# UNIQUE METHODS OF VIRAL COLLECTION AND IDENTIFICATION

FINAL REPORT
1 January 1966 - 30 June 1967

Contract No. AF41(609)-3020
Project Task No. 775402

MRI Project No. 2927-B



For

USAF School of Aerospace Medicine
Aerospace Medical Division (AFSC)
Brooks Air Force base, Texas 78235

Attn: SMSPP

MIDWEST RESEARCH INSTITUTE
425 VOLKER BOULEVARD/KANSAS CITY, MISSOURI 64110/AC 816 LO 1-0202

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by

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## PREFACE

This final report describes for the first time the research performed during the last three and a half months of Contract No. AF41(609)-2439, Project Task No. 775402. It also summarizes the work completed during the preceding 14-1/2 months and already presented in five informal reports dated 1 April 1966, 8 July 1966, 6 October 1966, 5 January 1967, and 6 April 1967.

The experiments described in this report were conducted by Dr. Louis H. Goodson (Project Leader), Dr. John O. MacFarlane, Dr. David A. Ringle, Mrs. Anne Monley, Mrs. Betty Herndon, Mr. Duane Lamb, and Mr. Joe Utt. Dr. Jerome P. Schmidt of the School of Aerospace Medicine has served as consultant and project monitor.

The principles of animal care employed on this program were in compliance with the recommendations of the Animal Care Panel and the Institute of Laboratory Animal Resources, National Academy of Sciences.

Approved for:

MIDWEST RESEARCH INSTITUTE

W. B. House, Director
Biological Sciences Division

11 August 1967

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## SUMMARY

Six unique methods for the rapid identification of virus have been devised and investigated to determine their potential suitability for use in the fabrication of a new virus identification system. In the phagocytosis *in vitro* studies we prepared immune WBC and observed their response when challenged with virus. Impedance measurements on phospholipid bilayer membranes in buffer solutions have been used to detect the immune reaction. The effects of virus challenge on the electrophoretic migration rate of antibody coated plastic particles and tanned sheep red blood cells have been measured. Hydrogen overvoltage measurements have been performed on solutions containing antibody and antigen-antibody complexes. Fluorescent tagged antibody has been employed in the single and double immuno-diffusion techniques in an effort to visualize the "soluble" virus-antibody conjugates. Virus has been tagged with rhodamine to permit its use with immobilized antibody for a viral identification process. Molecular sieves and ion exchange resins have been employed in studies for the separation and detection of "soluble" virus-antibody complexes.

From these studies we have concluded that the *in vitro* phagocytosis approach is likely to lead to a very sensitive virus identification system if methods for obtaining uniformly sensitive WBC can be developed. The phospholipid bilayer membrane approach also suffers from the difficulty of being difficult to control or reproduce. Fundamental research on both of these approaches is recommended although immediate success is not anticipated.

The use of fluorescent antibodies in combination with the immunodiffusion process is not expected to be valuable for the identification of virus; however, this approach for the detection of insoluble antigen-antibody conjugates is expected to yield at least a tenfold increase in sensitivity over the usual immunodiffusion methods.

Our results with the "soluble" virus-antibody complexes are of a preliminary nature but they are most encouraging. We are recommending that a high priority be given to an extension of these studies and that the new studies include specifically:

1. The labeling of viruses with a multiplicity of fluorescent tags.

2. The use of molecular sieves for the separation of "soluble" virus-antibody complexes.

3. Quantitative estimates of the minimum level of virus which can be identified by the combined use of fluorescent-tagged virus and/or antibody and gel filtration techniques.

In addition, studies should be conducted to determine the suitability of fluorescent tagging of virus to replace isotopically-tagged virus in the identification system based on the use of immobilized antibodies.

# I. INTRODUCTION

On a previous contract (AF41(609)-2439) a series of 20 unique methods of virus collection and identification were devised and evaluated in a preliminary manner in order to select those methods which show promise for the identification of fewer than 100 virus particles in 6 hr. and which would be suitable for incorporation into an automatic identification system. Identification methods which require replication of the virus as the first stage of the identification process were not considered in this study since most viruses require more than 6 hr. for the replication process alone. On the basis of information found in the literature and also on the basis of exploratory studies, three of the 20 methods were selected as candidates for more intensive study. It was understood at the outset that if these methods became unpromising in terms of meeting the project objective that we would re-investigate some of the other methods or consider new methods not then under consideration.

For six months we concentrated our efforts on the application of the following four methods to the identification of virus:

1. Phagocytosis in vitro: Immune and hyperimmune white blood cells (WBC) were prepared and allowed to react with viral antigen to produce cellular changes or lysis which could be monitored either microscopically or by the release of fluorescent particles or other fluorescent materials.

2. Synthetic phospholipid membranes: Phospholipid bilayer membranes were prepared under the surface of a buffer solution from unoxidized egg lecithin and ox brain proteolipid dissolved in tetradecane. The effects of antibody, viral and non-viral antigens, and guinea pig complement on the electrical impedance of these membranes were investigated.

3. Particle electrophoresis: Plastic spheres in the 6 - 14 micron size range with antibody chemically bound to its surface and tanned sheep red blood cells coated with antibody were used to determine the change in electrophoretic mobility resulting when the particles were challenged by viral or non-viral antigens.

4. Hydrogen overvoltage: A gradually increasing direct current was applied to a pair of working platinum electrodes. The potential at the working cathode was measured against a standard hydrogen electrode to determine the hydrogen overvoltage produced by the presence of antibodies, antigens, or combinations of them. Although this approach was not originally included in the methods chosen for investigation in this period, it was re-investigated to tie up some loose ends remaining at the close of the preceding study.[1]

At the end of six months of intensive investigation on these four methods, the principal investigators together with the sponsor agreed that we should concentrate our remaining efforts of the phagocytosis in vitro approach. However, at the end of an additional eight months of study on the sensitization and lysis of white blood cells, there seemed little hope that we could develop a reliable and reproducible identification procedure within the remaining four months of our contract. Therefore, at a joint conference with the sponsor at Brooks Air Force Base a decision was made to consider approaches to identification which might detect $10^4$ virus particles or less in 6 hr. The two methods investigated during the last four months of the contract were as follows:

1. Immunodiffusion: We had postulated that "soluble" virus-antibody conjugates produced in either single or double immunodiffusion processes might be visible as fluorescent bands provided that fluorescent antibody was employed in place of non-labelled antibody. We also hoped that the use of fluorescent antibody would improve the sensitivity of those immunodiffusion processes in which precipitin reactions are normally observed. We therefore prepared fluorescent-tagged antibodies and investigated them for both viral and non-viral systems.

2. Studies of virus-antibody complexes: The standard immunodiffusion procedures are unsuited for virus identification because many of the viruses do not give visible bands or precipitates; this is in contrast to bacterial and other antigens which do give precipitates. Recognizing that we would need to measure "soluble" complexes of virus and their antibodies, we investigated methods for tagging, separating and identifying such complexes from the two individual components used to prepare the complexes.

## II. EXPERIMENTAL

In the following sections will be found descriptions of each of the identifications systems, the results obtained with that system and our recommendations for future work with some of these methods.

### A. Phagocytosis In Vitro

#### 1. Introduction

In the previous study[1] one of the more promising of a number of potential methods for the identification of small numbers of viral particles

was based upon the phagocytic changes observed when immune white blood cells were exposed to the antigen to which they (the WBC) were sensitized. Sensitized WBC for such studies may be obtained by the active immunization of animals providing the WBC or the normal WBC of a normal animal may be "passively" sensitized in vitro by appropriate treatment with immune sera. These WBC changes (i.e., reaction with virus) occur "in vitro" as well as "in vivo" and the cellular reactions are very rapid. The efforts in the present study were aimed at developing an "in vitro" procedure which would be capable of meeting the goals outlined for the project.

Since the phagocytic changes in WBC produced by the antigen-antibody reaction are immunological in nature, the method offered the specificity needed to provide an identification of an unknown virus. The earlier data indicated that the method appeared promising since lytic and cellular changes were obtained in WBC exposed to the specific virus for which they were sensitized. The currently reported studies were made in an effort to improve the regularity, sensitivity, and ease of performing the in vitro test procedures. Emphasis has been placed on improving the techniques for the readout of the cellular lysis and/or changes.

Expansion of the preliminary studies to include multiple virus and cell systems was done in order to determine the suitability of the method for a broad application in viral identification.

As an integral part of the study many facets of the test proper, the properties of reagents, and the preparation of the sensitized WBC suspensions were investigated since it was hoped that the composite result would be a simple, rapid, easily monitored in vitro procedure.

The following sections present the methods, experimental approaches, results, discussion and conclusions of the studies on phagocytosis in vitro for viral identification.

Materials and methods: The basic methods for viral growth, titration and immunization of animals for the in vitro phagocytic studies were those used for the previous contract (AR41(609)-2439).[1] Also, the basic principles and methods for the current phagocytic tests were similar to those described in the quarterly and final reports of this same contract.

However, in these later efforts to develop virus identification techniques through use of phagocytic methods, we obtained and utilized a new cell line and a new immunological system for an arthropod-borne virus, Semliki Forest Virus.

a. WISH human amnion cell line: This cell line was obtained from the American Type Culture Collection and adapted to MRI cultivation techniques using Medium 199 and 2 percent calf serum. It was obtained so that some of the viruses being studied could be cultivated in two different cell systems. This permitted us to eliminate or prevent an antigen-antibody reaction in the phagocytic reactions due to cellular antigens. Calf serum was utilized in the propagation of the WISH cells for a similar reason. Our LLC-$MK_2$ (monkey kidney line) cells were cultivated in M-199 and horse serum. By use of these two different sera in the two-cell systems we did not have to worry about an antigen-antibody reaction in the phagocytic tests due to the serum used in cultivation of the cells.

The WISH cell line was satisfactorily stored in the frozen state by using glycerin as a stabilizer. This procedure was also used with the LLC-$MK_2$ monkey kidney cell line.

b. Preparation of new poliovirus pools and immunization of rabbits: We produced new virus pools using the WISH cell line and used these pools for the immunization of newly acquired rabbits. These were immunized by incorporating the appropriate virus in complete Freund's adjuvant and injecting the emulsions subcutaneously on each of two occasions. Four weeks after the second adjuvant dose the rabbits were given booster injections of an aqueous suspension of virus. These rabbits and several rabbits previously immunized during the previous study were the source of immune white blood cells (WBC) for our phagocytic studies. Normal control rabbits were also kept as a source of normal WBC.

c. Application of WBC procedure for Semliki Forest Virus system: Since we needed to expand our previous phagocytic observations (made with poliovirus) to another viral system, we obtained Semliki Forest Virus and prepared the appropriate antisera, virus, and immune animals for additional phagocytic tests. Semliki Forest Virus (SFV), a member of the anthropod-borne virus group, is a virus which can be utilized as a simulant for one group of viruses of biologic importance both in biological warfare and in clinical virology; yet, it may be handled with relative ease and freedom in the laboratory.

Although we studied Semliki Forest Virus in a number of cell systems, we found that primary chick embryo cultures were the best for our work; therefore, we prepared Semliki Forest Virus pools in chick embryo tissue cultures. Virus in the form of mouse brain suspensions was obtained by intracerebral inoculation of mice and harvest of the infected brains. Thus we had two distinct tissue sources of SFV for our phagocytic work and for the immunization of animals.

A group of rabbits were immunized with chick embryo tissue culture grown SFV virus. The virus, in Freund's complete adjuvants was used for the primary antigenic stimulus, but the rabbits were boostered with aqueous virus.

Semliki Forest Virus infects adult white rats, generally without apparent disease, yet produces antibody and immunity demonstrable by standard complement fixation, hemagglutination inhibition and neutralization techniques.[2,3] Young rats, less than 23 days, usually succumb to infection. We have taken advantage of this inapparent infection in adult rats as a means of obtaining immune animals as a source of immune sera and WBC. A group of albino rats (Carworth All Purpose Albino Rats) were infected intraperitoneally with $3.3 \times 10^7$ $LD_{50}$'s (determined intracerebrally in mice) of Semliki Forest Virus in the form of an infected mouse brain suspension. No illness was observed in these rats but four such rats were titered for neutralizing antibody in primary chick embryo tissues and had antibody levels of 1 - 32 or higher when tested against 3170 $TCID_{50}$'s (tissue culture infective dose 50 percent) of Semliki Forest Virus. Sera from control, non-infected rats did not neutralize the virus. Thus, these rats immune to Semliki Forest Virus were used as a source of sera and immune WBC for phagocytic tests. Control non-infected rats were also used.

