Effect of Lecithin, Cholesterol and Cardiolipin on Mumps Virus Hemolysin.* (21236)

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The hemolysin of mumps virus(1) has been shown to exhibit many of the characteristics ascribed to enzymes(2,3) and to be distinct from the mumps hemagglutinin(2-5). In the course of experiments studying the nature of the mumps hemolysin, it was found to possess certain properties in common with lecithinase A, such as similar pH and temperature optima, inhibition by urethane and by high concentrations of calcium and magnesium, and the failure of potassium cyanide and sodium fluoride to affect its hemolytic action. However, the mumps viral hemolysin differs from lecithinase A in that it is heat labile while lecithinase A is heat stable(5). Because of this similarity of the mumps viral hemolysin to a lecithinase, it was of interest to test the action of lecithin on the viral hemolysin. Cholesterol and cardiolipin were studied also to determine if they would affect the hemolytic action of mumps virus.

Materials and Methods. A strain of egg-adapted mumps virus was used(1-5). Embryonated hens' eggs, which had been incubated previously for 8 days at 37.5°C, were inoculated amniotically with 0.1 ml of a 10^{-2} dilution of the seed virus in broth. After inoculation, the eggs were incubated 5-6 days at 35°C, chilled in the refrigerator and the infected fluids harvested. These virus-infected fluids were placed in screw-cap test tubes and stored at –40°C until used. Before use these fluids were rapidly thawed and dialyzed for 24 hours at 4°C against phosphate buffered saline (pH 7.0-7.2). This buffered saline was used as the diluent for all reagents and tests in this study. Tests for hemolytic and hemagglutinating activities of the virus were carried out with the methods previously described (2,3), using a 0.5% suspension of chicken erythrocytes for the hemagglutination tests and a 2% suspension for the hemolysin tests. In previous experiments(2,3), percentage hemolysis readings of less than 10% were found to be unreliable so these values are disregarded and the hemolytic titer is taken as the highest dilution of virus producing at least 10% hemolysis. Furthermore, one-tube differences in the hemolysis or hemagglutination tests are within experimental error and therefore not significant. Stock solutions of lecithin, cholesterol and cardiolipin were prepared in absolute alcohol and were diluted for use in buffered saline with agitation to secure even dispersion of small size particles. Lecithin was prepared from egg yolk according to Pangborn's method(6,7). Cholesterol was purchased from Eastman Kodak Co., and the cardiolipin was generously supplied by Dr. M. C. Pangborn(8,9). These materials suspended in buffered saline were used as diluents for the virus and for the cell controls in the hemagglutination and hemolysis tests to determine their possible inhibitory effects. Similar dilutions of alcohol were used in all experiments to observe any possible action of alcohol on erythrocytes or virus or their interaction to affect either hemagglutination or hemolysis. In studies in which the test compounds were allowed to interact with the chicken erythrocytes before the virus was added, 20 ml volumes of washed erythrocytes were added to an equal volume of a buffered saline suspension of the test compound and mixed thoroughly. One aliquot was placed in the refrigerator at 4°C and the other at 37°C for 3 hours. At the end of this incubation period, the erythrocytes were washed 3 times and a 0.5% and a 2% suspension prepared which was added in equal volumes to each of two serial dilutions of virus for tests respectively of its agglutinative and its hemolytic activities. In certain experiments, virus was allowed to react with cells before the addition

* This investigation was supported by contract between the Office of Naval Research, Department of the Navy, and the University of Rochester and by the Ernest L. Woodward Fund.

TABLE I. Effects of Lecithin, Cholesterol, and Cardiolipin on Hemagglutination and Hemolysis by Mumps Virus. Eleven experiments.

