



A comparative analysis of enteroviral antigens produced by treatment with sodium dodecyl sulfate  
by Marlene Joyce Mackie

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of  
DOCTOR OF PHILOSOPHY in Microbiology

Montana State University

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**Abstract:**

Antisera were prepared in rabbits against whole Poliovirus I and degraded Poliovirus I protein that had been produced by treatment of whole capsids with sodium dodecyl sulfate, urea, acetic acid and 2-mercaptoethanol. The antisera were reacted using the Ouchterlony technique with whole and degraded viral proteins of Polioviruses I, II, III, ECHOviruses 1, 5, 18, 22, and Coxsackie viruses B1, A9 and A13. When whole Poliovirus I antiserum and degraded Poliovirus I antiserum were reacted with the whole viruses, precipitin reactions were observed only with the antisera and whole and degraded Poliovirus I proteins. When whole Poliovirus I antiserum was reacted with the degraded viral proteins, precipitin reactions indicating identity were observed with Polioviruses I, II, III, and ECHOvirus 5. The reaction of degraded Poliovirus I antiserum with degraded viral proteins produced precipitin lines with Polioviruses I, II, III, ECHOvirus 5 and Coxsackie viruses B1 and A13. The data indicate that the three subgroups of enteroviruses have antigenic determinants based on primary amino acid sequence in common.

Acute and convalescent sera of human volunteers that had been immunized with one dose of oral, trivalent, Poliovirus vaccine were reacted with whole viruses and degraded viral proteins. Precipitin reactions were observed with whole Poliovirus I and II and degraded Poliovirus I protein. The efficacy of using the double diffusion as a tool in the diagnosis of enteroviral diseases is discussed.

A COMPARATIVE ANALYSIS OF ENTEROVIRAL ANTIGENS PRODUCED  
BY TREATMENT WITH SODIUM DODECYL SULFATE

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MARLENE JOYCE MACKIE

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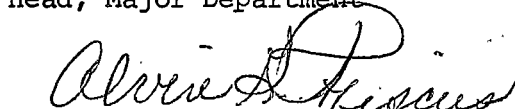
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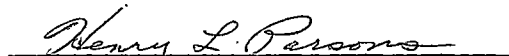
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## ABSTRACT

Antisera were prepared in rabbits against whole Poliovirus I and degraded Poliovirus I protein that had been produced by treatment of whole capsids with sodium dodecyl sulfate, urea, acetic acid and 2-mercaptoethanol. The antisera were reacted using the Ouchterlony technique with whole and degraded viral proteins of Polioviruses I, II, III, ECHOviruses 1, 5, 18, 22, and Coxsackie viruses B1, A9 and A13. When whole Poliovirus I antiserum and degraded Poliovirus I antiserum were reacted with the whole viruses, precipitin reactions were observed only with the antisera and whole and degraded Poliovirus I proteins. When whole Poliovirus I antiserum was reacted with the degraded viral proteins, precipitin reactions indicating identity were observed with Polioviruses I, II, III, and ECHOvirus 5. The reaction of degraded Poliovirus I antiserum with degraded viral proteins produced precipitin lines with Polioviruses I, II, III, ECHOvirus 5 and Coxsackie viruses B1 and A13. The data indicate that the three subgroups of enteroviruses have antigenic determinants based on primary amino acid sequence in common.

Acute and convalescent sera of human volunteers that had been immunized with one dose of oral, trivalent, Poliovirus vaccine were reacted with whole viruses and degraded viral proteins. Precipitin reactions were observed with whole Poliovirus I and II and degraded Poliovirus I protein. The efficacy of using the double diffusion as a tool in the diagnosis of enteroviral diseases is discussed.

## INTRODUCTION

The immunodiffusion technique of Ouchterlony has been used to study viral diseases since 1953 when Jensen and Francis (22) reported the presence of a specific reaction with influenza virus. Subsequently, numerous animal, plant, and human viruses have been studied with double diffusion, radial diffusion and more recently counter-immunoelectrophoresis techniques.

This work has been directed in part toward the development of a rapid, reliable method for diagnosis of viral diseases. Tanaka (47) demonstrated an increase of specific precipitating antibodies in patients with adenovirus disease and the influenza virus has been shown to react specifically with homologous antiserum (18,37,38). Infections with varicella-zoster have also been diagnosed by double diffusion in agar (4,49). Brunell developed a procedure using vesicular fluid and crusts which gave a specific reaction with varicella-zoster and negative results when reacted with vaccinia precipitin antigens. Diagnosis by immunodiffusion has become available for viruses in the California Encephalitis Virus group (50) and others such as myxoma and fibroma of rabbits, canine distemper, lymphocytic choriomeningitis and swine fever (30). Counterimmunoelectrophoresis has been used more recently in the rapid detection of precipitating antibody for the influenza virus (1).

Because of the specificity of the reaction between a virus and its homologous antiserum, relationships between antigenically related viruses have been demonstrated. Myxoma and fibroma viruses have been shown to have common antigens (30) and group antigens are present in adenoviruses (47). Immunization of rabbits with one injection produced specific precipitin reactions with individual members of the California Encephalitis Virus group. However, hyperimmunization produced sera which would react with all members of the group (50). Interactions have also been demonstrated between Influenza A virus strains with similar envelope proteins (38).

The Ouchterlony method has been used successfully in studies with various enteroviruses. Although it was originally intended to utilize double diffusion in the diagnosis of enteroviral diseases, the procedure has not been established and most of the information obtained has been used in determining antigenic relationships. Harris et al. (17) reacted hyperimmune anti-ECHOvirus 8 serum with ECHOvirus 1, Poliovirus I and Coxsackie virus B5, and Middleton et al. (31), Styk and Schmidt (45) and Forsgren (12,13) have used double diffusion to detect antigenic relationships between Polioviruses, ECHOviruses and Coxsackie viruses. These studies have shown that in all cases, the reactions of whole virus with the various antisera were found to be specific and few cross-reactions were noted except in the most closely related enteroviruses such as ECHOviruses 1 and 8.

It has been demonstrated that patients with ECHOvirus (6), Coxsackie virus (7) and Poliovirus infections (12,13) elicit a precipitin antibody response that is detectable by double diffusion. However, since the reactions are virus specific, specific anti-whole virus antiserum for each virus would be required for diagnosis, making this method impractical. Although neutralization tests are more time consuming, diagnosis of the ECHOviruses is less expensive through the use of the "intersecting serum pool scheme" devised by Schmidt et al. (40). Coxsackie viruses are diagnosed through similar intersecting schemes, and polioviruses have been classically diagnosed through the use of the three type-specific hyperimmune Poliovirus antisera in neutralization tests.

The Ouchterlony technique has also been used to study the antigenic components of different viruses. The subunits are produced by exposing the particular virus to various dissociating agents depending upon the virus being studied. Hosaka et al. (19) used ether to dissociate HVJ virus, heat was used for mumps virus (9), saponin for rabies virus (51), pyridine, urea and sodium dodecyl sulfate for Potato viruses X, S, and M (42) and sodium dodecyl sulfate plus dithiothritol for adenoviruses (23). The resulting degraded viral proteins were injected into animals and the antisera were used for studying antigenic relationships.

In one instance, that of Potato viruses X, S, and M, the use of degraded viral protein has been developed into a useful diagnostic test (43,44).

Poliovirus capsids have been dissociated in several ways. The most frequently used agents are high concentrations of urea (about 50 percent by volume), guanidinium salts, acetic acid and moderate concentrations of detergent (1-2 percent). The resulting polypeptides have been examined extensively by sodium dodecyl sulfate disc gel electrophoresis and density gradient centrifugation, but they have not been used to immunize animals for the purpose of studying antigenic relationships among the three subgroups of enteroviruses.

When purified Polioviruses are dissociated by treatment with sodium dodecyl sulfate, acetic acid and urea, 4 polypeptides, viral proteins (VP) 1, 2, 3, and 4, can be demonstrated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (34). The four viral proteins occur in roughly equimolar amounts. The molecular weights of VP 1, 2, 3, and 4 are 35,000, 28,000, 24,000, and 5,000, respectively.

The present study was designed to use degraded Poliovirus I capsid proteins as immunogens in rabbits. The antisera were used to compare the antigenic structure of Poliovirus I to members of the other enteroviral subgroups.

In viral capsomeres, monomers of viral proteins interact to produce a polymer in a way that involves part of their total surface

(32). Any antigenic determinant, which occurs in the area of interaction between two monomers, is hidden and becomes available only upon disaggregation of the polymer. Antigenic determinants occurring on the surfaces of both the polymers and monomers and those which are created by proper assembly of monomers into polymers are not hidden. It has been suggested that in the evolution of viruses, the most type specific antigens become located on the outermost parts of the viral structure (25). Following this concept, surface antigenic sites should have developed into highly type-specific antigenic determinants while internal or "hidden" antigens could be common for a certain group of viruses. The homogeneity of viral reactions with corresponding precipitating antibody, such as seen with enteroviruses (12, 13), adenoviruses (47) and influenza viruses (38), seems to bear this out.

It was believed that by dissociating the Polioviruses, ECHO-viruses and Coxsackie viruses with sodium dodecyl sulfate and reacting these dissociated proteins with antiserum prepared against the Poliovirus I whole and dissociated capsid protein, antigenic relationships between the enteroviral subgroups may exist that had not been previously observed. Conceivably, antigenic sites common to all the enteroviral subgroups could be exposed by the deaggregation of the viral capsid.

Considering these possibilities, it seemed advisable to further examine these relationships, therefore the following study was undertaken.

## MATERIALS AND METHODS

### Cell Cultures

Two cell lines were used. KB (human carcinoma of the nasopharynx) was obtained from Microbiological Associates and maintained on Hank's Lactalbumin supplemented with 10 percent bovine serum, penicillin (20,000 units/ml)-streptomycin (20,000 ug/ml) and 200 ug/ml kanamycin (Kantrex, Bristol). VERO (kidney, African Green Monkey) cells were obtained from the Rocky Mountain Laboratory, Hamilton, Montana, courtesy of Mr. Jack Cory. The line was maintained on Medium 199 with Earle's Balanced Salts (Medium 199/EBSS, Flow Laboratories) to which 0.8 percent sodium carbonate, 10 percent bovine serum, penicillin (20,000 units/ml)-streptomycin (20,000 ug/ml) and kanamycin (200 ug/ml) had been added. The cells were grown in 16 ounce prescription bottles with an area of 88 cm<sup>2</sup> available for cell attachment.

