STUDIES ON MUMPS VIRUS: THE RELATIONSHIP OF COMPLEMENT FIXING AND HEMAGGLUTINATING PROPERTIES TO VIRUS PARTICLES

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STUDIES ON MUMPS VIRUS: THE RELATIONSHIP OF COMPLEMENT-FIXING AND HEMAGGLUTINATING PROPERTIES TO VIRUS PARTICLES

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INTRODUCTION

From the days of Hippocrates to the present, man has been the victim of what is commonly referred to as "the mumps". The infection, as it is ordinarily encountered clinically, produces swelling of the parotid and/or submaxillary glands, which may be unilateral only, bilateral, or infection of first one gland followed within a period of three to five days by infection of the second gland. Classical mumps was described in the writings of Hippocrates (Adams-1886) in 500 B.C., and this manifestation of the disease is today the most commonly recognized symptom of infection. Recently it has been shown that mumps virus may be responsible for pancreatitis, orchitis, oophritis, and meningoencephalitis.

The agent responsible for infection was not definitely known, and at one time it was believed to be caused by a spirochete, as reported by Kermorgant (1925). These organisms were present in the sediment of washings of the buccal cavity of mumps patients. When cultures of the spirochetes were inoculated into Stensen's duct or into the parenchyma of the parotid gland of monkeys, a parotitis resulted which was not unlike that produced by inoculation of untreated saliva of the patients. He was not able to pass the infection serially in monkeys.

The role of the spirochete as the etiologic agent of mumps was short lived. Pontano (1930) and Rocchi (Gordon, 1940) both refuted this theory.

They proved that spirochetes were present in the saliva of individuals not infected with mumps and that the organisms were a frequent inhabitant of the buccal cavity.

With the turn of the twentieth century there gradually appeared a number of publications with reference to mumps. Granata (Gordon, 1940) was probably the first to suggest that the causative agent was viral in nature. Employing rabbits as a host, he was able to produce fever and swelling of the parotid glands by inoculation of bacteriologically sterile filtrates of saliva obtained from individuals suffering from the disease. Nicolle and Conseil (1913) were able to achieve what they considered typical swelling of the parotid gland of a monkey, accompanied by fever and a monocytosis of the blood by inoculation of aspirated fluid from an infected patient. This work was closely followed by that of Gordon (1914) who also used monkeys for test. Intracerebral inoculations of saliva from patients with mumps, rendered free of bacteria by filtration produced a fatal meningitis. Subsequent attempts to pass the agent to other monkeys resulted in failure. Intraperitoneal inoculation of a monkey produced typical parotid swelling.

Definite progress in cultural methods was achieved by Wallstein (1916). Using a bacteria free filtrate of saliva from children in the acute infectious stage of parotitis, she was able to reproduce in cats, by intraparotid and intratesticular injections, swelling of the respective gland. Rise in temperature and leucocytosis preceeded the onset of swelling. Serial passage through cats increased the virulence of the agent. Neutralization tests were also performed employing serum from animals which had recovered from the infection and an emulsion of either infectious testicle or parotid gland. Injection of the serum and virus greatly reduced or prevented completely the changes which had been noted previously when antigen was inoculated singularly. At a later date, through intrathecal injection of filtered saliva, a meningitis was induced. The condition was transmissible to other animals by injection of cell free cerebrospinal fluid.

It remained for Johnson and Goodpasture in 1934 to prove definitely that the infectious agent of mumps was viral in nature. Saliva was secured from patients in the acute stage of infection, filtered through Burkefeld V and N filters, and inoculated into Stenson's duct of the parotid gland of rhesus monkeys. Following inoculation, parotitis resulted in the monkeys from four out of six specimens inoculated. Specimens of saliva from so-called normal individuals did not produce parotitis. Serial passage of the virus in monkeys was effected by inoculation of suspensions of infected parotid gland removed from animals in the height of disease.

Monkeys which had recovered from the infection were found to be immune to reinfection with mumps. Very limited neutralization tests employing serum from mumps convalescent patients did show some inhibition of the virus.

The possibility that the virus being studied was that of herpes simplex was obviated by cutaneous, corneal, and intracerebral inoculation of rabbits. No nuclear inclusions were observed in the lesions. These experiments also ruled out possible vaccinia virus, as monkeys immune to mumps remained susceptible to vaccinia. Histologically, the virus showed a predilection for parenchymal cells of the parotid gland of monkeys. No structures were observed which could be called microorganisms. Spirochetes were not found in sections stained by Levaditi's method, nor were they found in dark field preparations of fresh lesions.

