BIKEN'S JOURNAL 1, 90-99 (1958)

On the Hemolytic Activity of HVJ.[*1, 2]

II. Decrease of Sphingomyelin in Red Blood Cells Hemolysed by HVJ.

YASUHIRO HOSAKA

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

(Received for publication, October 31, 1958)

SUMMARY

Paper chromatography of the phospholipids extracted from red cells hemolysed by virus showed a decrease in concentration of sphingomyelin as compared to that in the control. Further by quantitative chemical analysis a decrease of sphingomyelin in the virus-hemolysed red cells was shown. This decrease was not caused by liberation of sphingomyelin from the cells, but by a decomposition of the substance. As lecithin and cephalin are concerned any change was not able to be detected. This change in the cells seems to be correlated only with the hemolytic activity of the virus.

INTRODUCTION

In the previous report (Hosaka 1958) differences between the hemolytic activities of HVJ and lecithinase were discussed.

While the optimal pH range for the reaction and the presence of two stages in the reaction are similar for HVJ and lecithinase, discrepancies have been found in their heat stability, and in the effect of Ca ions and phospholipids on the hemolysis.

As discussed in the previous paper to investigate whether the hemolytic activity of HVJ is the same as that of lecithinase it is necessary to determine the alteration of phospholipid components in the red cells hemolysed by HVJ. While the author's studies were in progress Moberly *et al.* (1958) reported an alteration of "a component of erythrocytes similar to sphingomyelin" in the hemolytic reaction of mumps virus. The present results with HVJ are consistent with their finding. The amount of altered sphingomyelin in red cells was also quantitatively determined.

Materials and Methods

Virus: HVJ, Z strain, was used. Purification of the virus was carried out

*1 Synonyms: *Mixovirus parainfluenzae I,* Sendai virus.

*2 Supported by a Grant in Aid of the Advance of Scientific Research from the Ministry of the Education of the Japanese Government.

as described in the previous report (Hosaka 1958).

Hemagglutination test: A pattern method was employed using 0.5 ml. of 0.5 per cent red cells (Hosaka 1958).

Red blood cells: Blood was withdrawn by cardiac puncture of roosters and centrifuged at 300×g for 5 minutes. The packed cells were washed 3 times with isotonic saline (pH 6.8) and then resuspended in the same solution at a concentration of 10 per cent for the hemolysis test, or in phosphate buffered saline (pH 7.2) at a concentration of 0.5 per cent for the hemagglutination test.

Lipid extracts for paper chromatography: Sixty ml. of 10 per cent red cells were mixed with 24 ml. of the virus preparation containing 20,000 HAU/ml. and incubated at 37°C for 2 hours. Then the reaction mixture was centrifuged at 1200×g for 10 minutes. The sediment was used for step (a) and the supernatant fluid was used for steps (b) and (c) as follows.

a) *Extraction of phospholipids from hemolysed red cells:* Hemolysed cells were treated with 10 volumes of acetone. After drying, the material was extracted with 20 ml. of chloroform-methanol mixture (1 : 1, v/v) at 50°C for 10 minutes and then centrifuged at 1200×g for 10 minutes. The sediment was further extracted with 10 ml. of chloroform-methanol. The first and second extracts were combined and the solvent was evaporated to dryness at 45°C under reduced pressure. The residue was dissolved in 10 ml. of chloroform-methanol and the solvent was re-evaporated *in vacuo*. This process was repeated. The final residue was dissolved in 1 ml. of chloroform-methanol. As a control, 60 ml. of 10 per cent normal red cells were incubated at 37°C for 2 hours and then treated in a similar manner.

b) *Extraction of phospholipids from eluted virus:* The supernatant fluid of the above mentioned hemolytic reaction mixture was further centrifuged at 44,000×g for 30 minutes. The pellet, considered as the eluted virus fraction, was washed with acetone and extracted twice with 10 ml. of chloroform-methanol (1 : 1, v/v) at 50°C for 10 minutes. The solvent was removed from the extract *in vacuo* and the residue was dissolved in chloroform. The chloroform was evaporated and the final residue was taken up in 0.5 ml. of chloroform-methanol.

