BIKEN'S JOURNAL 1, 70-89(1958)

On the Hemolytic Activity of HVJ.

I. An Analysis of the Hemolytic Reaction

YASUHIRO HOSAKA

Department of Preventive Medicine, Research Institute for Microbial Diseases, Osaka University

(Received for publication, October 28, 1958)

SUMMARY

The hemolytic activity of HVJ was found to be enhanced in various ways. Centrifugal fractionation showed that the enhanced activity was still combined with virus particles and isolation of free hemolysin was not successful. A comparison of several properties showed the "enhanced" hemolytic activity is not essentially different from that of original virus.

The hemolytic process in this virus was a first order reaction and the hemolytic activity was quite different from the hemagglutinating or eluting activity.

In the hemolytic reaction, an initial and a later stage could be differentiated when the hemolysis developed at low temperature or in the presence of antiserum. Although the hemolytic activity of HVJ is like that of lecithinase in some properties, its true nature seems to be different.

INTRODUCTION

The hemolytic activity of HVJ has been described by Fukai and Suzuki (1955). Several properties of HVJ reported in the paper indicated that it belongs to MNI group.

If this group of viruses is divided into the hemolytic (i.e. Newcastle Disease Virus, Mumps virus and HVJ) and the non-hemolytic group (influenza viruses), there are considerable differences in the properties of the two groups. For example while the latter group can produce the incomplete form when a large inoculum is used, (von Magnus, 1951) the former does not. (Granoff, 1955., Fukae, 1958., and Tadokoro, 1959) Furthermore when a high concentration of these hemolytic viruses are mixed with appropriate host cells, infected cells fuse rapidly in groups of tens and as the result many polynuclear giant cells are formed. (Henle *et al.*, 1954, Okada *et al.*, 1957 and Kim, 1958). This phenomenon was considered to be caused by the viral hemolytic activity. Thus the hemolytic activity of these viruses is very interesting not only from the view-point of viral multiplication but also for an analysis of the pathogenicity of the virus.

This paper describes the results of some analysis on HVJ hemolysis.

Materials and Methods

Virus: HVJ, Z strain was used throughout the experiments. Infected allantoic fluid of 10-day-old embryonated eggs was collected after 72 hours incubation and centrifuged at 3,000 r. p. m. for 30 minutes. The supernatant was further centrifuged at 22,500 r. p. m. for 40 minutes in a #40 rotor of a Spinco L ultracentrifuge and the pellets were resuspended in isotonic phosphate buffered saline (PBS) of pH 7.2. This virus suspension was centrifuged at 3,000 r.p.m. for 20 minutes and the upper four-fifths was employed in the experiments.

Red blood cells: Blood was withdrawn by cardiac puncture of roosters using sodium citrate as anti-coagulant and centrifuged at 1,200 r.p.m. for 5 minutes. The sedimented red cells were washed 3 times with PBS. The cells were then resuspended in PBS at a concentration of 0.5 per cent for the hemagglutination test and of 2 per cent for the hemolysis test.

Hemagglutination test: Salk's pattern method (1944) employing 0.5 ml. of 0.5 per cent red blood cells was used with isotonic citrate saline as the diluent.

Hemolysis test: One ml. of ice-cold virus sample was mixed with 2 ml. of 2 per cent red blood cell suspension. The tube containing the mixture was kept cold for 30 minutes to allow virus adsorption onto the red cells and then kept in 37°C water bath for 60 minutes with mechanical agitation. At the end of incubation the tube was centrifuged at 1,500 r.p.m. for 5 minutes. The supernatant fluid was transferred to a colorimeter cuvette and the amount of hemoglobin liberated was determined in a Coleman spectrophotometer at 5400 Å. All determinations were expressed as optical densities. A series of experiments was carried out using the same suspension of red cells and the same virus preparation. As the control one ml of PBS was mixed with 2 ml of 2 per cent red cells and treated in the same manner. All readings given are those which subtracted control readings from the direct readings.

Freezing and thawing: A small amount of broth (*ca.* 1/1000) was added to the virus suspension. One part of this was repeatedly exposed to freezing at −70°C and thawing at 30°C. The other half was used as a control without any treatment. In this paper freeze-thawed virus is assigned as "treated virus" while control virus as "untreated virus".

Preparation of "white cell fraction": Blood was withdrawn by cardiac puncture of roosters using sodium citrate as anti-coagulant and the blood cells were washed 3 times with PBS. After resuspension in PBS the fraction was centrifuged at 800 r.p.m. for 5 minutes. The sediment was again resuspended and centrifuged at the same speed. This step was repeated 3 times. The supernatant fluids from these low speed sedimentations were combined and centrifuged at 3,000 r.p.m. for 5 minutes. The final sediment was resuspended in PBS and used as the "white cell fraction". It contained leucocytes and platelets.

Preparation of stroma from red cells: Fifty ml. of 10 per cent red cells in saline was mixed with 2 ml. of 2 per cent saponin. The mixture was allowed to stand for 30 minutes with intermittent shaking and then centrifuged at 3,000 r.p.m. for 10 minutes. The sediment was washed with saline until it became white.

Extraction of phospholipid from red cell stroma: Crude phospholipid from stroma was extracted by the method of Lea *et al.* (1955) from two sources; one was red cell stroma prepared by saponin treatment and the other the residue of the cells hemolysed in distilled water.

RESULTS

I. *Effects of some physical agencies on the hemolytic activity*

Chu and Morgan (1950a) reported the enhancing effect of freeze-thawing on the hemolytic activity of mumps virus and Granoff and Henle (1954) a similar

effect of various agencies on that of N.D.V.. Therefore the effect of these agencies on the hemolytic activity of HVJ was studied.

a) *Freeze-thawing:* The effects of freezing and thawing on the hemolytic activity were compared when the virus was suspended in various solutions. Concentrated virus was diluted to the same degree in the following solution:

1) PBS containing 5/100 volumes broth
2) PBS containing 0.5/100 volumes broth
3) PBS containing 2 per cent casein hydrolysate
4) PBS containing 0.3 per cent lysine
5) PBS
6) 0.3 M sucrose solution

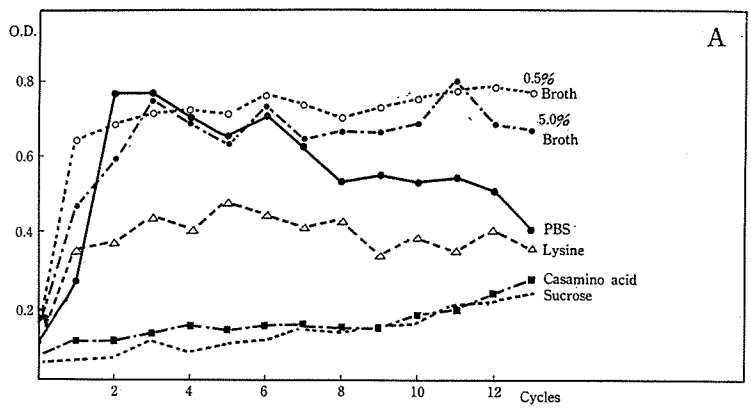


Fig. 1 A. Effect of freeze-thawing on the hemolytic activity of HVJ suspended in various solutions (Virus concentration: 512 HAU/ml).
ordinate: liberated hemoglobin (O.D.)
abscissa: cycles of freeze-thawing