Johnson and Goodpasture (1935) continued their experiments in serial passage, using a single strain of virus through 14 successive generations in monkeys in a period of 16 months. An acute, non-suppurative parotitis was produced by the mumps virus. At the end of 14 months and 11 generations in monkeys, human volunteers were inoculated by spraying the oral cavity with an emulsion of infected parotid gland of a monkey. Susceptible individuals for tests were selected from an area believed to have been free of mumps. Persons which had had typical clinical parotitis were included as immune controls. Of the 13 theoretically susceptible children inoculated, 6 developed typical mumps, 3 showed questionable symptoms, and 4 showed no apparent symptoms as was the case also with four immune volunteers, which were inoculated.

These investigators had thus been successful in isolating mumps virus from saliva of persons ill with clinical mumps by inoculation of monkeys and transmitting the infection serially in monkeys. Through inoculation of human volunteers with infected parotid gland of the monkey they proved that the virus in experimental parotitis in the monkeys was analogous to the agent causing mumps infection in man.

Findlay and Clark (1934) confirmed the findings of Johnson and Goodpasture. They obtained two specimens of saliva from mumps patients, one collected 48 hours after onset and the second obtained 72 hours after onset. The specimens were filtered and inoculated directly into Stenson's duct of rhesus monkeys. From the specimen collected at 48 hours after onset, parotitis resulted, while the 72 hour specimen was not infective. They were able to pass the virus successfully through six generations in monkeys. Swelling of the parotid glands and histological changes similar to those seen in man were observed accompanied by a leucopenia. Injection of the viral material into the tunica vaginalis produced an orchitis. Intracerebral inoculation of mice, rats, and guinea pigs proved to be innocuous as no symptoms or changes were observed.

A group of French scientist, Levaditi, Martin, Bonnefoi, and Schoen (1935) were rather skeptical regarding the results they obtained with experimental parotitis. They used monkeys and chimpanzees for test, and confirmed the histological findings of Findlay and Clarke (1934). Difficulty was encountered in serial passage of the viral material, but it was accomplished. Intravenous and intranasal inoculation of the viral material was not effective in producing parotitis. When healthy chimpanzees were placed in contact with infected animals, they did not contract the disease. Inoculation of a single parotid gland did not result in bilateral infection. Since indefinite and minor changes were observed when presumable inert material was inoculated, this group of workers agreed only to the effect that a non-suppurative parotitis transmissible in series could be produced when saliva from patients ill with mumps was inoculated into monkeys.

Means of propagation of the virus were limited to the monkey until the work of Habel (1945). He was able to show multiplication of the virus when the yolk sac, amniotic cavity and allantoic cavity of embryonated eggs were injected with infected inoculum. This work was confirmed by Levens and Enders (1945) and also by Beveridge, Lind and Anderson (1946).

Hirst (1941), working with influenza viruses types A and B showed that red blood cells were agglutinated by these viruses. The amount of virus present could be measured by the hemagglutination titers obtained, and it was observed that hemagglutination was inhibited by specific antisera (1942). Hirst also was able to adsorb the virus onto erythrocytes (1942). Burnet (1942) found that there was a striking resemblance between Newcastle disease virus and that of influenza in that Newcastle disease virus also agglutinated red blood cells from several species of animals which had been found to be true of the influenzas. The two viruses were neutralized by specific immune sera. In addition to the viruses already named, Clark and Nagler (1943) added vaccinia virus as another one capable of agglutinating fowl cells. It was soon learned that mumps possessed physical properties very similar to those of the Newcastle disease-influenza group. Enders <u>et al</u> (1946) showed that the hemagglutinating and complement fixing antigens appeared at different times in the embryonated egg, but that a correlation could be drawn between the two titers. Since no lesions are produced on the membranes and the embryo is not killed by mumps virus, a rapid test for infectivity can be performed by testing supposedly infected fluids for hemagglutinins. This procedure was used by Beveridge, Lind and Anderson (1947) and Leymaster and Ward (1947) as a means of detecting infectivity. Both groups of scientist isolated mumps virus by inoculating saliva from patients in the acute stages of clinical mumps directly into embryonated eggs. Patients were from widely separated geographical areas and strain variations were not observed, such as those which have been shown for instance with influenza (Friedewald, 1944)