2. Experiments

a. Semliki Forest Virus tests using Rabbit WBC: Three rabbits hyperimmunized with SFV grown in chick embryo tissue were tested for their SFV antibody levels. Two rabbits had serum titers of 1 - 32 and the third rabbit had a 1 - 64 level of antibody when tested against 50 $TCID_{50}$ (tissue culture infectious doses -- 50 percent) of SFV. These neutralization tests were done in primary chick embryo cultures, and end-points were read by observing the cytopathologic changes of stationary tube cultures. The levels of antibody cited were obtained after two doses of adjuvant-emulsified virus at two-week intervals and two subcutaneous doses of aqueous virus 45 and 63 days after the first injection of virus. The sera tested were obtained 11 days after the last virus injection. (These rabbits were later re-injected in order to maintain their hypersensitive state.)

Phagocytic tests were made, using WBC from the SFV immunized rabbits. In each test WBC from a normal rabbit were run simultaneously with the immune WBC. Since the SFV immune rabbits had been immunized with chick embryo tissue culture grown virus, the test SFV for the phagocytic tests was a brain suspension from mice infected with SFV (except

for several special trials). Therefore, the brain suspension of SFV was used to circumvent such problems relating to common antigens in the immunizing and test antigens.

The first test of the WBC from an SFV immune rabbit was preliminary in nature and the results were observed by both bright field and fluorescent microscopy. For the fluorescent observations acridine-orange was added and fluorescence was observed by means of a microscope equipped with a dark field condenser and appropriate filters. (These fluorescent methods will be discussed more fully later in the report.) Immune WBC separated by several different methods were tested by mixing them in hanging drop preparations with 0.25 x $10^5$ $TCID_{50}$ doses of SFV (mouse brain suspension). Granulation, swelling, clumping and lysis were observed with the bright field microscope. Such changes were not observed with preparations of WBC from a normal rabbit. Although the results were similar to those with poliovirus and reported earlier,[1/] the cellular changes were not quite as pronounced. By fluorescent microscopy cellular changes were also noted, but the fluorescent changes which could be seen were only in the nuclei. The nuclei of immune WBC in the presence of SFV became larger, more yellow, and more intensely fluorescent than the nuclei of normal WBC treated with SFV. This fluorescence intensity later faded. In some immune WBC exposed to virus, red (RNA) fluorescence appeared, but this was irregular in quantity, size, and occurrence. The red RNA fluorescence was not observed in normal WBC treated with SFV. We think that these fluorescent observations are exceedingly preliminary, and considerable experience needs to be obtained before interpretations can be considered as valid.

In another test, WBC from a SFV immune rabbit were tested for their phagocytic reactivity 33 days after a booster dose of antigen. The WBC preparation was observed to contain much fewer large mononuclear cells than usual. When this WBC suspension was exposed to SFV in the form of 1 - 10 mouse brain suspension, the results were quite equivocal. When the antigen (SFV mouse brain) was diluted 1 - 100, the WBC from the SFV immune rabbit were affected more than WBC from a control normal rabbit. Similar preparations examined by fluorescence microscopy using 0.001 percent acridine-orange as the dye also exhibited minor differences between the WBC from immune and normal WBC upon exposure to SFV. These fluorescent changes were mainly in the nuclei, and consisted of more intense yellow and more red RNA granules in the immune cells in contrast to a yellow-green fluorescence in the normal cells. The results of this test seem to indicate the importance of booster antigenic stimulus shortly before the WBC are used. Minor differences in WBC will be difficult to observe by fluorescence microscopy unless the changes are in the nuclei.

After the test results reported above were available, we thought that an additional booster dose of virus would improve the quality of the phagocytic tests. Consequently each SFV immune rabbit was given an intravenous injection of aqueous SFV (grown in chick embryo tissue culture), and the WBC reactions were studied six days after this additional immunization. Tests were made with WBC from each of the three SFV immune rabbits and a normal (control) rabbit using a number of dilutions of mouse brain virus and other antigens. The results were exceedingly encouraging since all three immune rabbits demonstrated the type of cellular changes which we had observed in our earlier studies with poliovirus, yet the WBC from the normal rabbit were not affected. These observations were made using bright field microscopy and are difficult to describe; however, the results were corroborated by two separate individuals. Table 1 summarizes the variables and observations. We would like to stress that microscopic observations are very difficult to incorporate into a table of this type, and we recognize the subjective nature of such observations. This is one of the handicaps of the phagocytic tests and a factor to be considered in evaluating various methods of viral identification.

Tests with the WBC of all three SFV immune rabbits were easier to read when the virus was more dilute, and we observed positive changes even when the 1 - 100,000 mouse brain virus was added to the immune cells; yet the WBC from the normal rabbit did not react. Added complement was not essential to the phagocytic test, but the results were somewhat faster and easier to read when guinea pig complement was added.

Two tests with WBC from Semliki Forest Virus (SFV) immune rabbits illustrate the variability of results of in vitro WBC viral identification tests. A test performed with the WBC of three rabbits on August 2, 1966, yielded moderately good results but another test on August 11, 1966, of the WBC from one of the same rabbits failed to produce satisfactory identification of Semliki Forest Virus.

In the test of August 2, 1966, the WBC from each of three SFV immune rabbits reacted with 250 $TCID_{50}$ and higher concentrations of SFV. These tests were performed in the absence of guinea pig serum (complement) since the WBC were found to be lysed by guinea pig serum. The interpretation of these tests was complicated by the fact that the WBC count had dropped rapidly in the first few minutes of the test -- presumably by specific lysis. Morphological changes in the WBC were observed and the WBC counts continued to drop over the 2 to 3 hr. period that the slide preparations were observed. Normal rabbit WBC did not demonstrate similar morphological changes and WBC count reductions when exposed to SFV. Data for part of the slides run on the 2 August 1966 test for the identification of SFV are incorporated into Table 2.

TABLE 1

IN VITRO PHAGOCYTIC TESTS[a] WITH SEMLIKI FOREST VIRUS

Section A: Tests with 1 - 10 Dilution of SFV[b]

| Slide No. | WBC Tested: Rabbit Source | WBC Tested: No. of WBC[c] | Results: Interpretation[d] | Results: Microscopic Observations |
|---|---|---|---|---|
| 1 | SFV immune rabbit #1 | 82,500 | Positive | Swollen, granular cells (2 times normal size); very few clumps in 20 - 40 min.; lysis within 2 hr. of many cells. |
| 2 | SFV immune rabbit #2 | 58,000 | Positive | Similar to slide #1 but more swollen cells and more lysis (fewer remaining cells) in 2 hr. Few cells in pairs and triplets. |
| 3 | SFV immune rabbit #3 | 178,000 | Positive | Numerous swollen granular cells and clumps of cells within 20 min. Reaction became more pronounced with time and by 2 hr. very few cells left, therefore, lysis. Rosettes of granular cells clumped together. |
| 4 | Normal rabbit #1 | 53,000 | Negative | No swollen or granular cells within 2 hr. No clumps. Cells spread over slide in uniform manner. |

a/ Procedure: One drop of WBC suspension plus 1 drop of SFV mixed and observed microscopically.

b/ 1 - 10 dilution of mouse brain suspension of SFV. The one drop of this 1 - 10 virus used in the test contained approximately $10^7$ mouse intracerebral $LD_{50}$ doses of SFV.

c/ Numbers of WBC per drop are approximate and should be considered as an average value. Count may be 0.5 to 2.0 times the values listed.

d/ Interpretation: Swelling, granulation, clumping and lysis of WBC considered as positive. No significant changes in WBC considered as negative.

TABLE 1 (Continued)

IN VITRO PHAGOCYTIC TESTS[a] WITH SEMLIKI FOREST VIRUS

Section B: Tests with 1 - 100 Dilution of SFV[b]

| Slide No. | WBC Tested: Rabbit Source | WBC Tested: No. of WBC[c] | Results: Interpretation[d] | Results: Microscopic Observations |
|---|---|---|---|---|
| 5 | SFV immune rabbit #1 | 82,500 | Positive | Swollen, granular cells (2 times normal size). Fair numbers of clumps in 20 - 40 min. Lysis within 2 hr. of many cells. Similar to slide #1 except more clumping. |
| 6 | SFV immune rabbit #2 | 58,000 | Positive | Similar to slide #5 (see above). |
| 7 | SFV immune rabbit #3 | 178,000 | Positive | Reaction much more pronounced than in slides 5 and 6 (see above) and slide 3 (see Section A). Clumping of cells very prominent during early part of reaction. Lysis great by 2 hr. |
| 8 | Normal rabbit #1 | 53,000 | Negative | No swollen or granular cells within 2 hr. No clumps. Cells spread over slide in uniform manner. |

a/ See Section A for description of procedure.
b/ 1 - 100 dilution of mouse brain suspension of SFV. The one drop of this 1 - 100 virus used in the test contained approximately $10^6$ mouse intracerebral $LD_{50}$ doses of SFV.
c/ See Section A for description.
d/ See Section A for description.

TABLE 1 (Continued)

## IN VITRO PHAGOCYTIC TESTS[a] WITH SEMLIKI FOREST VIRUS

### Section C: Tests with 1 - 10 Dilution of SFV from CETC[b]

| Slide No. | WBC Tested: Rabbit Source | WBC Tested: No. of WBC[c] | Results: Interpretation[d] | Results: Microscopic Observations |
|---|---|---|---|---|
| 9 | SFV immune rabbit #1 | 82,500 | Positive | Swollen, granular cells (2 x normal size) but no clumps. Lysis within 2 hr. of many cells. Precipitate formed on slide. (Due to common antigens used for immunizing rabbit and in test antigen.) |
| 10 | SFV immune rabbit #2 | 58,000 | Positive | Similar to Slide #9 but somewhat stronger reaction and more precipitate. |
| 11 | SFV immune rabbit #3 | 178,000 | Positive | Similar to Slide #9 but much stronger reaction, more clumping and very heavy precipitate. |
| 12 | Normal rabbit #1 | 53,000 | Negative | No clumping, granulation or precipitate. All cells singular and no evidence of lysis. Cells evenly distributed on slide. |

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a/ See Section A for description of procedure.

b/ 1 - 10 dilution of chick embryo tissue culture suspension of SFV. The one drop of the 1 - 10 virus used in the test contained approximately 7 x $10^6$ $TCID_{50}$ (tissue culture infections dose - 50 per cent) of SFV.

c/ See Section A for description.

d/ See Section A for description.

TABLE 1 (Continued)

IN VITRO PHAGOCYTIC TESTS[a] WITH SEMLIKI FOREST VIRUS

Section D: Tests with 1 - 100 Dilution of S/V and Complement Present During Test[b]

| Slide No. | WBC Tested: Rabbit Source | WBC Tested: No. of WBC[c] | Results: Interpretation[d] | Results: Microscopic Observations |
|---|---|---|---|---|
| 13 | SFV immune rabbit #1 | 82,500 | Positive | Similar to Slide 5 (Section B) except granulation, swelling, clumping and lysis occurred much faster. |
| 14 | SFV immune rabbit #2 | 58,000 | Positive | Similar to Slide 6 (Section B) except granulation, swelling, clumping and lysis occurred much faster. |
| 15 | SFV immune rabbit #3 | 178,000 | Positive | Similar to Slide 7 (Section B) except granulation, swelling, clumping, and lysis occurred much faster. Lysis was much more pronounced than in Slide #7. |
| 16 | Normal rabbit #1 | 53,000 | Negative | Similar to Slide 4 (Section A) and Slide 8 (Section B). No granulation, swelling, clumping or lysis. |

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a/ See Section A for description of procedure.

b/ These tests run with the same SFV virus ($10^6$ mouse intracerebral LD50 doses) as was used in slides reported in Section B of this table. In addition to WBC and virus, 1 drop of undiluted guinea pig serum was added as a source of complement. (Guinea pig serum stored at -80°C from immediately after bleeding until use.)

c/ See Section A for description.

d/ See Section A for description.