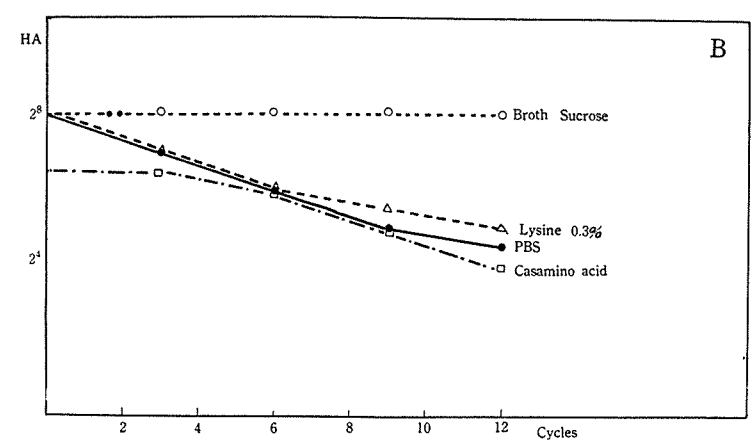


Fig. 1 B. Effect of freeze-thawing on the hemagglutinating activity of HVJ suspended in various solutions.
ordinate: hemagglutination titre
abscissa: cycles of freeze-thawing

The results in Fig. 1 A and Fig. 1 B show the effect of freeze-thawing on the hemolytic activity and hemagglutinin. The hemolytic activity was found to be enhanced after repeated freezing and thawing. In diluted broth and PBS the hemolytic activity was markedly enhanced while in sucrose and casein hydrolysate solution there was only slight enhancement, moderate enhancement was observed in lysine solution.

When freezing and thawing was repeated twice in lysine, broth, or PBS, the hemolytic activity reached a maximum and after 7 to 8 times treatments the activity began to be reduced except the case with diluted broth. This reduction seems to be correlated with reduction in the hemagglutinating activity, since only in the broth solution was the hemagglutinating activity unaltered even after 12 times freezing and thawing.

b) *Sonic vibration:* The hemolytic activity of HVJ was also enhanced when the virus suspension was exposed to sonic vibration. After various lengths of exposure aliquots of virus suspension were removed for hemolysis and hemagglutination titrations. Fig. 2 shows the results. Throughout the periods of exposure the hemagglutinin titer was unaltered or slightly enhanced.

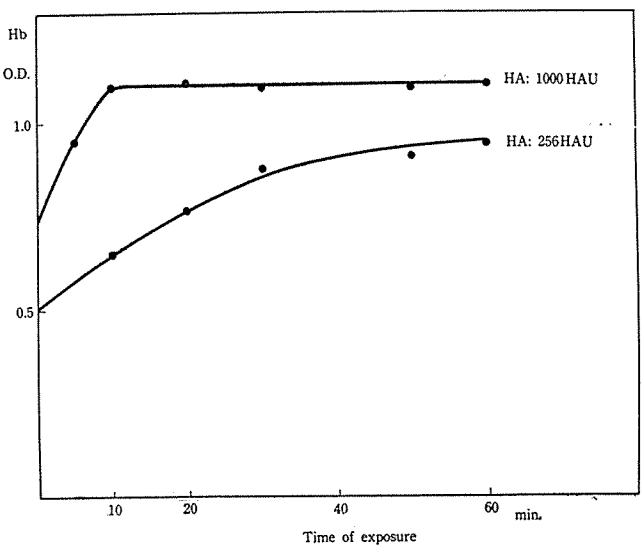


Fig. 2. Effect of sonic vibration on the hemolytic activity of HVJ (in Raytheon sonic oscillator, Model S-102, 9K.C.).
ordinate: liberated Hemoglobin (O.D.)
abscissa: Time of exposure

c) *Osmotic pressure change:* The virus was suspended for various periods in cold hypertonic solutions of NaCl and glycerol and then diluted rapidly in distilled water to lower the osmotic pressure to isotonic conditions. With 6, 3, and 0.6 M glycerol the dilution was performed by addition of 19, 9, and 1 volumes of distilled water respectively. In both cases in which 3 M and 0.6 M glycerol were used the shockate treatment was followed by secondary dilution with 0.3 M glycerol to equalize the virus titers.

As control the virus in 0.3 M glycerol was diluted 20 times with the same solution. Then for hemolytic tests each 1 ml of treated virus suspension, devised to contain the same titer (32 HA units), was mixed with 2 ml of red cell suspension.

In 3, 1.5 and 0.15 M NaCl, the virus was treated and tested in the same manner as that used for glycerol solution. (As the final media 0.15 M solution was used in this case.)

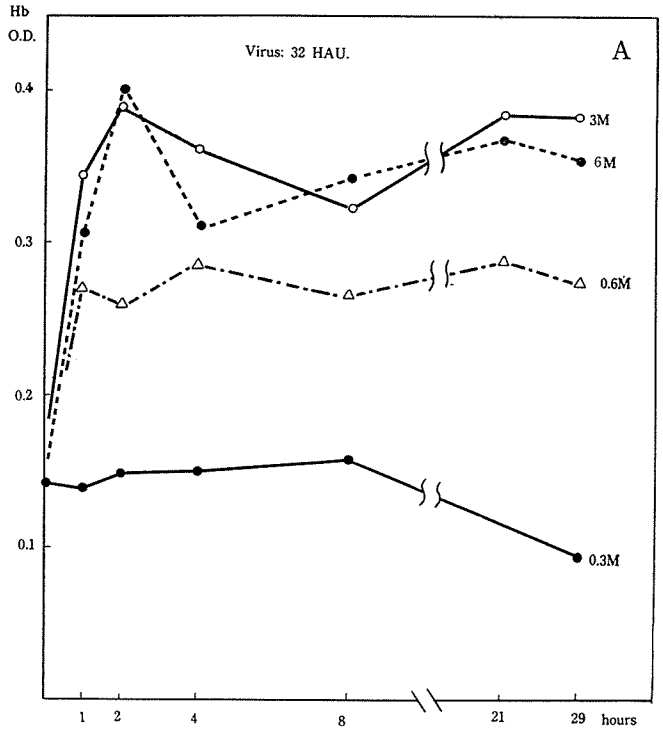


Fig. 3A. Effect of change in osmotic pressure on the hemolytic activity of HVJ. (in glycerol solution)
abscissa: Suspension period of virus in hypertonic solution

The results are presented in Fig. 3A and B. In both glycerol and NaCl, there is a tendency that the heavier the grade of the reduction in osmotic pressure, the greater the enhancement of the hemolytic activity, except in 6 M glycerol where the effect is the same as that of 3 M glycerol. The suspension periods giving maximal effects were 4 hours for NaCl, 2 hours for 6 M glycerol, 2 hours for 3 M glycerol, and 1 hour for 0.6 M glycerol. Generally glycerol had a greater effect than NaCl.

To analyze in more detail the effect of osmotic pressure changes on the virus a "slow dilution method" was adopted. The virus suspensions in hypertonic solutions of NaCl, sucrose, and glycerol were divided in two, and one was treated osmotically by rapid dilution with 20 volumes of distilled water, the other was dialyzed against 20 volumes of distilled water in a cellophane membrane, and after 24 hours dialysis the internal and external fluids were mixed. This procedure could be considered as a very slow dilution.

A comparison of the hemolytic activities of rapidly diluted virus and of slowly diluted virus is presented in Fig. 4. The control is virus suspended in PBS and diluted with PBS in a similar manner.

