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ABSTRACT

LATEX AGGLUTINATION OF POLIOVIRUS INFECTED

AND UNINFECTED TISSUE CULTURE FLUIDS

By William F. Hill

Neutralization, complement fixation, and precipitin tests have been applied to studies of poliovirus. The first two have been used most often in epidemiologic and diagnostic work. The precipitin test has been little used. Reports have appeared in the literature (Smith et al, 1956; LeBouvier et al, 1957; Grasset et al, 1958) which indicated that under certain conditions poliovirus antigens were precipitable when mixed with specific antisera. The application of a carrier particle technic to the poliovirus system was thought to be feasible. The recent introduction of the latex fixation test for demonstrating a variety of precipitable antigen-antibody reactions suggested that the latex particle could serve as the carrier for poliovirus. The major premise that underlies the kinetics of the latex fixation test was not incompatible with the kinetics of antigen-antibody precipitation reactions. Experiments were undertaken to study the problems associated with developing a serologic latex agglutination reaction for the identification of polioviruses.

At the beginning, results with the latex agglutination reaction suggested the presence of group specific poliovirus antigens.

These results were consistent with many reports in which a similar finding was obtained with polioviruses in the complement fixation test. Subsequent experiments indicated that the latex agglutination cross-reactions were due to host cell antigen-antibody systems. It was concluded that common antigens existed among HeLa, Hep 2, human amnion, and monkey kidney tissue cells. A latex agglutination reaction with poliovirus and its antibody could not be absolutely discounted but was not recognized in the presence of the cross-reacting host cell-antihost cell systems. Procedures designed to remove the host cell antigen-antibody system (ultracentrifugation, salt fractionation, pH adjustment) were unsuccessful. Absorption of the antisera with Hep 2 cell antigen removed the host cell antibodies. The absorbed antisera was latex agglutination negative when tested with poliovirus antigens.

Heterophil antibody activity was observed with antisera prepared in rabbits against the polioviruses and HeLa and Hep 2 cells. Latex agglutination could not be attributed to the presence of the heterophil antibody. The antibody agglutinated human red blood cells of A, B, and O groups. In addition, red blood cells of monkey, sheep, chicken, and Guinea pig were agglutinated. Absorption of the HeLa and Hep 2 cell antisera with guinea pig kidney antigen (Difco) removed the hemagglutinin completely. This indicated that the heterophil antibody was of the Forssman type.

A latex agglutination reaction adapted to the poliovirus-antibody system was not considered impossible. The results indicated that two conditions must be met before the technic may be successfully applied:

(1) concentrated viral antigens free from host cell antigenic components; and (2) specific viral antisera. Conceivably, with highly purified and concentrated viral antigens, a latex agglutination reaction specific for polioviruses would be predictable.

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By

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INTRODUCTION

Neutralization, complement fixation, and precipitin tests have been applied to studies of poliovirus. The first two have been used most often in epidemiologic and diagnostic work. The precipitin test has been little used. Reports have appeared in the literature which indicate that under certain conditions poliovirus antigens were precipitable when mixed with specific antisera. The application of a carrier particle technic to the poliovirus system was thought to be feasible. The recent introduction of the latex fixation test for demonstrating a variety of precipitable antigen-antibody reactions suggested that the latex particle could serve as the carrier for poliovirus. The major premise that underlies the kinetics of the latex fixation test (Oreskes and Singer, 1961) was not incompatible with the kinetics of antigen-antibody precipitation reactions. The essential difference was the visible manifestation of agglutination of the latex particles. This was a result of the antigen-antibody interaction at the particle surface.

The use of a carrier particle technic to increase the physical dimensions of a precipitable antigen-antibody reaction should be serologically profitable. There are certain limiting attributes associated with standard precipitation reactions: (1) low antibody titers cause difficulty in determining endpoints; (2) zone phenomena over a narrow range (excess antigen and/or excess antibody) inhibit the visible reaction; and (3) when the antigen mass is small as with viral antigens, an aggregate may not be visible. The use of a carrier (such as latex

particles) could eliminate some of the limiting characteristics of the standard precipitation reaction. Greater visibility would be obtained by the aggregated particles. Adsorption of viral antigens onto carrier particles could increase mass so that visible agglutination would result from the antigen-antibody union. If antigen mass is not critical then a carrier particle technic should increase the sensitivity so that less antibody would be required at a given antigen concentration. When the antigens are of viral dimensions, a carrier system could be a method for demonstrating specific anti-viral antibodies with a high degree of sensitivity. The use of latex particles as a carrier to show viral identity has not been reported. The three poliovirus types (Bodian et al, 1949) were selected for the investigation. The term antigen as used does not signify singular identity as such, but was used for convenience in designating a fluid containing virus or host cell substance.

In the beginning, it was hoped that cross-reactions of host cell antigens could be eliminated by propagating the viruses on different cell lines. Poliovirus used to prepare antibodies was propagated in HeLa cell cultures while poliovirus used for latex agglutination testing was propagated in Hep 2 or Rhesus monkey kidney cell cultures.

Preliminary determinations indicated that the $\log_{10}$ tissue culture dose 50 per cent (TCD_{50}) (Reed and Muench, 1938) was as acceptable for this study as the plaque forming unit (PFU) (Dulbecco and Vogt, 1954). Values for the virus concentration and neutralization index (50% NI) were obtained of all reagents.

REVIEW OF LITERATURE

The basis for undertaking this investigation was not without precedent. In 1922, Jacques Loeb established the groundwork for carrier systems when he described a method for preparing a stable suspension of collodion particles. He showed that the collodion particles acquired a surface deposit of protein and assumed many of the properties of the protein itself when the particles were suspended in a solution of protein. In studies on antigen-antibody "Agglutination by Precipitin", Jones (1927 and 1928) demonstrated that cow serum antigen adsorbed onto collodion particles was rendered agglutinable in the presence of specific antibody. Furthermore, Jones showed that the collodion particles would adsorb as many as five distinct antigens simultaneously and would agglutinate in the presence of each individual specific antibody. Delves (1937) also reported on the agglutination of collodion particles coated with human albumin and human pseudoglobulin antigens by their homologous antibodies. Cannon and Marshall (1940) utilized collodion particles for determining precipitative titers of antisera. They applied the method of resuspension-agglutination in order to shorten the period of testing and to increase the avidity of the test. This method involved the centrifugation of the suspension of antigen coated collodion particles; if specific antibody was present it would combine with the protein on the particles whereby they could not be readily resuspended by shaking. Eisler (1941) studied the influence of collodion particles on the visible end-point in antibody titrations. He concluded that

collodion methods effected increased titers of both low and high titered sera. Lowell (1942) compared collodion particle technic with other methods of measuring antibody and concluded that the collodion particle technic consistently permitted greater serum dilutions without loss of visibility. Cavelti (1943) evaluated the collodion particle technic with tissue antigens and homologous rabbit antisera and also serum antigens and pneumococcus polysaccharide. He concluded that the influence of particle size and age of the collodion as well as the proportion of admixture of antigen and collodion to serum had to be considered in order to obtain optimal results.

The above studies indicated that a carrier agglutination reaction may be substituted for the precipitin test. Belyavin (1955) described the direct precipitation of influenza virus with specific rabbit antisera. His results demonstrated serological specificity. He also showed that the precipitation of influenza virus could be inhibited by factors present in normal allantoic fluids. More pertinent, was the report by Smith and coworkers (1956) in which the direct precipitation of poliovirus antigens and rabbit antiserum was presented. The reaction was shown to be poliovirus type specific. In addition, specific viral precipitates with the use of double diffusion agar methods has been reported with the poliovirus antigen-antibody system (LeBouvier et al, 1957; Grasset et al, 1958). More recently, Hummeler and coworkers (1962) and Hummeler (1963) demonstrated the specific agglutination of poliovirus particles with the electron microscope.

Equally important to the background of this investigation, were the reports concerned with the use of carrier particles for viral

antigen-antibody reactions. For example, Roberts and Jones (1941) reported on the use of bacterial cells (Serratia marcesens) as a carrier for encephalitis virus. The bacterial cells coated with encephalitis virus were agglutinated by specific viral antiserum. Goodner (1941) used collodion particles as a carrier for virus-antibody agglutination. He referred to the reaction as collodion fixation. He reportedly has applied the technic successfully to the viruses of influenza A and B, yellow fever, and poliovirus. The concluding comment of Goodner was; "collodion fixation is a method of great delicacy; about 1000 times that of any heretofore described reaction."

Particles other than collodion have been used as carriers for viral or rickettsial antigen-antibody identification. Smorodintsev and Fradkina (1944) demonstrated that chemically pure carmine particles could be coated with typhus vaccine as the antigen and rendered agglutinable in the presence of specific antibody. They suggested that the test was more sensitive if antibody was also adsorbed onto the carmine particles. This was an example in which both antigen and antibody were artificially increased in size. Segre (1957) described a method for identifying the viruses of hog cholera and vesicular stomatitis. He coated basic anion exchange resin particles with specific antibody. The particles so treated would agglutinate in the presence of specific antigen. This procedure represented a change in the usual approach in that antibody was adsorbed onto the carrier particles rather than the antigen. Of interest, was the study reported by Roberts (1949) in which protamine sulfate was used as a

carrier for poliovirus antigens. According to Roberts, the results of the protamine flocculation test agreed with those obtained in monkey neutralization and immunity experiments. He suggested that the positively charged protamine molecules adsorb a few negatively charged virus particles thus serving as an antigenic mass sufficient to give a visible reaction in the presence of specific antibody. Subsequently, the work of Roberts has been discounted (Mack, personal communication). Bradley (1952) described a rapid method of testing plants in the field for Potato Virus X. His method took advantage of the presence of chloroplasts in the plant sap. The combination of chloroplasts and virus from infected plants were specifically agglutinated with homologous rabbit antiserum; the chloroplasts functioning as a carrier for the antigen-antibody reactants.

The selection of a suitable carrier particle for antigen-antibody reactions has been less difficult with the recent introduction of biologically inert polystyrene latex particles. The latex particles have been standardized in terms of particle size. The basic latex agglutination technic as described by Singer and Plotz (1956) and modified by Rheins and coworkers (1957) consisted of coating latex particles with gamma globulin. Latex particles so prepared were used to detect rheumatoid diseases. Singer and Plotz (1956) referred to the latex agglutination reaction as the latex fixation test. The test has been extended to a variety of antigen-antibody identities. For example, Muraschi (1958) applied the latex particle technic to the serodiagnosis of leptospirosis. He stated that the latex agglutination

test compared favorably in sensitivity and specificity with standard tests for serodiagnosis of leptospirosis. The test was rapid and simpler to perform. Kelen and Labzoffsky (1960) also described a latex agglutination test for leptospirosis. They extended the studies of Muraschi by using soluble antigens of leptospirae prepared by pyridine extraction rather than whole organisms. They supported Muraschi's report by concluding that the latex agglutination test possessed a high degree of specificity and sensitivity and compared equally with other standard serologic tests for leptospirosis. Carlisle and Saslaw (1958) developed a latex agglutination test for histoplasmosis by coating the particles with histoplasmin. They compared the histoplasmin-latex agglutination test with the histoplasmin-collodion agglutination test. They concluded that the former showed comparable results from the standpoint of specificity and sensitivity in sera from infected dogs, monkeys, and immunized rabbits. Znamirowski and coworkers (1959) reported on the application of latex agglutination with extracts of staphlococci. They indicated that the results with the latex agglutination test paralleled closely those obtained with hemagglutination technics. Christian and coworkers (1958) employed latex particles for serodiagnosis of disseminated lupus erythematosus. They used an extracted calf thymus nucleoprotein for coating the latex particles. Patients having clinical symptoms compatible with the disease were shown to give a positive latex agglutination. According to the authors, the latex agglutination test correlated closely with results of lupus erythematosus (L. E.) cell preparations from the same patient. The application of latex agglutination with crude extracts of thyroid gland to studies involving thyroid disease has also been reported

(Senhauser et al, 1962; Philp et al, 1962). These technics determine the level of circulating thyroglobulin antibodies which relate to thyroid disorders. It was concluded that the sensitivity of the latex agglutination test was intermediate between agar diffusion and tanned cell hemagglutination technics.

The technical difficulties inherent to carrier particle methods for virus-antibody systems was undoubtedly multifold. There were at least three factors to be considered: (1) relatively pure viral antigens in a sufficient mass to represent an antigenic moiety; (2) specific viral antisera of sufficient titer to be reactive and free from non-specific inhibitors; and (3) a suitable carrier particle standardized in terms of particle size, concentration, and adsorptive characteristics.

From the above, it would seem that the use of carrier particles for demonstrating antigen-antibody reactions provides a sensitive, simple, rapid, and specific test. The application of a carrier particle technic for detecting virus-antibody reactions would also seem to be feasible. The reported characteristics of latex particles in terms of adsorption, particle size homogeneity, and stability would indicate that latex particles would be the best choice as a viral antigen carrier. An investigation oriented toward developing a carrier particle technic for identifying poliovirus was planned. Such a method, if successful, would be a new serologic procedure and should prove useful in the diagnostic laboratory.

MATERIALS AND METHODS

A. Phosphate Buffered Saline Solution, pH 7.5.

This solution was used for washing and preparing tissue cultures for versene treatment. The solution had the following composition:

NaCl	8.0	g
KCl	0.2	g
Na_2HPO_4	1.15	g
KH_2PO_4	0.2	g
H_2O (distilled) to make	1000	ml

(above figures for anhydrous salts)

B. Versene (Ethylenediamine-tetra-acetic acid).

Versene was used throughout the investigation for removing cultured cells from the walls of the bottles. The versene was prepared as follows: Add 0.6 ml of commercial versene (34 per cent) to 1000 ml of phosphate buffered saline solution. The solution was sterilized at 121° C for 15 minutes and stored at 4°C.

C. Tissue Cultures.

Monkey kidney cells (Macaca rhesus), HeLa cells (Gey et al, 1936) derived from human carcinoma of cervix, and Hep 2 cells (Moore et al, 1955) derived from human epidermoid carcinoma of the larynx were originally obtained commercially from Microbiological Associates, Bethesda, Maryland. Subsequent requirement for HeLa and Hep 2 cells was satisfied by serial cultivation in the laboratory. Ten per cent calf serum was added to the medium as a growth factor.

D. Virus.

The poliovirus used in this investigation was obtained from the Virology Unit of the Medical Laboratory Center of the USAF Hospital

Andrews, Andrews Air Force Base, Washington, D . C. The viruses were propagated in HeLa, Hep 2, and monkey kidney cell cultures.

E. Preparation of Stock Polioviruses.

Preparation of the three types of poliovirus (Younger, 1955; Bodian et al, 1949) was accomplished by inoculating 0.3 ml of each virus type into a tissue culture. Thirty-two ounce prescription bottles were used and contained 30 ml of fluid; thus effecting a 1/1000 dilution of original virus seed. The infected cultures were incubated at 37°C until the cytopathic effect was complete. The tissue culture virus was frozen and thawed three times and then centrifuged at 1500 rpm for 30 minutes (size #1 International Centrifuge SBR). Virus harvest so clarified was placed in screw-cap tubes and stored at -60°C until used in each experiment. All tissue cultures were maintained with medium 199 (Morgan, Morton, and Parker, 1950) plus antibiotics. No serum was added to the maintenance medium. For example:

medium 199 (10X)	10.0 ml
4% $NaHCO_3$ with phenol red	2.0 ml
penicillin (100,000 units per ml)	1.0 ml
streptomycin (100,000 units per ml)	0.8 ml
mycostatin (50,000 units per ml)	0.2 ml
sterile distilled water	86.0 ml
total volume	100.0 ml

F. Poliovirus and Control Host Cell Antigens Used for Preparing Antisera.

(1) Poliovirus Antigens.

HeLa cell propagated poliovirus types were used. The clarified infected fluids of each virus type served as the inoculum. (Designation: HeLa poliovirus type 1, 2, and 3).

(2) HeLa and Hep 2 Cell Antigens.

The cell cultures were not standardized but represented moderately heavy suspensions of versene treated cells. The cells were washed three times with sterile phosphate buffered saline solution; centrifuged at 2000 rpm for 30 minutes (size #1 International Centrifuge SBR) and then resuspended in 0.6% formol-saline solution. Sterility tests were conducted after permitting the cell suspension to stand 48 hours at room temperature. Thioglycollate broth was used for bacterial control.

(3) Partially Purified Poliovirus Antigens.

HeLa cell propagated poliovirus of each type was subjected to a two-cycle ultracentrifugation as follows:

a. Infected cell culture fluid (ICCF) was centrifuged at 1500 rpm for 30 minutes (size #1 International Centrifuge SBR) to remove gross cellular material.

b. The clarified ICCF from a. above was centrifuged at 17,500 rpm (27,710 X g) for 30 minutes (Spinco Model L Centrifuge - rotor No. 40) to remove additional extraneous material.

c. The supernatant fluid from b. above was centrifuged at 35,000 rpm (110,800 X g) for 2 hours (Spinco Model L Centrifuge - rotor No. 40) to sediment the virus.

d. The supernatant fluid from c. above was discarded and the pellet resuspended to the original volume in sterile 0.85% saline solution containing antibiotics. The resuspended virus was held at room temperature for 1 hour and then placed at 4°C overnight.

e. The partially purified virus antigen was tested for infectivity in HeLa cells and frozen at -60°C until used.

G. Preparation of Sera.

(1) Normal Rabbit Serum.