Inhibitor, mg/ml	Hemolytic test: Dilutions of virus fluid 8	16	32	64	128	256	Cell control	Hemagglutination test: Titer	Cell control
Lecithin									
.0	72*	53	34	23	12	0	0	2048†	—
.3	73	52	33	21	13	0	0	2048	—
.0	43	33	23	18	11	10	0	1024	—
.4	25	16	9	0	0	0	0	1024	—
.0	55	41	28	21	16	13	0	1024	—
.9	30	20	9	6	0	0	0	1024	—
.0	55	43	29	18	11	0	0	512	—
5.0	21	12	0	0	0	0	0	256	—
Cholesterol									
.0	42	37	26	18	13	0	0	1024	—
.05	34	22	14	10	0	0	0	>2048	±
.0	55	41	28	21	16	13	0	1024	—
.09	44	28	19	13	0	0	0	>2048	+
.0	55	43	29	18	11	0	0	4096	—
.5	23	0	0	0	0	0	0	>8192	+
Cardiolipin									
.0	58	42	28	18	12	0	0	2048	—
.04	53	33	23	15	11	0	0	>2048	+
.0	24	17	11	0	0	0	0	256	—
.05	14	0	0	0	0	0	0	>2048	+
.0	28	21	15	10	0	0	0	512	—
.1	16	11	0	0	0	0	0	>2048	+

* % of hemolysis determined colorimetrically.
† Reciprocal of highest dilution of virus producing hemagglutination.
—, No hemagglutination; ±, slight hemagglutination; +, clear-cut hemagglutination.

of the various compounds by making serial dilutions of virus in a 2% suspension of erythrocytes and placing these suspensions in the refrigerator for 3 hours. At the end of this period, the cells were washed 3 times with cold buffered saline and resuspended in buffered saline to make a 0.5% and a 2% suspension of virus-adsorbed red cells to which an equal volume of fluid containing the test compound could be added and its effect on hemagglutination and hemolysis, respectively, observed.

Results. 1. *Effect of lecithin, cholesterol and cardiolipin on mumps virus hemagglutination and hemolysis.* Table I presents data showing that lecithin, cholesterol and cardiolipin inhibit the hemolytic action of mumps virus. Controls on these tests showed that the effect was not due to the alcohol used to dissolve these compounds, nor was their action the result of a change in pH, which is known to affect the mumps hemolysin(2,3), since pH measurements of the fluids revealed no significant alterations. The lowest concentration of lecithin exhibiting at least a four-fold decrease in hemolysin titer contained 0.4 mg/ml (0.00514 M) and increasing the concentration up to 5 mg/ml greatly enhanced this inhibitory action. None of the quantities of lecithin tested produced any significant effect on the hemagglutinating activity of the virus. Cholesterol caused a 4-fold reduction of viral hemolysis at a concentration of 0.09 mg/ml (0.000232 M), and smaller quantities had no significant effect. At this level (0.09 mg/ml) cholesterol produced agglutination of the erythrocytes *per se*. Cardiolipin in the concentrations of 0.04 mg/ml to 0.1 mg/ml caused spontaneous agglutination of the red cells, though it inhibited the viral hemolysin when levels of 0.05 mg/ml were reached, but concentrations of cardiolipin exceeding 0.1 mg/ml were hemolytic *per se*.

In other experiments it was shown that the

TABLE II. Results of Reaction of Inhibitor and Virus before Addition of Erythrocytes and Hemolysis.

Inhibitor		Hr at 4°C	Hemolytic test — Dilutions of virus fluid: 2	4	8	16	32	64	128	256	Cell control	Hemagglutination test: Titer	Cell control
	mg/ml												
0	0	0	68*	52	43	33	23	18	11	10	0	1024†	—
	0	18	65	53	40	29	20	14	10	10	0	"	—
Lecithin	.4	0	55	29	25	16	11	10	0	0	0	"	—
		18	52	26	10	0	0	0	0	0	0	"	—
	.7	0	48	31	18	10	0	0	0	0	0	"	—
		18	33	10	0	0	0	0	0	0	0	"	—
Cholesterol	.05	0	67	54	41	29	20	14	10	0	0	>2048	±
		18	63	48	32	17	10	0	0	0	0	"	±
	.09	0	48	48	36	26	17	10	0	0	0	"	+
		18	46	46	27	13	0	0	0	0	0	"	+

* % of hemolysis determined colorimetrically.
† Reciprocal of highest dilution of virus producing hemagglutination.
—, No hemagglutination; ±, slight hemagglutination; +, clear-cut hemagglutination.

inhibitory activity of lecithin, cholesterol and cardiolipin at their minimal effective concentrations was markedly enhanced when they were present simultaneously. This additive effect enhanced their suppressive action on viral hemolysis at least 16 times.

