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Studies on Measles Virus Hemagglutination

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SUMMARY

The conditions for measles virus hemagglutination and some of the physical properties of the hemagglutinin were investigated.

1. Cynomolgus monkey red blood cells were less susceptible to measles virus than green monkey red blood cells. A suitable pH range of measles hemagglutination was pH 7.0 to 8.0.

2. The hemagglutinin lost almost all its activity when incubated at 56°C for 40 hours, but at 4°C the activity was retained for at least 3 weeks and at 37°C for 9 days. After 128 minutes UV irradiation, the hemagglutinating titer of the irradiated sample decreased to 1/32 of that of the control. Periodate completely inactivated the hemagglutinin.

3. Treatment of the hemagglutinin sample with ether alone resulted in almost complete disappearance of the hemagglutinin from the aqueous phase but it was recovered from the intermediate phase. On treatment with ether and Tween 80, relatively pure hemagglutinin was obtained in high titer in the aqueous phase.

When used as the antigen for the hemagglutination-inhibition reaction, ether-Tween 80 treated hemagglutinins had a 2 to 4 fold higher antibody titer of antisera than untreated hemagglutinin.

4. On sucrose density gradient centrifugation, the hemagglutinating and complement fixing activities of native and ether-Tween 80 treated samples appeared in the same density fractions.

On CsCl density gradient equilibrium centrifugation, the hemagglutinating activity of the native sample had a peak at a density of 1.186 in one case and that of another native sample showed two peaks at densities of 1.190 and 1.240, while the hemagglutinating activity of the ether-Tween 80 treated material had a sharper peak at a density of 1.260.

After the treatment of the viral material by sonic vibration, the hemagglutinating activity appeared as a broad band with a density of 1.190 to 1.240 and showed no prominent peak.

5. The complement fixing activity of the native material had a peak at a density of 1.215, but after ether-Tween 80 treatment, the activity was found in two peaks, the main one being of the same density 1.260 as the hemagglutinating activity and the other satellite peak having a density of 1.190.

6. In guinea pigs immunized with the fraction separated by CsCl density gradient centrifugation of an ether-Tween 80 treated sample, the response of neutralizing antibody corresponded to the response of hemagglutination-inhibiting antibodies.

INTRODUCTION

Since Periés (1960) found that measles virus agglutinated the red blood cells of the mountain baboon, little has been reported on the nature of measles virus hemagglutinin and the hemagglutination phenomena, though some workers have reported on the applications of hemagglutination and its inhibition by specific sera of convalescent subjects in epidemiological surveys of measles infection (Rosen, 1960; DeMeio and Gower, 1961; Rosanoff, 1961).

So little measles virus hemagglutinin appears in infected tissue culture fluid that it was difficult to obtain a suitable preparation of hemagglutinin to work on.

Moreover the conditions for the hemagglutination test varied so much in different laboratories that it was necessary to find the essential conditions necessary for measles virus hemagglutination. Further the characteristics of the measles virus hemagglutinin are uncertain so that various methods of analysis were tested applying the ether disintegration technique.

MATERIALS AND METHODS

1. *Virus*

The Toyoshima strain of measles virus (Toyoshima *et al.*, 1959, 1960a) was used. The FL cell adapted strain was passaged in KB cells for more than ten generations before the experiments.

2. *Cell culture*

KB cells were grown in monolayer cultures in 1,000 ml Roux bottles. As growth medium, yeast extract-lactalbumin hydrolysate medium (YLH) containing 20 per cent bovine serum was used and for maintenance, 3 per cent bovine serum was added to YLH. KB cells formed monolayers 4-5 days after dispersion and at this time the total cell number per bottle was 2-5 x 10^7.

3. *Preparation of stock measles virus sample*

Monolayers of KB cells were inoculated with Toyoshima strain virus at an input of 10^7 $TCID_{50}$ per bottle and virus adsorption was allowed to proceed for 1 hour at 37°C. Then maintenance medium was added and the cultures were incubated at 37°C. The infected cells and fluid were harvested when the cells showed maximum cytopathic effects. After adjustment of the pH to 7.0-7.2 by the addition of 7 per cent bicarbonate solution, the harvested samples were stored at −20°C. The supernatant obtained after centrifugation at 2,500 rpm for 10 minutes was used as the stock preparation of hemagglutinin.

4. *Concentration of hemagglutinin*

Concentration by centrifugation: The stock samples were centrifuged at 36,000 g for 1 hour in a Hitachi 40P ultracentrifuge with a No.30 rotor. The pellets were resuspended in M/15 phosphate buffered saline, pH 7.2 (PBS), placed at 4°C for 18-24 hours and clarified at 3,000 rpm for 30 minutes. The supernatant was designated as centrifugal concentrate (CC).

Carbowax concentration: The stock samples were concentrated by forced dialysis against a thick solution of Carbowax 6,000 for an appropriate time at 4°C and designated as Carbowax concentrate, (CWC).

5. *Red blood cells (rbc)*

Several species of ape were used. Monkeys were bled from the femoral vein using an equal volume of sterile Alsever's solution as anticoagulant and the blood was stored at 4°C. Before use, the rbc were washed three times with M/15 PBS, pH 7.2, and suspended in the same solution at a concentration of 1 per cent (V/V). The suspension contained 1.2-1.4 x 10^6 rbc per 0.1 ml and had an optical density of 0.145-0.155 at 540 mμ in a Coleman spectrophotometer.

6. *Hemagglutination (HA) test*

Two fold serial dilutions of virus were made in M/15 PBS, pH 7.2, in volumes of 0.4 ml. One tenth ml of 1 per cent rbc was then added to each and after thorough mixing the tubes were incubated at 37°C for 3 to 4 hours, or at 4°C, overnight. The titers were expressed as the reciprocals of the highest dilution which showed complete agglutination.

7. *Hemagglutination inhibition (HI) test*

Serial two fold dilutions of sera were prepared in 0.2 ml volumes of M/15 PBS and then 4 units of hemagglutinin in 0.2 ml PBS were added. After incubation at 37°C for 1 hour, the virus - serum mixtures were mixed with 0.1 ml of 1 per cent rbc and reincubated at 37°C for 3 hours. The reciprocals of the serum dilution in the last tube in which hemagglutination was completely inhibited was taken as the HI titer.

To eliminate the non-specific inhibitor and/or serum hemagglutinin to monkey rbc, the procedures of Rosen (1961) were applied unless otherwise specified: Five fold diluted sera were mixed with the same volume of 25 per cent Kaolin (Wako Pharmaceuticals Co., Japan) suspension in PBS, pH 7.2. The mixture was vigorously shaken for 20 minutes and then centrifuged at 2,500 rpm for 20 minutes. The supernatants were then absorbed for 30 minutes at 37°C with an equal volume of the same 3 per cent rbc suspension as used for the HI test. After low speed centrifugation to eliminate the added rbc, supernates were used for the test.

8. *Complement fixation test*

A modification of Kolmer's method was used.

9. *Infectivity titration*

Titrations were carried out in KB cell tube cultures according to the method of Toyoshima *et al.* (1959).