TABLE 1 (Concluded)

IN VITRO PHAGOCYTIC TESTS[a] WITH SEMLIKI FOREST VIRUS

Section E: Tests with 1-1,000, 1-10,000, 1-100,000 Dilutions of SFV and Chick Embryo Produced SFV (Complement Present During Tests)[b]

| Slide No. | Test Virus | WBC Tested: Rabbit Source | WBC Tested: No. of WBC[c] | Results: Interpretation[d] | Results: Microscopic Observations |
|---|---|---|---|---|---|
| 17 | 1-1,000 Mouse Brain SFV | SFV immune rabbit #3 | 178,000 | Positive | Many granular, swollen cells, many in clumps, lysis of large mononuclear cells, but many cells left on slide even after 1 hr. |
| 20 | 1-10,000 Mouse Brain SFV | SFV immune rabbit #3 | 178,000 | Positive | Some granular, swollen cells, numerous clumps of 2, 3 or 4 cells within 20 min. but definitely positive by 45 min. |
| 21 | 1-100,000 Mouse Brain SFV | SFV immune rabbit #3 | 178,000 | Positive | Few clumps of 2, 3 or 4 cells - swollen and granular. Weaker than reaction in slide 20 (1-10,000 SFV) but definitely positive in 45 min. |
| 18 | 1-10 CETC SFV | SFV immune rabbit #3 | 178,000 | Positive | Granular, swollen cells, and some lysis but not as great as slide 17. Heavy precipitate on slide due to common antigen in immunizing and test antigens. |
| 19 | 1-10 M199 Spent tissue Culture Medium | SFV immune rabbit #3 | 178,000 | Negative | Very few granular cells, no swollen cells, no lysis, and no clumping. No precipitate. Uniform distribution of WBC over slide. |

a/ See Section A for description of procedure.

b/ Tests made with three high dilutions of mouse brain suspension of SFV. 1 drop of 1-1,000 dilution = $10^5$ mouse intracerebral $LD_{50}$ doses of SFV, 1 drop of 1-10,000 dilution = $10^4$ $LD_{50}$ doses of SFV, 1 drop of 1-100,000 dilution = $10^3$ $LD_{50}$ doses of SFV, and 1 drop of 1-10 CETC suspension of SFV = $7 \times 10^6$ $TCID_{50}$ of SFV. 1 drop of undiluted guinea pig serum added to each slide to supply complement.

c/ See Section A for description.

d/ See Section A for description.

TABLE 2

## IDENTIFICATION REACTIONS FOR SEMLIKI FOREST VIRUS

Test Date: 8-2-66

| Slide No. | Reagents: VEC | Reagents: Test Virus or Agent | Reagents: Accessory Factors | Cells/Field: Original | Cells/Field: After 2-3 Hr. | Microscopic Observations | Identification as Semliki Forest Virus |
|---|---|---|---|---|---|---|---|
| 1 | SFV Rabbit #1 | SFV 25,000 $TCID_{50}$ | 0.85% Saline | 70 | 3-15 | Uneven distribution, debris large, swollen granular cells | Positive |
| 2 | " " #2 | " " " | " " | 90-100 | 40 | Uneven distribution, debris large, swollen, granular cells | " |
| 3 | " " #3 | " " " | " " | " | 10-20 | Uneven distribution, debris large, swollen, granular, spiked cells | " |
| 4 | Normal Rabbit | " " " | " | 50-60 | 40-50 | Even distribution, mostly smooth, normal cells | Negative |
| 13 | SFV Rabbit #1 | " 2,500 " | -- | 70 | 30-35 | Uneven distribution, pairs large, swollen, granular, spiked cells | Positive |
| 14 | " " #2 | " " " | -- | 90-100 | 45 | Uneven distribution, cell clumps large, swollen, spiked, irregular cells | " |
| 15 | " " #3 | " " " | -- | " | 40-45 | Uneven distribution, nuclei, spiked, irregular cells | " |
| 16 | Normal Rabbit | " " " | -- | 50-60 | 40 | Distribution somewhat irregular, round large, some spiked cells | ? Negative |
| 17 | SFV Rabbit #1 | " 250 " | -- | 70 | 15 | Uneven distribution, clumps, large swollen cells, debris | Positive |
| 18 | " " #2 | " " " | -- | 90-100 | 30 | Uneven distribution, pairs, large swollen granular cells | " |
| 20 | " " #3 | " " " | -- | " | " | Uneven distribution, nuclei large, swollen spiked cells | " |
| 21 | Normal Rabbit | " " " | -- | 50-60 | 40-50 | Fairly uniform distribution, smooth small cells | Negative |
| 22 | SFV Rabbit #1 | " 25 " | -- | 70 | 35 | Fairly uniformly distributed, few large, few granular cells | " |
| 23 | Normal Rabbit | " " " | -- | 50-60 | " | Even distribution, smooth, small cells, some spiked cells | " |
| 24 | SFV Rabbit #1 | " 2,500 " | SFV Rabbit AF* | 20-40 | 5-6 | Uneven distribution nuclei, large swollen cells, lysis | Positive |
| 25 | Normal Rabbit | " " " | Normal Rabbit Serum* | 20-30 | 10-12 | Fairly even distribution, round smooth and irregular cells | ? Negative |

* Normal or Immune Serum from same rabbit supplying the VEC

The negative results of August 11, 1966, for identifying SFV by immune WBC cannot be explained. The rabbit supplying the WBC was one of the same ones used in the 6 August 1966 test; therefore, it had been bled and the WBC tested just nine days earlier. It also had been given an intravenous-booster dose of SFV on August 5, 1966, just six days before the second groups of WBC were used. The booster dose of virus may have adversely affected the response of the WBC rather than enhanced their sensitivity to virus. Much needs to be learned relating to the proper timing of antigenic stimulation and use of WBC for viral identification.

b. Semliki Forest Virus tests using rat WBC: The WBC from one Semliki Forest Virus immune rat and one normal rat were studied for their ability to show phagocytic changes when exposed to the antigen, Semliki Forest Virus. The Semliki Forest Virus used was a chick embryo tissue culture virus preparation. Since this was only a preliminary test, we did not fully determine the extent of the reactions. Specific cellular changes and lysis occurred with the immune rat WBC that did not occur with the WBC from the normal rat. The extent of changes was not as great as observed earlier with polio immune cells but we attributed this partly to the bleeding and WBC separation techniques used. Use of a 50-fold dilution of the virus antigen increased the cellular changes and made them more easily observed.

Earlier in this report, we indicated that white rats infected with SFV developed antibody to the virus but did not develop clinical symptoms of disease. The WBC of one such rat were tested for their reaction to SFV in in vitro phagocytic tests. Specific cellular changes and lysis occurred with the immune rat WBC that did not occur with WBC from a normal rat. The extent of the immune cell changes was not as great as had been observed with poliovirus.

Tests were subsequently completed on three more rats possessing neutralizing antibody to SFV. These tests were made four months after the rats had been infected with SFV. No booster immunization had been attempted since we desired to determine if WBC sensitivity existed in animals which had been infected, and thereby had not been hyperimmunized. The WBC from each of the three rats tested exhibited some cellular changes when exposed to SFV, but these changes were not very pronounced and were not sufficiently strong to be used to identify a virus. The changes in WBC from the immune rats were apparent only when compared with the morphology of normal rat WBC tested with the same virus. Immune WBC from SFV rats reacted more strongly when tested against SFV in the form of a mouse brain suspension than when SFV from chick embryo tissue culture was used. From these results we believe that the infection in rats had not led to a hypersensitivity reaction even though specific antibody was formed during the infections.

c. WBC from rats after SFV infection and re-injection of antigen: In the previous section we indicated that the WBC obtained from white rats after infection with SFV exhibited cellular changes upon in vitro exposure to SFV. However, the changes were much less than observed with poliovirus immune WBC and the rat WBC were not considered sensitive enough for use in viral identification procedures. We studied WBC from one of these same white rats after further antigenic stimulation. Seven days after an additional antigenic stimulation by intravenous injection of SFV (mouse brain tissue suspension) the WBC from the SFV immune rat reacted to SFV and normal rat WBC did not. The reaction was not sufficiently strong for use in viral identification procedures. In attempting to enhance the SFV reaction by addition of guinea pig serum (complement) it was observed that WBC from rats were very quickly lysed by guinea pig serum. This will be discussed more fully later in this report.

Seven days following a second intravenous injection of SFV, the WBC's of the same rat were again studied for their phagolytic reactions. The extra antigenic stimulation still did not enhance the reactions of the rat immune WBC to SFV; therefore, further study of WBC from infected rats does not seem warranted at this time.

These SFV results extended the phagocytic technique to at least two viral systems, and offered encouragement to the possibility of applying the technique to other viruses to which we can hyperimmunize animals.

The SFV rabbit and rat studies suggest the importance of the hypersensitive state of the animals providing the WBC for phagocytic tests. Booster doses of antigen are important, and it may be necessary to keep the animals highly immunized by regular booster doses of antigen.

With the sensitivity of the WBC high, as was the case with the SFV rabbit, WBC (and in the earlier poliovirus studies), it may be possible to identify 1,000 or perhaps fewer viral particles within a few hours, perhaps even within a few minutes after the test is set up. The development of methods to increase the sensitivity and reliability of the read-out are necessary, particularly if any type of instrumented unit is to become possible.

d. Poliovirus tests using rabbit WBC: Using WBC from the new groups of poliovirus immune rabbits, additional tests (previous tests under Contract AF41(609)-2439) were performed to verify the earlier observations and in an attempt to utilize a fluorescent dye in the read-out.

These newly immunized rabbits were injected with poliovirus grown in the WISH human amnion cell line cultivated in Medium 199 (M-199) supplemented with calf serum during the cell growth phase. For virus to challenge the WBC preparations, the poliovirus strains were grown in the LLC-$MK_2$ monkey kidney cell line. The LLC-$MK_2$ cells were cultivated in M-199 supplemented with horse serum during the cell growth period. Thus (as pointed out earlier in this report), we maintained separate culture conditions for the antigens used for immunization and for *in vitro* challenge of the WBC. This was done to eliminate any false results due to antigens other than the virus itself.

In one test we reconfirmed our earlier results that poliovirus 1 will cause swelling, granular changes and lysis of WBC from a poliovirus 1 immune rabbit. Similar changes were not obtained when poliovirus 1 was added to WBC from a normal rabbit. By bright-field microscopy these changes occurred within 15 - 60 min., and the results were easier to observe when more dilute virus was added to the WBC. Changes were observed with about 10,000 $TCID_{50}$ of poliovirus 1, but no tests were made using less virus. These tests were made four weeks after an aqueous booster dose of poliovirus 1 had been given to the immune rabbits. We are certain that immune status of the rabbit can be improved over that possessed by the rabbit used for this test. The rabbit had received two doses of virus in adjuvant and one aqueous booster dose. Aliquots of these same WBC preparations from the poliovirus 1 immune rabbit and the normal rabbit were reacted with virus and spent tissue culture fluid in the presence of acridine-orange (0.001 percent) and observed by fluorescence microscopy. Fluorescent changes were observed in the immune WBC treated with poliovirus that did not occur in normal WBC. The nuclear material in the immune WBC became enlarged and more diffuse, and bright yellow granules appeared, then faded. Normal WBC treated with virus did not become bright yellow but remained a more pale yellow-green. Their color also faded with time. Evidently the acridine-orange (AO) did not prevent cellular changes.

Similar results were obtained in a test performed 35 days after a rabbit had been given a booster dose of poliovirus. Photographic techniques for the fluorescent examination were only partially successful. The cytoplasm did not fluoresce and appeared as a dark (black) halo around the yellow-green nucleus.