2. *Action of inhibitor on virus before addition of cells.* In their suppressive action on viral hemolysis these compounds might exert an effect primarily on the virus, the erythrocyte, or on their interaction to produce lysis of the erythrocyte. Experiments were therefore undertaken to determine if their addition to the virus prior to its admixture with the erythrocytes would enhance the inhibitory action. A typical experiment in which lecithin and cholesterol were allowed to interact with serial dilutions of virus overnight at 4°C is presented in Table II.

Exposure of virus to the inhibitors for 18 hours at 4°C enhanced their suppressive action on the viral hemolysin, though control tests indicated that the viral hemolysin did not deteriorate under these conditions. Lecithin at 0.4 mg/ml exhibited the most marked increase of inhibitory activity for the viral hemolysin, though the viral hemagglutinin was unimpaired, indicating that the virus still combined as readily with erythrocytes. The action of cholesterol on the viral hemolysin also increased under these conditions, but any possible action on the viral hemagglutinin was obscured by its own agglutinative property.

3. *Action of inhibitors when added to erythrocytes before exposure to virus.* Since the compounds under study might produce their inhibitory actions on viral hemolysis by altering the surface of the erythrocytes, the effect of treating the red cells with inhibitors on their subsequent hemolysis by the virus was studied by the method described and results, using lecithin, are summarized in Table III. Pretreatment of erythrocytes with lecithin (0.4 mg/ml) at 37°C caused a 4-fold decrease in viral hemolysin titer, but interaction of lecithin and red cells at 4°C produced no effect. Neither type of treatment had any effect on the viral hemagglutinin, thus indicating that the virus could adsorb to the erythrocytes in an unimpaired manner. Furthermore, since hemagglutination tests on the supernatant fluids in the hemolysin tests failed to show any impairment of release of virus from the lecithin-treated cells, the elution process was not interfered with. Lecithin apparently interacts with erythrocytes at 37°C but not at 4°C in a manner which interferes with their hemolysis by mumps virus, but it does not affect the capacity of the virus to adsorb to and elute from these erythrocytes. Since the erythrocytes were thoroughly washed after contact with the lecithin before use, it may be suggested that they were "coated" with lecithin.

TABLE III. Influence of Pretreatment of Erythrocytes with Lecithin upon Hemolytic Activity of Virus.

Treatment of erythrocyte for 3 hr		Hemolytic test								Hemagglutination test	
		Dilutions of virus fluid							Cell		Cell
mg/ml	Temp., °C	2	4	8	16	32	64	128	control	Titer	control
.0	37	84*	70	58	40	25	16	10	0	2048†	—
		64‡	16	16	4	2	—	—			
.4	"	67	52	37	23	14	0	0	0	"	—
		128‡	32	16	4	—	—	—			
.0	4	90	81	64	43	30	21	15	0	"	—
		64‡	16	8	4	2	—	—			
.4	"	86*	75	56	39	24	14	10	0	"	—
		128‡	64	8	4	2	—	—			

* % of hemolysis determined colorimetrically.
† Reciprocal of highest dilution of virus producing hemagglutination.
‡ Reciprocal of hemagglutination titer of supernatant fluid after hemolysis test.
—, No hemagglutination.

Similar treatment of erythrocytes with cholesterol (0.14 mg/ml) and cardiolipin (0.04 mg/ml) at 4°C or 37°C had no effect on the hemolytic activity of mumps virus.

Since it was thought that lecithin might be present on the erythrocyte surface as a "coating" after washing of the erythrocytes, it was of interest to see if there was any specificity of inhibition of viral hemolysis associated with the nature of a substance coating the surface of an erythrocyte. A purified polysaccharide† prepared from *Escherichia coli,* which was shown to coat erythrocytes by rendering them agglutinable by a homologous *E. coli* immune serum, was allowed to react with chicken erythrocytes in the same manner as lecithin. These coated erythrocytes were hemolyzed and agglutinated by mumps virus in an unimpaired manner though their surfaces were coated by the polysaccharide, since they were readily agglutinated by the *E. coli* antiserum.

