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MEMS REAGENT AND SAMPLE HANDLING PROCEDURE

Feasibility of Viral Antibody Detection by Passive Immune Agglutination

G. D. Bailey, M.A.
H. J. Tenoso, Ph.D.
Organon Diagnostics

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LYNDON B. JOHNSON SPACE CENTER
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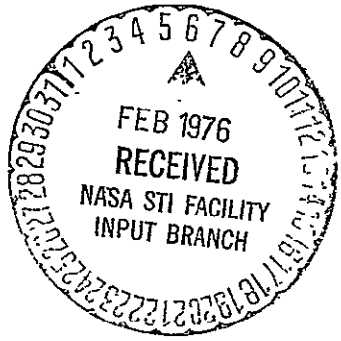

Organon Diagnostics
A DIVISION OF ORGANON INC. / A PART OF Akzona
9060 EAST FLAIR DRIVE • EL MONTE, CALIFORNIA 91731
TELEPHONE 213-572-6601 TELEX 674-502

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16. Abstract

An attempt was made to develop a test requiring no preadsorption steps for the assessment of antibodies to rubella and mumps viruses using the Passive Immune Agglutination (PIA) method. Both rubella and mumps antigens and antibodies were prepared. Direct PIA tests, using rubella antigen-coated beads, and indirect PIA tests, using rubella antibody-coated beads, were investigated. Attempts, using either method, were unsuccessful. Serum interference along with nonspecific agglutination of beads by the rubella antigen resulted in no specific response under the test conditions investigated. A new, highly sensitive approach, the enzyme immunoassay (EIA) test system, is recommended to overcome the nonspecificity. This system is a logical outgrowth of some of the solid phase work done on MEMS and represents the next generation test system that can be directly applied to early disease detection and monitoring.

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PREFACE

The work described herein was an attempt to increase the versatility of the Microbial Ecology Measurement System (MEMS) to include the detection of rubella and mumps antibodies in human sera. Mumps and rubella virus were propagated in tissue culture and specific antigens were isolated and purified. The purified rubella antigens were attached to latex beads and attempts were made to develop a test for the direct detection of rubella antibodies by passive immune agglutination (PIA). Means of attaching antigens to latex beads, other than simple adsorption, were investigated. An alternative method of rubella antibody detection, the indirect PIA, was examined. The extent of human serum interference on the PIA test was determined and potential methods of overcoming the interference were developed.

Because of the lack of a specific response, and the nonspecific agglutination of beads by rubella antigens, the detection of rubella antibodies by either direct or indirect PIA was unsuccessful under the conditions investigated on this program.

A new, highly sensitive technique, enzyme immunoassay, is recommended as a method for detecting viruses and viral antibodies while minimizing the nonspecificity.

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1.0 INTRODUCTION

This report describes work completed by Organon Diagnostics on Contract No. NAS 9-13672 for the National Aeronautics and Space Administration (NASA) from the period January 1, 1975 to September 30, 1975. During this time an attempt was made to develop a simplified method of assessing viral antibodies using Passive Immune Agglutination (PIA). The viruses chosen for study were rubella and mumps. It was hoped that the use of the PIA would eliminate the time-consuming serum treatment procedures now required in conventional tests, e.g., hemagglutination inhibition (HI) and complement fixation (CF). The HI test requires that the serum sample be treated to remove both nonspecific inhibitors of hemagglutination and nonspecific hemagglutinating factors. The CF test requires highly skilled technicians to perform in that a careful balance of reagents is required, e.g., complement, test antigen, hemolysin and indicator erythrocytes. Because of these tests, the present work was undertaken.

The original program at Organon Diagnostics (formerly Aerojet Medical and Biological Systems) for developing systems for the detection and quantitation of viruses was sponsored by the U.S. Army. This effort consisted of a comprehensive evaluation of newer techniques for rapid detection and identification of viral agents in captured biological aerosol samples; i.e., aqueous collector fluid samples. Those methods selected for investigation were evaluated for sensitivity, selectivity, logistic simplicity, economy, rapidity, and capability for ultimate automation. Nine basic assay concepts were evaluated for applicability to 7 different biological agents (including 4 viruses). Of the various rapid cellular, fluorometric, radioimmune and passive immunochemical techniques evaluated, one method in particular emerged as most promising. That method involved the use of spherical latex particles, coated (either by absorption or covalent bonding techniques) with highly reactive and purified immune globulin, to isolate specific agent antigen from suspending aqueous fluids. Attachment of the specific antigen unit, whether whole infectious agent, inactivated particles or components (e.g., capsid fragments, soluble components, etc.) to the sensitized particle effectively increased the biological particle size to that of the bead. The resulting amplification allowed such simple techniques as filtration and low-speed centrifugation to effectively

separate the isolated agent from its carrier fluid (water, media, cell and tissue lysates). Clumping of such antibody-coated beads in the presence of homologous antigen formed the basis of a sensitive viral detection system, termed Passive Immune Agglutination (PIA).

The feasibility of the PIA for use in the Microbiol Ecology Measurement System (MEMS) for NASA was shown with five respiratory viruses and Mycoplasma pneumoniae on Contract No. NAS 9-9740. At this time, a manual microscopic readout of bead agglutination was being used which required a great amount of technician involvement.

Work continued on Contract No. NAS 9-11371 wherein the PIA test conditions were optimized for the detection of influenza virus and the mycoplasma. In addition, means of automating the PIA readout were investigated. The photometric readout method was chosen which permitted the measurement of small changes in the degree of bead agglutination and eliminated the subjective aspects of the manual method. Along with this work, the group antigen concept of virus detection was investigated. The group antigen of adenoviruses, the hexon, was isolated and purified and group reacting antibodies were produced in rabbits.

Three prototype photometric test beds were fabricated under Contract No. NAS 9-13126 and were delivered to NASA for evaluation.

Highly purified antigens were prepared from several influenza virus types and an adenovirus on Contract No. NAS 9-13672. These were used to obtain high quality antisera in animals. The immunoglobulins were isolated from these antisera, purified and delivered to NASA. In addition, a detailed study and development of sample handling techniques took place. The work reported herein was an extension of that contract.

During the course of this program, antigens from both viruses were isolated and purified. A portion of these antigen preparations were used to immunize laboratory animals. The main usage of the purified rubella antigens was in sensitizing latex beads for the direct detection of antibodies in serum samples by PIA. Because of problems which were encountered while attempting to develop a rubella antibody test, work with the mumps virus reagents consisted only of reagent preparation.

The direct PIA method showed promise in preliminary studies when adenovirus hexons were attached to beads. The test was able to detect low concentrations of adenovirus antibodies in rabbit sera while giving little or no response to a rabbit antiserum directed toward influenza. Throughout the development of the rubella antibody test there were problems of nonspecificity. Rubella negative sera, as determined by conventional means, constantly displayed agglutination patterns not unlike those obtained with positive sera. Only when the beads were pre-coated with an extraneous protein, bovine serum albumin (BSA), were there differences between positive and negative sera. There were, still, excessive responses with the negative sera. The agglutination responses, both from the positive and the negative sera, could be eliminated by the simple addition of protein in the form of normal rabbit serum.

Bead sensitization studies were also conducted but were not conclusive. None of the methods tried, including three different ways of chemically binding rubella antigen, provided any improvement over absorbed beads.

Because of the inherent problem of prozone formation in agglutination reactions, an indirect rubella antibody PIA test was also investigated. Beads used in these tests were coated with rubella immunoglobulins. The test relied upon the ability of test serum antibodies to combine with rubella antigen in a preincubation and prevent the agglutination of the antibody sensitized beads. These tests were also hindered by nonspecific reactions. The antibody-coated beads did not agglutinate, specifically, in the presence of the homologous antigen. The antigen, in fact, agglutinated adenovirus antibody-coated beads just as well.

Because of the lack of any real specific agglutination, studies on the effects of human serum in the PIA test were also inconclusive. It was found, however, that the pattern of nonspecific agglutination in the presence of serum differs depending upon the composition of the buffer used. Prospective methods of correcting and eliminating nonspecific agglutination were developed on this program. The feasibility of these methods could not be proven because of the lack of a specific response in the test system.

Although PIA testing was hindered by both nonspecific agglutination and the absence of a specific response, other tests showed that the rubella

antigens and antibodies were reactive. The antigens were highly reactive in the hemagglutination test and their action could be inhibited in the HI test using commercially obtained antisera. CF testing also demonstrated their immunological reactivity.

It appears that the lack of success in our efforts was the result of the interference of serum and the nonspecific agglutination of beads by the rubella antigens. These problems were not solved under the conditions tried during the course of the present program.

Organon Diagnostics, as the result of MEMS technology, has developed other detection methods which use proteins immobilized on latex beads. The major effort has been the development of solid phase radioimmunoassays (RIA) for viruses. However, there is another technique, in the early stages of development at Organon, which does not require the use of radioisotopes. Rather, antibodies are labeled with enzymes and the method is called Enzyme Immunoassay (EIA). In the EIA, the sample is reacted with antibody-coated beads. After washing the sample from the beads, the enzyme labeled antibody is allowed to react. This forms a sandwich when it reacts with the antigen bound to the beads. After the excess enzyme-labeled antibody is washed away, a substrate containing a dye is added. A color change is the result of the enzymatic decomposition of the substrate which is in direct proportion to the amount of enzyme attached to the beads. These tests have been shown to equal the sensitivities attained using RIA methods. Because of the constraints posed by MEMS regarding the use of radioisotopes, the EIA methodology appears to be an ideal solution to the problems associated with detection of viruses and their antibodies.

Sections 2.0 through 6.0 of this report contain a technical discussion of the work performed. Section 7.0 includes a discussion of the results, conclusions which may be made, and recommendations for further studies.

2.0 TASK I - PREPARATION OF RUBELLA AND MUMPS ANTIGENS

2.1 RUBELLA

2.1.1 Rubella Propagation

Rubella is an ether sensitive, enveloped virus possessing RNA as genetic material. The virus has been classified, in the past, as a paramyxovirus but most recently as a togavirus.

Rubella strain M33, obtained from the American Type Culture Collection, was adapted for growth in Vero cells, a continuous line of African Green Monkey kidney cells. The use of this cell line for rubella virus propagation has been described by Liebhaber, et al (1967). These investigators showed that Vero cells produced rubella virus in higher titers than the commonly used BHK-21 line of hampster kidney cells.

The cells were grown at 37°C in 690 cm^2 roller bottles rotating at 0.25 revolutions per minute and were fed with Eagle's Minimum Essential Medium, buffered with Earle's balanced salts solution (EBSS), and supplemented with 10% fetal bovine serum and L-glutamine. Fungizone and Gentamycin were added to prevent fungal and bacterial contamination. When the cell monolayers had formed, usually after four days, the growth medium was removed and the cells were washed with Puck's Saline. Rubella virus seed, in a ten ml aliquot, was allowed to adsorb to the cells for two hours while rotating at 37°C. The inoculum was removed and the cells were washed with EBSS. The infected cells were maintained with 100 ml of Serumless Medium (GIBCO).

The growth of rubella virus was monitored by the appearance of the cytopathic effect (CPE). After a period of seven to nine days the CPE reached a maximum and the cells were harvested by scraping into the medium. At this point the infected cell suspension was utilized for further passaging. HA titrations were performed using one-day old chick erythrocytes by the method of Schmidt, et al (1971). After three passages, when the HA titer was sufficient, large scale propagation was initiated. Table 2-1 shows the data for the various rubella virus harvests.

TABLE 2-1 SUMMARY OF RUBELLA VIRUS HARVESTS

Passage	HA Titer	Volume (ml)
1	0	100
2	1:8	100
3	1:16	100
4	1:32	400
5	1:16	1200
6	1:32	2100
7	1:32	800

2.1.2 Rubella Antigen Purification

The virus and viral antigens were released from the cells by sonication for two minutes with a probe-type sonifier. In early preparations, the cellular debris was removed by centrifugation at 650 x g for ten minutes at 4°C. The clarified tissue culture fluids or, later, the unclarified fluids were mixed with Tween 80 (0.125% final concentration). The mixture was shaken for five minutes at room temperature. Diethyl ether was then added to 0.33% concentration and this was mixed on a magnetic stirrer for 15 minutes at 4°C (Norrby, 1966). It was later found that by increasing the time of both treatments to 45 minutes at 4°C a two- to four-fold increase in HA titer could be achieved over that obtained with the earlier treatment times.

The Tween-ether treated fluids were then centrifuged for 20 minutes at 12,000 x g at 4°C. The gel-like upper phase was removed by aspiration. If unclarified fluids were treated; the cellular debris was found in a pellet at the bottom of the centrifuge tubes. The viral antigens were located in the aqueous phase which was collected by decantation. This fluid was then concentrated 20 to 30 times by dialysis against polyethylene glycol having a molecular weight of 20,000 (PEG 20,000). The antigens were then purified by exclusion chromatography on an agarose column (BioGel A-1.5 m, BioRad Laboratories) equilibrated with 0.02 M Tris buffer, pH 8.4 containing 0.03 M sodium ethylenediaminetetracetate (EDTA). The dimensions of the column were 2.5 cm x 80 cm.

Typically, 30 ml volumes of the concentrated, Tween-ether treated tissue culture fluids were chromatographed at a flow rate of 1.5 ml per minute. Elution was with the same buffer. Fractions of about four ml were collected and tested for HA and complement fixation (CF) activities. A typical chromatographic run is shown in Figure 2-1. The HA activity was contained in the first peak, exclusively. This material also exhibited CF activity. The second peak had CF activity but no HA.