10. *Titration of neutralizing antibody*

The neutralizing antibody titer was tested according to the method of Toyoshima *et al.* (1959). One volume of 100 $TCID_{50}$/0.1 ml of measles virus was added to one volume of serial two fold dilutions of sera. After one hour's incubation at room temperature, five tubes of each dilution were inoculated with 0.2 ml of the mixture. The antibody titer was expressed as the highest serum dilution which caused neutralization of measles virus in over 50 per cent of the tubes after two weeks observation.

RESULTS

Production of measles hemagglutinin and its relation to other viral activities

KB cell passaged measles virus was inoculated into KB cell monolayers in 200 ml milk bottles at a multiplicity of infection of 1 $TCID_{50}$/cell. Two bottles

were harvested at appropriate intervals and immediately frozen. Later the contents of the series of bottles were titrated for hemagglutination, complement fixation, and infectivity. As shown in Fig. 1, 30 hours after infection, hemagglutinin was detected and it gradually increased until 72 hours.

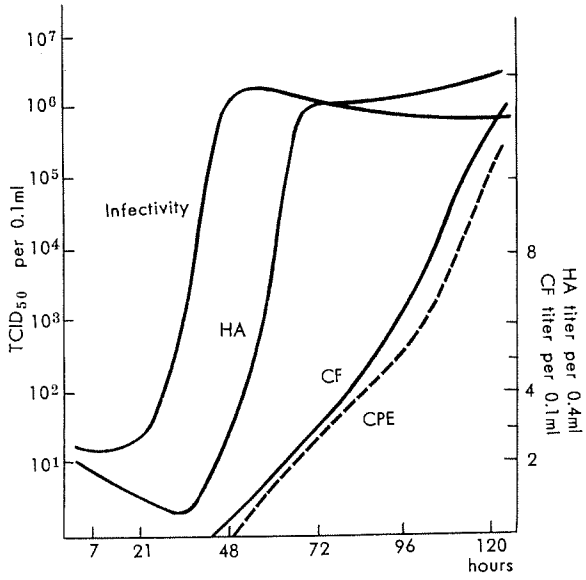

Fig. 1. Growth Curves of Hemagglutinin and Other Components of Measles Virus in KB cells

After this, the hemagglutinin remained at a constant level. The infectivity became detectable and reached a maximum earlier than the hemagglutinin. Complement fixing antigen appeared later, and increased slower than hemagglutinin. In one series of experiments, hemagglutinin appeared in the fluid at the 68th hour, though cell associated hemagglutinin was detected as early as the 30th hour after infection.

The conditions which regulate measles virus hemagglutination

1) *pH dependency of hemagglutination*

With the stock suspension, the pH dependency of hemagglutination was not clearly shown over a pH renge of from 6.1 to 7.8. On the other hand, with CWC and CC, a high and constant HA titer was obtained when HA titration was performed at between pH 7.0 and 8.0. Under pH 7.0 the titer decreased gradually with pH.

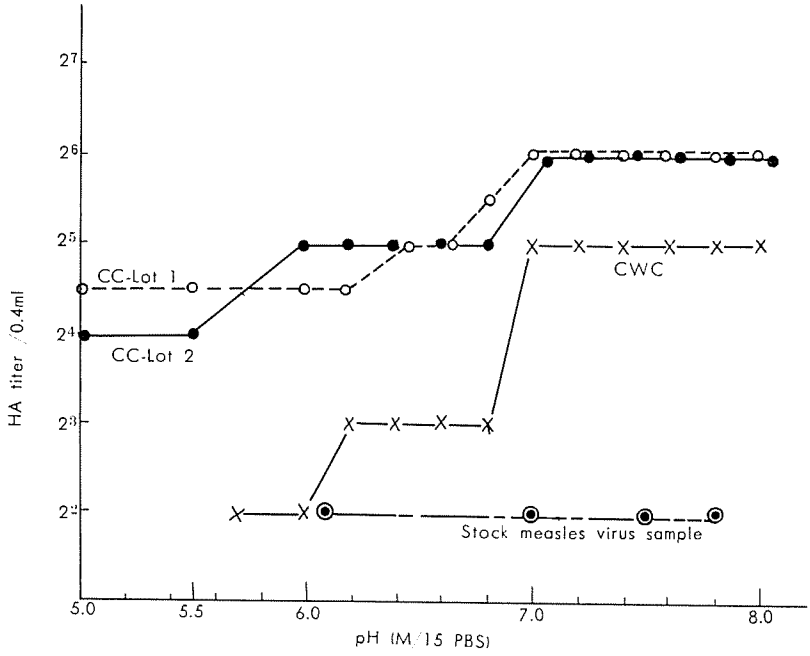

Fig. 2. pH Dependency of Measles Hemagglutination
CC : Centrifugally concentrated sample
CwC: Carbowax-concentrated sample

2) *Incubation temperature*

When the routine technique of HA titration was used, there was not much difference between the HA titers after incubation at 37°C for 3 hours and those after incubation at 4°C overnight, as shown in Table 1. When, however, the virus samples were immersed in an ice bath prior to being mixed with rbc, the HA titer was 2 to 4 fold lower after 4°C overnight incubation than after incubation for 3 hours at 37°C.

3) *Susceptibility of red blood cells*

i) A comparison of the rbc of several individual Cynomolgus monkeys indicated a wide variation in their susceptibilities to hemagglutinin. The most susceptible rbc were agglutinated at a dilution of 1:1024 of one hemagglutinin sample but the least susceptible rbc showed agglutination at a dilution of 1:64 the same lot of hemagglutinin. With another hemagglutinin sample, they were agglutinated at dilution of 1:128 and 1:8 respectively.

After bleeding the agglutinability of rbc did not change even when stored for

Table 1. Comparison of HA Titers Tested at 37°C and 4°C

Virus material	Condition of HA test			
	at 37°C for 3 hrs		at 4°C overnight	
	Complete agglutination	Partial agglutination	Complete agglutination	Partial agglutination
TCF*	1:32	1:64	1:32	1:64
TCF	1:32	1:64	1:32	1:32
CC**	1:256	1:512	1:256	1:256
CC	1:256	1:256	1:256	1:256
CC	1:64	1:128	1:64	1:64
CWC***	1:256	1:512	1:256	1:256
CWC	1:512	5:1024	1:512	1:512

* Infected tissue culture fluid
** Centrifugally concentrated virus material
*** Carbowax concentrated virus material

Table 2. Variation in Hemagglutinating Titer of Cynomolgus Red Blood Cells of Different Individuals

Monkey	days after bleeding	No. of red blood cell per 0.1 ml	HA titer per 0.4 ml	
			KB_7 measles virus	KB_{12} measles virus
Cynomolgus 1	27	PBS 1.2×10^6 DGV 1.4×10^6	1:64 1:64	1:16 1:16
Cynomolgus 2	27	PBS 1.4×10^6 DGV 1.1×10^6	1:256 1:256	1:32 1:32
Cynomolgus 3	16	PBS 1.1×10^6 DGV 1.1×10^6	1:1024 1:1024	1:128 1:128
Cynomolgus 4	13	PBS 1.2×10^6 DGV 1.3×10^6	1:255 1:256	1:32 1:32
Cynomolgus 5	7	PBS 1.3×10^6 DGV 1.1×10^6	1:64 1:64	1:16 1:16
Cynomolgus 6	2	PBS 1.4×10^6 DGV 1.2×10^6	1:64 1:64	1:8 1:8

