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On the Hemolytic Activity of HVJ*
III. Further Analysis on the Decrease in the Cell Sphingomyelin Contents by HVJ and NDV

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SUMMARY

In further studies on the hemolysis by HVJ and NDV, the problem of whether a decrease of cell sphingomyelin contents is invariably associated with the viral hemolytic activity was studied.

1) When fowl red cells reacted with various myxoviruses, a decrease in the sphingomyelin content of the cells only occurred with the hemolytic viruses, HVJ and NDV, and not with non-hemolytic viruses, influenza viruses.

2) When human and guinea pig red cells were hemolysed by HVJ and NDV, there was no change in the sphingomyelin or other phospholipid contents of the cells.

3) A comparison of the kinetics of hemolysis and of sphingomyelin decrease in fowl red cells by HVJ and NDV showed that the hemolysis proceeded more slowly than the sphingomyelin decrease.

4) HVJ did not affect purified sphingomyelin isolated from fowl red blood cells.

5) No significant change of sphingomyelin content in Ehrlich ascites tumor cells was detected during the fusion and the cytolysis phenomena of the cells by HVJ.

INTRODUCTION

In the previous study (Hosaka, 1958b), it was found that a decrease of sphingomyelin in fowl red blood cells occurred with hemolysis of the cells by HVJ but not with simple adsorption and elution of HVJ without hemolysis. These results indicated a close relationship between the decrease in cell sphingomyelin and the hemolytic activity of HVJ in the case of the hemolysis of fowl red cells by the virus. It is interesting to see whether such a relationship is general in viral hemolysis. Thus a study was made of whether viral hemolysis was invariably associated with a similar decrease of cell sphingomyelin using various myxovirus-red cell systems. The decrease in cell sphingomyelin in the fusion and the cytolysis of Ehrlich ascites tumor cell, which was considered to be caused by the hemolytic activity of HVJ (Okada, 1958), was also examined.

The results are described in the present paper.

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*1 Synosym: Sendai virus

MATERIALS AND METHOD

1. *Virus*

Influenza virus: PR8 and NWS strains, HVJ: Z strain and NDV: Osaka strain were employed. Influenza viruses were inoculated into 11 day old developing eggs and incubated for 48 hours. HVJ and NDV were inoculated into 10 day old developing eggs and incubated for 72 and 48 hours respectively. Infected allantoic fluids were centrifuged at 22,000 rpm for 30 minutes in Spinco L ultracentrifuge with No. 30 after low speed centrifugation at 3,000 rpm for 30 minutes. The pellets were suspended in isotonic saline and centrifuged further at 3,000 rpm for 30 minutes. The supernatant was used for experiments.

2. *Red blood cells*

Fowl and guinea pig blood was withdrawn by cardiac puncture using sodium citrate as anti-coagulant. Human blood (type 0) was supplied from Nippon Blood Co. Ltd., Osaka. Red blood cells were washed 3 times with isotonic saline and suspended in the same solution.

3. *Ehrlich ascites tumor cells*

The cells were harvested 7–8 days after intraperitoneal transplantation, washed with Hanks' solution and suspended in Hanks' solution or Hanks' solution containing one tenth volume of mouse ascitic fluid (with 0.05-0.1 mg/ml heparin as anticoagulant).

4. *Determination of sphingomyelin*

The principle was according to the method of Sribney and Kennedy (1958) and is presented in Table 1.

Table 1. Determination of sphingomyelin

Reaction mixture (1 ml of virus + 3 ml of cell suspension)
↓ Extracted 3 times with 10 ml of methanol at 60°C for 5 minutes.
Combined methanol extract (ca. 30 ml)
↓ 8 ml of methanolic N-NaOH added and then hydrolysed at 37°C for 60 minutes.
Hydrolysate
↓ Cooled and neutralized with 4 ml of 2N HCl. 20 ml of chloroform and 30 ml of 2M KCl added, and mixed throughly and then centrifuged.
Chloroform layer
↓ Washed twice with 60 ml of 2M KCl and then with 60 ml of distilled water to remove water-soluble phosphoryl compounds and filtered.
Chloroform layer
Containing only alkali-stable lipids.

The phosphorus of alkaline-stable lipids in the final chloroform layer were regarded as sphingomyelin–P.

5. *Determination of phosphorus and nitrogen*

Phosphorus was determined by the method of Harris and Popat (1954) and nitrogen by the micro-Kjeldahl method.