A number of studies were undertaken with regard to immunity in which complement fixation tests were used to determine the amount of antibody present. Enders & Cohen (1942) found that sera from monkeys drawn prior to inoculation with mumps failed to fix complement, while sera drawn during convalescence showed varying capacities to fix complement. Habel (1945) using the complement fixation technique described by Enders, (1942), tested human sera with antigens derived from embryonated eggs for presence of antibody and virus. He concluded that virus propagated in chick embryos was not only a good source of antigen but useful also as a source for diagnostic skin test antigen. A series of studies on immunity was begun by Enders _et_ _al_ (1945). Using monkeys as test animals, he found that a specific antibody appeared in the blood in from 8 to 14 days following infection. A decrease was shown with lapse of time, and some antibody persisted over a period of many months. With this information, the resistance of vaccinated monkeys was estimated and then challenged with a vaccine prepared from formolized infected parotid gland. Approximately 60 percent of the monkeys showed increased resistance while others failed to show resistance even though antibody was present. They concluded that resistance cannot be based solely on the presence of complement fixing antibody, but that it could not be excluded as a possible factor.

Further studies by Enders (1945) revealed that complement fixing antibody regularly appeared or increased in the sera of humans in the acute or convalescent phases of mumps. Upon injection of heat-inactivated mumps virus, convalescent mumps patients developed dermal hypersensitivity. Both of these reactions were observed in clinically inapparent mumps infections. These findings proved to be of particular diagnostic value with regard to acute aseptic meningo-encephalitis which were not accompanied by enlargement of the salivary gland in many instances as shown by Kane and Enders (1945). In a study of 51 patients, 33 individuals developed complement fixing antibodies in titers compatible with recent infection with mumps virus. Of the 33 patients, 16 showed symptoms of meningo-encephalitis only, while in the remaining 17, the same condition was subsequently accompanied by enlargement of the salivary glands. Eighteen patients in the group studied gave no evidence of recent mumps infection with the complement fixation test. Indications of meningo-encephalitis having been caused by mumps were reported earlier on the basis of clinical picture, history of exposure and spinal fluid findings by Paddock (1932), Montgomery (1934), Silwer (1936), Finkelstein (1938), Harris and Bethel (1938), and Tabor and Newman (1940).

Swan and Mawson (1943) recovered mumps virus from spinal fluid and passed it serially in monkeys, but complement fixation tests were not done. Thus Kane and Enders proved by the use of complement fixation tests what had been believed to have been true for several years. Henle et al (1947) reported the presence of a soluble mumps antigen which could be removed by differential centrifugation. In addition, Henle and McDougal (1947) isolated mumps virus from spinal fluid in two instances of meningo-encephalitis and in the first infection confirmed their isolation with complement fixation tests, neutralization tests in eggs, and after the 5th passage of the virus in eggs they subjected four supposedly susceptible individuals to the virus with resultant parotitis developing in two of the individuals. Other instances of mumps meningo-encephalitis confirmed by complement fixation have been reported more recently (Kravis et al, (1951); Kilham, (1949).

These are some of the most interesting highlights with regard to isolation of the etiologic agent, cultural methods of propagation, and development of diagnostic procedures for laboratory diagnosis of the infection.

The purpose of this study is to observe the complement fixing and hemagglutinating properties of mumps virus in relation to infectivity capacities of infected untreated fluids, ultracentrifuged infected fluids and sonic vibrated-ultracentrifuged infected fluids, using embryonated hen eggs as hosts.

MATERIALS AND METHODS

An egg adapted strain of mumps virus was obtained from Dr. John F. Enders, Childrens Hospital, Boston, Massachusetts. Varying dilutions and amounts of seed virus were inoculated via the amniotic route into seven-day old embryos. To reduce contamination, 100µg streptomycin sulfate and 500 units penicillin G were added per ml physiological saline-virus solution inoculum. The antibiotics in this amount had no effect on the virus or host cells. Eggs were incubated at 38 C prior to inoculation, followed by incubation at 35.5 to 36 C after inoculation for a period of 96 hours. The embryos were then placed in the refrigerator at 4 C overnight and the amniotic and/or allantoic fluids harvested on the following morning.

