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Purification and Some Antigenic Properties of Measles Virus Hemagglutinin*

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SUMMARY

Various purification procedures, such as ammonium sulfate precipitation, differential centrifugation, DEAE cellulose column chromatography and sucrose density gradient centrifugation, were applied in the purification of measles virus hemagglutinin. By combination of these various purification procedures, a highly purified measles virus hemagglutinin preparation was obtained.

The purified hemagglutinin showed antigenicity for hemagglutination inhibiting and neutralizing antibodies. This suggests that the protein coat of virus particles, split off by ether-tween 80 treatment, may have antigenicity both for hemagglutination inhibiting and neutralizing antibodies.

INTRODUCTION

In the previous study, it was observed that neutralizing (NT) and comlement fixing (CF) antibodies as well as hemagglutination inhibiting (HI) antibody were formed in guinea pigs inoculated with ether-tween 80 disrupted measles virus, which showed a single hemagglutinin (HAnin) peak in sucrose density gradient (pesudo-equilibrium) and in CsCl density gradient equilibrium centrifugation (Funahashi and Kitawaki, 1963). About the same time, Waterson *et al.* (1963), also reported that the injection of an ether-tween treated preparation of measles virus stimulated the production of NT, HI and CF antibodies in the rabbit. On the other hand, a close relationship between NT and HI antibody titers was observed by several workers (Black and Rosen, 1962; Cutchins, 1962; Kunita *et al.*, 1963). It is interesting to study the relation between HI and NT antigen of measles virus using purified HAnin.

However, the purity of the HAnin preparation used in the previous study (Funahashi and Kitawaki, 1963) seemed to be insufficient for studies on the relation

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between the two antigenicities. It was thought that a combination of various purification procedures might be useful for further purification of measles virus HAnin.

Highly purified, crystalline preparations of plant viruses have been obtained by precipitation with ammonium sulfate, since Stanley (1935), first succeeded in the purification and crystallization of tobacco mosaic virus. However, this precipitation technique has been little used in the purification of animal viruses, except in the purification of the poliovirus (Brown and Kolmer, 1937; Clark *et al.*, 1941; Herrarte and Francis, 1943).

Recently, ion-exchanger cellulose, such as DEAE or ECTEOLA have become excellent tools for virus purification, since the first successful application of these ion-exchangers, in the purification of several animal viruses by Hoyer *et al.* (1958).

This report describes studies on purification methods and the antigenicity of purified measles virus HAnin. For the purification of measles virus HAnin, the precipitation method with ammonium sulfate and cellulose column chromatography applied in combination with other procedures described previously.

MATERIALS AND METHODS

1. *Virus*

The Toyoshima strain of measles virus (Toyoshima *et al.*, 1959), which was adapted to KB cells was used. The virus was grown in monolayer cultures in 1-l Roux bottles. The fluid and cells were collected after complete cytopathic effect had developed and stored at −20°C.

2. *Tissue culture*

KB cells were cultivated in medium which consisted of 0.5 per cent lactoalbumin hydrolysate, 0.1 per cent yeast extract, antibiotics and Hanks solution (YLH), supplemented with 15 per cent or 3 per cent bovine serum for growth or maintenace, respectively.

3. *Methods for purification of measles virus HAnin*

a) *Precipitation with ammonium sulfate*

The measles virus was precipitated by addition of an appropriate volume of saturated ammonium sulfate (pH 7.2) to give the required saturation at room temperature (about 26°C). After low speed centrifugation, the supernatants were mixed with further saturated ammonium sulfate and treated as previously. The precipitates from each step were dissolved in 1/4 volume of the original solution of 1/15 M phosphate buffered saline (PBS).

b) *Treatment with streptomycin*

The virus material was mixed with 1/10 volume of 15 per cent streptomycin sulfate "Meiji" (final concentration 1.5 per cent) and incubated at room temperature for 30 min. Then the mixture was clarified by low speed centrifugation.

c) *Differential centrifugation*

Crude or partially purified measles virus preparations were centrifuged at 36,000 *g* for 1 hour in a Spinco model L ultracentrifuge with a type 30 roter. The pellets were resuspended in 1/15 M PBS, allowed to stand overnight at 4°C and then clarified by low speed centrifugation.

d) *Ether and tween 80 treatment*

Measles virus preparations were mixed with 1/10 volume of 20 mg/ml tween 80. After several minutes, an equal volume of ether was added and the mixtures were shaken in an ice bath for 15

min. After low speed centrifugation, the measles virus HAnin was recovered from the aqueous phase.