Fractions from the first peak were pooled for HA antigen. In some experiments the second peak fractions were pooled for CF antigen.

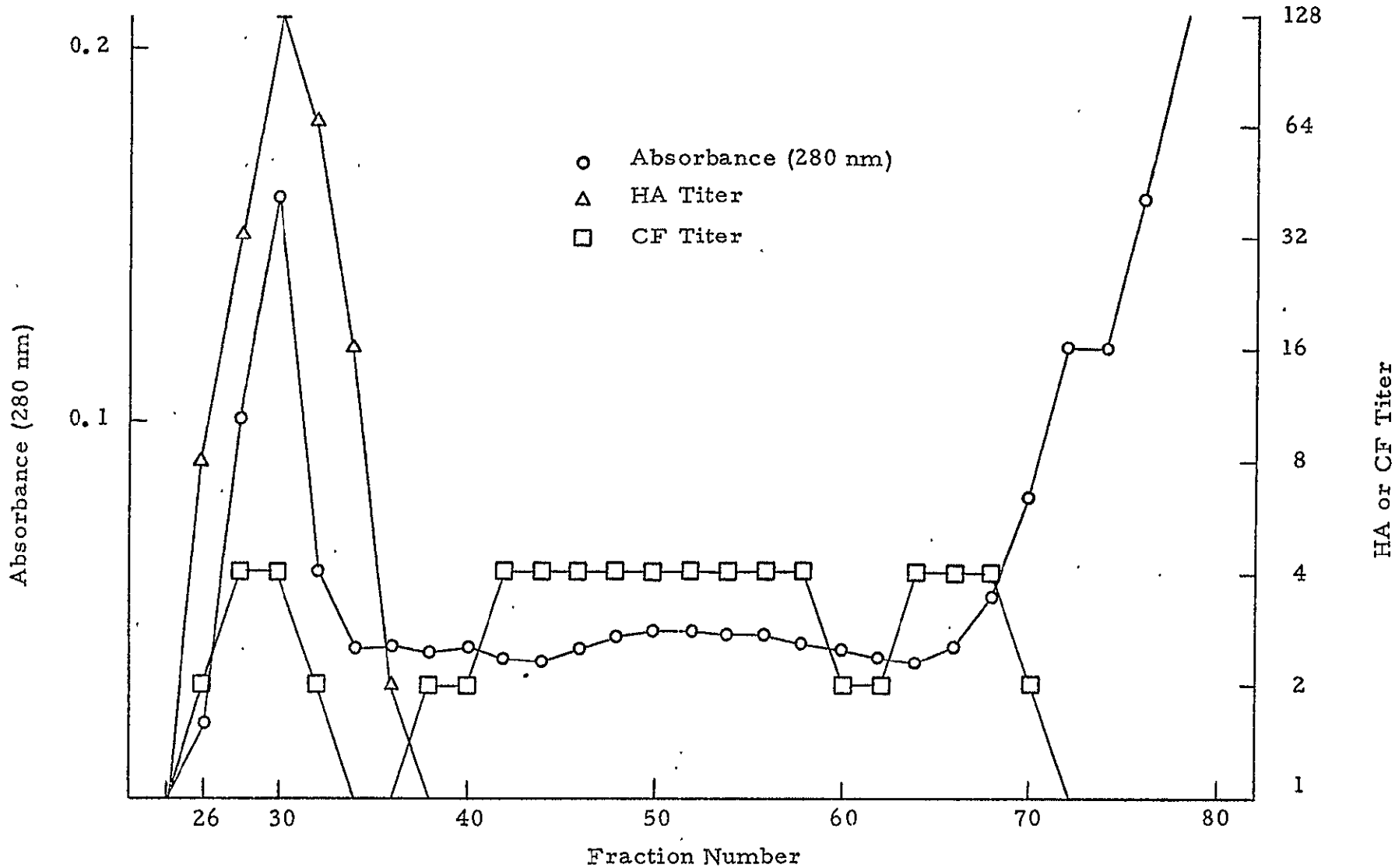

Figure 2-1 Chromatography of Tween 80-Ether Treated Rubella on BioGel A-1.5 m

The pools of each type of antigen were further concentrated by dialysis against PEG 20,000. A run wherein both the HA and CF antigens were purified is given in Table 2-2. HA and CF titers were determined at each stage in the purification process as was the protein concentration. A ratio was then obtained between the biological titer (HA or CF) and the protein content (P). An increase in the HA/P or CF/P ratio was desirable since this indicated the removal of unwanted protein from the antigen preparations. When the HA antigens were concentrated, both after Tween-ether treatment and after chromatography, the increase in protein was not accompanied by a similar increase in HA titer. This was most likely caused by aggregation of the HA antigens. Such aggregates would behave like single particles in the HA test.

Following the major purification steps, ie, Tween-ether treatment and column chromatography, there were significant increases in purity as judged by the HA/P ratios. The data also showed that most of the CF activity appeared in the second peak after chromatography. A summary of the rubella antigen preparations produced on this program is given in Table 2-3.

2.2 MUMPS

2.2.1 Mump Propagation

Mumps virus is characterized by being ether sensitive, enveloped, and possessing RNA. It is classified as a paramyxovirus. The Jones strain of mumps was obtained from the American Type Culture Collection and was adapted for growth in Vero cells. The cultural conditions for cell growth were identical to those used for rubella. After cell monolayers had formed, they were washed with Puck's Saline and ten ml of virus seed was allowed to adsorb to the cells for two hours at 37°C. The cells were maintained with Serumless Medium. Maximum CPE was attained after three to four days at which time the remaining attached cells were scraped into the medium. As with rubella virus, the infective fluids were sonicated to release the viruses from the cells. HA titrations were performed using guinea pig erythrocytes by the standardized method of Hierholzer and Suggs (1969). After clarification by low speed centrifugation or later, without clarification, the tissue culture fluids were treated with Tween 80-ether. The conditions for this treatment

TABLE 2-2 PURIFICATION OF RUBELLA ANTIGENS

TREATMENT	HA Titer	CF Titer	Protein µg/ml	HA/P Ratio	CF/P Ratio
Tissue Culture Fluid	32	4	333	0.10	0.01
Tween-Ether	128	16	74	1.73	0.22
Concentrated (22X)	256	- -	1,620	0.15	- - -
A-1.5 m Chromatography					
First Peak	256	2	120	2.13	0.02
Concentrated (20X)	2,048	- -	2,280	0.90	- - -
Second Peak	0	16	60	- -	0.27
Concentrated (32X)		512	2,010	- -	0.26

TABLE 2-3 SUMMARY OF RUBELLA ANTIGEN PREPARATIONS

Antigen Lot Number	HA Titer	Protein (μg/ml)
RuV2	256	600
RuV4	1024	5460
RuV5	256	1620
RuV6-1	2048	2280
RuV6-2*	256	126
RuV6-3*	256	114

*Not concentrated.

were identical to those used for rubella. Early Tween 80 treatment was for five minutes at room temperature and later for 45 minutes at 4°C while mixing. Ether treatment was originally for 15 minutes but in later preparations was 45 minutes at 4°C.

After the Tween 80-ether treatment the fluids were concentrated three to four fold by PEG 20,000 dialysis. It was found that further concentration at this step did not result in a satisfactory increase in HA or CF titer and was probably the result of aggregation of the antigens.

Purification of the mumps antigens was accomplished by chromatography on a BioGel A-1.5 m column. A typical chromatographic run is shown in Figure 2-2. The HA antigens appeared in the first peak. A second, broad peak contained CF antigen with no HA activity. The HA antigen showed a slight amount of CF activity. These results were similar to those obtained with rubella virus.

A different source of CF antigen was obtained from non-Tween 80-ether treated infectious tissue culture fluids. The clarified fluids were centrifuged at 30,000 x g for 2 hours. The supernatant fluid (400 ml) was concentrated 28 times to 14 ml and passed through BioGel A-1.5 m. A sharp peak appeared which contained a large amount of CF activity. Additional CF activity was evident in a large number of the fractions which followed. This chromatographic separation is graphically depicted in Figure 2-3. The fractions containing the CF antigen in the first peak were pooled ($\sim$70 ml) and concentrated to 3 ml (28 times). The concentrate had a protein content of 2.6 mg/ml and a CF titer of 1:1028. The remaining CF fractions were discarded. A summary of the mumps antigen production is given in Table 2-4.

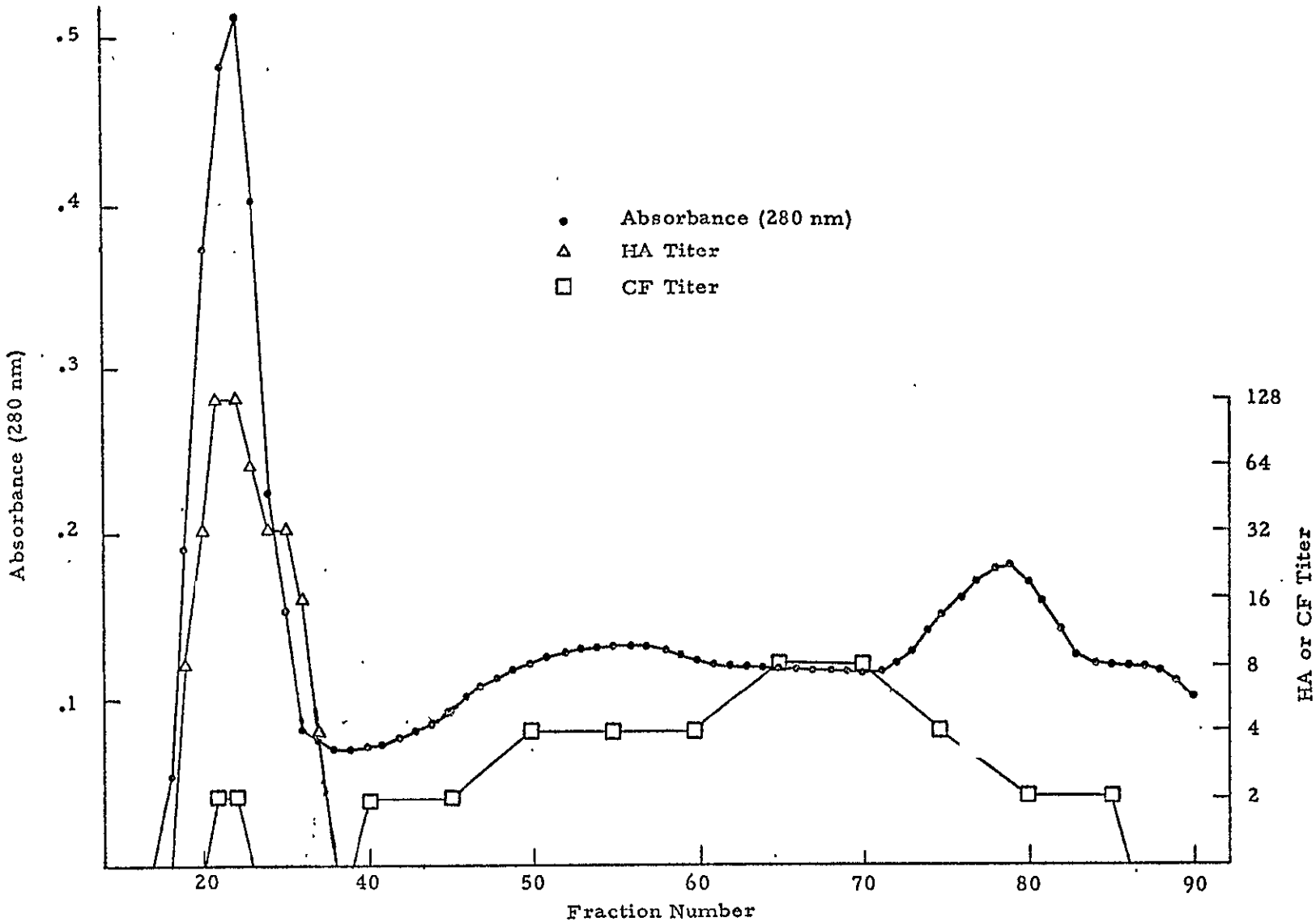

Figure 2-2 Chromatography of Tween 80-Ether Treated Mumps Virus on BioGel A-1.5 m