6. *Paper chromatography*

Paperchromatography of phospholipids was carried out according to the method of Marinetti *et al.* (1957). The method was more suitable than that of Lea *et al.* (1955) which was used in the previous study (Hosaka, 1958b).

Twenty ml of red blood cell suspension in isotonic saline containing 3 ml of the packed cells was mixed with each 3 ml of HVJ and NDV (20,000 HA/ml) and incubated at 37°C for 60 minutes. Then the red cells were washed 3 times with isotonic saline. Most of the virus was removed during the washing because it had already been eluted from the red cells during incubation at 37°C. The lipids was extracted from the sedimented red cells 3 times with 10 ml of methanol-chloroform (5:1, v/v) at 60°C and the combined extracts were evaporated *in vacuo* at 55°C. The residues were dissolved in chloroform, evaporated *in vacuo* and then taken up in 0.1 ml of isoamyl alchol-benzene (1:1, v/v).

Five μl of each sample was applied to paper (Toyo No. 51).

The relative amounts of each phospholipid was determined by the area of color development with Rhodamine 6G and choline reagents (Marinetti *et al.*, 1957). Identification of phospholids by chemical reagents was according to the method described in the previous report (Hosaka, 1958b).

7. *Isolation and purification of sphingomyelin*

The sediment of the hemolysate of 300 ml of fowl red blood sells obtained by adding 3 1 of distilled water containing several drops of acetic acid was washed several times with distilled water and finally with cold acetone to remove non-phospholipid material. The phospholipids of the stroma were successively extracted 3 times at 40°C with 100 ml and twice with 50 ml of ether-methanol (1:1, v/v) in a Waring blender. The combined extract was evaporated to dryness *in vacuo* and ether was added to the residue. The solution was kept in the cold overnight. The precipitate formed was separated by centrifugation.

Sphingomyelin was purified from the ether-insoluble phospholipid by Rapport and Lerner's method (1958), but the last precipitation steps were replaced by a single precipitation with ethylacetate-methanol (10:1, v/v). The sphingomyelin thus obtained gave a spot on a paperchromatogram and the N/P ratio of it was 1.95.

RESULTS

1. *Reaction of fowl red blood cells with HVJ, NDV and Influenza virus*

In the previous study, it was found that the hemolysis of fowl red cells by HVJ resulted in a decrease of sphingomyelin in the cells. To ascertain the close

Table 2. Change in sphingomyelin content*1 with Influenza virus, HVJ and NDV

		inactivated virus*2	active virus	Change in sphingomyelin content
Influenza virus	PR8	13.0μg	13.5μg	−
	NWS	10.5	11.0	−
HVJ		16.5	12.0	+
NDV		17.0	14.0	+

*1 After incubation at 37°C for 60 minutes, the total sphingomyelin content of both reaction mixtures of each virus were determined.

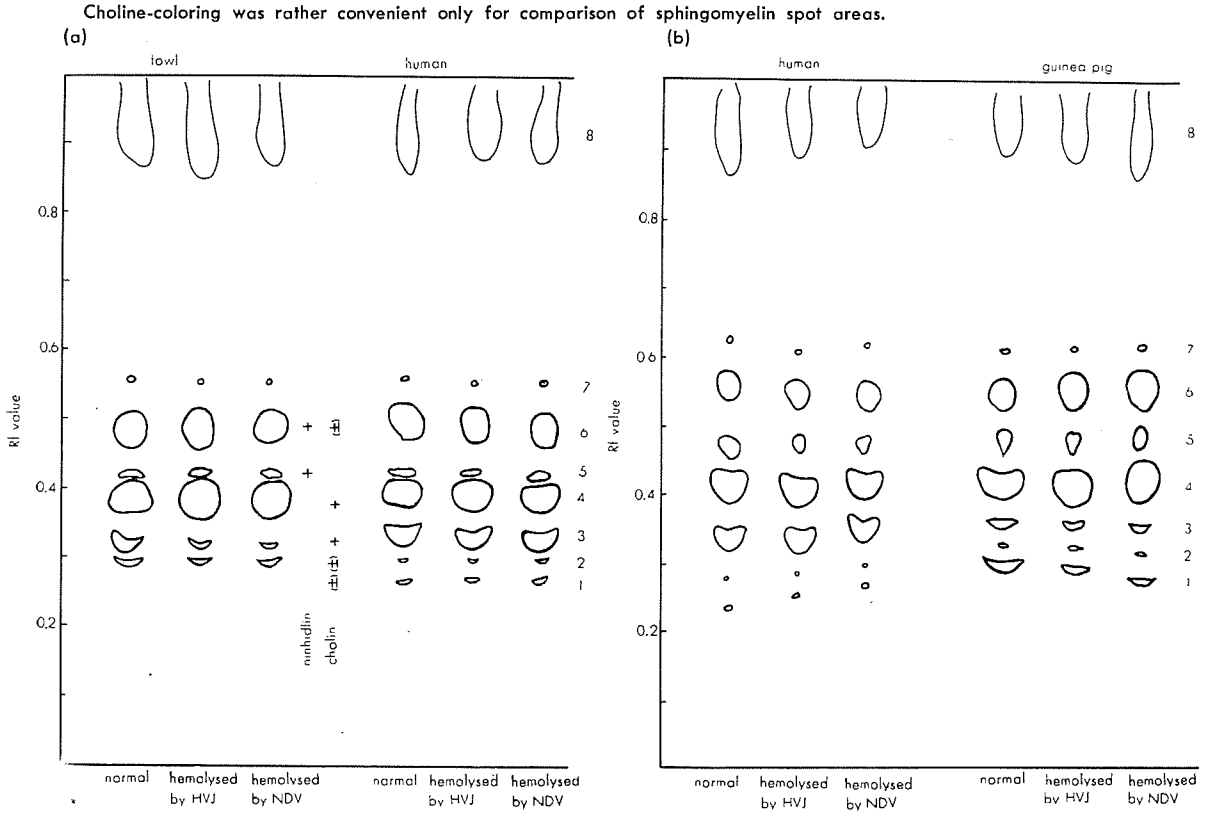
*2 Inactive virus was heat-treated one at 60°C for 15 minutes. Since the inactivated virus had no effect on cell phospholipids, it was used as a control.