Unfortunately we failed to get any results when an additional repeat was tried using WBC from a rabbit which had been given a booster dose of antigen seven days before the test. The WBC from both the normal rabbit and the immune rabbit had badly deteriorated, and it was impossible to use them for either fluorescent or bright field microscopic studies.

We have no explanation for this failure to get usable WBC, but the test illustrates the problems occasionally encountered in WBC work.

(1) Poliovirus type 1: Two additional series of experiments with WBC from poliovirus 1 immune rabbits were performed and illustrate the variation in results obtained with cells from the same animals. Data obtained with WBC tests run July 26, 1966, were the most favorable we obtained, yet the results observed in tests run on September 9, 1966, with WBC from the same rabbits were equivocal and definitely not of the quality necessary to help solve the virus identification problem within the limits defined in the scope of the project. On July 26, 1966, two rabbits bled 14 days after their last injection of poliovirus 1 antigen (intravenously administered) provided WBC sensitive to poliovirus 1. Cells separated from the bloods by use of a Locke-Citrate solution[4]/ were relatively free of platelets. These cells when exposed to virus showed minor changes, clumping, swelling, and changes in cellular morphology; but not much evidence of lysis was obtained. Such changes were observed in tests with $5 \times 10^6$, $5 \times 10^5$ and $5 \times 10^4$ $TCID_{50}$ (50 percent tissue culture infectious dose) of poliovirus 1 but not with spent tissue culture medium (control). When the WBC from these same two rabbits were removed by centrifugation from their serum-Locke-Citrate suspending fluid and resuspended in 0.85 percent NaCl, the WBC were highly sensitive to poliovirus 1 and phagolytic reactions were observed (Table 3). The cells from one rabbit reacted positively when only 250 $TCID_{50}$ of virus was added to the WBC. Unfortunately we did not test below 250 $TCID_{50}$. These tests were run in the presence of guinea pig serum as a source of complement, but the complement and/or spent tissue culture medium used in control slides did not cause WBC changes or lysis. The addition of homologous poliovirus immune serum from the rabbit used as the source of WBC seemed to enhance the lytic response. In these studies we attempted to quantitate the reaction. The average number of WBC present per microscopic field was determined for each slide. Lysis then could be more reliably assessed through use of such counts. Data in Table 3 indicate the reduction of WBC in positive reactions. This counting procedure was extended in later studies.

WBC from the same two rabbits were obtained on September 9, 1966, and used in viral identification tests. The rabbits had been injected intravenously 23 days earlier with poliovirus 1 in an effort to raise their antibody level. The WBC in these tests were only weakly reactive and the lytic response was essentially absent. Cellular changes were observed when $5 \times 10^5$ of poliovirus 1 was added, but the changes observed were not pronounced enough to be satisfactory for an identification test. During this test (September 9, 1966) we observed that guinea pig serum (complement?), was lytic for the rabbit WBC. This nonspecific lytic action will be discussed more fully later in the report.

TABLE 5

IDENTIFICATION REACTIONS FOR POLIOVIRUS TYPE 1

Test Date: 7-26-66

| Slide No. | Reagents: HBC | Reagents: Test Virus or Agent | Reagents: Accessory Factors | Cells/Field: Original | Cells/Field: After 2-3 1/2 Hr. | Microscopic Observations | Identification as Poliovirus 1 |
|---|---|---|---|---|---|---|---|
| 13 | Normal Rabbit | Polio 1 25,000 $TCID_{50}$ | G. P. Serum[a] | 20 | 20 | Even distribution, normal cells | Negative |
| 14 | Polio 1 Rabbit #1 | " " " " | " " | 60-70 | < 20 | Lysis | Positive |
| 15 | Polio 1 Rabbit #2 | " " " " | " " | " | Few | Uneven distribution, large, swollen cells | " |
| 16 | Normal Rabbit | 1-10 Spent T.C. Fluid[c] | " " | 30-40 | 30-40 | Even distribution, normal cells | Negative |
| 17 | Polio 1 Rabbit #1 | " " " " | " " | 60-70 | 60-70 | " " " " | " |
| 18 | " " " #2 | " " " " | " " | " | 40-50 | " " " " | " |
| 19 | " " " " | Polio 1 25,000 $TCID_{50}$ | " " | " | 8-15 | Uneven distribution, granular, swollen, pairs | Positive |
| 20 | " " " " | " " 2,500 $TCID_{50}$ | " " | " | 20 | Uneven distribution, clumps, granular, swollen cells | " |
| 21 | " " " " | " " 250 $TCID_{50}$ | " " | " | 10-12 | Uneven distribution, round, large, glassy (lysis) | " |
| 22 | " " " " | 1-10 Spent T.C. Fluid | " " | " | 30-40 | Even distribution, normal cells | Negative |
| 23 | Normal Rabbit | Polio 1 25,000 $TCID_{50}$ | " " Normal Rabbit Serum[b] | 30-40 | " | Even distribution, Normal cells | " |
| 24 | Polio 1 Rabbit #1 | " " " " | G. P. Serum Polio 1 Rabbit AB[b] | 60-70 | 10 | Uneven distribution, cells spiked, swollen | Positive |
| 25 | " " " #2 | " " " " | G. P. Serum Polio 1 Rabbit AB[b] | " | Few | Uneven distribution, granular, spiked, swollen | " |
| 26 | " " " " | 1-10 Spent T.C. Fluid | G. P. Serum Polio 1 Rabbit AB[b] | " | 40-50 | Even distribution, normal cells | Negative |

a/ Undiluted guinea pig serum
b/ Serum or antibody from rabbit supplying HBC
c/ Spent tissue culture medium - no virus

(2) Poliovirus type 2: Two additional sets of data were obtained for WBC preparations from poliovirus 2 immune rabbits. The WBC from two rabbits obtained seven days after an intravenous antigenic booster failed to detect 5 x $10^6$ virus particles. We used a different anticoagulant on these bleedings, 4 percent citrate-saline, rather than citrate only; yet we cannot be sure the negative results were due to the anticoagulant. The 4 percent citrate-saline is slightly hypertonic but it has been used by others in WBC work,[5] and we removed the WBC from the anticoagulant-plasma mixture before they were used in the tests. The WBC in this study were less affected by guinea pig complement than WBC in other similar tests. Specific immune rabbit serum (antipolio 2) from the same rabbit supplying the WBC did not enhance the specificity or sensitivity of the tests. The possibility exists that the intravenous virus injection seven days earlier may have reduced the WBC reactivity rather than enhanced the phagolytic WBC reaction.

Similar tests were run just two days later on the WBC from one older rabbit (from our earlier studies) which had been given an intravenous booster dose of virus seven days before but which had had a prolonged period with no booster doses before that. WBC from this animal after washing and resuspending in 0.85 percent NaCl were lysed by normal guinea pig serum (complement). However, even in the absence of complement, these WBC were affected by $10^3$ $TCID_{50}$ and higher concentrations of virus. Table 4 summarizes the results of the experiment and illustrates the WBC changes observed. Lysis occurred in the various mixtures of WBC and virus but it was not by any means complete. Cellular changes were observed in the WBC exposed to virus but these were not as clearly discernible as in previous tests. We did not consider the slides on this test to be nearly as easy to read or as positive as several previous tests; yet the effects were sufficient to permit observation of differences between the controls and the virus treated cells when as little as $10^3$ $TCID_{50}$ were used.

e. Tests with WBC from immune rabbits using cell counts: Five tests were performed with WBC from immune rabbits utilizing cell counts to follow the reactions. In some of our earlier work we had obtained a significant drop in the number of immune WBC reacted with the virus for which the WBC were sensitive; therefore, we wanted to investigate such reactions with quantitative methods. In four of these tests the cell counts for the WBC -- antigen mixtures and appropriate controls were made using hemacytometer slides and actually determining the number of WBC by counts with the bright field microscope. The various WBC preparations were counted immediately after mixing of the appropriate reagents and again after designated incubation periods. Some counts were even made after the WBC mixtures had been incubated overnight at 37°C.

TABLE 4

IDENTIFICATION REACTIONS FOR POLIOVIRUS TYPE 2
Test Date: 8-25-66

| Slide No. | Reagents: WBC | Reagents: Test Virus or Agent | Reagents: Accessory Factors | Cells/Field: Original | Cells/Field: After 2 1/2 Hr. | Microscopic Observations | Identification as Poliovirus 1 |
|---|---|---|---|---|---|---|---|
| 1 | Polio 2 Rabbit | Polio 2 80,000 $TCID_{50}$ | -- | 20-30 | 6-7 | Numerous small and few large round cells | Positive |
| 2 | Normal Rabbit | " " " | -- | 20 | 25-30 | Even distribution, normal cells | Negative |
| 3 | Polio 2 Rabbit | 1-10 Spent T.C. Fluid[a] | -- | 20-30 | 12-35 | Fairly evenly distributed, mostly normal cells | " |
| 4 | Normal Rabbit | " " " " | -- | " | 15 | Fairly evenly distributed, normal cells | " |
| 5 | Polio 2 Rabbit | Polio 2 8,000 $TCID_{50}$ | -- | " | 13-15 | Some very large round, many small spiked cells, pairs | Positive ? |
| 6 | " " " | " " 800 " | -- | " | 6-15 | Irregular distribution, many small, some smooth & large | Positive ? |
| 7 | " " " | " " 80 " | -- | " | 25 | Irregular distribution pairs, small jagged and swollen cells | Negative ? |
| 8 | " " " | " " 8,000 " | Polio 2 Rabbit AB[b] | " | 6-7 | Irregular distribution, large round swollen and irregular cells | Positive |
| 9 | Normal Rabbit | " " " " | Normal Rabbit Serum[b] | 12-20 | 13-14 | Irregular distribution, most normal cells | Negative ? |

a/ Spent Tissue Culture Fluid - No Virus
b/ Serum or Antibody From Rabbit Supplying WBC

Table 5 depicts the counts for WBC from one normal and two SFV immune rabbits, immediately and at various periods of time after the cells were exposed to saline or SFV (four different concentrations). For these experiments, WBC were obtained from citrated whole blood. After washing the WBC in a Locke-Citrate solution, the WBC were mixed with the appropriate dilution of virus and incubated at 37°C. The erratic results depicted in Table 5 illustrate the variability we have encountered and we conclude that the data in Table 5 represent a failure to identify SFV with WBC from two rabbits immune to SFV. The WBC counts varied greatly between operators, between counts on the same mixture, and for different concentrations of virus (no effect of dilution noted) and the counts with normal WBC-virus mixtures also gave widely varying results. These rabbits had been given a subcutaneous booster dose of virus (aqueous) just nine days before the WBC were obtained for these tests. When aliquots of these same suspensions of immune WBC (for SFV) were mixed with SFV and observed for morphologic changes only a few granular, round cells were observed; otherwise, there were no cellular changes as a result of the viral-WBC interaction.

Data from the four experiments in which the hemacytometers were used are recorded in Tables 6, 7, 8, and 9. These will be discussed more fully in the following paragraphs. We feel that such data are much more reliable than that which we obtained in earlier tests in which the counts were obtained by averaging the number of cells in five microscopic fields of coverslip preparations similar to those used in these tests.

Unfortunately, none of the tests we ran during this period were made with highly sensitive test systems; in fact, most of the results must be considered as unsatisfactory identifications. This is true in spite of the fact that we know the WBC were obtained from rabbits possessing neutralizing antibody. For three experiments we also tested the same WBC suspensions by the coverslip methods used in our previous work and by which we had been able to detect virus as low as 250 to 1,000 $TCID_{50}$'s (tissue culture cytopathic infectious dose -- 50 percent end point). By these simultaneous microscopic observations of coverslip preparations for morphologic changes in the immune WBC exposed to the homologous virus we only were able to detect virus when relatively large amounts of virus were added, and in some cases we failed to detect virus when we should have had a positive reaction. These results are further illustrations of the variability encountered with immune WBC detection of viral antigen.