As a control, phospholipid was similarly extracted from the concentrated purified virus (40,000 HAU/ml.) without incubation with red blood cells.

c) *Extraction of phospholipid from the supernatant of high speed centrifugation:* The supernatant of high speed centrifugation in step (b) was precipitated with 10 per cent trichlor acetic acid (TCA) and the phospholipid in the precipitate was extracted in the same manner as that of red cells.

Paper chromatography: Paper chromatography was carried out on the silica-impregnated paper (TOYO No. 51), using methanol-chloroform water (10 : 30 : 1, v/v) as developer, according to the method of Lea *et al.* (1955). Six to ten microlitres of the sample containing about 0.2 micromoles of phospholipid were applied to the origin. The chromatography was run at 18-20°C for 2 to 3 hours until the solvent front had travelled down about 20-25 cm. After development, the papers were air-dried and then washed with running water for 10

minutes to remove certain water-soluble substances (Rouser *et al.*, 1956) and then dried.

The method used for detection was the same as that of Lea *et al.* (1955); the strips, sprayed on both sides with 0.5 per cent ninhydrin in *n*-butanol were heated at 100°C for 5 to 10 minutes and the spots containing free amino group were marked. Then the papers were treated with the phosphomolybdate $SnCl_2$ reagent for the detection of choline-containing substances (Levine and Chargaff, 1951). Sphingomyelin was demonstrated by the presence of a chloroform-soluble, ninhydrin reacting base (sphingosine) in acid hydrolysates of the eluate from the corresponding spots (McKibbin, 1949).

Though the mobilities were not compared with those of standard compounds, because no sufficiently pure standards were available, the analytical detection and a comparison of Rf values with Lea's data (1955) permit the identification of the phospholipids. While Rf values of each substance were variable, separation of spots was clear (Lea *et al.*, 1955, Marinetti and Stotz, 1956, Turner, 1957). Sometimes difficulties were encountered in separating cephalin and lecithin but these were not critical. The relative amounts of phospholipids were estimated by comparing the colored areas of corresponding spots in normal and hemolysed samples.

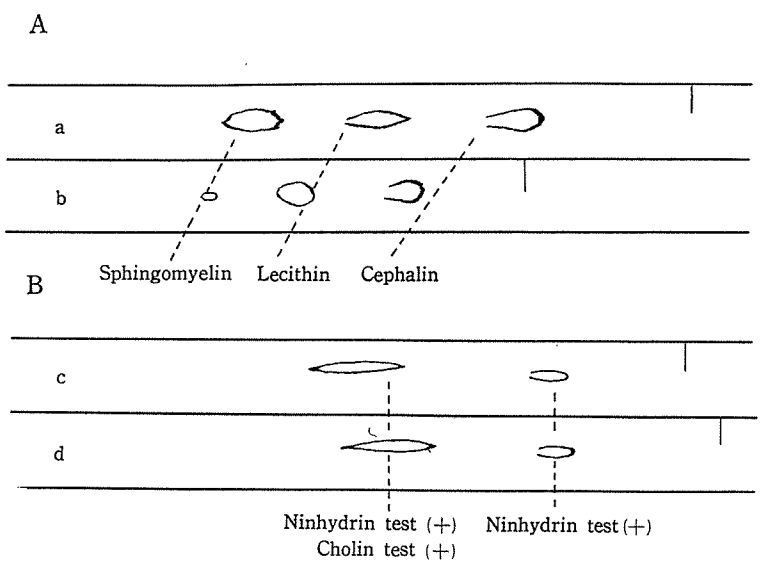


Fig. 1. A. Paper chromatograms of Phospholipids extracted from fowl red blood cells.
a. Normal red blood cells
b. Virus-hemolysed red blood cells

B. Paper chromatograms of Phospholipids extracted from HVJ.
c. Before adsorption-elution
d. After adsorption-elution
Developing solvent: Methanol-chloroform-water mixture (10: 30: 1, v/v) at 20°C.