The subculturing procedure for both cell lines included treatment with versene-trypsin followed by centrifugation for 5 minutes at 400 to 500 revolutions per minute (RPM). Fresh medium was added and both lines were subcultured at a 1:4 expansion ratio. Average inoculum per bottle consisted of  $1 \times 10^6$  cells/bottle of KB cells and  $4 \times 10^6$  cells/bottle of VERO cells.

### Infection of Cells

Two methods were used to infect the cells. KB monolayers were infected by adding 0.2 ml of virus suspension containing  $1 \times 10^7$  plaque forming units (PFU) of virus per ml, 20 ml Earle's Lactalbumin with penicillin (20,000 units/ml)-streptomycin (20,000 ug/ml) and 3 percent bovine serum. The cell sheets were incubated at 37°C. until the entire monolayer showed cytopathogenic effect. This method was utilized exclusively for the preparation of all the viral antigens used in the comparative experiments.

A second method of infection was used for the preparation of the larger amounts of Poliovirus I necessary for rabbit immunization. When KB cells had become a complete monolayer, cells were stripped from the bottle with versene-trypsin. Cells from one bottle were re-suspended in 1-2 ml of Earle's Lactalbumin giving a final concentration of cells of  $3-6 \times 10^6$  cells per ml. The number of cells in the total suspension culture was counted by hemocytometer and 15-20 PFU Poliovirus I/cell was added to the suspension. The suspension culture was incubated at 37°C. for 6 hours while being constantly agitated at low speed on a magnetic stirrer (29).

### Recovery of the Virus

The recovery of the viruses from the infected cells in monolayers or suspension cultures was the same. The infected cells and

the suspending medium were frozen and thawed 3-5 cycles (-70°C. to 37°C.) to disrupt intact cells and release virus.

Concentrated viral antigens were prepared from wild type viruses including Polioviruses I, II, and III, ECHOviruses 1, 5, 18, and 22, Coxsackie viruses A9, A13 and B1, and Adenovirus I which was used as a control. These antigens had a concentration of  $2.0 \times 10^7$  to  $2.0 \times 10^9$  PFU/ml. They were prepared by pooling the cell debris and supernatant fluid from sixteen or more infected monolayers of KB cells. The viruses were released from intact cells by rapid cycles of freezing and thawing. The supernatant fluid was centrifuged for 10 minutes, 4°C. at 10,000 RPM (Sorvall Centrifuge). The supernatant was removed and centrifuged at 30,000 RPM for 2 hours at 4°C. (Spinco, Model L). The supernatant fluid was discarded and 1.0 ml of 0.02 M phosphate buffered saline (PBS) pH 7.2 was added to the viral pellet. The pellet was evenly suspended in the PBS by drawing the material through successively smaller hypodermic needles (16g to 25g). Large particles were removed from the viral suspension by centrifugation at 10,000 RPM for 10 minutes at 4°C. The supernatant was centrifuged once again for 2 hours at 4°C. at 30,000 RPM. The resulting clear viral pellet was suspended in 1.0 ml PBS pH 7.2 and used for subsequent precipitin reactions (3).

### Purification of Poliovirus I

Further purification of Poliovirus I was necessary since the virus was to be used for disruption and rabbit immunization. The virus was layered on a density gradient of cesium chloride (1.33 gm/ml) and banded overnight by centrifugation (SW-50 rotor, 40,000 RPM, 4°C., Spinco, Model L). The enteroviruses have a buoyant density of 1.33 in cesium chloride; therefore, the purified virus was banded on the surface of the gradient. It was recovered with a syringe and suspended in 1-2 ml 0.02 M PBS pH 7.2. The virus was dialyzed overnight at 4°C. with 1000 volumes of 0.02 M PBS pH 7.2 to remove residual cesium chloride.

Purity was determined by ultraviolet spectrophotometry. The characteristic 260/280 ultraviolet absorption spectra, with maxima at 260 and minima at 240, of 1.69 for a poliovirus suspension containing 1 mg virus/ml was obtained (41). Purity was also demonstrated through the use of electron micrographs taken of the purified viral preparation.

### Degradation of Poliovirus I

Solubilizing of Poliovirus I capsid proteins was accomplished by adding 10 percent acetic acid, 1.0 percent sodium lauryl sulfate (Sigma Chem. Co., St. Louis, Mo.) (SDS) and 0.5 M urea to the cesium chloride gradient purified viral suspension. After incubation for

1 hour at 37°C., the viral sample was dialyzed overnight at room temperature against 2000 volumes of 0.01 M PBS containing 0.1 percent SDS, 0.5 M urea and 0.1 percent 2-mercaptoethanol (Sigma Chem. Co., St. Louis, Mo.) (46). The degraded virus was rechecked by ultraviolet spectrophotometry. The 260/280 ratio was 1.0 with most of the absorbancy occurring between 220 and 240 nm. The degraded Poliovirus I protein was used in the immunization of animals and in precipitin studies in agar.

All other viruses used in the study were degraded in the same manner and resulting viral proteins were used in comparative precipitin studies with the Ouchterlony technique.

#### Immunization of Animals

Production of antiserum to whole Poliovirus I. Five ml of infected tissue culture fluid (TCF) (Earle's Lactalbumin containing 3 percent bovine serum, penicillin (20,000 units/ml)-streptomycin (20,000 ug/ml) and approximately  $9.3 \times 10^7$  PFU Poliovirus I/ml) plus 5 ml of incomplete Freund's adjuvant was emulsified and injected intramuscularly into a rabbit in four different sites. At the same time, the animal was given 1.0 ml infected TCF intravenously. Each week for the next two weeks each rabbit was given 1.0 ml infected TCF intravenously. At the beginning of the third week, the initial procedure was repeated. One week later, the rabbits received a final

intravenous injection of 1.0 ml infected TCF. Seven to 10 days later the rabbits were exsanguinated by cardiac puncture. Serum was separated from the cells by centrifugation for 10 minutes at 10,000 RPM at 4°C. (28).

Preparation of antiserum to degraded Poliovirus I protein.

Each rabbit received a total of approximately 3.0 mg of degraded viral protein. The amount of protein was calculated from the spectrophotometric reading at 260 nm and the extinction coefficient of the virus (41). The first two intramuscular injections, given 1 week apart, consisted of 2.5 ml degraded viral protein and 2.5 ml complete Freund's adjuvant which had been emulsified. These injections were followed by three weekly intramuscular injections of 2.5 ml degraded viral protein and 2.5 ml incomplete Freund's adjuvant. The rabbits were exsanguinated by cardiac puncture a week after the final injection and the serum was later used in double diffusion studies.

Immunization of Human Volunteers

Twelve volunteers, all of which had been immunized with one dose of oral, trivalent Poliovirus vaccine within one year, were bled at day 0. They were given one dose of live, oral, trivalent Poliovirus vaccine (Orimmune, Lederle) and bled again at the end of 2 weeks. Sera were tested for activity against all whole viruses and degraded viral proteins by the Ouchterlony technique.

Viral Plaquing Experiments

The plaque forming units (PFU)/ml of all Polioviruses and ECHOviruses used in the study were determined by exposing monolayers of VERO cells in 3 ounce prescription bottles for 1.5 hours to 0.5 ml of a virus dilution which had been made in Medium 199/EBSS to which 2 percent fetal calf serum and 0.8 percent sodium bicarbonate had been added. After the adsorption period, the monolayers were overlaid with 7 ml of a mixture of half 2 percent ionagar in distilled water and half double-strength Medium 199/EBSS with 4 percent fetal calf serum added. The monolayers were incubated 48 hours for the Polioviruses and up to 96 hours for ECHOviruses 1 and 5. A second overlay of 4 ml containing half 2 percent ionagar in distilled water, half double-strength Medium 199/EBSS, 4 percent fetal calf serum and 1.0 ml of 1.0 percent neutral red per 100 ml of overlay medium was added to the monolayer. The neutral red was dissolved in Medium 199/EBSS and autoclaved for 15 minutes (121°C., 15 lbs. pressure). The monolayers were incubated overnight in the dark at 37°C. and the plaques were counted against a white paper background with conventional fluorescent room lighting (8).

Plaque forming unit information for the Coxsackie viruses was obtained from Hsuing (20) and used in the present study.

### Disc Gel Electrophoresis

SDS polyacrylamide gel electrophoresis was used for determination of the molecular weights of the degraded Poliovirus I proteins (11). Poliovirus I protein, degraded by the previously described method was electrophoresed on SDS gels having a final concentration of 10 percent acrylamide plus 0.1 percent SDS (42). Gels were stained with 0.025 percent Coomassie brilliant blue (Colab, Chicago Heights, Ill.) and destained with 7.5 percent acetic acid plus 5 percent methanol (5). Molecular weights of degraded viral proteins were estimated by comparing their mobilities to the mobilities of known proteins prepared in the same manner. The following proteins were used: bovine serum albumin (BSA, M.W. 67,000), ovalbumin (oval, M.W. 45,000), chymotrypsinogen (chymo, M.W. 25,000 and ribonuclease (RNase, 13,700)).

### Serology

Antisera were titrated for whole virus and degraded viral protein activity by double diffusion methods. Two-fold dilutions of antisera against whole Poliovirus I and against degraded Poliovirus I proteins were made in 0.02 M PBS pH 7.2. The double diffusion tests were carried out in 0.9 percent ionagar dissolved in 0.05 M tris HCl pH 7.2 containing 0.85 percent NaCl. The serum titrations were placed in the smaller outer wells (3 mm in diameter) and whole or degraded

viral protein was placed in the center well (5 mm in diameter). A diffusion distance of 3 mm between serum and antigen wells afforded maximal reactivity without a loss of definition (31).