Infectivity was measured by hemagglutination tests. Preliminary tests were made on the fluids of each embryo, using an equal volume of amniotic and/or allantoic fluid and 0.5 percent chicken red blood cells. Controls for the test consisted of normal allantoic chicken red blood cell suspension and saline solution-chicken red blood cell suspensions. The tubes were incubated at room temperature and read when the controls had settled to form a dense button in the bottom of the round bottomed tubes. Those fluids which were found to be positive, exhibiting a thin even layer of cells over the entire bottom of the tube, were pooled from the various dilutions. Portions of the pool were separated for further testing, and the remainder of the pool stored at -70 C in a mechanical deep freeze. Serial two-fold dilutions of the infected fluid portions were made using physiological saline solution as a diluent. To 0.5 ml of each dilution of infected fluid was added 0.5 ml of 0.5 percent chicken red blood cells. The tubes were incubated at room temperature and read when the controls had settled.

It was found that 0.1 ml of $10^{1.9}$ dilution of the 42nd passage of chorio-allantoic seed virus produced the greatest infectivity curves as determined by the hemagglutination titers. This procedure was employed to obtain infected virus material for study.

At the time of test, fluids which had shown a hemagglutination titer of 1:128 or higher were removed from the deep freeze, and thawed in cold running water. In order to obtain a homogenous suspension for test, all the fluids were pooled. Clearing of urates and gross products of metabolism was accomplished by centrifugation in a refrigerated No. 2 International centrifuge equipped with a multispeed attachment at a speed of 6,900 RPM at 4 C for 10 minutes. The clear supernate was removed from the sediment and used for subsequent test.

Sonic treatment of the infected fluid was performed with a Raytheon type R-22-3, Serial No. D 305. The infected material was placed in the oscillation cup, and vibrated for one hour at maximum frequency with voltage maintained at 130. The capacity of the oscillation cup was approximately 60 ml, and thus it was necessary to make two runs in order to obtain a sufficient amount of material for ultracentrifugation.

The sonic vibrated fluid and untreated fluid were transferred to tubes for ultracentrifugation. The tubes had previously been sterilized overnight by exposure to ultraviolet light in a dust proof chamber. Two tubes were filled with each of the two fluids, balanced, and placed in the chilled Rotor B of a Spinco Ultracentrifuge model E. The specimens were spun at a speed of 42,000 RPM (117,000 x g) for one hour. Temperature variation was between 4 and 7 C.

At the end of one hour, one tube of sonic vibrated fluid and one tube of untreated fluid was removed. From each tube approximately 5 ml of the top supernatant fluid was removed for assay. The remaining fluid was then withdrawn, after which the sediments were resuspended in approximately 5 ml of the lower supernatant fluid. Hereafter, these fractions will be designated as Ultracentrifuge Supernate 1, Sonic-vibrated Supernate 1, Ultracentrifuge Sediment 1 and Sonic-vibrated Sediment 1. These portions were placed immediately in the deep freeze.

The entire supernatant fluid of each of the remaining specimens was removed and the sedimented pellets were then resuspended in cold physiological saline solution. The tubes were balanced, placed in the ultracentrifuge rotor, and recentrifuged for one hour as indicated previously. Temperature variation of this run was between 5 and 9 C.

After one hour centrifugation, the supernatant fluid was removed from each tube and the pellets resuspended in approximately 5 ml of the lower supernatant fluid. These various fractions will be referred to as Ultracentrifuge Supernate 2, Sonic-vibrated Supernate 2, Ultracentrifuge Sediment 2, and Sonic-vibrated Sediment 2. All specimens were placed in the deep freeze until infectivity, hemagglutination, and complement fixation tests were completed.

Eggs for infectivity test were prepared for inoculation in the following manner. The eggs were incubated at 38 C for 6.5 days. At this time the temperature of incubation was reduced to 35.5 to 36 C, and continued for 12 hours. Relative humidity was in the neighborhood of 62 percent. The embryos were not rotated during incubation.

Eggs were candled using transillumination. Infertile or dead embryos were discarded. The position of the live embryos was marked and the air space outlined in relation to the shell. Holes for insertion of the inoculating needle were made about ½ inch above the embryo in the air sac area with a ball shaped carborundum needle attached to a dental drill. Care was taken to grind through the shell only, so that the underlying membrane was not penetrated and remained intact. The eggs were labeled as to dilution and fraction of material to be inoculated. Five eggs were prepared for each dilution. The eggs were placed in the incubator while appropriate dilutions of viral material were prepared for inoculation.