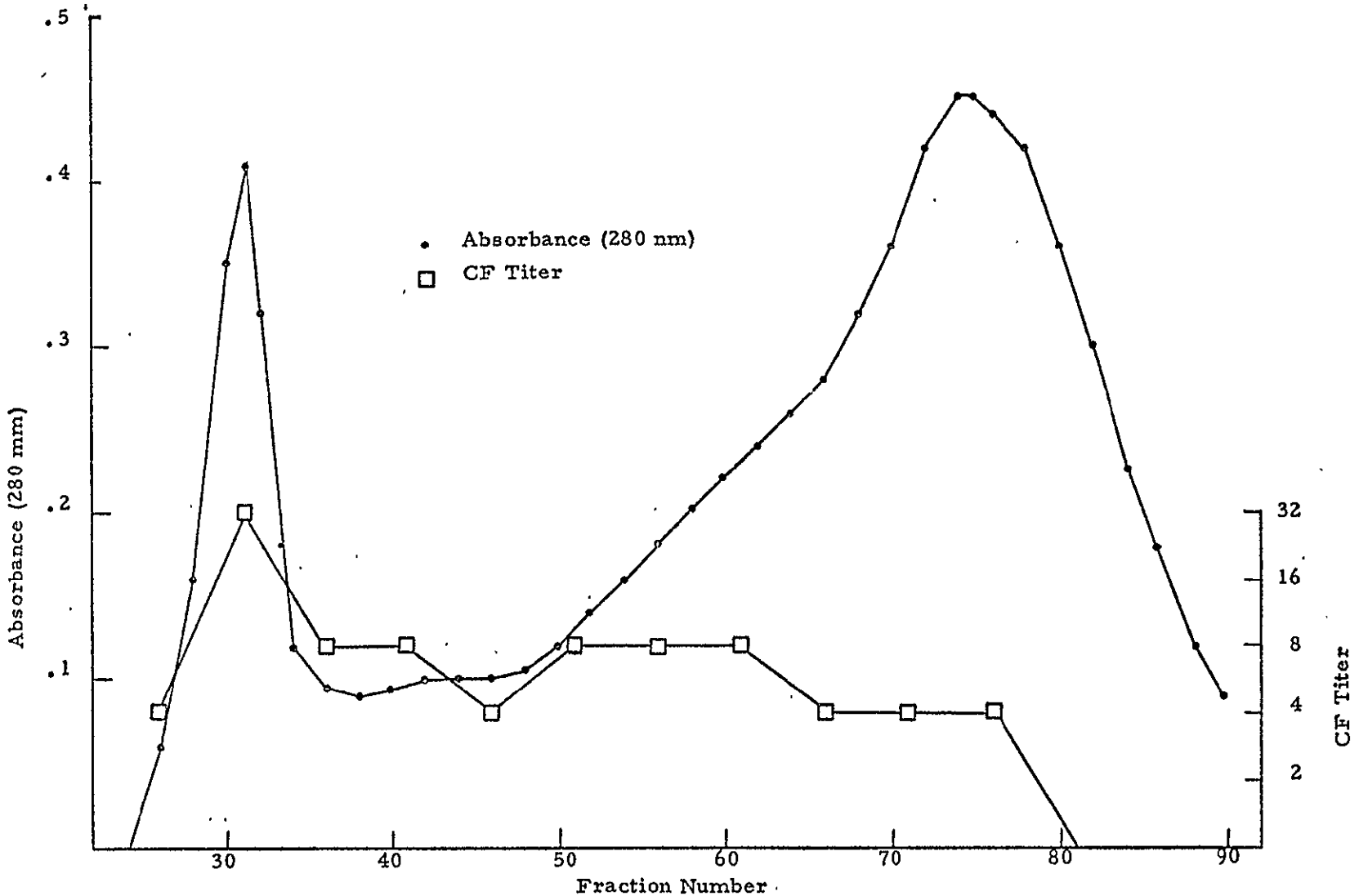

Figure 2-3 Chromatography of Mumps Soluble Antigens on BioGel A-1.5 m

TABLE 2-4 SUMMARY OF MUMPS ANTIGEN PRODUCTION

ANTIGEN PREPARATION	HA TITER	CF TITER	PROTEIN (mg/ml)	VOLUME (ml)
HA Antigen 26 June 1975	1024	- -	10.9	4.0
Soluble CF Antigen 22 July 1975	- -	1028	2.6	2.0
Tween-Ether CF Antigen 27 August 1975	- -	128	- -	12.5

3.0 TASK II - DEVELOPMENT OF A PIA TEST FOR VIRAL ANTIBODIES

The main thrust of this program was to develop direct PIA tests for the determination of rubella and mumps antibodies. In these tests, viral antigens were to be attached to beads which, in turn, would be reacted with dilutions of antisera. If specific antibodies were present, they would react with the antigen-sensitized beads and cause agglutination to occur. Agglutination would be measured by photometric means.

Since rubella and mumps were not available, preliminary tests were performed with beads sensitized with adenovirus hexons, the adenovirus group reactive antigen. Polystyrene beads (1.1 μm) were sensitized by the method described elsewhere (Final Report, Contract NAS 9-13672) and suspended at a 1:25 dilution in glycine buffered saline (GBS) containing 0.2% gelatin. Tests were performed against rabbit anti-adenovirus hexons and rabbit anti-influenza sera produced on the same previous MEMS program. In these tests the antisera were diluted in GBS. To 0.2 ml of antiserum dilution 0.2 ml of beads diluted 1:100 were added. Duplicate tests were incubated for 18 hours at 37°C and for 4 hours at 45°C. After incubation, the beads were diluted to 1:2,000 for reading in the MEMS photometric test bed by the addition of 3.6 ml of GBS. Controls consisted of GBS in place of the serum. The results, as shown in Figure 3-1, indicated a definite response with the adenovirus antiserum while none was evident against the influenza antiserum. If one takes a reference to sample ratio (R/S) of 0.9 as the positive cutoff, the tests incubated at 45°C were positive at a 1:80 dilution while those incubated at 37°C were positive at a 1:320 dilution. The adenovirus antiserum tested had a CF titer of 1:16 against hexons. Similar tests performed at the same time using the IgG fractions of these antisera resulted in no agglutination. However, in later tests, both the adenovirus and influenza IgG preparations gave similar agglutination results as shown in Table 3-1.

When purified rubella HA antigen was available, beads were sensitized and attempts were made to develop a direct PIA test for detection of rubella antibodies.

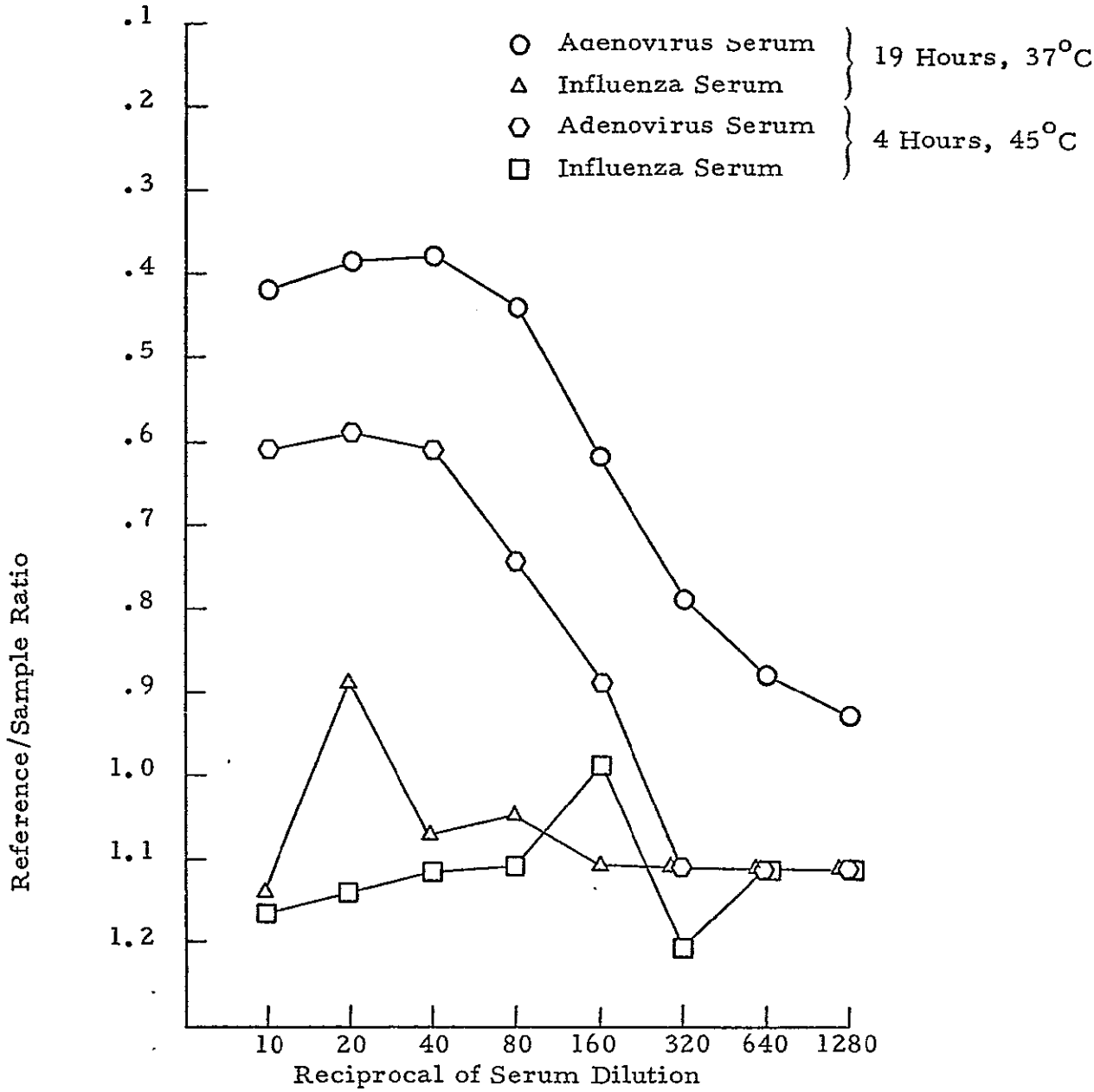

Figure 3-1 Detection of Rabbit Adenovirus Antibodies by Direct PIA

TABLE 3-1 DIRECT PIA FOR ADENOVIRUS ANTIBODIES-TESTING OF IgG PREPARATIONS

Dilution	Reference/Sample Ratio	
	Adenovirus IgG	Influenza IgG
1:2	0.57	0.59
1:4	0.65	0.51
1:8	0.79	1.06
1:16	1.07	1.13
1:32	1.35	1.35
1:64	1.41	1.38

Initial testing was performed with human antisera. The positive serum had an HI titer of 1:64 and the negative <1:8. Unfortunately, all the initial work was invalid since the testing was performed at a less than optimum pH (7.2). However, some good came of this work. A concept of correcting for colorimetric differences in serum samples was devised. This work will be described more fully in Section 6.0. Briefly, this method consisted of reacting dilutions of a given serum sample both with specific antigen coated beads and with beads coated with bovine serum albumin (BSA). By comparing the R/S ratio of the BSA beads vs. the R/S ratio of the specific beads (BSA beads/specific beads) a new ratio was obtained which eliminated interference due to color. Also unfortunately, this procedure was not utilized in later experiments as it may have corrected for nonspecific bead agglutination as well.

After the pH problem had been corrected, testing was performed against human rubella IgG with no success. Although agglutination occurred, there was no logical response. Usually, the ratios obtained remained at one level throughout the entire range of dilutions.

At this point, it was decided to use means other than the HI test to determine if there was an antigen-antibody reaction taking place. The methods chosen were counterelectrophoresis and uptake of ^{125}I-labeled human rubella IgG.

Counterelectrophoresis is a technique which is based upon the ability of a specific antigen-antibody complex to precipitate in agar gel and upon the characteristic mobility of proteins in an electrical field. Under the proper conditions of pH and ionic strength, antibody molecules migrate towards the negative electrode (cathode) when electric current is applied. This is the result of both the above conditions and electroendosmosis. Many antigens, however, migrate toward the positive electrode (anode) under the same conditions. When current is applied, the antigen and antibodies meet and form a precipitate. The procedure is more sensitive then immunodiffusion since the reactants are not diluted while moving towards each other, under the influence of the electric field.

In these tests, agarose (1%) was prepared in Tris-barbital-sodium-barbital, pH 8.8 (HR buffer, 0.05 ionic strength) obtained from Helena Laboratories. The agarose was dissolved in a boiling water bath then filtered through Whatman #1 filter paper. Three ml of the hot, melted agarose were placed on a standard microscope slide and allowed to harden. Wells were then punched in the gel, 2 mm in diameter and 4 mm between centers. Opposite wells were filled with rubella antigen and antibody. The slides were then inverted so the gel made contact with filter paper wick electrodes on a Helena electrophoresis system. Current was applied, 8 mA per slide, for one hour. No precipitin lines were evident at this time. However, when the slides were incubated overnight in a moist chamber, faint precipitin lines formed immediately adjacent to the antibody wells in some of the tests. In tests performed with commercial rabbit anti-rubella serum, lines formed against both the HA antigen and the CF rubella antigen. No lines formed when human anti-rubella serum was used. Lines did form between the HA antigen and concentrated human IgG. These results are shown, diagrammatically, in Figure 3-2.

The other method attempted utilized human rubella IgG labeled with ^{125}I by the method of Hunter and Greenwood (1962) as modified by Hutchinson and Ziegler (1974). The original experiments utilized beads sensitized with rubella HA antigen by absorption. Several bead preparations were tried. To 0.1 ml of 1:50 beads, 0.1 ml of ^{125}I-rubella human IgG containing 100,000 counts per minute (cpm) was added. After an 18 hour incubation at room temperature the beads were centrifuged and washed twice with GBS. The radioactivity was then determined in a well-type gamma counter. The results (Table 3-2) indicated that a large amount of the radioactivity was adsorbed nonspecifically to the control beads which were coated with normal rabbit IgG. Although a wash buffer was available (having been developed on another program) which would diminish the adsorbed cpm, it was not used with the beads sensitized by absorption because it contained the non-ionic detergent Triton N101. It was feared that the Triton would wash the absorbed antigens from the beads.