TABLE 5

WBC REACTIONS: SEMLIKI FOREST VIRUS AS TEST ANTIGEN
COVERSLIP PREPARATIONS COUNTED BY TWO OPERATORS

| Reagent Applied to WBC* | SFV Immune Rabbit WBC (Rabbit No. 1) | | | SFV Immune Rabbit WBC (Rabbit No. 3) | | | Normal Rabbit WBC | | |
|---|---|---|---|---|---|---|---|---|---|
| | Incubation Time (min) | No. Cells Per Field | Operator | Incubation Time (min) | No. Cells Per Field | Operator | Incubation Time (min) | No. Cells Per Field | Operator |
| Saline (control) | 30 | 250 | AM | 30 | 135 | DL | 1 | 249 | AM |
| | 57 | 155 | DL | 100 | 249 | AM | 80 | 224 | DL |
| $2.5 \times 10^6$ $TCID_{50}$** SFV | 20 | 224 | DL | 5 | 199 | AM | 20 | 202 | DL |
| | 140 | 217 | AM | 96 | 176 | DL | 90 | 280 | AM |
| $2.5 \times 10^5$ $TCID_{50}$ SFV | 40 | 227 | AM | 39 | 140 | DL | 15 | 156 | AM |
| | 65 | 146 | DL | 110 | 257 | AM | 85 | 268 | DL |
| $2.5 \times 10^4$ $TCID_{50}$ SFV | 43 | 216 | DL | 20 | 182 | AM | 32 | 230 | DL |
| | 145 | 239 | AM | 110 | 215 | DL | 100 | 275 | AM |
| $2.5 \times 10^3$ $TCID_{50}$ SFV | 55 | 246 | AM | 46 | 170 | DL | 25 | 197 | AM |
| | 75 | 143 | DL | 120 | 200 | AM | 90 | 200 | DL |

* 0.5 ml. of WBC suspension mixed with 0.5 ml. of saline or 0.5 ml of virus dilution containing the number virus of particles listed in the first column of table. After incubation, approximately 0.1 ml. of the mixture of WBC and virus was made into a slide and the average WBC per high power microscopic field (470x) determined.

** $TCID_{50}$ = Tissue culture cytopathic infectious dose - 50 percent end point.

TABLE 6

WBC REACTIONS USING SEMLIKI FOREST VIRUS AS TEST ANTIGEN
(Hemacytometer Counting Procedure)

| WBC Source | Reaction Time (hr.) | WBC Counts* and Percent** of Control | | | | | |
|---|---|---|---|---|---|---|---|
| | | Saline (control) | Semliki Forest Virus Added to Cells ($TCID_{50}/ml$) | | | | |
| | | | $5 \times 10^6$ | $5 \times 10^5$ | $5 \times 10^4$ | $5 \times 10^3$ | $5 \times 10^2$ |
| SFV Immune Rabbit | 0 | 5.62* (100.0)** | 4.70 (82.6) | 5.36 (95.4) | 5.60 (99.7) | 5.60 (99.7) | 5.22 (92.8) |
| | 1¼ - 1½ | 5.18 (92.2) | 4.94 (87.8) | 5.32 (95.5) | 5.78 (102.8) | 5.04 (89.7) | 5.02 (88.2) |
| | 24 | 4.72 (84.0) | 4.66 (83.0) | | 5.42 (96.3) | | |
| Normal Rabbit | 0 | 3.84* (100.0)** | 3.68 (95.8) | 3.48 (90.6) | 3.40 (88.6) | 3.60 (93.8) | 3.24 (85.3) |
| | 1¼ - 1½ | 3.94 (102.6) | 3.50 (91.2) | 3.46 (90.1) | 3.20 (83.3) | 3.32 (86.5) | 3.08 (80.2) |
| | 24 | 2.94 (76.0) | 3.42 (88.1) | | 3.26 (85.9) | | |

* WBC counts expressed as millions ($10^6$) cells/ml.

** Percent of cell survival is based on cell count of saline control at 0 hr.

TABLE 7

WBC REACTIONS USING POLIOVIRUS 2 AS TEST ANTIGEN
(Hemacytometer Counting Procedure)

| WBC Source | Reaction Time (hr.) | WBC Counts* and Percent** of Control | | | | | |
|---|---|---|---|---|---|---|---|
| | | Saline (control) | Spent Tissue Culture Medium | Poliovirus 2 added to Cells ($TCID_{50}/ml$) | | | |
| | | | | $1.5 \times 10^5$ | $1.5 \times 10^4$ | $1.5 \times 10^3$ | $1.5 \times 10^2$ |
| Poliovirus 2 Immune Rabbit | 0 | 3.90* (100.0)** | 4.02 (103.7) | 3.76 (96.5) | 4.06 (104.0) | 3.82 (98.2) | 4.42 (113.2) |
| | 1½ | 3.94 (101.0) | 4.04 (103.5) | 4.10 (105.1) | 3.96 (101.6) | 3.62 (92.8) | 3.84 (98.5) |
| Normal Rabbit | 0 | 2.94* (100.0)** | 3.18 (108.3) | 2.88 (98.2) | 2.50 (85.1) | 2.56 (86.1) | 3.00 (102.1) |
| | 1½ | 2.64 (89.8) | 2.80 (98.7) | 2.72 (92.4) | 2.64 (89.8) | 2.78 (94.6) | 3.00 (102.1) |

* WBC counts expressed as millions ($10^6$) cells/ml.

** Percent of cell survival is based on cell count of saline control at 0 hr.

TABLE 8

WBC REACTIONS USING POLIOVIRUS 1 AS TEST ANTIGEN
(Hemacytometer Counting Procedure)

| WBC Source | Reaction Time (hr) | WBC Counts* and Percent** of Control | | | | | |
|---|---|---|---|---|---|---|---|
| | | Saline (control) | Spent Tissue Culture Medium | Poliovirus 1 Added to Cells ($TCID_{50}/ml$) | | | |
| | | | | $5 \times 10^5$ | $5 \times 10^4$ | $5 \times 10^3$ | $5 \times 10^2$ |
| Normal Rabbit | 0 | 2.96* (100.0)** | 2.90 (97.8) | 3.14 (106.1) | 3.10 (104.6) | 3.02 (102.0) | 3.02 (102.0) |
| | 1½ | 2.78 (95.8) | 2.84 (95.8) | 2.88 (97.3) | 3.04 (102.7) | 2.90 (97.8) | 3.06 (103.2) |
| Poliovirus 1 Immune Rabbit | 0 | 2.40* (100.0)** | 2.38 (95.1) | 2.24 (93.3) | 2.24 (93.3) | 2.56 (111.0) | 2.48 (103.3) |
| | 1½ | 2.30 (95.8) | 2.30 (95.8) | 2.30 (95.8) | 2.14 (89.2) | 2.42 (100.7) | 2.16 (90.0) |

* WBC counts expressed as millions ($10^6$) cells/ml.
** % of cell survival based on cell count of saline control at 0 hr.

TABLE 9

WBC REACTIONS USING POLIOVIRUS 1 AND POLIOVIRUS 2
AS TEST ANTIGENS
(Hemacytometer Counting Procedures)

| WBC Source | Reaction Time (hr.) | WBC Counts* and Percent** of Control | | | | | |
|---|---|---|---|---|---|---|---|
| | | | Virus Added to Cells (type and $TCID_{50}$/ml) | | | | |
| | | | Poliovirus 1 | | | Poliovirus 2 | |
| | | Saline (control) | $5 \times 10^5$ | $5 \times 10^4$ | $5 \times 10^3$ | $1.5 \times 10^5$ | $1.5 \times 10^3$ |
| Poliovirus 1 Immune Rabbit | 0 | 3.02* (100.0)** | 2.24 (74.3) | 2.36 (78.2) | 2.16 (71.5) | 2.60 (81.2) | 3.60 (119.2) |
| | $2\frac{1}{2}$-$3\frac{1}{2}$ | 1.30 (43.2) | 1.00 (33.1) | 1.16 (38.4) | 1.46 (48.4) | 1.14 (37.8) | 1.22 (40.4) |
| Poliovirus 2 Immune Rabbit | 0 | 3.46* (100.0)** | 2.76 (79.8) | 3.36 (97.1) | 3.68 (106.2) | 2.76 (79.8) | 4.18 (120.7) |
| | $2\frac{1}{2}$-$3\frac{1}{2}$ | 2.38 (68.8) | 1.64 (47.4) | 1.82 (54.3) | 1.88 (54.3) | 2.00 (57.8) | 1.92 (55.5) |

* WBC counts expressed as millions ($10^6$) cells/ml.
** Percent of cell survival based on cell count of saline control of 0 hr.

Table 6 illustrates data obtained when WBC counts were made using a hemacytometer chamber immediately and 1-1/4 to 1-1/2 hr. after the cells were mixed with saline or SFV. Five different concentrations of virus were tested against the WBC from a normal rabbit and against the WBC from an SFV immune rabbit. The WBC preparations used in this experiment were obtained from citrated whole blood, but the WBC had been washed and resuspended in Locke-Citrate plus serum from the rabbit supplying the WBC. (Final concentration of serum = 50 percent of diluent.) The WBC-virus and WBC-saline mixtures were slowly rolled at 37°C during the 1-1/4 to 1-1/2 hr. incubation. (A standard tissue culture roller drum was used for this mixing.) This method of testing did not permit us to obtain any observations of clumping since the cells were completely dispersed for the counts. Any change in count reflected a phagolytic effect, which is the type of reaction we had observed in the past. The data in Table 6 do not suggest any significant lytic reaction. In fact, the WBC counts are remarkably close when compared to the standard errors usually encountered in WBC counts. In the later counts made after the mixtures had been incubated, platelets were reduced and some of the SFV immune cells exposed to SFV were swollen or smaller than in earlier observations. The WBC counts made after 24 hr. at 37°C indicated good stability of these two WBC preparations. This WBC stability has not always been observed with incubated WBC.

Data for the reactions of poliovirus 2 and appropriate control solutions with WBC from a poliovirus 1 immune rabbit and WBC from a normal rabbit are presented in Table 7. These WBC counts were made using the hemacytometer technique and again no lytic effect of poliovirus 2 on immune WBC was detected. In fact, the WBC counts probably all fell within the standard error encountered in such tests. From this test we might consider the standard error to be about 15 percent, although we did not feel it was necessary to calculate the value. The WBC from this test were nearly all dead when checked with methylene blue on the following morning; therefore, they were not as stable as those used for the tests recorded in Table 6. The poliovirus 1 immune rabbit used for the WBC in the tests recorded in Table 7 had been injected with an aqueous suspension of antigen 20 days before she was bled.

Similar data are presented in Table 8 for WBC tests with poliovirus 1 using (1) normal rabbit WBC, and (2) WBC from a poliovirus 1 immune rabbit six days after an antigenic booster with poliovirus 1 in incomplete Freund's adjuvant. Again we obtained no evidence of a lytic action when immune WBC were exposed to virus. However, in coverslip preparations made from these same cells and virus preparations, the poliovirus 1 immune WBC demonstrated morphologic changes when exposed to a 1-100 dilution of poliovirus 1. Many of these cells became granular and had a gray sheen typical of the WBC damaged by virus (as observed in

earlier studies); however, high dilutions of poliovirus 1 and control preparations, saline or spent tissue culture medium, did not cause these WBC changes. Thus by morphologic changes we saw a positive reaction with a large amount of virus but still got no lytic changes in the immune cells.

Table 9 summarizes the data from another similar study in which we studied the homologous and heterologous reactions between poliovirus 1 and poliovirus 2 and the corresponding two types of immune rabbit WBC. In these studies the WBC were obtained from citrated blood with the aid of 2 percent (final concentration) of dextran (molecular weight 200,000 to 300,000). Again we obtained no significant evidence of a specific lytic action when cell counts were made. However, the observer of these slides felt that "there appears to be some reaction with the poliovirus 1 and poliovirus 1 immune cells." A second microbiologist set up the corresponding virus-cell mixtures in coverslip preparations and observed a positive morphologic change in the WBC in the poliovirus 1 and poliovirus 1 immune cell mixture but not in the other test mixtures. Thus, this test again gives a suggestion that WBC can be used to identify virus but the optimum conditions must exist for these observations to be possible. Dextran separated cells appear to be much more active (motile) and contain a larger variety of WBC types; however, they may be more fragile -- note the lower counts of WBC after 2-1/2 to 3-1/2 hr.