All rabbits selected for antibody production were bled by intracardiac puncture prior to the inoculation schedule. The blood was allowed to stand overnight at 4°C and the serum removed from the clot following centrifugation at 1500 rpm for 15 minutes (size #1 International Centrifuge SBR). The sera were held at -20°C until used. These were considered to be the normal rabbit sera. Just before use, the sera were inactivated at 56° C for 30 minutes unless otherwise indicated.

(2) Antisera.

HeLa poliovirus or HeLa or Hep 2 cell antigen was inoculated, via the marginal ear vein, into each of two rabbits. Inoculations were scheduled three times a week for a period of four weeks. Seven days following the last inoculation and after food was withheld for 24 hours, the animals were bled by intracardiac puncture. The antisera were recovered and handled in an identical manner to the normal rabbit serum.

H. Determination of Tissue Culture Dose 50 (TCD_{50}).

The fifty per cent endpoint method of Reed and Muench (1938) was used for all virus titrations and virus neutralization tests. All culture tubes showing evidence of cytopathic effect (CPE) were considered positive. The appearance of normal and poliovirus infected Hep 2 cell cultures are shown in Figures 1, 2, 3, and 4.

I. Virus Titration (TCD_{50} per ml).

Tenfold serial dilutions of stock virus suspensions were prepared in medium 199 containing $NaHCO_3$ and antibiotics. For purposes of titration, each of 4 culture tubes was inoculated with one ml of a given dilution. Cultures were incubated (stationary) at 37°C and examined daily for evidence of CPE. The HeLa cell propagated poliovirus types had the following $\log_{10}$ TCD_{50} per ml titers in HeLa cell cultures:

HeLa poliovirus type 1	$10^{7.66}$
HeLa poliovirus type 2	$10^{7.50}$
HeLa poliovirus type 3	$10^{7.66}$

The Hep 2 cell propagated poliovirus types had the following $\log_{10}$ TCD_{50} per ml titers in Hep 2 cell cultures:

Hep 2 poliovirus type 1	$10^{8.50}$
Hep 2 poliovirus type 2	$10^{7.00}$
Hep 2 poliovirus type 3	$10^{5.66}$

J. Globulin Fractionation.

A modified method of Thurston et al, (1957) was used to fractionate whole serum:

1. Add 9 ml of 20% Na_2SO_4 solution to 1 ml of whole serum, slowly.

2. Incubate the mixture 3 hours at 37°C in a water bath.

3. Centrifuge the precipitate at ~500 rpm for 20 minutes. (size #1 International Centrifuge SBR).

4. Remove and discard the supernatant fluid and wash precipitate 3 times carefully with 5 ml of glass triple distilled water (layer over precipitate).

5. Restore globulin fraction to original volume with 0.85% sterile saline solution.

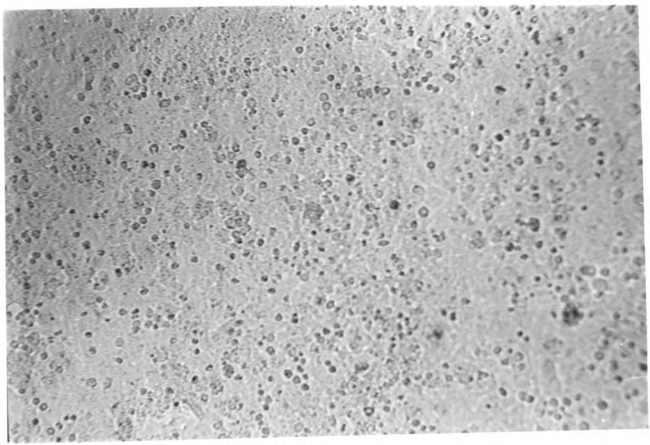


FIG. 1. Normal Hep 2 cell culture. X 50.

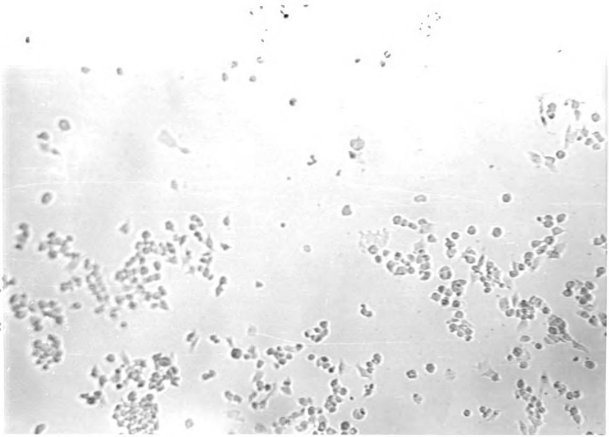


FIG. 2. Poliovirus infected Hep 2 cell culture, 2+ CPE. X 50.

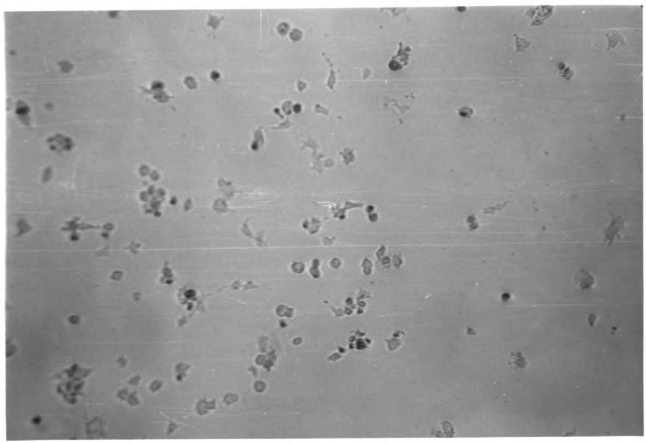


FIG. 3. Poliovirus infected Hep 2 cell culture. 3+ CPE. X 50.

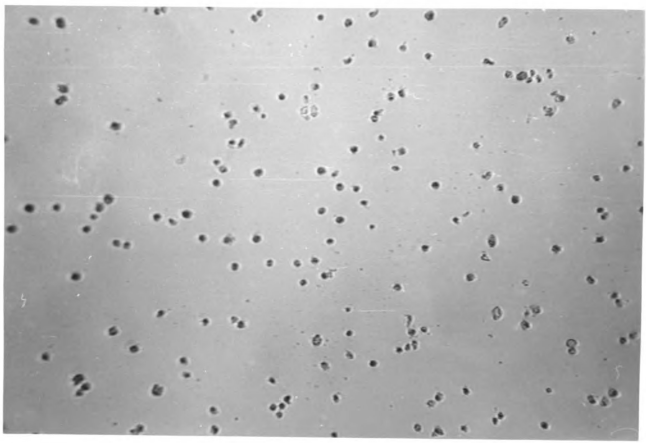


FIG. 4. Poliovirus infected Hep 2 cell culture, 4+ CPE. X 50.

K. Validity of Calculated TCD_{50}.

Sometimes changes occur in tissue cell cultures which adversely effect the physical appearance of the cells. This may resemble CPE. Also, since 1+ CPE readings are questionable when compared to uninoculated controls, it is possible to call a culture positive (1+ CPE) when no true CPE exists. A one tube error term variance table is included to illustrate the TCD_{50} $\log_{10}$ confidence level (see Table 1). The example indicates that a one tube error in a titration could be in error by 0.16 to 0.34 $\log_{10}$ units. To test the example, Hep 2 cell propagated poliovirus type 1 was titrated in Hep 2 cell cultures using eight replicate titrations. Table 2 indicates that the one tube error term was in agreement with the standard deviation of eight replicate titrations; i. e., the one tube error term allows an error of 0.16 to 0.34 $\log_{10}$ TCD_{50} units while the standard deviation of eight replicate titrations was shown to be 0.33 $\log_{10}$ TCD50 units. The 95% confidence level gave a value of 0.27 $\log_{10}$ TCD_{50} units.

TABLE 1. Validity of Calculated TCD_{50}

Typical distribution of CPE versus no-CPE as exhibited by cell cultures using four tubes per virus dilution					
Virus dilution	Possible tissue culture $dose_{50}$ scores				
10^{-4}	4/0*	4/0	4/0	4/0	4/0
10^{-5}	4/0	4/0	4/0	4/0	4/0
10^{-6}	2/2	3/1	1/3	0/4	4/0
10^{-7}	0/4	0/4	0/4	0/4	0/4
10^{-8}	0/4	0/4	0/4	0/4	0/4
Calculated TCD_{50} per ml	$10^{6.00}$	$10^{6.33}$	$10^{5.66}$	$10^{5.50}$	$10^{6.50}$

* Numerator represents tubes exhibiting CPE and denominator represents tubes exhibiting no-CPE.

Log difference variable for a one tube error term

Actual TCD_{50} per ml	TCD_{50} per ml readings as recorded				
	$10^{6.50}$	$10^{6.33}$	$10^{6.00}$	$10^{5.66}$	$10^{5.50}$
$10^{6.50}$		0.17**			
$10^{6.33}$	0.17		0.33		
$10^{6.00}$		0.33		0.34	
$10^{5.66}$			0.34		0.16
$10^{5.50}$				0.16	

** Log error.

TABLE 2. Statistical Analysis of Eight Replicate Infectivity Titrations

No.	log TCD_{50} per ml	$(TCD_{50}$ per ml$)^2$	Statistics
1	8.75	76.5625	$s^2 = 0.107143$
2	8.50	72.2500	$s = 0.3271085$
3	8.75	76.5625	(rounded = 0.33)
4	8.75	76.5625	SE = 0.115629
5	8.50	72.2500	
6	8.00	64.0000	95% CL = $\bar{X} \pm$
7	8.00	64.0000	0.27288
8	8.75	76.5625	(8.227 to 8.773)
	$\Sigma X = 68.00$	$\Sigma X^2 = 578.7500$	$\bar{X} = 8.50$ log TCD_{50}

$(\Sigma X)^2 = 4624$

$$s^2 = \frac{\Sigma X^2 - \frac{(\Sigma X)^2}{n}}{n-1} = \frac{578.75 - \frac{4624}{8}}{7} = \frac{0.75}{7}$$

$s^2 = 0.107143$ log TCD_{50} $\quad s = \sqrt{s^2} = \sqrt{0.107143}$

$= 0.3271088$

$$SE = \frac{s}{\sqrt{n}} = \frac{0.327}{2.828} = 0.115629 \text{ log } TCD_{50}$$

95% Confidence limits = (SE) (2.36) = 0.27288 log $TCD_{50} \pm \bar{X}$

$= 8.50 \pm 0.273 = 8.227$ to 8.733

L. Complement.

Guinea pig complement was obtained from Markham Laboratories, Chicago, Illinois. The complement was reconstituted with the restoring fluid provided by the manufacturer and then frozen at -60°C until used.

M. Neutralization Testing of Antisera.

Neutralization testing of prepared antisera was conducted by constant virus serum dilution technic according to Diagnostic Procedures for Virus and Rickettsial Diseases, 2nd Ed., 1956, pages 80 to 82 and Procedures for Routine Laboratory Diagnosis of Virus and Rickettsial Diseases by Kalter, S. S., and Hillis, W. D., 1961.

Poliovirus antisera and control host cell antisera were inactivated at 56°C for 30 minutes prior to testing. The procedure for Hep 2 cell propagated poliovirus type 1 will be covered in detail for illustration; all titrations against homologous and heterologous poliovirus antisera and control host cell antisera were carried out in a like manner with all virus types; i. e., checker-board titrations.

The virus was diluted so as to contain 100 TCD_{50} per 0.1 ml final concentration after mixing with an equal volume of diluted antiserum. Therefore, 2000 TCD_{50} per ml concentration of virus was required. Volumes used were 0.5 ml for serum and for virus. Controls consisted of serum, cell cultures, and virus titration with each test. The virus-serum mixture was incubated for one hour at room temperature and then 0.2 ml of the mixture was inoculated into each of four Hep 2 cell cultures. The control virus titration was conducted by mixing

one ml of the 2000 TCD_{50} virus suspension and one ml of 1/5 normal rabbit serum followed by tenfold dilutions in medium 199.

Only homotypic antiserum showed neutralizing capacity against the particular type of poliovirus tested. HeLa and Hep 2 cell antisera exhibited no neutralizing capacity against the three poliovirus types. Table 3 illustrates the calculations necessary for obtaining the neutralization index (NI) for type 1 poliovirus antiserum. Table 4 illustrates the control virus titration for each neutralization test and consists of data obtained with Hep 2 poliovirus type 1.

In all neutralization tests, cultures were examined daily. Recordings were made on the 4th day post-inoculation. The lowest dilution of antiserum used was 1/5 (initial dilution). Rabbit antisera prepared with HeLa poliovirus types had the following 50% NI:

HeLa poliovirus type 1 antiserum = 1/3214

HeLa poliovirus type 2 antiserum = 1/2560

HeLa poliovirus type 3 antiserum = 1/505

TABLE 3. Hep 2 Poliovirus Type 1 Neutralization with Homotypic Rabbit Antiserum (condensed)

Final serum dilution	Final log serum dilution	CPE readings		CPE subtotals		TCD
		CPE	no-CPE	CPE	no-CPE	% no-CPE
1/1280	$10^{-3.107}$	0	4	0	7	100
1/2560	$10^{-3.408}$	1	3	1	3	75
1/5120	$10^{-3.709}$	4	0	5	0	0

Formulae :

1. Proportional distance = $\frac{\text{Log dilution above 50\% - 50}}{\text{Log dilution above 50\% - dilution next below 50\%}}$

Thus: $\frac{75 - 50}{75 - 0} = \frac{25}{75} = 0.33$

2. Log dilution factor times proportional distance = factor

$0.33 \times -0.3 = -0.099$

3. Log dilution above 50% no-CPE + factor = 50% log NI

log 1/2560 $= 10^{-3.408}$
\+ factor $= -0.099$

50% log NI $= 10^{-3.507}$

4. 50% NI = antilog of $10^{-3.507}$ = 1/3214 (final serum dilution)

TABLE 4. Control Virus Titration with Hep 2 Poliovirus Type 1

Virus dilutions from 1/5 normal rabbit serum		Hep 2 cell culture tube number			
per 0.1 ml	dilution	1	2	3	4
100 TCD_{50}	10^{0}	4+*	4+	4+	4+
10 TCD_{50}	10^{-1}	3+	3+	1+	0
1 TCD_{50}	10^{-2}	0	0	0	0
0 TCD_{50}	10^{-3}	0	0	0	0

*CPE rating.

N. Tris Buffer, pH 8.2.

The salts used in the buffering system were chosen in order to furnish the ions known to favor complement fixation reactions. The final molarity was adjusted to maintain optimal isotonicity as described by Mayer et al, (1946); Mayer et al, (1948); and Osler et al, (1951).

The buffer was prepared as follows:

Stock solutions.

a. 0.2 M solution of tris (hydroxymethyl aminomethane) 24.2 g in 1000 ml.

b. 0.2 M HCL.

Mix 50 ml of a plus 21.9 ml of b and dilute to 200 ml final volume. The final pH is 8.2

Saline solution.

NaCl	8.5 g per liter	0.14500 M
$CaCl_2$	0.02 g per liter	0.00018 M
$MgCl_2$	0.10 g per liter	0.00050 M
Final molarity		0.14568 M

The pH chosen to use in this study was 8.2 except where indicated. This pH was reported as used with other latex agglutination tests. The Tris buffering system selected for this study was chosen in order to obtain maximum buffering capacity to the reaction. The pKa of tris was calculated to be 8.0799 based on published data in the Merck Index (1963).

Experiment #7 indicated that complement was not necessary for the test. The salts added to the buffering system were changed in subsequent experiments. A 0.15 M salt solution containing NaCl and $CaCl_2$ was used for the remainder of the investigation.

O. Latex Suspension.

Polystyrene latex particles 0.81 u in diameter were used throughout the investigation. The latex suspension was obtained commercially from Difco Laboratories, Detroit, Michigan. Latex stock was prepared by diluting the commercial preparation 1/5 with distilled water, filtering through Whatman No. 40 filter paper and storing at 4°C until used for preparing the reagent latex suspension.

Reagent latex was prepared by diluting the stock latex with Tris buffered saline solution at pH 8.2 to make a suspension exhibiting approximately 6.25 per cent transmission (% T) on a Coleman Junior Spectrophotometer at a wave length of 650 mu using 19 X 150 mm round cuvettes. After all reagents were added to the test, the final % T was 50. The following protocol will serve to illustrate:

% Transmission	O.D. *	Volumes used per tube in the test
6.25	1.204	0.2 ml reagent latex
25.00	0.602	0.2 ml poliovirus antigen
39.75	0.401	0.2 ml antiserum
50.00	0.301	0.2 ml complement or additional diluent

*Optical density.

In actual practice because of the large volumes required, reagent latex and antigen (either poliovirus infected or uninfected tissue culture fluid) were added in equal volumes sufficient to complete an entire titration; i. e., 0.4 ml of latex-antigen mixture was added to each tube. Antisera dilutions were also prepared as master dilutions prior to distribution to the proper tubes in either 0.2 ml when complement was used or as 0.4 ml when complement was omitted. The final volume in each tube was 0.8 ml and provided a final concentration of latex per tube equivalent to 50% T spectrophotometrically.

P. Performing Tests and Reading the Reaction.

The test proper was made by preparing dilutions of antigen and then adding an equal volume of latex suspension. The tubes were held at least 30 minutes at room temperature or overnight at 4°C to allow adsorption of the antigen to the latex particles. During the adsorption period, the antisera dilutions were made and distributed to the proper tubes for the titration. The latex-antigen mixture was added last using 0.4 ml representing each antigen dilution. (Note: when complement was used, the appropriate volume of complement was added before the latex-antigen mixture). The tubes were shaken well and incubated at 37°C for 1 hour in a water bath and then refrigerated at 4°C overnight.