RESULTS

Paper chromatography of the phospholipids from virus-hemolysed red blood cells.

Fig. 1 shows chromatograms of the phospholipid fractions from intact and hemolysed red cells. No difference was observed in the amount of lecithin and cephalin of control and hemolysed red cells but a marked decrease in the size of sphingomyelin spot was observed in the hemolysed sample. A spot correspoinding to lysolecithin was not detectable. No difference was found in the chromatograms of phospholipids extracted from the virus itself before and after the adsorption-elution cycle. There is a possibility that HVJ has special phospholipids but at present no detail is available on this point.

In the supernatant fluid from high speed centrifugation no phosphatides were detected by paper chromatography. Therefore the decrease of sphingomyelin in the red cells was probably due to decomposition of the substance of the cells and was not to liberation of sphingomyelin containing substances from the cells.

Quantitative analysis of the phospholipids in red cells during the hemolytic reaction:

i) *Method for the simultaneous determination of hemolysis and the sphingomyelin content.*

a) Five ml of HVJ suspension containing 8,000 HAU/ml. were mixed with 10 ml of 10 per cent red cells in saline and incubated in 100 ml. centrifuge tubes at 37°C for various periods. Then the tubes were placed in an ice bath at 4°C to stop the reaction and centrifuged in the cold. One ml. of the supernatant from each tube was used for the determination of liberated hemoglobin by measuring the optical density at 540 mμ in a Coleman spectrophotometer. These values were plotted against the incubation time to obtain a hemolysis curve.

The other part of the reacting mixture was used to estimate the amount of sphingomyelin in hemolysed cells.

b) For the determination of the amount of sphingomyelin, extraction of phospholipids from reaction mixtures was carried out as shown in Table 1.

After each centrifugation the supernatant was removed carefully to avoid contamination by the sediment. Ten per cent TCA was employed to remove water soluble phosphate compounds which might be contaminants (Marinetti *et al.* 1957) and to detect the phosphate compounds, if any, derived from decomposition of sphingomyelin. Extraction of the nonphospholipid from the sediment with 90 per cent acetone resulted in the removal of much brown material but the final chloroform solution still had a slightly brownish color.

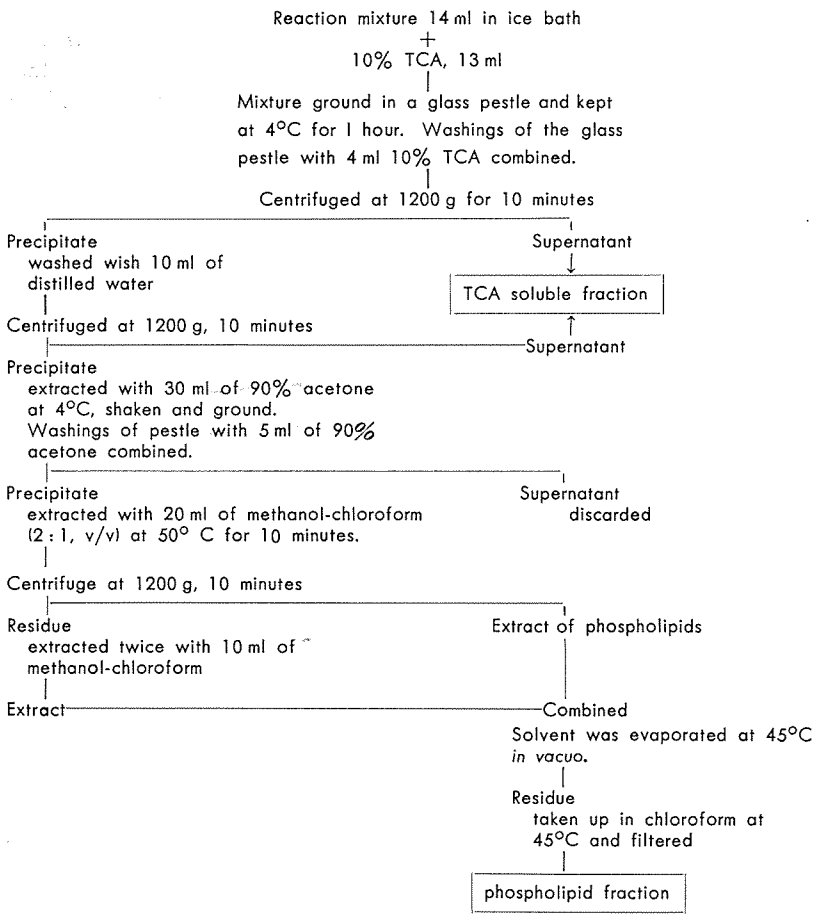
The final solution of phospholipid in chloroform, extracted from each reaction mixture was divided into two equal parts. One part was used to determine total phospholipid P and the other for mono-amino phosphatide P in the KOH hydrolysate (Schmidt *et al.* 1946). The difference between these two values was a measure of sphingomyelin P.