e) *DEAE cellulose column chromatography*

Anion-exchange DEAE cellulose was sieved, the 100 to 200 mesh being used as adsorbent. The DEAE cellulose was equilibrated with 0.02 M phosphate buffer (PB) pH 7.2 after washing with 1 N NaOH and 1 N HCl. Columns (1.4×15 cm or 2.0×20 cm) were loaded with virus materials which had previously been dialysed against 0.02 M PB (pH 7.2) and eluted in stepwise or gradient elution with varying concentration of sodium chloride in 0.02 M PB. In stepwise elution, 50 to 70 ml volumes of sodium chloride of 0.1, 0.15, 0.2 and 0.5 M were added after washing the column with 0.02 M PB. A linear gradient was produced by using two identical flasks mounted at the same level and connected by tubing so as to maintain hydrostatic equilibrium. The flow rate was 10-20 ml/hour for gradient elution and 30-40 ml/hour for stepwise elution. The effluent was collected in 5 ml or 10 ml fractions. All experiments were carried out at 5—10°C. The NaCl concentration was determined by Mohr's method.

f) *Sucrose density gradient centrifugation*

Density gradients were set up in 1×3 inch Lusteroid tubes by layering 3.5 ml of 40, 35, 30, 25, 20, 15 and 10 per cent (w/w) sucrose solutions in distilled water. After standing the tubes at 4°C for several hours, to allow the formation of a continuous gradient, 3.0 ml of the partially purified HAnin material was layered on the top of each density gradient and the tubes were centrifuged in a Spinco model L ultracentrifuge using the SW 21.5 at 17,500 rpm for 1 hour. After centrifugation, the tubes were cutted into four fractions from the top using a Beckman tube slicer.

g) *Concentration of purified material*

The purified samples were force dialyzed in visking tubes against Sephadex at 4°C to concentrate them.

5. *Hemagglutination (HA) and hemagglutination inhibition (HI) tests*

HA and HI tests were carried out using green monkey red blood cells, as previously described (Funahashi and Kitawaki, 1963).

6. *Infectivity titration*

Titration was carried out in KB cell tube cultures and observed for 2 weeks before the final reading.

7. *Complement fixation tests*

Drop method techniques was used, as previously described (Kunita *et al.*, 1963).

8. *Neutralization test*

Neutralizing antibody was titrated as previously described (Funahashi and Kitawaki, 1963)

9. *Treatment of sera*

Sera were inactivated at 56°C for 30 min. for CF and NT tests. For the HI test, sera were treated with kaolin and cynomolgus red blood cells as previously described (Kunita *et al.*, 1963).

10. *Protein assay*

Protein was assayed by Lowry's method and optical densities (OD) were measured with a Coleman Junior Spectrophotometer at 660 *mμ* using 0.02 M PB as the blank. The specific activity was expressed in HA units per mg protein.

11. *Nucleic acid assay*

The virus materials were mixed with an equal volume of cold 1 N perchloric acid (PCA) in an ice bath and after centrifugation, the precipitates were washed twice with cold 0.5 N PCA. Then,

the precipitates were resuspended in 1/4 of the original volume of 0.5 N PCA and heated at 70°C for 20 min. and the OD at 260 $m\mu$ was determined.

RESULTS

1. *Precipitation of measles virus with ammonium sulfate*

Crude measles virus material, clarified by low speed centrifugation, was fractionated by stepwise sedimentation with ammonium sulfate. A saturated solution of ammonium sulfate (pH 7) was added drop by drop to the starting material to give a 20 per cent saturated solution, and then centrifuged at low speed. The precipitate was resuspended in 1/4 of the original volume of 1/15 M PBS (20 per cent AS fraction) and the supernatant was mixed with further ammonium sulfate solution to give a 25 per cent saturated solution. By repeating this procedure, various fractions precipitating at between 20 and 60 per cent saturation were obtained. The biological activities of the measles virus in these fractions are shown in Fig. 1.

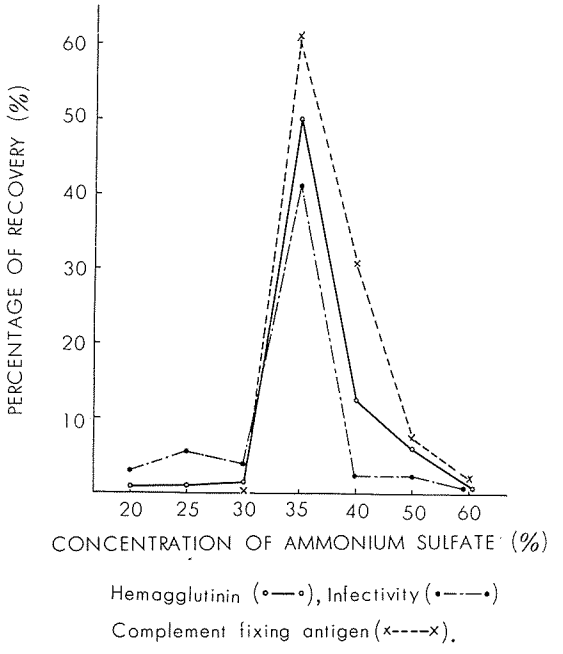


Fig. 1 Precipitation of Measles Virus with Ammonium Sulfate

Most of the biological activities were precipitated in the 35 per cent AS fraction, that is, the recovery rates of infectivity, HAnin, and CF antigen in this fraction, were 41 per cent, 50 per cent and 62 per cent, respectively. In other fractions, the recoveries of HAnin and other activities were very low. So, the

35 per cent AS fraction was employed for further purification. For this purpose, the 35 per cent AS fraction was obtained, after the 30 per cent AS fraction had been eliminated. Table 1 shows the recovery rates of HAnin and infectivity in various experiments. About 50-80 per cent of the HAnin and 50-74 per cent of the infectivity were recovered in the 35 per cent AS fraction. The specific activity of HAnin showed a 2-6 fold increase in this fraction. In the 30 per cent fraction, about the same infective titer was recovered as in the 35 per cent fraction in two experiments, while HAnin recovery was less than 27 per cent in the 30 per cent fraction.