22 days in Alsever's solution at 4°C and the hemagglutinability of rbc taken successively from the same individual remained constant. The susceptibility to measles hemagglutinin did not seem to be dependent on the physiological conditions of the monkeys but to be determined by individual characteristics of the animals.

ii) When rbcs of several species of apes were tested, a remarkable difference in the HA titer of the same hemagglutinin sample was found. Using the same CC sample, rbc of 8 out of 9 green monkeys, and 8 out of 10 Rhesus monkeys showed

Table 3. Comparison of the Susceptibilities of Red Blood Cells from Different Species of Monkey

Monkey	No.	HA titer per 0.4 ml							Mean	>128
		12	24	48	69	128	256	512		
Green	9	0	0	0	1	5	2	1	268	8/9
Cynomolgus	9	1	1	2	3	1	1	0	112	2/9
Rhesus	10	0	0	0	2	8	0	0	121	8/10
Patas	2	0	0	0	0	1 1	0	0	160	2/2
Skyes	2	0	0	0	0	0	0	0	256	2/2
Vervet	2	0	0	0	0	0 1	1	0	224	2/2
						128	256			

Carbowax concentrated sample was used as the virus material.

HA titers of more than 128, whereas only 2 out of 9 Cynomolgus monkeys had this level. This suggests that the two former species are more susceptible. With regard to patas, skyes and vervet monkeys, though the rates are apparently high, no conclusion can be drawn from the small number of cases tested.

iii) When the adsorption capacities for measles hemagglutinin of the rbc of green and Cynomolgus monkeys, representing rbcs with high and low susceptibilities were compared the results shown in Fig. 3 were obtained. In the adsorp-

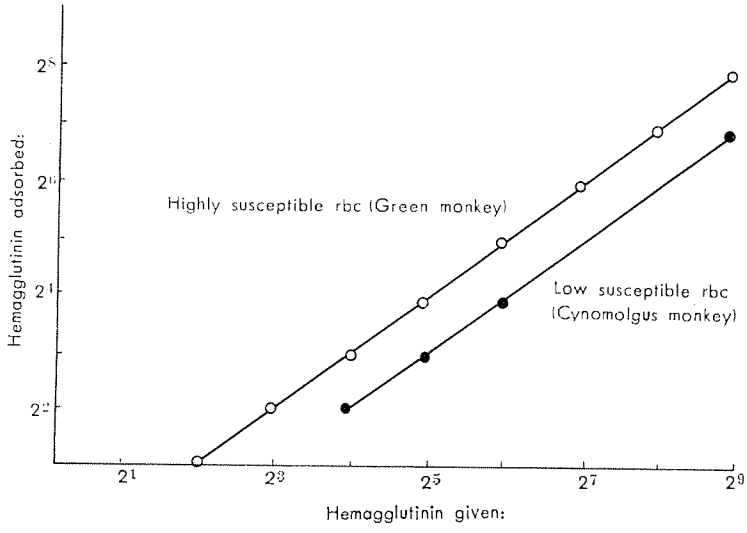

Fig. 3. Hemagglutinin Adsorbing Capacity of High and Low Susceptible Red Blood Cells* of Apes**

* 1% rbc solution (1.2-1.5 × 10^6 cells/0.1 m) was used.

** When highly susceptible rbc was used, the HA titer was 64 times higher than with low susceptible rbc.

tion experiment carried out under the routine conditions for HA titration, the former adsorbed 50 per cent of the hemagglutinin while the latter adsorbed only 25 per cent.

The susceptibility of monkey rbc to measles hemagglutinin did not seem to be affected by HI, complement fixing, and neutralizing antibodies against measles agent which were present in the original individual apes.

4) *Red blood cell concentration*

As shown in Fig. 4, the HA titer of the same hemagglutinin sample was linearly dependent on the rbc concentration. When the cell concentration was doubled the HA titer decreased to one half. A final concentration of $2\text{-}3 \times 10^5$rbc/ml was found to be the optimum for HA titration as described in the section on materials and methods.

Some characteristics of measles virus hemagglutinin

1) *Heat stability*

Concentrated hemagglutinin suspended in PBS, pH 7.2, was found to withstand inactivation at 4°C at least for 3 weeks and at 37°C for 9 days.

When heated at 56°C, complement fixing antigen was apparently most resistant. The second most resistant is hemagglutinin and the infectivity is most labile showing an exponential inactivation curve.

These differences in the inactivation rate, however, may reflect differences in the sensitivities of the method used for titration of the three activities. Still it can be said that complement fixing- and hemagglutinating-agents are more resistant than infectivity.

There is a small difference in the inactivation curves of CC (centrifugal

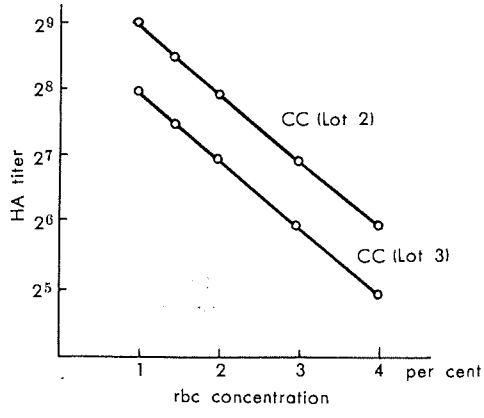

Fig. 4. Relationship between Red Blood Cell Concentration and Hemagglutinin Titer

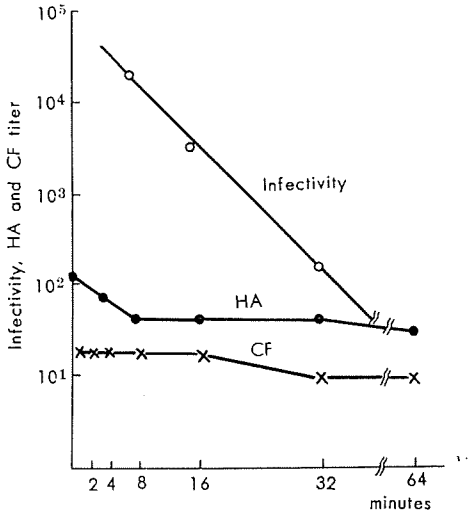

Fig. 5a. Inactivation of Carbowax Concentrated Virus Material by Heating at 56°C

concentrate) and CWC (Carbowax concentrate) at 56°C (Fig. 5b). While the hemagglutinating activity of CC remained constant for the first six hours of heating