The hemolytic activity was greater with the "slowly diluted" virus than with

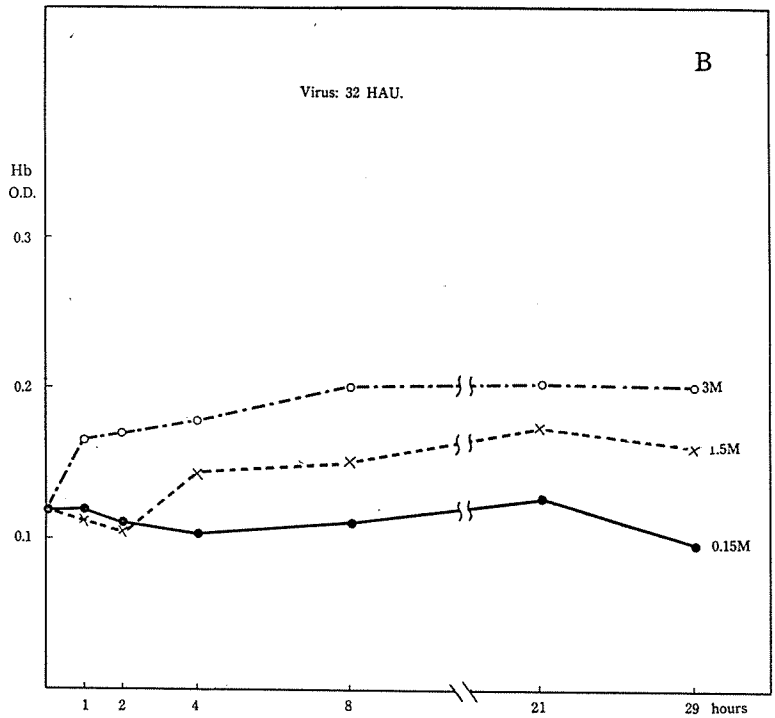


Fig. 3B. Effect of change in osmotic pressure on the hemolytic activity of HVJ. (in NaCl solution)
abscissa: Suspension period of virus in hypertonic solution

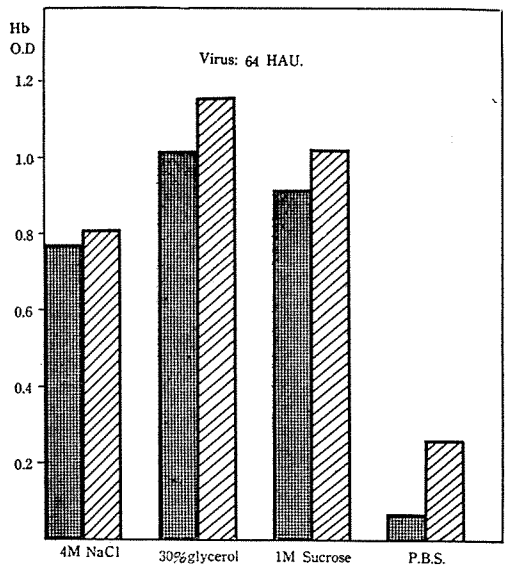


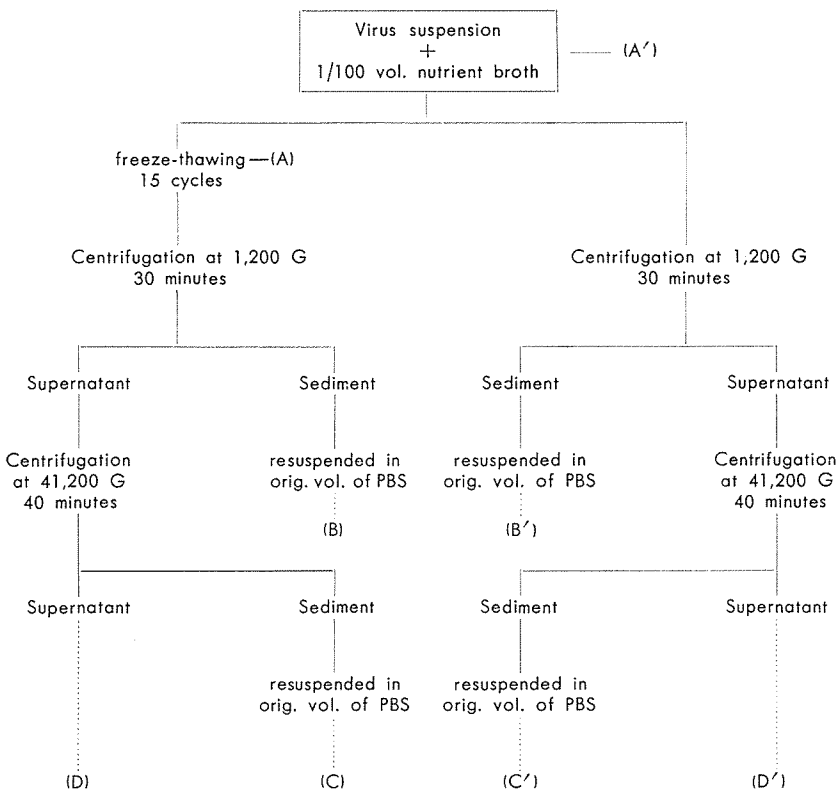
Fig. 4. Comparison of the enhancement of hemolytic activity of "rapid" and "slow" dilution of the virus suspension. "Slow dilution" was attained by dialysis against 20 volumes of distilled water for 24 hours, after which the dialysed fluid and dialysate were mixed.
black : rapidly diluted virus
hatched: slowly diluted virus

the "rapidly diluted". Glycerol seems the most effective reagent for osmotic experiments. The enhancement of hemolysis is attained by simple "slow dilution"; shockate treatment is not essential. The hemagglutinating activity was not changed by any of these treatments.

II. *Fractionation of freeze-thawed virus*

As described above, viral hemolytic activity was enhanced in several ways. Is the virus particles disintegrated into a number of subunits of hemolysin by these treatments, or how does the virus particle liberate the free hemolysin ? To answer these questions the correlation between hemolytic activity and virus fractionated by centrifugation was investigated.

Table 1. Centrifugal fractionation of freeze-thawed and untreated (control) virus preparation



A virus suspension containing a little amount of broth was fractionated as shown in Table 1. Hemolysis, hemagglutination, and the protein content (Lowry *et al.*, 1951) were determined in 4 fractions: the starting material, sediment of low speed centrifugation, sediment and supernatant of high speed centrifugation. (Fig. 5)

Most of the hemolytic activity after freeze-thawing was found in the sediment of high speed centrifugation that corresponds to the fraction of virus particles.

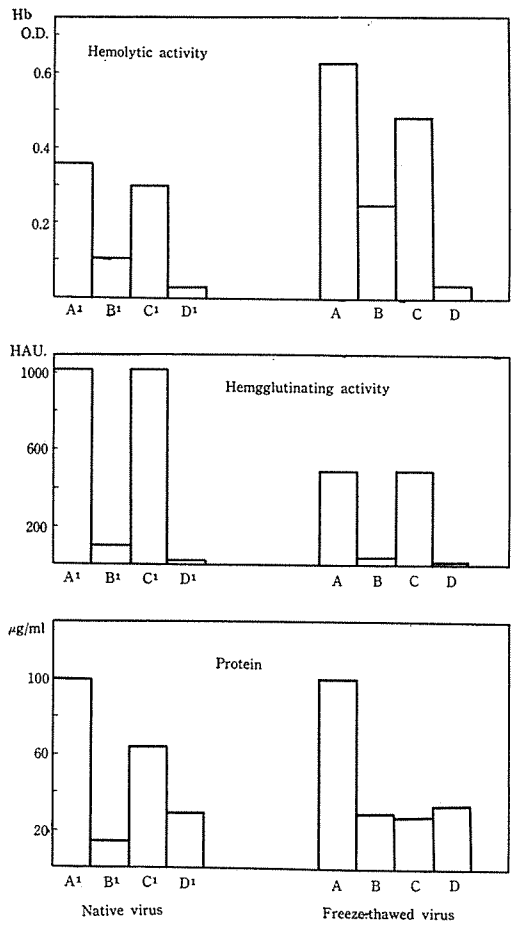


Fig. 5. Distribution of hemolytic activity, hemagglutinating activity, and protein content in the fractions separated by centrifugation from freeze-thawed and native (untreated) virus. (cf. Table 1)
A: Starting material
B: Sediment of low speed centrifugation
C: Sediment of high speed centrifugation
D: Supernate of high speed centrifugation

Hemagglutinating activity was reduced to one half of the control value after freeze-thawing. This decrease seemed to be due to the easily sedimentable aggregate of virus caused by the treatment. Electronmicroscopy of the low speed sedimentable fraction showed the existence of aggregates and this fraction showed low hemagglutinating activity although it contained a considerable amount of protein and viral hemolysin. The shape and size of individual virus particles in the suspension were not changed after this apparently drastic freeze-thawing treatment.