Cold sterile physiological saline solution was used as diluent for the infected viral fluid. It has been shown that polysaccharides which tend to inhibit multiplication of the virus by alteration of the host cells in such a way that multiplication does not occur, may or may not also affect the hemagglutination component (Ginsberg <u>et al</u>, 1948 a and b). Racks were filled with tubes containing volumes of saline solution for serial dilutions in what was anticipated to be the appropriate range for the various fractions to be inoculated. The dilution tubes in racks were refrigerated until used. The fraction of virus material to be inoculated into eggs was removed from the deep freeze, thawed in cold running water and the dilutions made. One-hundred µg streptomycin sulfate and 500 units penicillin G were added per ml of inoculum.

Immediately before inoculation, the crown of the egg was swabbed with a 5 percent solution of tincture of iodine. The shell membrane was penetrated with a sterile tapered dissecting needle. Sterile 26 gauge, two inch needles, and 1 ml tuberculin syringes graduated in hundredths were used for inoculation. To avoid possible transfer of contaminants from one dilution to another, a sterile needle and syringe was used for each dilution.

The egg was held in front of the egg candler at a 45 degree angle, the needle inserted into the amniotic sac and 0.1 ml inoculum was injected. When all of the eggs were inoculated, the crown of the egg was again swabbed with disinfectant. The holes in the shell were sealed with a thin layer of melted paraffin after which the embryos were placed in the incubator.

All eggs were candled daily, and any dead embryos were placed overnight in the refrigerator and the amniotic and/or allantoic fluids harvested on the following day. The procedure used for harvesting is described as follows. The upper half of the egg was first swabbed with disinfectant. The shell over the air sac was removed by cutting with curved surgical scissors slightly above the line made on the shell at initial candling. The shell membrane was grasped with a pointed forcep and removed. Fluid from the allantoic sac was removed using sterile 17-19 gauge needle and 5 ml syringe. This left the amniotic sac more or less suspended, and by lifting the chorioallantoic membrane with a pair of forceps, the fluid from the amniotic sac could be removed with sterile needle and syringe. The fluid from each egg was kept separate until preliminary hemagglutination tests were done as described earlier. During the time of harvest and preliminary testing, the fluids were maintained in an ice bath so that hemagglutination and complement fixation titers would not be lowered. Fluids which exhibited hemagglutination were pooled and serial two-fold hemagglutination tests performed on each dilution of the various fractions as indicated earlier.

Complement fixation tests were performed as follows. Commercial amboceptor and lyophilized guinea pig complement were used in the tests. Preliminary tests for amboceptor activity were first done using serial 10-fold dilutions of 1:1,000 through 1:10,000 in the presence of an excess of complement diluted 1:30. Two-tenths ml complement was pipetted into 12 Kahn tubes, after which 0.1 ml of each dilution of amboceptor was added. One-tenth ml freshly prepared 2 percent sheep red blood cells was added to each tube. To standardize this procedure with that of the test proper, 0.2 ml buffered saline solution was added to each tube, to replace the antigen-antiserum volume. The test was incubated for 30 minutes in the 37 C water bath and read immediately. The highest dilution showing complete hemolysis was taken as one unit. Two exact units were used in the complement titration.

Having determined the dilution of amboceptor to use, the complement titration was then carried out. Equal volumes of the appropriate dilution of amboceptor and 2 percent sheep red blood cells were mixed and incubated at room temperature for 15 minutes. Two rows of twelve tubes were placed in a rack. One-tenth ml antigen was added to each tube in the second row. Complement was prepared in serial dilutions of 1:10 to 1:100. Two-tenths ml of each dilution of complement was added, 0.2 ml sensitized cells, and then 0.2 ml saline solution were added to each tube in the first row, and 0.1 ml saline solution to each tube in the second row. The test was placed in the 37 C water bath, incubated for 30 minutes and read. The end point of complement activity or the highest dilution effecting complete hemolysis was noted. To allow for decreased activity in the overnight incubation test, two full units, or twice the amount of the next lower dilution than that exhibiting complete hemolysis was used in the test proper. The complement titration in the presence of antigen should coincide with the result obtained by straight complement titration.