relationship of viral hemolysis to the decrease of sphingomyelin in fowl red cells, a study was made of whether such a decrease was observed only in the reaction of the fowl red cells with the hemolytic myxoviruses, HVJ and NDV, or whether even the non-hemolytic myxoviruses, Influenza viruses produced a similar effect.

One volume of packed fowl red cells was suspended in two volumes of isotonic saline. The pH was adjusted to 7.0 with Tris buffer. Three ml of the suspension were mixed with 1 ml of each virus preparation (20,000 HA/ml) and as controls virus heated at 60°C for 15 minutes were used. After incubation at 37°C for 60 minutes, the total sphingomyelin content of the reaction mixtures of the active and the heat inactivated viruses were compared.

The results are presented in Table 2. No significant change of sphingomyelin content was found in the reaction of fowl red cells with Influenza viruses while a considerable decrease was found with HVJ and NDV. Similar results were obtained with these viruses in reactions with the stroma of fowl red cells prepared

Fig. 1. Paperchromatograms of phospholipids of fowl, human and guinea pig red cells hemolysed by HVJ and NDV. Chromatography was carried out at 23°C on silicic acid- impregnated paper. The solvent was diisobutylketone : acetic acid : water (40 : 25 : 5). (Marinetti *et al.*, 1957)
The spots were stained with Rhodamine 6G and were all yellow except spot 5 (purple). The identity of the spots are as follows; 1, 2, inositol-phosphatide (tentative). 3, sphingomyelin, 4, lecithin, 5, phosphatidylserine, 6, phosphatidylethanolamine, 7, unidentified, 8, non-phosphatids.
Choline-coloring was rather convenient only for comparison of sphingomyelin spot areas.



in distilled water. Thus it was shown that the decrease of sphingomyelin in fowl red cells was caused only by the hemolytic viruses of myxovirus.

2. *Hemolysis of human and guinea pig red cells by HVJ and NDV*

In our experiences, the hemolysis of human and guinea pig red cells by HVJ appears greater than that of fowl red cells.

Burnet and Lind (1960) and Chu and Morgan (1950), employing NDV and mumps virus respectively, reported that the viral hemolytic titers of red blood cells from various animals differed depending upon the species of the animals used. Thus it was very interesting to examine whether the hemolysis of red cells of other species by HVJ and NDV also results in a decrease of the cell sphingomyelin and, if it is so, whether the extent of the decrease was dependent on the intensity of viral hemolysis.

The phospholipids of human, guinea pig and fowl red cells hemolysed by HVJ and NDV and also the phospholipids of untreated red cells were compared by paperchromatogram.