The use of rubella IgG covalently bound to solid supports allowed for a more rigorous wash and thus removed excess adsorbed radioactivity. Two means of support were tried. In the first, 1.1 μm polystyrene beads

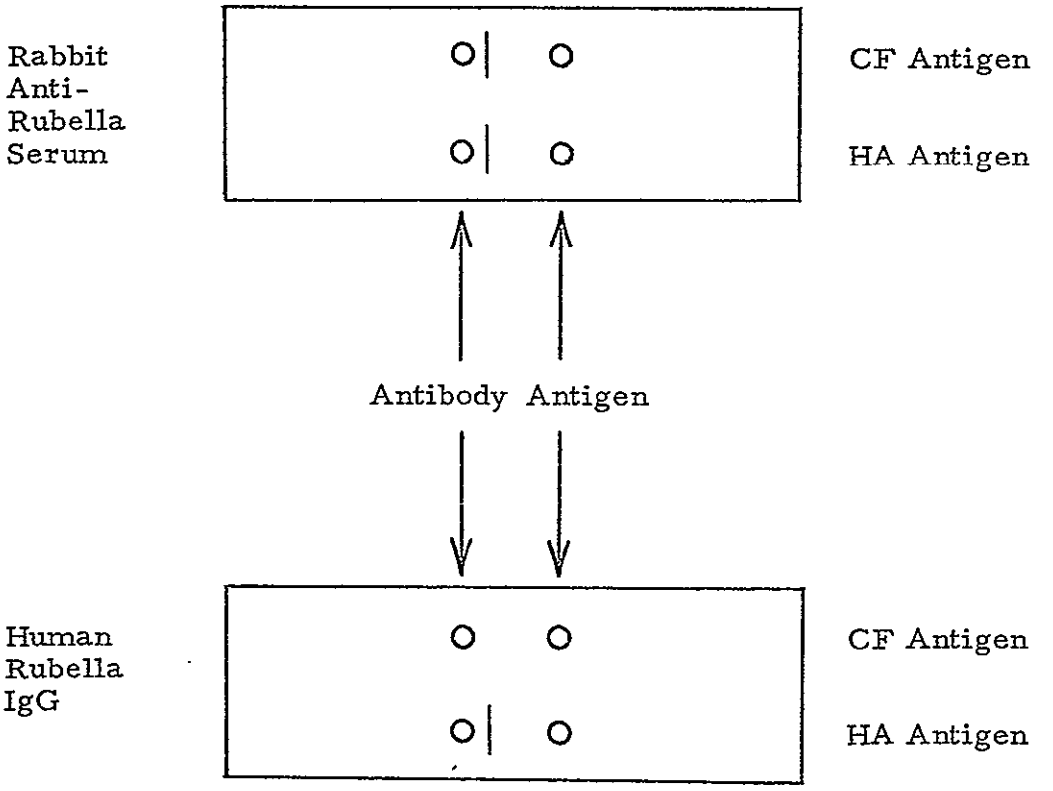

Figure 3-2 Counterelectrophoresis of Rubella Antigens and Antibodies

TABLE 3-2 UPTAKE OF ^{125}I-LABELED RUBELLA HUMAN IgG BY RUBELLA HA ANTIGEN SENSITIZED BEADS

Bead Preparation	Counts/Minute	Ratio of Counts Sample/Control
#1	3970	1.21
#2	4630	1.41
#3	3750	1.14
#4	4330	1.32
#5	3880	1.18
Control Beads	3280	- - -

were nitrated with fuming nitric acid. The nitro groups were then reduced to amines with sodium dithionite. Human rubella IgG was then attached to the beads via the diazonium linkages formed with nitrous acid (Tenoso and Smith, 1972). Rubella HA antigen (HA titer 1:128) was diluted in .1 M ethylene-diamineacetate (EDA), 1 mM $MgCl_2$, 1% NaCl, 1% filtered normal rabbit serum, pH 7.4. This buffer was developed for use in radioimmunoassays (RIA) on another program and is called RIA buffer. The HA antigen dilutions (0.4 ml) were reacted with 0.1 ml of beads overnight at 45°C. After incubation, the beads were washed by centrifugation with RIA wash fluid which consisted of 0.1 M EDA, 1 mM $MgCl_2$ and 0.01% Triton N-101. Two-tenths ml of ^{125}I-IgG containing 100,000 cpm was then added and incubated for 2 hours at 45°C. If the antigen was bound, the labeled IgG attached to the antigen forming a sandwich. The beads were then washed twice with RIA wash buffer and counted in the gamma counter. The results indicated that a specific reaction had taken place (Table 3-3) in that the radioactivity of three dilutions tested (in triplicate) gave a dose response relationship. Only one such experiment was attempted and no attempt was made to optimize the reaction conditions.

Additional testing was performed using antibody sensitized filter paper disks in a sandwich RIA. Human rubella IgG was bound to 5 mm disks of Whatman #4 filter paper activated with CnBr. The method used for binding the IgG to the disks was that of Ceska and Lundkvist (1972). This test was performed by incubating 0.4 ml of test antigen (in RIA buffer) in a test tube which contained an antibody sensitized disk. After a 3-hour incubation at 37°C, the test fluid was withdrawn by aspiration. The disks were then washed twice with RIA wash fluid by adding 2.5 ml to the tube, shaking the tube, then aspirating the liquid. After washing, 0.2 ml of radiolabeled rubella IgG, containing 100,000 cpm, was added and allowed to incubate for 1 hour at 37°C. After this second incubation period, the disks were washed twice and the radioactivity bound was determined. Tests were performed with both the rubella HA and CF antigens. The results (Table 3-4), again, showed a dose response relationship, thus suggesting that a specific reaction had taken place. Other tests performed with dilutions of the bacteriophage F_2 showed no such response (Table 3-5).

TABLE 3-3 BEAD SANDWICH RADIOIMMUNOASSAY OF RUBELLA HA ANTIGEN

HA Antigen Dilution	Average cpm (3 Determinations)	Sample/Control
1:10	2041	2.28
1:20	1474	1.65
1:40	1086	1.21
Control	896	- - -

TABLE 3-4 DISK SANDWICH RADIOIMMUNOASSAY OF RUBELLA HA AND CF ANTIGENS

Antigen Dilution	HA Antigen		CF Antigen	
	Counts/Minute	Ratio of Counts Sample/Control	Counts/Minute	Ratio of Counts Sample/Control
Non-diluted	696	3.25	461	2.15
1:10	351	1.64	353	1.65
1:20	331	1.55	335	1.57
1:40	245	1.14	247	1.15
1:80	246	1.15	221	1.03
1:160	227	1.06	208	0.97
Control	214	- - -	214	- - -

TABLE 3-5 RUBELLA DISK SANDWICH RADIOIMMUNOASSAY OF BACTERIOPHAGE F_2

Antigen Dilution	Counts/Minute	Ratio of Counts Sample/Control
1:10	205	0.98
1:20	211	1.00
1:40	202	0.96
1:80	198	0.94
1:160	222	1.06
Control	210	- - -

With the above suggestions that an antigen-antibody reaction was taking place with the rubella reagents, work was continued in the development of the direct PIA test for rubella antibodies. It was suggested that the beads be pre-coated with BSA prior to sensitization with the rubella antigens. It was felt that specificity might be enhanced by protecting the beads from non-specific proteins which might adsorb to the "open" spaces and cause agglutination. Polystyrene beads were pre-coated with BSA at three levels of concentration, 0.01%, 0.1% and 0.25%. The stock beads were washed once with distilled water and resuspended in GBS to the original concentration. One ml of bead suspension was incubated with 25 ml of each of the BSA solutions for 1 hour at room temperature. The beads were then washed twice with GBS then resuspended in 24 ml GBS. A 1.0 ml amount of rubella HA antigen (5.5 mg protein, HA = 1:1024) was added and allowed to incubate overnight at $4^{\circ}C$ with constant stirring. The beads were then centrifuged and resuspended to 25 ml with GBS + gelatin as before.

At this time, it was found that the MEMS Photometric Test Bed was malfunctioning. The PIA readout was then conducted in a Beckman DB dual-beam spectrophotometer. A wavelength of 475 nm was used. The instrument was adjusted to 100% Transmittance (T) by placing identical control bead preparations in each cuvette. The test beads were then placed in the reference cuvette. The reading obtained on the %T scale of the instrument was the ratio of the amount of light passing through the control beads to that passing through the test beads (R/S). All remaining PIA tests were read on this instrument.

The three bead preparations were tested with rabbit anti-rubella and anti-influenza sera in the PIA test. A final bead reaction dilution of 1:200 was used. These data are shown in Table 3-6 and indicated less non-specific agglutination in the beads pre-coated with 0.10% and 0.25% BSA.

A block titration was performed with these two lots of beads to determine an optimum bead reaction dilution. From these results, shown in Tables 3-7 and 3-8, it was decided that a 1:100 bead reaction concentration was best although at all bead dilutions there were nonspecific reactions. Also, the responses of the influenza serum in the block titrations with both bead lots at a 1:200 bead dilution were quite different from those in the original titrations where there were much less nonspecific responses.

TABLE 3-6 PIA OF BEADS PRE-COATED WITH VARIOUS CONCENTRATIONS OF BOVINE SERUM ALBUMIN (BSA)

Serum Dilution	Reference/Sample Ratio					
	0.01% BSA		0.10% BSA		0.25% BSA	
	Positive	Negative	Positive	Negative	Positive	Negative
1:8	.87	.33	.98	.86	.80	.90
1:16	.22	.67	.37	.98	.35	.95
1:32	.69	.48	1.02	1.10	.90	.89
1:64	.81	.59	1.10	1.02	.79	.99
1:128	1.05	.88	1.10	1.10	.74	.98
1:256	.90	.96	.94	.88	.75	.96
Buffer Control	1.00	1.00	1.00	1.00	1.00	1.00

Positive = Rabbit anti-rubella

Negative = Rabbit anti-influenza

TABLE 3-7 BLOCK PIA TITRATION-RUBELLA BEADS PRE-COATED WITH 0.1% BSA

Serum Dilution	Reference/Sample Ratio					
	1:100*		1:200*		1:400*	
	Positive	Negative	Positive	Negative	Positive	Negative
1:8	.42	.78	.43	.55	.37	.58
1:16	.46	.82	.36	.74	.36	.79
1:32	.47	.95	.35	.75	.35	.83
1:64	.82	1.03	.37	.71	.35	.85
1:128	.82	1.05	.71	.80	.47	.91
1:256	.88	1.09	.96	.91	1.06	.94

* = Final bead reaction dilution

Positive = Rabbit anti-rubella

Negative = Rabbit anti-influenza

TABLE 3-8 BLOCK PIA TITRATION - RUBELLA BEADS PRE-COATED WITH 0.25% BSA

Serum Dilution	Reference/Sample Ratio					
	1:100*		1:200*		1:400*	
	Positive	Negative	Positive	Negative	Positive	Negative
1:8	.54	.85	.39	.59	.50	.75
1:16	.50	.96	.38	.81	.47	.85
1:32	.62	1.00	.39	.85	.45	.92
1:64	.63	.92	.36	.82	.45	.98
1:128	.88	.94	.63	.88	.48	1.01
1:256	.90	1.10	.87	.94	.98	.98

* = Final bead reaction dilution

Positive = Rabbit anti-rubella

Negative = Rabbit anti-influenza

The two bead lots were then tested at a 1:100 dilution against commercial human positive (HI = 1:512) and negative rubella antisera with an overnight incubation at 37°C. These results are shown graphically in Figures 3-3 and 3-4. There was a great deal of agglutination in the negative serum. Noticeable differences, however, were evident with the positive serum with both bead preparations. The differences were greater with the 0.25% BSA pre-coated beads, but there was less agglutination in the negative serum with 0.10%.

Tests were performed with the two bead lots in order to determine the optimum reaction temperature. Rubella positive and negative rabbit antisera diluted 1:8 in GBS were incubated overnight at 4°C, 25°C, 37°C and 45°C. The bead reaction dilution was 1:100. It appeared that both beads lots reacted best with a 45°C incubation as shown in Table 3-9. However, when this temperature was used in a titration of human rubella positive serum, no agglutination occurred so an 18 hour incubation at 37°C was used in further tests of the human positive serum. Only the beads pre-coated with 0.10% BSA were tested further. Similar agglutination patterns were evident in the month these beads were tested with the positive serum. However, one month later, or two months after the beads were sensitized and used in the original tests with human rubella positive and negative sera, much nonspecificity was evident. Table 3-10 shows the data of tests performed at this time. The agglutination response with the negative serum was greater than that with the positive serum.

A new bead preparation, pre-coated with 0.10% BSA was tested against a 1:8 dilution of human rubella positive serum at 4°C, 25°C, 37°C, and 45°C for various times. These results, Table 3-11, again indicated, that a four hour incubation at 45°C should give a good response. However, again, this was found not to be the case when titrations were performed. Results similar to those found with the original beads were obtained with an 18 hour incubation at 37°C.

During the course of all these PIA studies it was realized that the GBS was made incorrectly. The concentrations of both the glycine and the NaCl were found to be twice what they should be, i.e., 0.2 M glycine, 0.1 M NaCl. The pH, however, was correct, 8.2.