Table 1. Precipitation of Measles Virus by Ammonium Sulfate

Experimental No.	Concentration of saturated ammonium sulfate (%)					
	Recovery rate of hemagglutinin(%)			Recovery rate of infectivity (%)		
	30%*	35%	40%	30%	35%	40%
1	10**	80	10	47.2	47.2	6.0
2	9	73	18		nt	
3	17	67	17	49.6	49.6	0.7
4	27	53	20		nt	
5	0.5	50	53	8.9	74	5.0

nt: not tested
* per cent of saturated ammonium sulfate in virus material.
** Recovery rates are expressed in per cent.

2. *Treatment with streptomycin*

The crude measles virus material or the material purified and concentrated with ammonium sulfate was mixed with 1/10 volume of a 15 per cent solution of streptomycin sulfate (the pH of which was adjusted to 7.2). The mixture was incubated at room temperature for 30 min. After low speed centrifugation, the pellets were resuspended in the original volume of 1/15 M PBS. Although treatment of measles virus with streptomycin did not singificantly affect the HA titers, 50 per cent of the CF antigen was sedimented by this treatment. The supernatants and resuspended pellets were treated with PCA for determination of their nucleic acid contents. About 30 per cent of the nucleic acid was precipitated by treatment with streptomycin. (Table 2).

3. *Fractionation by DEAE cellulose column chromatography*

a) *Non ether-tween treated material (active material)*

The active material (total protein: 25.3 mg) which had been partially purified and concentrated with ammonium sulfate fractionation, was dialyzed against 0.02 M PB and applied to the DEAE column (1.4×15 cm). By stepwise with sodium chloride of different molarities buffered to pH 7.2, the measles HAnin was eluted in the 0.15 M and 0.2 M fractions. The infectivity was also eluted in the same two

Table 2. Effect of Streptomycin Treatment of Measles Virus

Sample	HA titer	CF titer	O D
Crude virus material			
Before treatment	128	2	0.275
Supernatant of SM treated material	128	1	0.199
Precipitate of SM treated material	<4	<1	0.099
Material partially purified by ammonium sulfate			
Before treatment	256	4	0.384
Supernatant of SM treated material	256	2	0.275
Precipitate of SM treated material	4	1	0.106

OD: Optical density at 260 mμ, HA titer: Units per 0.4 ml,
CF titer: Units per 0.1 ml, SM: Streptomycin sulfate.

fractions and in the 0.5 M fraction. The OD at 660 mμ was determined by Lowry's method. The protein, which seemed to be of cellular components was mainly eluted in 0.02 M PB and in the 0.1 M fraction. The protein content at higher NaCl

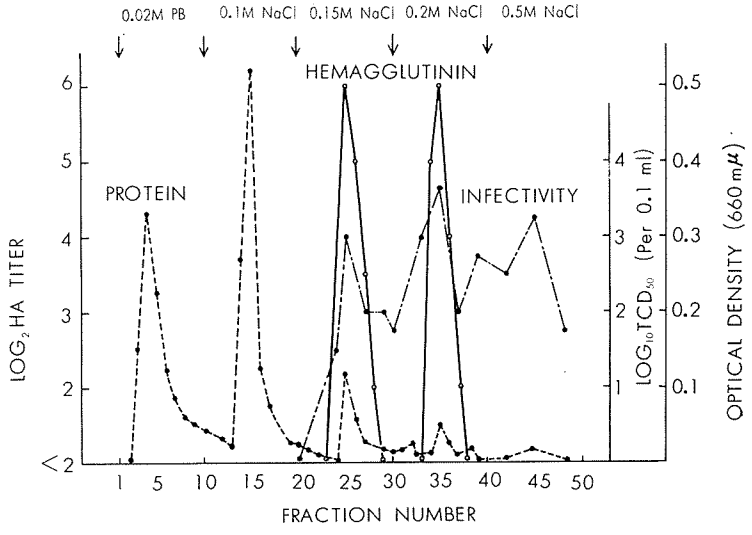


Fig. 2 Chromatography of Active Measles Virus Material on DEAE Column by Stepwise Elution

concentrations was very low and the specific activity of the lst and 2nd peaks showed increases of about 6 and 13 fold, respectively. The total recovery of HAnin in the two peaks was about 73 per cent.

b) *Ether and tween 80 treated material*

The 35 per cent AS fraction precipitated with ammonium sulfate was treated with ether and tween 80, dialyzed against 0.02 M PB was applied to the DEAE column under the same conditions as above. Stepwise elution resulted in two peaks as in the case of active material, but the lst peak was eluted in the 0.1 M fraction and the 2nd peak in the 0.15 M fraction. By gradient elution, as shown in Fig. 3, the measles HAnin began to be eluted at about 0.1 M NaCl concentration, forming a sharp initial peak with a tail. It seemed that cellular protein was mainly eluted by washing with 0.02 M PB. The recovery rate of measles HAnin was about 32 per cent and the specific activity of HAnin showed a 2.4 fold increase in this experiment. Infectivity in this case was completely lost by ether and tween 80 treatment.

