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Studies on the Purifiction of Newcastle[1] Disease Virus by Agar Gel Filtration

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Introduction

A number of methods have been applied to the virus purification. These are differential centrifugation(Luria 1953), precipitation of non-virus material by using chemicals such as protamine sulfate (Warren et al. 1949), virus adsorption and elution on erythrocytes (Francis et al. 1942), virus adsorption and elution on cation exchange resin (Muller et al. 1952) and agar gel filtration (Steere et al. 1962, Ackers et al. 1962).

Agar gel filtration method for the purification of a crude virus material was first introduced by Polson in 1961, and it has been used extensively in plant virus purification by Steere et al. in 1962. However, a limited number of reports, on the method in animal virus purification, have been reported.

The advantage of this method over other methods is not only simple on the view of apparatus and operation but economic. The technique is based on the fact that the pores of agar are so large that virus particles diffuse into the gel pores and retarded as they pass through the column with the degree of retardation increasing as particle size decreases.

In these studies, a possible application of the agar gel filtration on Newcastle disease virus purification was attempted.

Materials and Methods

1. Agar Gel Column :

Suspensions of extra pure agar powder (Kanto Chemical Co.; Inc) were made in 4 to 8 percent in distilled water autoclaved at 121°C. for 30 minutes, and allowed to form gel at 22°C. The agar was chopped into small pieces by means of Waring blendor for 5 to 20 minutes and sorted by washing through sieves to obtain the fraction which will pass through 40-mesh but not a 60-mesh screen.

Glass cylinders, used as column, were allowed to pack to within 10 cm from the top of each column and each core was covered with a filter paper disk to prevent disturbance of its surface when various solutions were applied. Packed agar gel was washed with sufficient buffered saline solution to remove traces of soluble material from the agar. Column sizes were varied from 1.5×100 cm. to 2×60 cm.

2. Newcastle Disease Virus:

Throughout the studies, B_1 strain of Newcastle disease virus was used. The seed virus material was mixed with 200 units of penicillin and 100 μg of dihydrostreptomycin per ml. and incubated at room temperature for 20 minutes before inoculation.

More than five normal chicken embryos of 10 days age were inoculated 0.2ml. of the inoculum

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into the allantoic cavity. After 72 hours incubation at 37°C. they were kept at refrigerator for 60 minutes to prevent a possible hemorrhage in the harvesting process.

Allantoic fluid was harvested, and aliquots of 5 ml. were dispensed in screw cap tubes, and stored at -60°C. until to use. Before use, it was thawed and centrifuged at about 2,500 rpm for 10 minutes and the supernatant was subjected to the filtration. The normal allantoic fluid was also treated, in the same way, as the infected material and used as control group.

3. Buffered Saline Solution :

With the exception of the study on the effect of buffered saline solution, of the virus filtration through agar gel column, phosphate-NaCl buffered solution was used throughout the studies. Phosphate-NaCl solution was prepared as described in Methods for the Examination of Poultry Biologics. The ingredients of NaCl 170 gm, KH_2PO_4 13.6 gm, NaOH 3.0 gm were dissolved in 1,000ml. of distlled water and kept in refrigerator as a stock solution, and a 1 : 20 dilution of the stock solution of which pH 7.1 to 7.2 was made and used throughout the experiments.

4. Filtration :

In the filtration of virus as well as control material, they were diluted 1 : 5 in buffered saline and 10 ml. of each sample was applied to the top of the column. After the virus material is disappeared from the top of column, buffered saline solution was poured to the top of column so that the elution of virus material is accelerated. All columns were operated by gravity flow and were adjusted to give a flow rate of 5 to 10 ml. per hour and was accomplished at row temperature. First 30ml. of the filtrate was discarded but the later filtrate was received in test tube in amount of 5ml. and their hemagglutinating titers of Newcastle disease virus contained in the filtrates were tested.

5. Hemagglutination Test :

Chicken erythrocytes were collected from more than 3 adult chickens using 4 percent sodium citrate as an anticoagulant. The anticoagulant was mixed with blood in ratio of 1 : 9. The pooled cells were washed more than three times with physiological saline at 1,000 rpm for 10 minutes, and the packed cells were used immediately or stored as a 50 percent suspension for 5 days at refrigerator. For use, the cells were suspended in saline as a 0.5 percent suspension by volume.

Virus suspension was made in 2 fold dilutions beginning 1/5 and 1/15 and allowed to react RBC for 30 to 60 minutes at room temperature (20°to 25°C). Hemagglutinating titers were read when the cells of control had sedimented to form a central buttom-like deposit. A positive test is one in which the agglutinated cells form an even but thin deposit over the entire buttom and shoulders of the tube. Some times the edges of the deposit become ragged and tiny streams of cells cascade to the center. The specificity of the reaction can be tested by tilting the rack of tubes. The agglutinated cell deposit does not flow as does the sediment in the erythrocyte control tube. The tests were always conducted with a control which had known HA titer. The titers were expressed as the end dilution, e g., a titer of 1280.

