STUDIES OF THE HEMOLYSIS OF RED BLOOD CELLS BY MUMPS VIRUS

IV. Quantitative Study of Changes in Red Blood Cell Lipides and of Virus Lipides*

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The hemolysis of chicken red blood cells by mumps virus (1) has been shown to exhibit many of the properties of an enzymatic reaction (2, 3). Although subsequent studies showed that the hemolysin possessed certain properties in common with lecithinase A (2–5), the last study in this series, by Moberly, Marinetti, and Morgan (6), demonstrated conclusively that the mechanism of hemolysis was not the conversion of red cell lecithin by mumps virus to the hemolytic compound lysolecithin. Evidence was offered however that another specific change was taking place in the cell stroma with hemolysis. This was manifest as a decrease in the size of the sphingomyelin spot on phosphatide chromatograms prepared from lipide extracts of hemolyzed chicken red blood cells, when compared to unhemolyzed controls.

The present study is an attempt to clarify the role of sphingomyelin in which the phosphatides and non-phosphorous-containing lipides of chicken and human red blood cells were examined quantitatively to detect possible alterations following hemolysis. In addition the lipides of the virus itself were examined, and the virus was reacted with isolated phosphatide substrates in an attempt to define a specific enzyme-substrate system.

Materials and Methods

Virus.—The Enders strain of mumps virus was inoculated into the amniotic sac of 8-day embryonated eggs which were then incubated for 96 hours. Surviving eggs were refrigerated for 12 hours and the amniotic fluid harvested. Pooled fluids with a hemagglutination titer of at least 1:512 were concentrated by differential centrifugation at 0°C. The supernatant fluid obtained after centrifugation at 3,000 R.P.M. for 20 minutes was spun in the Spinco ultracentrifuge (model L) with No. 40 rotor at 30,000 R.P.M. for 20 minutes. The white pellet obtained was resuspended in 10 per cent of the original volume of 0.16 molar buffer with a

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pH of 7.2. No precipitate is obtained when uninfected amniotic fluid is processed in this manner.

Virus for control studies was either heated to 50°C. for 2 hours or suspended in buffer of pH 6.0. Either procedure reduces hemolytic titer at least 20-fold without reducing the hemagglutinin titer. The reduction in hemolytic titer produced by heating is paralleled by a similar reduction in elution of the virus, but lowering the pH of the reaction to 6 has no effect on virus elution. Thus this latter virus could adsorb to the erythrocytes and subsequently elute from them under the experimental conditions employed (3).

Red Blood Cells.—Blood was drawn into Alsever's solution, either by cardiac puncture of 3- to 9-month-old White Leghorn roosters or by venipuncture from a young, adult male subject. Heparin cannot be used since it alters the chromatographic properties of the phosphatides. The cells were immediately washed 3 times in buffered saline, made up to 5 per cent suspension by microhematocrit, and used within 4 hours.

Hemolytic System.—Cells and virus were combined in the proportion of 1.0 ml. concentrated virus for each 2 ml. of 5 per cent red blood cells, mixed by vigorous shaking, and allowed to stand in a 37°C. water bath for 3 hours. The result was usually 80 to 90 per cent hemolysis of chicken red cells and 60 to 70 per cent hemolysis of human red cells, as determined by colorimetric tests. Control samples, with heated virus or buffer of pH 6.0, treated similarly produced hemolysis of 0 to 5 per cent. It was found late in the study that mumps viral hemolysis increases with temperature at least up to 41°C., but 37°C. was used throughout these experiments.

Lipide Extraction Procedures.—1. Total lipide: Each volume of the 5 per cent erythrocyte and virus suspension was extracted with 5 volumes of ethyl ether–methanol 2:1 at 37°C. for 15 minutes. The residue was concentrated by centrifugation and reextracted by the same solvent twice more. The pooled centrifuged extracts were evaporated to dryness, adding methanol as required, under reduced pressure in an atmosphere of water-pumped nitrogen at 37°C., filtered through sintered glass, and stored under nitrogen.

2. Unbound lipide: Each volume of the 5 per cent erythrocyte suspension was extracted 5 times with 2 volumes of iso-octane, by vigorous shaking in a separatory funnel. The octane extracts were decanted, pooled, washed 3 times with distilled water, and evaporated as above.

Phosphatide Chromatography.—The methods used were essentially those of Marinetti, Erbland, and Kochen (7).