Following incubation, the tubes were centrifuged at 2000 rpm for 5 minutes (size #1 International Centrifuge SBR). The tubes were then flipped gently and read in the usual manner from 4+ to - depending upon the degree of agglutination. For example:

4+ = Clear supernatant fluid with large agglutinated clumps.

3+ = Clear supernatant fluid with agglutinated clumps smaller than 4+.

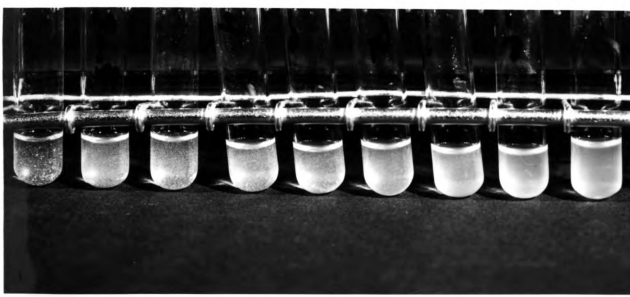
2+ = Clear supernatant fluid with small agglutinated clumps.

1+ = Cloudy supernatant fluid with small agglutinated clumps.

$\pm$ = Cloudy supernatant fluid with a fine granularity present.

- = Cloudy supernatant fluid with no clumping evident.

In actual practice, for purposes of classification, the 1+ and $\pm$ reactions were recorded but were considered as questionable and were grouped with the negatives. Figure 5 illustrates tubes allowed to stand at room temperature (following centrifugation) for several hours and then flipped again gently. The various agglutination reactions were typical for all reactions recorded. The tube on the far right is a latex control. If tubes were held at room temperature for longer periods (up to 4 days), control tubes exhibited a fine granularity suggestive of a $\pm$ to a 1+ reaction. Definite 2+ reactions were considered as endpoint readings, as read immediately following centrifugation. Differences in viscosity between the greater serum concentrations may introduce an element of error when grading the degree of reaction and should be taken into account when comparing



4+ 3+ 3+ 2+ 2+ 1+ ± - -

FIG 5. Top: Tubes allowed to stand at room temperature following centrifugation and gently flipping.

Bottom: Tubes gently flipped. Control on right.

results of separate titrations. Occasionally, it was found that the high dilutions of latex-antigen controls exhibited a fine granularity. These questionable reactions were recorded but as previously noted were classed as negative.

RESULTS

Experiment #1

Specificity of Latex Agglutination with Hep 2 Poliovirus Antigens.

The first experiment was conducted to ascertain whether or not the latex agglutination test would demonstrate: (1) activity with the virus-antibody system; and (2) viral specificity. The titrations were done to provide maximum viral antigen concentration for each antiserum tested. The mechanics of the serological adhesion phenomenon have been shown to require complement (Lamanna, 1955; Davis, 1957) and so complement diluted 1/30 was added to each tube containing antigen and antibody. Tables 5a, 5b, 5c, and 5d represent the results of latex agglutinations in which all three Hep 2 poliovirus types served as antigens. Latex agglutination was observed with all the HeLa poliovirus antisera. In two of the titrations (Tables 5b and 5c), the Hep 2 cell antiserum reacted at a serum dilution of 1/8. Control tests were also done with one of the reagents missing from the tube (Table 5d). These results indicated that the latex agglutination reaction had revealed: (1) group specific antigens among the polioviruses; and (2) antigens to Hep 2 cells. Common group specific poliovirus antigens have been reported in the complement fixation (CF) test (Casals et al, 1951, 1952; Svedmyr et al, 1952; Black et al, 1954, 1955; LeBouvier, 1954, 1955; Melnick, 1955; Schmidt et al, 1956; and Mayer, 1957).

TABLE 5a. Specificity of Latex Agglutination with Hep 2

Poliovirus Type 1

Rabbit antisera type	Hep 2 poliovirus type 1 antigen final log TCD_{50} per ml $10^{7.9}$ Final sera dilutions						
	1/8	1/16	1/32	1/64	1/128	1/256	Control
HeLa polio 1	4+	3+	1+	-	-	-	-
HeLa polio 2	3+	2+	-	-	-	-	-
HeLa polio 3	3+	2+	-	-	-	-	-
HeLa cell	-	-	-	-	-	-	-
Hep 2 cell	-	-	-	-	-	-	-
Normal	-	-	-	-	-	-	-
Antigen control							-

TABLE 5b. Specificity of Latex Agglutination with Hep 2

Poliovirus Type 2

Hep 2 poliovirus type 2 antigen
final log TCD_{50} per ml $10^{6.4}$

Rabbit Antisera type	Final sera dilutions						
	1/8	1/16	1/32	1/64	1/128	1/256	Control
HeLa polio 1	2+	2+	2+	3+	3+	-	-
HeLa polio 2	2+	3+	1+	-	-	-	-
HeLa polio 3	3+	3+	3+	-	-	-	-
HeLa cell	-	-	-	-	-	-	-
Hep 2 cell	3+	-	-	-	-	-	-
Normal	-	-	-	-	-	-	-
Antigen control							-

TABLE 5c. Specificity of Latex Agglutination with Hep 2

Poliovirus Type 3

Rabbit antisera type	Hep 2 poliovirus type 3 antigen final log TCD_{50} per ml $10^{5.06}$						
	Final sera dilutions						
	1/8	1/16	1/32	1/64	1/128	1/256	Control
HeLa polio 1	1+	4+	4+	4+	1+	-	-
HeLa polio 2	3+	4+	4+	1+	-	-	-
HeLa polio 3	4+	4+	4+	1+	-	-	-
HeLa cell	-	-	-	-	-	-	-
Hep 2 cell	3+	1+	-	-	-	-	-
Normal	-	-	-	-	-	-	-
Antigen control							-

TABLE 5d. Latex Agglutination Reagent Control Tests

Antigens used	Antisera 1/8	Complement 1/30	Results
Hep 2 polio 1	HeLa polio 1	-	2+
Hep 2 polio 2	HeLa polio 2	-	2+
Hep 2 polio 3	HeLa polio 3	-	1+
Hep 2 polio 1	--	present	-
Hep 2 polio 2	--	"	-
Hep 2 polio 3	--	"	-
--	HeLa polio 1	"	-
--	HeLa polio 2	"	-
--	HeLa polio 3	"	-
--	--	"	-

In most of the reports, cross-reactions with the CF test were referable to antigen handling prior to testing. Reference was also made to the possible existence of group specific poliovirus antigens. The HeLa cell antiserum and the normal rabbit serum failed to elicit a reaction. It should be recalled that HeLa cell propagated poliovirus types were used for preparing the specific viral antisera. Thus, any common host cell antibodies present in the specific viral antisera should have reflected the HeLa cell. This was not the case.

The reagent control tests (Table 5d) indicated that at least two components (antigen and antibody) were necessary for latex agglutination. The need for complement remains questionable since latex agglutination occurred in its absence. The presence of complement seemed to intensify agglutination and produce more clear-cut reactions. All other control tubes were negative.

Experiment #2

Latex Agglutination of Hep 2 Poliovirus Antigens with Homotypic Antiserum (Block-Titration).

The latex agglutination cross-reactions observed in experiment #1 suggested a need for delineating the parameters of the latex reaction of each viral antigen-antibody preparation. Latex agglutination testing (block-titration) was done for each poliovirus type and its antibody. Complement diluted 1/30 was added to each tube. The results are shown in Table 6. Latex agglutination appeared to follow reaction zones typical of classical precipitation titrations; i. e., zonal inhibition phenomenon. Antigen excesses caused inhibition of the reaction at the high serum dilutions or antibody excesses caused inhibition at the high antigen dilutions. Inspection of the three separate titrations indicated that quantitative latex agglutination differed among the three types of poliovirus and their antiserum. The Hep 2 poliovirus type 1 antigen was agglutinated to a dilution of 1/256. The other poliovirus antigen-antibody systems agglutinated at an antigen dilution of 1/64. All control tubes were negative. These results indicated that the observed latex agglutination was produced by an antigen-antibody union. The presence of zone phenomenon would seem to support the suggestion by Singer and Plotz (1956) that latex agglutination was a precipitation reaction occurring at the surface of the latex particles.

TABLE 6. Latex Agglutination of Hep 2 Poliovirus Antigens with Homotypic Antisera

Final * antiserum dilution	Final antigen dilutions 1/8	1/16	1/32	1/64	1/128	1/256	Serum control
Hep 2 poliovirus type 1 antigen							
1/8	4+	4+	4+	4+	3+	3+	-
1/16	4+	4+	4+	4+	3+	3+	-
1/32	3+	4+	4+	4+	3+	3+	-
1/64	-	4+	4+	4+	3+	2+	-
1/128	-	1+	4+	4+	3+	2+	-
1/256	-	-	2+	4+	4+	2+	-
Antigen controls	-	-	-	-	-	-	Latex C** -
Hep 2 poliovirus type 2 antigen							
1/8	4+	3+	3+	2+	±	±	-
1/16	4+	4+	2+	2+	±	-	-
1/32	3+	3+	4+	4+	±	-	-
1/64	1+	4+	4+	4+	±	-	-
1/128	-	2+	3+	3+	1+	-	-
1/256	-	-	1+	2+	1+	-	-
Antigen controls	-	-	-	-	-	-	Latex C** -
Hep 2 poliovirus type 3 antigen							
1/8	4+	4+	2+	1+	-	-	-
1/16	4+	4+	2+	1+	-	-	-
1/32	4+	4+	3+	2+	±	-	-
1/64	4+	4+	3+	2+	-	-	-
1/128	-	4+	4+	3+	1+	-	-
1/256	-	1+	2+	2+	1+	±	-
Antigen controls	-	-	-	-	-	-	Latex C** -

*The final serum dilutions resulted from the addition of an appropriate volume of complement diluted 1/30.
** Latex control.

Experiment #3

Specificity of Latex Agglutination with Hep 2 Poliovirus Antigens (Block-Titration).

The results observed in experiment #1 and #2 suggested that cross-reactions among the poliovirus types might be due to antigen-antibody ratios. Consequently, viral antigen-antibody specificity as determined by block-titration was undertaken. Hep 2 poliovirus type 1 antigen was tested with the three viral antisera. Complement diluted 1/30 was added to each tube containing antigen and antibody. The results showed (Table 7) cross-reactions. The homotypic titration differed by only two tubes when compared with the heterotypic antisera at a particular antigen dilution. These results indicated that: (1) a group specific antigen existed among the polioviruses; or (2) a common antigen not related to the viral antigens was responsible for the latex cross-reactions. At the limiting dilutions, there appears to be an observable degree of viral antigen-antibody specificity; particularly at an antigen dilution of 1/1024. Whether or not these results were due to actual viral antigen-antibody specificity at the antigen optimum or to strength differences among the respective antisera was undetermined. To test this, three Hep 2 poliovirus type antigens were tested with one antiserum. The results are shown in Table 7a. The Hep 2 poliovirus antigens reacted with the one antiserum in almost an identical manner. These results show that antisera titers caused the differences noted in the titrations recorded in Table 7. It was concluded that no definitive type specificity existed among the three Hep 2 poliovirus antigens.

TABLE 7. Specificity of Latex Agglutination with Hep 2

Poliovirus Type 1 Antigen

	Hep 2 poliovirus type 1 antigen						
HeLa poliovirus type 1 antiserum	Final antigen dilutions						
	1/32	1/64	1/128	1/256	1/512	1/1024	Serum controls
1/20*	4+	4+	4+	4+	4+	3+	-
1/40	4+	4+	4+	4+	4+	3+	-
1/80	4+	4+	4+	4+	3+	3+	-
1/160	4+	4+	4+	4+	2+	±	-
1/320	2+	4+	4+	3+	±	-	-
1/640	-	2+	3+	2+	±	-	-
1/1280	-	-	2+	2+	±	-	-
HeLa poliovirus type 2 antiserum							
1/20	4+	4+	4+	4+	2+	-	-
1/40	4+	4+	3+	2+	2+	-	-
1/80	4+	4+	4+	2+	2+	-	-
1/160	2+	2+	4+	2+	2+	-	-
1/320	-	±	2+	1+	1+	-	-
1/640	-	-	±	1+	±	-	-
1/1280	-	-	-	±	±	-	-
HeLa poliovirus type 3 antiserum							
1/20	4+	4+	4+	2+	1+	±	-
1/40	4+	4+	4+	2+	1+	-	-
1/80	4+	4+	4+	2+	1+	-	-
1/160	2+	3+	4+	2+	1+	-	-
1/320	1+	2+	3+	2+	1+	-	-
1/640	-	-	2+	1+	1+	-	-
1/1280	-	-	-	1+	±	-	-
Antigen controls	-	-	-	-	±	±	LC** -

* Final serum dilution after addition of complement diluted 1/30.
** latex control.

TABLE 7a. Specificity of Latex Agglutination with HeLa Poliovirus Type 3 Antiserum

HeLa poliovirus type 3 antiserum	Final antigen dilutions					
	1/16	1/32	1/64	1/128	1/256	Serum controls
	Hep 2 poliovirus type 1 antigen					
1/10*	4+	4+	4+	4+	2+	-
1/20	4+	4+	4+	4+	3+	-
1/40	4+	4+	3+	3+	3+	-
1/80	4+	4+	2+	±	±	-
1/160	4+	4+	2+	-	-	-
Antigen controls	-	-	-	±	±	Latex control -
	Hep 2 poliovirus type 2 antigen					
1/10	4+	4+	4+	4+	3+	
1/20	4+	4+	4+	4+	2+	
1/40	4+	3+	3+	3+	2+	
1/80	3+	3+	1+	±	±	
1/160	3+	3+	1+	±	-	
Antigen controls	-	-	-	-	±	
	Hep 2 poliovirus type 3 antigen					
1/10	3+	4+	4+	4+	4+	
1/20	4+	4+	4+	4+	3+	
1/40	3+	3+	2+	2+	2+	
1/80	3+	3+	2+	1+	±	
1/160	2+	3+	2+	±	-	
Antigen controls	-	-	-	-	±	

* Final serum dilution. This titration was conducted at a later date than the results shown in Table 7 and therefore no complement was added to the tubes.

Experiment #4

Latex Agglutination of Poliovirus Type Antigens with HeLa and Hep 2 Cell Antisera.

Experiment #1 showed that host cell antigen could function in the latex agglutination reaction with specific antiserum. Zone phenomenon was also exhibited in the system as shown in experiment #2. Therefore, it was decided to determine what part cellular antigens and zone phenomena were playing in the latex reaction. Titrations were done consisting of Hep 2 poliovirus types 1, 2, and 3 antigens and HeLa poliovirus types 1, 2, and 3 antigens. These antigens were titrated with antisera prepared against Hep 2 and HeLa cells. The titrations were done so as to reflect the host cell derivation with no reference to the virus type. The results are presented in Tables 8 and 8b (heterotypic host cell combinations) and in Tables 8a and 8c (homotypic host cell combinations). Inspection of the tables indicated that a common antigen or antigens existed between HeLa and Hep 2 cells. Zone inhibition was apparent in all the titrations. These results suggested two possibilities: (1) latex agglutination in previous experiments was due exclusively to the cellular antigen present; titration differences resulting from quantitative differences in the antigen-antibody ratios of the cellular antigen system; and (2) latex agglutination in previous experiments was due to a mixed antigen-antibody system composed of cellular antigens and viral antigens reacting with their respective antibodies at certain antigen-antibody ratios; overlaping of reactions occurring in such a manner as to mask individual specificities. It was concluded that HeLa and Hep 2 cells

have common antigens. No final conclusion may be made as to the latex agglutinability of the virus-antibody system.

TABLE 8. Latex Agglutination of Hep 2 Poliovirus Type Antigens with HeLa Cell Antiserum

HeLa cell antiserum final dilution*	Final antigen dilutions							
	1/4	1/8	1/16	1/32	1/64	1/128	1/256	Serum control
Hep 2 poliovirus type 1 antigen								
1/8	-	±	1+	2+	3+	3+	3+	-
1/16	-	±	±	1+	3+	3+	2+	-
1/32	-	-	±	±	±	±	±	-
Antigen controls	-	-	-	-	-	-	- LC**	-
Hep 2 poliovirus type 2 antigen								
1/8	±	±	2+	3+	3+	3+	3+	
1/16	±	±	1+	1+	2+	2+	2+	
1/32	-	-	-	±	1+	1+	±	
Antigen controls	-	-	-	-	-	-	-	
Hep 2 poliovirus type 3 antigen								
1/8	-	±	1+	2+	2+	3+	4+	
1/16	-	±	±	1+	2+	2+	3+	
1/32	-	±	±	1+	2+	2+	±	
Antigen controls	-	-	-	-	-	-	-	

* Final serum dilution after addition of complement diluted 1/30.
** Latex control.

TABLE 8a. Latex Agglutination of HeLa Poliovirus Type Antigens with HeLa Cell Antiserum

HeLa cell antiserum final dilution*	HeLa poliovirus type 1 antigen						
	Final antigen dilutions						
	1/4	1/8	1/16	1/32	1/64	1/128	1/256
1/8	±	2+	3+	4+	4+	4+	4+
1/16	±	±	2+	3+	3+	3+	3+
1/32	-	±	±	3+	3+	2+	1+
Antigen controls	-	-	-	-	-	-	-
	HeLa poliovirus type 2 antigen						
1/8	-	2+	3+	3+	3+	3+	2+
1/16	±	±	2+	3+	3+	4+	2+
1/32	-	±	±	2+	2+	2+	1+
Antigen controls	-	-	-	-	-	-	-

* Final serum dilution after addition of complement diluted 1/30.