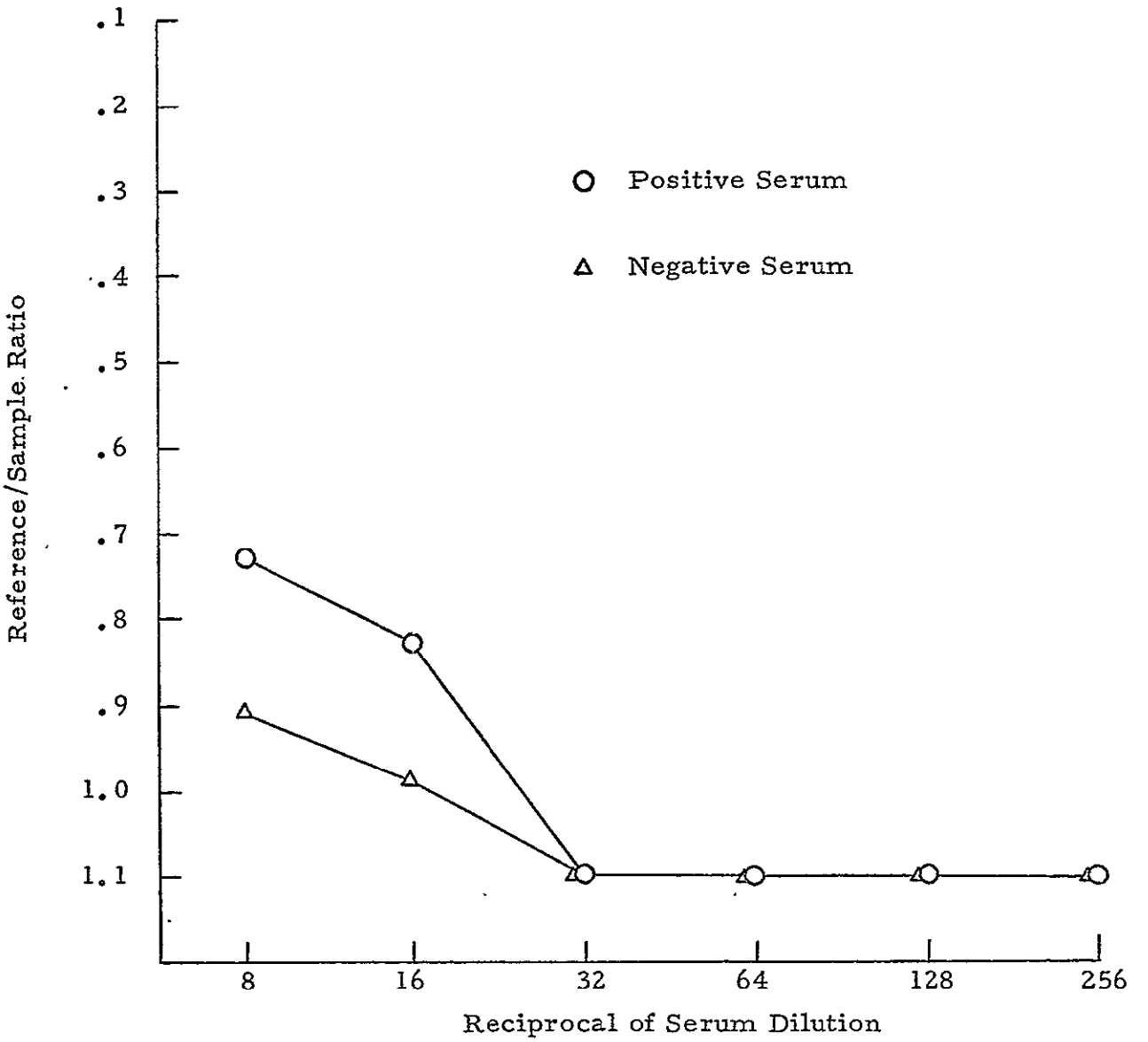

Figure 3-3 Rubella PIA Tests of Human Sera with Beads Pre-Coated with 0.1% BSA

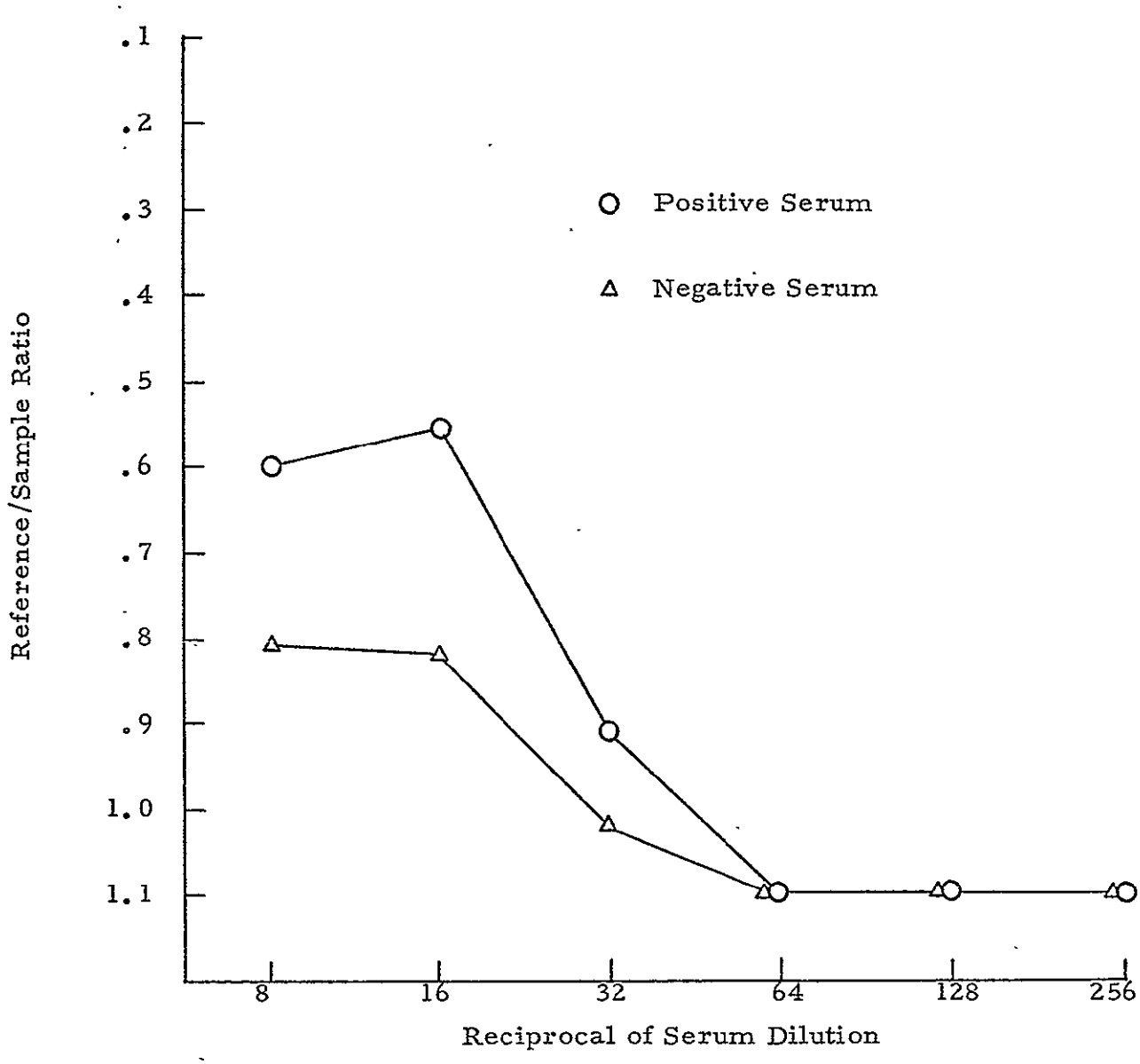

Figure 3-4 Rubella PIA Tests of Human Sera with Beads Pre-Coated with 0.25% BSA

TABLE 3-9 PIA USING BEAD PRE-COATED WITH 0.10% OR 0.25% BSA AT VARIOUS INCUBATION TEMPERATURES

Incubation Temperature	Reference/Sample Ratio			
	0.10% BSA		0.25% BSA	
	Positive	Negative	Positive	Negative
4°C	.43	.94	.51	1.02
25°C	.46	.76	.54	0.81
37°C	.65	1.10	.57	1.04
45°C	.30	.97	.44	1.10

Positive = Rabbit anti-rubella 1:8

Negative = Rabbit anti-influenza 1:8

TABLE 3-10 PIA TEST OF RUBELLA ANTIGEN SENSITIZED BEADS TWO MONTHS AFTER PREPARATION (HUMAN SERA)

Serum Dilution	Reference/Sample Ratio	
	Positive	Negative
10	.80	.64
20	1.04	.75
40	1.04	.91
80	1.02	.95
160	.94	.95
320	.91	.89
640	.82	.86

TABLE 3-11 TIME AND TEMPERATURE STUDY ON THE DIRECT TEST FOR RUBELLA ANTIBODIES

Time (Hours)	Reference/Sample Ratios			
	4°C	25°C	37°C	45°C
0	1.00	1.00	1.00	1.00
1	1.01	.94	.90	.81
2	.95	.89	.77	.67
3	.81	.87	.72	.64
4	.79	.82	.73	.59
5	- - -	.80	.56	.60
6	.77	.60	.56	.51
23	.53	.32	.57	.58

Since the direct tests, so far, were so insensitive (PIA titer = 1:16, HI titer = 1:256) it was felt that the use of the proper buffer concentration i.e., 0.1 M glycine, but with 1% NaCl might improve the test by promoting agglutination. Results of a titration using this buffer is given in Table 3-12. The results showed no specific response. Agglutination, apparently, was a result of diluting the serum since both the positive and negative samples gave similar patterns.

Since the dilution of the serum sample results in a constant decrease in the protein concentration, tests were performed after the addition of a constantly increasing amount of normal rabbit serum. The rabbit serum was first diluted 1:10 in glycine buffered saline (GBS) then filtered through a 0.45 μm membrane filter. Solutions containing 50%, 75%, 87.5% and 93.75% of the 1:10 rabbit serum were prepared in GBS. Equal amounts (0.1 ml) of the proper concentration of rabbit serum were then added to each test serum dilution as outlined in Table 3-13. To the starting dilution of test serum (1:10) an equal volume of GBS was added. To the second (1:20) an equal volume of 50% 1:10 rabbit serum solution was added and so on. At the higher dilutions (1:320 and higher), 100% rabbit serum solution was added since the differences in protein concentration were minimal. The reference control contained 100% rabbit serum solution and GBS. This procedure resulted in the complete obliteration of agglutination and there were no differences between positive and negative sera as shown in Table 3-14. The addition of NaCl in increments up to 2% in all reagents had no effect in promoting agglutination (Table 3-15). All these tests were incubated 16 hours at 37°C. From these results, it appears that the agglutination patterns reported earlier were non-specific since they could be removed by equalizing the protein content of the various dilutions of test sera.

A final attempt to develop a direct PIA test for rubella antibodies also was without success. It was felt that perhaps there was no cross-linking between beads after the initial antigen-antibody reaction had taken place. For this reason, antibodies directed toward the Fc fragment of human IgG was added after the beads had reacted with the test sera. A 1:10 dilution of positive or negative human sera was allowed to react with

TABLE 3-12 RUBELLA DIRECT PIA USING GLYCINE BUFFER WITH 1% NaCl (HUMAN SERA)

Serum Dilution	Reference/Sample Ratio	
	Positive	Negative
10	1.05	.98
20	.98	1.03
40	.97	.90
80	.95	.86
160	.78	.79
320	.77	.75
640	.74	.80

TABLE 3-13 SCHEME FOR THE EQUALIZATION OF PROTEIN CONTENT IN TEST SERUM DILUTIONS

Reciprocal of Test Serum Dilution	Concentration of 1:10 Rabbit Serum Added (%)
10	0
20	50
40	75
80	87.5
160	93.75
320	100
640	100

TABLE 3-14 EFFECT OF EQUALIZING PROTEIN CONTENT ON THE RUBELLA DIRECT PIA (HUMAN SERA)

Serum Dilution	Reference/Sample Ratio			
	Rabbit Serum		No Rabbit Serum	
	Positive	Negative	Positive	Negative
102	.94	.99	1.00	.93
20	1.02	.96	.97	.96
40	.97	.97	.94	.89
80	.91	.90	.87	.81
160	.90	.93	.84	.84
320	.92	.91	.84	.83
640	.94	1.01	.79	.80

TABLE 3-15 EFFECT OF NaCl ON DIRECT RUBELLA PIA (HUMAN SERA)

Serum Dilution	Reference/Sample Ratio					
	1% NaCl		1.5% NaCl		2% NaCl	
	Positive	Negative	Positive	Negative	Positive	Negative
10	1.05	1.04	.99	.90	1.10	.92
20	1.10	1.10	1.03	1.00	1.09	1.10
40	1.10	1.10	1.04	.99	1.04	1.08
80	1.01	1.12	1.08	.88	1.05	1.10
160	1.01	.96	.88	.96	1.04	1.10
320	1.10	.94	.90	.99	1.08	1.05
640	1.06	.92	.88	.97	1.10	1.05

rubella antigen coated beads for one hour at 37°C. At this time, dilutions of anti-human Fc serum were added and allowed to react for 16 hours at 37°C. Dilutions of anti-Fc were tested with hopes of finding an optimum dilution which would result in specific agglutination. A control series was included which contained dilutions of anti-Fc with GBS substituted for test serum. The results (Table 3-16) indicated that no specific agglutination occurred since there were no differences between the positive or negative test sera or the anti-Fc control series. These results tend to indicate that either the primary reaction did not take place, i.e., antibody with rubella antigen, or that the Fc portions of the IgG molecules were not exposed and could not react with the anti-Fc serum.