Controls consisted of uninfected cells, Earle's Lactalbumin plus 3 percent bovine serum (TCF), PBS pH 7.2 and disruption fluid containing 0.1 percent 2-mercaptoethanol and 0.1 percent SDS in 0.01 M PBS pH 7.2.

## RESULTS

### Determination of Purity of Poliovirus I Antigen Preparation

Purity of the Poliovirus I antigen preparation was determined by two methods. Figure 1 shows an electron micrograph of Poliovirus I purified by the procedure outlined in Materials and Methods. Since little cell debris or other non-viral material was observed in the electron micrographs, purity of the virus preparation was confirmed by this method.

Another method of determining virus purity was through the use of ultraviolet spectrophotometry. The virus preparation gave the characteristic ultraviolet absorption spectra with maxima at 260 nm, minima at 240 nm and a ratio of E260/E280 of 1.69 to 1.72 (41) (Figure 2). After degradation was achieved, the virus preparation was rechecked with ultraviolet spectrophotometry to insure that the reaction was complete (Figure 2). A marked change in the ultraviolet absorption spectrum indicated that a change in the composition of the antigen preparation had occurred. The degradation of the viral capsids accompanied by the release of viral ribonucleic acid produced a spectrum with the maxima at 230 and minima at 260 nm.

### Separation of the Subunits with Sodium Dodecyl Sulfate Disc Gel Electrophoresis

Previous studies have reported subunits with molecular weights of 35,000, 28,000, 24,000 and 5,000 when whole capsids are degraded

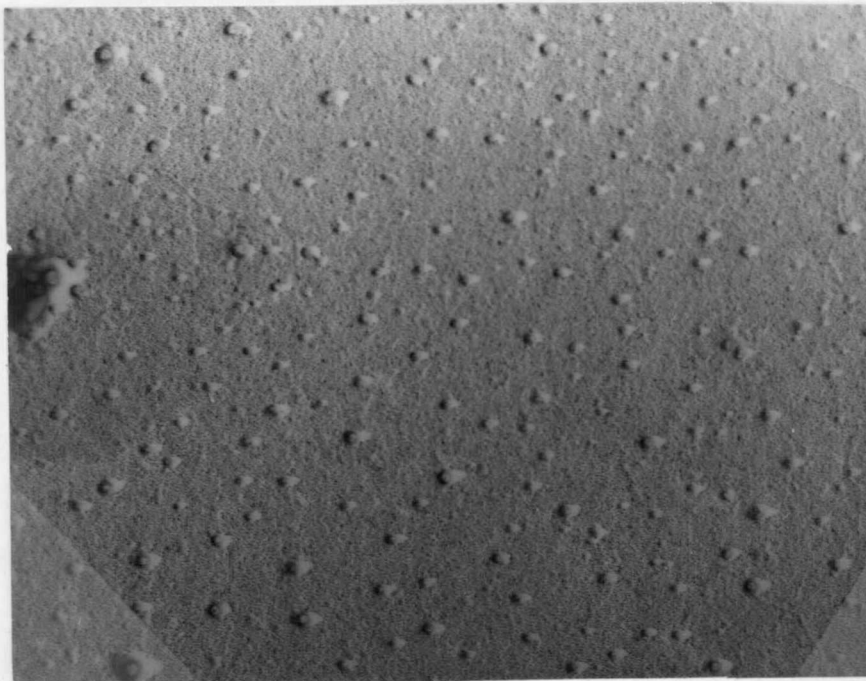


Figure 1. Electron micrograph of Poliovirus type 1 purified by the method described in Materials and Methods x 20,000.

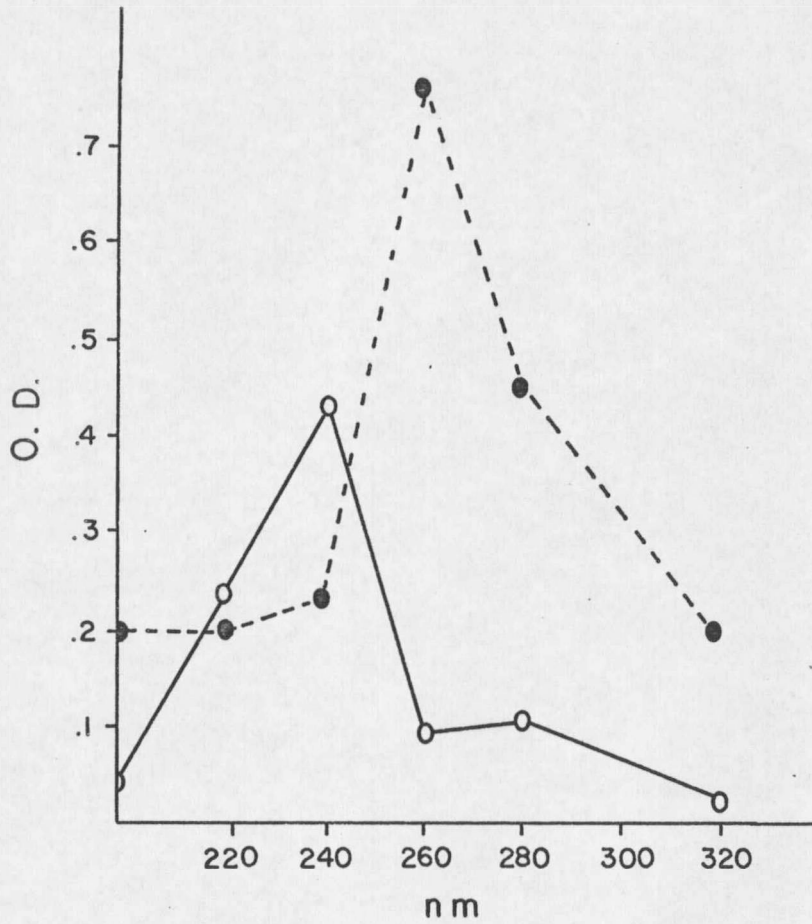


Figure 2. A comparison of the ultraviolet absorption spectra of Poliovirus I before and after degradation with SDS, urea and acetic acid. ● = whole Poliovirus I; ○ = degraded Poliovirus I protein.

using sodium dodecyl sulfate (SDS), urea, acetic acid and 2-mercaptoethanol (46). In the present study, viral capsid proteins produced by similar degradation procedures were separated on SDS disc gel electrophoresis. Molecular weights of the subunits were estimated from an SDS gel electrophoresis molecular weight curve (Figure 3) constructed from data with known protein standards (Figure 4). Proteins with molecular weights of 95,000, 80,000, 71,000 and 37,000 were observed (Figure 5). It is thought that incomplete degradation or re-aggregation may be responsible for the higher molecular weight proteins observed in the gel and that these may be composed of either incompletely degraded fragments of viral capsid or proteins composed of subunits which have reaggregated during the electrophoretic procedure.

#### Titering Hyperimmune Rabbit Antiserum

Tables I and II show the titers of rabbit hyperimmune sera for whole Poliovirus I and degraded Poliovirus I proteins during the course of immunization. The titers were performed as outlined in Material and Methods and were considered as the last dilution to produce a visible precipitin line in agar.

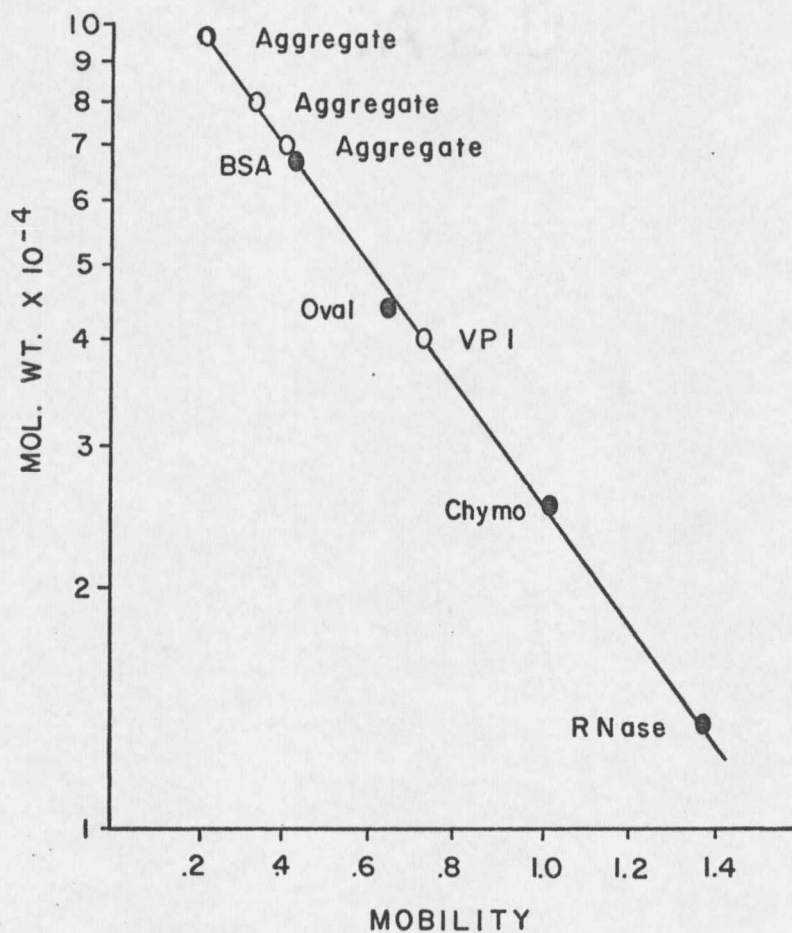


Figure 3. Estimation of molecular weights utilizing the SDS gel electrophoresis system. (Aggregates) aggregated Poliovirus I proteins, (VPI) Poliovirus I viral protein 1. Mobility equals the ratio of the distance the protein migrates to the distance chymotrypsinogen migrates.