The enhanced hemolytic activity of virus after freeze-thawing was also inhibited by the specific antiserum.

III.) *Hemolytic activity of freeze-thawed and untreated virus.*

As shown in preceding paragraphs the hemolytic activity of HVJ could be enhanced in several ways. On fractionating the virus by centrifugation, isolation of the free hemolytic components other than virus particles was unsuccessful. In the following section some comparative experiments are described to establish the identity of the "enhanced" hemolytic activity of the virus.

a) *The isoelectric point:* The treated and untreated virus preparations were diluted with distilled water and 5 ml aliquots were distributed in several tubes. A calculated volume of 1.5×10^{-3} M HCl was added to each tube to give pH values of from 4.0 to 7.2. The pH values were confirmed by Beckman model G pH meter. The tubes were kept cold for 24 hours and then 1 ml aliquots of the contents were mixed with 2 ml of red cells in PBS of pH 7.2 which contained 1.28 per cent NaCl to make the final reaction mixture isotonic. Then hemolysis measurements were made. At the same time hemagglutinin was titrated.

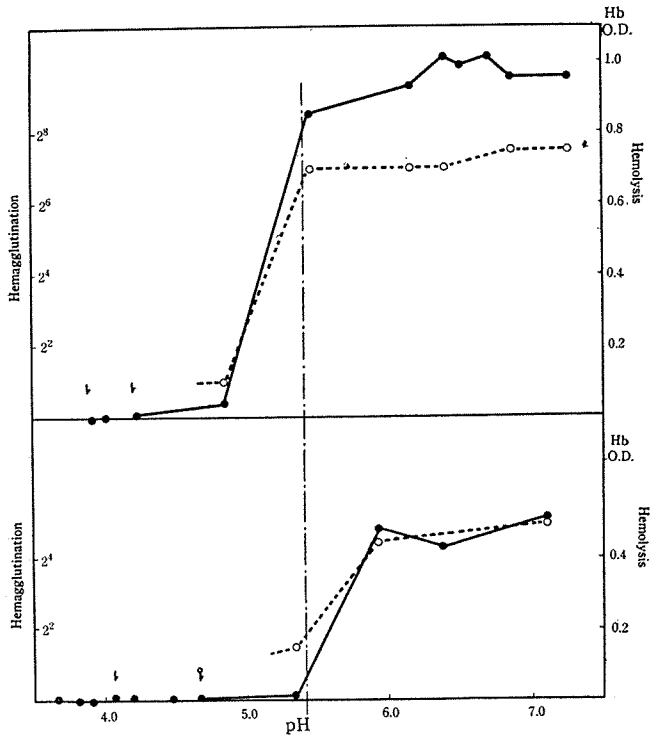


Fig. 6. Isoelectric point of freeze-thawed and untreated virus.
Aliquots of virus suspension were kept for 24 hours at various pH and then the pH at which the viral activities disappear was determined.
above: freeze-thawed virus below: untreated virus
solid line: hemolysis broken line: hemagglutination

The results are shown in Fig. 6. Above pH 5.5 hemagglutinating and hemolytic activities remained unchanged whereas the activities were not detectable at below pH 5.4.

This abrupt change in activities could be considered as due to an isoelectric point of the virus, though it is not so exact as the one determined physicochemically. From this experiment it may be concluded that the isoelectric point of the treated virus coincides with that of the untreated virus.

b) *Reaction rate:* A virus preparation was divided in two. One part was exposed to 10 cycles of freeze-thawing and the other control part was untreated. Appropriate dilutions of the treated virus and the untreated virus were prepared with PBS. One ml. aliquots of the diluted sample were distributed in a series of tubes. These aliquots were mixed with 2 ml. of 2 per cent red cells and placed in a water bath at 37°C after the adsorption had been established in cold. After various incubation periods the tubes were centrifuged and the optical density of the hemoglobin

liberated in the supernatant fluid were plotted against the incubation time.

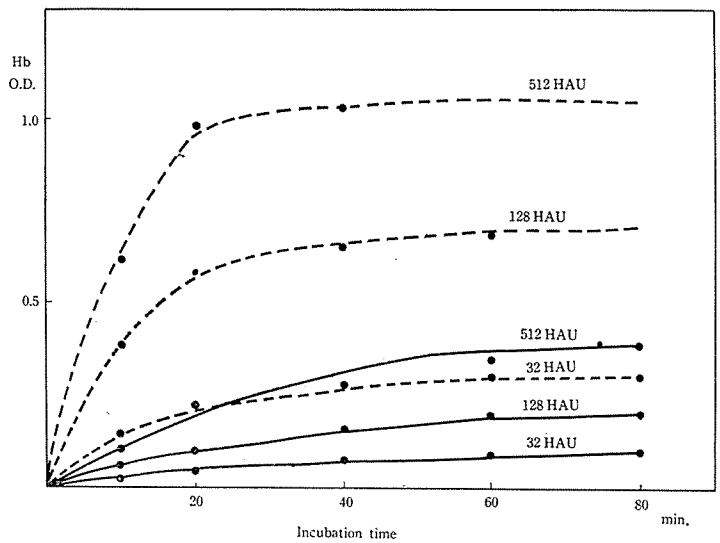


Fig. 7A. Comparison of the process of the hemolytic reaction of freeze-thawed and untreated virus.
broken line: freeze-thawed virus
solid line : untreated virus

The results are presented in Fig. 7A showing that liberation of hemoglobin by the treated virus is *more rapid* than that of the untreated virus at the early stage of the hemolytic reaction and *slower* in the following stages. The liberation of hemoglobin reached a plateau in 60 to 80 minutes; in 80 minutes the reaction reaches completion. Expressing the amount of hemoglobin liberated in 80 minutes "a" as the final product and assuming this value to be the total amount of hemoglobin, which was originally present inside the used red blood cells, and which was subsequently involved in the hemolytic reaction, a formula for the reaction rate can be applied to analysing the hemolytic reaction. If X is the hemoglobin liberated during reaction time t, then (a-X) is that which is liberated from time t to 80 minutes. When log (a-X) was plotted against time t, a linear correlation was observed. The results are shown in Fig. 7B. The lines for each virus are parallel and the rate constant of the freeze-thawed virus is a little higher than that of the untreated virus. The hemolytic reaction shown as the liberation of hemoglobin seems to be a first order reaction under these conditions.

c) *Rate of heat inactivation:* The virus was treated thermally to investigate heat inactivation of the hemolytic activity. Aliquots of a virus preparation were distributed in a number of ampoules which were sealed and placed in a water bath at 38°-45°C. At this temperature the hemagglutinating activity was unaffected. After various time intervals the grade of hemolysis by thermally treated virus was determined.