In order to determine the complement fixing titer of various fluids in this study, it was necessary to obtain sera from mumps patients. These sera were diluted 1:2, inactivated at 60 C for 20 minutes, and absorbed. The viral antigen used for standardization of antisera was from commercial source. Serial two-fold dilutions of the antisera were prepared and 0.1 ml of each dilution pipetted per tube. One-tenth ml viral antigen, which had been standardized previously, was then added to each dilution of antisera. Two-tenths ml of complement was added per tube. Controls consisted of each antigen, antiserum, and known positive antiserum combined with the antigens, complement in 2 units, one unit and ½ unit, and also lysin. The reagents were mixed by shaking the rack, and then placed in the refrigerator at 10 C for 15 to 18 hours. On the following day the test was completed.

Since the sensitivity of the red blood cells varies from day to day, hemolysin titrations were repeated approximately one hour before refrigerator incubation was completed. Three rows of ten tubes containing 0.2 ml complement in 2 units, 1 unit, and ½ unit were prepared the day before with the controls. To these tubes were added 0.2 ml sensitized sheep red blood cells in serial 10 fold dilutions of 1:1,000 to 1:10,000 which had been incubated at room temperature for 15 minutes. The tubes were incubated in the 37 C water bath for 30 minutes and read. The highest dilution which elicited complete hemolysis in the presence of two units and one unit of complement, and little or no hemolysis in the presence of one-half unit of complement was the dilution used to complete the test.

Sufficient volume of sensitized cells were prepared. While they were incubating, the test itself was removed from the refrigerator and placed at 37 C for 15 minutes. Two-tenths ml sensitized cells were then added to each tube, after which the test was incubated in the 37 C water bath for 30 minutes, and read. The titers of antisera obtained in this test were used in subsequent tests for determining complement fixing activity of ultracentrifuged and sonic-vibrated antigens.

Fluids harvested from the eggs inoculated with the original infected fluid as well as both the ultracentrifuged and sonic vibrated-ultracentrifuged material were tested for complement fixing properties using the method just described.

RESULTS

Mumps infected fluid was used as a control for the ultracentrifuged and the sonic vibrated-ultracentrifuged fluids. Complement fixation tests on the control fluid showed that complete fixation occured in the 1:2 dilution with viral antisera. The supernatant fluids of both cycles of ultracentrifuged fluid failed to fix complement. The first sediment showed complete fixation in the 1:16 dilution with viral antisera. The second sediment failed to fix complement in the presence of antiserum.

The first sediment of the sonic vibrated-ultracentrifuged material showed a titer of 1:4 with viral antisera. Complete hemolysis resulted in tests on the first and second supernates and the second sediment. (Table I).

These results indicate that with weak virus suspensions the complement fixing antigens are removed from the supernatant fluid with a resultant eight-fold concentration of the virus in the sediment of first cycle ultracentrifugation as compared with the control. Recentrifugation appears to destroy the complement fixing properties under the condition of test. Sonic vibration followed by ultracentrifugation greatly reduces the property to a two-fold increase over the original suspension.

Infectivity tests were computed according to Reed and Muench (1938). The original control fluid produced in eggs contained $ID_{50\%}$ $10^{2.5}$.

TABLE I

RESULTS OF COMPLEMENT FIXATION TEST ON MUMPS VIRUS FRACTIONS

Virus Fraction	Reciprocal of virus dilution showing fixation with virus antiserum
Untreated control	2
Ultracentrifuge Supernate 1	-
Ultracentrifuge Sediment 1	16
Ultracentrifuge Supernate 2	-
Ultracentrifuge Sediment 2	-
Sonic vibrated Supernate 1	-
Sonic vibrated Sediment 1	4
Sonic vibrated Supernate 2	-
Sonic vibrated Sediment 2	-
Non-infected Control	-
Viral antigen Control	16
Soluble antigen Control	-

The first supernatant fluid of the ultracentrifuged material was infective to $ID_{50\%}10^{1.48}$, while the first sediment had an $ID_{50\%}$ $10^{3.0}$. Thus in the first cycle of ultracentrifugation, infectivity in the supernatant fluid was decreased to one-tenth that of the control, while the sediment was increased slightly over three-fold. The second supernatant fluid showed no infectivity in eggs. The second sediment was infective to $ID_{50\%}$ $10^{2.7}$. Comparison with the first concentration shows that only half of the virus was recovered when it was subjected to recentrifugation.