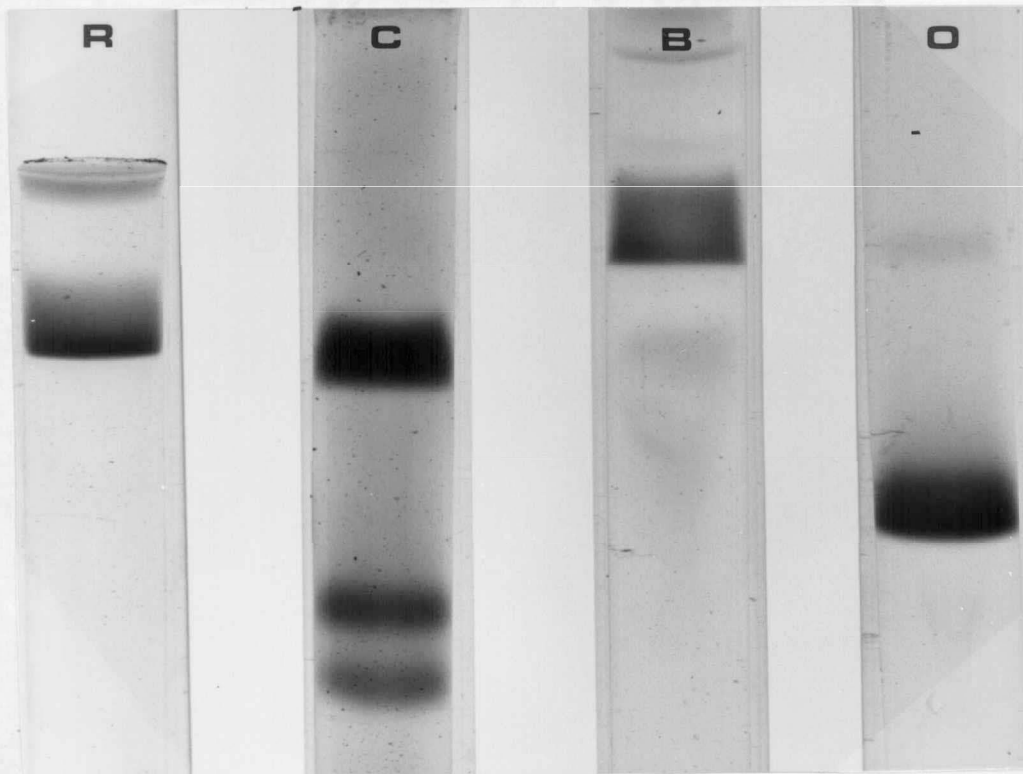


Figure 4. SDS gels of four standards used to construct the molecular weight estimation curve shown in Figure 3. C=chymotrypsinogen, R=ribonuclease, O=ovalbumin, B=bovine serum albumin.

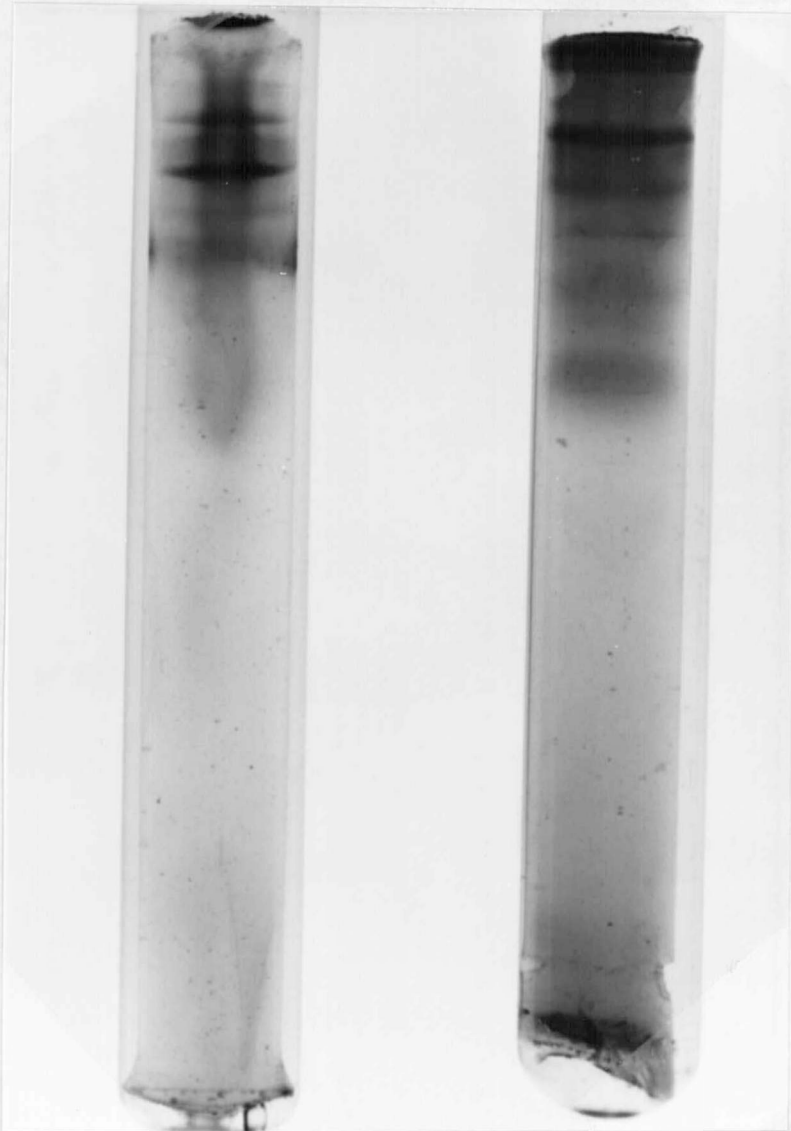


Figure 5. SDS gels showing the proteins separated from the preparation of degraded Poliovirus I protein. Molecular weights of the separated bands of protein are given in Figure 3.

Table I. Immunization schedule for the production of anti-degraded Poliovirus I serum.

Date	Material Injected	Route	Titer Against Degraded Protein	Titer Against Whole Virus
11/ 4/72	2.5 ml D. virus + 2.5 ml CFA*	I.M.	--	--
11/10/72	2.5 ml D. virus + 2.5 ml CFA	I.M.	--	--
11/17/72	2.5 ml D. virus + 2.5 ml IFA**	I.M.	Undilute	--
11/24/72	2.5 ml D. virus + 2.5 ml IFA	I.M.	1:2	1:2
12/ 3/72	2.5 ml D. virus + 2.5 ml IFA	I.M.	1:2	1:2
12/11/72	--	--	1:4	1:2

\* Complete Freund's adjuvant

\*\* Incomplete Freund's adjuvant

Table II. Immunization schedule for the production of anti-whole Poliovirus I serum.

Date	Material Injected	Route	Titer Against Whole Virus	Titer Against Degraded Protein
6/ 2/72	5 ml TCF* + 5 ml IFA**	I.M.	--	--
	1 ml TCF	I.V.		
6/ 9/72	1 ml TCF	I.V.	--	--
6/16/72	1 ml TCF	I.V.	1:2	1:2
6/23/72	5 ml TCF + 5 ml IFA	I.M.	1:2	1:4
	1 ml TCF	I.V.		
7/ 2/72	1 ml TCF	I.V.	1:8	1:4
7/10/72	--	--	1:16	1:4

\* Tissue culture fluid with virus

\*\* Incomplete Freund's adjuvant

Determination of Plaque Forming Units of  
Viruses used in the Study

The plaque forming units (PFU)/ml of each virus used in the study were determined by plating viral dilutions on monolayers of VERO cells by the method previously described. Each virus was plaqued 6 times and the average of these data are included in Table III. In addition to determining the PFU/ml of each virus, an attempt was made to compare the efficiency of virus production which occurs when monolayers of cells are infected as opposed to the suspension method of infection described earlier. It was found that viral production could be increased 10,000 times by concentrating the cells and infecting them with 15-20 virus particles per cell in suspension culture. Monolayer infected cells produced  $9.3 \times 10^7$  PFU/ml while suspension infected cells produced  $1.07 \times 10^{12}$  PFU/ml.

In order to insure that approximately the same amount of viral protein would be available for reacting with the antisera in the comparative experiments, it was necessary to react dilutions of whole and degraded Poliovirus I antigen preparations with the hyperimmune sera prepared against these respective antigens to determine the minimum number of PFU necessary to produce a precipitin line. These data were used to determine the dilutions of all other viruses used in the study needed to equal the minimum reactive level of whole and degraded Poliovirus I antigens (Table IV).

Table III. Plaque-forming units of the viruses used in the present study.

Virus	Plaque-forming Units/ml
Poliovirus I	$9.3 \times 10^7$
Poliovirus II	$1.3 \times 10^8$
Poliovirus III	$1.5 \times 10^8$
ECHO virus 1	$4.4 \times 10^8$
ECHO virus 5	$1.13 \times 10^7$
ECHO virus 18	$6.0 \times 10^6$
ECHO virus 22	$5.4 \times 10^8$
Coxsackie virus B1	$5.0 \times 10^6^*$
Coxsackie virus A9	$2.0 \times 10^7^*$
Coxsackie virus A13	$1.0 \times 10^7^*$

\* Values obtained from Hsuing (20).

Table IV. Determination of dilution of each virus required to equal the minimum reactive number of Plaque-forming units of Poliovirus I as determined by double diffusion in agar.