ii) Alteration in sphingomyelin content:

Typical results for the disintegration of the phosphatides in red cells during viral hemolysis are shown in Fig. 2.

The total phospholipid P decreases as viral hemolysis proceeds but the

Table 1. Extraction of Phospholipids from the reaction mixture of hemolysis.



amount of mono-amino phosphatide P, remains constant; consequently this reduction which represents the sphingomyelin P also decreases corresponding to the change of total phospholipid P. The same result was obtained in duplicate experiments.

iii) Alteration of the content of TCA soluble P:

Changes in the amount of TCA soluble P during viral hemolysis were studied with samples employed for the estimation of phospholipid P.

Distilled water was added to the TCA soluble fraction of Table 1 to give final volumes of 50 ml. Five ml of these diluted samples were used to determine total P and inorganic P by the method of Fiske and Subbarow.

As shown in Fig. 3 the total P and inorganic P of the TCA soluble fraction increases during hemolysis. Further, the increase of total P (TCA soluble) exceeds much the decrease in sphingomyelin P. Therefore there is no direct correlation between these two. The increase of TCA soluble P might be, in part, due to some

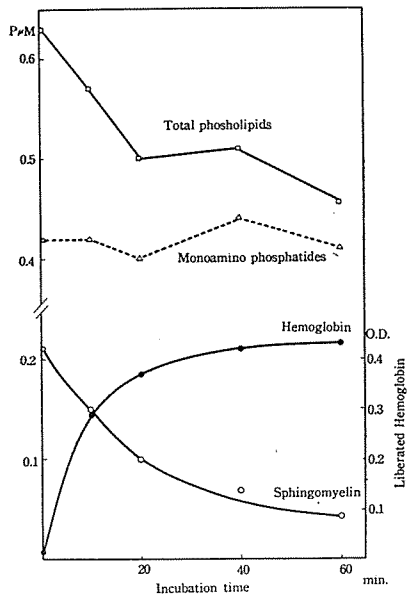


Fig. 2 Change in the amount of sphingomyelin in hemolytic reaction.
The sphingomyelin content is presented as: (Totol phospholipid P) − (Monoamino phosphatide P)

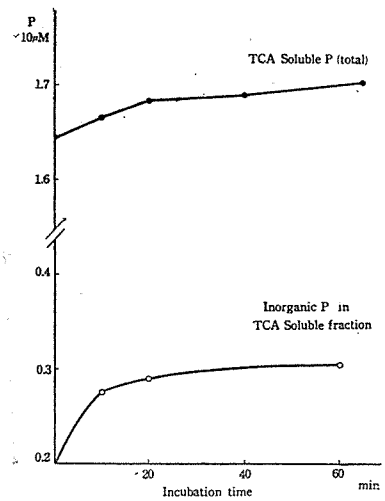


Fig. 3 Changes in the amount of TCA soluble P in red blood hemolysed by virus

nucleotides which became soluble in TCA during hemolysis.

iv) Effect of heat inactivated virus:

The alteration of sphingomyelin has been studied quantitatively in the preceding paragraphs. It remained to be decided whether these changes were specifically caused by the hemolytic activity of HVJ. Tadokoro *et al.* (1957) reported that heating at 45°C only decreased the hemolytic activity of HVJ and had no effect on the hemagglutinating and eluting activity. After inactivation of hemolytic activity by heating at 45°C for various periods (0, 30, and 120 minutes) the virus was tested for its sphingomyelin decomposing activity.