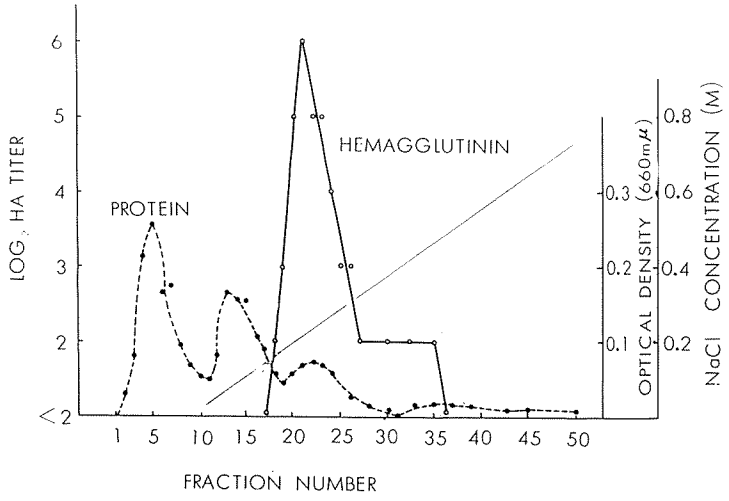
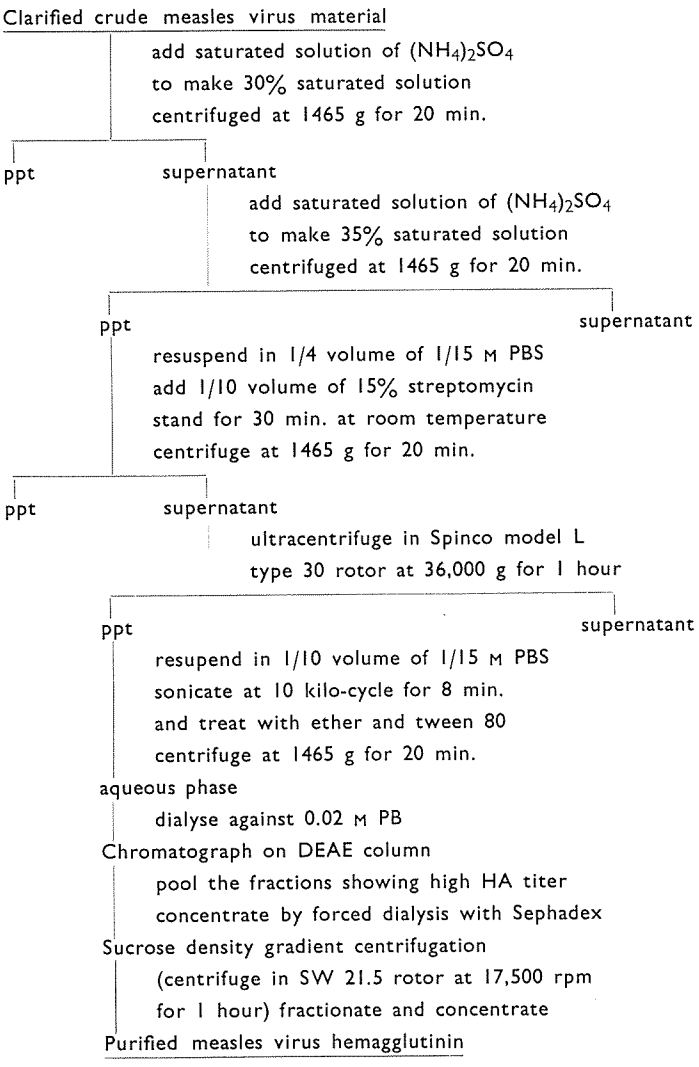


Fig. 3 Chromatography of Ether-Tween 80 Treated Material on DEAE Column by Gradient Elution

4. *Purification of measles virus HAnin by combination of various purification procedures*

To obtain a more highly purified sample of measles HAnin, various purification procedures were combined. Table 3 shows one combination of purification procedures used. The crude virus material was first precipitated with ammonium sulfate. After removal of the precipitate of the 30 per cent AS fraction, the 35 per cent AS fraction was obtained. After low speed centrifugation, the precipitate of the 35 per cent AS fraction was resuspended in 1/4 of the original volume of 1/15 M PBS. This fraction was treated with streptomycin sulfate (final concentra-