An additional phagocytic test for the identification of virus will be described later in this report under the discussion of fluorescent-labeled cells. (See page 37.)

f. Phagocytic tests with mouse WBC: In our previous studies using WBC from immune animals, we employed WBC obtained from rats after subclinical viral infection and from rabbits following hyperimmunization. In order to study WBC from another species and also WBC isolated from animals following a rapid active immunization, we investigated the use of mice. Separate groups of mice were immunized with poliovirus 1, poliovirus 2, and with spent tissue culture medium. Each mouse was given an intravenous injection on 30 January, 1 and 3 February 1967. Eleven days after the last injection, WBC were obtained by cardiac bleeding and used in phagolytic tests. The WBC from each mouse were separated by the dextran-citrate method described in our previous work.[1] These WBC suspensions were then tested in slide tests for their sensitivity to the homologous and heterologous antigens. Due to the small numbers of cells, the reactions could only be evaluated by the observation of morphological changes using 350 X bright field microscopy. Specific data will not be presented since no significant results were obtained. Occasional morphological changes similar to those seen in the earlier rabbit WBC tests were observed but no viral identification was possible by these cells. Because of the

difficulties of WBC preparation from mice, we feel that this method is not worthy of further evaluation.

g. Passive sensitization of WBC for viral identification: One partially successful experiment encouraged us to give further consideration to the passive sensitization of WBC for use in viral identification. WBC from a normal rabbit were divided and one-half incubated at 36°C with poliovirus 1 immune serum (rabbit). The other half of the WBC were treated with normal rabbit serum. When these cells were exposed to poliovirus 1 in microscopic hanging drop preparations, the WBC treated with poliovirus 1 immune serum behaved as if they were from a poliovirus 1 immune rabbit. The WBC treated with normal rabbit serum behaved as WBC from a normal rabbit. Spent tissue culture medium added to both types of treated WBC failed to elicit a response. Both a 1 - 10 and 1 - 100 poliovirus dilution produced the phagocytic WBC changes with the poliovirus antiserum treated WBC. Thus, $10^5$ and $10^6$ $TCID_{50}$ of poliovirus 1 produced the changes. Studies of the treated WBC with more dilute virus were prevented by clotting problems in the WBC preparation. Passive sensitization of WBC has the advantage of eliminating the need for immunized animals as a source of immune WBC.

It would greatly simplify the techniques and increase the possibility of rapid viral identification if WBC could be made sensitive to viral action by means of passive sensitization with known antisera. In the previous paragraph we indicated that one attempt at passive sensitization of WBC by poliovirus 1 immune rabbit serum was partially successful. Four more attempts were made to passively sensitize WBC with immune sera. Two experiments were made with poliovirus 2 antibody, one experiment with ppliovirus 1 and one experiment with SFV antisera.

In none of these studies were we successful in developing a method for passive sensitization of WBC capable of permitting viral identification; however, many procedures for studying this phenomenon were not tried. For poliovirus 2 identifications, we tried a partially purified immune globulin and a nonpurified rabbit antiserum as agents for passively sensitizing the WBC from a normal rabbit. The results were negative for all attempts including mixtures containing guinea pig complement. In another experiment, we attempted to sensitize WBC with rabbit antipoliovirus 2 antibody obtained on the same day the WBC were used, yet the WBC treated with this immune sera failed to provide viral identification. Neutralization on this antipolio serum demonstrated it to have a respectable antibody level; a 1 - 1280 dilution neutralized approximately 562 $TCID_{50}$ of virus. The sera and gamma globulins used in all the other tests had appreciable neutralizing titers. Thus, it may not be the level of virus

neutralizing antibody that is important but the type of antibody may be the critical factor. In the passive sensitization tests we have continued to encounter problems of clotting when the WBC are incubated with the sera. The use of a Locke-Citrate diluent[4] helps eliminate this problem but its action on the immune phenomena must be investigated further.

The antipoliovirus 1 antisera used for passive sensitization experiments on September 15, 1966, were obtained from the same bloods that served as the source of the immune WBC which were sensitive to poliovirus 1 in lytic tests on August 26, 1966. Thus, our negative results with passive sensitization were independent of the fact that the antisera used in the tests had come from the same rabbits that had demonstrable activity in WBC lytic tests. The temperature, time, and method of passive sensitization of WBC need considerable investigation. The temperature and times of incubation of WBC in antisera for antibody adsorption were limited by the clotting problems mentioned earlier.

The rabbit antiserum to SFV used in the passive sensitization experiments had not been titered for neutralizing antibody, but should have contained significant antibody. It was obtained from a rabbit which had earlier been demonstrated to have neutralizing antibody. This rabbit had been given a number of booster doses of SFV between the original antibody titration and time it was bled for antibody to be used for the passive sensitivity WBC tests. This serum also came from the same rabbit which had given highly sensitive WBC in the lytic reactions reported previously. As reported in the previous paragraph (which discussed poliovirus 1 passive sensitization) the presence of SFV neutralizing antibody in a serum does not mean that it is a good reagent for passive sensitization of WBC.

h. Use of fluorescent dyes: As an approach to help or even eliminate the subjective observations of these microscopic tests, we investigated the use of two fluorescent dyes: acridine-orange (AO) and fluorescein diacetate (FDA). Acridine-orange has been used for following cellular changes particularly of neoplastic cells.[6] It causes the WBC in blood to fluoresce, yet the red blood cells in the same smears do not fluoresce.[7] (The red cells may give a faint red-brown background, depending upon the light conditions.)

Fluorescein diacetate has been observed to be taken up by cells of a number of types.[8] The FDA is then hydrolyzed and the accumulation of the fluorescein within the cell results in the fluorescence of the cell. FDA treated cells are still viable and may be cloned after fluorescent treatment.[8]

Bertalanffy[6] and others have used acridine-orange as a fluorescent cellular dye but most, if not all, of the work has been on fixed preparations. We tried to use the fluorescent dye as a label for viable cells.

Preliminary fluorescent dye studies were done with three types of cells: WBC, WISH human amnion cells, and peritoneal exudate cells from L1210 leukemia in DBA mice. Saline (0.85 percent NaCl), M/15 phosphate buffer (pH 6.0), pH 7.0 saline-glycerine buffer, and M-199 have been satisfactorily used as diluents for AO; but pH 7.4 tris buffer was not satisfactory. With tris buffer the cells were fluorescent, but a fuzzy halo effect around each cell was obtained and caused difficulty in observation. For most of our AO fluorescent studies we used 0.85 percent saline as diluent. Phosphate buffered saline was used as the diluent for the fluorescein diacetate studies because it was used by the original investigators.[7] It appeared to work quite satisfactorily for staining WBC. For microscopic observation of fluorescent cells we used the dark field condenser and a mercury arc lamp equipped with an ultraviolet filter transmitting light at 365 mμ (B & L #7-51 excitation filter). T-2 barrier filters (B & L) are used in the eye-pieces.

By titration studies we found that an acridine-orange concentration of 0.001 percent produced good fluorescence when mixed with an equal volume of cells. 0.0002 percent AO dyed the cells but not as strongly as did 0.001 percent and the fluorescence required a few minutes to develop. Only an occasional cell became fluorescent after 0.00004 percent AO treatment, and this was weak and required a half hour or so to develop. Similar data on FDA indicated that either 0.5 μg. or 0.1 μg/ml produced good fluorescence in cells. The fluorescence was observed immediately with the higher concentration, but 2 or 3 min. were required with the 0.1 μg/ml dye. Even after a number of minutes 0.02 μg/ml FDA only caused an occasional cell to fluoresce. Thus at least 0.1 μg/ml FDA and 0.0002 percent AO were necessary for good specimen observations. The fluorescence observed with FDA appeared to be slightly superior to AO, but much needs to be learned concerning both reagents.

Cells treated with either of the two fluorescent dyes appeared to be viable since we watched cellular changes take place and amoeboid movement was not stopped.

Earlier in this report we mentioned the fluorescent read-out of the various WBC reactions. These were made possible by incorporating one drop of the 0.001 percent AO in each slide preparation used for fluorescent observation. We feel that considerably more data are needed by this method before it can be adequately evaluated. The biggest handicap of these

fluorescent microscopic read-outs of WBC reactions was the inability to see the cytoplasmic changes that occurred but which could be seen by bright field microscopy.

Several investigations were made to determine if the two fluorescent dyes could be incorporated into cells, and a subsequent dye release monitored. Use of the Aminco-Bowman Spectrophotofluorometer for following such reactions was studied since it permits an exceedingly sensitive read-out of a fluorescent material.

WISH human amnion cells were treated with AO, washed twice, and then frozen and thawed. After centrifugation to sediment the cell fragments, acridine-orange fluorescence was observed in the supernatant of the frozen and thawed cells.

In several other experiments, (1) WBC treated with fluorescent dye and (2) WBC treated with diluent only were washed twice, submitted to three cycles of freezing and thawing, and the cell sediments removed by centrifugation. The washes and the supernatants from the disrupted cells were checked for fluorescence by means of the Aminco-Bowman Spectrophotofluorometer (SPF). Plots of the emission spectra were made by means of an automatic recorder (Electro Instruments, Inc.). The charts thus obtained permitted comparison of the various fractions. In some cases it was possible to compute the relative intensity of the fluorescence.

With acridine-orange the optimal excitation wavelength was found to be 365 mμ and the peak emission was obtained at 525 mμ. Saline-glycine buffer for the acridine-orange studies caused some problems since an extra fluorescence peak at 425 mμ occurred. However, the presence or absence of AO could be detected by the occurrence of a "hump" or second peak at the 525 mμ emission band. This problem was circumvented by use of M/15 phosphate buffer. In the WBC tests with AO dye the fluorescent intensities were fairly low but we succeeded in demonstrating the release of the fluorescent dye from the intact cells by the freezing and thawing procedure. It appeared that 0.00005 percent AO was close to the minimum AO detectable by the SPF. Although we were able to detect release of AO from frozen and thawed cells, we were measuring that amount of dye released from a large number of cells ($50 \times 10^6$ cells).

In similar WBC studies with FDA as the fluorescent dye, the fluorescence measurements were more intense, easier to quantify, and perhaps more adaptable to smaller numbers of WBC. In addition, ways exist to increase the sensitivity of fluorescein assay by several orders of magnitude over those used in the experiments. As pointed out earlier, the FDA was hydrolyzed by the cells to yield fluorescein, which was the product observed

or measured. Thus our measurements with the SPF were done by excitation at 490 mμ and the peak of emission was observed to be between 510 to 525 mμ. At these excitation and emission wavelengths control (non-dyed) cells exhibited no fluorescence.

From the FDA studies it was very apparent that the WBC readily picked up the FDA and hydrolyzed it rapidly. The WBC containing the fluorescein could be separated and the fluorescein liberated by freezing and thawing. The cell stroma or sediment did not retain any fluorescence and was negative by the SPF assay. A complication encountered in these studies must be mentioned. Immediately after the WBC were treated with FDA, the supernatant became fluorescent. This was apparent even to the naked eye. Part of this conversion of FDA may have been due to WBC enzymes and part may have been due to esterases present in the serum. Additional studies are necessary to determine the effects of the various enzyme systems on the conversion of the non-fluorescent FDA to the fluorescent fluorescein. Studies are also needed on washed WBC which are free of the esterases from serum.

Although we cannot yet cause a consistent release of a fluorescent material from WBC by viral action, we now have two compounds which can be worked with at the same time and assayed fluorometrically. The assays can be done spectrophotometrically by using two different excitation wavelengths. Thus these two compounds, AO and FDA, might be used as labels for WBC immune to different viruses if such WBC can be used for viral identification. These two dyes must be considered along with the fluorescent plastic particles previously studied[1] as possible means for monitoring WBC lysis.

i. Fluorescent-labeled protein pickup by WBC: In order to learn more about the pickup of protein by WBC, we have studied the adsorption and/or absorption of fluorescent-labeled proteins by rabbit WBC. Information from such studies was desired in order to better control the passive sensitization of normal WBC. It also had potential value in our labeling of immune WBC since a fluorescent read-out could enhance the sensitivity of the lytic or other WBC reactions for viral identification. In earlier studies we observed that WBC could be made fluorescent with acridine-orange and with fluorescein diacetate. Acridine-orange has the disadvantage that it may be damaging to the WBC. Fluorescein diacetate is hydrolyzed by the cell esterases and this is difficult to control or monitor in actively metabolizing WBC.