Because of the problems associated with the direct test for rubella antibodies no work was attempted with mumps.

TABLE 3-16 EFFECT OF ANTI-HUMAN Fc SERUM ON THE DIRECT RUBELLA PIA (HUMAN SERA)

Reciprocal of Anti-Fc Dilution	Reference/Sample Ratio		
	Positive	Negative	Anti-Fc Control
10	.93	.90	.82
50	.93	.88	.94
100	.99	.91	.93
200	1.09	1.04	1.02
400	1.09	1.05	.99
800	1.10	1.09	.94

4.0 TASK III BEAD SENSITIZATION STUDIES

Experiments were performed to determine better methods of sensitizing beads for the PIA test. These experiments included attempts to increase the quality of beads sensitized by absorption and to investigate some methods of chemically bonding rubella HA antigen to the latex beads.

Initial experiments were directed toward the determination of the best procedure to wash stock 1.1 μm beads. During manufacture, a certain amount of detergent adsorbs onto the surface of the latex beads. It was felt that to get the best protein absorption, the detergent should be removed. Six 0.5 ml aliquots of stock latex beads were washed by centrifugation at 12,000 x g for 5 minutes. Two aliquots were washed with 0.2 N HCl, two with reagent alcohol and two with distilled water. Washing with the same solutions was repeated except for those washed with alcohol which were washed with 0.2 N HCl. Alcohol-treated beads showed bead destruction after this wash and were discarded. The remaining beads were then washed twice with water. The bead aliquots washed with HCl and water were resuspended in 5.0 ml GBS containing 0.75 or 1.0% NaCl. To each aliquot was added 0.5 ml of rubella HA antigen containing 810 μg protein (1.62 mg/ml stock beads). Absorption of the antigen to the beads took place at 4°C for 18 hours while stirring slowly with a magnetic stirrer. At this time 7.5 ml of GBS containing the proper concentration of NaCl was added. The beads were then pelleted in the centrifuge, the supernatant fluids saved for protein determinations, and the beads resuspended to 12.5 ml with the proper GBS. The protein concentration of the supernatant fluids was determined and the amount adsorbed to the beads calculated. This data is given in Table 4-1 and indicates no advantage of either wash method or NaCl concentration as to protein uptake by the beads since all were about 10 μg/mg of beads.

Beads were sensitized later using the HCl wash, but with 5.46 mg HA antigen protein per ml of stock beads. These beads adsorbed 27 μg protein per mg of beads. By increasing the protein added, more than twice the amount attached to the beads.

Experiments were also performed on methods of chemically bonding rubella HA antigen to beads.

TABLE 4-1 EFFECT OF HCl WASH AND NaCl CONCENTRATION ON THE ABSORPTION OF RUBELLA HA ANTIGEN TO LATEX BEADS

Beads	0.2 N HCl Wash	% NaCl in GBS	Protein (μg/mg Beads)
1	Yes	0.75	10.5
2	Yes	1.0	9.9
3	No	0.75	10.2
4	No	1.0	10.2

Although a consultant from Dow Chemical claimed cyanogen bromide (CNBr) attachment of proteins to polystyrene beads was doubtful, it was felt that it might be possible. The CNBr technique utilizes free hydroxyl groups for covalently binding to the amino groups of proteins. According to Dow literature, some hydroxyl groups remained on the surface of the beads, thus providing possible sites for CNBr activation and consequently covalent attachment of the proteins to the beads. When the CNBr activated beads were produced, an aliquot was reacted with ^{125}I-labeled IgG to determine the amount of protein incorporated on the beads. The protein attached was less than the absorbed beads. Some of the remaining CNBr activated beads were coated with rubella HA antigen and tested for ^{125}I-labeled human rubella IgG uptake. They failed to yield favorable results. The remaining beads were then coated with unlabeled rubella IgG and reacted with HA antigens. Radiolabeled rubella IgG was then added to test for uptake. These, too, failed to show significant uptake.

Diazotized latex beads were also prepared both with rubella IgG and with HA antigen. The IgG-coated beads were used in a sandwich RIA (Section 3.0) and showed some uptake of radiolabeled rubella IgG. The beads, coated with HA antigen by diazotization, did not take up ^{125}I-rubella IgG.

Polystyrene beads with bound surface carboxyl groups were obtained from Dow Chemical. The beads were 0.8 μm in diameter. These carboxylate modified particles have surfaces whose absorption characteristics are different from polystyrene latex surfaces. The monolayer of carboxyl groups permit covalent bonding of antigens, antibodies, enzymes or other biochemicals to the surface of latex particles. Consequently, this latex should permit the formation of amide bonds between the amino groups on the biochemical species and the carboxylic acid groups on the latex particle. Accordingly, a catalyst for the covalent binding of the antigen was needed. Carbodiimide was utilized in binding rubella HA antigen to the carboxylate beads. This compound has been used extensively in protein chemistry to form peptide chains under mild conditions (pH 4.8, 25°C), and has been shown to be very effective in forming amide bonds in gels for affinity chromatography.

One milliliter of carboxylate beads were washed once with distilled H_2O and resuspended in 20 ml of H_2O adjusted to a pH of 4.8. A 0.5 gm of carbodiimide was dissolved in 3.0 ml of H_2O and maintained at 4°C. One milliliter of HA antigen (5.46 mg/ml, pH 4.8) was added to the carboxylate bead suspension and stirred with a magnetic stirrer at 4°C. The chilled carbodiimide solution was added slowly, dropwise to the antigen and carboxylate bead solution. The pH was maintained at 4.8 with 0.2 N HCl. After one hour, the pH had ceased its trend towards basicity and the solution was stirred overnight (18 hours) at 4°C.

The beads were then centrifuged and the supernatant was discarded. Protein determination was impossible because of the carbodiimide present. The bead pellet was resuspended in glycine buffered saline (GBS) with 0.2% gelatin.

The sensitized carboxylate beads were reacted with ^{125}I-rubella IgG. There was no specific uptake observed. PIA titrations were performed against rabbit anti-rubella and rabbit anti-influenza. The results (Table 4-2) showed no differences between the two antisera.

The best results were obtained with beads precoated with BSA. This work was described in Section 3.0.

TABLE 4-2 PIA USING RUBELLA HA ANTIGEN-SENSITIZED CARBOXYLATE BEADS

Reciprocal of Serum Dilution	Reference/Sample Ratio	
	+	-
8	0.46	0.49
16	0.55	0.58
32	0.61	0.78
64	0.92	0.97
128	0.99	1.10
256	1.00	1.05

+ = Rabbit anti-rubella serum

- = Rabbit anti-influenza serum

5.0 TASK IV ALTERNATE PIA DETECTION METHODS

The direct PIA is susceptible to the formation of prozones whenever the sought for agent, antigen or antibody, is in excess. This is brought about by the beads being saturated and unable to form bead-to-bead connections. Negative results are possible, therefore, in samples which should be highly reactive.

The use of an indirect test would eliminate the prozone problem. In this test, which may be called a PIA inhibition test, the test antigen would first be titrated with antibody-sensitized beads. A quantity of antigen which which gave a proper PIA response in that titration would be incubated with dilutions of patient's serum. Antibody sensitized beads would be added to each serum dilution and incubated. If the serum contained antibodies, agglutination would be inhibited. When the dilution of the serum was high enough that excess antigen was present, the beads should agglutinate. The PIA inhibition titer would be the highest dilution of serum that prevented agglutination of the beads.

Rabbits were immunized for the purpose of providing antisera to rubella and mumps. The immunoglobulins were to be used for sensitizing beads for the PIA inhibition test. Rubella and mumps antisera were produced in a similar manner. Intramuscular injections were first given with purified antigens emulsified with Freund's Complete Adjuvant (FCA). Two weeks later similar injections were given. After an additional two weeks, intraperitoneal injections of purified antigen without FCA were given. Seven days later, the rabbits were exsanguinated by cardiac puncture, the sera separated and titered. The data for the antisera produced is given in Table 5-1.

The immunoglobulins were isolated from a portion of the serum obtained from rubella rabbit number one by ammonium sulfate precipitation. The IgG fraction was then purified by ion-exchange chromatography on DEAE Sephadex. This IgG was used to sensitize 1.1 μm latex beads by the usual method, i.e., with no pre-coating.

Initial indirect PIA tests were performed with a rubella positive human serum (HI = 1:256). The test consisted of incubating 0.1 ml of serum

TABLE 5-1 RESULTS OF FINAL BLEEDINGS OF RABBITS IMMUNIZED WITH RUBELLA OR MUMPS ANTIGENS

Rubella:

Rabbit No.	CF Titer	HI Titer
1	160	160
2	160	160
3	640	320
4	160	80
5	160	80
6	N.D.	160

Mumps:

Rabbit No.	CF Titer
1	32
2	32
3	256
4	128
5	256
6	32

dilutions with 0.1 ml of HA antigen at a dilution which gave a good PIA response. The antigen used was diluted 1:64 which gave a 0.81 reference to sample radio in an earlier PIA titration. After a 30-minute incubation, 0.2 ml of 1:100 beads were added and the tubes were incubated further to allow for agglutination. Table 5-2 shows data obtained under different time and temperature conditions for this second incubation. The first incubation, i.e., with the serum dilutions and HA antigen was for 30 minutes at the temperature indicated. The indirect PIA titer was determined as the highest serum dilution which displayed a reference to sample ratio higher than the HA antigen control. It appeared that incubating at 45°C gave the best results.

Also during this period other bead reaction concentrations were tried, i.e., 1:1000 and 1:2000. These tests, however, showed no differences between the serum dilutions and the HA antigen controls.

In additional tests, a titration was performed with a 5-hour incubation at 45°C against purified rubella HA antigen having a titer of 1:1024. The data for this titration is given in Table 5-3 and is graphically demonstrated in Figure 5-1. This test indicated detection at a dilution of 1:1600 or about 1.5 times more dilute than the HA titer (1:1024). Using these data, a PIA inhibition test was performed. Equal volumes (0.1 ml) of rubella human serum dilutions and three dilutions of HA antigen (1:800, 1:1600, and 1:3200) were reacted for one hour at 45°C. Anti-rubella beads were then added (0.2 ml diluted 1:100) to give a bead reaction dilution of 1:200. After a 5-hour incubation at 45°C, 3.6 ml of GBS was added for reading in the photometer. The results are given in Table 5-4. Oddly, all three showed similar responses It appeared that about 1:160 was the titer in all three tests. One would expect that the higher the dilution of HA antigen, the more sensitive the test would be. Comparative tests with more concentrated levels of HA antigen gave similar results. The amount of antigen in the first incubation had no effect on the outcome of the test. Additionally, tests performed against the positive human serum without HA antigen or with a negative human serum with HA antigen gave results identical to those previously mentioned.

At this time the HA antigen was being stored at 4°C. It was thought that, perhaps, this storage condition was unsuitable so 0.1 ml aliquots from the original HA antigen were prepared and stored frozen at -80°C. A new.

TABLE 5-2 RUBELLA INDIRECT PIA WITH DIFFERENT CONDITIONS FOR BEAD INCUBATION

Reciprocal of Serum Dilution	Reference/Sample Ratio		
	16 hrs. 37°C	5 hrs. 37°C	5 hrs. 45°C
4	.31	--	--
8	.65	--	--
16	1.02	--	--
32	1.10	.92	1.10
64	1.09	.97	1.10
128	.94	.77	1.00
256	.85	.95	.78
512	.73	.75	.78
1024	--	.82	.62
2048	--	.79	.62
4096	--	.84	.59
Ag Control	.69	.78	.61

TABLE 5-3 RUBELLA HA ANTIGEN PIA TITRATION

Reciprocal of HA Antigen Dilution	Reference/Sample Ratio
100	0.93
200	0.69
400	0.58
800	0.56
1,600	0.75
3,200	0.90
6,400	0.89
12,800	0.97
25,600	1.01