As a control, HVJ which had been heated at 60°C for 15 minutes was used. This sample did not show any hemolytic or hemagglutinating activity. Five ml. of each preparation (containing 8,000 HAU/ml) were added to 10 ml. of 10 per cent red cells and incubated at 37°C for 80 minutes. Then hemolysis was estimated and phospholipid was extracted from each reaction mixture for the determination of sphingomyelin. The results are presented in Fig. 4.

The decrease of sphingomyelin in red cells was inversely proportional to the time of heating at 45°C: in other words the hemolytic activity is the factor which is responsible for the decrease of sphingomyelin.

Also in this experiment, the increase in TCA soluble P was proportional to the hemolytic activity of the treated virus preparations. The results are presented

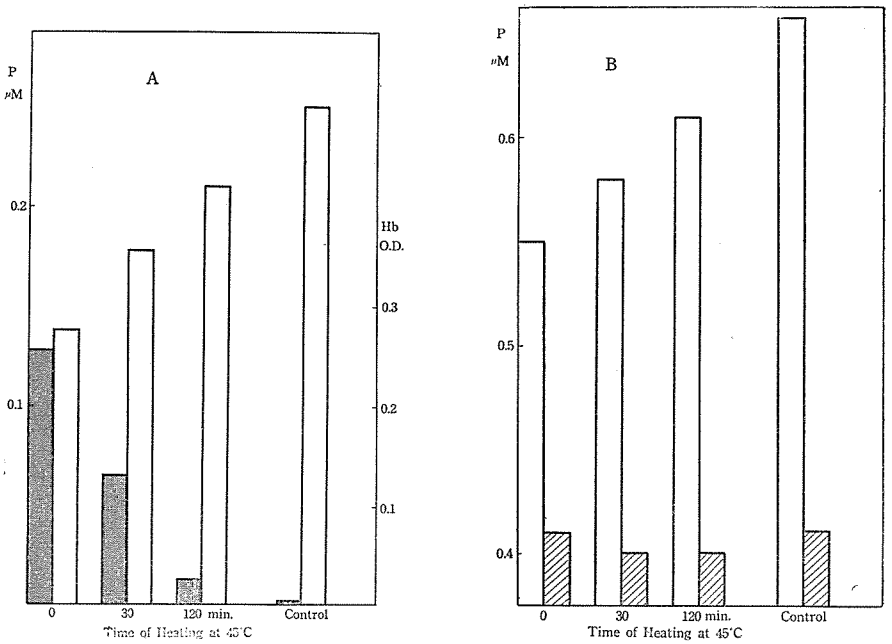


Fig. 4. Effect of heat inactivation of hemolytic activity of the virus on the alteration of phospholipids in the red blood cells.

A. Relationships between the hemolytic activity of the heated virus and the decrease in sphingomyelin in red blood cells.

black: hemoglobin liberated white: sphingomyelin content

B. Relationship between the total phospholipid P and mono-aminophosphatide P.

white: total phospholipid P hatched: mono-amino phosphatide P

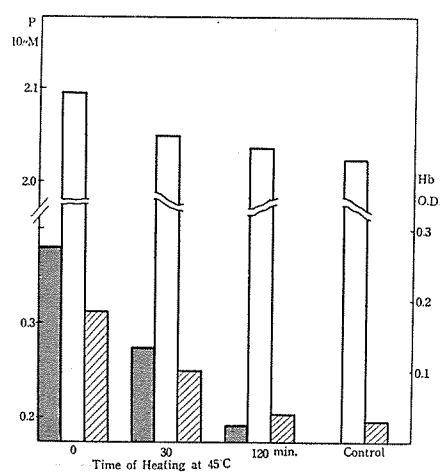


Fig. 5. Correlation between the hemolytic activity of heated virus and inorganic P in the TCA soluble fraction of the hemolysed red blood cells.

black: hemoglobin liberated

white: total phosphatide P in TCA soluble fraction

hatched: inorganic P in TCA soluble fraction

in Fig. 5.

v) Effect of modified conditions :

The hemolytic activity of HVJ was enhanced by freeze thawing and sonic vibration (Hosaka 1958) and decreased in the presence of Ca ions. The relation between the extent of hemolysis and the decrease of sphingomyelin in the cells was investigated under these modified conditions.