Virus	PFU/ml	PFU/Bohle	No Bohles	Total PFU/ml	Whole Polio-virus I-anti-whole P.V. I active PFU	Dil.	Degraded Polio-virus I-anti-degraded P.V. I active PFU	Dil.
Polio-virus I	$9.3 \times 10^7$	$1.9 \times 10^9$	16	$3.0 \times 10^{10}$	$3.7 \times 10^9$	1:8	$7.5 \times 10^9$	1:4
Polio-virus II	$1.3 \times 10^8$	$2.6 \times 10^9$	16	$4.2 \times 10^{10}$		1:11		1:5
Polio-virus III	$1.5 \times 10^8$	$3.0 \times 10^9$	16	$4.8 \times 10^{10}$		1:14		1:7
ECHO virus 1	$4.4 \times 10^8$	$8.8 \times 10^9$	12	$1.1 \times 10^{11}$		1:28		1:14
ECHO virus 5	$1.13 \times 10^7$	$23 \times 10^8$	18	$4.1 \times 10^9$		Und	$8.2 \times 10^9$	Und*
ECHO virus 18	$6.0 \times 10^6$	$1.2 \times 10^8$	30	$3.6 \times 10^9$		Und	$7.2 \times 10^9$	Und*
ECHO virus 22	$5.4 \times 10^8$	$1.1 \times 10^{10}$	30	$3.3 \times 10^{11}$		1:90		1:45
Coxsackie virus B1	$5 \times 10^6$	$1 \times 10^8$	37	$3.7 \times 10^9$		Und	$7.4 \times 10^9$	Und*
Coxsackie virus A9	$2 \times 10^7$	$4 \times 10^8$	18	$7.2 \times 10^9$		1:2		Und
Coxsackie virus A13	$1 \times 10^7$	$2 \times 10^8$	18	$3.6 \times 10^9$		Und	$7.2 \times 10^9$	Und*

\* Whole virus antigen preparation was concentrated two fold by ultracentrifugation before disruption occurred.

Reaction of Whole Viruses with Anti-whole  
Poliovirus I and Anti-degraded Poliovirus I  
Sera

Approximately the same number of PFU/ml for each whole virus was reacted with whole Poliovirus I antiserum in a double diffusion system. The only precipitin reaction obtained was the specific reaction between whole Poliovirus I antigen and anti-whole Poliovirus I serum (Figure 6). When whole virus antigens were reacted with degraded Poliovirus I antiserum, no cross reactions were seen (Figure 7). The only precipitin line formed was that which occurred when whole Poliovirus I was reacted with degraded Poliovirus I antiserum. No common or "group" antigens were observed in precipitin tests reacting either whole Poliovirus I or degraded Poliovirus I antisera with whole viruses other than Poliovirus I.

These observations did not change when the other virus antigens were used undiluted giving a considerable increase in total viral antigen available for precipitation.

Reaction of Degraded Viruses with Whole  
Poliovirus I and Degraded Poliovirus I  
Antisera

Initial experiments utilized approximately the same PFU/ml of each virus as the previously determined minimum PFU/ml required for degraded Poliovirus I protein to react with antiserum prepared against it in rabbits. Cross-reactions were observed with degraded

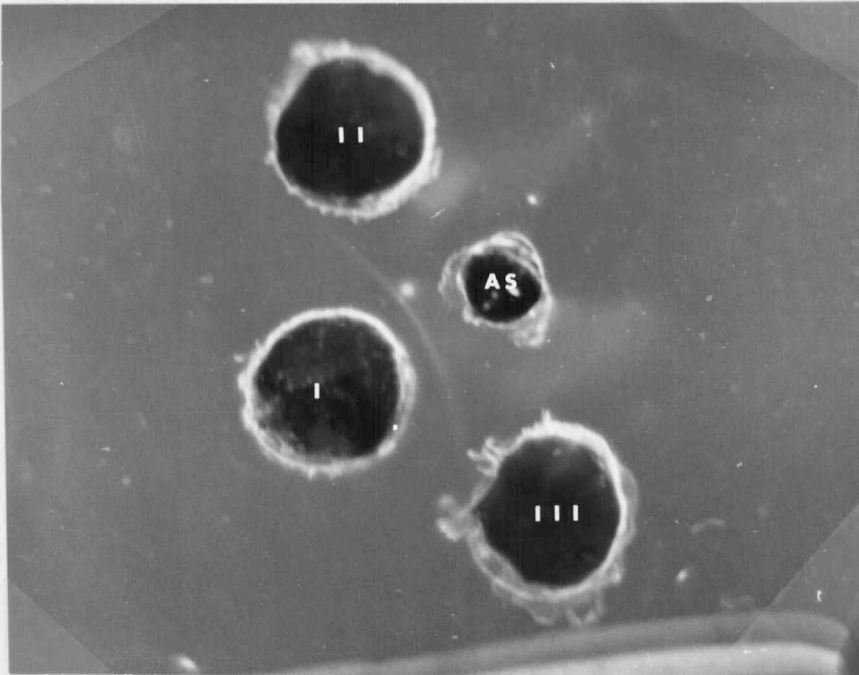


Figure 6. Ouchterlony double diffusion test comparing whole Poliovirus I antiserum to whole Poliovirus I and two other whole viruses. (AS) whole Poliovirus I antiserum, (II) whole Poliovirus II, (I) whole Poliovirus I, and (III) whole Poliovirus III.

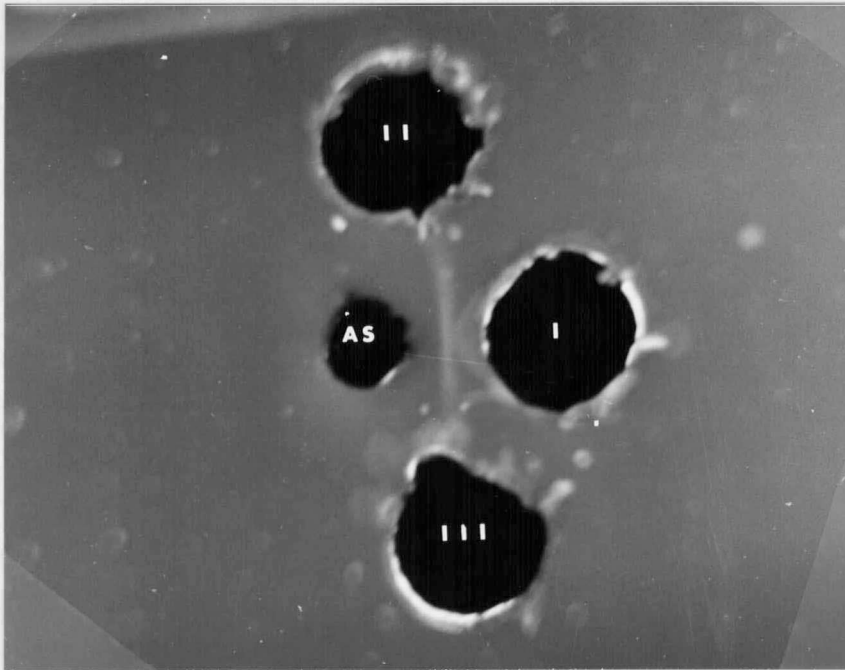


Figure 7. Ouchterlony double diffusion test comparing degraded Poliovirus I antiserum to whole Poliovirus I and two other whole viruses. (AS) degraded Poliovirus I antiserum, (II) whole Poliovirus II, (I) whole Poliovirus I, and (III) whole Poliovirus III.

Polioviruses II and III, ECHOvirus 5 and Coxsackie viruses A13 and B1 (Figures 8-10). The precipitin lines in all cases showed fusion indicating identity. No spurs were present which would indicate antigens with partial identity.

When degraded viral proteins were reacted with whole Poliovirus I antiserum, cross reactions were observed with Polioviruses II and III and ECHOvirus 5 (Figures 11 and 12). When the virus preparations were used undiluted, thereby increasing the protein available for reaction, the viruses cross reacting did not change but the precipitin lines were increased in size. Once again, the precipitin lines demonstrated fusion and absence of spurs indicating immunologically identical antigens.

Human Acute and Convalescent Sera Reacted  
with Whole and Degraded Virus Antigen  
Preparations

Twelve human volunteers, all of which had been immunized orally with trivalent Poliovirus vaccine within one year, were bled. This serum specimen will be referred to as the acute specimen. They were given one dose of live, oral, trivalent Poliovirus vaccine (Orimmune, Lederle) and bled again in 10 days. The second serum specimen is referred to as the convalescent specimen. These sera were reacted with whole and degraded Poliovirus I antigen preparations using the Ouchterlony technique. Six volunteers responded with

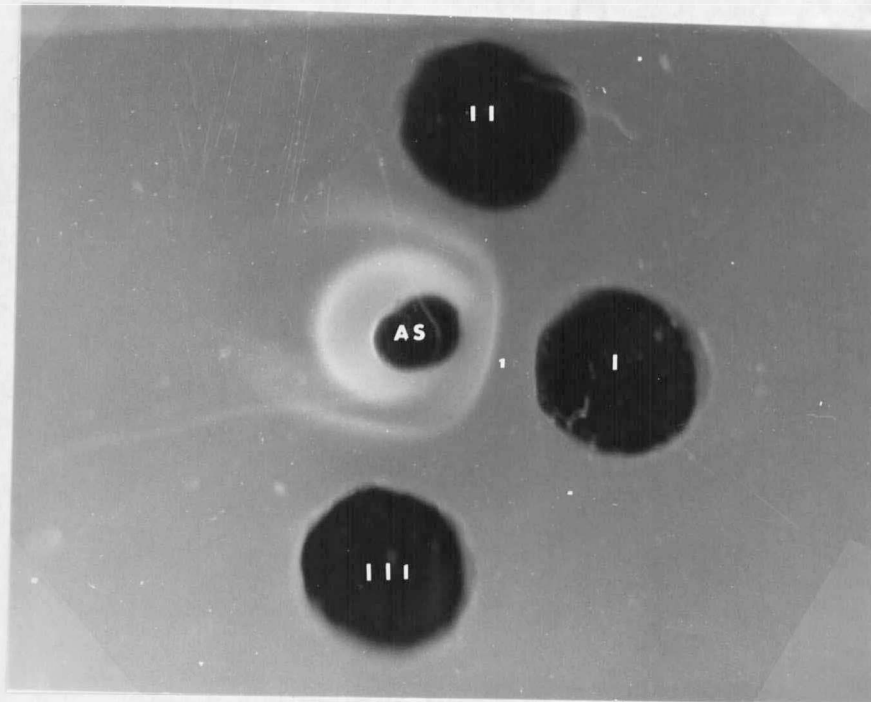


Figure 8. Ouchterlony double diffusion test comparing degraded Poliovirus I protein antiserum with other degraded viral proteins (degraded Poliovirus I protein). (AS) degraded Poliovirus I antiserum, (II) degraded Poliovirus II, (I) degraded Poliovirus I, and (III) degraded Poliovirus III.