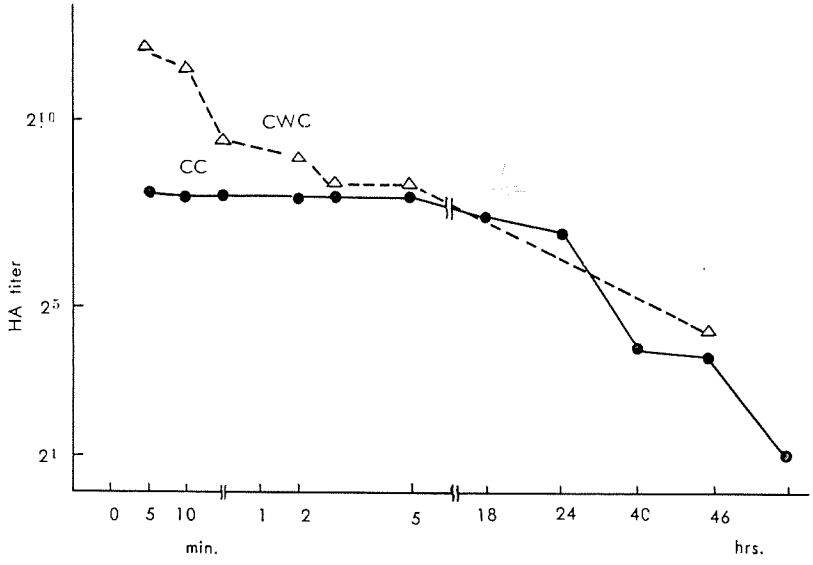

Fig. 5b. Heat Inactivation of Carbowax Concentrated and Centrifugally Concentrated Virus

and then began to decrease, the hemagglutinating activity of CWC decreased

greatly during the first two hours of heating. After two hours, the inactivation curves of the two samples almost coincided and at ca. the 40th hour, the hemagglutinating titer had decreased to $1/2^5$ to $1/2^8$ of the original titer.

This suggests that there may be two kinds of hemagglutinin: the one is rapidly inactivated and the other is more resistant to heat treatment at 56°C. While CWC contained whole substances originally present in the stock sample, the "rapidly inactivated hemagglutinin" in CC might be lost into the supernatant fluid during the centrifugation required for its concentration. But the nature or existence of this hemagglutinin is still open to question and a more appropriate explanation for the apparent resistance to thermal inactivation of CWC may be the existence of some protecting substances of a non-dialysable nature.

2) *Trypsin treatment*

CC, CWC and the ether-Tween 80 treated sample (cf. later) were treated with trypsin solution at a final concentration of 0.001 to 0.1 per cent and the mixtures incubated at 37°C for 30 minutes. The treated samples were then diluted 10 times with PBS containing 0.1 per cent bovine serum and HA titration was performed with these diluted samples.

The HA activities of CC and the ether-Tween 80 treated sample were greatly affected by trypsin and decreased when even as little as less than 0.006 per cent

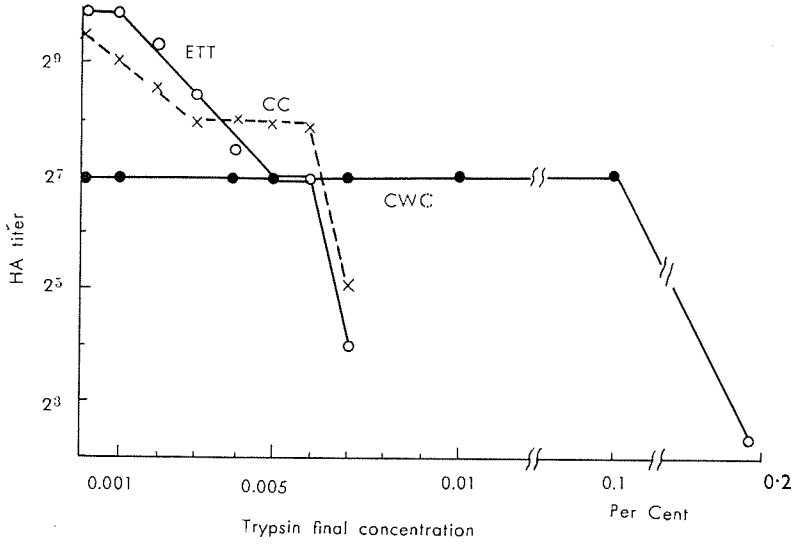

Fig. 6. Trypsin Treatment of Centrifugally Concentrated, Carbowax Concentrated, and Ether-Tween 80 Treated Virus Samples

Virus samples were treated with same volume of trypsin solution at 37°C for 30 mins

was added. On the contrary, CWC retained its original titer with as much as 0.1 per cent trypsin but when the trypsin concentration was raised, complete inactivation occurred. This apparent resistance might be caused by some protein present in the CWC sample. The data obtained showed that the hemagglutinating activity was highly sensitive to trypsin treatment.

3) *Periodate treatment*

Hemagglutinating activity was completely destroyed by the addition of KIO_4 at a final concentration of 0.46 mg per ml.

4) *UV irradiation of hemagglutinin*

UV irradiation was performed using a "National" UV lamp, GL-15W, of 15 watts, as the UV source. Two ml aliquots of CC and the ether-Tween 80 treated sample (cf. later) were placed in two Petri dishes of 5 cm diameter, and irradiated from a distance of 40 cm.

The HA titer of CC decreased to one fourth of the original titer after 40 minutes irradiation and to 1/32 after 128 minutes. On the other hand, the HA titer of the ether-Tween 80 treated sample decreased more slowly and was 1/8 of the original titer after 128 minute's irradiation.

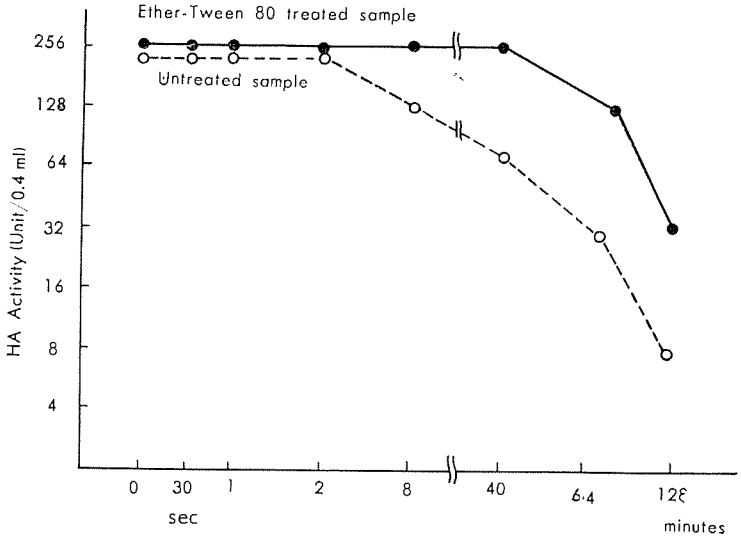

Fig. 7. Ultra Violet Irradiation of Ether-Tween 80 Treated and Untreated Samples

Effects of ether treatment of measles virus hemagglutinin

1) *Ether treatment*

CC was mixed with the same volume of ether at 0-4°C and was vigorously

shaken for the required time. When the mixture was centrifuged at 2,500 rpm for 15 minutes, it separated into three phases: an ether phase, an intermediate phase, and an aqueous phase. Hemagglutinin originally present in the water phase was trapped in the intermediate phase after prolonged shaking and after 30 minute shaking the HA activity of the aqueous phase disappeared completely while a considerable amount of hemagglutinin was recovered from the intermediate phase. No infectivity was detected in either phase after ether treatment.

2) *Effects of surface active agents on the ether treatment of hemagglutinin*

The virus material was mixed with an equal volume of ether in the presence of Tween-80 and shaken vigorously at 0-4°C. In this treatment hemagglutinating activity was retained in the aqueous phase and the titer increased 2 to 20 fold. Increase in the Tween-80 concentration usually caused an increase in the HA titer while the infectivity was completely lost as a result of treatment with ether alone. Treatment by Tween-80 alone had no effect on the HA titer even when empolying 1 hour's shaking at 4°C.