The results are shown in Fig. la and lb. The areas of the spots of sphingomyelin of fowl red cells hemolysed by HVJ and NDV are much less than those of untreated fowl red cells but unexpectedly those of human and guinea pig red cells hemolysed by HVJ and NDV show no significant differences from those of untreated red cells. There was no significant change in other phospholipid content of hemolysed and untreated red cells of the 3 species. The extent of the hemolysis of the human and guinea pig red cells was so great that the red cells became white, while the hemolysed fowl red cells remained still a little reddish.

Further it was shown that the composition and the contents of phospholipids of red cells differed from species to species; thus human red cells had only a little inositol phosphatide 1 and 2, guinea pig red cells contained a little sphingomylin but much inositol phosphatide 1 and fowl red cells contained a little inositol phosphatide 1.

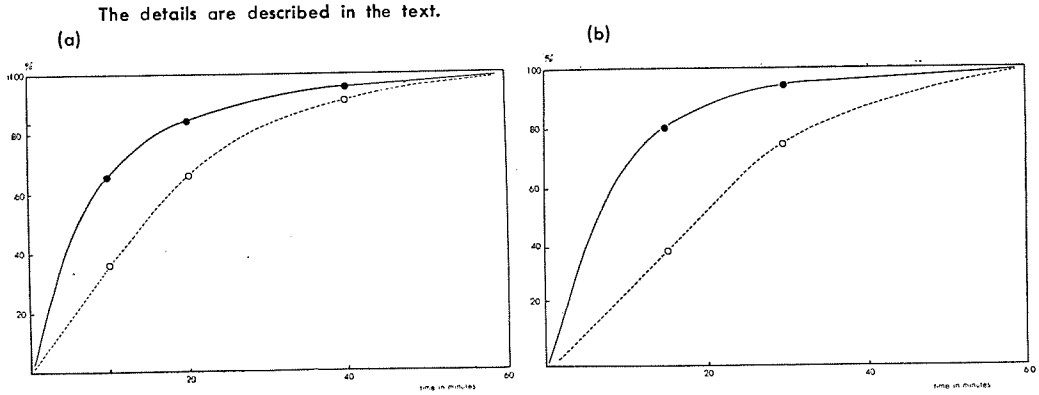
Our finding that guinea pig red cells have a little sphingomyelin is consistent with the report of Turner *et al.* (1959).

3. *Comparison of the kinetics of hemolysis and sphingomyelin decrease in fowl red cells by HVJ and NDV*

In the previous study (Hosaka, 1958b), a comparison was made of the kinetics of hemolysis and sphingomyelin decrease of fowl red cells by HVJ. It was found that the latter proceeded more slowly than the former. Thus, as illustrated in Fig. 2a, if the per cent hemolysis and the per cent decrease of sphingomyelin are plotted against incubation time at 37°C, it is clear that there is a considerable difference between hemolysis and sphingomylin decrease and the latter reaction proceeds more slowly than the former.

The kinetics of these two reactions of fowl red cells by NDV was also compared. The reaction mixture consisted of 3 ml of a suspension of fowl red cells in isotonic saline containing 1 ml of the packed cells and of 1 ml of NDV (20,000 HA /ml). After various periods of incubation at 37°C, the hemolysis and the decrease

Fig. 2. Comparison of kinetics of hemolysis ond sphingomyelin decrease of fowl red cells by HVJ (a) and NDV (b).
solid line : hemolysis
broken line : sphingomyelin decrease
The details are described in the text.



in sphingomyelin were determined. In Fig. 2b, the per cent hemolysis and the per cent decrease of sphingomyelin are plotted against the incubation time. As seen in the figure, a similar difference between the two reactions was also observed in this virus-red cell system.

Table 3. Effects of various conditions on the interactions of isolated sphingomyelin with HVJ.

	HVJ	sphingomyelin	additions
Exp. 1	2 ml of 20,000 HA/ml in 0.4% saline	2 ml containing 1 μM-P in distilled water	none
Exp. 2 effect of Ca or Mg ion	〃	〃	2.0～40μM of Ca or Mg ion Tris buffer, pH 7.0
Exp. 3 effect of serum albumin	〃	〃	bovine serum albumin 2 mg. 2.0～40 μM of Ca or Mg ion Tris buffer, pH 7.0
Exp. 4 effect of surface active agents	〃	〃	Emasol*1 1130, 10～20 mg or Emasol 4130, 10～20 mg or both, 10～20 mg Tris buffer, pH 7.0
Exp. 5 effect of CMP*2	〃	〃	CMP 1μM $MgCl_2$ 1μM others as in Exp. 4

*1 Emasol was kindly supplied from KAO Soap Co., Ltd.
Emasol 1130 and 4130 are surface active agents like Tween 20 and 80 respectively.