In the case of freeze-thawed virus heat inactivation of the hemolysis was shown

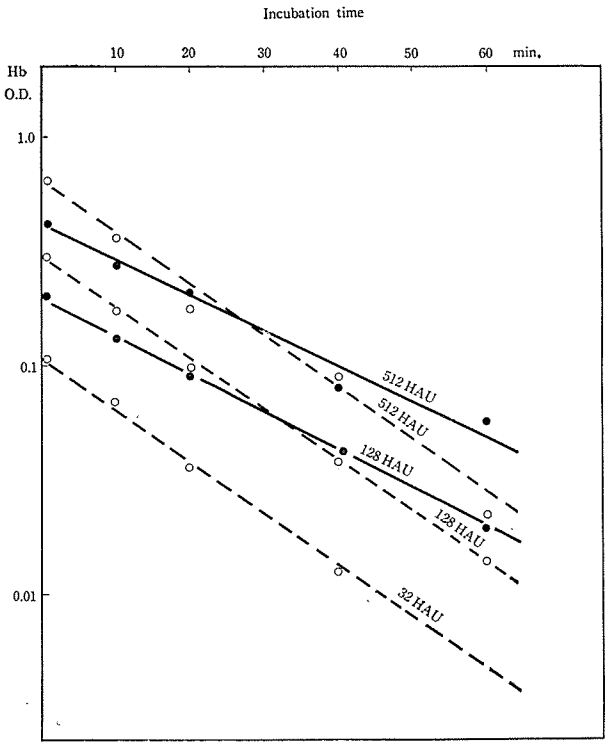


Fig. 7B. Comparison of relative reaction rate of the hemolytic reaction of freeze-thawed and untreated virus. Values of log (a-X) (refer to the text) were plotted against the incubation time.
broken line: freeze-thawed virus
solid line : untreated virus

to be a first order reaction while that of untreated virus had an initial shoulder, particularly apparent at 43°C, and then was linear. (Fig. 8.)

The activation energy of heat inactivation of hemolytic activity in both virus samples calculated from Arrhenius' equation was equal: 27.3 Kcal/mol. (Fig. 9).

d) *Eluting activity:* Aliquots of the treated and untreated virus preparations were distributed in several tubes and 2 per cent red blood cell suspension was added to each tube. After pretreatment in the cold to assure the complete adsorption of the virus onto the cells, all tubes were incubated at 37°C for the desired period. Then the tubes were centrifuged and the supernatants were tested for both liberation of hemoglobin and elution of hemagglutinin. The results are presented in Fig. 10.

The rate of the elution of the treated virus was slower than that of the untreated but its hemolysis was greater. When 0.1 M phosphate buffer was used as the reacting medium, some prolongation in the elution time was observed in comparison with that in PBS.

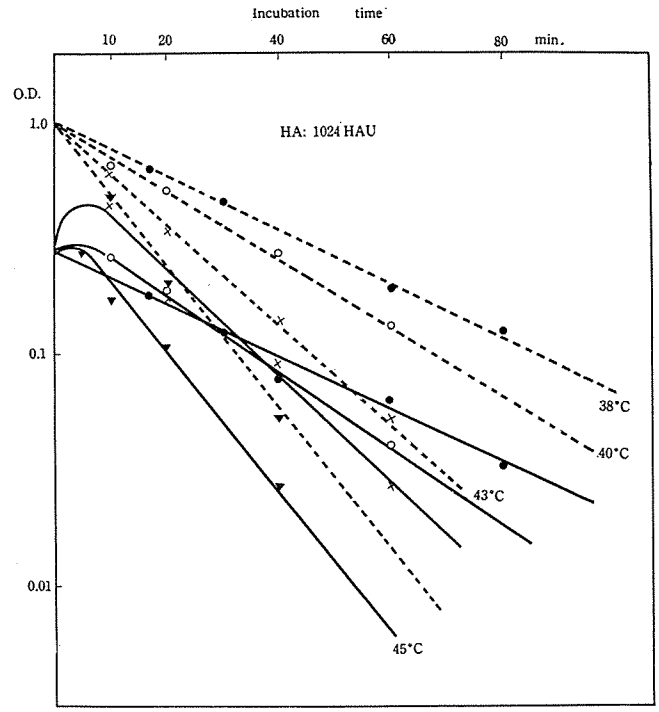


Fig. 8. Heat inactivation of hemolytic activity
Solid line: untreated virus,
Broken line: freeze-thawed virus

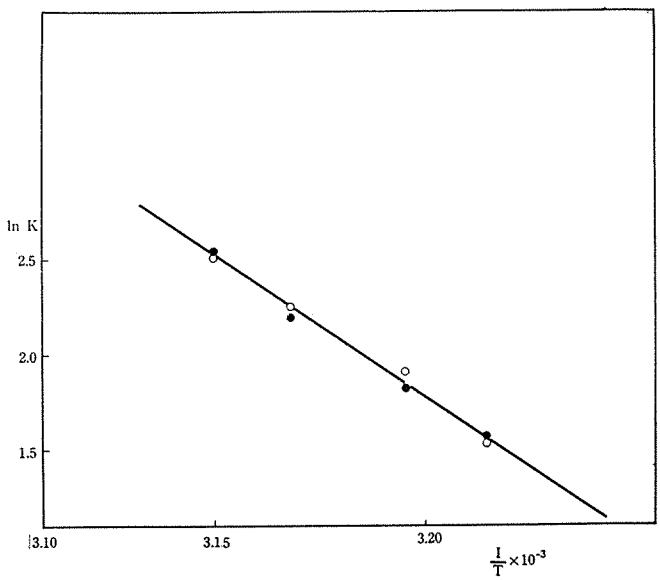


Fig. 9. Calculation of activation energy, K, of the heat inactivation reaction of the hemolytic activity of HVJ by Arrhenius' equation.
solid circle: untreated virus
open circle: freeze-thawed virus

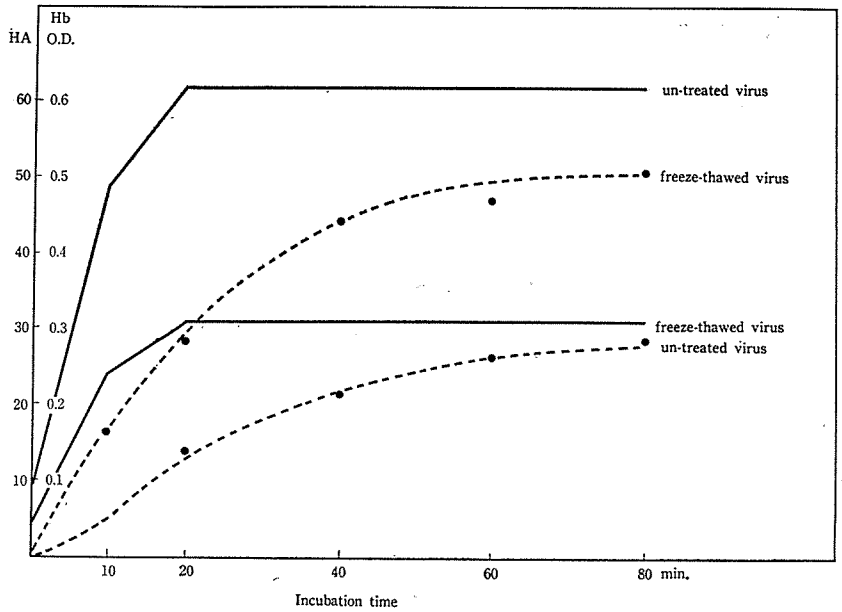


Fig. 10. Eluting activity of freeze-thawed and untreated virus.
solid line: hemagglutinin titer of eluted virus
broken line: liberated hemoglobin

IV. *Analysis of the mechanism of the hemolytic reaction*

In sections I, II and III the several properties of the hemolytic activity by HVJ were studied. In this section the mechanism of the reaction is studied.

a) *Optimal pH range for hemolysis and elution:* Two ml. aliquots of 2 per cent red cell suspension in saline were washed with 0.1 M phosphate buffer of various pH values (4.5-9.0) and resuspended in 2 ml. of the same buffer of the corresponding pH. Aliquots of concentrated virus were diluted in 10 volumes of 0.1 M phosphate buffers at corresponding pH values to those of the red cell suspension. Then 2 ml. red cell suspensions and 1 ml. of the diluted virus suspensions with the corresponding pH values were combined respectively. After adsorption at 4°C for 30 minutes the mixtures were placed in a 37°C water bath. After various times the tubes were centrifuged and hemolysis and eluted hemagglutinin in the supernatant were determined. The pH of the supernatant was measured in a Beckman model G pH meter after incubation.