4. *Action of inhibitors after virus was adsorbed to erythrocytes.* In the experiments described to this point, the inhibitors were always present at the time of adsorption of virus to the erythrocyte, and it is of importance to determine whether or not they can interfere with the viral hemolytic reaction after this combination has occurred. Virus was allowed to interact with erythrocytes, as described herein, at 4°C which permits adsorption to red cells but no hemolysis(2,3). These erythrocytes were then washed to remove any virus not attached to the red cells and the inhibitors added in appropriate concentrations with the results presented in Table IV.

With addition after adsorption of virus to the erythrocyte, none of the inhibitors had any effect on viral hemolysis in concentrations studied, though they were present in the suspending fluid during the period of incubation at 37°C when hemolysis occurred.

Discussion. Since cholesterol and lecithin are believed to be components of the outermost surface of the red cell wall, their inhibitory effects on hemolysis by mumps virus are of special interest. In concentrations of 0.4 mg/ml of lecithin and 0.09 mg/ml of cholesterol these compounds reduced the hemolytic activity of mumps virus. The specificity of this inhibitory action is subject to question, however, since cardiolipin, a phospholipid isolated from heart tissues, also has been shown to inhibit the mumps hemolysin.

If, as earlier studies suggest, this viral hemolysin is an enzyme(1-5), it may be that in attacking the cell wall of the erythrocyte it has some special affinity for the constituent cholesterol or lecithin. These substances, if present in the reactive hemolytic system, possibly combine with the viral hemolysin first and thus render it unable to affect the erythrocyte surface. Since hemagglutination takes place in an unimpaired manner in the presence of lecithin, it is again suggested, in line

† Obtained through the courtesy of Mr. B. B. Wiley.

TABLE IV. Effect of Preadsorption of Virus to Erythrocytes upon Inhibitory Capacity of Compounds.

Erythrocytes	Inhibitor		Hemolysis test: Dilutions of virus fluid 2	4	8	16	32	64	128	Cell control	Hemagglutination test: Titer	Cell control
		mg/ml										
Virus preadsorbed	0	.0	52*	44	40	37	20	16	10	0	512†	—
Idem	Lecithin	.4	50	39	33	22	16	12	0	0	256	—
	Cholesterol	.07	52	43	35	24	19	13	10	0	>512	±
	Cardiolipin	.05	56	46	36	26	19	11	0	0	>512	+

* % of hemolysis determined colorimetrically.
† Reciprocal of highest dilution of virus producing hemagglutination.
—, No hemagglutination; ±, slight hemagglutination; +, clear-cut hemagglutination.

with previous experimental evidence(2,3) that hemolysis occurs subsequent to hemagglutination during elution of virus from the cell. When the inhibitors are allowed to come in contact with the virus before addition of the erythrocytes, their suppressive reaction is enhanced and most markedly with lecithin, which suggests that the viral hemolysin does react with lecithin and cholesterol. Furthermore, lecithin appears to act by binding the virus particle in some way to prevent hemolysis without affecting its adsorption to the red cell surface, resulting in hemagglutination.

If lecithin is an important reactive constituent of the erythrocyte surface for viral hemolysis, "coating" of the cell with excess lecithin should reduce hemolysis. Experiments carried out in this study showed that pretreatment of erythrocytes with lecithin at 37°C rendered them less susceptible to hemolysis by mumps virus, but similar exposure at 4°C was without effect, as was also similar treatment at 37°C with cholesterol or cardiolipin. If lecithin does "coat" the erythrocytes, the temperature at which exposure occurs appears important. Absence of any effect on the susceptibility of the erythrocytes treated with lecithin to hemagglutination by the virus indicated that virus could still combine with the erythrocytes. In addition, these experiments demonstrated that virus also eluted in an unimpaired manner from these treated red cells. Furthermore, a polysaccharide prepared from *E. coli,* which could be shown to "coat" the erythrocyte, had no effect on viral hemolysis, indicating some specificity for the action of lecithin.