Note: Serum controls and latex control are the same as those tabulated in Table 8.

TABLE 8b. Latex Agglutination of HeLa Poliovirus Type Antigens with Hep 2 Cell Antiserum

Hep 2 cell antiserum final dilution*	HeLa poliovirus type 1 antigen							
	Final antigen dilutions							
	1/4	1/8	1/16	1/32	1/64	1/128	1/256	Serum control
1/8	±	1+	3+	3+	4+	4+	2+	-
1/16	-	1+	2+	4+	4+	3+	2+	-
1/32	-	±	2+	4+	2+	2+	2+	-
Antigen controls	-	-	-	-	-	-	- LC**	-
	HeLa poliovirus type 2 antigen							
1/8	±	1+	2+	3+	4+	4+	1+	
1/16	-	1+	2+	4+	4+	4+	1+	
1/32	-	±	2+	2+	2+	2+	1+	
Antigen controls	-	-	-	-	-	-	-	
	HeLa poliovirus type 3 antigen							
1/8	-	1+	2+	2+	3+	4+	ND***	
1/16	-	1+	1+	2+	4+	4+	ND	
1/32	-	-	1+	2+	2+	4+	ND	

* Final serum dilution after addition of complement diluted 1/30.
** Latex control.
*** ND = Not done.

TABLE 8c. Latex Agglutination of Hep 2 Poliovirus Type Antigens with Hep 2 Cell Antiserum

Hep 2 cell antiserum final dilution*	Hep 2 poliovirus type 1 antigen					
	Final antigen dilutions					
	1/4	1/8	1/16	1/32	1/64	1/128
1/8	-	1+	3+	4+	4+	4+
1/16	-	1+	2+	4+	4+	4+
1/32	-	±	1+	4+	4+	4+
Antigen controls	-	-	-	-	-	-
	Hep 2 poliovirus type 2 antigen					
1/8	±	3+	3+	4+	4+	4+
1/16	-	2+	3+	3+	4+	4+
1/32	-	2+	4+	4+	4+	4+
Antigen controls	-	-	-	-	-	-
	Hep 2 poliovirus type 3 antigen					
1/8	1+	3+	3+	3+	4+	4+
1/16	±	2+	3+	4+	4+	4+
1/32	-	1+	2+	4+	4+	3+
Antigen controls	-	-	-	-	-	-

* Final serum dilution after addition of complement diluted 1/30.

Note: Serum controls and latex control are the same as those tabulated in Table 8b.

Experiment #5

Latex Agglutination of Hep 2 Cell Antigen with Various Antisera.

Experiment #4 indicated that a common antigen existed between HeLa and Hep 2 cells. It was apparent that latex agglutination of the uninfected Hep 2 cell antigen system be studied. Hep 2 cells were prepared as described previously under materials and methods. The clarified fluids were used as the antigen for titration with various antisera. The results are shown in Table 9. The HeLa cell antiserum and HeLa poliovirus antiserum showed antigen excess inhibition zones more than the specific Hep 2 antiserum. Normal rabbit serum was negative. The comparison between the uninfected Hep 2 cell culture fluids (Table 9) and the poliovirus infected Hep 2 cell culture fluids (previous experiments) was difficult to interpret because of the inability to measure the relative concentration of Hep 2 antigenic components present in both systems. The results suggested that caution must be exercised when interpreting previous experiments. Cellular antigens could have existed as antigen excesses in the uninfected system and not be in excess in the poliovirus infected system. The results suggested that inoculation of rabbits with poliovirus infected HeLa cell culture fluids stimulated a greater antibody response to host cell antigens than when HeLa cells were injected alone. The HeLa cell antiserum has been consistently of lower activity than the Hep 2 cell antiserum.

TABLE 9. Latex Agglutination of Hep 2 Cell Antigen with Various Antisera

	Hep 2 cell antigen						
HeLa poliovirus type 1 antiserum	Final antigen dilutions						
	1/8	1/16	1/32	1/64	1/128	1/256	Serum controls
1/20*	-	-	3+	4+	4+	3+	-
1/40	-	-	±	4+	4+	4+	-
1/80	-	-	±	1+	4+	4+	-
1/160	-	-	-	-	2+	4+	-
1/320	-	-	-	-	-	1+	-
Hep 2 cell antiserum							
1/20	2+	2+	3+	3+	4+	4+	-
1/40	2+	2+	3+	3+	4+	4+	-
1/80	1+	1+	1+	2+	2+	2+	-
1/160	-	-	-	-	±	±	-
1/320	-	-	-	-	-	-	-
HeLa cell antiserum							
1/20	-	-	±	2+	3+	3+	-
1/40	-	-	-	1+	2+	2+	-
1/80	-	-	-	-	-	-	-
1/160	-	-	-	-	-	-	-
1/320	-	-	-	-	-	-	-
Normal rabbit serum							
1/20	-	-	-	-	-	-	-
1/40	-	-	-	-	-	-	-
1/80	-	-	-	-	-	-	-
1/160	-	-	-	-	-	-	-
1/320	-	-	-	-	-	-	-
Antigen controls	-	-	-	-	-	-	Latex control -

* Final serum dilution after addition of complement diluted 1/30.

Experiment #6

Heterophil Test on All Antisera Used in the Investigation.

According to Wilson and Miles (1946), animals not containing heterophil antigen in their tissues are capable of producing heterophil antibody in response to injection of suitable tissues. The heterophil antibody, in addition to causing hemolysis of sheep red blood cells, also forms precipitates and fixes complement when mixed with tissue fractions containing heterophil antigen (Wilson and Miles, 1946). Therefore, the possibility of a heterophil antigen-antibody system causing the latex agglutination cross-reactions was considered. Accordingly, a standard Davidsohn's Presumptive Heterophil Test (Air Force Manual 160-47, 1960) was conducted on all the antisera including the normal rabbit serum.

The results of the standard heterophil test are shown in Table 10. No significant heterophil activity was observed. The HeLa cell antiserum caused a weak reaction (1+) at a dilution of 1/7 and 1/14 with the sheep red blood cells. The weak reactions observed with the HeLa cell antiserum suggested that further study should be undertaken. It was decided to do heterophil tests with Referencells (commercial human red blood cells marketed by Knickerbocker Biologics, Pfizer Labs. Div., Chas. Pfizer & Co., Inc.) which are used for human blood grouping. In addition, red blood cell (RBC) suspensions (5% concentration by volume) of various animals were also tested. The technic for conducting the test was as recommended for Referencells. The tube test

was as follows:

1. Place 2 drops of serum being tested into each test tube.

2. Add 1 drop of red blood cell suspension to each tube.

3. Mix well and centrifuge at 1500 rpm for 1 minute. (size #1 International Centrifuge SBR).

4. Gently resuspend the cell button and record the presence or absence of agglutination.

The results of this test are recorded in Tables 10a through 10g. All the rabbit antisera (undiluted) agglutinated the Referencells (Table 10a). Normal rabbit serum was negative. Titration of the various sera with human O, Rh- and O, Rh+ RBC's showed minor quantitative endpoint differences among the sera (Table 10b). Absorption of three antisera with Referencells indicated that the RBC antigens were not of the human blood groups. Absorption with one cell type (human group) effectively removed all the hemagglutinins to the other groups (Table 10c). These results indicated that other animal RBC's should be tested for agglutinability by host cell antisera. Various animal RBC's were tested with host cell antisera and normal rabbit serum. The results showed wide-spread activity with all the RBC's tested (Table 10d). Mouse, guinea pig, and monkey (Rhesus) RBC's were agglutinated by undiluted normal rabbit serum. Titration of the host cell antisera with the various animal RBC's indicated low activity with most RBC's (Table 10e). Monkey and human (O, Rh+) RBC's were agglutinated over all dilutions used in the test. Absorption of HeLa cell antiserum with monkey, human, and guinea pig RBC's indicated

TABLE 10. Davidsohn's Presumptive Heterophil Test with Various Sera and Sheep RBC

Serum types	Final serum dilutions									
	7*	14	28	56	12	224	448	896	1792	Saline control
HeLa polio 1	-	-	-	-	-	-	-	-	-	-
HeLa polio 2	-	-	-	-	-	-	-	-	-	-
HeLa polio 3	-	-	-	-	-	-	-	-	-	-
HeLa host cell	1+**	1+	-	-	-	-	-	-	-	-
Hep 2 host cell	-	-	-	-	-	-	-	-	-	-
Normal rabbit	-	-	-	-	-	-	-	-	-	-

* Reciprocal of serum dilution.

** Weak reactions.

Note: The procedure was done according to those outlined in Laboratory Procedures in Clinical Serology (Air Force Mannual 160-47, 1960).

TABLE 10a. Serum Hemagglutinin Activity with Commercial Referencells

Serum types*	Referencell type				
	H**	A_1	A_2	B	O
HeLa poliovirus type 1	+***	+	+	+	+
HeLa poliovirus type 2	+	+	+	+	+
HeLa poliovirus type 3	+	+	+	+	+
Hep 2 cell	+	+	+	+	+
Normal rabbit	-	-	-	-	-

* Serum types were used undiluted.

** H= Hemantigen. (Pooled human blood).

*** + = hemagglutination.

TABLE 10b. Titration of Hemagglutinins against Human O; Rh- and O, Rh+ Red Blood Cells*

Serum types	O (Rh-)	O (Rh+)
	CDE/ce ** ooo/++	CDE/ce ++o/o+
HeLa poliovirus type 1	64***	128
HeLa poliovirus type 2	32	128
HeLa poliovirus type 3	16	64
HeLa cell	32	64
Hep 2 cell	32	64
Normal rabbit	0	0

* 5% RBC concentration used.

** Blood group genotype.

*** Reciprocal of serum dilution before addition of RBC's.

TABLE 10c. Absorption of Antiserum with Human Blood Group Substance and Titration using Commercial Referencells

Serum types	Final serum dilution	Absorbed with	Referencell activity				
			H*	A_1	A_2	B	O
HeLa poliovirus 1	1/10	O	-	-	-	-	-
		A_1	-	-	-	-	-
		A_2	-	-	-	-	-
		B	-	-	-	-	-
		H	-	-	-	-	-
HeLa cell	1/10	O	-	-	-	-	-
		A_1	-	-	-	-	-
		A_2	-	-	-	-	-
		B	-	-	-	-	-
		H	-	-	-	-	-
Hep 2 cell	1/10	O	-	-	-	-	-
		A_1	-	-	-	-	-
		A_2	-	-	-	-	-
		B	-	-	-	-	-
		H	-	-	-	-	-

* H = Hemantigen (pooled human blood).

Note: Procedure: 0.5 ml of serum (diluted 1/5) was mixed with 0.5 ml of each Referencell type and held for 1 hour at room temperature; then the mixture was centrifuged at 1500 rpm for 10 minutes.

TABLE 10d. Red Blood Cell Agglutinin Spectra of Host Cell Antisera and Normal Rabbit Serum

Red blood cell type	HeLa cell antiserum	Hep 2 cell antiserum	Normal rabbit serum
mouse (swiss)	+	+	+
rabbit	-	-	-
human (O, Rh+)	+	+	-
guinea pig	+	+	+
sheep	+	+	-
chicken	+	+	-
monkey (Rhesus)	+	+	+

Note: All sera inactivated at 56°C for 30 minutes and used undiluted.

RBC's washed three times with 0.9% saline solution and resuspended to a 5% concentration by volume.

TABLE 10e. Titration of Hemagglutinin Against Various Animal Red Blood Cells

Red blood cell type	HeLa cell antiserum	Hep 2 cell antiserum	Normal rabbit serum
mouse (swiss)	8*	8	8
rabbit	0	0	0
human (O, Rh+)	32**	32**	0
guinea pig	16	16	2
sheep	4	2	0
chicken	2	2	0
monkey (Rhesus)	32**	32**	2

* Reciprocal of serum dilution.

** Last tube of the dilution series.

Note: All sera inactivated at 56°C for 30 minutes. RBC's washed three times with 0.9% saline solution and resuspended to a 5% concentration by volume.

that the hemagglutinin was not related to human blood group antigens (Table 10f). It also was observed that the efficacy of absorption was dependent upon the dilution of serum. For example, the addition of 5% RBC's to undiluted antiserum (equal volumes) failed to remove the antibodies. The addition of 5% RBC's to antiserum diluted 1/5 (equal volumes) removed the antibodies completely. Raposa and coworkers (1964) reported the presence of RBC agglutinins to human A, B, and O as well as to monkey, guinea pig, and rat but not to chicken and sheep RBC's with antisera prepared in rabbits against simian adeno- and simian enteroviruses. These findings are undoubtedly important; particularly as regards to studies concerned with developing hemagglutination technics for virus identification.

Absorption of the HeLa and Hep 2 cell antisera with guinea pig kidney antigen (GPK, Difco) and beef erythrocyte antigen (BE, Difco) as used in the Davidsohn's Differential Heterophil Test (Air Force Mannual 160-47, 1960) indicated that the hemagglutinin present in the host cell antisera was directed against an antigen of the Forssman type (Table 10g).

The influence of the heterophil antigen-antibody system on the latex agglutination reaction was determined. Table 10h shows the results of a homotypic and heterotypic Hep 2 poliovirus antigen latex titration with unabsorbed and GPK absorbed HeLa poliovirus type 2 antiserum. No change was observed indicating that the heterophil system was not contributing to the latex agglutination cross-reactions. It appeared that dilution was sufficient to remove the heterophil influences.

TABLE 10f. Absorption of HeLa Cell Antiserum with Animal RBC's and Tested for Agglutinins to Commercial Referencells

Red blood cell used for absorption	Final serum dilution	Before absorption	Agglutinin activity						
			H*	A_1	A_2	B	O	M**	GP***
monkey (Rhesus)	1/2	+	+	+	±	+	+	±	+
guinea pig	1/2	+	+	+	±	+	+	±	±
human (O Rh+)	1/2	+	+	+	±	+	+	+	±
monkey (Rhesus)	1/10	+	-	-	-	-	-	-	-
guinea pig	1/10	+	-	-	-	-	-	-	-
human (O Rh+)	1/10	+	-	-	-	-	-	-	-

* H = Hemantigen (pooled human blood).
** = monkey RBC's.
*** = guinea pig RBC's.

Note: Procedure. 0.5 ml of serum undiluted or diluted 1/5 in saline solution was added to 0.5 ml 5% RBC type for 1 hour at room temperature; then the mixtures were centrifuged at 1500 rpm for 10 minutes.

TABLE 10g. Hemagglutinin Titer Before and After Absorption with Guinea Pig Kidney (GPK) and Beef Erythrocyte (BE) Antigens

Red blood cell type	HeLa cell antiserum absorbed by:			Hep 2 cell antiserum absorbed by:		
	None	GPK	BE	None	GPK	BE
mouse (swiss)	10*	0	10	10	0	10
rabbit	0	0	0	0	0	0
human (O Rh+)	40	0	40	40	0	20
guinea pig	20	0***	20	20	0***	10
sheep	5	0	5	5	0	5
chicken	+5**	0	0	+5**	0	0
monkey (Rhesus)	40	0***	40	40	0***	40

* Reciprocal of serum dilutions,

** ± = weak reaction in 1st tube.

*** Lysis of the 1/5 and 1/10 dilution after overnight incubation (4°C).

Note: Absorption was done according to procedures recommended by Difco.

TABLE 10h. Latex Agglutination of HeLa Poliovirus Type 2 Antiserum Absorbed with Guinea Pig Kidney (GPK) Antigen

Final unabsorbed antiserum dilutions	Antigens							
	Hep 2 poliovirus type 2				Hep 2 poliovirus type 1			
	Final antigen dilutions							
	1/8	1/16	1/32	1/64	1/8	1/16	1/32	1/64
1/20	2+	4+	4+	2+	2+	4+	3+	3+
1/40	2+	4+	4+	1+	2+	4+	2+	1+
1/80	2+	3+	4+	1+	2+	4+	2+	±
1/160	-	1+	1+	1+	±	2+	1+	-
Antiserum absorbed with GPK antigen								
1/20	2+	4+	4+	1+	2+	4+	3+	2+
1/40	2+	4+	4+	1+	2+	4+	3+	2+
1/80	2+	2+	4+	±	2+	4+	2+	-
1/160	±	1+	2+	±	-	2+	1+	±
Antigen controls	-	-	-	-	-	-	-	-

Note:

Serum controls and latex control were negative.

Whole serum inactivated at 56°C for 30 minutes.

Absorption procedure: 0.8 ml of serum was added to 3.2 ml of commercial guinea pig kidney antigen and incubated for 1 hour at room temperature; the mixture was centrifuged at 2000 rpm for 10 minutes.

Experiment #7

Latex Agglutination of Poliovirus Complement Fixation (CF) Antigen.