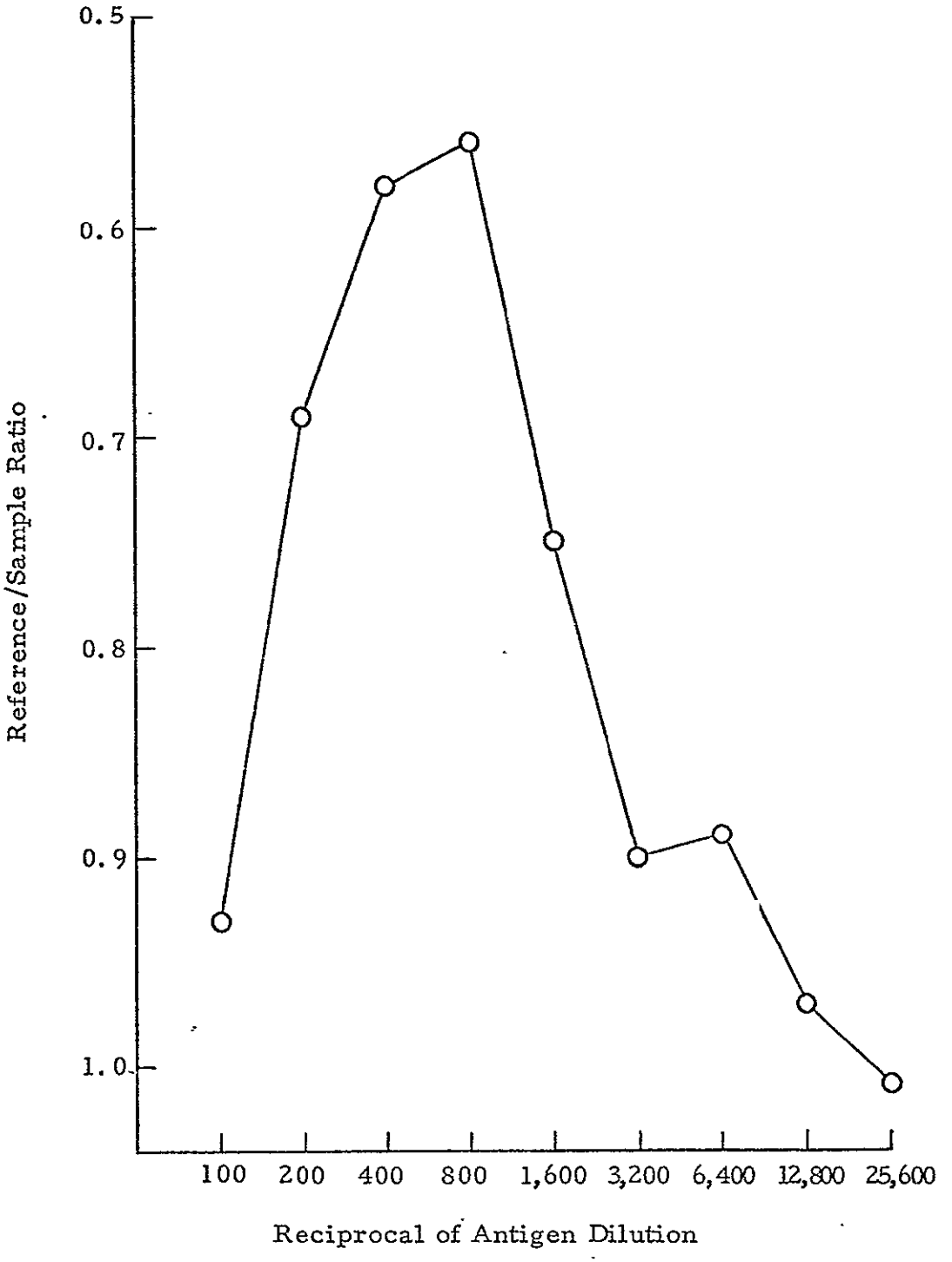

Figure 5-1. Rubella HA Antigen PIA Titration

TABLE 5-4 RUBELLA PIA INHIBITION TEST WITH VARIOUS DILUTIONS OF HA ANTIGEN

Reciprocal of Serum Dilution	Reference/Sample Ratio HA Antigen Dilution		
	1:800	1:1600	1:3200
10	1.07	1.03	1.08
20	1.04	1.02	1.00
40	1.02	0.99	1.02
80	0.89	0.89	0.84
160	0.82	0.82	0.79
320	0.76	0.71	0.73
640	0.81	0.75	0.79
1,280	0.78	0.78	0.73
2,560	0.75	0.80	0.81
Antigen Control	0.72	0.79	0.77

aliquot was retrieved from the freezer, thawed and diluted for each test from then on. This provided no benefit, however.

The above results were obtained with beads sensitized with IgG obtained from commercial rabbit anti-rubella serum. When the sera produced on the present program were available, beads sensitized with this IgG were used in hope that the homologous antigen-antibody system would work better.

Antigen titrations were performed with these beads with both an overnight incubation at 37°C and a 5-hour incubation at 45°C. As a comparison, the antigen was titered under the same conditions using beads sensitized with adenovirus IgG produced on an earlier program. The results (Table 5-5 and Figure 5-2) showed no real differences and further indicated that both parts of the indirect test, both the antigen titration and the response when tested against serum dilutions, were nonspecific.

In one last futile attempt, both titrations were performed using the rabbit serum dilution scheme outlined in Section 3.0 to provide for a constant level of protein. Agglutination was eliminated in all cases.

TABLE 5-5 RUBELLA HA ANTIGEN PIA TITRATION WITH RUBELLA AND ADENOVIRUS ANTIBODY-COATED BEADS

Reciprocal of HA Antigen Dilution	Reference/Sample Ratio			
	Rubella Beads		Adenovirus Beads	
	37°C	45°C	37°C	45°C
100	0.69	0.78	0.79	0.90
200	0.64	0.73	0.72	0.74
400	0.67	0.72	0.77	0.82
800	0.90	0.85	0.84	0.75
1,600	1.01	0.95	0.94	0.90
3,200	1.00	0.91	1.03	0.90

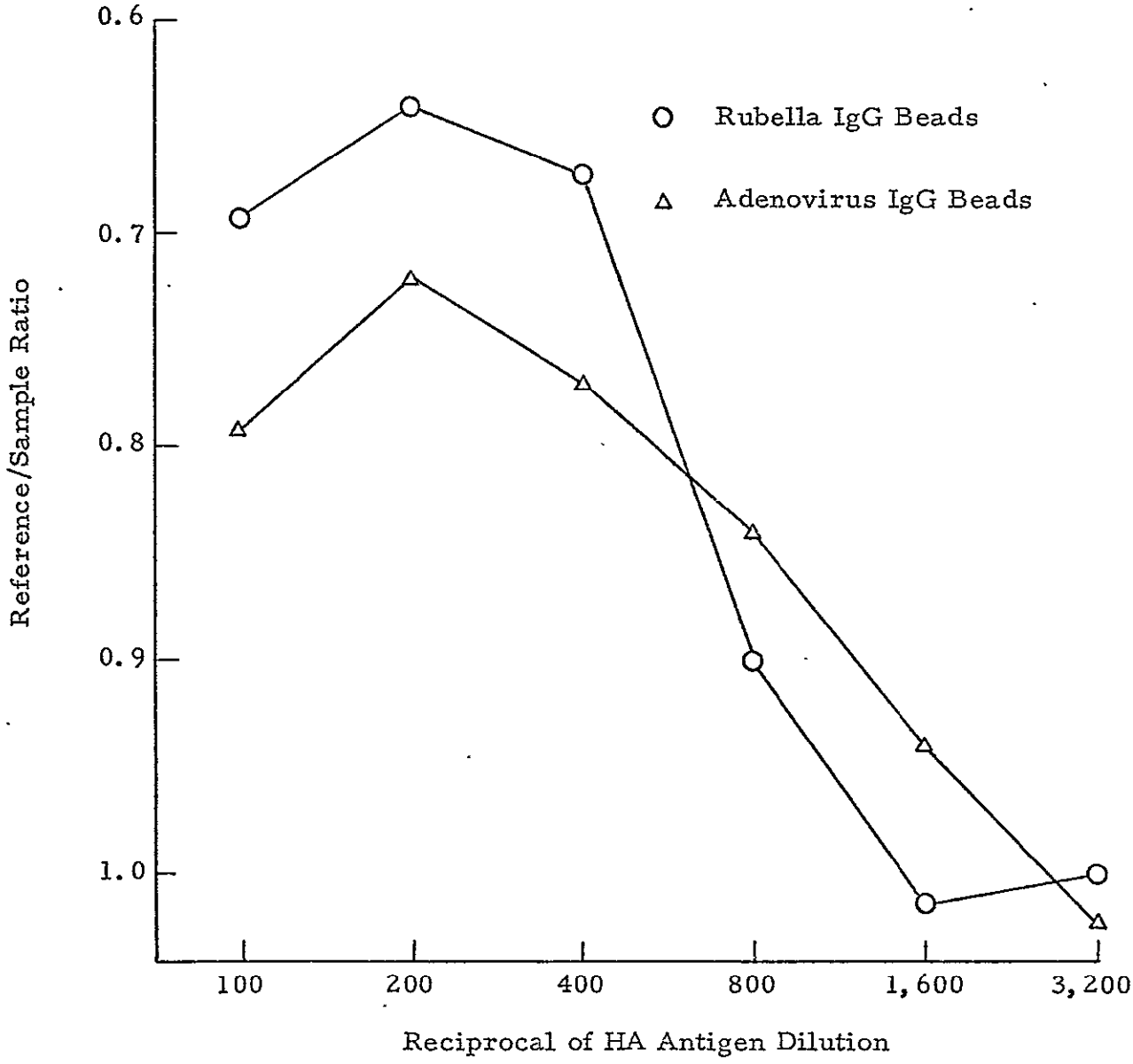

Figure 5-2. Rubella HA Antigen PIA Titration with Rubella and Adenovirus Antibody-Coated Beads -- Overnight Incubation, 37°C

6.0 TASK V EFFECTS OF HUMAN SERUM ON THE PIA TEST

Initial PIA tests utilized adenovirus hexon-coated beads and rabbit anti-hexon and rabbit anti-influenza sera. In these tests, there was little agglutination caused by the hexon negative (influenza) serum while a good response was evident with the positive serum. These results have been presented, graphically, in Section 3.0 (Figure 3-1). The reference to sample ratios of the titration which was incubated overnight at 37°C are presented here in Table 6-1. These results were quite encouraging, however, when beads were sensitized with rubella HA antigen, the same influenza antiserum (i.e., rubella negative) gave nonspecific agglutination throughout the entire program (e.g., Tables 3-6, 3-7, 3-8). It appears, from a comparison of the hexon and rubella systems, that something inherent in the rubella HA antigen coated beads resulted in the nonspecific response with the rabbit influenza antiserum. Nonspecific responses were also evident when human rubella negative sera were tested (e.g., Figures 3-3, 3-4; Table 3-10). Unfortunately, the adenovirus antibody direct PIA test was never performed with human sera so no comparison could be made. However, if the results with the rabbit anti-hexon sera are valid, similar results might be expected with human negative sera.

As noted in Section 5.0, the rubella HA antigen nonspecifically agglutinated adenovirus antibody-coated beads. Perhaps, under certain conditions of protein content and ionic strength the rubella antigens attached to the beads also form aggregates.

It appears that nonspecific serum reactions took two forms depending upon the composition of the GBS used. In the earlier experiments wherein 0.2 M glycine and 0.1 M NaCl were used, nonspecific agglutination appeared at lower serum dilutions. However, when the buffer was changed to 0.1 M glycine and 1% (0.17 M) NaCl, the lower serum dilutions produced no nonspecific agglutination but as the serum was diluted, an increasing amount of agglutination occurred. A comparison between the two buffers is given in Table 6-2. The nonspecific response with the second buffer could be eliminated by providing for approximately equal protein concentrations in each dilution by the addition of increasing amounts of normal rabbit serum as was previously shown in Table 3-13. The agglutination response of the positive serum, however, was also eliminated in these tests (Table 3-14). Providing for a constant protein

TABLE 6-1 DETECTION OF ADENOVIRUS ANTIBODIES BY DIRECT PIA - REFERENCE TO SAMPLE RATIOS

Reciprocal of Serum Dilution	Reference/Sample Ratio	
	Hexon Serum	Influenza Serum
10	0.43	1.14
20	0.40	0.90
40	0.38	1.07
80	0.44	1.05
160	0.58	1.11
320	0.80	1.08
640	0.88	1.11
1,280	1.03	1.15

TABLE 6-2 RUBELLA DIRECT PIA TESTS OF A HUMAN NEGATIVE SERUM USING BUFFERS OF DIFFERENT COMPOSITION

Reciprocal of Serum Dilution	Reference/Sample Ratio	
	0.2 M glycine 0.1 M NaCl	0.1 M glycine 1% NaCl
10	0.64	0.93
20	0.75	0.96
40	0.91	0.89
80	0.95	0.81
160	0.95	0.84
320	0.89	0.83
640	0.86	0.80

concentration in the serum dilutions would appear to be beneficial by eliminating the nonspecific response in negative serum samples. Unfortunately, in the tests performed, the response from the positive serum was nonspecific as well.

In addition to the effect of protein on PIA testing of serum samples, interference due to color intensity was also evident. Variations in absorbance at 475 nm of six different human serum samples, diluted 1:10, is shown in Table 6-3. This variation was the result of different amounts of hemoglobin in the samples. A method of correcting for color variations was conceived on this program. It involved the use of beads coated with a nonspecific protein, e.g., BSA. In practice, aliquots of every serum dilution would be reacted both with specific antigen coated beads and with BSA-coated beads having the same concentration. The sample aliquot reacted with the BSA beads would serve as the reference control and thus correct for color differences. Although encouraging in theory, in actual use the BSA-coated beads displayed much nonspecific agglutination when compared with a buffer reference control. The same serum reacted with rubella antigen coated beads showed no agglutination. The results with a negative serum are given in Table 6-4. The results with a positive serum were almost identical. If this method were to be used, the beads in the reference control must not nonspecifically agglutinate in the serum tested. This method was not pursued since, at the time, there was no specific reaction with positive sera. In retrospect, this concept should have been developed further using the adenovirus system.