Sonic vibrated-ultracentrifuged material, when inoculated into eggs, produced $ID_{50\%}$ $10^{1.5}$ from the first supernatant fluid, and $ID_{50\%}$ $10^{2.6}$ from the first sediment. No infectivity resulted upon inoculation of the second supernatant fluid into eggs. The second sediment had an $ID_{50\%}$ $10^{1.9}$. It appears that ultravibration disrupts the virus particles to incomplete degradations which no longer possess the characteristic of infectivity. Part of the reduction in activity is to be expected from the techniques of handling. See Table II.

Hemagglutination and complement fixation tests were performed on the fluids harvested from the various fractions which were inoculated for infectivity tests. Hemagglutination titers were found to vary considerably in fluids harvested from different dilutions, however, complement fixation titers were much more consistent.

It is of interest to note that the second supernatant fluid of the sonic vibrated-ultracentrifuged suspension showed no infectivity nor hemagglutination; however, complement fixing antigens were present

TABLE II

INFECTIVITY TEST OF VIRUS FRACTION TESTED IN EGGS

Fraction	Log of virus dilution									
	$10^{1.0}$	$10^{1.3}$	$10^{1.6}$	$10^{1.9}$	$10^{2.2}$	$10^{2.5}$	$10^{2.8}$	$10^{3.1}$	$10^{3.4}$	$ID_{50\%}$
Untreated Control			5/5	5/5	5/5	2/5	1/5			$10^{2.5}$
Ultracentrifuged Supernate 1	5/5	5/5	1/5							$10^{1.48}$
Ultracentrifuged Sediment 1			5/5	5/5	4/5	5/5	5/5	2/5	0/5	$10^{3.0}$
Ultracentrifuged Supernate 2	0/5	0/5	0/5							0
Ultracentrifuged Sediment 2			5/5	5/5	5/5	5/5	3/5	3/5	0/5	$10^{2.7}$
Sonic vibrated Supernate 1	5/5	4/5	2/5							$10^{1.5}$
Sonic vibrated Sediment 1			4/5	3/5	4/5	1/5	1/5	1/5	1/5	$10^{2.6}$
Sonic vibrated Supernate 2	0/5	0/5	0/5							0
Sonic vibrated Sediment 2			2/5	1/5	0/5	0/5	0/5	0/5	0/5	$10^{1.9}$

in this fraction. Whether this antigen is incomplete virus which has separated from the particle itself and capable only of complement fixation, or if it is a soluble antigen cannot be said with definity.

The hemagglutination titer of the original control fluid was 1:128. Ultracentrifugation at 117,000 times gravity for one hour completely removed the hemagglutinative agent from the supernatant fluid as tests on this fraction were negative on the undiluted fluid. The titer of the first sediment was 1:512, showing that concentration by ultracentrifugation increased four times the hemagglutinative property. The second supernate failed to agglutinate the cells, as was true of the first supernate, indicating that enough virus particles, causing hemagglutination, were removed by centrifugation to prevent hemagglutination. The second sediment had a titer of 1:32. These results are shown in Table III.

Sonic vibrated-ultracentrifuged fluid reacted in the same over-all manner as the plain ultracentrifuged fluid but to a much higher titer. Both the first and second supernate were negative in the undiluted fluid. The first sediment showed complete agglutination of red cells in the 1:2048 dilution, while the second sediment had a titer of 1:128. These titers were four times as great as those found in the sediments of the same fluid which had been treated by ultracentrifugation only.

Results of complement fixation, hemagglutination and infectivity tests performed on various fractions are summarized in Table IV.

TABLE III

HEMAGGLUTINATION TESTS ON SONIC AND NON-SONIC ULTRACENTRIFUGED FRACTIONS OF MUMPS VIRUS

Fraction	Reciprocal of Virus Dilution												
	Und.	2	4	8	16	32	64	128	256	512	1024	2048	4096
Untreated Control	+	+	+	+	+	+	+	+	-	-	-	-	-
Ultracentrifuge Supernate 1	-	-	-	-	-	-	-	-	-	-	-	-	-
Ultracentrifuge Sediment 1	+	+	+	+	+	+	+	+	+	+	-	-	-
Ultracentrifuge Supernate 2	-	-	-	-	-	-	-	-	-	-	-	-	-
Ultracentrifuge Sediment 2	+	+	+	+	+	+	-	-	-	-	-	-	-
Sonic vibrated Supernate 1	-	-	-	-	-	-	-	-	-	-	-	-	-
Sonic vibrated Sediment 1	+	+	+	+	+	+	+	+	+	+	+	+	-
Sonic vibrated Supernate 2	-	-	-	-	-	-	-	-	-	-	-	-	-
Sonic vibrated Sediment 2	+	+	+	+	+	+	+	+					
Non-infected Control	-	-	-	-									