Previous experiments indicated that the presence of host cell antigens in the virus-antibody system were responsible for latex agglutination cross-reactions. Poliovirus propagated in a different host cell might eliminate this effect. A commercial complement fixation (CF) poliovirus antigen made by propagating the virus in human amnion cells was selected for the experiment. To determine the role of complement, parallel latex agglutination titrations were done with the commercial viral CF antigen with and without complement. The amnion cell control (CF) antigen, provided by the manufacturer, was also tested. The results are shown in Tables 11 and 11a. Latex agglutination between type specific poliovirus antiserum and control CF antigen showed some differences when complement was used as compared to the tests without complement (Table 11). Whether or not the differences noted were significant remains inconclusive. No significant difference was shown between the amnion cell control CF antigen and the heterotypic poliovirus antiserum with or without added complement (Table 11a). No significant differences were observed between poliovirus CF antigen with either homotypic (Table 11) or heterotypic (Table 11a) viral antisera with or without added complement. It was concluded that complement does not affect the latex agglutination reactions. This conclusion was consistent with the reports of Smith and coworkers (1956) and Belyavin (1955), regarding direct precipitation of poliovirus and other viruses. It was observed that the antigen excess inhibition zones were more prevalent among the control CF antigen

latex agglutination titrations than among the viral CF antigen. The reason for this effect was unknown. The Hep 2 cell antiserum also caused latex agglutination with the viral CF antigen (Table 11a). This indicated that HeLa, Hep 2, and human amnion cells have common antigens. The conclusion from this and previous experiments was that the latex agglutination cross-reactions were due to common antigens among the different cell lines.

TABLE 11. Latex Agglutination of Poliovirus Type 2 CF Antigen with Homotypic Antiserum in the Presence and Absence of Complement

HeLa poliovirus type 2 antiserum	Poliovirus type 2 CF antigen					
	Final antigen dilutions					
No complement added	1/32	1/64	1/128	1/256	1/512	Serum controls
1/20*	4+	4+	4+	3+	2+	-
1/40	4+	4+	4+	3+	2+	-
1/80	2+	4+	4+	4+	±	-
1/160	-	4+	4+	3+	±	-
1/320	-	1+	4+	3+	±	-
Complement added**						
1/20*	4+	4+	4+	4+	4+	-
1/40	4+	4+	4+	3+	2+	-
1/80	4+	4+	3+	2+	-	-
1/160	4+	4+	3+	2±	=	=
1/320	±	3+	4+	2+	-	-
Antigen controls	-	-	-	-	± Latex control	-
No complement added	Amnion cell control CF antigen					
1/20*	±	4+	3+	±	-	
1/40	-	±	1+	1+	±	
1/80	-	±	±	±	±	
1/160	-	-	±	±	±	
1/320	-	-	-	±	±	
Complement added**						
1/20*	4+	4+	3+	1+	-	
1/40	1+	4+	4+	2+	±	
1/80	-	±	4+	2+	±	
1/160	-	-	±	2+	±	
1/320	-	-	-	1+	±	
Antigen controls	-	-	-	-	-	

* Final serum dilutions.

** Complement added to each tube at a dilution of 1/30.

TABLE 11a. Latex Agglutination of Poliovirus Type 2 CF Antigen with Heterotypic Antiserum in the Presence and Absence of Complement

HeLa poliovirus type 1 antiserum	Poliovirus type 2 CF antigen					
	Final antigen dilutions					
No complement added	1/32	1/64	1/128	1/256	1/512	Serum controls
1/20*	4+	4+	4+	4+	4+	-
1/40	4+	4+	4+	4+	2+	-
1/80	4+	4+	3+	2+	2+	-
1/160	-	4+	4+	3+	1+	-
1/320	-	1+	4+	4+	1+	-
Complement added**						
1/20*	4+	4+	4+	4+	2+	-
1/40	4+	4+	4+	3+	2+	-
1/80	4+	4+	4+	2+	±	-
1/160	±	4+	4+	3+	±	-
1/320	-	1+	4+	2+	±	-
No complement added	**Amnion cell control CF antigen**					
1/20*	1+	4+	3+	3+	2+	
1/40	-	1+	3+	2+	±	
1/80	-	-	3+	2+	±	
1/160	-	-	-	1+	±	
1/320	-	-	-	±	±	
Complement added**						
1/20*	2+	4+	4+	3+	±	
1/40	-	3+	4+	4+	±	
1/80	-	±	2+	2+	±	
1/160	-	-	±	1+	1+	
1/320	-	-	-	1+	1+	
Hep 2 cell antiserum**	**Poliovirus type 2 CF antigen**					
1/20*	1+	4+	4+	3+	3+	-
1/40	-	4+	4+	3+	3+	-
1/80	-	2+	4+	4+	3+	-
1/160	-	-	3+	3+	2+	-
1/320	-	-	1+	1+	1+	-

* Final serum dilutions

** Complement added to each tube at a dilution of 1/30.

Experiment #3

The Influence of Electrolytes on the Stability of Latex Particles.

Loeb (1922) reported upon the effects of electrolytes on the stability of collodion particles. He emphasized that divalent cations were more effective than univalent cations in causing agglutination of the collodion particles. He attributed this effect to reduced electrostatic repulsion (zeta potential forces) as caused by the valency of the cation upon the negatively charged collodion particles. Latex particles also carry a negative charge (Difco) similar to collodion particles. Singer and Plotz(1956) stated that calcium chloride and magnesium chloride did not increase the agglutination of latex in the presence of positive rheumatoid serum. In previous experiments, a tris buffered diluent containing calcium and magnesium ions was used. It was necessary to determine whether or not these cations entered into the reaction. Maximum latex sensitivity in the absence of non-specific agglutination was important. Therefore, the influence of electrolytes on latex particle stability was studied prior to any additional antigen-antibody latex agglutinations.

The influence of various protein precipitants on latex stability was also studied. This test was included since future experiments may require fractionated (globulin) rather than whole serum. Rheins et al (1957) reported that albumin could function as a protective colloid and inhibit latex agglutination even in the presence of known positive rheumatoid serum.

The results of this experiment are recorded in Tables 12, 13, and 14. The divalent cation containing salts were done in duplicate. Electrolytes exceeding a certain concentration caused the latex particles to agglutinate (Table 12); divalent cations being more effective than univalent cations. This was in agreement with the results of Loeb (1922). Calcium chloride caused non-specific latex agglutination at a molar strength of 1.2×10^{-2} (0.13%). Magnesium chloride caused non-specific agglutination at a molar strength of 4.9×10^{-2} (0.46%). Sodium chloride caused non-specific agglutination of latex particles at a molar strength of 3.7×10^{-1} (2.16%). The protein precipitants, Na_2SO_4 and $(NH_4)_2SO_4$, also caused non-specific agglutination of latex particles (Tables 13 and 14). Sodium sulfate caused the latex particles to agglutinate at a concentration of 10 per cent (Table 13). Ammonium sulfate caused the latex particles to agglutinate at a relative concentration of 1/32 saturation (Table 14).

The results of conducting a latex agglutination test with Hep 2 poliovirus type 1 antigen and homotypic antiserum are recorded in Table 15. The diluent in this titration contained added calcium ions calculated as salt at a molar concentration of 2.26×10^{-3} (0.025%). This concentration of calcium chloride was below the level shown to produce non-specific agglutination of latex particles (0.13%). Examination of the reaction tubes indicated increased sensitivity in that the intensity of the agglutinated particles was more clear-cut. It was concluded that divalent cations (Ca^{++}) increased the sensitivity of antigen-antibody latex agglutination.

TABLE 12. Influence of Certain Salts on Stability of Latex Particles

Tube No.	Dilution factor	Final molarity calculated as salt*	Final latex particle concentration ** 50% light transmission				
			NaCl	$MgCl_2$	$MgCl_2$	$CaCl_2$	$CaCl_2$
1	1/1	1.5×10^{0}	ND***	1+	1+	ND	ND
2	1/2	7.5×10^{-1}	4+	3+	3+	4+	4+
3	1/4	3.7×10^{-1}	2+	4+	4+	4+	4+
4	1/8	1.9×10^{-1}	-	4+	4+	4+	4+
5	1/16	9.9×10^{-2}	-	4+	4+	4+	4+
6	1/32	4.9×10^{-2}	-	3+	2+	4+	3+
7	1/64	2.5×10^{-2}	-	±	±	3+	3+
8	1/128	1.2×10^{-2}	-	-	-	3+	3+
9	1/256	6.2×10^{-3}	-	-	-	1+	±
10	1/512	3.1×10^{-3}	-	-	-	-	-
11	1/1024	1.5×10^{-3}	-	-	-	-	-
12	1/2048	7.7×10^{-4}	-	-	-	-	-
13	1/4096	3.8×10^{-4}	-	-	-	-	-
14	-	0.85% NaCl	-	-	-	-	-
15	-	Dist. H_2O	-	-	-	-	-

* $CaCl_2$ and $MgCl_2$ were diluted in 0.85% NaCl. All diluents buffered with tris at pH 8.2. Molarity of initial salts = 2 M.

** Measured spectrophotometrically in distilled water.

*** ND = not done.

Note: Procedure: Tubes were incubated at 4°C overnight to obtain ionic equilibria and then centrifuged at 2000 rpm for 5 minutes before reading results.

TABLE 13. Influence of Na_2SO_4 on the Stability of Latex Particles

Tube No.	Dilution factor	Concentration * of Na_2SO_4 in per cent	Results
1	1/1	20	2+
2	1/2	10	2+
3	1/4	5	1+
4	1/8	2.5	±
5	1/16	1.25	-
6	1/32	0.625	-
7	1/64	0.3125	-
8	1/128	0.15625	-
9	1/256	0.078125	-
10	control**	-	-

* Salt concentration calculated before adding latex particles.

** Tris buffered diluent pH 8.2 (0.145 M NaCl + 0.00226 M $CaCl_2$).

Note: Procedure: Tubes incubated at $4^{\circ}C$ overnight; then centrifuged at 2000 rpm for 5 minutes before reading results.

TABLE 14. Influence of $(NH_4)_2SO_4$ on the Stability of Latex Particles

Tube No.	Dilution factor	Relative concentration* of $(NH_4)_2SO_4$	Results
1	1/2	S/2	3+
2	1/4	S/4	4+
3	1/8	S/8	4+
4	1/16	S/16	3+
5	1/32	S/32	2+
6	1/64	S/64	1+
7	1/128	S/128	±
8	1/256	S/256	-
9	1/512	S/512	-
10	Control**	-	-

* Stock $(NH_4)_2SO_4$ was a saturated solution; salt concentration calculated before adding latex particles.

** Tris buffered 0.145 M NaCl solution at pH 8.2

TABLE 15. Latex Agglutination Sensitivity of Hep 2 Poliovirus Type 1 Antigen and Homotypic Antiserum with a Modified Diluent Containing 0.145 M NaCl and 0.00226 M $CaCl_2$ (Tris Buffered pH 8.2)

	Hep 2 poliovirus type 1 antigen					
HeLa poliovirus type 1 antiserum	Final antigen dilutions					
	1/16	1/32	1/64	1/128	1/256	Serum controls
1/10*	4+	4+	4+	4+	2+	-
1/20	4+	4+	4+	4+	2+	-
1/40	4+	4+	4+	4+	2+	-
1/80	3+	4+	4+	4+	2+	-
1/160	±	2+	4+	4+	3+	-
1/320	-	±	1+	3+	3+	-
1/640	-	±	±	3+	3+	-
1/1280		-	±	1+	2+	-
Antigen controls	-	-	-	-	-	Latex control -

*Final dilutions.

Note: Antiserum not inactivated.

Salt concentration was prepared by adding 8.5 g NaCl plus 0.25 g $CaCl_2$ per liter to tris buffer pH 8.2.

Experiment #9

Latex Agglutination of Antisera Absorbed with Hep 2 Cell Antigen.

Latex agglutination cross-reactions among the reagents in previous experiments had shown that HeLa, Hep 2, and human amnion cells have common antigens. Removal of the common antibodies in the various antisera to host cells by serum absorption was undertaken. The antisera were absorbed with Hep 2 cell antigen. It was found that host cell antibodies could be removed from the antisera by conducting latex agglutination of Hep 2 cell antigen with the antisera. The host cell antigen-antibody-latex complex was removed by centrifugation. The supernatant fluids were then re-tested for remaining antibody.

Latex agglutination of Hep 2 poliovirus type 2 and Hep 2 cell antigens are shown in Tables 16 and 17 with unabsorbed homotypic and heterotypic poliovirus antisera and Hep 2 cell antiserum. The tubes making up the titrations from Table 17 (Hep 2 cell antigen) were then centrifuged at 2200 rpm for 20 minutes and the clear supernatant fluids (Hep 2 cell absorbed antisera) retested with the Hep 2 poliovirus type 2 antigen as used in Table 16 (control reaction). The results are shown in Table 18. The Hep 2 cell antiserum lost most of its antibodies. This indicated effective absorption of the host cell antibodies. Results with the Hep 2 cell absorbed HeLa poliovirus antisera were difficult to interpret. Whether or not the obvious changes observed between the latex agglutinations of Table 16 and Table 18 were due to narrowed optimal zones and still represented host cell cross-reactions was difficult to assess. Why the absorption was less complete at antigen dilutions of 1/16 and 1/32 as observed in

Table 18 was unknown. Were poliovirus group specific antigens involved? This was the first evidence suggestive of mixed antigen-antibody systems; i. e., the cellular system and the viral system. It was possible that the relative titer of the host cell antibody in the different antisera coupled with the effect of antigen excess inhibition caused the observations. The absence of viral antigen-antibody type specificity indicated that the serum absorption procedure should be modified.

Goldsworthy and Rudd (1935) emphasized the importance of optimal proportions to obtain maximum absorption of unwanted antibodies and/or antigens in a mixed system. The optimal zone in the latex agglutination reaction was unknown. Consequently, prior to absorption, the antisera to be absorbed was diluted from 1/5 through 1/160 and then mixed with an equal volume of latex particles and Hep 2 cell antigen and Hep 2 cells. These mixtures were incubated for 1 hour at 37°C and then placed at 4°C overnight. Following incubation, the tubes were centrifuged at 2200 rpm for 20 minutes and the supernatant fluids titrated with Hep 2 cell and Hep 2 poliovirus type 2 antigens. The results are shown in Tables 19 and 19a. The Hep 2 cell antigen titrations (Table 19a) were negative with the absorbed antisera. This indicated effective absorption of the host cell antibody from the antisera (at all dilutions). The Hep 2 poliovirus type 2 antigen (Table 19) was latex positive at the 1/20 and 1/40 dilution with specific antiserum. This may be interpreted as viral antigen-antibody specificity. One tube (antigen dilution of 1/16) showed latex agglutination with the heterotypic

TABLE 16. Latex Agglutination of Hep 2 Poliovirus Type 2 Antigen with Various Antisera (Before Absorption)

HeLa poliovirus type 2 antiserum	Hep 2 poliovirus type 2 antigen					
	Final antigen dilutions					
Final dilutions	1/16	1/32	1/64	1/128	1/256	Serum controls
1/20	4+	4+	4+	4+	3+	-
1/40	4+	4+	4+	3+	3+	-
1/80	4+	3+	2+	2+	1+	-
1/160	3+	3+	2+	±	-	-
1/320	2+	3+	±	-	-	-
HeLa poliovirus type 3 antiserum						
1/20	4+	4+	4+	4+	2+	-
1/40	4+	3+	3+	3+	2+	-
1/80	3+	3+	1+	±	±	-
1/160	3+	3+	1+	±	-	-
1/320	2+	3+	1+	±	-	-
Hep 2 cell antiserum						
1/20	4+	4+	4+	4+	3+	-
1/40	4+	4+	4+	3+	2+	-
1/80	2+	3+	3+	2+	2+	-
1/160	-	2+	2+	1+	±	-
1/320	-	-	1+	1+	±	-
Antigen controls	-	-	-	±	± Latex control	-

TABLE 17. Latex Agglutination of Hep 2 Cell Antigen with Various Antisera (Before Absorption)

HeLa poliovirus type 2 antiserum	Hep 2 cell antigen				
	Final antigen dilutions				
Final dilutions	1/16	1/32	1/64	1/128	1/256
1/20	3+	3+	4+	4+	2+
1/40	3+	3+	4+	4+	3+
1/80	3+	3+	2+	1+	-
1/160	1+	1+	±	-	-
1/320	1+	1+	±	-	-
HeLa poliovirus type 3 antiserum					
1/20	3+	3+	3+	2+	±
1/40	3+	3+	2+	1+	±
1/80	3+	3+	2+	±	-
1/160	3+	3+	1+	-	-
1/320	1+	3+	±	-	-
Hep 2 cell antiserum					
1/20	3+	4+	4+	3+	2+
1/40	3+	3+	3+	3+	2+
1/80	±	3+	3+	3+	2+
1/160	-	2+	±	±	±
1/320	-	-	±	-	-
Antigen controls	-	-	±	±	±

TABLE 18 Latex Agglutination of Hep 2 Poliovirus Type 2 Antigen with Hep 2 Cell Absorbed Antisera

Absorbed HeLa poliovirus type 2 antiserum	Hep 2 poliovirus type 2 antigen				
	Final antigen dilutions				
Final dilutions	1/16	1/32	1/64	1/128	1/256
1/40	3+	3+	2+	2+	1+
1/80	4+	4+	1+	±	-
1/160	1+	4+	1+	±	-
1/320	-	4+	1+	-	-
1/640	-	±	±	-	-
Absorbed HeLa poliovirus type 3 antiserum					
1/40	3+	3+	±	-	-
1/80	3+	3+	1+	-	-
1/160	1+	3+	±	-	-
1/320	-	3+	±	-	-
1/640	-	-	-	-	-
Absorbed Hep 2 cell antiserum					
1/40	-	2+	±	±	±
1/80	-	±	±	±	-
1/160	-	-	-	-	-
1/320	-	-	-	-	-
1/640	-	-	-	-	-
Antigen controls	-	-	-	±	±