1. Quantitative paper chromatography: Whatman No. 1 paper was impregnated with Mallinckrodt silicic acid (7), and washed thoroughly with deionized distilled water. Lipide extracts were made up to known phosphatide concentration on the basis of phosphorous analysis, and applied to the paper in amounts varying between 2 and 10 μg. of lipide P per spot. The chromatograms were run in both diisobutyl ketone–*n*-butyl ether–acetic acid–water 20:20:20:3 at 2°C. and in diisobutyl ketone–acetic acid–water 40:25:5 at 23°C. for 16 hours. Rhodamine 6G dye was used for detecting the spots under ultraviolet light. Further identification of the phosphatides was accomplished by specific spot tests for free amino groups, for acetal phosphatide, and for choline, and by running known phosphatides on the same chromatograms.

For quantitation several chromatograms of the same extract were run in the same solvent system, stained lightly with rhodamine 6G, corresponding spots cut out, combined, and extracted 3 times by refluxing for ½ hour with 0.8 N HCl in redistilled methanol. The extracts were filtered through Whatman No. 44 filter paper to remove silicic acid, which inhibits color development in the P analysis.

2. Phosphorous analysis: The extracts were evaporated to dryness and digested by refluxing for 15 minutes with 0.9 ml. 70 per cent perchloric acid and 3 drops of concentrated nitric acid. P analysis was done by the method of Harris and Popat (8), and, in later experiments, by the following more sensitive method. To each digested sample was added 7.1 ml. water, 1 ml. 2.5 per cent ammonium molybdate, and 1 ml. 10 per cent ascorbic acid. The samples

were incubated at 37°C. for 90 minutes, allowed to cool, and compared to a 2 μg. standard in a Beckman spectrophotometer model B at 820 mμ.

3. Column chromatography: Much finer separations of phosphatides can be accomplished by paper than by column chromatography, making paper the medium of choice for quantitation. However, column chromatography was used for the fractionation and partial purification of larger amounts of chicken red blood cell lipides. Lipide extracts were applied to 10 gm. silicic acid columns and eluted with increasing concentrations of methanol in chloroform (7). Specific phosphatides were located in the fractionated eluate by paper chromatography.

Non-phosphatide Lipide Chromatography.—0.2 mg. of lipide was spotted on silicic acid-impregnated paper and run in *n*-heptane–diisobutyl ketone 96:6 at 23°C. for 4 hours. Staining with rhodamine 6G developed well defined fatty acid, neutral fat, cholesterol, and cholesterol ester spots.

Fatty Acid and Cholesterol Analyses.—Lipide extracts were fractionated into phosphatide and non-phosphatide components on 1 gm. silicic acid columns. The extract was put on the column in chloroform, and the non-phosphatides were eluted with chloroform followed by ethyl ether. The phosphatides were eluted with chloroform-methanol 1:1 followed by methanol. An aliquot of the non-phosphatide fraction was taken for colorimetric determination of total cholesterol by the Liebermann-Burchard reaction. The remaining lipide was dried, dissolved in absolute ethanol, and titrated against 0.0037 N NaOH in a nitrogen atmosphere, using a phenolphthalein indicator.

RESULTS

Studies with Chicken Red Blood Cells.—

1. Effect of Hemolysis on Total Red Blood Cell Phosphatides.—In a series of 3 experiments chicken red blood cells were hemolyzed with mumps virus at pH 7.2, total lipide extracts of the virus and erythrocyte system made, and the phosphatides studied by quantitative paper chromatography. In all of these experiments the control system was different only in that the virus had been heated. In the third experiment an additional control was the use of active hemolytic virus, whose hemolytic reaction was inhibited by the use of a buffer of pH 6.0.

In all 3 experiments the major visible change on the chromatograms of the hemolyzed cells was a marked decrease in the size of the sphingomyelin spot (Fig. 1 *a*). Quantitative analysis confirmed this observation. The results of one such experiment (*i.e.*, the third experiment) are presented in Table I. The data show a 65 per cent decrease in sphingomyelin following hemolysis, with a proportionate decrease in total lipide P, and no other striking changes. In this experiment citrate buffers were used, and the amounts of P recovered in the 3 groups represent actual recovery from equal volumes of red blood cells. About 5 to 10 per cent of the lipide recovered was of viral origin. In the first 2 experiments phosphate buffers were used, and variable amounts of inorganic P were carried over into the total lipide extracts. Although this P did not move on the chromatograms (9), and hence did not interfere with the measured proportions of the phospholipides within a group, it was difficult to calculate the actual total recovery from a given red blood cell volume. Therefore the data from the 3 experiments were summarized in relative, rather than in absolute, values. If,

as was indicated by the sizes of the spots and by experiment 3, only the sphingomyelin changes significantly with hemolysis, then if lecithin is assumed to be recovered in a ratio of 1 to 1 the other phosphatides should also be recovered in ratios close to 1.0. Lecithin was chosen as the reference point because it is the largest component, and hence most accurately determined, and because the work of Moberly *et al.* (6) with lecithin demonstrated no change in this