*2 CMP : Cytidine monophosphate
Exp. 3 and Exp. 5 were referenced for lipoprotein lipase (Korn, 1955) and ceramide-phosphorylchline transferase (Sribney and Kennedy, 1959) respectively.

4. *Effect of HVJ on purified sphingomyelin*

To study the relation between the viral hemolysis and decrease of cell sphingomyelin further, it is necessary to see whether HVJ or NDV can directly attack sphingomyelin. Therfore purified sphingomyelin was mixed with HVJ and, also with the virus inactivated by heating at 60°C for 15 minutes. Both test and control mixtures were incubated at 37°C for 60 minutes under the varions conditions shown in Table 3.

After incubation, cold perchloric acid was added to the reaction mixture to a final concentration of 5 per cent and the phosphorus and nitrogen contents of the perchloric acid soluble fraction were determined. In another series of larger scale experiments, the phospholipids in the reaction mixture were compared to the those of the control by paperchromatography. In no experiments, a significant change was detected from control. Therefore it seems that HVJ is unable to attack sphingomyelin itself directly under these conditions.

5. *Sphingomyelin content of Ehrlich ascites tumor cells during the fusion and cytolysis of the cells by HVJ*

Okada (1958) reported that on incubation of Ehrlich ascites tumor cells with HVJ, fusion and lysis of the cells occurred *in vitro* with and without shaking respectively and the hemolytic activity of HVJ seemed to be responsible for the fusion and cytolysis. Therefore it is interesting to investigate whether the change in cell sphingomyelin contents occurs during fusion and cytolysis by HVJ.

It was observed that fused and lysed cells appeared simultaneously, in various proportions to one another, depending upon such factors as the mode and speed of shaking, the incubation medium and the extent of the hemolytic activity of the

Table 4. Fusion and lysis of Ehrlich ascites tumor cells by HVJ under various conditions #

virus	medium of cell suspension	conditions of shaking		
		shaking *2	moderate *3 shaking	no shaking
native virus	ascitic solution *1	F ###	F ##	F + L +
	Hanks' solution	F ###	F ## L +	L ##
freeze-thawed § virus	ascitic solution *1	F ###	F ## L +	L ###
	Hanks' solution	F ### L +	F + L ##	L ###

F and L are indication of fusion and lysis, respectively.

*1 Hanks' solution containing one tenth volume of mouse ascitic fluids.

*2 Centrifuge tubes were pitched up and down 50—60 times/minutes.

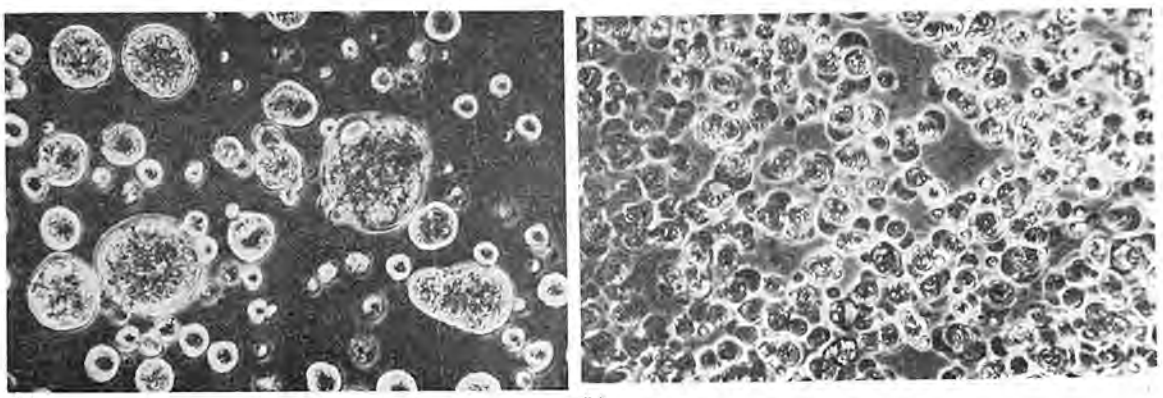
*3 Centrifuge tubes were shaken 50—60 times/minutes vertically.

1 ml of HVJ (20,000 HA/ml) was mixed with 2 ml of the tumor cells ($4-7\times10^7$ cells/ml) and the deformation of the cells was observed microscopically after incubation at 37°C for 60 minutes.

§ The hemolytic activity of HVJ was increased by freezing-thawing (Hosaka, 1058a).

virus. Therefore at first the conditions, in which either fusion or cytolysis occurred uniformly, had to be determined in order to get clear cut results.