6. Determination of Total Nitrogen

Determination of total nitrogen of the filtrate was conducted in the way of micro-Kjeldahl method. Before analysis, all the samples were retitrated. The ammonia fomed were carried into four percent boric acid solution containing few drops of Brunswick reagent as an indicator and titrated with 1/50 N hydrochloric acid solution. Other detailed procedures were followed to the method of Bock and Benedict (Hawk and Oser et al. 1954).

Experimental Results and Discussion

1. Effect of Buffered Saline

The effects of various buffered saline on the filtration of NDV through agar gel column were compared. Phosphate-NaCl, Veronal-NaCl and EDTA-NaCl buffered saline were prepared and adjusted to pH 7.0. Phosphate-NaCl buffered

saline was prepared as described in Materials and methods, while Veronal-NaCl and EDTA-NaCl buffered solutions were prepared as follows:

Veronal-NaCl buffered solution

5,5-diethyl barbituric acid in amount of 6.40 gm was dissolved in 500 ml. of hot water (approx. 73°C.) and the other ingredients such as 48 75 gm of NaCl, 10.28 gm of sodium 5,5-diethyl-barbiturate, 0.22gm of calcium chloride (dihydrate) and 1.02gm of magnesium chloride were added to 1,000 ml of water. The solution cooled to approximately 25°C. and made up to 2,000ml. with distilled water. For the final dilution, one part of the stock buffer solution added into 4 parts of distilled water.

Table I. Effect of Buffered Saline on the Agar Gel filtration of Newcastle Disease Virus

Effluent (ml)	Hemagglutinating Titer		
	Phosphate-NaCl	Veronal-NaCl	EDTA-NaCl
45	0	0	0
50	0	0	0
55	5	5	20
60	40	40	80
65	160	160	160
70	320	320	160
75	320	80	40
80	160	80	20
85	80	40	20
90	40	20	20
95	20	20	10
100	10	20	10
105	0	20	10
110	0	20	10
115	0	20	10
120	0	20	10

Unfiltered virus material used in this study showed its HA titer of 2560.

EDTA-NaCl buffered solution

Sodium ethylenedinitrile tetra-acetate (EDTA) in amount of 18.61 gm and 42.50 gm of sodium chloride were dissolved in 1,000 ml. of distilled water. The stock solution was further diluted in 1:4 with distilled water before use(Steere 1962). Four percent agar columns were washed sufficiently with the above buffers. Then ten ml. of samples diluted 1 to 5 with 3 different buffer were applied at the top and eluted with the same buffered saline.

The results are illustrated in Table I. The results show that the highest HA titer and the shortest trailing could be obtained wtih phosphate-NaCl buffer while the other buffered solution resulted in a lower HA titer and longer trailing of virus.

2. Effects of pH Variation

In the previous experiment it has been shown that phosphate-NaCl buffer was adequate to obtain the higher HA titer. In this experiment, the pH variations of phosphate-NaCl buffer were tested. Phosphate-NaCl buffer were adjusted to pH 6.0, 7.0 and 8.0 with 1 N sodium hydroxide and 1 N hydrochloric acid. Virus samples were diluted, in the same way as mentioned previously, with phosphate-NaCl buffers having different pH values and filtered through agar

Table II. Effect of Hydrogen Ion Concentration of Phosphate Buffered Saline on the Agar Gel Filtration of Newcastle Disease Virus

Effluent (ml)	Hemagglutinating Titer		
	Hydrogen Ion Concentration		
	6.0	7.0	8.0
45	0	0	0
50	0	0	0
55	0	0	10
60	40	40	80
65	160	160	160
70	160	320	320
75	160	320	320
80	160	160	80
85	40	80	80
90	40	40	10
95	40	10	10
100	40	5	10
105	40	0	0
110	40	0	0
115	40	0	0
120	40	0	0

Unfiltered virus material used in this study showed its HA titer of 255)

gel column prepared with 4 percent agar.

The results are presented in Table II and the results indicate that at pH 7.0 and pH 8 0 showed the highest HA titer and the narrowest trailing of virus, while at pH 6.0 the titer and trailing of virus were unfavorable.

3. Effect of Agar Concentration :

It has been experienced that the porocity of agar gel may influence on the HA titer of filtrate and trailing of virus. Therefore, in this experiment, the effect of agar concentration in the preparation of solid agar was studied. A number of different concentration namely two to eight percent agar were prepared and chopped before sieving as mentioned previously, packed agar gel column was sufficiently washed with phosphate NaCl buffered solution(pH 7·0) and conducted virus filtratien.