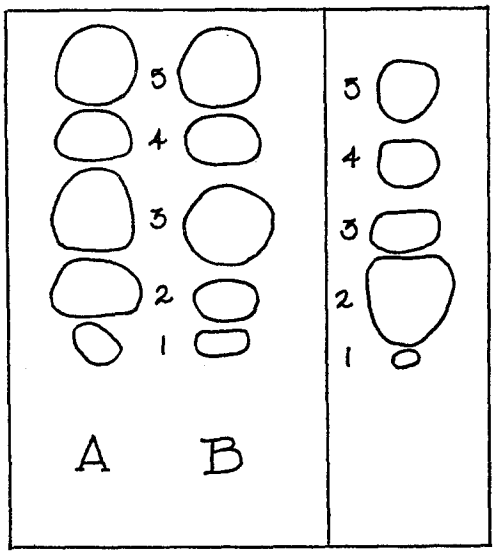

FIG. 1 *a* FIG. 1 *b*

FIG. 1 *a*. Chromatograms of phosphatides of chicken erythrocytes. A, control cells plus heated virus; B, cells hemolyzed by mumps virus. Spots 1 and 4 stained blue with rhodamine 6G, the others stained yellow. Spots 2 and 3 gave positive choline reactions. Spots 4 and 5 gave positive ninhydrin reactions. Spot 1 corresponds to inositol phosphatides, 2 to sphingomyelin, 3 to lecithin, 4 to phosphatidyl serine, and 5 to phosphatidyl ethanolamine.

FIG. 1 *b*. Chromatogram of phosphatides of mumps virus preparation. Spots labelled as in Fig. 1 *a*.

component by hemolysis. The data of the 3 experiments are presented in this manner in Table II, and it is evident from the averages that only sphingomyelin recovery is significantly changed by hemolysis. Statistical analysis of the 3 experiments revealed no significant alterations in any of the other phosphatides.

An experiment was performed, identical with the experiments described above, except that the lipide extracts were fractionated on a silicic acid column instead of on paper. Both extracts were eluted in 80 fractions and each fraction was analyzed for P, and peak fractions were chromatographed on paper. However, as has been reported before (7) there is considerable heterogeneity of

peaks in this system, and adequate quantitation of individual phosphatides was not possible. A distinct loss of sphingomyelin with hemolysis was evident, however.

TABLE I

*Effect of Mumps Virus Hemolysis on Chicken Red Blood Cell Phosphatides**

Virus Buffer	Heated pH 7.2	Active pH 6.0	Active pH 7.2
Hemolysis, *per cent*	0	0.6	76
	μg.P	*μg.P*	*μg.P*
Inositol phosphatides	0.8	0.8	0.8
Sphingomyelin	2.3	2.5	0.8
Lecithin	3.6	3.5	3.4
Phosphatidyl serine	1.3	1.1	1.0
Phosphatidyl ethanolamine	1.7	2.0	1.8
Total lipide P recovered, μg.	9.7	9.9	7.8

* Lipides extracted from 5 ml. volumes of 5 per cent cell suspensions.

TABLE II

Summary of 3 Experiments Showing the Effect of Hemolysis on the Relative Recoveries of Chicken Red Blood Cell Phosphatides

Experiment	Inositol phosphatides	Sphingomyelin	Lecithin	Phosphatidyl serine	Phosphatidyl ethanolamine
1*	1.52‡	0.37	1.00	1.22	1.08
2*	0.74	0.34	1.00	1.21	1.02
3*	1.04	0.36	1.00	0.81	1.13
3§	1.00	0.32	1.00	0.94	0.93
Average	1.07	0.35	1.00	1.04	1.04

In each experiment the arbitrary assumption was made that no actual change occurred in lecithin, and the data were adjusted accordingly, *i.e.*, proportions were unchanged, but all data were divided by the factor required to bring the lecithin figure to 1.00; *e.g.*, factors of 3.6, 3.5, and 3.4 for the groups in experiment 3 (Table I).

* Compared to heated virus control.

§ Compared to pH 6 inhibited control.

‡ The figures represent the recovery ratio: hemolyzed/control.