When a high concentration of Tween-80 was used, the separation of the

Table 4. Increase in HA Titer in Aqueous Phase after Ether-Tween 80 Treatment

Fxp.	conditions of ether treatment*		HA titer		increment
	concentration Tween 80	Treatment time	Before	After	
1	10 mg/ml	10 min	1:24	1:384	16
	10	60	1:24	1:256	10.6
	10	90	1:24	1:256	10.6
	0	10	1:24	1:12	0.5
2	2	20	1:256	1:512	2
3	2	20	1:512	1:1024	2
4	10	10	1:24	1:256	10.6
5**	30	30	1:128	1:2548	16
	15	30	1:128	1:1536	12
	7.5	30	1:128	1:1024	8
	3.75	30	1:128	1:1024	8
	1.87	30	1:128	1:1024	8
	0.9	30	1:128	1:1024	8
	0.4	30	1:128	1:512	4
6	10	15	1:64	1:1280	20
	10	10	1:32	1:96	3

* 1/10 volume of Tween 80 solution was added to the CC samples and then the same volume of ether was added to this mixture except in Exp. no. 5

** An equal volume of Tween 80 solution was added to the CC Sample

intermediate and aqueous phases became blurred. Moreover, the samples obtained could not be titrated for hemagglutinating activity because monkey rbc began to hemolyze in 4.5 mg/ml Tween 80.

When Tween 80 was added before ether, the recovery in hemagglutinating titer was high but the addition of Tween 80 more than 6 minutes after the ether had been added reduced the hemagglutinin titer of the aqueous phase.

Emasol, a surface active agent which has been successfully used to liberate Myxovirus hemagglutinin, when used in the ether treatment of measles hemagglutinin, increased the hemagglutinating activity as well, or better than Tween 80 did.

Unfortunately the hemolytic action of Emasol, however, was more than that of Tween 80 and so Emasol was unsuitable for these experiments. From these observations the conditions of ether-Tween 80 treatment was standardized in succeeding experiments as followings:

One ml of concentrated measles virus sample was mixed with an equal volume of ether and 0.1 ml of Tween 80 solution (the final concentration of Tween 80 was 2 mg per ml). After shaking in an ice bath for 15 minutes, the sample was centrifuged at 2,000 rpm for 10 minutes. Then the aqueous phase was drawn out by a 20 ml syringe with a long needle. The treated samples were stored in a refrigerator of 4°C.

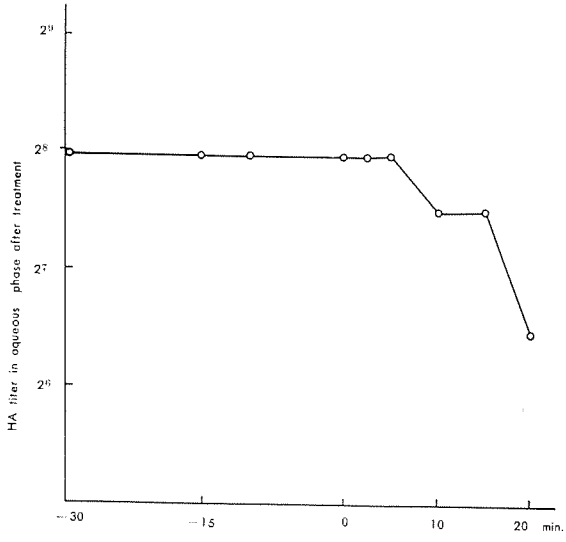

Fig. 8. Effect of Tween 80 on Hemagglutinin

Zero time indicates the time when Tween-80 was mixed with ether.
The HA titer before ether-Tween 80 treatment was 24 HAU/0.4 ml.
The final concentration of Tween 80 was 2 mg per ml.

3) *The difference between native and ether-Tween 80-treated measles hemagglutinin*

Half a stock sample of hemagglutinin was untreated and the other half was treated with ether and Tween 80 following the above procedure with an hemagglutinin yield of 4 time that of the native sample.

The native and ether-Tween 80-treated hemagglutinin were used as the antigen to determine the HI titer of several samples of sera of subjects convalescing from measles to compare their specificities against measles antibody. For the test 4 HAU of the two kinds of hemagglutinin were used per tube and the results are shown in Table 5.

There is a parallerism in the HI titer with native hemagglutinin and ether-Tween 80-treated hemagglutinin. Moreover, when ether-Tween 80-treated hemagglutinin was used, the HI titers were 2 to 4 fold higher than with native hemagglutinin.

Table 5 contains the corresponding complement fixing antibody titers of the sera and there is a good agreement between the HI and CF titers.

4) *Examination of the antigenic nature of ether-Tween 80-treated measles virus samples*

Table 5. Comparison of the HI Titer of Convalescent Sera of Children with Ether-Tween 80 Treated and Untreated Virus Sample

Child	HI titer		CF antibody titer
	Ether-Tween 80 treated HAnin	Untreated HAnin	
T. K.	>320	192	16
S. K.	96	48	8
S. Y.	>1280	1280	128
T. M.	640	160	128
J. A.	384	160	32
T. H.	192	160	32
Y. A.	768	384	128
H. N.	384	384	128
N. N.	768	320	256
S. K.	40	<10	4
T. A.	40	<10	4
K. H.	384	64	16
Y. S.	320	160	16

i) Examinations were performed by testing the response of guinea pig antibody to the hemagglutinin fractions.

The CC was treated with ether and Tween 80 and fractionated by CsCl density gradient equilibrium centrifugation in three 1/2″×2″ lusteroid tubes. The columns were divided into various fractions and the fractions at the same heights in the tubes were pooled. After dialysis against PBS, two or three guinea pigs were subcutaneously inoculated three times at weekly intervals with 0.5 ml of each fraction.

Table 6. Antibody Response to Ether-Tween 80 Treated Sample Fractionated by CsCl Density Gradient Equilibrium Centrifugation

Immunogen		Antibody Titer		
Fraction No.	HA unit	HI	NT	CF
1	32	<40	16	16
2	32	<40	4	4
3	192	160	512	128
4	960	640	512	512
5	4096	320	256	512
6	4096	320	256	512
7	2048	640	1024	256
8	960	80	128	8
9	32	20	<4	64
10	<2	10	<4	512

Seven days after the last immunization, the guinea pigs were bled and the titers of hemagglutination inhibition, neutralizing, and complement fixing antibodies were determined (Table 6). The HI and CF antibody showed a high titer with fractions which had high HA and CF titers and the response of the NT antibody was more in gunea pigs with a high HI antibody titer.

ii) When standard monkey antiserum was absorbed with the fraction showing the highest hemagglutinating titer, the HI and NT antibodies decreased simultaneously from 256 HI units/0.4 ml to 96 HI units/0.4 ml and 256 NT units/0.1 ml to 64 NT units/0.1 ml, respectively. This decrease in the titers means that 63 per cent of the HI-and 77 per cent of the NT antibody of the serum was absorbed by the fractionated material.