We studied rhodamine-labeled bovine serum albumin, fluorescein isothiocyanate (FITC) labeled human serum albumin, FITC labeled rabbit serum, and FITC labeled calf serum. The latter two were prepared by us at

MRI for this project. The FITC-labeled human serum albumin was prepared by Dr. D. A. Ringle for another MRI project. The rhodamine-labeled bovine albumin used was the commercial product of Microbiological Associates and it is regularly used as a counter stain for fluorescence microscopy.

Normal rabbit WBC separated by four different procedures, including citrate, dextran, and combinations of the two, were treated with rhodamine-labeled bovine albumin at two different temperatures and the cells observed with the fluorescence microscope. Rhodamine bovine albumin was successfully used to label WBC. The method of WBC separation did not affect the fluorescent labeling. The rhodamine is probably both on the surface and taken up internally by the WBC. The labeling was rapid and occurred at both room temperature and at 37°C. It was stronger and more complete at 37°C and also stronger after longer incubation, i.e., 1 hr. stronger than 20 min. By proper light filtration only the rhodamine-labeled cells were observed through the fluorescent microscope (T-2 and 7-37 filters, B & L). By substituting the D-2 (density filter) for the 7-37 filter, the WBC can be differentiated into several types; also platelets and the red blood cells can be observed. This last combination of filters (T-2 and D-2) permits observation of both natural and rhodamine fluorescence. Also by the use of the D-2 and T-2 filter combination, the natural fluorescence of the unlabeled cells was visible in the fluorescence microscope. This method of observation may be valuable for WBC work even in the absence of a fluorescent compound such as rhodamine.

The normal rabbit WBC failed to pick up the FITC fluorescent-labeled human serum albumin; therefore, it was not useful for passive labeling of WBC.

Normal rabbit serum and normal calf serum were conjugated with FITC by a modification of the method of Spendlove[9] and were freed of unconjugated FITC by dialysis and passage through a Sephadex-G25 column. In our studies neither of these two conjugated proteins was picked up by normal rabbit WBC during incubation at room temperature or 37°C (1-3/4 hr.). The addition of normal rabbit serum as a source of "accessory" factors for the phagocytic reaction did not help bring about the uptake of the labeled serum. Thus, the FITC conjugated sera, one homologous for rabbit WBC (rabbit serum) and one heterologous to rabbit WBC (calf serum) were neither one adsorbed onto or taken into the normal rabbit WBC. The FITC-labeled sera, therefore, behaved as did the FITC-labeled human albumin. These results are in contrast to positive phagocytosis by WBC of rhodamine-labeled bovine albumin.

The significance of these observations must await further investigations. However, Parker and Schmid[10] reported that particulate complexes of labeled γ-globulin and rheumatoid factor were required before phagocytosis occurred. They found that fluorescein-labeled globulin alone was not phagocytized. The rhodamine bovine albumin used by us did have a slight turbidity but none of the FITC-labeled proteins had noticeable turbidity. Our results might thus be correlated with this difference in phagocytosis depending upon the presence or absence of particulates. If this is true, then we can readily overcome the failure to label the WBC by using particulate complexes. Plastic particles, bentonite, or antigen-antibody complexes such as were used in our earlier studies,[1] combined with or reacted with a fluorescent-labeled antibody may be readily phagocytized. Thus by this method passive sensitization or labeling of WBC may be accomplished.

After the above observations, we repeated our previous study of trying to label the normal WBC with 1.1 μ ellipsoidal fluorescent plastic particles. This was rapidly accomplished and the WBC still appeared to be viable.

j. Use of rhodamine-labeled bovine albumin as an indicator for WBC reactions: WBC from one poliovirus 1 and one poliovirus 2 immune rabbit were obtained by the citrate-2 percent dextran method, and these were used in viral identification tests. Rhodamine-labeled bovine albumin (as described in previous section) was added to each WBC preparation. After 25 to 85 min. at room temperature -- to permit fluorescent labeling of the WBC -- these rhodamine-labeled cells were used to make coverslip preparations by mixing immune cells and virus. These WBC preparations were not highly reactive when tested with virus. The poliovirus 2 immune WBC were affected by 1 - 10 poliovirus 2 and this could be observed in the fluorescence microscope by following cellular changes with the rhodamine fluorescence. WBC disappeared and those which remained were elongated and swollen, and clumps were observed. WBC changes also were observed by fluorescence microscopy for poliovirus 2 immune WBC treated with 1-100 poliovirus 2 but these changes were certainly insufficient for viral identification purposes. Similar morphologic changes in non-fluorescent-labeled poliovirus 2 immune WBC were observed by bright field microscopic observations set up simultaneously with the tests using the rhodamine-labeled WBC.

Complement lysis and cellular changes in rabbit WBC were successfully followed using rhodamine-labeled bovine albumin treated WBC.

k. Phagocytic tests with coated and uncoated particles: Earlier in this report we presented evidence and cited literature indicating that WBC did not appear to pick up antibody or labeled protein unless the foreign material was particulate in nature. Experiments were conducted to determine whether it might be possible to transport serum proteins into the WBC, i.e., passively sensitize the WBC, by using bentonite or fluorescent plastic particles as particulate carriers of the protein.

Bentonite particles were prepared as described by Bozicevich et al., 11/ using Bentone 38 (National Lead Company) as the starting material. Bentone 38 particles are 0.5 to 1.0 μ in size. Aliquots of these bentonite particles were coated with (1) fluorescein isothiocyanate (FITC) labeled normal rabbit serum, and (2) FITC-labeled normal calf serum. Room temperature adsorption for 30 min. was used. This procedure was similar to that employed by Potter and Stollerman in their studies on phagocytosis of bentonite-coated particles. 12/ In slide reactions between normal rabbit WBC and both the FITC-labeled normal rabbit serum and FITC-labeled normal calf serum, we failed to observe a significant phagocytic pickup of the coated bentonite particles. Our observations were made by means of the fluorescent microscope and the WBC were observed after incubation for 1 hr. at room temperature had been allowed for the particle pickup to take place. Thus, our results are somewhat different than Potter and Stollerman obtained with non-FITC-labeled γ-globulin. They had obtained phagocytosis of globulin-coated particles. However, they observed wide variations in phagocytosis of globulin-coated bentonite particles depending upon the source and method of processing the globulin.

We made a similar study using WBC from a normal rat. Bentonite particles in three different buffers (glycine, phosphate, and Locke-Citrate) were coated with normal rabbit serum. The serum-coated bentonite particles were then added to aliquots of the normal rat WBC. The phagocytic pickup was checked immediately and again after 1-1/2 to 2 hr. incubation. With the bright field microscope, we observed the bentonite particles in and around the cells immediately after mixing. After incubation, methylene blue staining of the WBC demonstrated numerous bentonite particles in the large WBC and lymphocytes. The WBC suspended in the glycine and Locke-Citrate buffers were more active than WBC in the phosphate buffer. The WBC pickup of the serum-coated particles was also checked by fluorescence microscopy. To do this we added a drop of FITC-labeled antirabbit globulin to the WBC mixtures and observed the fluorescent staining of the particles and cells. By this fluorescent staining procedure, we could demonstrate that the serum-coated particles were quickly attached to the outside of the rat WBC and by 1-1/2 to 2 hr. large aggregates of particles were observed inside the WBC.

Based on the varied results obtained with these bentonite particles, it appears that the FITC treatment of the serum before it is adsorbed on the bentonite particles may prevent the phagocytic pickup of the coated particles. A second factor may have also influenced the WBC reactions with the coated particles. This related to the use of serum homologous or heterologous to the WBC for coating the particles. In the one experiment rabbit WBC failed to pick up bentonite particles coated with rabbit serum. Thus, the WBC might have considered the rabbit serum as homologous; therefore, the particles were not picked up. In the rat WBC studies, the rabbit serum (on the bentonite particles) was heterologous; therefore, the rat WBC phagocytized the coated particles due the "foreign" protein. However, we had expected the WBC used in the first experiment to phagocytize the bentonite particles coated with calf serum, since the calf serum protein was "foreign" to the WBC. Yet this did not occur.

In two other experiments WBC were tested for the pickup of fluorescent plastic particles. Contrasting results were obtained. WBC from a normal rabbit were tested against three concentrations (0.1 mg/ml, 0.01 mg/ml and 0.001 mg/ml) of plastic particles in pH 8.2 glycine buffer. Practically no particles were phagocytized. When similar tests were made with WBC from a normal rat, phagocytosis of the fluorescent plastic particles was observed nearly immediately after the addition of the particles to the cells and after 1-1/2 hr. incubation at 37°C the WBC contained numerous fluorescent particles. These phagocytized particles were released when the rat WBC cells were lysed by the addition of guinea pig complement. It should be pointed out that about 50 percent of the rat WBC containing phagocytized fluorescent plastic particles were still viable after 20 hr. at 37°C.

It became readily apparent from these studies with fluorescent plastic particles that considerable variation in phagocytic action occurred.

Shortly after these phagocytic tests were done, the article by Bona et al.[13] appeared. Their interesting results demonstrated that phagocytic cells are capable of recognizing as "foreign" altered autologous constituents but the alteration must be "adequately" displayed. We believe that to have satisfactory passive pickup of antibody by WBC the antibody will have to be "foreign" to the WBC; therefore, we should use a heterologous antibody for the WBC, i.e., antibody from one species and WBC from another species.

1. Use of antiglobulins to study WBC: Two experiments were performed to test whether FITC-labeled antirabbit globulin (of goat origin) could be used to locate rabbit globulin in normal rabbit WBC and in rat WBC passively sensitized to rabbit serum. WBC were obtained from a normal rabbit using two different WBC separation procedures. Dextran (200,000 - 300,000 molecular weight) was used to separate the WBC in one fraction of citrated blood. The WBC suspension was then divided and part of the cells were washed to get rid of the dextran and plasma; the other fraction of WBC was left in the dextran-citrate diluent. WBC were also obtained by differential centrifugation of citrated whole rabbit blood. Suspensions of each of the three WBC fractions were then exposed to the action of FITC-labeled antirabbit globulin. Immediately after the addition of the labeled antiglobulin, aliquots of each suspension were observed under the fluorescent microscope and only a rare WBC exhibited fluorescence. After 1-1/2 hr. of incubation on a roller drum at 37°C, aliquots of each suspension were again observed and all three preparations had large clumps of fluorescent material. The cells were even obscured by the material. This material was granular and was a typical antigen-antibody precipitate; thus the antiglobulin had reacted with the normal rabbit serum (globulin) present in the suspension and on the surface of the WBC. However, the precipitate prevented adequate determination of where the reaction took place. After 20 hr. incubation at 37°C, fluorescent observations were again made of the treated WBC suspensions and fluorescent particulates were observed inside the WBC. All cells separated by the differential centrifugation citrate method were dead but many of the dextran separated cells were viable and contained fluorescent granules both inside and on the surface of the WBC. Thus we did get a reaction between the FITC-labeled antirabbit globulin and the globulin present in or around the normal rabbit WBC; however, the results of these tests were of little value in our passive sensitization work.

In studies with rat WBC passively sensitized with normal rabbit serum, three different suspending fluids were used for the WBC. (These WBC were obtained by the dextran-citrate procedure.) WBC packed and re-suspended in (1) Locke-Citrate buffer; (2) medium 199, pH 7.0; and (3) 0.85 percent NaCl were each passively sensitized with normal rabbit serum by incubation at 37°C for 1-1/2 hr. Aliquots of each suspension of WBC were treated with a 1-100 dilution of FITC-labeled antirabbit globulin. This FITC-labeled antirabbit globulin was used to serve as an indicator of the location of the normal rabbit serum used to passively sensitize the WBC. By fluorescent microscopy, we were able to show that the normal rabbit serum appeared to be attached to the surface of many of the WBC. The small WBC (lymphocytes) appeared to pick up more serum (or stain brighter) than the larger WBC.