TABLE IV

SUMMARY OF INFECTIVITY, HEMAGGLUTINATION AND COMPLEMENT FIXATION TESTS ON VARIOUS SONIC-NONSONIC ULTRACENTRIFUGED FRACTIONS

Antigen Fractions	Egg Infectivity $ID_{50\%}$	Hemagglutination	Complement Fixation
Control	$10^{2.5}$	1:128	1:2
Ultracentrifuge Supernate 1	$10^{1.48}$	-	-
Ultracentrifuge Sediment 1	$10^{3.0}$	1:512	1:16
Ultracentrifuge Supernate 2	-	-	-
Ultracentrifuge Sediment 2	$10^{2.7}$	1:32	-
Sonic vibrated Supernate 1	$10^{1.5}$	-	-
Sonic vibrated Sediment 1	$10^{2.6}$	1:2048	1:4
Sonic vibrated Supernate 2	-	-	-
Sonic vibrated Sediment 2	$10^{1.9}$	1:128	-

DISCUSSION

It has been shown that mumps infected allantoic-amniotic fluid of embryonated eggs when treated by ultracentrifugation is rendered free of demonstrable hemagglutinating and complement fixing components in the first supernatant fluid. Active virus remains which is capable of producing one-tenth the infectivity titer of the original untreated fluid when inoculated into embryonated eggs. The sedimented viral pellets exhibit infectivity, hemagglutination and complement fixation. In the second cycle supernatant fluid, hemagglutination, complement fixation and infectivity are all absent. Hemagglutination and infectivity are both demonstrated in the second cycle sediment, but to lower titers than those found in the first sediment. Complement fixing antigen is no longer active.

From these results, the infectious particle and component responsible for hemagglutination are sedimented in a high gravitational field in a similar manner. These two agents appear to be very closely related. Complement fixing antigen is also concentrated in like manner, but under the conditions of test it could not be demonstrated after recentrifugation. This is undoubtedly due to the relative instability of the infectious particle when subjected to ultracentrifugation.

Mumps virus fluid when treated with ultravibration-ultracentrifugation produced much higher hemagglutination titers than those encountered in ultracentrifuged fluid. It is believed that the increase is caused by freeing of the hemagglutinative component from the virus particle as well as the effect of high frequency waves on destruction of inhibitors present in non-infected amniotic-allantoic fluids. This is substantiated by Penttinen (1951) who observed similar results.

Infectivity titers of the sediments of vibrated fractions were lower than those present in plain ultracentrifuged fractions. Even though the fluids were kept cold during oscillation, it would appear that the disruption of virus by ultravibration combined with ultracentrifugation tends to reduce infective properties. The infectivity titer of the first supernatant vibrated fluid was slightly higher than that of the ultracentrifuged suspension. The presence of complement fixing antigens in the second supernatant fluid may be attributed to incomplete viral material which after alteration by ultravibration was not sedimented in the gravitational field. Relatively high hemagglutination titers were present in the supernatant fluids of both fractions. Under the conditions of test, these results closely parallel those of Henle _et_ _al_ (1947).

SUMMARY

Complement fixing properties of mumps virus were studied with regard to its relationship to virus particles as determined by infectivity and hemagglutination. Amniotic-allantoic infected fluids were treated by ultracentrifugation and sonic vibration-ultracentrifugation.

Complement fixing antigens, hemagglutination components, and infective particles were all concentrated in high gravitational fields. Supernatant fluids of both fractions were negative for complement fixation and hemagglutination, however, infectious particles remained in the first supernatant fluids. First cycle sediments of both fractions exhibited complement fixation, infectivity and hemagglutination. Second cycle supernatant fluids of the two fractions were negative in hemagglutination and infectivity tests. The ultracentrifuged fraction was also negative in complement fixation tests, but positive complement fixation tests were demonstrated in the vibrated suspension. Recentrifugation of the fractions resulted in positive hemagglutination and infectivity reactions, but negative complement fixation results.

Mumps virus is relatively unstable when subjected to ultracentrifugation and ultravibration. Complement fixing antigens, hemagglutinative components and infective particles, although very closely related, appear to be distinct and separable entities.

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