TABLE 19. Latex Agglutination of Hep 2 Poliovirus Type 2 Antigen With Hep 2 Cell Absorbed Antisera

Absorbed HeLa poliovirus type 2 antiserum	Hep 2 poliovirus type 2 antigen					
	Final antigen dilutions					
Final dilutions	1/16	1/32	1/64	1/128	1/256	Serum controls
1/20	4+	2+	±	-	-	-
1/40	4+	4+	3+	2+	-	-
1/80	±	1+	2+	1+	-	-
1/160	-	-	-	-	-	-
1/320	-	-	-	-	-	-
1/640	-	-	-	-	-	-
Absorbed HeLa poliovirus type 3 antiserum						
1/20	±	±	1+	±	-	-
1/40	2+	1+	±	-	-	-
1/80	±	±	-	-	-	-
1/160	-	-	-	-	-	-
1/320	-	-	-	-	-	-
1/640	-	-	-	-	-	-
Absorbed Hep 2 cell antiserum						
1/20	-	-	±	±	-	-
1/40	-	-	-	-	-	-
1/80	-	-	-	-	-	-
1/160	-	-	-	-	-	-
1/320	-	-	-	-	-	-
1/640	-	-	-	-	-	-
Antigen controls	-	-	-	±	±	Latex control -

TABLE 19a. Latex Agglutination of Hep 2 Cell Antigen with Hep 2 Cell Absorbed Antisera

Absorbed HeLa poliovirus type 2 antiserum	Hep 2 cell antigen				
	Final antigen dilutions				
Final dilutions	1/16	1/32	1/64	1/128	1/256
1/20	-	-	-	-	-
1/40	-	-	-	±	-
1/80	-	-	-	±	-
1/160	-	-	-	-	-
1/320	-	-	-	-	-
1/640	-	-	-	-	-
Absorbed HeLa poliovirus type 3 antiserum					
1/20	-	-	-	-	-
1/40	-	-	-	-	-
1/80	-	±	-	-	-
1/160	-	-	-	-	-
1/320	-	-	-	-	-
1/640	-	-	-	-	-
Absorbed Hep 2 cell antiserum					
1/20	-	-	-	-	-
1/40	-	-	-	-	-
1/80	-	-	-	-	-
1/160	-	-	-	-	-
1/320	-	-	-	-	-
1/640	-	-	-	-	-
Antigen controls	-	-	-	-	±

HeLa poliovirus type 3 antiserum. The control Hep 2 cell antiserum was negative at all serum and antigen dilutions (Table 19). These were encouraging results. However, the significance of this titration will have to remain speculative. The antibody titer of the several antisera used for absorption and their eventual loss of detectability with subsequent latex testing may have contributed to the results suggestive of viral antigen-antibody specificity. From previous experiments, it was also apparent that antigen excesses inhibit latex agglutination. It was possible that antigen excesses relative to the various antibody titers after absorption caused the results.

A third antiserum absorption test was performed. This absorption consisted of absorbing one antiserum (HeLa poliovirus type 3 antiserum) with homotypic and heterotypic Hep 2 poliovirus and with Hep 2 cell antigens. The results are presented in Tables 20, 21, and 22. Table 20 shows latex agglutination of HeLa poliovirus type 3 antiserum before absorption with the various antigens (control titration). Table 21 shows the results of re-testing for remaining antibody in the supernatant fluids (from Table 20) with heterotypic poliovirus antigens. The loss of latex agglutination at the high dilutions of antigen with Hep 2 poliovirus antigens should be noted. The loss of latex agglutination with the control Hep 2 cell antigen system was at the low dilutions of antigen. This indicated absorption phenomenon. The supernatant fluids of the twice absorbed viral antiserum was re-tested with Hep 2 cell antigen, (from Table 21). The results are shown in Table 22. Latex agglutinations were suggestive of the Hep 2 cell antigen control

titration as performed in Table 20. The HeLa poliovirus type 3 antiserum twice absorbed with Hep 2 cell antigen failed to react when re-tested with specific viral antigen.

The results of this experiment were inconclusive. The results observed in one absorption test (Tables 16, 17, and 18) indicated group specific poliovirus antigens. The results of the second absorption test (Tables 19 and 19a) indicated poliovirus antigen-antibody type specificity. The results of the third absorption test (Tables 20, 21 and 22) indicated that the virus-antibody system was non-latex reactive.

TABLE 20. Latex Agglutination of Hep 2 Poliovirus Types 1 and 3 and Hep 2 Cell Antigens with HeLa Poliovirus Type 3 Antiserum

HeLa poliovirus type 3 antiserum	Hep 2 poliovirus type 1 antigen					
	Final antigen dilutions					
Final dilutions	1/16	1/32	1/64	1/128	1/256	Serum controls
1/10	4+	4+	4+	4+	2+	-
1/20	4+	4+	4+	4+	3+	-
1/40	4+	4+	4+	4+	3+	-
1/80	4+	4+	2+	±	±	-
1/160	4+	4+	2+	-	-	-
Antigen controls	-	-	-	±	±	Latex control -
	Hep 2 poliovirus type 3 antigen					
1/10	3+	4+	4+	4+	3+	
1/20	4+	4+	4+	4+	3+	
1/40	3+	3+	2+	2+	2+	
1/80	4+	4+	2+	1+	±	
1/160	2+	3+	2+	±	-	
Antigen controls	-	-	-	±	±	
	Hep 2 cell antigen					
1/10	4+	4+	4+	4+	4+	
1/20	±	4+	4+	4+	3+	
1/40	-	±	4+	4+	4+	
1/80	-	-	±	4+	4+	
1/160	-	-	-	1+	4+	
Antigen controls	-	-	-	-	-	

TABLE 21. Latex Agglutination of Hep 2 Poliovirus Types 1 and 3 Antigens with Cross-Absorbed HeLa Poliovirus Type 3 Antiserum

Absorbed HeLa poliovirus type 3 antiserum	Hep 2 poliovirus type 3 antigen				
	Final antigen dilutions				
Final dilutions	1/16	1/32	1/64	1/128	1/256
Poliovirus type 3 antiserum absorbed with Hep 2 poliovirus type 1					
1/20	4+	4+	2+	1+	1+
1/40	4+	4+	2+	±	±
1/80	2+	4+	2+	±	-
1/160	-	3+	3+	±	-
1/320	-	2+	3+	±	-
Poliovirus type 3 antiserum absorbed with Hep 2 poliovirus type 3					
	Hep 2 poliovirus type 1 antigen				
1/20	4+	4+	1+	±	-
1/40	4+	3+	1+	±	-
1/80	4+	3+	2+	±	-
1/160	1+	3+	3+	±	-
1/320	-	2+	3+	-	-
Poliovirus type 3 antiserum absorbed with Hep 2 cell antigen					
	Hep 2 cell antigen				
1/20	-	-	3+	4+	4+
1/40	-	-	-	4+	4+
1/80	-	-	-	-	2+
1/160	-	-	-	-	±
1/320	-	-	-	-	-

TABLE 22. Latex Agglutination of Hep 2 and Hep 2 Poliovirus Type 3 Antigens with Twice Absorbed HeLa Poliovirus Type 3 Antiserum

Twice absorbed HeLa poliovirus type 3 antiserum	Hep 2 cell antigen				
	Final antigen dilutions				
Final dilutions	1/16	1/32	1/64	1/128	1/256
Polio type 3 antiserum absorbed with Hep 2 poliovirus types 1 and 3*					
1/40	±	2+	4+	4+	4+
1/80	-	±	3+	4+	4+
1/160	-	-	-	2+	4+
1/320	-	-	-	-	2+
1/640	-	-	-	-	-
Polio type 3 antiserum absorbed with Hep 2 poliovirus types 3 and 1*					
1/40	-	-	4+	4+	4+
1/80	-	-	-	4+	4+
1/160	-	-	-	1+	3+
1/320	-	-	-	-	1+
1/640	-	-	-	-	-
Antigen controls	-	-	-	-	-
Polio type 3 antiserum twice absorbed with Hep 2 cell antigen					
	Hep 2 poliovirus type 3 antigen				
1/40	-	-	-	-	-
1/80	-	-	-	-	-
1/160	-	-	-	-	-
1/320	-	-	-	-	-
1/640	-	-	-	-	-
Antigen controls	-	-	±	±	-

* Sequence of absorption (see Tables 20 and 21).

Experiment #10

Latex Agglutination of Hep 2 Poliovirus Type 1 and 2 Antigens following Ultracentrifugation.

Experiment #9 showed that when specific poliovirus antiserum was twice absorbed with Hep 2 cell antigen, the antiserum failed to produce a positive latex agglutination with specific poliovirus antigen. These results suggested that the Hep 2 poliovirus antigens might contain non-specific latex agglutination inhibitors. In addition, there was the problem of host cell antigen cross-reactions. In an attempt to remove part of the antigens, Hep 2 poliovirus type 1 antigen was ultracentrifuged. (Spinco Model L Centrifuge - rotor No. 40). Tables 23, 24, and 25 show the results of latex agglutination tests on the supernatant fluids following a specified time and force of centrifugation. No significant changes in the latex agglutination results were observed. The results indicated that the latex agglutination cross-reacting antigen was smaller than 100 mu since the force and time of centrifugation would have sedimented particles of this size (Spinco Model L Operating Manual). Virus infectivity was not determined in the supernatant fluids following this initial ultracentrifugation.

A second ultracentrifugation series was conducted with Hep 2 poliovirus type 2 antigen. The supernatant fluids and the final pellet were tested for latex agglutinability and virus infectivity (TCD_{50} per ml). The methods used and results are outlined in Table 26. The latex agglutination reactions were not tabulated since the reactions were similar to the titrations as recorded for Hep 2 poliovirus type 1 (Table 24). These results indicated

TABLE 23. Latex Agglutination of Hep 2 Poliovirus Type 1 Antigen Supernatant Fluid after Ultracentrifugation at 15,000 rpm (20,360 X g) for 30 Minutes

HeLa poliovirus type 1 antiserum	Hep 2 poliovirus type 1 antigen					
	Final antigen dilutions					
Final dilutions	1/16	1/32	1/64	1/128	1/256	Serum controls
1/10	4+	4+	4+	4+	2+	-
1/20	4+	4+	4+	4+	3+	-
1/40	3+	4+	4+	4+	3+	-
1/80	3+	3+	4+	4+	3+	-
1/160	3+	2+	2+	2+	2+	-
HeLa poliovirus type 3 antiserum						
1/10	3+	3+	4+	4+	2+	-
1/20	3+	3+	3+	3+	2+	-
1/40	3+	3+	2+	2+	2+	-
1/80	3+	3+	±	±	±	-
1/160	3+	3+	±	-	-	-
Hep 2 cell antiserum						
1/10	2+	3+	3+	2+	±	-
1/20	2+	2+	2+	1+	±	-
1/40	2+	1+	1+	±	±	-
1/80	-	1+	1+	±	±	-
1/160	-	-	-	-	-	-
Antigen controls	-	-	-	±	±	Latex control -

TABLE 24. Latex Agglutination of Hep 2 Poliovirus Type 1 Antigen Supernatant Fluid after Ultracentrifugation at 20,000 rpm (36,190 X g) for 30 Minutes

HeLa poliovirus type 1 antiserum	Hep 2 poliovirus type 1 antigen					
	Final antigen dilutions					
Final dilutions	1/16	1/32	1/64	1/128	1/256	Serum controls
1/10	4+	4+	4+	4+	2+	-
1/20	4+	4+	4+	4+	3+	-
1/40	3+	4+	4+	4+	4+	-
1/80	3+	3+	4+	4+	4+	-
1/160	3+	3+	3+	3+	3+	-
HeLa poliovirus type 3 antiserum						
1/10	4+	4+	4+	4+	3+	-
1/20	4+	4+	4+	4+	2+	-
1/40	3+	3+	3+	4+	3+	-
1/80	3+	3+	±	±	±	-
1/160	2+	3+	±	±	±	-
Hep 2 cell antiserum						
1/10	2+	4+	4+	2+	±	-
1/20	2+	4+	4+	4+	1+	-
1/40	2+	2+	3+	3+	2+	-
1/80	±	2+	3+	3+	2+	-
1/160	-	±	±	±	±	-
Antigen controls	-	-	-	-	± Latex control	-

TABLE 25. Latex Agglutination of Hep 2 Poliovirus Type 1 Antigen Supernatant Fluid after Ultracentrifugation at 30,000 rpm (81,430 X g) for 30 Minutes

HeLa poliovirus type 1 antiserum	Hep 2 poliovirus type 1 antigen					
	Final antigen dilutions					
Final dilutions	1/16	1/32	1/64	1/128	1/256	Serum controls
1/20	4+	4+	4+	4+	2+	-
1/40	4+	4+	4+	4+	2+	-
1/80	3+	4+	4+	4+	2+	-
1/160	2+	2+	3+	3+	±	-
1/320	2+	2+	2+	2+	±	-
HeLa poliovirus type 3 antiserum						
1/20	3+	3+	4+	3+	1+	-
1/40	2+	2+	4+	4+	1+	-
1/80	2+	2+	2+	2+	1+	-
1/160	2+	2+	-	-	-	-
1/320	2+	2+	-	-	-	-
Hep 2 cell antiserum						
1/20	2+	3+	4+	3+	2+	-
1/40	2+	2+	4+	3+	2+	-
1/80	2+	2+	2+	1+	±	-
1/160	±	1+	1+	-	-	-
1/320	-	-	-	-	-	-
Antigen controls	-	-	-	±	±	Latex control -

TABLE 26. Hep 2 Poliovirus Type 2 Antigen Centrifugation Protocol and Test Results Outlined

Centrifugation protocol

Step #1. 1500 rpm for 30 minutes (500 X g);(Size #1 International Centrifuge SBR);Clarified supernatant fluid recovered and used for step #2.

Step #2. 20,000 rpm for 30 minutes (36,190 X g);(Spinco Model L Centrifuge - rotor No. 40); Supernatant fluid recovered and used for step #3 and for testing; Pellet restored to original volume and tested for virus infectivity.

Step #3. 35,000 rpm for 30 minutes (110,800 X g);(Spinco Model L Centrifuge - rotor No. 40); Supernatant fluid recovered and used for testing; Pellet restored to original volume and used for testing.

Results outlined

	Supernatant fluid		Pellet	
Protocol number	Latex agglutination reaction	Virus titer	Latex agglutination reaction	Virus titer
1	ND*	$10^{5.50}$	ND	ND
2	+++**	$10^{5.45}$	ND	$10^{4.66}$
3	+++	-***	-***	$10^{6.00}$

* ND = Not done.

** +++ = Latex agglutination similar to other titrations conducted previously; see text.

*** - = No infectivity (CPE) observed at the lowest dilution of virus used (10^{-3}). No latex agglutinations observed.

Note: In the latex agglutination titration, final antigen dilutions were 1/16 through 1/256; final antisera dilutions were 1/20 through 1/320.

that the latex agglutinable antigen was soluble. The absence of latex agglutination in the final 35,000 rpm pellet paralleled the results of the serum absorption tests, in that no poliovirus-antibody latex agglutination was observed. Virus infectivity determinations of the various supernatant fluids and pellets indicated that virus was sedimented at the 20,000 rpm cycle. During the final 35,000 rpm cycle, the infective virus was completely sedimented as evidenced by its absence in the supernatant fluid. The 35,000 rpm pellet showed an infectivity titer equivalent to the original clarified harvest material. The infectivity titer as found in the resuspended pellet following the 20,000 rpm cycle indicated the existence of a close association of the poliovirus with host cell material since little virus should have sedimented at the centrifugal force and time used (Smith et al, 1956).

Why the final 35,000 rpm pellet material failed to cause latex agglutination with antisera was unknown. However, perhaps inhibitors were involved? It was also possible that the virus concentration was below the critical level required for latex agglutination with specific antibody? Further study will be required to answer the above questions.

Experiment #11

Latex Agglutination of Hep 2 poliovirus Type 2 Antigen precipitated with Ammonium Sulfate.

Experiment #10 indicated that the latex agglutinating antigen was soluble. It was possible that the host cell antigen substance might be separable by salt fractionation. Clark and coworkers (1941) and Loring and Schwerdt (1942) reported that poliovirus can be precipitated by one third to one half saturated ammonium sulfate. One half saturation with ammonium sulfate of Hep 2 poliovirus type 2 antigen was used for the experiment. Fractionated serum (globulin) was also used as the source of antibody rather than whole serum. The salt was added in the following manner: (1) a volume of saturated ammonium sulfate was added to an equal volume of clarified viral harvest; (2) the precipitate was allowed to develop overnight at 4°C and then recovered by centrifugation at 2000 rpm for 20 minutes; (3) the supernatant fluid was decanted carefully; and (4) the precipitate was dissolved in tris buffered saline solution at pH 8.2 to its original volume. The reconstituted precipitate was titrated for virus infectivity in Hep 2 cell culture. The TCD_{50} per ml was $10^{6.33}$ and was equivalent to the infectivity of the original clarified harvest. The results of the latex agglutination titration are shown in Table 27. Latex agglutinations characteristic of previous experiments were observed. An interesting aspect of this study was the parallel virus infectivity determination. The results were consistent with the report of Clark et al (1941) and Loring et al (1942); i.e., half saturation with ammonium sulfate of poliovirus

TABLE 27. Latex Agglutination of Ammonium Sulfate Precipitated Hep 2 Poliovirus Type 2 Antigen

HeLa poliovirus type 2 antibody globulin*	Hep 2 poliovirus type 2 antigen				
	Final antigen dilutions				
Final dilutions	1/8	1/16	1/32	1/64	Antibody globulin controls
1/20	4+	4+	4+	2+	-
1/40	±	4+	4+	2+	-
1/80	-	±	4+	4+	-
1/160	-	-	±	3+	-
HeLa poliovirus type 1 antibody globulin					
1/20	4+	4+	4+	4+	-
1/40	4+	4+	4+	3+	-
1/80	-	3+	4+	2+	-
1/160	-	-	3+	2+	-
Hep 2 cell antibody globulin					
1/20	±	4+	3+	3+	-
1/40	-	2+	3+	3+	-
1/80	-	-	3+	3+	-
1/160	-	-	±	2+	-
Antigen controls	-	-	-	-	Latex control -

* Globulin fractions reconstituted (by volume) equivalent to whole antisera.

infected tissue culture fluid precipitated the virus almost completely as evidenced by the infectivity titer of the reconstituted floc.