Analysis of native and ether-Tween 80-treated material by density gradient centrifugation

1) *Sucrose density gradient centrifugation*

Native hemagglutinin which was concentrated by centrifugation had a hemagglutinating titer of 256 units/0.4 ml and a complement fixing titer of 16 units/0.1

ml. The hemagglutinating and complement fixing titers of the ether-Tween 80-treated material used were 128 units/0.4 ml and 8 units/0.1 ml respectively. Density gradients were prepared in 1/2"×2" lusteroid tubes layering 0.4 ml of sucrose solutions in PBS of 4 per cent differences from 25 per cent to 57 per cent. Samples of 0.8 ml of hemagglutinin were placed on the top of the gradient columns and the tubes were then centrifuged at 15,000 rpm for 8 hours in a Hitachi 40P refrigerated ultracentrifuge using a RPS 40 rotor. After centrifugation, the tubes were punctured at their base with a hypodermic needle and fractions of 0.5 ml collected. The hemagglutinating titers were determined immediately after four fold dilution of the fractions with distilled water. Before complement fixing titrations, the diluted fractions were dialyzed against PBS, pH 7.2, for more than 12 hours at 4°C.

In the two samples the distribution of the hemagglutinating activity in the columns was almost the same and peaks were found in fractions 4 and 5. The peak of the ether-Tween 80-treated sample was the sharper. The complement fixing activity was in fractions 5 to 8 with a single peak in the case of the native sample, and 2 peaks in the ether-Tween 80-treated sample located in fractions 1 and 5. (Fig. 9)

The recovery of hemagglutinin in this experiment was 103 per cent for the native sample and 80 per cent for the ether-Tween 80-treated sample.

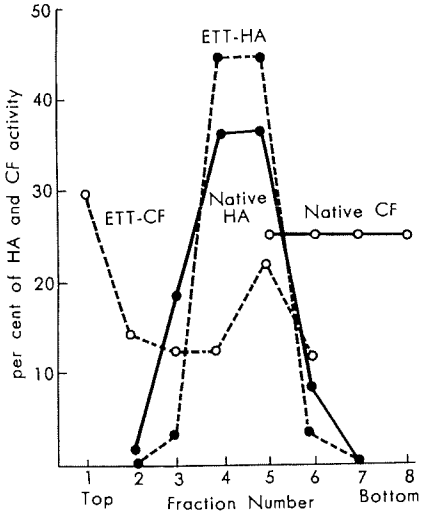

Fig. 9. Distribution of Hemagglutinating and Complement Fixing Activity of Native and Ether-Tween-80 Treated Samples Fractionated by Sucrose Density Gradient Centrifugation (Centrifuged at 15,000 rpm for 8 Hours)

2) *CsCl density gradient equilibrium centrifugation*

a) The same samples as for the sucrose density gradient centrifugation were used. A stock solution of CsCl with a density of 1.801 was prepared by dissolving CsCl crystals in distilled water. One volume of the test sample was mixed with several volumes (four or five) of CsCl stock solution to obtain the desired initial density. Usually the hemagglutinating activity was not reduced by incubation at 4°C for 3 days in 5M CsCl solution but as a stabilizer, a small amount of bovine serum was added to the mixture. The mixture was spun at 32,500 rpm for 65 to 72 hours until density equilibrium was reached, using a Hitachi 40P refrigerated ultracentrifuge with a RPS 40 rotor. After centrifugation, the fractions were obtained as described above. The densities of the fractions were calculated from their refractive indices, n, measured in an Abbe's refractometer (Hitachi Model PRA-B) at 25°C applying the formula (Ifft, 1961):

$\varrho^{25°}=10.8601\ n_{0}^{25°}-13.4974$

In calculations of density, the refractive increments of solutes other than CsCl in the test samples were neglected as their concentrations were very minute.

A representative test sample mixture was as follows:

1)	Native sample	4 ml
	CsCl stock solution	1.3 ml
	Bovine serum	0.1 ml
	Initial density: 1.2234	
2)	Ether-Tween 80-treated sample	5 ml
	CsCl stock solution	3.3 ml
	Bovine serum	0.1 ml
	Initial density: 1.2667	

The peak of hemagglutinating activity of the native sample was seen in fraction 6 (density 1.186) but with the ether-Tween 80-treated sample the peak shifted to a fraction of higher density (density 1.260) and was sharper (Fig. 10, 11, 12). Recoveries of hemagglutinin were 83 per cent and 84 per cent respectively. The complement fixing activity of the native sample was in a single peak in fraction 4 (density 1.215) but that of the ether-Tween 80-treated sample was in two peaks: the major one, coincided with the hemagglutinin peak (fraction 5, density 1.260) and the minor one was located in the top of the tube (fraction 9, density 1.190). The recovery of complement fixing antigen was nearly 100 per cent.

Thus, after treatment with ether and Tween 80, the peak of hemagglutinating activity in the CsCl density gradient column of KB_7 passaged measles virus shifted to a heavier fraction than the native sample.

On CsCl density gradient equilibrium centrifugation, while the hemagglutinin of the KB passaged measles virus sample had a clear peak at a density of

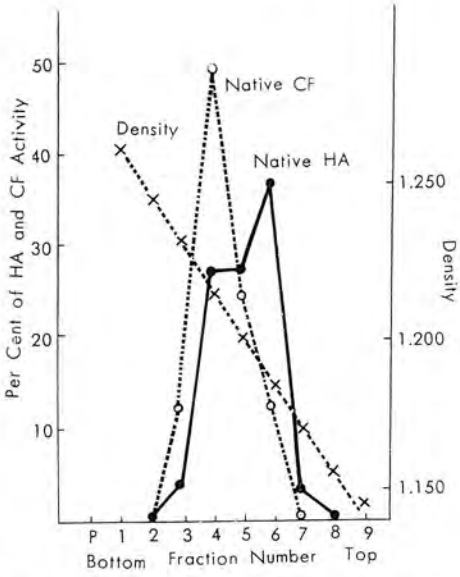

Fig. 10. Distribution of Hemagglutinating and Complement Fixing Activity of Native Sample on CsCl Density Gradient Equilibrium Centrifugation (Centrifuged at 32,500 rpm for 65 Hours) Initial Density 1.223

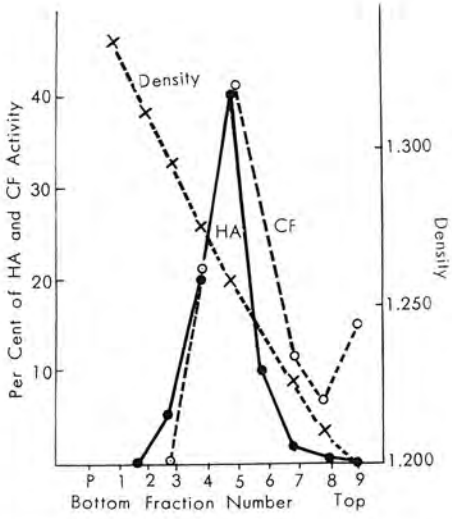

Fig. 11. Distribution of Ether-Tween-80 Treated Sample on CsCl Density Gradient Equilibrium Centrifugation (Centrifuged at 32,500 rpm for 65 Hours) Initial Density 1.267