A virus preparations containing 8,000 HAU/ml. was divided in 4 parts which were treated as followings.

1) Heated at 60°C for 15 minutes (negative control).
2) Freeze-thawed 3 times.
3) Untreated (native control).
4) $CaCl_2$ was added at 0.1 per cent final concentration.

Five ml. of each preparation were mixed with 10 ml. of 10 per cent red cells and incubated at 37°C for 90 minutes. Then hemolysis and the change in sphingomyelin P content were determined. The results are presented in Fig. 6A. In this experiment again, the stronger the hemolytic activity the more decrease of sphingomyelin is observed. Fig. 6B shows the changes in TCA soluble P in this experiment.

vi) Phospholipids of red cells hemolysed by distilled water :

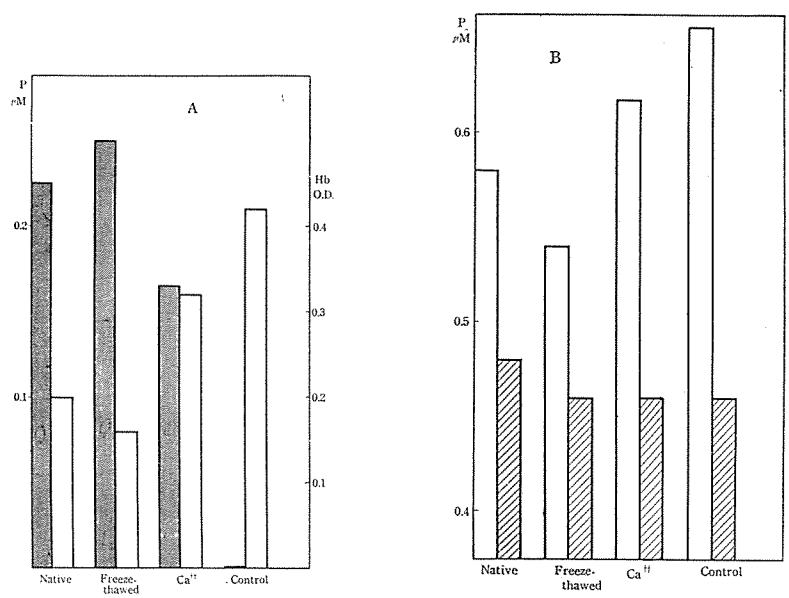


Fig. 6. A. The extent of hemolytic activity and the decomposition of sphingomyelin by native, freeze-thawed, and Ca-treated virus.
black : hemoglobin liberated
white : sphingomyelin content
B. Relation between the total phospholipid P content and monoamino phosphatide P content.
white : total phospholipid P
hatched : mono-amino phosphatide P

From the preceding experiments the decrease in sphingomyelin seems to be consistent in the virus-hemolysed red cells. This change could be secondary to the decomposition of the red cells. Therefore the amount of phospholipids in red cells hemolysed by distilled water was compared with that of normal red cells.

Two 10 ml. aliquots of 10 per cent red cells in saline were centrifuged at 800× g for 15 minutes. The supernatant was removed carefully and 10 ml. of saline was added to one aliquot and 10 ml of distilled water to the other. The tubes were incubated at 37°C for 60 minutes and then the phospholipids were extracted from the lysed cells and the control. Table 2, shows that there was no difference in the amount of sphingomyelin or monoamino-phosphatide.