Table 3. Purification of Measles Virus Hemagglutinin by the Combination of Various Purification Procedures



tion 1.5 per cent) at room temperature for 30 min, and then centrifuged at 2,500 rpm for 20 min. to remove nucleoprotein. The supernatant was centrifuged at 18,000 rpm for 1 hour in a Spinco model L ultracentrifuge using type 30 rotor. The pellets were resuspended in 1/10 volume of 0.02 M PB and placed at 4°C overnight. The sample partially purified and concentrated by centrifugation was sonicated at 10 kilo-cycle for 8 min. and then shaken with ether and tween 80 in an ice bath. After low speed centrifugation, the aqueous phase was obtained. Forty ml of ether-tween 80 treated sample (total protein 122 mg) was loaded onto a

DEAE column (2.0 × 20 cm) and eluted by an NaCl gradient in 0.02 M PB. Fractions of 10 ml were collected. The elution pattern of HAnin is shown in Fig. 4. The 1st peak of protein, which seemed to be of cellular origin, was completely separated from the HAnin peak and the 2nd peak of protein was eluted a little later than the HAnin peak. Fractions 18, 19, 20 and 21, which showed high specific activity were collected and concentrated by forced dialysis against Sephadex.

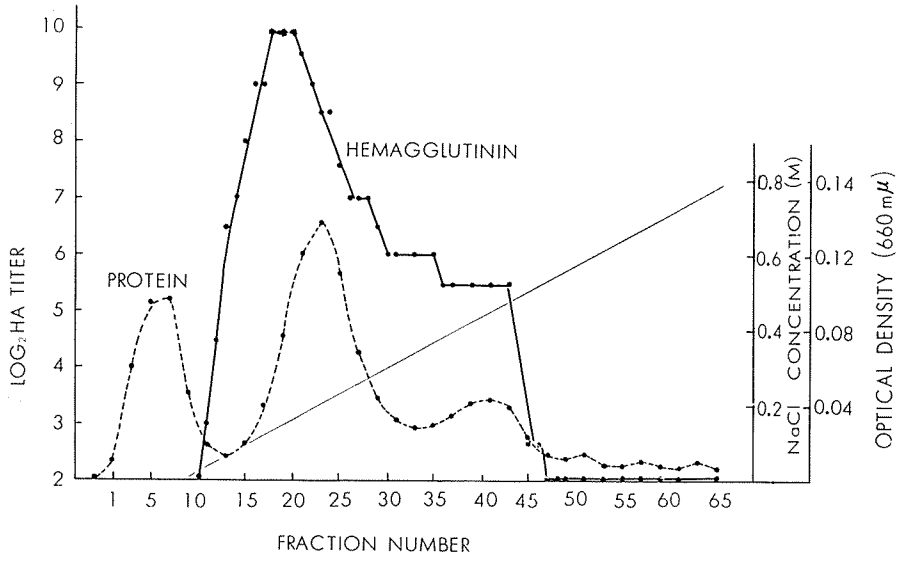


Fig. 4 Gradient Elution of Ether-Tween 80 disrupted Measles Virus Hemagglutinin Purified by a Combination of Various Procedures

Three ml of the concentrated sample was layered on the top of a sucrose density gradient (10-40 per cent, w/w) and centrifuged in a SW 21.5 rotor at 17,500 rpm for 60 min. After centrifugation, the tubes were separated into four fractions.

Table 4. Fractionation of Purified Hemagglutinin by Sucrose Gradient Centrifugation

Fraction No.	Volume (ml)	HA titer
1	3	320
2	11	160
3	7	20
4	4.5	20

HA titer: Units per 0.4 ml

The HA titer of each fraction is shown in Table 4. Fractions 1 and 2 were pooled and concentrated by forced dialysis against Sephadex.

The biological activities, specific HAnin activity (HAU/mg. protein) and recovery rate at each step in this purification procedure are shown in Table 5. Infectivity was completely lost after ether-tween treatment and CF antigen could not be detected in the HAnin fraction after DEAE column chromatography. The increase of specific activity was about 2 fold after precipitation with ammonium sulfate, about 14 fold after ultracentrifugation, about 74 fold after ether-tween treatment, about 180 fold after chromatography and about 300 fold after sucrose density gradient centrifugation. In this case, the HA titer increased 2 fold on sonication and a further 2 fold on ether-tween treatment. So, the false increase of specific activity of HAnin was also included. The recovery of HAnin was about 32 per cent after ultracentrifugation, 129 (63) per cent after ether-tween 80 treatment, 21 (11) per cent after chromatography and 15 (7.5) per cent after sucrose density gradient centrifugation. The figures in parentheses represent the true recovery rates corrected by considering the false increase of activity.