2. Effect of Hemolysis on Unbound Red Blood Cell Lipides.—To investigate the effect of hemolysis caused by mumps virus on lipoprotein bonding in the chicken red blood cell wall, iso-octane extracts of hemolyzed cells were studied. Iso-octane, being a non-polar solvent, is not considered to break lipide-protein bonds, and hence extracts mainly unbound lipide. The results are shown in

Table III. The striking increase seen in free phosphatide with hemolysis was shown on paper chromatography to be due to a large increase in sphingomyelin, with no change in the lecithin and phosphatidyl ethanolamine components also present.

3. Reaction of Virus with Isolated Red Blood Cell Lipides.—To investigate the possibility that mumps virus is able to act directly on isolated sphingomyelin, 125 mg. of phosphatides were extracted from chicken red blood cells and fractionated on a silicic acid column into partially purified components. 0.75 ml. portions of concentrated virus, both active and heated, with a hemagglutinin titer of 1:8200 were added to fine suspensions of from 0.15 to 0.60 mg. of the various phosphatide fractions, and incubated for 3 hours. These mixtures were then reextracted and chromatographed. No decrease in sphingomyelin or in any other component was seen after treatment with active hemolytic virus.

TABLE III

*The Effect of Viral Hemolysis on the Release of Unbound Lipides in the Chicken Red Blood Cell**

Virus	Total weight	Fatty acid	Total cholesterol	Phosphatide
	mg.	*mg.*	*mg.*	*mg.*
Control (heated).....	3.9	0.78	2.7	0.075
Hemolytic.....	4.7	0.72	2.9	0.217

* Lipides extracted from 57 ml. volumes of 5 per cent cell suspensions.

Studies with Human Red Blood Cells.—

The experimental conditions which gave a 65 per cent loss of sphingomyelin in chicken cells were repeated using human red blood cells. No change in sphingomyelin or in any other phosphatide component was demonstrated. Using heated virus 3 per cent hemolysis was produced, and sphingomyelin constituted 24.1 per cent of the total phosphatide P. When active hemolytic virus was used, producing 63 per cent hemolysis, sphingomyelin accounted for 23.9 per cent of the total phosphatide P. No increase in the sphingomyelin content of iso-octane extracts was demonstrable after hemolysis.

Studies with Sheep Red Blood Cells.—

Turner has reported (10) that sheep red blood cells contain no lecithin. Because of the bearing of this finding on the possible role of a lecithinase in mumps virus hemolysis, this work was repeated and his finding was confirmed. The major phosphatide components appear to be sphingomyelin and phosphatidyl serine, with smaller amounts of phosphatidyl ethanolamine and inositol phosphatides. No spot was seen having an R_f value identical with that of authentic lecithin which was run simultaneously. The spot having the R_f

value of sphingomyelin was stained yellow with rhodamine 6G and gave positive choline and negative ninhydrin reactions, which are compatible with either sphingomyelin or lecithin, but also gave a positive dinitrophenylhydrazine test for acetal phosphatide, which was not expected with sphingomyelin.[1] Therefore, to rule out the possibility that this spot represented a unique acetal type lecithin, an extract was heated at 100°C. for 15 minutes with concentrated acetic acid, to hydrolyze any choline plasmalogen to lysolecithin, which could be identified chromatographically. This treatment produced no change in the chromatogram. The nature of this sphingomyelin component in sheep erythrocytes requires further elucidation.

TABLE IV

The Phosphatide Distribution in Mumps Virus

Phosphatide	Lipide P (duplicate analyses)		Average per cent of total lipide P
	μg.	μg.	
Sphingomyelin	1.58	1.55	60
Lecithin*	0.28	0.28	11
Phosphatidyl serine	0.26	0.28	10
Phosphatidyl ethanolamine 1	0.34	0.40	14
Phosphatidyl ethanolamine 2	0.07	0.17	5

* This fraction also includes a small unidentified component running between sphingomyelin and lecithin.

Studies of Mumps Virus.—

1. Attempts at Purification.—Because differential centrifugation is a relatively inefficient fractionation procedure producing losses of up to 85 per cent, 4 chemical methods of virus purification were applied to mumps-infected amniotic fluid. The methods of Cox (11) and Pollard (12), precipitating virus from 30 per cent methanol at −10°C., completely destroyed the mumps hemolysin. Utilizing the selective elution of virus from cellulose anion exchange columns (DEAE-SF and ECTEOLA-SF, Brown and Co.) by the method of Hoyer *et al.* (13), only 4 per cent virus recovery was achieved. A single treatment by the fluorocarbon method of Manson *et al.* (14) reduced the hemagglutinin titer 128-fold.