The results are illustrated in Table III, and the results indicate that the lower the agar concentration is the slower the virus elution at first stage of filtration and longer the virus trailng. However, in cases of four to six per cent group, fairly high titer of HA activity and shorter trailing of virus was observed. At the above seven percent group, agar gel may not allow the virus to diffuse into agar particles and may resulted in the quick virus elution and shorter virus trailing.

Table III. Effect of Agar Concentration on the Preparation of Agar Gel Column for Newcastle Disease Virus Filtration

Effluent (ml)	Hemagglutinating Titer						
	Agar Concentration(%)						
	2	3	4	5	6	7	8
45	0	0	0	0	0	20	20
50	0	0	0	0	0	160	80
55	0	0	0	40	80	160	160
60	0	10	20	320	160	320	320
65	20	40	80	640	640	640	640
70	80	80	320	320	640	320	320
75	80	160	320	180	80	320	320
80	160	160	160	40	40	160	160
85	180	160	80	10	20	80	80
90	80	80	80	5	10	40	40
95	40	80	40	0	0	20	20
100	40	40	10	0	0	10	0
105	20	40	0	0	0	5	0
110	20	10	0	0	0	5	0
115	10	10	0	5	0	5	0
120	10	10	0	0	0	5	0

Unfiltered virus material used in this study showed its HA titer of 2560

4. Total Nitrogen and HA Titer of Filtrate

Due to the fact that the purpose of the studies is not only to secure a high titer of virus material but relatively a pure state of virus material, a comparison of HA and nitrogen content of the filtrate was attempted.

Virus material and normal allantoic fluid were diluted to 5 with phosphate-NaCl buffer(pH 7.0). Each ten ml. of effluents from 6 percent agar column was collected and determined total nitrogen and HA activity.

The results were illustrated in Table IV. The results indicate that the egg material contained in allantoic fluid and virus particles were seemed

Table IV. Hemagglutinating Titer and Nitrogen Content of Agar Gel Filtrate of Newcastle Disease Virus Infected Allantoic Fluid

Effluent (ml)	Total Nitrogen (ug/ml)	HA Titer
40— 50	2.55	0
50— 60	6.08	120
60— 70	22.75	640
	0.02(FNAF)*	
70— 80	12.36	320
80— 90	5.15	60
90—100	3.85	0
100—110	3.34	0
110—120	6.87	0
120—130	2.43	0
130—140	1.57	0
140—150	0.98	0
150—160	2.94	0
160—170	5.89	0
170—180	28.25	0
180—190	39.83	0
190—200	48.07	0
NFV**	642.64	2560
NFNAF***	533.74	0

FNAF* : Filtered Normal Allantoic Fluid
NFV** : Non Filtered Virus
NFNAF*** : Non Filtered Normal Allatntoic Fluid

to be seperated : The HA titer of the filtrate reacted to peak with 60-70 ml. group effluent and the first peak of nitrogen content also reached with 60-70 ml. After the flow out of 60-70 ml. effluent, HA titer and nitrogen content gradually decreased in parallel. This may indicate the nitrogen may be originated from virus material.

After the flow out of 140-150 ml. effluent, nitrogen content of filtrate again increased and a higher amount of nitrogen, compare to the first peak, was detected at the flow out of 190-200 ml. but no HA activity has been shown. This may indicate the second peak of nitrogen content possibly originated from egg material.

Conclusion

Throughout the studies the following conclusions were made. Agar gel filtration may be simply applied for the purification of Newcastle disease virus. And the following conditions or ingredients may influence the filtration. These are types of elution buffer and its pH, degree of porosity of agar particles and amount of effluent.

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寒天 겔 濾過法에 의한 뉴켓슬病바이러스의 純化試驗

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純化된 뉴켓슬病 바이러스를 얻기 爲한 基礎的인 實驗으로 바이바스 材料를 緩衝液의 種類 및 水素이온 濃度, 寒天濃度를 달리한 寒天겔칼럼에 通過시켰다. 그라고 바이러스 材料의 血球凝集力價와 總窒素量을 比較 測定하여 다음과 같은 結果를 얻었다.

1. 寒天겔 濾過法을 뉴켓슬病 바이러스의 純化에 簡單히 利用 할수 있다.
2. pH 7.0 以上의 燐酸食鹽 緩衝液과 5~8%의 寒天濃度 粒子를 使用할때 가장 좋은 結果를 얻을 수 있었다.
3. 每時 5~10ml의 流出速度 일때 55ml 부터 80ml의 流出液中에서 高濃度의 바이러스를 얻을 수 있었다.