Table 2. phospholipids of fowl red blood cells hemolysed with distilled water

Cells	Total phospholipids	Mono-amino phosphatides	Sphingomyelin
	μM	μM	μM
Hemolysed with distilled water	0.59	0.39	0.20
Normal	0.58	0.40	0.18

DISCUSSION

These experiments show that the hemolysis by HVJ results in a decrease of sphingomyelin in the red cells. The decrease of sphingomyelin was produced by the hemolytic activity but not by the hemagglutinating or eluting activity. The phenomenon seems to be common for the hemolysis of HVJ, mumps virus (Mobery *et al.* 1958) and NDV., although the last has not yet been confirmed. Therefore these three viruses seem to be classified as another group different from the influenza virus group.

The decrease of sphingomyelin probably means its decomposition by an enzyme on the virus other than the so called receptor destroying enzyme. At present it is uncertain whether sphingomyelin is directly split by a certain enzyme of HVJ.

Few reports have been published on the enzymatic decomposition of sphingomyelin, which results in the liberation of phosphoryl-choline (MacFarlane, 1948: Fujino, 1952). It is of interest from the point of view of lipid metabolism to study the decomposition of sphingomyelin by HVJ and the further interaction between host cell lipid and HVJ. It is of more interest that giant cells were formed as a response of host cells to HVJ, possibly by the hemolytic activity of the virus (Okada *et al.* 1957).

Acknowledgement: The author wishes to thank to Dr. Konosuke Fukai for his constant encouradgement and advice, and to Miss kazuko Kawai and Miss Yaeko Shimizu for their technical assistance.

REFERENCES

Fujino, Y., (1952). Studies on conjugated lipids, 4. On the enzymatic hydrolysis of sphingomyelin. *J. Biochem.* **39,** 55-62.

Hosaka, Y., (1958). The hemolytic activity of HVJ. I. An analysis of the hemolytic reaction. *Biken's J.* **1,** 70-89.

Lea, C. H., Rhoades, D. N., and Stoll, R. D. (1955). Phospholipid III. On the chromatographic separation of glycerophospholilpids. *Biochem. J.* **60,** 353-363.

Levine, C., and Chargaff, E. (1951). Production for the microestimation of nitrogenous phosphatide constituents. *J. Biol. Chem.* **192,** 465-478.

Macfarlane, M. G. (1948). The biochemistry of bacterial toxins. 2. The enzymic specificity of *Clostridium welchii* lecithinase. *Biochem. J.* **42,** 587-590.

Marinetti, G. V., and Stotz, E. H. (1956). Chromatography of phosphatides on silicic acid impregnated paper. *Biochim. et Biophys. Acta* **21,** 168-170.

Marimetti, G. V., Erbland, J., Albrecht, M. and Stotz, E. (1957). The in vitro incorporation of P^{32}-labelled orthophosphate into the phosphatides of isolated rat liver mitochondria. *Biochim. et Biophys. Acta* **26,** 131-143.

McKibbin, J. M. and Taylor, W. E. (1949). The nitrogenous constituents of the tissue lipides. II. The determination of sphingosine on tissue lipide extracts. *J. Biol. Chem.* **178,** 29-35.

Mobery, M. L., Marinetti, G. V., Witter, R. F. and Morgan, H. F. (1958). Studies of the hemolysis of red blood cells by mumps virus. III. Alteration in lipoprotein of the red blood cell wall. *J. Exp. Med.,* **107,** 87-94.

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.

Rouser, G., Marinetti, G. V., Witter, R. F., Berry, J. F. and Stotz, E. (1956). Paperchromatography of phospholipides. *J. Biol. Chem.* **223,** 485-497.

Schmidt, G., Benotti, J., Hershman, B. and Thannhauser, S. H. (1946). A micromethod for the quantitative partition of phospholipide mixtures into monoaminophosphatide and sphingomyelin. *J. Biol. Chem.* **166,** 505-5 .

Tadokoro, J., Suzuki, T. and Fukazawa, R. (1958). On the heat stability of hemagglutinating and hemolytic activities of HVJ (Z) virus. *Med. J. Osaka Univ.* **8,** 699-709.

Turner, J. D. (1957). Absence of lecithin from the stroma of the red blood cells of certain animals and its relation to venom hemolysis. *J. Exp. Med.* **105,** 189-193.