Table 5. The Biological Activities, Specific Activity and Recovery Rate at Each Step in the Purification of Measles Virus Hemagglutinin

Sample	HA titer	CF titer	TCD_{50}	Protein	HA per mg protein	Recovery Rate (%)
Crude virus material (Starting material)	96	8	6.5	3.51	68.5	
Supernatant of streptomycin treated material	128	8	5.5	2.12	150	
Precipitate of ultra-centrifuged material	1280	32	6.5	3.25	985	32
After sonication	2560	32	6.3	3.56	1235	
After ether-tween 80 treatment	5120	16	—	3.06	5000	127(63)
Pooled fractions of DEAE column chromatography	1280	<1	—	0.256	12300	21(11)
Combination of fractions 1 & 2 after sucrose density gradient centrifugation	1024	<1	—	0.125	20230	15(7)

The figures in parentheses represent the true recovery rate corrected by considering the false incresse of activity.
HA titer: Units per 0.4 ml, CF titer: Units per 0.1 ml
TCD_{50} are expressed as the reciprocal of $\log_{10}$ per 0.1 ml

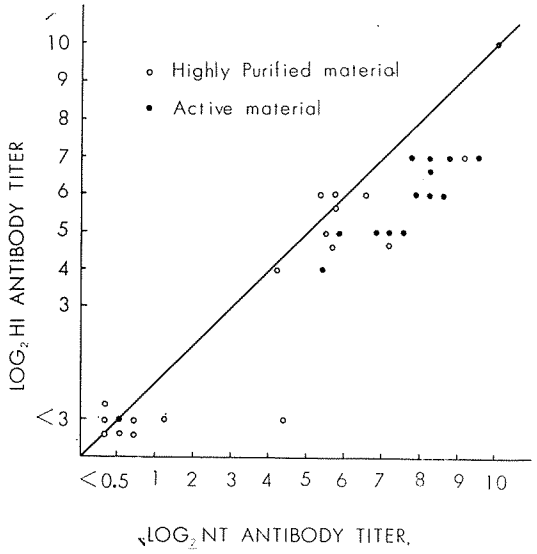


Fig. 5 Correlation of Hemagglutination Inhibiting and Neutralizing Antibody Responses of Guinea Pigs which were Inoculated with Active Measles Virus Material and Highly Purified Measles Virus Hemagglutinin

5. *The antigenic nature of the purified HAnin.*

a) *Immunization of guinea pigs with active virus materials and purified measles virus HAnin*

The following four samples were prepared for immunization of guinea pigs: crude virus material (active), centrifugally concentrated active material, centrifugally concentrated ether-tween 80 treated material and measles HAnin purified by DEAE column chromatography. The last sample was prepared by fractionation of the centrifugally concentrated and ether-tween disrupted measles virus on a DEAE cellulose column.

Guinea pigs were inoculated intracutaneously with these preparations three times at weekly interval. The animals were bled one week after the last injection. As shown in Table 6, the HI and NT antibody responses in the guinea pigs inoculated with these four antigens were nearly parallel. CF and NT ratios were lower in guinea pigs immunized with the partially purified HAnin than in other animals. CF antibodies were also detected with host cell antigen which was disrupted by sonication. By absorption with this host cell antigen, CF antibody titers were reduced markedly.

b) *Immunization of guinea pigs with measles HAnin which was highly purified by a combination of various purification procedures*

Highly purified measles HAnin obtained by a combination of various purification procedures, was diluted serially four fold from 1024 to 4 HAU/0.4 ml. One ml of each dilution was inoculated intracutaneously into five guinea pigs.

Table 6. Antibody Responses of Guinea Pigs Inoculated with Partially Purified Measles Virus Hemagglutinin, Ether-Tween 80 Treated Virus Material and Active Virus Material.

Sample	Guinea pig No.	Antibody titers*			
		HI	NT	CF	
				Pre-absorption	Post-absorption
Partially purified measles virus hemagglutinin (Fractionated with DEAE column)	A—1	9	8.7	6	4
	2	9	8.0	2	<2
	3	10	9.7	5	4
	4	10	10.5	<2	<2
	5	10	9.7	4	<2
	6	10	9.0	4	<2
	7	9	7.5	<2	<2
	8	7	5.5	<2	<2
	9	9	6.3	<2	<2
G. M.		9.2	8.3	2.3	0.9
Centrifugally concentrated ether-tween 80 treated material	B—1	9	8.0	5	3
	2	6	4.7	5	2
G. M.		7.5	6.4	5	2.5
Crude active material	C—1	10	8.5	4	2
	2	10	8.5	7	6
	3	10	10.5	8	5
	4	9	7.5	5	<2
G. M.		9.8	8.8	6	3.3
Centrifugally concentrated active material	D—1	10	9.8	6	4
	2	11	11	6	3
	3	11	11.3	7	5
G. M.		10.7	10.7	6.3	4

G.M.: Geometric Mean
* Antibody titers are expressed as the reciprocal of $\log_2$

Some guinea pigs died by accident. After 4 weeks, the animals were bled. As controls, guinea pigs were inoculated with centrifugally concentrated, active virus material containing the same HA units as the purified HAnin. The HI and NT antibody titers were nearly parallel in each animal and were not correlation with purity of the antigens or the antigen doses inoculated. However the NT antibody titers were higher than the HI antibody titers, because the challenge virus used for the neutralization test was as lower a concentration as 30 TCD_{50}/0.1 ml. (Table 7 & 8)