A serum control system used in the indirect test consisted of incubating the rubella antibody-coated beads with duplicate sets of serum dilutions. One set was first incubated with the rubella HA antigen and the second set was incubated with buffer in place of the antigen. The use of this control corrected for nonspecific reactions by using the same beads and the same serum dilutions as in the positive test. An example of the use of this control is given in Table 6-5. The use of this control was the first indication that the indirect rubella antibody test was nonspecific. If the test was specific, the results of the tests not incubated with HA antigen should have been negative at all dilutions, i.e., reference/sample ratios about 1.0. The ratios obtained between the tests incubated with HA antigen to those without would be an indication of the specific agglutination.

TABLE 6-3 ABSORBANCES AT 475 nm OF VARIOUS HUMAN SERUM SAMPLES

Serum Number	Absorbance (475 nm) 1:10 Dilution
1	0.44
2	0.54
3	0.35
4	0.24
5	0.52
6	0.36

TABLE 6-4 COMPARISON OF BSA-COATED BEADS AND RUBELLA ANTIGEN-COATED BEADS IN THE PIA TEST

Reciprocal of Serum Dilution	Reference/Sample Ratio	
	BSA Beads	Rubella Ag Beads
10	0.75	1.30
20	0.69	1.33
40	0.69	1.26
80	0.84	1.44
160	1.13	1.41
320	1.14	1.30
640	1.12	1.29
1,280	1.20	1.09

TABLE 6-5 RUBELLA INDIRECT PIA - TESTS PERFORMED WITH AND WITHOUT INCUBATION WITH RUBELLA HA ANTIGEN

Reciprocal of Serum Dilution	Reference/Sample Ratio		Ratio of Results
	HA Antigen	No HA Antigen	HA Antigen / No HA Antigen
10	1.07	1.09	0.98
20	1.04	1.10	0.95
40	1.02	1.04	0.98
80	0.89	0.97	0.92
160	0.82	0.81	1.01
320	0.76	0.73	1.04
640	0.81	0.70	1.16
Antigen Control	0.72		--

The method of correcting for dilution of the protein content serum was described in Section 5.0. This method involved adding increasing concentrations of normal rabbit serum solution to the tubes containing increasing dilutions of test serum. Again, this resulted in the elimination of agglutination of both positive and negative serum samples.

7.0 DISCUSSION, CONCLUSIONS, AND RECOMMENDATIONS

The objective of this program was to develop a simplified method for measuring viral antibodies using the PIA approach. Hopefully, such a test would not require tedious and time consuming manipulations such as serum treatment and titration of reagents. Two methods of approach were investigated with rubella virus. The first, called the direct PIA, used latex beads coated with virus. These beads should agglutinate in the presence of specific antibodies. In the second approach, called the indirect PIA, rubella antigen was first reacted with serum. If antibodies were present, they would bind the antigen. Then when rubella antibody-coated beads were added, agglutination would be inhibited. This method would eliminate the negative response caused by prozone formation when the antibodies were in high concentration.

The detection, by PIA, of rubella antibodies in human serum using either the direct or indirect approach was without success. Although somewhat favorable results were obtained in the direct test when the antigen-coated beads were pre-coated with BSA, the test was insensitive and there was excessive agglutination in the negative serum. In addition, agglutination in both positive and negative samples was eliminated by equalizing the protein content in the serum dilutions.

In the indirect test, rubella IgG, obtained both commercially and on the present program, was attached to the beads. Testing of these beads with dilutions of rubella HA antigen exhibited a typical agglutination-type response. There was the ever present prozone in the area of antigen excess, followed by a good increase in agglutination which then dropped to the level of the control. This same response was obtained, however, with adenovirus IgG-coated beads. The curves were quite similar with the rubella IgG-coated beads giving a slightly greater positive response.

The indirect test was performed by incubating dilutions of the positive and negative human sera with an equal volume of the dilution of HA antigen which gave a maximum response in the HA antigen PIA titration. Again, the responses with both negative and positive sera were identical indicating total nonspecificity.

Conventional testing of the rubella antigens by HA, HI and CF showed them to be immunologically reactive. However, little specific response was

obtained when they were attached to latex beads. This could have been the result of several factors. For example, the interference of the serum may have overwhelmed the specific response. Secondly, the methods used to attach the antigens to the beads may have rendered them nonreactive. An indication of this was the lack of a specific response when purified rubella IgG was tested.

A major problem with the indirect test appears to have been caused by the nonspecific agglutination of beads by the rubella HA antigen. This problem is similar to that found with the parainfluenza viruses on a contract supported by the National Institute of General Medical Sciences. These viruses are also "enveloped" and are somewhat similar to rubella. The PIA test was nonspecific in that equivalent results were obtained no matter what beads were used. In addition, the development of a RIA method for the detection of the parainfluenza viruses was hindered by the nonspecific adsorption of parainfluenza antigens to heterologous IgG coated beads and to the walls of the plastic test tubes. This RIA method was a competitive type assay. Only when a sandwich-type RIA was developed did favorable results occur. In these tests, specific IgG was radiolabeled. Nonspecific attachment of the virus to plastic surfaces still occurred but as long as the homologous labeled IgG was used, specificity was maintained. Heterologous labeled IgG did not react with nonspecificially absorbed parainfluenza viruses. It was possible to separate adenovirus, parainfluenza type 2 and parainfluenza type 4 by this method. The two parainfluenza viruses did not cross-react. Equal specificities and sensitivities were obtained for all three viruses whether tested under controlled buffer conditions or in simulated throat wash samples.

Many factors are involved in the specific agglutination of beads in the PIA test. These factors, ionic strength, pH, protein content, etc., may vary greatly between sample types, and different samples of the same type. This presents a serious problem even in the PIA detection systems which work well; e.g., adenovirus. A PIA detection system has been successfully developed for NASA to monitor reclaimed water for the potential presence of viruses. In this system, a marker virus, bacteriophage F_2, will be added upstream from the purification system. Its presence after purification would indicate a system malfunction. The PIA method used in this instance has two important factors in its favor, namely, the F_2 phage-antibody system appears to be a good one

This work has been described fully in the Water System Virus Detection Final Report (Contract No. NAS 9-14002).

A brief investigation was performed to determine the feasibility of the EIA to detect adenovirus hexons. Adenovirus group IgG was labeled with horseradish peroxidase (HRPO) by the method of Nakane and Kawaoi (1974). The test consisted of incubating 0.5 ml of sample for 2 hours at 37°C with beads to which adenovirus IgG was chemically bound. The samples tested were dilutions of hexons in buffer, various adenovirus types, several adenovirus negative throat washes, rubella HA antigen, parainfluenza type 4 HA antigen, influenza HA antigen, human serum, and undiluted HeLa cell extract. After sample incubation, the beads were washed once with RIA wash fluid by centrifugation. The HRPO-labeled adenovirus IgG (0.1 ml) was then added and allowed to incubate one hour at 37°C. The excess enzyme conjugate was removed by two washes with RIA wash fluid. Three ml of substrate (H_2O_2) containing ABTS color indicator (2,2'-azino-diethylbenzthiaoline-sulfonic acid) was then added. Color development was allowed to take place for 1 hour at 37°C. The color is the result of the coupled enzymatic decomposition of H_2O_2 and oxidation of substrate which is proportional to the amount of labeled antibody on the beads.

After color development the results were read in a dual beam spectrophotometer at 425 nm. The instrument was adjusted to 0.0 absorbance with buffer controls which were colorless. The absorbance of each sample was then determined. The response of hexons diluted in buffer is presented graphically in Figure 7-1. This hexon preparation which had a CF titer of 1:160, was detectable at a 1:12,800 dilution by RIA and only 1:1280 by PIA. In the EIA, the hexons diluted 1:64,000 still showed an absorbance of 0.1. Conservatively, the EIA was at least as sensitive as the RIA. Table 7-1 shows the results of the other samples tested. Only one of the samples shows a significant non-specific response in the test, throat wash #1. All of the adenoviruses gave a strong positive response. An additional test was performed (not shown) in which a chicken chorioallantoic membrane extract was tested. This sample contained much particulate debris, red blood cells and tissue cells, which pelleted during centrifugation. This material caused a strong color change even in the absence of the enzyme. This was caused by endogenous peroxidases present in the cells and demonstrated that the wash method used, i.e.

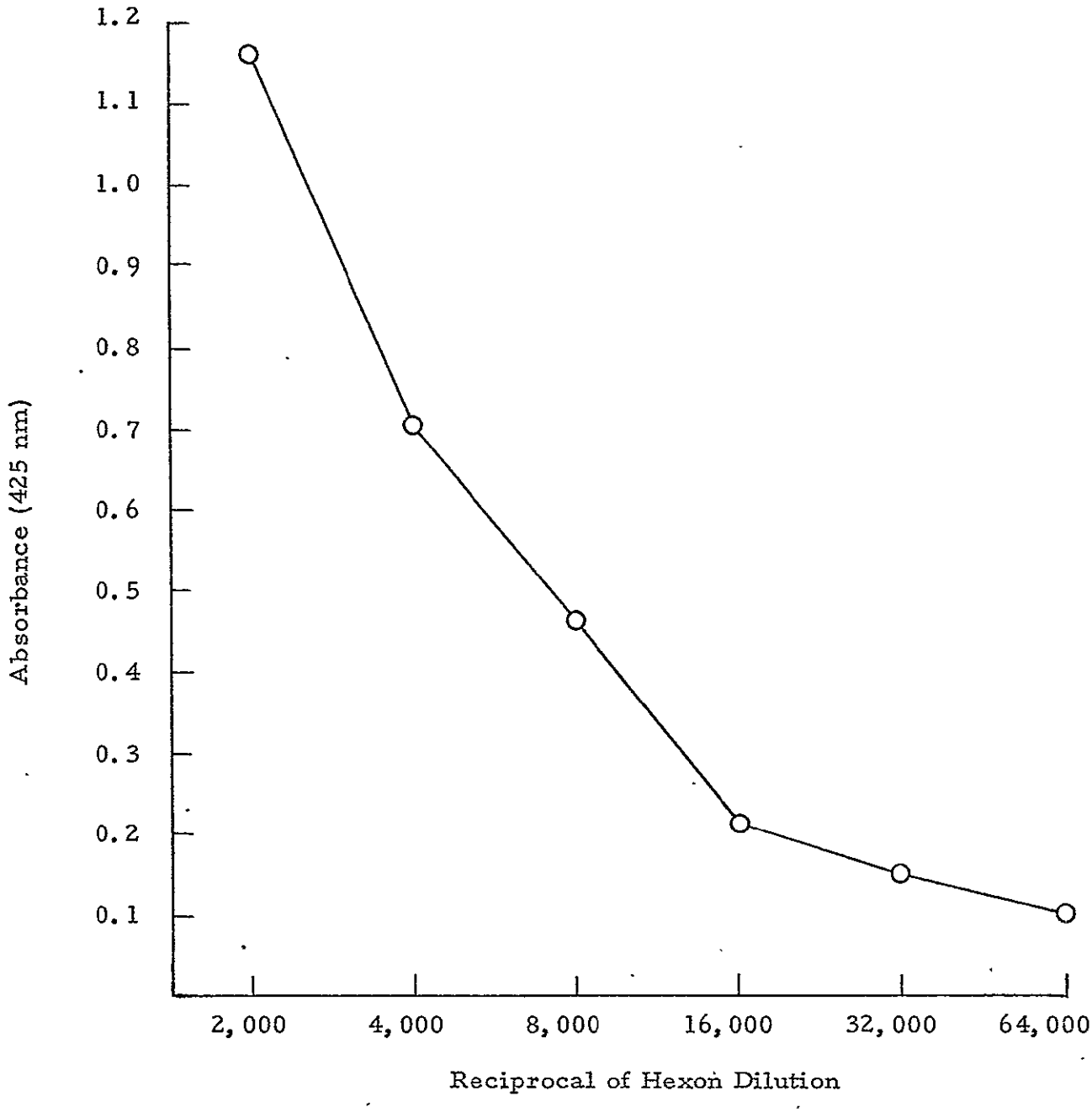

Figure 7-1. Adenovirus EIA Detection Sensitivity Hexons in RIA Buffer

TABLE 7-1 ADENOVIRUS EIA - RESULTS FROM VARIOUS SAMPLES

Sample	Absorbance
Throat Wash #1	0.14
Throat Wash #2	0.03
Throat Wash #3	0.00
Throat Wash #4	0.00
Rubella HA Antigen	0.07
Parainfluenza HA Antigen	0.00
Influenza HA Antigen	0.03
Human Serum	0.09
HeLa Extract	0.04
Adenovirus Type 1	0.56
Adenovirus Type 2	0.49
Adenovirus Type 3	1.50
Adenovirus Type 5	0.90
Adenovirus Type 7	1.10

centrifugation, was not optimum. This was most likely the cause of the positive result obtained with throat wash #1.

These tests may serve as an indication of the sensitivity and specificity attainable with the EIA method of assay. Even though no attempt was made to optimize the test conditions, the results were quite favorable.

Given the proper conditions, no doubt PIA methods could be developed for the detection of most viruses and their antibodies. However, because of the problems associated with the PIA test in most instances, it is recommended that it be dropped in favor of the EIA system. Since a change in color intensity is the result of positive tests, the MEMS photometer would be a likely method of measuring these changes. In addition, most of the antigens and antibodies, which would be required, have already been produced on previous MEMS programs. The method of bead sensitization was also developed on a previous MEMS program; i.e., by covalent bonding. It seems, therefore, that rather than abandon the solid phase technology, it should be extended to a next-generation test system which would be more specific, more sensitive and more rapid than presently available methods.

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