It was concluded that the latex agglutinating antigen was a protein of the globulin type. This confirmed the soluble nature of the antigen as indicated in the ultracentrifugation experiment. There was also evidence suggestive that common host cell antigens exist as part of the complete virus particle. It should be recalled that serum cross-neutralization tests showed only homotypic specificity among the three poliovirus types. Latex agglutination could be conceivably demonstrating a different viral antigen-antibody reactive grouping. It should be noted that Grasset and coworkers (1958) reported the presence of neutralizing antibody to poliovirus in the absence of precipitating antibody and vice-versa. These authors suggested the possibility of group specific precipitating antigens and antibodies existing among the poliovirus types similar to those found in the complement fixation test.

Experiment #12

Latex Agglutination of Hep 2 Poliovirus Type 1 Antigen Adsorbed to Latex Particles at pH 4.0 with Various Antisera.

The isoelectric point of polioviruses was stated to be pH 4.5 (Mack, personal communication). Schwerdt and Schaffer (1955 and 1956), in studies on the purification of polioviruses, adjusted the pH of their infected tissue culture fluids to pH 4.0 to 4.5 prior to precipitating the virus with cold methanol. This adjustment of pH was added evidence that the isoelectric point of polioviruses was between 4.0 to 4.5. According to Oreskes and Singer (1961), maximum adsorption of proteins to latex particles probably occurs among the nonpolar chemical groupings of the protein. Thus, there was a rational basis for adjusting the pH prior to admixing the latex particles with the substance to be adsorbed. It was hoped to selectively adsorb out the poliovirus antigens and leave the host cell antigens remaining in the supernatant fluid. The experimental design of this experiment was done in the following manner:

1. Doubling dilutions of Hep 2 poliovirus type 1 antigen were made in 0.85% saline solution (unbuffered).

2. pH was adjusted to 4.0 (pH paper) with 0.2 M acetic acid solution.

3. Equal volumes of reagent latex (6.25% T) was added to each antigen dilution. Latex was suspended in unbuffered 0.85% saline solution.

4. The mixtures were incubated for 1 hour at 37°C in a water bath and then placed overnight at 4°C.

5. Following the incubation time allowed for adsorption, the mixtures were centrifuged at 2400 rpm for 30 minutes (size #1 International Centrifuge SBR).

6. The supernatant fluid was decanted and the sedimented latex particles resuspended in tris buffered pH 8.2 diluent, as previously used, to the original volume.

The results of this experiment are shown in Table 28. No selective latex agglutination suggestive of viral antigen-antibody specificity was observed. The results indicated that antigen was adsorbed onto the latex particles at the pH used. This was consistent with the report of Oreskes and Singer (1961). The results indicated that the latex agglutinable substance in the infected tissue culture fluids was at or close to the isoelectric point of the polioviruses. Here again, was evidence suggestive that common host cell antigens exist as part of the complete virus particle as speculated in experiment #11.

TABLE 28. Latex Agglutination of Hep 2 Poliovirus Type 1 Antigen Adsorbed to Latex Particles at pH 4.0 with Various Antisera

HeLa poliovirus type 1 antiserum	Hep 2 poliovirus type 1 antigen					
	Final antigen dilutions*					
Final dilutions	1/16	1/32	1/64	1/128	1/256	Serum controls
1/10	4+	4+	4+	2+	1+	-
1/20	4+	4+	4+	2+	1+	-
1/40	4+	4+	4+	2+	±	-
1/80	4+	4+	4+	2+	-	-
1/160	2+	2+	3+	3+	±	-
HeLa poliovirus type 2 antiserum						
1/10	4+	4+	2+	1+	-	-
1/20	4+	4+	3+	2+	-	-
1/40	2+	3+	3+	2+	±	-
1/80	1+	1+	2+	2+	±	-
1/160	-	-	±	1+	±	-
Hep 2 cell antiserum						
1/10	2+	2+	2+	1+	±	-
1/20	3+	2+	2+	1+	±	-
1/40	3+	3+	3+	±	±	-
1/80	3+	3+	3+	±	±	-
1/160	-	±	±	1+	±	-
Antigen controls	-	-	-	-	-	Latex control -

* Dilution factors based on dilutions before pH adjustment.

Experiment #13

Latex Agglutination of Monkey Kidney Cell (MK) Propagated Poliovirus Type Antigens with Various Antisera.

Previous experiments had shown that latex agglutination of Hep 2 poliovirus antigens with various antisera was due to the common origin of host cells. The presence of the host cell antigen-antibody system caused latex agglutination cross-reactions. It was postulated that host cells obtained from a different animal may eliminate the problem. It was therefore decided to propagate the poliovirus types in monkey kidney cells (Macaca rhesus).

To minimize the influence of Hep 2 cell antigens, the stock Hep 2 poliovirus types were diluted 1/10 in medium 199 prior to seeding. Three tenths ml of a 1/10 dilution was introduced into 32 oz size prescription bottles containing MK cells. This procedure represented a final dilution factor of 1/1000. After 36 hours of incubation at 37°C, the poliovirus infected tissue culture fluids were harvested. An additional 30 ml of medium 199 was added to each bottle and the bottles re-incubated until the cytopathic effect was complete (5 days). The viral harvests were handled in a manner identical to the Hep 2 poliovirus harvests; i.e., frozen and thawed three times; clarified at 1500 rpm for 30 minutes; and then stored at -60°C until used for testing.

Virus infectivity titrations of the MK poliovirus types were conducted in MK cell culture or Hep 2 cell culture as indicated in Table 29. Latex agglutination was performed with the 36 hour and the 5 day harvest used as antigen with HeLa poliovirus type 1 antiserum.

TABLE 29. Infectivity Titration of Monkey Kidney Poliovirus Types in Monkey Kidney and Hep 2 Cell Cultures

Virus Identification	TCD_{50} per ml Titered in	
	MK cells	Hep 2 cells
36 hour MK poliovirus type 1	$10^{7.50}$	ND*
36 hour MK poliovirus type 2	$10^{6.00}$	ND
36 hour MK poliovirus type 3	$10^{6.00}$	
5 day MK poliovirus type 1	$10^{6.66}$	$10^{7.00}$
5 day MK poliovirus type 2	$10^{6.50}$	$10^{7.33}$
5 day MK poliovirus type 3	$10^{6.50}$	$10^{7.00}$

* ND = Not done.

The results of the 36 hour harvest MK poliovirus type antigens are shown in Table 30. They were suggestive of virus-antibody activity. The control, Hep 2 cell antiserum (globulin) was essentially negative when tested with MK poliovirus type 1 antigen (Table 30a). Latex agglutination of the 5 day MK poliovirus type antigens were negative (not tabulated) under the same conditions of testing. The conclusion was that the 36 hour viral harvests contained sufficient Hep 2 cell antigen to effect a positive latex agglutination. It was reasoned, at the outset, that a 1/1000 dilution of the Hep 2 poliovirus types used as seed would effectively eliminate the presence of these host cell antigens. If these results were due to poliovirus-antibody latex agglutination, then the poliovirus selected for this investigation were manifesting group specificity. The absence of latex agglutination with the 5 day MK poliovirus type antigens suggested that host cell material might also function to inhibit latex agglutination. To test the latter postulation, MK cell cultures were infected with a high multiplicity of virus (original Hep 2 poliovirus types; undiluted initially) and allowed to attach to the cells for 1 hour at 37°C. Following the incubation period, the MK cell cultures were washed three times with medium 199. The fluids from the MK infected cultures were then removed at intervals of 12, 24, 36, 48, and 72 hours and at 6 days when the cytopathic effect was complete. These timely harvests were tested for: (1) latex agglutination with various antisera; and (2) virus infectivity titer. The latex agglutinations (using MK poliovirus type 1 as the model) were negative.

TABLE 30. Latex Agglutination of 36 Hour MK Poliovirus Type Antigens with Antibody Globulin

HeLa poliovirus type 1 antibody globulin*	MK poliovirus type 1 antigen					
	Final antigen dilutions					
Final dilutions	1/8	1/16	1/32	1/64	1/128	Antibody globulin controls
1/20	2+	4+	4+	2+	-	-
1/40	-	3+	4+	2+	-	-
1/80	-	2+	3+	3+	±	-
1/160	-	-	1+	2+	±	-
Antigen controls	-	-	-	-	-	Latex control -
	MK poliovirus type 2 antigen					
1/20	2+	2+	±	-	-	
1/40	1+	2+	±	-	-	
1/80	-	2+	1+	-	-	
1/160	-	-	1+	±	-	
Antigen controls	-	-	-	±	±	
	MK poliovirus type 3 antigen					
1/20	4+	4+	4+	±	-	
1/40	4+	4+	4+	1+	-	
1/80	-	3+	2+	2+	-	
1/160	-	-	2+	2+	-	
Antigen controls	-	-	-	-	-	

* Globulin fraction reconstituted (by volume) equivalent to whole serum.

TABLE 30a. Latex Agglutination of 36 hour MK Poliovirus Type 1 Antigen with Hep 2 Cell Antibody Globulin

Hep 2 cell antibody globulin*	MK poliovirus type 1 antigen					
	Final antigen dilutions					
Final dilutions	1/8	1/16	1/32	1/64	1/128	Antibody globulin controls
1/20	-	±	2+	1+	-	-
1/40	-	-	1+	1+	-	-
1/80	-	-	-	1+	±	-
1/160	-	-	-	-	±	-
Antigen controls	-	-	-	-	-	Latex control -

* Globulin fraction reconstituted (by volume) equivalent to whole serum.

Virus infectivity titers in MK cell cultures were as follows:

MK poliovirus type 1	TCD_{50} per ml
12 hour harvest	no CPE observed
24 hour harvest	$10^{5.50}$
36 hour harvest	$10^{5.50}$
48 hour harvest	$10^{6.00}$

The negative latex agglutination results with the various MK poliovirus type 1 harvests again suggested that non-specific latex agglutination inhibitors might be involved. The question of latex agglutination inhibitors existing in either the antigenic fluids and/or the antisera was therefore explored. Belyavin (1956) reported the presence of precipitation inhibitors in normal serum as observed in direct precipitation of influenza virus. His results indicated that the inhibitor could be destroyed by heating the serum at 56°C for 1 hour. Heating the rabbit antisera used in this investigation for 1 hour at 56°C failed to produce a positive latex agglutination reaction with MK poliovirus type antigens previously negative with various antisera. Kaolin absorption of the antisera was also explored since lipoproteins have been shown to inhibit viral hemagglutination (Clark and Casals, 1958). It was possible that lipoproteins may inhibit latex agglutination. Kaolin absorbed antisera failed to uncover non-specific latex agglutination inhibitors. Combination of heat treatment and kaolin absorption of the antisera was also unsuccessful. In addition, heating the MK poliovirus type antigens at 56°C for 30 minutes as well as trypsinization (Corbo, 1959) of the MK infected fluids failed to

uncover non-specific inhibitors to latex agglutination. These efforts were therefore abandoned.

The results of this experiment suggest the following conclusions: (1) the original 36 hour MK poliovirus antigen contained sufficient contaminate Hep 2 cell antigen to cause latex agglutination with the poliovirus antisera. The reason the control Hep 2 cell antiserum was not capable of causing a significant latex antigen-antibody reaction was unknown; and (2) MK propagated poliovirus antigens have no antigens common to the Hep 2 or HeLa cell systems.

Experiment #14

Latex Agglutination of Monkey Kidney Cell Poliovirus Type Antigens Following Ultracentrifugation.

The absence of latex agglutination of clarified monkey kidney (MK) poliovirus type antigens with specific antisera (with the exception of the 36 hour harvest) of experiment #13 indicated that higher concentration of viral substance might be required to effect a virus-antibody latex agglutination reaction. Accordingly, MK poliovirus type 1, 2, and 3 antigens were subjected to ultracentrifugation cycles. The procedure applied were those described by Smith and coworkers (1956). For illustration, Table 31 compares the virus material used and the centrifugation procedure used by Smith et al (1956) and those applied in this experiment. The poliovirus antisera used in this experiment were prepared in rabbits injected with partially purified HeLa poliovirus antigens (see Materials and Methods). In addition, the sera were fractionated with sodium sulfate and the globulin fraction used as the antibody source.

The results of the latex agglutination titrations are shown in Tables 32, 33, and 34. The latex agglutinations among the various MK poliovirus type antigens and antisera showed extensive cross-reaction activity. The control Hep 2 cell antiserum also exhibited latex agglutination with all the MK poliovirus type antigens. These results were unexpected and indicated that a need for re-interpretation of the relationship between MK cells and Hep 2 cells was required. It now was apparent that MK cells and Hep 2 cells had antigens in common.

TABLE 31. Comparison of Poliovirus Preparations and Differential Centrifugation Procedures Between Smith and Coworkers (1956) and Those Used in Experiment #14

As described by Smith et al (1956)	As used in experiment #14
TCD_{50} titers:	TCD_{50} per ml titers:
Vaccine virus before centrifugation:	
HeLa poliovirus type 1 $10^{6.24}$	HeLa poliovirus type 1 $10^{7.66}$
HeLa poliovirus type 2 $10^{5.38}$	HeLa poliovirus type 2 $10^{7.50}$
HeLa poliovirus type 3 $10^{5.80}$	HeLa poliovirus type 3 $10^{7.66}$
Reagent virus before centrifugation:	
Same as vaccine virus	MK poliovirus type 1 $10^{6.66}$
	MK poliovirus type 2 $10^{6.50}$
	MK poliovirus type 3 $10^{6.50}$
Differential centrifugation data:	
1. 1500 rpm for 15 minutes. (horizontal centrifuge) RCF* = unknown	2000 rpm for 30 minutes. (Size #1 International Centrifuge SBR) RCF = 900 X g
2. 15,000 rpm for 42 minutes. (Spinco Model L Centrifuge - rotor No. 21) RCF = 30,160 X g	15,000 rpm for 45 minutes. (Spinco Model L Centrifuge - rotor No. 30) RCF = 26,390 X g
3. 30,000 rpm for 5 hours. (Spinco Model L Centrifuge - rotor No. 30) RCF = 105,500 X g	30,000 rpm for 3 hours. (Spinco Model L Centrifuge - rotor No. 30) RCF = 105,500 X g
Nominal concentration factor of pellet:	
38.5 or 77 X	19 X

* RCF = Relative centrifugal force.

TABLE 32. Latex Agglutination of MK Poliovirus Type 1 Antigen Pellet after Ultracentrifugation with Various Antibody Globulin

HeLa poliovirus type 1 antibody globulin*	MK poliovirus type 1 antigen					
	Final antigen dilutions					
Final dilutions	1/4	1/8	1/16	1/32	1/64	Antibody globulin controls
1/8	3+	4+	4+	4+	1+	-
1/16	±	3+	4+	4+	1+	-
1/32	-	±	4+	4+	1+	-
1/64	-	-	±	2+	2+	-
HeLa poliovirus type 2 antibody globulin						
1/8	4+	4+	4+	2+	-	-
1/16	-	4+	4+	2+	±	-
1/32	-	1+	3+	3+	±	-
1/64	-	-	±	2+	±	-
HeLa poliovirus type 3 Antibody globulin						
1/8	4+	4+	4+	3+	2+	-
1/16	±	4+	4+	3+	2+	-
1/32	-	2+	4+	4+	1+	-
1/64	-	-	2+	3+	1+	-
Hep 2 cell antibody globulin						
1/8	-	3+	4+	4+	3+	-
1/16	-	-	3+	3+	2+	-
1/32	-	-	1+	2+	2+	-
1/64	-	-	-	2+	2+	-
Antigen controls	-	-	-	-	+ Latex cont.	-

* Globulin fractions reconstituted (by volume) equivalent to whole serum. Antisera was prepared from the partially purified HeLa poliovirus antigens (see under Materials and Methods).

TABLE 33. Latex Agglutination of MK Poliovirus Type 2 Antigen Pellet after Ultracentrifugation with Various Antibody Globulin

HeLa poliovirus type 1 antibody globulin*	MK poliovirus type 2 antigen				
	Final antigen dilutions				
Final dilutions	1/4	1/8	1/16	1/32	1/64
1/8	4+	4+	4+	4+	2+
1/16	1+	4+	4+	4+	1+
1/32	±	±	4+	4+	1+
1/64	±	±	3+	4+	1+
HeLa poliovirus type 2 antibody globulin					
1/8	4+	4+	3+	1+	±
1/16	1+	4+	4+	2+	±
1/32	±	1+	3+	3+	±
1/64	-	-	1+	2+	±
HeLa poliovirus type 3 antibody globulin					
1/8	4+	4+	4+	2+	1+
1/16	2+	4+	4+	3+	1+
1/32	-	2+	3+	3+	1+
1/64	-	-	2+	3+	1+
Hep 2 cell antibody globulin					
1/8	±	3+	4+	4+	2+
1/16	±	1+	4+	4+	3+
1/32	±	±	2+	2+	2+
1/64	-	-	1+	2+	1+
Antigen controls	-	-	-	±	±

* Globulin fractions reconstituted (by volume) equivalent to whole serum. Antisera was prepared from the partially purified HeLa poliovirus antigens (see under Materials and Methods).