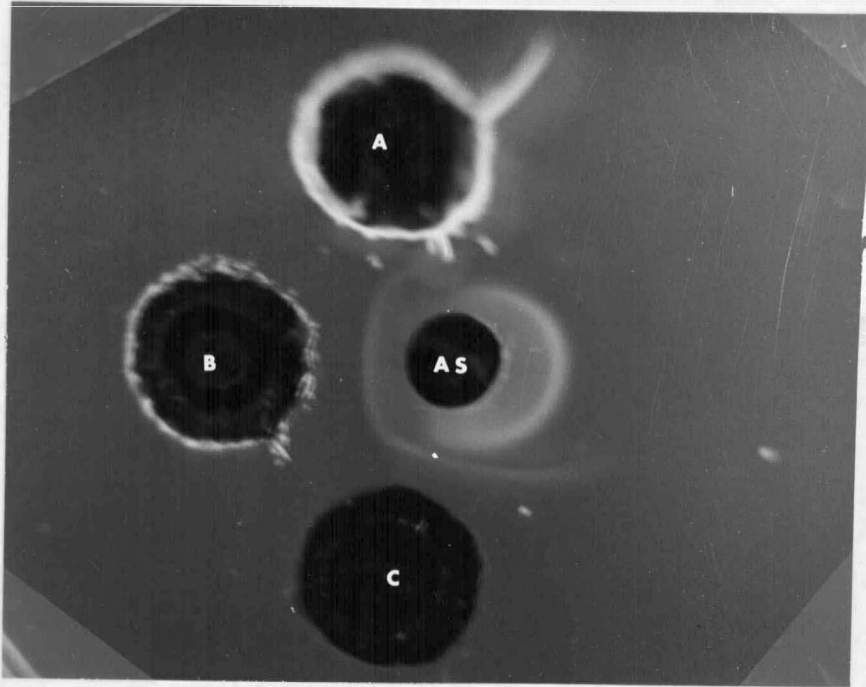


Figure 9. Ouchterlony double diffusion test comparing degraded Poliovirus I protein antiserum with degraded Poliovirus I protein and two other degraded viruses. (AS) degraded Poliovirus I protein antiserum (A) degraded ECHOvirus 1, (B) degraded Poliovirus I protein, and (C) degraded ECHOvirus 5 protein.

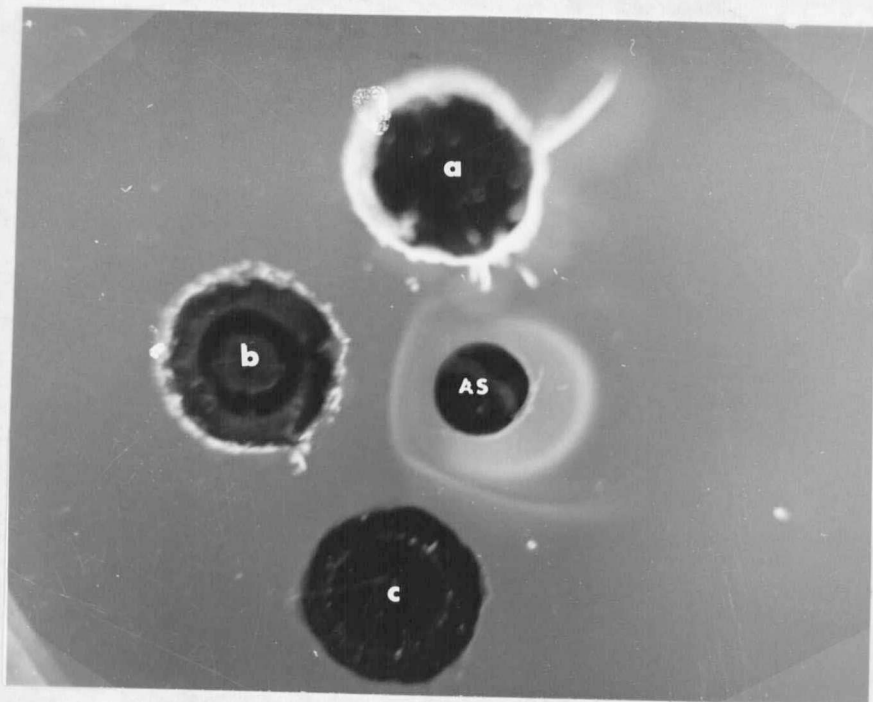


Figure 10. Ouchterlony double diffusion test comparing degraded Poliovirus I protein antiserum with Poliovirus I degraded protein and two other degraded viruses. (AS) Poliovirus I degraded protein antiserum, (a) degraded Cocksackie virus A9 protein, (b) degraded Poliovirus I protein, and (c) degraded Cocksackie virus A13 protein.

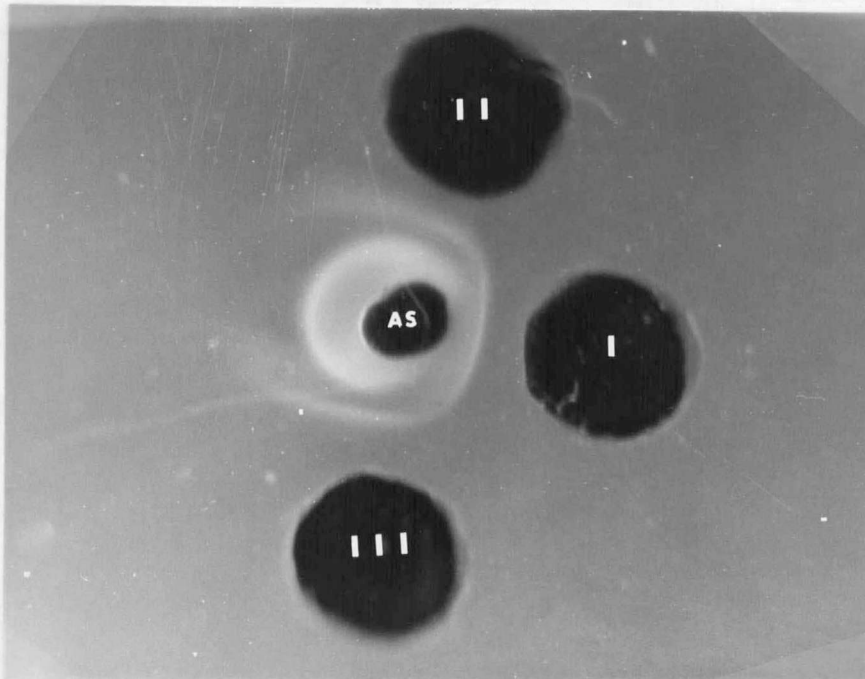


Figure 11. Ouchterlony double diffusion test comparing whole Poliovirus I antiserum with Poliovirus I degraded protein and two other degraded viruses. (AS) whole Poliovirus I antiserum, (II) degraded Poliovirus II protein, (I) degraded Poliovirus I protein, (III) degraded Poliovirus III protein.

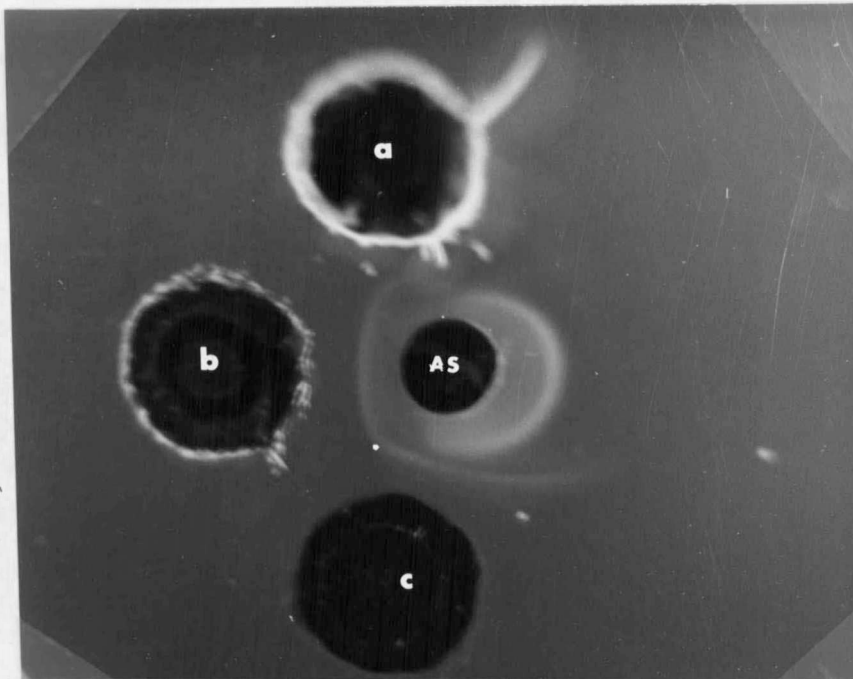


Figure 12. Ouchterlony double diffusion test comparing whole Poliovirus I antiserum to degraded Poliovirus I protein and two other degraded viruses. (AS) whole Poliovirus I antiserum, (a) degraded ECHOvirus 1 protein, (b) degraded Poliovirus I protein, (c) degraded ECHOvirus 5 protein.

antibodies which reacted with whole Poliovirus I. Three of these had negative acute sera when reacted undiluted, while antibodies were present in the convalescent sera when reacted undiluted (Figure 13).

Ten volunteers had antibodies in their sera which reacted with degraded Poliovirus I proteins. In six of these, antibodies were observed only in the convalescent sera (Figure 14).

When the 12 paired sera were reacted with the other whole viruses utilized in the study, only 2 were found to react. Both of these reacted with whole Poliovirus II and in both cases, the antibodies were present in the convalescent sera (Figure 15).

When the 12 paired sera were reacted with the other degraded viral proteins, no reactions other than the previously reported reactions between the sera and degraded Poliovirus I protein were observed.