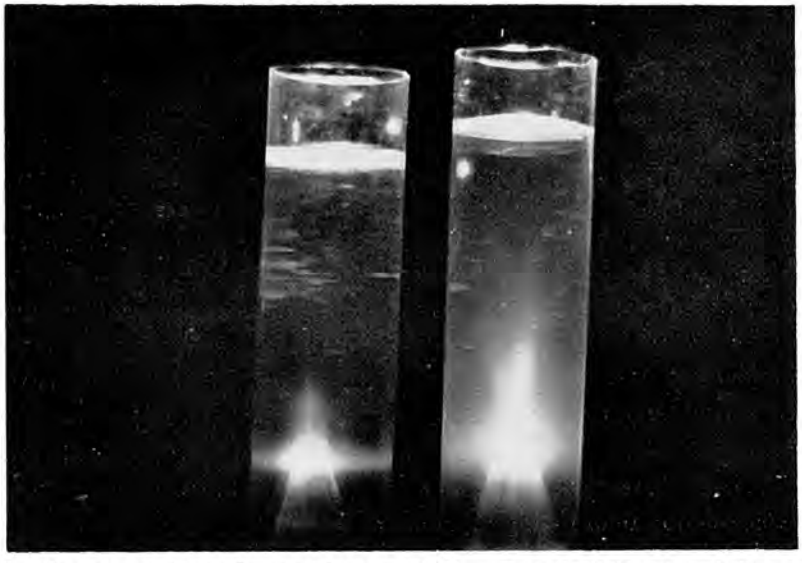

Fig. 12. Banding of Ether-Tween 80 Treated Virus Sample (left tube, cf. Fig. 11.) and Native Sample (right tube, cf. Fig. 10.)

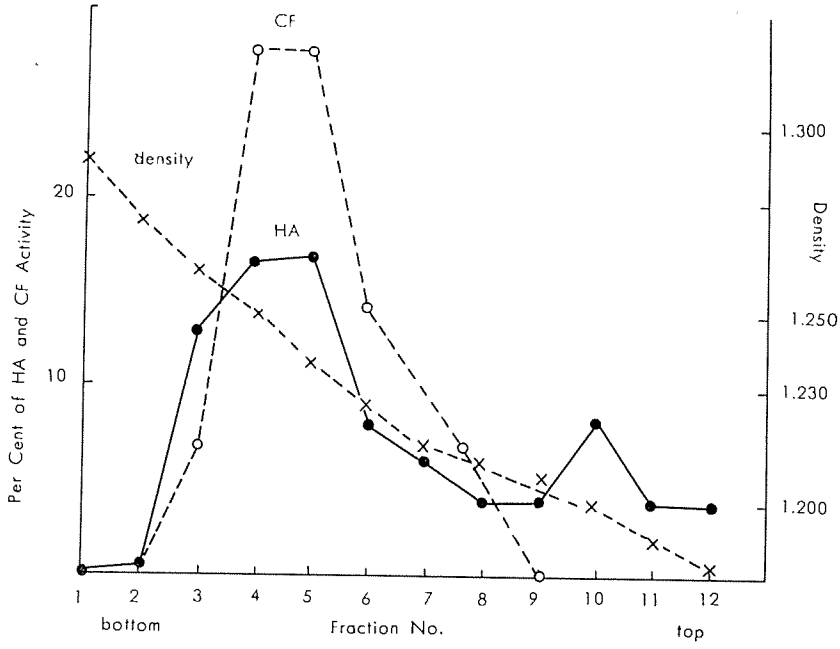

Fig. 13. Distribution of Hemagglutinating and Complement Fixing Activity of Centrifugally Concentrated Sample on CsCl Density Gradient Column (30,000 rpm for 48 hrs. Initial Density 1.258)

1.186, ERK (embryonated rabbit kidney) cell passaged measles virus sample showed a broad distribution of hemagglutinin with a density of about 1.260. These facts suggest that the characters of the hemagglutinin varies with the host from which the sample is obtained.

b) The initial density of $KB_{12}ERK_1$ passaged measles virus was raised to 1.258 with CsCl and the solution centrifuged at 30,000 rpm for 48 hours. Two peaks of hemagglutinating activity were detected. One peak was in fractions 4 and 5 with an average density of 1.24 and the other peak in fraction 10 with a density of 1.190 (Fig. 13). Fractions 3 to 7 and fractions 8 to 12 respectively and both pools were recentrifuged at the same speed and for the same time as were pooled before. The high density portion showed a sharp hemagglutinin peak at a density of 1.215 (Fig. 14).

On the contrary, the hemagglutinating activity of the low density pool was distributed widely around a density of 1.19 (Fig.15). The hemagglutinin in this portion seemed to be composed of various components, while the high density pool contained rather homogeneous material.

c) Analysis of sonicated hemagglutinin

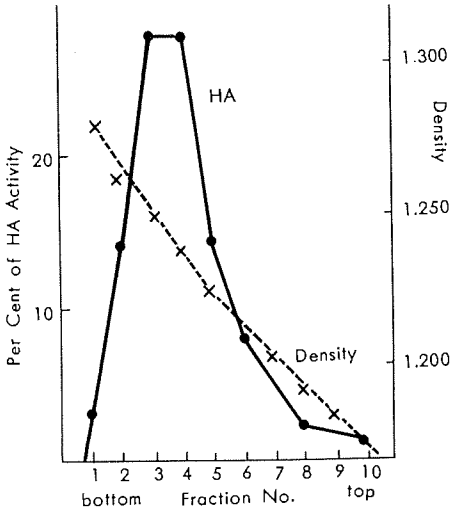

Fig. 14. Distribution of Recentrifuged Heavier HA Components on CsCl Density Gradient Equilibrium Centrifugation (30,000 rpm for 48 hrs. Initial density 1.249)

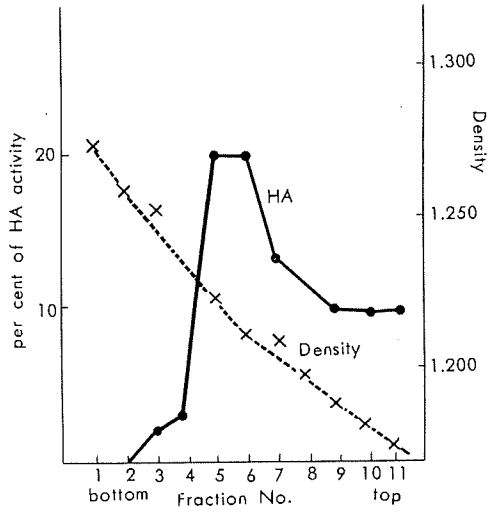

Fig. 15. Distribution of Recemtrifuged Lighter HA Conponent on CsCl Density Gradient Equilibrium Centrifugation (30,000 rpm for 48 hrs. Initial density, 1.251)

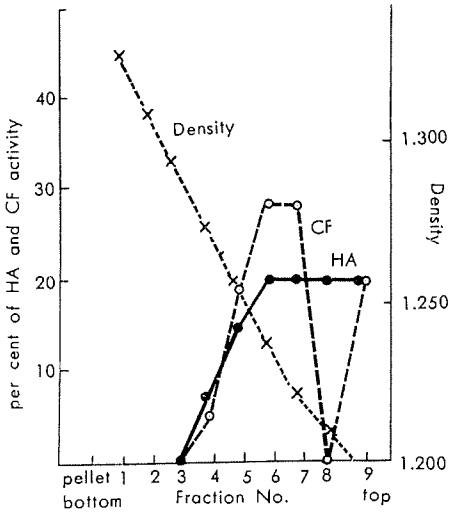

Fig. 16. Distribution of Hemagglutinating and Complement Fixing activity of Sonicated Virus on CsCl Density Gradient Equilibrium Centrifugation (32500 rpm for 65 hrs. Initial density, 1.273)

The distribution of hemagglutinin in the CsCl column was slightly displaced towards a higher density after sonication. When CC was sonicated at 10 KC for 30 minutes, the hemagglutinating activity was found in a density range from 1.190 to 1.240 after CsCl density gradient equilibrium centrifugation at 32,500 rpm for 65 hours. The density of the hemagglutinating peak of untreated CC was less than this and distributed at a density of between 1.186 and 1.215. (Fig. 16).