TABLE 34. Latex Agglutination of MK Poliovirus Type 3 Antigen Pellet after Ultracentrifugation with Various Antibody Globulin

HeLa poliovirus type 1 antibody globulin *	MK poliovirus type 3 antigen				
	Final antigen dilutions				
Final dilutions	1/4	1/8	1/16	1/32	1/64
1/8	4+	4+	3+	3+	2+
1/16	1+	4+	4+	4+	1+
1/32	±	1+	4+	4+	1+
1/64	±	±	2+	3+	2+
HeLa poliovirus type 2 antibody globulin					
1/8	4+	4+	3+	2+	±
1/16	±	4+	3+	2+	±
1/32	-	2+	3+	2+	1+
1/64	-	-	1+	2+	1+
HeLa poliovirus type 3 antibody globulin					
1/8	4+	4+	4+	4+	2+
1/16	3+	4+	4+	3+	2+
1/32	±	3+	4+	4+	2+
1/64	-	-	4+	4+	1+
Hep 2 cell antibody globulin					
1/8	1+	4+	4+	4+	2+
1/16	±	±	4+	4+	2+
1/32	-	±	2+	2+	2+
1/64	-	-	±	2+	1+
Antigen controls	-	-	-	-	±

* Globulin fractions reconstituted (by volume) equivalent to whole serum. Antisera was prepared from the partially purified HeLa poliovirus antigens (see under Materials and Methods).

The results indicated that the relative concentration of the common antigenic substance was of low order in the MK preparations; only the concentrated material showed latex agglutination antigen-antibody cross-reactions. These results negated the conclusion made in the previous experiment (experiment #13) regarding the relationship of Hep 2 and MK cells.

It was difficult to evaluate the results of this experiment with those reported by Smith and coworkers (1956). They reported poliovirus type specificity with their preparations. They did not include host cell antiserum in their tests.

DISCUSSION

Experiments were undertaken to study the problems associated with developing a serologic latex agglutination reaction for the identification of polioviruses. Observations of latex agglutination of poliovirus infected tissue culture fluids as compared to uninfected cell fluids have been described.

At the beginning, results with the latex agglutination reaction suggested the presence of group specific poliovirus antigens. These results were in agreement with many reports in which group specific complement-fixing antigens have been described for the polioviruses. (Casals et al, 1951, 1952; Svedmyr et al, 1952; Black et al, 1954, 1955; LeBouvier, 1954, 1955; Melnick, 1955; and Schmidt et al, 1956). Latex agglutination of poliovirus antigens with Hep 2 cell antiserum indicated that host cell antigens present in the poliovirus infective fluids were also latex agglutinable. Block-titration design proved to be more definitive in making accurate interpretations. The presence of antigen excesses in control Hep 2 cell antigen preparations could have led to erroneous conclusions if the constant antigen varying antiserum titration method was continued. This discovery was made early in the investigation. Subsequent experiments indicated that the latex agglutination cross-reactions were due to host cell antigen-antibody systems. Whether or not the antigen (s) responsible for the reaction was due to host cell antigens merely associated, in a

physical sense, with the viral antigens or indeed was an actual antigenic component of the complete poliovirus particle remains speculative. It was conceivable that virus passage history (continued passage in cell line cultures) could cause changes in the viral antigenic architecture. Host cell antigens could become an integral part of the virus during the maturation process. This has not been proven. Studies conducted in parallel showed that virus infectivity and latex agglutination were associated. Latex agglutination could be demonstrating viral antigen-antibody groupings different from the neutralizing antigen-antibody system. Grasset and coworkers (1958) reported the presence of neutralizing antibody to poliovirus in the absence of precipitating antibody and vice-versa. These authors suggested that group specific precipitating antigens and antibodies exist among the poliovirus types similar to those found in complement fixation tests. It was possible that the so-called group specific antigens and certain host cell antigens were one and the same? Nevertheless, specific poliovirus antisera (prepared with HeLa cell propagated poliovirus) showed latex agglutination with uninfected Hep 2 cell antigen. These results indicated: (1) latex agglutination cross-reactions were due, at least in part, to host cell antigens; and (2) HeLa and Hep 2 cells have common antigens.

Studies designed to reveal possible heterophil antigen-antibody substances were successful. The antibody found was of the Forssman type. It was possible that the combination of specific host cell antigen-antibody reactions coupled with the heterophil system could

have added to cross-reactions. It was concluded that no significant influence of the heterophil system could be attributed to the latex agglutination reactions. In retrospect, it was obvious that rabbits were a poor choice for preparing poliovirus antisera. It was now apparent that the antisera contained at least three types of antibody; (1) specific viral neutralizing antibody; (2) host cell precipitins as observed by latex agglutination; and (3) heterophil antibody.

The substitution of commercial complement fixing poliovirus antigens propagated in human amnion did not alter the latex agglutination specificity. The one feature that was common to all the cells, was their common human origin. Hep 2 and HeLa cells were originally obtained from human malignant tissues. Human amnion cells were originally obtained from normal tissues. Antigenically, they all react the same as observed by latex agglutination with specific antiserum.

Attempts to separate the host cell antigens from specific viral antigens by ultracentrifugation, salt fractionation, and pH adsorption technics were all unsuccessful in-so-far as latex agglutination with antiserum was concerned. The results from these experiments did contribute useful information to the overall study that was not appreciated at the outset. For example, the ultracentrifugation experiment indicated that the antigens were soluble. Ammonium sulfate fractionation of poliovirus infected tissue culture fluids indicated that the reacting antigen was globulin-like. This confirmed the soluble nature of the antigen. The poliovirus being tested was also

shown to be precipitated by the ammonium sulfate. Adsorption of the antigen onto latex particles was obtained at pH 4.0. The undertaking of this experiment was based on methods used to purify poliovirus (Schwerdt and Schaffer, 1955, 1956). It was reasoned that if the polioviruses could be selectively adsorbed onto the latex particles exclusive of host cell antigens, then viral antigen-antibody specificity may be forthcoming. However, latex agglutinations were not considered specific for virus. Two conclusions were obvious: (1) latex agglutinable antigens behave similarly to the viral antigens as far as pH was concerned; and (2) latex agglutinable antigens do adsorb onto the latex particles.

The major problem in interpreting the serum absorption results was directly referable to the experimental design used for the absorption. The absorption test in which one specific poliovirus antiserum was cross-absorbed with Hep 2 poliovirus antigens and Hep 2 cell antigen and then re-tested for remaining antibody indicated, in the final analysis, that the virus-antibody system was not causing latex agglutination. The question of latex agglutination inhibitors was also suggested for the first time.

The influence of various salts on latex particle stability was explored. The results showed that salt concentrations above a certain level caused spontaneous agglutination of the latex particles. At a certain predetermined salt ($CaCl_2$) concentration, it was concluded that an increase in the sensitivity of the latex agglutination antigen-antibody reaction was evident. This conclusion was in

disagreement with the findings of Singer and Plotz (1956). Conceivably with a precipitating antigen-antibody system such as might be expected with viral substances, the presence of a measured quantity of calcium or magnesium ions could make the difference between success or failure. This position was consistent with what is generally accepted about the kinetics of antigen-antibody precipitation reactions (von Krogh, 1916; Northrop and DeKruif, 1921-22; Eagle, 1932; Hooker, 1937; Heidelberger, 1934, 1939; Mason, 1922; Follensby, 1939; and Cann, 1956).

The latex agglutination reaction differences observed in the majority of the titrations between poliovirus antigens and control host cell antigens with various antisera may be attributable to zone phenomena (protein antigen excess). This suggests that latex agglutinations were due to host cell protein quantities rather than to other more speculative considerations. Holland (1964) reported that the results of a number of investigators consistently showed that host cell protein synthesis was drastically inhibited in poliovirus infected cells. This information suggested that protein determinations be done on the various antigenic fluids used in this study. Accordingly, the antigenic fluids were tested for protein by the Folin-Ciocalteau method (Andersch, 1960). The following information was obtained:

Hep 2 poliovirus type 1	44 mg % protein
Hep 2 poliovirus type 2	65 mg % protein
Hep 2 poliovirus type 3	53 mg % protein
Hep 2 cell antigen	708 mg % protein

The protein concentration of the Hep 2 poliovirus infected and Hep 2 uninfected cell fluids confirmed the postulation and was consistent with

the report of Holland (1964). The protein concentration was approximately 16 times greater in uninfected Hep 2 cell fluids. Thus, the basis for the observation, at certain antigen concentrations, whereby poliovirus antigens were latex agglutination positive while control host cell antigens were latex agglutination negative was established.

Latex agglutination of monkey kidney cell (MK) propagated poliovirus type antigens with various antisera originally suggested group specific poliovirus antigens. The latex agglutinations were confined to a particular virus harvest (36 hour MK poliovirus). The virus infectivity determinations on the 36 hour and the 5 day MK poliovirus antigens indicated that the infective virus concentration was equivalent in both harvests. Latex agglutination of the 5 day MK poliovirus harvest with various antisera was negative. These results were therefore inconclusive as to virus-antibody latex agglutination. Removal of possible serum precipitation inhibitors was undertaken. The treated sera were tested with clarified MK poliovirus antigens previously negative for latex agglutination. The treatment of the sera included: (1) kaolin absorption (Clark and Casals, 1958); (2) inactivation for 1 hour at 56°C (Belyavin, 1956); and (3) combination of the two methods. The results were unsuccessful in that no latex agglutination was evident with MK poliovirus antigens and specific antisera. The final results indicated that MK cells had no common antigens with HeLa and Hep 2 cells.

Differential centrifugation of the MK cell propagated poliovirus types was undertaken for the purpose of concentrating the virus. The centrifugation cycles paralleled, for the most part, the procedures reported by Smith and coworkers (1956). The final pellet, reconstituted so as to represent approximately a concentration factor of 19X caused latex agglutinations with homotypic and heterotypic antiserum (globulin). The control Hep 2 cell antiserum (globulin) also reacted with all of the MK poliovirus type antigens. These results negated previous conclusions relative to the absence of MK antigens common to HeLa and Hep 2 cell preparations. Concentrated MK poliovirus type antigens reacted while simply clarified MK poliovirus type antigens were latex agglutination negative. A comparison of this differential centrifugation run (Experiment #14) with the second differential centrifugation of Experiment #10 is illustrated in Table 35. This comparison was done because a contradiction seemed to exist concerning the sedimentability of the antigen. Comparing the methods and results in parallel should serve to define the situation. It was concluded that the MK antigens common to HeLa and Hep 2 cells were heterogeneous in reference to size and/or concentration. This conclusion was based on the comparative results of the two separate experiments.

The problems associated with a latex agglutination reaction for poliovirus have been described. They consisted of: (1) the presence of host cell common antigens in the poliovirus antigenic fluids; (2) multiple antibodies in the rabbit antisera; and (3) insufficient viral antigens so that the virus-antibody could not be detected.

TABLE 35. Comparison of Two Differential Centrifugation Preparations of Poliovirus Type 2

Experiment #10		Experiment #14	
Host cell propagation data:			
Hep 2 cell culture		MK cell culture	
TCD_{50} per ml = $10^{5.50}$		TCD_{50} per ml = $10^{6.50}$	
Centrifugation data: (RCF* and time)			
1. 500 X g for 30 minutes.		900 X g for 30 minutes	
2. 36,190 X g for 30 minutes		26,390 X g for 45 minutes	
3. 110,800 X g for 5 hours		105,500 X g for 3 hours	
Latex Agglutination:			
Clarified harvest from 1 above	+++**	Clarified harvest from 1 above.	--**
Supernatant fluid from 3 above.	+++	Supernatant fluid from 3 above	ND***
Pellet from 3 above (Concentration factor = 1X)	---	Pellet from 3 above (Concentration factor = 19 X)	+++

* RCF = Relative centrifugal force.
** +++ = Latex agglutinations similar to other titrations conducted previously; see text. --- = No latex agglutinations observed.
*** ND = Not done.

Latex agglutination adapted to the poliovirus-antibody system was not considered impossible. The results obtained in this investigation suggested that two conditions must be met before the technic may be successfully applied: (1) concentrated viral antigens free from host cell antigenic components; and (2) specific viral antisera. The first condition could be conceivably resolved by applying the methods of Mayer and coworkers (1957) for purifying and concentrating poliovirus antigens. The second condition could be resolved by using highly purified viral antigens. The use of animals other than rabbits for producing type specific viral antibodies would be indicated. Alternatively, human antiserum obtained following infection should also serve as a good source of antibody.

The rationale that served as the basis for undertaking this investigation was not supported by the results. For example, it was postulated at the outset that artificially increasing the physical dimensions (by the use of a carrier) of the viral antigens would effectively create an antigenic mass sufficient to produce an agglutination reaction. This did not work under the conditions of this investigation. The use of several host cell lines to eliminate host cell-antihost cell reactions was in error. The observed latex agglutination cross-reactions from poliovirus infected tissue culture fluids with various antisera resulted from host cell-antihost cell systems common to the virus propagating cells. These findings served to define some of the problems associated with developing a serologic latex agglutination test for polioviruses. Conceivably, with highly

purified and concentrated viral antigens (Merrill, 1936), a latex agglutination reaction specific for polioviruses would be predictable.

SUMMARY

1. An antigen was found in poliovirus infected tissue culture fluids that suggested the presence of group specific poliovirus antigens. Subsequent experiments indicated that the latex agglutination cross-reactions were due to host cell antigen-antibody systems. Common antigens could be postulated to exist between HeLa and Hep 2 cells. A Latex agglutination reaction with poliovirus and its antibody could not be absolutely discounted but was not recognized in the presence of the cross-reacting host cell-antihost cell systems.

2. Heterophil antibody activity was observed with antisera prepared in rabbits against the polioviruses and HeLa and Hep 2 cells. Latex agglutination could not be attributed to the presence of the heterophil antibody. The antibody agglutinated human red blood cells of A, B, and O groups. In addition, red blood cells of monkey, sheep, chicken, and guinea pig were agglutinated. Absorption of the HeLa and Hep 2 cell antisera with guinea pig kidney antigen (Difco) removed the hemagglutinin completely. This indicated that the heterophil antibody was of the Forssman type.

3. Commercial complement fixation poliovirus antigen made by propagating the virus in human amnion cells cross-reacted in the latex agglutination test similar to the Hep 2 poliovirus antigens. This indicated the presence of common antigens in the system. The common antigens were referable to the source of host cells used to propagate the polioviruses. All the host cells; HeLa, Hep 2, and human amnion had a common human origin.

4. Certain concentrations of electrolytes caused the latex particles to agglutinate spontaneously. Divalent cations were more effective than univalent cations. Salts used to precipitate proteins also caused the latex particles to agglutinate spontaneously. Appropriate adjustment of salt concentration of divalent cations ($CaCl_2$) increased the sensitivity of the latex agglutination antigen-antibody reaction.

5. Absorption of the antisera with Hep 2 cell antigen removed the host cell antibodies. Antiserum absorbed twice with Hep 2 cell antigen was latex agglutination negative when re-tested for remaining antibody with specific poliovirus antigen.

6. The supernatant fluids of ultracentrifuged Hep 2 poliovirus antigens was latex agglutination positive when tested with various antisera. These results indicated that the antigen was soluble. It appeared that latex agglutination could not be attributed to the virus-antibody system. The pellet obtained following ultracentrifugation at 35,000 rpm (110,800 X g) for 5 hours was latex agglutination negative when tested with antisera. The same pellet showed a virus infectivity titer of $10^{6.00}$ TCD_{50} per ml.

7. The latex agglutinative component present in Hep 2 poliovirus antigenic fluids was precipitated by half saturation with ammonium sulfate. Poliovirus was also precipitated by the ammonium sulfate treatment. The reconstituted precipitate showed a virus infectivity titer of $10^{6.33}$ TCD_{50} per ml.

8. Adjustment of Hep 2 poliovirus antigen to pH 4.0 prior to mixing with latex particles failed to demonstrate the virus-antibody system exclusive of the host cell system. Antigen did adsorb onto the latex particles. Latex agglutination was observed by the resuspended particles with antisera.

9. Monkey kidney cell propagated poliovirus antigens failed to cause latex agglutination with antisera prepared in rabbits against HeLa poliovirus types and Hep 2 cells. This suggested the absence of any monkey kidney antigens held in common with HeLa and Hep 2 cells. The pellet obtained from ultracentrifuged monkey kidney poliovirus antigens caused latex agglutination with viral antisera. Hep 2 cell antiserum was also positive. This indicated common antigens existed among HeLa, Hep 2, and monkey kidney cells.

10. A latex agglutination reaction adapted to the poliovirus-antibody system was not considered impossible. Results of this study indicated that clarified infected tissue culture fluids contained host cell antigens so that rabbit antisera showed antibodies to the host cells. The use of various cell lines failed to eliminate host cell cross-reactions. Host cells normally used to propagate poliovirus have common antigens. A latex agglutination poliovirus-antibody system would predictably require highly purified and concentrated viral antigens.

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