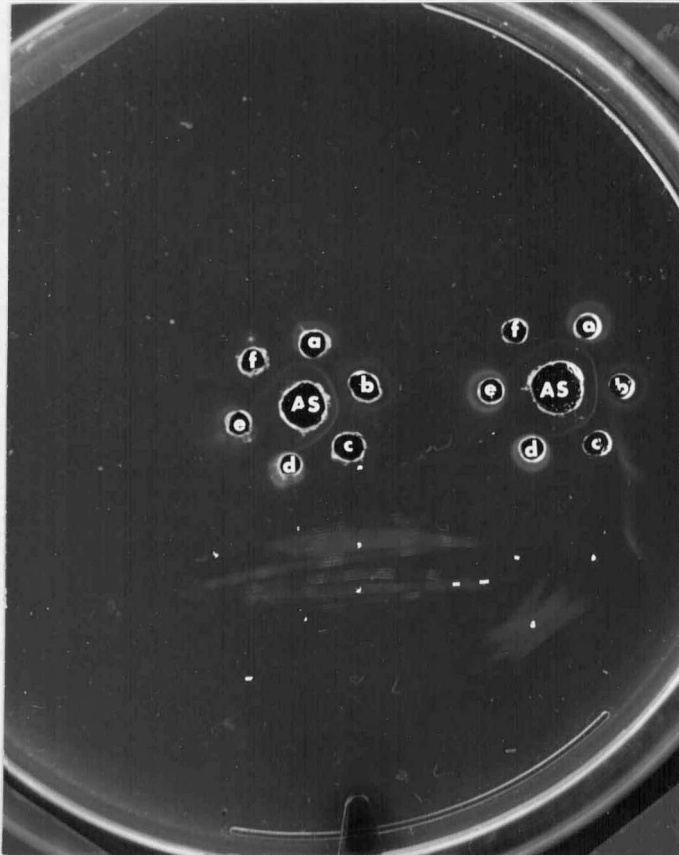


Figure 13. Ouchterlony double diffusion test comparing immunized human serums to whole Poliovirus I. (AS) whole Poliovirus I, (a,b,d, and e) convalescent human serum, and (c and f) whole Poliovirus I antiserum. The template on the right is exactly the same except that all human sera have been absorbed with tissue culture fluid + 3 percent bovine serum and cell fragments.

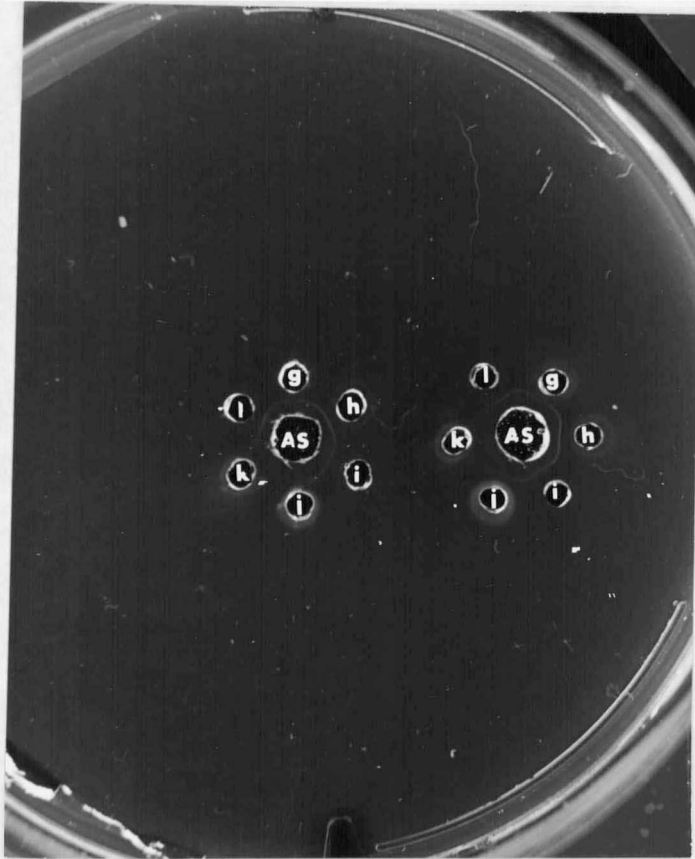


Figure 14. Ouchterlony double diffusion test comparing immunized human serums with degraded Poliovirus I protein (a) degraded Poliovirus I protein, (g,h,j, and k) convalescent human serums, (i,l) whole Poliovirus I anti-serum. The template on the right is exactly the same except that the human sera have been absorbed with tissue culture fluid + 3 percent bovine serum and cell fragments.

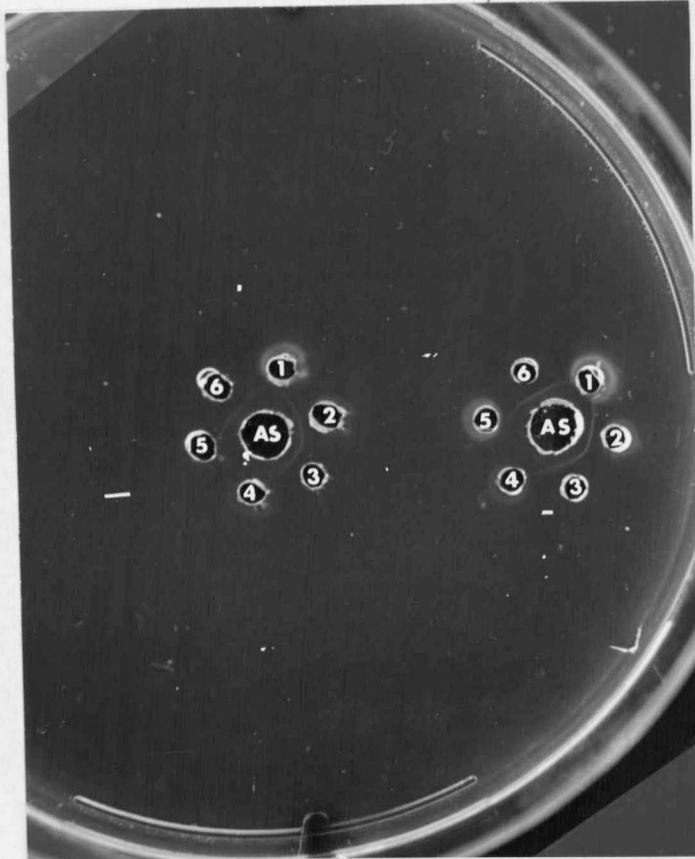


Figure 15. Ouchterlony double diffusion test comparing convalescent human serums with whole Poliovirus II. (a) whole Poliovirus II, (1,2,4 and 5) convalescent human serums, (3,6) whole Poliovirus II antiserum. The template on the right is exactly the same except that the human sera have been absorbed with tissue culture fluid + 3 percent bovine serum and cell fragments.

## DISCUSSION

Treatment of Poliovirus I capsomeres with sodium dodecyl sulfate (SDS), urea and 2-mercaptoethanol at an acid pH causes depolymerization and produces 4 monomers, VP 1, VP 2, VP 3, and VP 4 with molecular weights of 35,000, 28,000, 24,000, and 5,000, respectively. In the present study, aggregates with molecular weights of 95,000, 80,000 and 71,000 were observed. It is thought that these are the result of incomplete degradation or reaggregation of the subunits. One of the contributing factors to reaggregation may be that the electrophoresis was performed in a system with a pH of 7.1 while initial degradation and separation of the Poliovirus capsid subunits requires an acid pH. SDS (a non-ionic detergent) breaks electrostatic, hydrogen and hydrophobic bonds between monomers which results in loss of 2° and 3° structure of the capsid but does not alter the primary amino acid sequence of the monomers. Urea acts similarly by also destroying the quaternary and tertiary and secondary structures of the capsid but leaves the amino acid sequential arrangement intact. 2-mercaptoethanol prevents reaggregation of the monomers by reducing the disulfide bonds to free sulfhydryl groups.

The influence of the conformation of protein molecules on their antigenic specificity is well documented (15). There is a growing list of viruses, which when depolymerized by various means, undergo a transconformation which results either in alteration or loss

of original antigenic specificity (9,42,48). It is reasonable to postulate, therefore, that the loss of the native conformation of Poliovirus I capsids during depolymerization is accompanied by the formation of a set of antigenic sites which give a new immunogenic specificity to the degraded viral protein. It might be expected that much of the whole virus specificity would be lost by alteration of native tertiary structure which defines the antigenic determinants present on the surface of the nucleocapsid. It follows that the different immunogenic specificity of degraded viral protein would be due to new antigenic sites which were non-existent in the previous conformational state of the capsid.

It has been suggested that in the evolution of viruses, the type specific antigens become located on the outermost parts of the viral structure (25). The enteroviruses are composed of three subgroups of viruses which are all fairly uniform in molecular weight, shape, size and buoyant density. They do, however, elicit specific antibody responses when they are used as immunogens. These specific antibodies are probably formed in response to antigenic sites present on the surface of the capsid. Internal antigens are likely to be not as specific because of less pressure for evolution from external conditions. Thus, in a group of viruses, such as the enteroviruses, antigenic sites which are "hidden" may be similar or identical in all three subgroups and, in fact, the present study seemed to indicate

that this was the case. It is also possible that conformational determinants are stronger antigens than those dictated by primary structure and these are responsible for the specificity of whole virus reactions. When tertiary and secondary structure are reduced by depolymerization, antigens consisting of the primary amino acid sequence become the dominant antigens and these are sufficiently similar to cause cross reactivity.