The results of this study are shown in Table 4. The fusion and lysis of the tumor cells occurred almost uniformly when cells suspended in Hanks' medium



(a) (b)

Fig. 3. Fusion (a) and lysis (b) of Ehrlich ascites tumor cells by HVJ. The virus was treated with 3 cycles of freezing-thawing. The tumor cells were suspended in Hanks' solution containing one tenth ascitic fluid and incubated at 37°C for 60 minutes with, (a) and without (b) shaking.

containing one tenth volume of ascitic fluid were incubated with the frozen-thawed virus (Hosaka, 1958a) with and without shaking, shown in the Fig. 3 a and b. After such a uniform fusion and lysis was at 37°C for 60 minutes, the sphingomyelin content of the reaction mixture was determined. As no quantitative method for preparation of cell membranes was available, the determination of the sphingomyelin content of the cellmembrane was not attempted.

Table 5. Comparison of sphingomyelin contents* of Ehrlich ascites tumor cells fused and lysed by HVJ

control	fused cells	lysed cells
100.0±8.4	102.0±7.4	104.3±9.8

control : Virus inactivated at 60°C for 15 minutes.

* The total sphingomyelin content of the reaction mixtures were determined. The reaction mixture consisted of 1 ml of HVJ (20,000 HA/ml) and 2 ml of the tumor cell ($4-7\times10^7$ cells/ml). Values presented are average of 15 experiments.

The results are presented in Table 5. As seen in the Table, no significant change in sphingomyelin contents was found.

DISCUSSION

In further studies on the hemolysis by HVJ and NDV, it has been found that there is another type of hemolysis which is not accompanied by a decrease

in the sphigomyelin content of the red cells. Therefore it is clear that a decrease of sphingomyelin is not essential for viral hemolysis. However during viral hemolysis of fowl red cells, the hemolytic activity of the viruses has been shown to be closely related to the degradation of sphingomyelin. Thus it is interesting to consider here what differences between the red cells are responsible for the presence or absence of cell sphingomyelin decrease on the viral hemolysis, although it is at present impossible to answer to this question definitely.

Burnet and Lind (1950) mentioned the following facts in connection with the difference in hemolysis by NDV of human and fowl red cells; 1) with human cells, the initial phase of hemolysis was more rapid with only a slow subsequent incrense in hemolysis and 2) hemolysis of fowl red cells was more strongly inhibited by the presence of Ca ions. However these differences give no explanation of the presence or absence of degradation of sphingomyelin.

If it is assumed that the cell sphingomyelin is degraded by an enzymatic reaction, the following possiblities are considered. One possibility is that differences in the structure of the phospholipid or phospholipid-protein complex of red cells as substrate for a viral enzyme might be related to the presence or absence of the sphingomyelin decrease. Another possibility is that, if the enzyme is in the red cells, its presence or absence, or a difference in its localization might account for the phenomenon.

Further it may be significant to consider the possibility that the absence and presence of viral degradation of sphingomyelin might be differentiated between hemolysis of nucleated and non-nucleated red cells by the viruses. However further study is required to answer these questions.

It was shown in the preceeding section that HVJ can not attack sphingomyelin isolated from fowl red cells. Therefore more complex mechanism on the decrease of sphingomyelin of fowl red cells must be considered.

Mobery *et al.* (1958) reported an alteration in the sphingomyelin content of fowl red cells in the hemolysis of the cells by mumps virus and Soule *et al.* (1959) reported no change in sphingomyelin content in the hemolysis of human red cells by mumps virus. Therefore the hemolytic activities of HVJ, NDV and mumps virus appear to be due to a common agent which is absent in Influenza viruses. Furthermore these viruses are different from Influenza virus in morphology (Bang, 1948; Fukai *et al.*, 1955) and in their inability to form an "imcomplete virus" (Granoff, 1955; Tadokoro, 1958 and Fukae, 1958). From all these findings it seems reasonable to classify these viruses in another subgroup of myxoviruses.

The interactions of HVJ with Ehrlich tumor cells resulted in the fusion and lysis of the cells but no change in sphingomyelin content of the cells. It seems that in the fusion and lysis of the cells by HVJ, a lytic action of HVJ on the cell membrane with no effect on cell sphingomyelin was also occuring as in the hemolysis of human and guinea pig red cells by HVJ. However further study will be required to determine how the fusing and the lytic activities of HVJ are related to its hemolytic activity.

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