A nearly linear dose response was obtained with the purified HAnin when the antibody titers were expressed as geometric means, while tailing of the dose

Table 7. Antibody Responses of Guinea Pigs Inoculated with Highly Purified Measles Virus Hemagglutinin

Antigen dose *	Guinea pig No.	Antibody titers***				
		HI	G.M	NT	G.M	CF
1024 * *	1	5		7.2		<2
	2	6	5.5	5.7	6.0	<2
	3	5		5.5		<2
	4	6		5.6		<2
256	1	4		4.2		<2
	2	<3	3.3	4.4	4.6	<2
	3	6		5.4		<2
64	1	<3		<0.5		<2
	2	<3	2.3	<0.5	3.0	<2
	3	7		2.3		<2
16	1	<3		1.2		<2
	2	6		6.5		<2
	3	<3	2.2	<0.5	2.7	<2
	4	5		5.7		<2
	5	<3		<0.5		<2
4	1	<3	0	<0.5	0	<2
	2	<3		<0.5		<2

* Each guinea pig was inoculate with one ml of antigen.
* * HA units per 0.4 ml
* * * Antibody titers are expressed as the reciprocal of $\log_2$
G.M: Geometric mean

response curve was observed with active material. No CF antibody response could be observed against the purified HAnin sample in any case.

DISCUSSION

Recently, the purification of measles virus has been described by several authors (Schluederberg, 1962; Norrby, 1963; Waterson *et al.*, 1963; Funahashi and Kitawaki, 1963) using a sucrose density gradient or CsCl density equilibrium centrifugation methods. In this study, attempts were made to purify measles HAnin further and to investigate the anigenicity of the measles HAnin.

The measles virus could be precipitated and partially purified by ammonium sulfate with good recovery. That is, by addition of saturated ammonium sulfate solution to the crude measles virus material to give a 35 per cent saturated solution,

Table 8. Antibody Response of Guinea Pigs Inoculated with Centrifugally Concentrated, Active Measles Virus

Antigen dose*	Guinea pig No.	Antibody titers***				
		HI	G.M	NT	G.M	CF
1024**	1	10	8.5	10	8.9	7
	2	7		7.7		4
256	1	6		<3.5		nt
	2	7	6.7	8.7	6.0	7
	3	7		9.5		7
64	1	5		7.5		3
	2	6		8.2		3
	3	7	5.0	8.2	6.4	<2
	4	7		8.2		3
	5	<3		<0.5		<2
16	1	6		8.5		3
	2	6	5.3	8.0	7.3	2
	3	4		5.4		<2
4	1	5		6.8		<2
	2	5	5.0	7.2	6.5	<2
	3	5		5.6		<2

* Each guinea pig was inoculated with one ml of antigen.
** HA units per 0.4 ml
*** Antibody titers are expressed as the reciprocal of $\log_2$
G.M: Geometric Mean

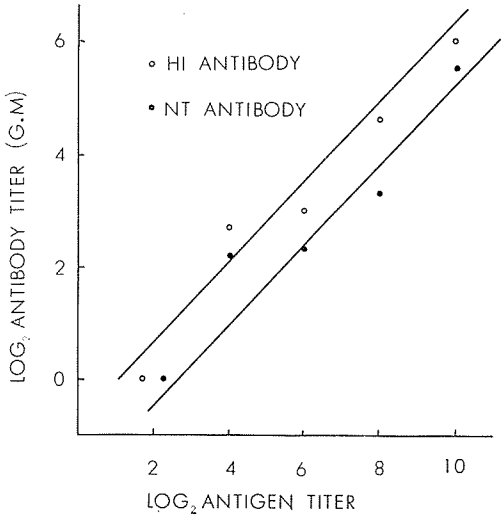


Fig. 6 Dose Response of Highly Purified Measles Virus Hemagglutinin

50 to 80 per cent of HAnin and 50 to 70 per cent of infectivity were recovered. The specific activity of HAnin in this fraction increased 2 to 6 fold. Thus, some of the non-viral protein could be removed by this procedure. Althouth considerable infectivity was recovered in the 30 per cent AS fraction, this may be due to the aggregation of virus, or precipitation with other precipitable protein or infectivity may be precipitated best in this fraction. In any case, precipitation with ammonium sulfate is one of the most useful procedures for concentration and purification of measles virus.

Nojima (1963) applied streptomycin treatment in the purification of Japanese encephalitis virus to precipitate the nucleoprotein. In the purification of measles virus, treatment of the virus material with streptomycin sulfate had little effect on the HA titer, but 50 per cent of the CF antigen was precipitated and 30 per cent of the nucleic acid was found in the pellets of streptomycin treated material. In this experiment, it was found that the nucleoprotein (probably mostly of cellular origin and partly of viral origin) could be partially eliminated by this procedure.