As shown in Fig. 11 the optimal pH for elution is 6.0 to 7.8 and for hemolysis 6.3 to 7.5. The optimal range for hemolysis is somewhat narrower than for elution and this is more apparent when the determinations were carried out at the end of the reaction (60-80 minutes incubation). Around pH 6.0 and 7.8 the hemolytic activity is much depressed, but there was still elution of the virus. This depression of hemolysis does not mean the destruction of the hemolytic activity because it was recovered when the solutions were neutralized.

b) *The effect of antiserum:* After viral adsorption at 4°C for 30 minutes, the tubes

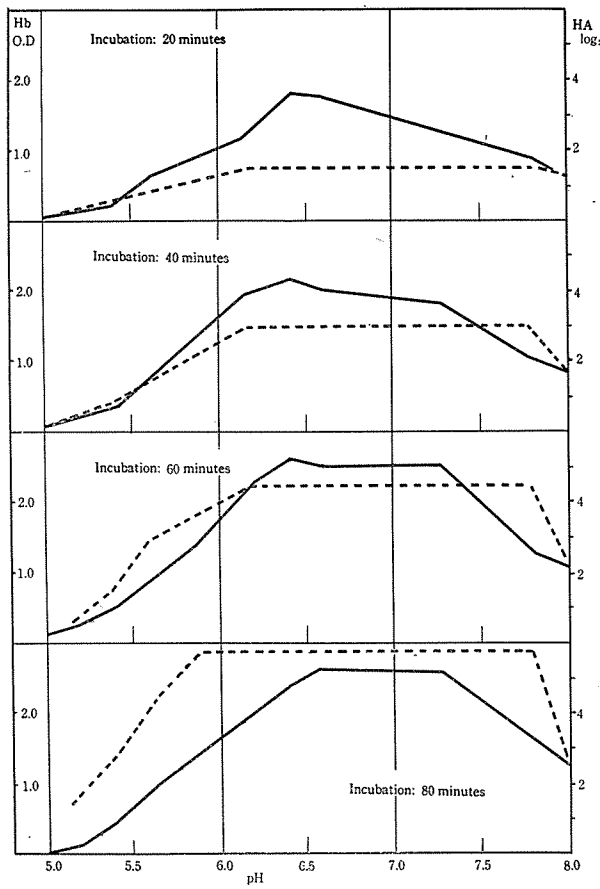


Fig. 11. Optimal pH range for hemolysis and elution.
The designated time indicates the incubation time in 0.1 M phosphate buffer.
solid line: hemolysis
broken line: elution

containing the virus-red cell mixture were placed in a water bath at 37°C. Immediately afterwards, and 6, 12 and 18 minutes later 0.5 ml of the antiserum enough to inhibit the virus hemagglutinin was added to each tube. After the same times of incubation, 0.5 ml of PBS was added to another series of control tubes. Fig. 12 indicates the results. Only when the antiserum was added at zero time was the hemolysis greatly suppressed; in other cases the serum was far less effective. On addition of serum after 12 minutes incubation the hemolytic reaction proceeded with no inhibition relative to the control.

Thus the hemolytic reaction could be differentiated in two stages. That is the initial stages (0-12 minutes) in which the reaction is sensitive to the inhibitory action of the antiserum and a later hemolytic stage in which the antiserum has no effect.

c) *The effect of low temperature:* By the same technique as used in the experiment described above at the various periods of incubation at 37°C, one group of tubes was kept in a 12-15°C bath and the other group of tubes in a 4°C bath. Control tubes were incubated at 37°C throughout the experiment. After 60 minutes and 120 minutes incubation hemolysis in each tube was compared with that in the controls. The results are presented in Fig. 13.

Tubes kept at low temperature from the start scarcely showed hemolysis. In the tubes which were kept at 12-15°C, after 6, 12 and 18 minutes of 37°C incubation, hemolysis proceeded slowly but steadily and reached a high value somewhat less than control, while the tubes at 4°C showed greatly inhibited hemolysis. These results suggest that there are two stages in the reaction: one the initial stage (0-6 minutes) in which the effect of low temperature is inhibitory

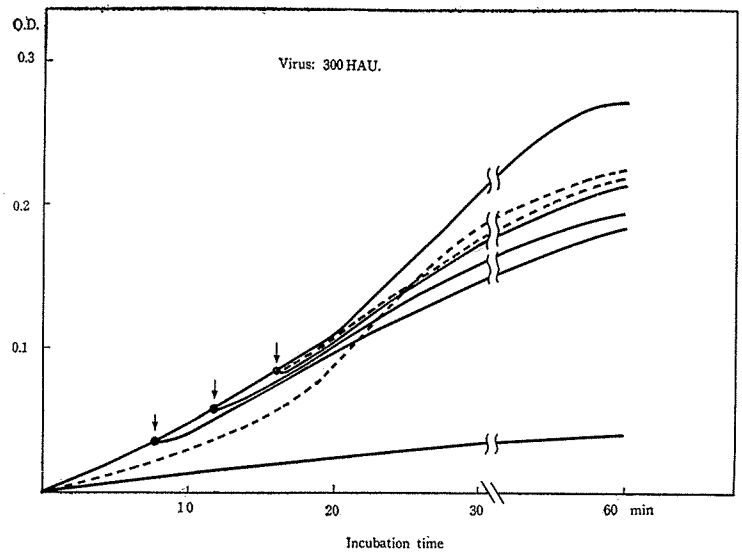


Fig. 12. Effect of anitserum on the process of the viral hemolysis.
The arrows indicate the addition of antiserum/or PBS into the hemolytic systems.
Solid line : process after the addition of antiserum
broken line : process after the addition of PBS (control)

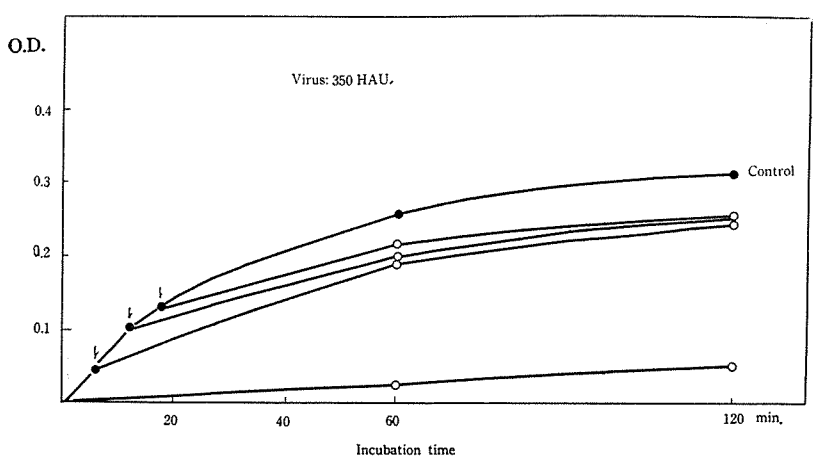


Fig. 13. Effect of low temperature on the process of the hemolysis.
The arrows indicate the time where the tubes containing the reaction mixture were transfered from a 37°C to a 12-15°C bath.

and the other, a later stage, hardly effected by the temperature. But this differentiation of the stages was not so clear cut as in the case of the antiserum effect.

Sato (1958) described similar effects of low temperature on the hemolytic reaction of HVJ.

d) *The effects of phospholipid etc.:* As shown by the experiments described in preceded paragraphs the hemolytic reaction is composed of two stages. This might reflect

two independent reactions: one enzymatic reaction, by which a hemolytic substance is produced, and the other a hemolytic reaction due to the product of the former reaction.