The role of the inhibitors was further clarified by the experiments in which virus was allowed to combine with erythrocytes before the inhibitors were added and the temperature raised to levels permitting the hemolytic action to occur. All 3 substances had no significant effect under these conditions. This suggests that the virus had already combined with the critical elements in the erythrocyte surface and thus hemolysis could occur as the virus eluted from the erythrocyte(2,3), in spite of the presence of the inhibitory compounds.

In all of these experiments the selective inhibitory effect of lecithin on the viral hemolysin as contrasted to the viral hemagglutinin supports the concept that these are separate and distinct properties of mumps virus.

Summary. 1. Lecithin, cholesterol, and cardiolipin reduce the activity of mumps virus hemolysin when present prior to but not after the combination of virus and erythrocyte and their inhibitory effects at minimal concentrations were shown to be additative. Lecithin does not affect the hemagglutinating activity of the virus, but the effects of cholesterol and cardiolipin cannot be ascertained as they agglutinate erythrocytes *per se.* Pretreatment of erythrocytes with lecithin but not with cholesterol or cardiolipin at 37°C rendered them less susceptible to lysis by mumps virus. 2. These results present additional evidence for the separate identity of the viral hemolysin and the viral hemagglutinin and suggest that, in the erythrocyte cell wall, lecithin and

cholesterol may be important constituents in its reaction with the viral hemolysin.

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Received July 14, 1954. P.S.E.B.M., 1954, v86.

Cholesterol Balance Studies in Mice with Modified Thyroid Activities.* (21237)

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It was observed recently in this laboratory (1), that in cholesterol-fed rats, thyroid administration significantly reduces plasma and liver cholesterol levels, in agreement with earlier findings(2-4). These effects cannot be attributed to a decrease in the rate of endogenous cholesterol formation, as thyroid hormone stimulates synthesis of the sterol(5-7). Therefore, the possibility was considered that mechanisms leading to an increased rate of sterol disposal are enhanced in the hyperthyroid state. Our work was undertaken to investigate, whether, in the mouse, the thyroid hormone accelerates the catabolism of cholesterol.

Methods. The total cholesterol balance was determined essentially as described recently (8). In the first experiment, female mice, 5-6 weeks old, were used.‡ All animals were fed purina chow supplemented with 1% sulfasuxidine and 0.05% streptomycin§ for one day prior to the experimental run, to prevent or reduce cholesterol destruction by the intestinal flora(8). During the entire balance period of 16 days, all mice of both experimental groups were kept individually in metabolism cages, and fed a diet containing the components mentioned above, 0.65% cholesterol, and other supplements as indicated.‖ In addition, the animals of the hyperthyroid group were given, at onset, one intraperitoneal injection of 50 μg of thyroxine, and then, fed 0.4% desiccated thyroid USP with their diet during entire balance period. In a second experiment, male mice (Swiss Colony Strain)¶ 5-6 weeks old were used. Prior to the experimental run, all animals were fed Purina Chow supplemented with 0.5% thiouracil** and the mentioned

* This investigation was supported in part by research grants from the National Heart Institute, National Institutes of Health, U. S. Public Health Service. The results were presented in part at the meeting of Pacific Slope Biochemical Conference, Los Angeles, Dec. 29-30, 1953.

† Predoctoral fellow of Life Insurance Medical Research Fund.

‡ Mice were obtained from California Caviary, Los Angeles.

§ Sulfasuxidine—Sharp and Dohme, Division of Merck and Co., Streptomycin hydrochloride—E. R. Squibb and Sons.

‖ Experimental diet: Purina chow (ground), 81.35%; yeast (strain G, Anheuser-Busch), 10%; dextrin, 2%; Wesson oil, 4.5%; cholesterol, .65%; desiccated ox bile (USP), .25%; sulfasuxidine, 1%; streptomycin, .25%. Cholesterol was dissolved in Wesson oil before being mixed with the diet.

¶ The authors are indebted to Dr. H. E. Pearson, Department of Medical Microbiology, for supplying these animals.

** The authors are obliged to the Lederle Laboratories Division, American Cyanamid Co., for providing this material (Deracil).