2. Phosphatide Content of Mumps Virus.—Mumps virus, as concentrated by differential centrifugation from amniotic fluid, was found to contain about 7 per cent phosphatide, based on the P analysis of lipide extracts and dry weight. Chromatography of lipide extracts (Fig. 1 *b*) in 4 experiments showed a constant and unusual phosphatide distribution, sphingomyelin being the major com-

[1] Turner noted a positive Schiff test for aldehyde after hydrolysis of the equivalent spot with $HgCl_2$.

ponent. This distribution was quantitated in duplicate paper analyses of the same extract (Table IV). The phosphatidyl ethanolamine spot appeared to consist of 2 fractions which were analyzed separately. Extracts of the material obtained by low speed centrifugation, of the supernatant fluid from which the virus had been separated by ultracentrifugation, and of uninfected amniotic fluid were all chromatographed and all showed a different phosphatide distribution from that of the virus preparation. Lecithin was the predominant component, the usual pattern seen with animal tissues.

DISCUSSION

These results clearly indicate two distinct effects of mumps virus during hemolysis of chicken red blood cells: a cleavage of bonds between sphingomyelin and protein, and a degradation of sphingomyelin to products which do not appear on phosphatide chromatography. The nature of these products is unknown. This attack only on sphingomyelin suggests a unique enzymatic specificity associated with the virus particle. Since no specific sphingomyelinase is known, contamination of the virus preparation with host enzymes does not appear to be an issue. The failure of virus to react with isolated sphingomyelin in aqueous suspension is not surprising, since the sphingomyelin in the red cell is virtually all bound to protein. The enzymatic hydrolysis of lipides only when they are bound to protein is a phenomenon which has been demonstrated with lipoprotein lipase (15).

Although the demonstrated disruption of the lipoprotein matrix of the chicken red blood cell stroma is quite adequate trauma to produce hemolysis in itself, and although it has been demonstrated to be associated specifically with hemolysis, independent of agglutination or viral elution, the failure to demonstrate a similar disruption in hemolyzed human erythrocytes strongly suggests that the primary mechanism of hemolysis by mumps virus has not yet been elucidated. However, the reaction has considerable intrinsic biochemical interest, and it is not surprising that the gross structural species differences in red cells are reflected in differences at the molecular level which prevent this reaction. The amount of viral phosphatide present in the system, 5 to 10 per cent, is far too small for any changes in this part of the system to play a significant role in the phosphatide changes demonstrated.

Another species difference at the molecular level, the absence of lecithin from sheep red blood cells, provides additional proof that a lecithinase is not responsible for hemolysis by mumps virus, since sheep erythrocytes are known to be hemolyzed by mumps virus (1).

The finding of 60 per cent sphingomyelin in the phosphatides of the partially purified mumps virus is unique among biological materials whose phosphatides have been studied, and the large amount present suggests that the phosphatides are not present simply as the result of non-specific adsorbtion to the virus

particles. Whether they are an integral part of the virus particle or whether the virus has an affinity for tissue sphingomyelin has not been established. Egg-adapted influenza virus cultivated in the egg allantoic cavity has recently been shown (16) to contain 11 per cent phosphatides, but only 30 per cent of this was found to be sphingomyelin, and the phosphatide proportions corresponded to those found in egg allantoic fluid.

Previous studies (6) did not detect any phosphatide in mumps virus preparations. Review of this work reveals that the chloroform extracts of the virus were filtered through filter paper rather than sintered glass. Phosphatides are adsorbed by the filter paper under the conditions which were used.

The hemolytic property of the virus may have a role in the release of mumps virus from the cell and it is conceivable that, if this enzymatic activity involves the splitting of sphingomyelin from protein binding, it could be responsible for the considerable quantity of sphingomyelin in the virus. In HeLa cell cultures, the growth of mumps virus has been associated with lesions which were dominantly cytolytic in nature (17) suggesting that the growth and release of the virus is associated with disruption of the cell wall. It may be that the lytic properties of the virus are important in its release from the host cell.

SUMMARY

Hemolysis of chicken red blood cells by mumps virus is associated with the release of sphingomyelin from the stromal lipoprotein and the destruction of 65 per cent of the sphingomyelin of the red cell stroma. However, the virus had no effect on isolated phosphatides extracted from the erythrocytes. The hemolytic action of the virus and changes in sphingomyelin content of the erythrocytes fail to occur at a pH of 6.0. The viral hemolysis of human erythrocytes is not associated with similar alterations in their content of sphingomyelin. The absence of lecithin from sheep erythrocytes, which are also lysed by mumps virus, is additional evidence that a viral lecithinase is not associated with the hemolytic property of mumps virus.

Mumps virus concentrated from the amniotic fluid of viral infected chick embryos contains about 7 per cent phosphatide, 60 per cent of which is sphingomyelin.

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