This suggestion is based on the fact that most of the adsorbed virus was eluted from red cells during the first 15-20 minutes of the incubation at 37°C while the hemolytic reaction reached a maximum after 60 minutes incubation. (Fig. 11) This lead to a comparison of HVJ and lecithinase hemolysis. Lecithinase is the only enzyme that has been studied as a causative agent of hemolysis.

It was reported that the hemolysis by lecithinase could be enhanced by the presence of leucocytes, phospholipid, and lecithin. (Turner, 1956 and Van Heyningen, 1954) The effect of these substances on HVJ hemolysis was studied. (Table 2)

Table 2. Effect of phospholipids and white cell fraction on the hemolysis of HVJ

virus	additions	Hemoglobin liberated *3 (O.D.)
256 HAU/0.5 ml	0.5 ml: PBS	.352
	0.5 ml: Phospholipids from saponin stroma*1	.588
256 HAU/0.5 ml	0.5 ml: PBS	.200
	0.5 ml: 2% white cell fraction	.152
512 HAU/0.5 ml	0.5 ml: PBS	.378
	0.5 ml: Phospholipids from red blood cells*2	.308

*1 5ml. of packed red cells were hemolysed with saponin. After washings with PBS crude phospholipid was extracted from the red cell stroma as ether-soluble aceton-insoluble fraction. (Lea et at. 1955) The phospholipid was emulsified in 5ml. of PBS.

*2 5ml. of packed red cells were hemolysed with distilled water containing a few amount of acetic acid. After washing with water, crude phospholipids were extracted from hemolysed cells as described in *1.

*3 Virus and additions were mixed and then 2ml. of 2 per cent red cells was added. After 60 minutes incubation at 37°C the liberated hemoglobin was determined as an index of hemolysis.

Phopholipid from the stroma of distilled-water-hemolysed red blood cells and the white fraction inhibited HVJ hemolysis but showed no effect on hemagglutinating activity. On the contrary the phospholipid extracted from the stroma of red cells hemolysed by saponin increased the hemolytic activity of HVJ. Because the phospholipid from saponin-treated stroma itself had some hemolytic activity when used in large quantity the latter finding can not be accepted without reservation.

e) *The effects of some chemical reagents:* Ethanol, ether, picric acid and formalin were diluted with PBS. These diluted reagents were mixed with the same volume of virus suspension and kept in the cold. At various intervals, aliquots were removed from each tube and diluted with 20 volumes of PBS to cut down the reaction. Hemolysis and hemagglutination were estimated on one ml of diluted virus. Another 1 ml of it was exposed to 10 cycles of freeze-thawing before measurements were made.

The reagents at the concentration used in this experiment had no effect on the hemagglutinating and the eluting activity of the virus. The hemolytic activity

blocked by this treatment could be largely recovered by freezing-thawing when the reaction was stopped within 4 hours, though no recovery was found after a long time.

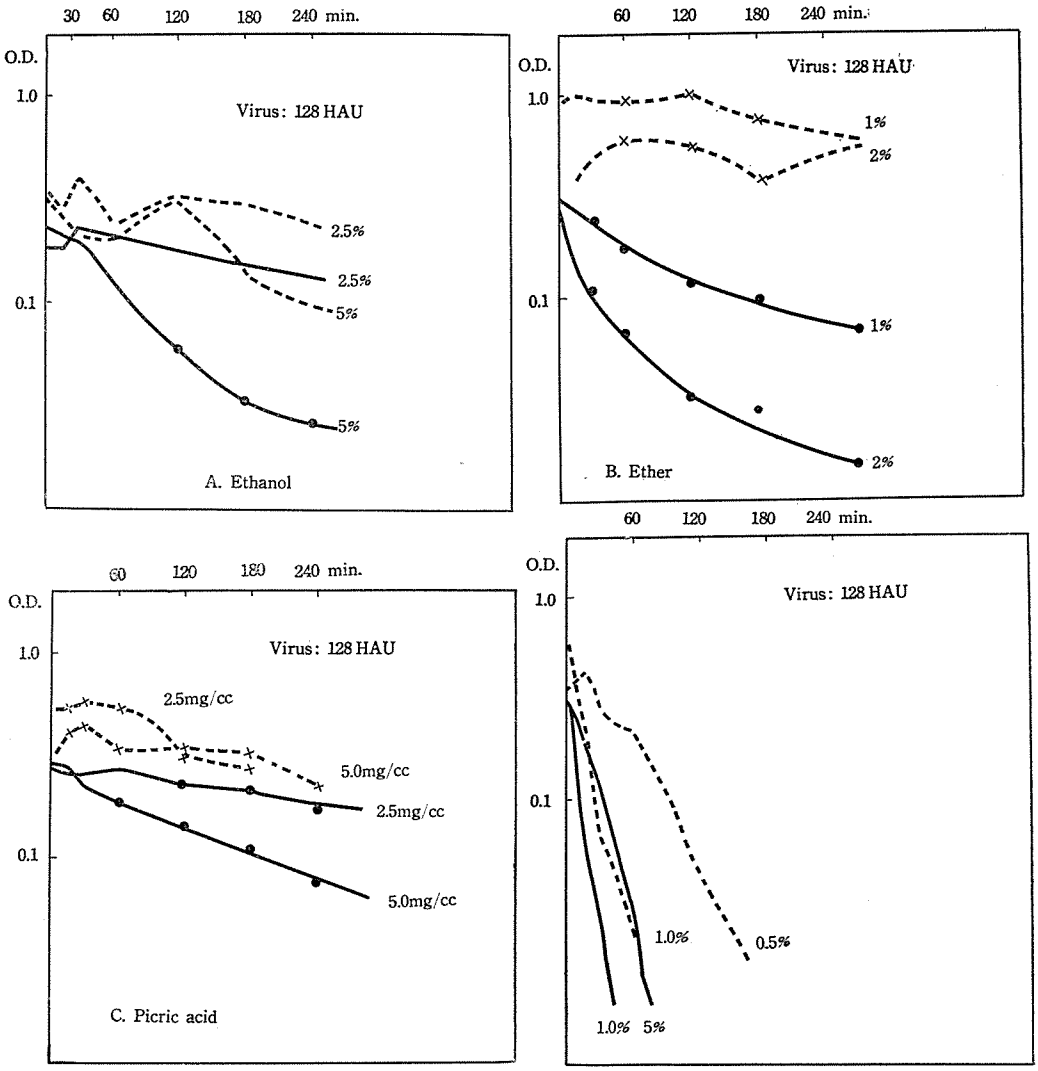


Fig. 14. Inactivation of the hemolytic activity by ethanol, ether, picric acid, and formalin and the recovery from inactive state after 10 cycles of freeze-thawing.
solid line : inactivation curve
broken line: recovery curve

DISCUSSION

The hemolytic activity of HVJ was easily enhanced by the several physical

agencies; freeze-thawing, sonic vibration, and changes in osmotic pressure. From fractionation experiments of freeze-thawed virus the enhancement of the hemolytic activity is considered to be due to an increase of the hemolysing capacity of individual virus particles, because these treatments produced neither hemolytic subunits nor split products from virus particles.

Chu and Morgan (1950a) and Granoff and Henle (1954) have already reported that the hemolytic activity of mumps virus and NDV could be enhanced by similar treatments. All these facts indicate that the hemolytic activities of these viruses have some common properties.

It is surprising that such a gentle treatment as "slow dilution" of virus suspension from hypertonic to isotonic conditions could enhanced the hemolytic activity more than more drastic treatments such as osmotic shock and glycerol was very effective in these series of experiments. Also the fact that broth prevented hemagglutinin from inactivation by freeze-thawing must be referred to the findings that broth protected the hemagglutinin from heat inactivation at 45°C. (Suzuki *et al.*, 1957) The author is inclined to consider these protective effects as due to the same mechanism.

