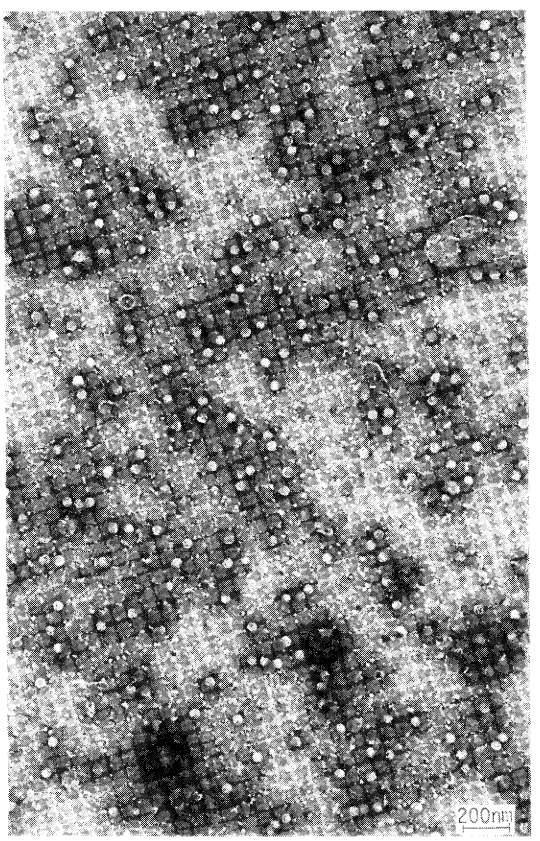

Photo. 1. The preparation of JE virus obtained two-phase system described in the text (magnification 1 : 40,000).

the examination of the specimens before the removal of DS by the method of Philipson *et al.* (1960). These results were usually obtained by the examination of the specimens containing the residue of DS in the specimens. Although the virus in interface of phase separation in the second step still contained a lot of cellular particles by electronmicroscopic observation but non of contaminates was found in the purified preparation of the top phase in this method (Table 5, Photo. 1). The sodium dextran sulfate (DS) in the purified preparation of JE virus was removed sufficiently by adding 3 M KCl as described by Philipson *et al.* (1960).

DISCUSSION

The concentration and purification of arboviruses was usually applied the ultracentrifugation technique. The difficulty to obtain satisfactory recovery of the virus was accompanied with this method. Recently, the polyethylen glycol (PEG) precipitation method was introduced for the partial purification of arboviruses by many workers (Horzinek, 1969; Klein *et al.*, 1970; Della-Porta *et al.*, 1972; Ingolt *et al.*, 1973; Igarashi *et al.*, 1973). So far as JE virus was concerned, the preparation precipitated with PEG was still contaminated with cellular particles or normal components derived from the extraction of mouse brain. On the other hand, the difficulty to remove the polymers from the partial purified JE virus preparation was remained. Consequently, it was required to make further purification of the specimen by density gradient sedimentation or column chromatography on cellulose.

Wesslen *et al.* (1959) and Nakai (1965) reported that the one step method of the phase separation in aqueous two phase system was available for the partial purification of vaccinia virus, mumps virus, newcastle disease virus, influenza virus, adenovirus, echo virus type 7 and 19 and JE virus. It was suggested that a efficient partial purification was possible even with one step method by proper choice of polymers. However, the preparation obtained with this method usually also contained the virus activity together with cellular debris and other contaminates.

The purification and concentration of the virus by the two step method of phase separation can avoid from the contamination of cellular particles and other components. Poliovirus (Norrby *et al.*, 1960), bacteriophage T2, adenovirus type 2, echo virus type 7 and 19 (Philipson *et al.*, 1960) were concentrated with fairly high degree of purity on the step of second concentration in the two step method.

In this study, the partial purification of JE virus was usually able to obtain effectively in the bottom phase of the initial step applied DS 0.5-PEG 8.0 (0.3 M NaCl) system. The highly purified JE virus was obtained in the top phase of the second concentration. Comparing two system of second concentration of DS 0.5-PEG 8.0 (0.8 M NaCl) and DS 0.5-PEG 8.0 (1 M NaCl), the virus distributed widely in bottom, interface and top phase in case of DS 0.5-PEG 8.0 (0.8 M NaCl). In contrast, the more efficient and satisfactory concentration of the virus could be obtained in case of DS 0.5-PEG 8.0 (1 M NaCl) (Table 4). The practical application for the purification of JE virus from the large

volume of starting material was accomplished using DS 0.5-PEG 8.0 (0.3 M NaCl) in initial step and DS 0.5-PEG 8.0 (1 M NaCl) in second concentration as shown in Table 5. It will be emphasized that the two step method of phase separation described in this study should be recommended for the concentration and purification of JE virus.

SUMMARY

The partial purification of JE virus was accomplished with PEG precipitation method. However, the preparation usually contaminated with cellular particles. Consequently, the further purification was required for the removal of the polymer and cellular particles by density gradient sedimentation or column chromatography. The two step method of phase seperation in the initial step with DS 0.5-PEG 8.0 (0.3 M NaCl) and the second concentration with DS 0.5-PEG 8.0 (1 M NaCl) was available for the concentration of JE virus with high degree of purity from the large volume of starting material.

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水性二層分配系による日本脳炎ウイルスの濃縮と精製

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日本脳炎（日脳）ウイルスの精製，濃縮は超遠心による沈澱法，密度勾配遠心法が通常の方法であるがウイルスの活性の保持や回収に難点がある．水性二層分配系による濃縮精製はウイルスの活性を失うことなく，良好な回収が得られ，且つ，大量の出発材料を処理しうる利点がある．組織培養日脳ウイルスを出発材料とし，ポリエチレングリコール（PEG）と硫酸デキストランナトリウム（DS-PEG）による二層分配を比較した．(1) PEG沈澱法では，5% または8% PEG (pH 8.0)-0.25M NaCl の場合，濃縮率と回収率はもっとも高い．しかし，濃縮ウイルス液中に細胞砕片の混入が認められ，且つ，PEG除去のため，さらに分画遠心法やカラムクロマトグラフィーを用いる必要性があった．
(2) DS-PEG を用いた二層分配系では第一段階 0.5% DS-8.0% PEG，0.3 M NaCl (pH 8.0) の場合，高度に濃縮され，第二段階で DS 層に 1M NaCl になるように NaCl を追加し，上層の DS 層にウイルスを集積した．このウイルス液中の DS は Philipson et al. (1960) の方法で塩化カリを加え，DS-KCl 沈澱として遠心で除去した．本二層分配法は大量の組織培養ウイルス液から高能率に，しかも少量の精製濃縮ウイルスを得るために極めて有効な方法である．

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