The low density complement fixing antigen, which could not be detected in the untreated sample, also appeared after sonication.

DISCUSSION

Rosen (1961) reported that hemagglutination tests carried out at 4°C gave lower values than those carried out at 37°C. We found little difference between the hemagglutination titers measured at 4°C and 37°C. However when virus samples were cooled in an ice bath before the red blood cell was added the hemagglutination titer at 4°C was 2 to 4 fold lower than at 37°C. This suggests that the hemagglutination phenomenon differs from that of myxoviruses; it seems that the effect of the hemagglutinating agent on the surfaces of the red blood cells decreased at low temperatures implying that the effect is not simply caused by adsorption.

Previously measles virus hemagglutination has only been studied with monkey red blood cells. However, the susceptibility of the red blood cells to measles hemagglutinin differed greatly, not only between species but between individuals of the same species and had no relation with the complement fixing and neutralizing antibodies in the monkey sera.

The different susceptibilities of red cells of individuals in a single species was shown in Vervet monkey red blood cells by Cutchins (1962). In our experiments, Cynomolgus monkey red blood cells showed remarkable individual differences in susceptibility while the susceptibilities of Rhesus monkeys were very similar.

The characteristics of measles hemagglutination differed from those of myxoviruses in other respects. The adsorption efficiency of measles hemagglutinin to the monkey red blood cells under the standard conditions of the hemagglutination test was consistently 50 per cent and was not related to the original hemagglutination titer. After red blood cells had been treated with RDE, they could still be agglutinated with measles hemagglutinin. Measles hemagglutinin was not eluted on incubation at 37°C or after RDE treatment. Thus the nature of the measles hemagglutinin differs from that of the myxoviruses although the two are morphologically similar (Waterson, 1961).

It was demonstrated that the hemagglutinin of measles virus was trypsin sensitive. The relatively high trypsin-resistancy of Carbowax concentrated samples

may be accounted for by the high concentration of coexisting protein in the samples. Measles hemagglutinin was more heat stable than infectivity but was sensitive to oxidizing agents, as already indicated by Periés (1962) and others.

After ether treatment, the measles hemagglutinin in the water phase was not completely destroyed but was transferred to the intermediate phase probably with denatured lipid components. However, when measles hemagglutinin was treated with ether and Tween 80, it remained in the aqueous phase even after 30 minutes treatment. In this case hemagglutinin seemed to be released from other components by the detergent. Contaminating substances were transferred from aqueous phase to the intermediate layer, so that, the hemagglutinin became dispersed as homogeneous smaller particles after ether-Tween 80 treatment and the titer increased considerably, this increase varying from case to case. This increase in the titer also seems to be due to the release of hemagglutinin particles from aggregates, from virus particles themselves or from some cellular components. This suggestion is supported by the results of the HI test using native and ether-Tween 80 treated hemagglutinin and the results of density gradient fractionation of the two samples. It must be remembered, however, that some inhibitor-like substance for measles hemagglutination in the original sample might be removed by this treatment.

It seems likely that lipid components derived from infected cells play an important role in the masking or aggregation of hemagglutinin. Much hemagglutinin was recovered from the intermediate phase of the ether treated sample and from the aqueous phase of the ether-Tween 80 treated sample, suggesting that hemagglutinin is resistant to ether in spite of its apparent sensitivity.

Complete loss of the infectivity after ether treatment alone or ether-Tween 80 treatment suggests that lipoprotein may be an essential constituent of active virus particles. Recently, Norrby (1963) reported nearly the same results as those described in this article on ether -Tween 80 treatment.

After sucrose density gradient centrifugation, the hemagglutininating activity of partially purified and centrifugally concentrated measles virus samples was distributed over a wide range of densities. The hemagglutinating activity of ether Tween 80 treated samples had a peak in the same position as that of the native sample. This result differs from Norrby's data (1963) showing that when a large hemagglutinin was treated with ether and Tween 80, it showed the same physical roperties as those of a small hemagglutinin. This discrepancy is probably due to differences in that sucrose gradients and conditions of centrifugation used. Experiments on sucrose density gradient centrifugation suggested the existence of various sizes, forms, and densities fo hemagglutinins, but the high viscosity of the sucrose solution used prevented sharp separation, and moreover the equilibrium may have been false.

To overcome these difficulties, CsCl density gradient equilibrium centrifuga-

tion was employed. By this procedure, the hemagglutinin in the centrifugally concentrated sample, CC, became distributed in two main fractions with densities of 1.19 and 1.24.

The hemagglutinating agent in the native sample seems to become subdivided into smaller subunits by ether-Tween 80 treatment, as in the case of ether treated myxoviruses (Hoyle, 1952; Hosaka *et al.*, 1960) and to become homogeneous particles with a bouyant density of 1.26. The increase in density of the hemagglutinin might be explained also as due to separation from denatured lipid substances.

Furthermore, the titers of the hemagglutination-inhibiting antibody of sera from patients convalescing from measles may be related to the nature of the hemagglutinin materials used for the test; the titers of the HI antibody were 2 to 4 fold higher with ether treated hemagglutinin than with native hemagglutinin, suggestint that the treated hemagglutinin, disintegrating into smaller particles has a smaller capacity to bind antibody. This agrees to some extent with the report of Peries (1962), that measles virus hemagglutinin is composed of large and small components.

The behavior of complement fixing agent coincided with the data described by Schleuderberg. After ether-Tween 80 treatment, a low density complement fixing antigen appeared, which was not detected in the native sample.

After ether treatment, the fact that the HA fractions had the capacity to produce and to adsorb neutralizing antibody regardless of the complete loss of the infectivity seemed to be due either to the coexistence of disrupted antigen for neutralizing antibody in the hemagglutinin fraction or to the coincidence of such disrupted antigen and hemagglutinin.

It is interesting to consider whether the measles virus hemagglutinin is one of the subunits which constitute the virus particles or a side product of virus multiplication. Although Schleuderberg reported that the infectivity was in the fraction with a bouyant density of 1.288, when fractionated by CsCl density gradient centrifugation, the infectivity of the fractionated sample could not be detected in fractions of a density of 1.288 in our experiments, but was detected in "the high density" hamagglutinin-complement fixing antigen fraction though its recovery rate was low.

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