In experiments to estimate the isoelectric point of the treated and untreated virus, though the method was indirect and rough, no significant difference was observed in the isoelectric points of two viruses.

HVJ hemolysis was found to be a first order reaction. Examinations of the hemolysis curve of hemolytic viruses by other investigators (Fukai and Suzuki, 1955, Burnet and Lind, 1950, and Granoff and Henle, 1954) revealed that these hemolytic reactions were all first order reactions when the data are arranged from the view of reaction rate analysis.

The reaction rate constant of the hemolysis of the virus treated by freeze-thawing was greater than that of untreated virus but the meaning for this difference is not yet known. The treated virus caused more rapid liberation of hemoglobin than the untreated virus at the early stages of the reaction.

The activation energy of heat inactivation of HVJ hemolysis was same, with or without freeze-thawing. This suggests that the mechanism of hemolysis by both viruses is the same. The value of this activation energy, (27.3 Kcal/mol.), is much smaller than that of heat inactivation of hemagglutinin, (83-340 Kcal/mol.), of the MNI group virus. (Woese, 1956) It is therefore concluded that the active group for hemolysis is different from that for hemagglutination.

Observation of the correlation between the development of the hemolysis and the elution showed that the hemolytic reaction required at least 60 minutes at 37°C to become maximal while the eluting reaction was completed within 15-20 minutes under the same conditions. HVJ of high hemolytic activity tended to be eluted more slowly than HVJ of low hemolytic activity. Therefore there is no direct correlation between hemolysis and elution.

The optimal pH range for the hemolysis of HVJ is somewhat wider, particularly on the acid side, than that for mumps virus determined by Chu and Morgan. The discrepancy in the optimal pH ranges for elution and hemolysis in the case of HVJ was not so marked as in the case of the mumps virus. (Chu

and Morgan, 1950b) These discrepancy might be due to the differences in the techniques used but it is possible to conclude from these experiments that the optimal pH range for hemolysis is different from that for the elution not only in mumps virus but also in HVJ.

Burnet and Lind (1950), in an investigation of the process of NDV hemolysis, described the initial stage and the hemolytic stage using RDE treatment and the effect of low temperature and stated that firmly adsorbed virus which could not be removed by RDE or antiserum would cause hemolysis and the firm irreversible chemical union was the initiating phase of a process leading to hemolysis. The effect of antiserum and low temperature on HVJ hemolysis seems to support these statements. It is possible to consider these two stages reflect to the two stages found in hemolytic reaction by lecithinase A.

Morimoto and Morgan (1954) reported on the inhibitory effects of lecithin, cholesterol, and cardiolipin on the mumps virus hemolysis. As described above the hemolysis of HVJ would also be inhibited by phospholipid fractions and this is not compatible with the assumption that the hemolytic agent of HVJ might be lecithinase. Furthermore there are some differences between the HVJ hemolytic agent and lecithinase. For example hemolysis of HVJ is inhibited by Ca ions (Fukai and Suzuki, 1955) while that of lecithinase is activated (Zeller, 1951) and the hemolytic activity of HVJ is inactivated by heating at 45°C while the activity of lecithinase is very heat stable. Morgan (1951) and Matsumoto and Yoshida (1957) discussed a comparison of the mumps virus and HVJ with lecithinase on the effects of inhibitors.

Thus for the identification of the hemolytic agent of HVJ, a detailed biological or chemical investigation, analogous to the analysis of the receptor destroying activity of the influenza viruses, is necessary.

Acknowledgements

The authors wish to thank Mr. Yoshimi Nishi and Miss Kazuko Kawai for their excellent technical assistance.

REFERENCES

Burnet, F. M., and Lind, P. E. (1950) Hemolysis by Newcastle Disease Virus. II. General character of hemolytic action. *Austral. J. Exp. Biol.*, **28**, 129–150.

Chu, L. W., and Morgan, H. R. (1950a) Studies on the hemolysis of red blood cells by mumps virus. I. The development of mumps virus hemolysin and its inactivation by certain physical and chemical agents. *J. Exp. Med.*, **91**, 393–404.

Chu, L. W., and Morgan, H. R. (1950b) Studies on the hemolysis of red blood cells by mumps virus. II. The relationships of hemagglutination, virus elution, and hemolysis. *J. Exp. Med.*, **91**, 406–415.

Fukae, S. (1958) Personal communication.

Fukai, K., and Suzuki, T. (1955) On the characteristics of a newly isolated hemagglutinating virus from mice. *Med. J. Osaka Univ.*, **6**, 1–15.

Granoff, A., and Henle, W. (1954) Studies on the hemolytic activity of Newcastle Disease Virus. *J. Immunol.*, **72**, 318–322.

Granoff, A. (1955) Noninfectious forms of Newcastle Disease and influenza viruses. *Virology*, **1**, 516–532.

Henle, G., Deinhort, F., and Girardi, A. (1954) Cytolytic effect of mumps virus in tissue cultures of epitherial cells. *Proc. Soc. Exp. Biol. and Med.*, **87**, 386-393.

Kim. (1958) Fusion of Ehrlich's tumor cells by N.D.V.. Personal communication.

Lea, C. H., Rhodes, D. N., and Stoll, R. D. (1955) Phospholipids 3. On the chromatographic separation of glycerophospholipids. *Biochem. J.*, **60**, 353-363.

Lowry, O. H., Rousebrouth, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275.

Matsumoto, T. and Yoshida, M. (1957) On the hemolytic activity of HVJ and its mutation. *Virus*, **7**, 446. (in Japanese)

Morgan, H.R., (1951) Factors interfering with the hemolytic activity of mumps virus. *Proc. Soc. Exp. Biol. and Med.*, **77**, 276-278.

Morimoto, M., and Morgan, H. R. (1954) Effect of lecithin, cholesterol, and cardiolipin on the mumps virus hemolysin. *Proc. Soc. Exp. Biol. and Med.*, **86**, 795-800.

Okada, Y., Suzuki, T., and Hosaka, Y. (1957) Interaction between influenza virus and Ehrlich's tumor cells. III. Fusion phenomenon of Ehrlich's tumor cells by the action of HVJ. Z strain. *Med. J. Osaka Univ.*, **7**, 709-717.

Salk, J. E. (1944) A simplified procedure for titrating hemagglutinating capacity of influenza virus and the corresponding antibody. *J. Immunol.*, **49**, 87-98.

Sato, S. (1958) On the hemolytic characteristics of Hemagglutinating Virus of Japan (Sendai Virus) X. *Virus*, **8**, 48-58. (in Japanese with English Summary).

Suzuki, T., Tadokoro, J., and Fukazawa, R. (1957) On the heat stability of both hemagglutinating and hemolytic activities of the Z virus. *Virus*, **7**, 291. (in Japanese with English Summary)

Tadokoro, J. (1959) Incomplete form of HVJ. (to be published)

Turner, J. C. (1956) On the relation of white blood cells and platelets to venom hemolysis. *J. Exp. Med.*, **104**, 517-523.

Van Heyningen, W. E. (1954) *The Proteins.* New York, Acadmic Press, 2A, Chapter 15.

von Magnus, P. (1951) Propagation of the PR8 strain of influenza A virus in chick embryo. 2. The formation of incomplete virus following inoculation of large doses of seed virus. *Acta Path. et Micro. Scand.*, **28**, 278-293.

Woese, C. R. (1956) Heat inactivation studies on animal viruses. I. The inactivation of virus hemagglutinin. *Arch. Biochem. and Biophys.*, **63**, 212-220.

Zeller, E. A. (1951) Phospholipase. *Enzyme* I (2), New York, Academic Press, 990.