By DEAE column chromatography of the measles virus material, which had been purified and concentrated by ammonium sulfate precipitation or differential centrifugation, measles HAnin was recovered at a rate of 20 to 80 per cent both on stepwise and on gradient elution. On stepwise elution, the both active and ether-tween 80 treated HAnin formed two peaks in the eluate. In ether-tween 80 treated material, the HAnin was eluted with a lower molarity of NaCl than in active material. However, the molarity of NaCl which elutes HAnin from DEAE column, seems to depend upon other factors, such as the conditions of the column. The two peaks of HAnin seen on stepwise elution may be partly due to the heterogeneity of the HAnin (small and large HAnin, Norrby, 1962) but more probably to the dissociation constant of virus and adsorbent which was proportional to the concentration of the cations in the salt solution (Taniguchi, 1962). On the other hand, infectivity did not form a sharp peak, but appeared in the HAnin peaks with tailing in higher NaCl concentrations. On stepwise elution of active material, infectivity was also eluted in the 0.5 M NaCl fraction, in which no HAnin could be detected. Since ether-tween 80 disrupted HAnin was apt to be eluted at a lower NaCl concentration than active material, the peak of HAnin in the 0.15 M and 0.2 M fractions of active material may consist of smaller, non-infective HAnin particles and intact virus particles. The infective virus in the 0.5 M NaCl fraction seems to be insuffient in amount to show HA activity. Thus, infectivity was not completely separated from HAnin. CF antigen was eluted in the HAnin peaks in some experiments but in very low titers (about 2 units) and could not be detected in most cases. This may be due to the low titer of CF antigen in crude virus material and the dilution effect of column chromatography.

By a combination of various purification procedures, that is, ammonium sulfate precipitation, streptomycin treatment, DEAE column chromatography, and sucrose density gradient centrifugation, highly purified measles HAnin was

obtained. The specific activity of this sample was 300 fold higher than that of the starting material. In this experiment, however, the HA titer increased 2 fold by both sonication of the preparation and by ether-tween 80 treamtent. The increase caused by sonication may be due to the dispersion of aggregated measles virus and partly to the splitting of virus particles. The increase of HA titer by ether-tween 80 treatment is mainly due to the splitting of virus particles into smaller particles. So, the specific activity and recovery rates shown in the results represent a false increase.

Waterson (1963) and Funahashi and Kitawaki (1963) reported that ether-tween 80 treated maesles virus produced HI, NT and CF antibodies in rabbits and in guinea pigs. In this experiment, guinea pigs showed HI and NT antibody responses after inoculation with partially purified measles HAnin as well as after inoculation with active materials. The HI and NT antibody responses were nearly parallel in all cases. Because the challenge virus used for the NT test was of as low a concentration as 30 TCD_{50}/0.1 ml, the NT antibody titers were higher than the HI antibody titers. A one log increase in challenge virus resulted in a two fold decrease in the serum NT titer (unpublished data). Correcting titers by this, the NT antibody titers were nearly the same level as the HI antibody titers.

Contrary to the previous report, CF antibody did not appear in guinea pigs inoculated with highly purified HAnin, while high levels of NT and HI antibodies were observed in these animals. After inoculation of crude or partially purified materials, high levels of CF antibody were detected. This antibody, however, also reacted with normal host cell component, when the cell antigens were prepared after complete disruption of cells by sonication, and this antibody decreased by absorption with highly concentrated cell antigens. This suggests that cellular protein contaminating the HAnin samples plays an important role in the production of CF antibody in experimental animals. This CF antibody, detected only when higly disrupted cell antigens were employed for the test, and this disruption was caused by measles virus infection or sonic oscillation, but not by mere freezing and thawing of intact host cells.

Schäfer (1957) and Davenport *et al.*, (1960) suggested that the HAnin of fowl plague virus and influenza isolated by ether treatment might be as effective as the corresponding virus for stimulating HI antibody formation. Measles HAnin also showed nearly the same serological behaviour as intact particles with regard to HI and NT antibody production. The HA titer of measles virus obtained from infected FL cells did not increase by ether-tween 80 treatment, but its antigenicity in the production of HI and NT antibody is comparable to that obtained from infected KB cells (unpublished data). This, and the presence of an HAless mutant of this virus (Waterson, 1963) suggest that the coat protein of virus is split off by ether-tween 80 treatment and the subunits show the antigenicity for producing NT antibody and may also contain a determinant site for HA activity in the case of small HAnin. Thus, NT antibody may act as HI antibody by combining with

the coat protein which also includes HA activity. On the other hand, the NT antibody level was higher than the HI antibody in the early convalescent period and vice versa or was at the same level a few years after natural infection and vaccination (Black and Rosen, 1962; Kunita *et al.*, 1963). This reverse of the NT/HI ratio of the antibody titer suggests that both NT and HI specific antibodies may exist in measles convalescent sera. The high HI activity in the serum in the early convalescent stage may depend on HI specific antibody and this antibody may have little or no neutralizing capacity. These problems should be investigated further.

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