In addition to presenting different antigenic sites, depolymerization has other effects on the immunogenicity of the degraded viral protein. Treatment with SDS not only changes all but the primary amino acid sequence of the monomers, it gives the monomers a high negative charge and alters the shape to round or elliptical, depending upon the molecular weight of the monomer (36). In this study, a reduction of molecular weight from that found in the whole capsid to that which is present in the subunits and alteration of shape and charge dramatically reduced the immunogenicity of the degraded viral proteins. Reduction of molecular weight is not always sufficient alone to reduce the antibody response to an antigen, but when it is coupled with a high negative charge which depresses the antibody response and an extreme change in molecular shape (2) increased amounts of antigen are necessary to elicit a detectable antibody response. The solubility of the antigen may also play an

important role in immunogenicity. Depolymerization solubilizes the subunits and it has been well documented that soluble antigens are less immunogenic than particulate antigens such as whole viruses (10). It was observed in this study that amounts of whole virus antigen necessary to immunize a rabbit could be determined in numbers of particles which comprised sub-microgram amounts of viral protein but the amount of degraded protein required for immunization consisted of milligram amounts (see Immunization, Materials and Methods).

Antiserum produced against whole Poliovirus I particles reacted in double diffusion with both whole virions and degraded viral protein. However, the lines did not exhibit fusion which indicated that different antigens were involved in both responses. It seems likely that the antigens present on the surface of the whole virion, which are reacting with the Poliovirus I antiserum, are those dictated by the tertiary structure of the capsid. However, degraded viral proteins do not possess the same tertiary structure, so antibodies reacting in this case are those which are formed against sequential determinants or possible determinants dictated by secondary structure. Antibodies may be formed in the rabbit against these when they become exposed during the enzymatic breakdown which occurs in the process of immunization (14,26).

When a rabbit is immunized with degraded Poliovirus I protein, antibodies, which react with both whole Poliovirus I and degraded

Poliovirus I protein, are produced. In this instance, however, lines of partial identity are seen indicating that at least some of the antigenic sites present after depolymerization are also present on the surface of the intact virion. The cross-reactivity could also be explained if the whole Poliovirus I and degraded Poliovirus I protein possessed similar but non-identical determinants capable of cross-reacting with a certain population of antibodies having lower degrees of specificity (10).

When whole Poliovirus I antiserum was reacted with the other whole Polioviruses (II and III), ECHOviruses 1, 5, 18, and 22, and Coxsackie viruses A9, A13, and B1, no reactions other than the homologous reaction with whole Poliovirus I were observed. This indicates that the conformational sites, as determined by the tertiary structure, are sufficiently different so that antiserum prepared against one virus is incapable of reacting with surface antigens present on another virus. While group specificity has usually been true of precipitin antibodies measured by immunodiffusion (12,13,31,45), intra-"group" antigens have been observed, particularly in the Polioviruses, when other methods of antibody assay have been used.

Hummeler and Hamparian (21) showed that adsorption of human serum with Poliovirus II native antigen removed complement fixing antibodies against Polioviruses I and III, whereas adsorption with Poliovirus I.

and III native antigens removed only homologous antibody. LeBouvier (27) found that when Poliovirus I antigen was used to immunize rabbits, precipitin and neutralizing antibodies for Poliovirus II and III were produced, and in a group of patients who had Poliovirus infections, Schmidt and Lennette (39) observed that the rate of heterotypic antibody formation was 55 percent for neutralizing antibodies, 17 percent for complement fixation antibodies and 21 percent for flocculating antibodies.

Reaction of anti-whole Poliovirus I serum with the other degraded viral proteins resulted in cross-reactions with Poliovirus II and III and ECHOvirus 5. The lines fused indicating that similar antigens were responsible for the precipitin bands. The data indicate that when enteroviruses are depolymerized, antigenic sites determined by a specific amino acid sequence are common to at least two of the three subgroups of enteroviruses are recognized and that these same antigenic sites either appear exposed on the surface of Poliovirus I or are exposed for antigenic recognition by enzymatic digestion within the animal. Korant (26) and Garfinkle and Tershal (14) have reported that degradation of Poliovirus I capsids does occur in vivo. This degradation process may expose sites similar to those exposed through artificial depolymerization.

When degraded Poliovirus I antiserum was reacted with the other whole viruses in the study, no reactions except that between

the anti-serum and degraded Poliovirus I protein occurred. This indicates that antibodies produced against the primary amino acid sequence of the monomers are incapable of reacting with the antigens when they are polymerized into the normal tertiary structure. Some or all of the antibody binding sites become obscured due to the conformation of the capsid. The opposite situation consisting of the reaction between anti-whole Poliovirus I serum and degraded viral proteins gave cross reactions between the other Polioviruses and ECHOvirus 5 and seems to be contradictory to the above statement. It is possible for both conditions to be true if an antigenic determinant consisting of an amino acid sequence exposed on the surface of the capsid elicits the production of an antibody against whole virus which is capable of reacting with the same amino acid sequence when it is present in the degraded viral protein.

When antiserum prepared against degraded Poliovirus I protein was reacted with the other degraded enteroviral proteins, cross-reactions between Polioviruses II and III, ECHOvirus 5 and Coxsackie viruses B1 and A13 were observed. The data indicate that probably all of these viruses contain an amino acid sequence sufficiently similar to react with antibody produced against a primary amino acid sequence present in degraded Poliovirus I protein. Furthermore, since the lines exhibited identity using the Ouchterlony technique, immunogenic

amino acid sequences of Poliovirus I are similar to amino acid sequences produced by degradation of wild type virus members of each of the subgroups of enteroviruses. This may indicate a much closer affiliation of some subgroup members to members of the same or other subgroups and may with additional study, offer information which could lead to a more accurate rearrangement of enteroviruses into more closely related subgroups.

The absence of precipitin bands in reactions between whole Poliovirus I and degraded Poliovirus I antisera and adenovirus type 1 whole and degraded capsids indicated that cross reactions occurring within the enteroviral groups were specific rather than non-specific reactions due to common treatments such as viral production, purification and degradation.

The studies with human volunteers offered information relative to the type of immunity elicited in response to oral vaccine and the efficacy of using immunodiffusion as a tool in the diagnosis of enteroviral diseases. Antibodies directed against whole Poliovirus I were detectable by double diffusion in 6 of the 12 volunteers, while only 2 persons produced antibody against any of the other whole viruses and both of these were in response to Poliovirus II. Since the vaccine was trivalent, a response to Poliovirus III would be expected also but this was not observed. Explanations could be either the

immune response to Poliovirus III consisted primarily of gut immunity and therefore an undetectable humoral response was generated or the challenge of the three types of Polioviruses in 1 dose of oral vaccine was insufficient to generate a detectable humoral response in most volunteers. In either situation, it is impossible to postulate the value of immunodiffusion as a diagnostic tool. There is information which indicates that humoral antibody levels detectable by double diffusion occur in active enteroviral disease (24,31,33) and that this response is specific for the virus involved. Therefore, diagnosis using double diffusion systems would require specific viral antigens for each enterovirus unless an "intersecting antigen pool scheme" based on the same principle as the "intersecting serum pool scheme" developed by Schmidt et al. (40) could be devised which would combine viral antigens in such a way that the point of intersection in a predetermined table would provide information concerning the virus responsible for the infection. One problem which would arise in this type of system, would be dilution of the antigen preparation that would occur when several viral antigens were pooled. This could possibly be overcome by a final high speed centrifugation after pooling had taken place.

The experiment reacting human serum with degraded viral proteins was inconclusive. In 10 of the 12 volunteers, antibody reacting with degraded Poliovirus I protein was observed. In 4 of these,

antibody was present in both acute and convalescent sera and in the remaining 6 antibody appeared in the convalescent serum only. The reactivity was not absorbed by treatment with cell fragments or tissue culture fluid with 3 percent bovine serum and would therefore seem to be specific. However, it seems unreasonable that a detectable antibody level for degraded viral protein would be sustained over the period of a year in view of the fact that in rabbits, titers had dropped to 0 by 1 month after the last injection of degraded viral protein. When the human sera were reacted with the other degraded viral proteins used in the study, no reactions were seen. The challenge available from the enzymatic digestion of the viral capsids presented in 1 dose of oral vaccine (about  $5 \times 10^8$  virions) cannot be adequate to induce an immune response in view of the fact that milligram quantities were required for rabbit immunization.

Patients with acute enteroviral diseases may produce antibodies against degraded enteroviral proteins but due to the cross-reaction observed between the three viral subgroups, the value as a presumptive diagnostic tool seems limited.

The present study may have established an acceptable method of analyzing immunologic interrelationships of enteroviruses. It could be expanded through the use of more hyperimmune sera against various whole and degraded enteroviruses. Information from these

experiments could establish entirely new subgroups of antigenically related enteroviruses. It has also presented information relative to immunodiffusion as a tool for diagnosis. Should new antigenically related subgroups be established due to additional research in this area, an immunodiagnostic scheme, including a presumptive test involving subgroup antigens (present on degraded viral proteins) and a confirmative test involving type specific antigens (present on whole virus capsids), could be evolved.

## SUMMARY

Antisera were prepared in rabbits against whole Poliovirus I and degraded Poliovirus I protein that had been produced by treatment of whole capsids with sodium dodecyl sulfate, urea, acetic acid and 2-mercaptoethanol. The antisera were reacted using the Ouchterlony technique with whole and degraded viral proteins of Polioviruses I, II, III, ECHOviruses 1, 5, 18, 22, and Coxsackie viruses B1, A9 and A13. When whole Poliovirus I antiserum and degraded Poliovirus I antiserum were reacted with the whole viruses, precipitin reactions were observed only with the antisera and whole and degraded Poliovirus I proteins. When whole Poliovirus I antiserum was reacted with the degraded viral proteins, precipitin reactions indicating identity were observed with Polioviruses I, II, III, and ECHOvirus 5. The reaction of degraded Poliovirus I antiserum with degraded viral proteins produced precipitin lines with Polioviruses I, II, III, ECHOvirus 5 and Coxsackie viruses B1 and A13. The data indicate that the three subgroups of enteroviruses have antigenic determinants based on primary amino acid sequence in common.

Acute and convalescent sera of human volunteers that had been immunized with one dose of oral, trivalent, Poliovirus vaccine were reacted with whole viruses and degraded viral proteins. Precipitin reactions were observed with whole Poliovirus I and II and degraded

Poliovirus I protein. The efficacy of using the double diffusion as a tool in the diagnosis of enteroviral diseases is discussed.

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LITERATURE CITED


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