From these two studies, we feel that the use of FITC antiglobulin will only be helpful in passive sensitivity studies for systems in which a heterologous serum is used to sensitize the cells. Even then the antiglobulin appears to us to have limited use or value.

m. Skin tests for assessment of rabbit immunity: We had wondered if there was a way to predict a good WBC rabbit source so we skin-tested all of our immune rabbits to determine if skin reactivity might be used an an indicator. Each immune rabbit was shaved and undiluted, 1-10 and 1-100 dilutions of the corresponding immunizing antigen were injected intradermally (0.1 ml. per injection). After 24, 48, 96, and 144 hr., erythema, edema, and hemorrhage were measured for each site of inoculation. No induration nor necrosis occurred at any injection site. The complete results will not be given in this report but merely summarized. All immune animals responded with erythema and most of them with edema; however, the degree of reaction was much more pronounced with the SFV immune rabbits. Only one poliovirus immune rabbit responded to the 1-100 dilution of virus. Approximate readings for the animals are tabulated as follows:

| | Virus Dilution Injected | | |
|---|---|---|---|
| | Undiluted | 1-10 | 1-100 |
| Poliovirus 1 - Immune Rabbits | | | |
| Erythema | 8-15 mm. | 6-10 mm. | 0-6 mm. |
| Edema | 5-15 mm. | 0-10 mm. | 0 mm. |
| Poliovirus 2 - Immune Rabbits | | | |
| Erythema | 7-16 mm. | 0-8 mm. | 0 mm. |
| Edema | 4-13 mm. | 0-Trace | 0 mm. |
| SFV Immune Rabbits | | | |
| Erythema | 29-33 mm. | 9-17 mm. | 3-9 mm. |
| Edema | 25-32 mm. | 7-12 mm. | 0-8 mm. |

It appears that all the rabbits had skin sensitivity to their corresponding antigens; however, we could not predict any difference in reactivity of WBC from any single rabbit upon the basis of these tests. The SFV immune rabbits were more skin sensitive probably due to the presence of a small amount of serum in the antigens used to immunize them. It would have been desirable to test the SFV rabbits with mouse brain SFV, but we did not want to cause them to develop a WBC sensitivity to brain tissue such as the SFV in mouse brain. These skin tests only indicated

that the animals were sensitive to the antigen injected. Whether this antigen was the virus per se was not proved. These data were not suprising since we knew the animals all possessed neutralizing antibody to the virus used to immunize them; however, skin sensitivity is more closely related to the hypersensitive state than the mere presence of antibody. We believe the WBC reactions will be more likely in a hypersensitized animal than in just an immune animal. Our rat WBC work previously reported bears on this point.

n. In vivo labeling of WBC: Two simple experiments were performed to see if it were possible to label the WBC in vivo by the intravenous injection of fluorescent particles or a fluorogenic compound. Thirty minutes after the intravenous injection of mice with 0.2 to 0.25 ml. of a suspension of fluorescent plastic particles (1.1 μ ellipsoidal particles) no fluorescent particles were observed in blood smears examined with the fluorescent microscope. However, smears prepared by mashing a small piece of spleen tissue between two slides revealed numerous fluorescent plastic particles in the splenic tissue. These particles were apparently in the splenic macrophages and also seemed to concentrate in the splenic nodules. These results are not surprising since the spleen is the main reticuloendothelial organ for removal of foreign materials. Liver smears prepared from these mice also contained fluorescent particles but not to the extent seen in the spleen. These results are quite interesting when one considers that the spleen became very fluorescent after the injection of only 20 μg. - 25 μg. of the plastic particles. We believe these fluorescent plastic particles will make ideal RES clearance materials but they will not label the circulating WBC for more than a few minutes.

In the other in vivo labeling study, we utilized a dilute solution of fluorescein diacetate (FDA). This compound is fluorogenic and fluorescein produced by its hydrolysis fluoresces. FDA was dissolved in acetone and then further diluted in 0.85 percent NaCl to a final concentration of 0.05 mg/ml. This solution (0.1 ml.) was injected intravenously into each mouse without obvious harm. Thirty minutes later blood smears and spleen pulp smears were prepared and examined with the fluorescent microscope. If the FDA had been taken up by the WBC and hydrolyzed we would have expected to see some fluorescence in the blood smears. This did occur but only a few cells were fluorescent. Since FDA has only limited solubility under aqueous conditions, we suspected that the material became insoluble in the blood stream and w s then taken up as a "particulate" which was later converted within the cells to the fluorescent compound. Similar fluorescent cells or aggregates were observed in the spleen smears, but again relatively few cells or nodules contained the fluorescent material.

The above results may have valuable applications in other work but not for reaching the goals of this project.

o. Lysis of WBC by guinea pig sera: In the previous sections of this report, we have frequently mentioned the use of guinea pig serum as a source of complement and/or other heat-labile components. The use of such normal guinea pig sera to enhance lytic reactions has been reported for many antigen-antibody systems and no attempt will be made at this time to review the literature on the effects of complement and/or guinea pig heat-labile substances. However our studies, particularly with the rat WBC, indicated that we must be concerned with the potential cellular changes induced in WBC by normal guinea pig sera and the effects of complement on the immune reactions involving WBC.

(1) Effect of guinea pig sera on rat WBC: Earlier in this report we noted that rat WBC were lysed rapidly by normal guinea pig sera. WBC from both normal and immune rats were affected and all the WBC were lysed within a matter of minutes after the guinea pig sera were added to the WBC. The guinea pig sera were obtained from normal guinea pigs and had been processed so as to retain a high complement level, i.e., the sera were separated rapidly and frozen immediately after they were separated from the cellular components of the blood. In the rat WBC studies reported, two separate guinea pig sera were tested and both were highly lytic to the rat WBC. WBC from two different rats at each of two different bleedings were lysed; therefore, we felt additional investigation was needed concerning the problem. Although the literature has not been fully checked, we have not found a specific reference to this particularly lytic phenomenon. Walford[14] in his book "Leukocyte Antigens and Antibodies" discusses leukolysins and mentions that a rapid leukolytic reaction occurs with the blood of normal humans when suspended in sera of certain other animals. However, these comments and the other references to leukolysins in Walford's book dealt mainly with human white cells.

The rats used in the SFV studies cited in the previous paragraph were Carworth Farms All-Purpose Albinos. The guinea pigs used as a source of complement were "run-of-the-mill" mixed breed animals. Since a group of normal guinea pigs from the Hartley Strain and a group of normal Charles River Albino rats were avilable, we conducted experiments to determine whether the lysis of rat WBC by guinea pig serum was observed with WBC and sera from animals of these other sources.

The WBC from each of six Charles River Albino rats were rapidly lysed by the two guinea pig preparations used in our SFV work. In most of these slides the WBC were completely lysed in 10-15 min. Inactivation of the guinea pig serum at 56°C for 30 min. prevented the lytic effect

of the guinea pig serum, but the inactivated serum caused a leukoagglutination of the WBC and large clumps of WBC were quickly formed when rat WBC were treated with inactivated serum. Dilutions of 1-10 or higher of the non-inactivated guinea pig serum eliminated the lytic and agglutinating property of the guinea pig serum.

Normal sera from each of four Hartley Strain guinea pigs and two run-of-the-mill guinea pigs lysed the WBC from two Carworth Farm All-Purpose Albino Rats. Therefore, it appears that the phenomenon of rat WBC lysis by normal guinea pig sera is not peculiar to the animals used in our SFV studies. This lytic problem was also one of the prime reasons for relegating rat WBC studies to a lower priority for viral identification.

(2) Effect of guinea pig sera on rabbit WBC: Problems with non-specific lysis of rabbit WBC were not encountered in our early work; however, during the later studies we had a number of rabbit WBC preparations which were lysed by normal guinea pig serum (as a source of complement and/or heat-labile components).

In our earlier studies, the WBC preparations were not packed and then resuspended in a solution free of anticoagulant. During these later studies most of the WBC suspensions were freed of the anticoagulant by an extra sedimentation, decantation, and resuspension in 0.85 percent NaCl (saline) or similar solutions. Such "washed" WBC suspensions were found to be quite susceptible to lysis by fresh normal guinea sera. This guinea pig serum effect was removed by heat inactivation (56°C for 30 min.) but inactivated serum retained some leukoagglutinating activity for rabbit WBC. A fivefold or higher dilution of the guinea pig serum prevented its lytic action on rabbit WBC.

The enhancing effect of guinea pig complement in some of our earlier studies was quite beneficial to the read-out; however, in the light of our later observations on the lytic effect of guinea pig sera on WBC, both rabbit and rat, care must taken in the planning and interpretation of WBC experiments. Our recent observations do not invalidate any of our earlier observations, since the controls run with each test were sufficient to delineate any non-specific guinea pig serum effects.

## 3. Conclusions

In vitro phagocytosis experiments have been conducted in an effort to develop a procedure which will be useful for the identification of 100 or fewer virus particles in 6 hr.

WBC from rabbits immune to poliovirus 1, poliovirus 2, and to Semliki Forest Virus have been studied for their ability to indicate the presence of the specific virus for which the cells had been actively sensitized. By the microscopic observation of the morphological changes of these immune WBC after they are exposed to the specific virus we have been able to identify 250 to 1,000 $TCID_{50}$ of polioviruses (both types 1 and 2) and Semliki Forest Virus. However, the regularity of such positive identifications has been poor. Numerous variables affect the capacity of the WBC to elicit such a specific identification. Many of these variables reflect the conditions used to immunize the animal supplying the WBC. Our studies have been conducted with WBC from animals known to contain antibody to the viruses, yet we cannot correlate our successes or failures with antibody levels. Still other variables relate to the in vitro portion of the procedure. Methods of WBC separation, the diluents, and other reagents are only a few of the variables encountered in the final phases of the experiments on viral identification. As mentioned in this report certain accessory factors such as guinea pig complement or serum have varied effects on the test system.

A number of attempts to quantify the WBC reactions between poliovirus 1, poliovirus 2, and Semliki Forest Virus with the corresponding immune WBC have not resulted in positive identification of the viruses studied. However, by use of a hemacytometer-counting technique we have reduced the variations in WBC tests due to the number of cells present at the time of read-out. We attribute the failure to identify the viruses in these studies to the lack of sensitivity of the WBC preparations and/or other factors which are as yet unknown. The WBC preparations also failed to exhibit the same degree of morphologic changes which have been observed in previous studies and by which we have been able to identify 250 to 1,000 $TCID_{50}$ of virus.

Data obtained by skin tests in the immune rabbits indicate that the animals are hypersensitive to the antigens but we have not yet been able to utilize this information to help improve the identification procedures by proper selection of WBC source animals.

We also have varied the timing and method of antigenic stimulation of the rabbits without finding the best method to maintain or increase the sensitivity of the WBC preparations. It appears that considerable investigation will be required to determine the optimum procedure for obtaining satisfactory immune WBC for in vitro viral identifications.

Data were obtained on the preparation and use of fluorescent-labeled proteins in the virus identification procedures using WBC. In studies with rhodamine-labeled bovine albumin we observed the active

phagocytosis of the labeled protein by normal WBC and morphologic changes in immune WBC when they were exposed to virus. In contrast to these positive results, our studies with three FITC-labeled proteins (human serum albumin, normal rabbit serum, and normal bovine serum) have been disappointing since the FITC-labeled proteins were not phagocytized by normal WBC.

Studies on the phagocytic pickup of serum-coated bentonite particles indicates that passive sensitization of WBC from normal animals by means of such bentonite particles will be influenced by the type of serum used. Apparently, the WBC recognize the difference between homologous and heterologous serum adsorbed to the particulates. Particles coated with heterologous serum (such as the antiserum to a virus) will be phagocytized better than particles coated with serum homologous to the WBC. The FITC labeling of serum may alter its ability to be used in passive sensitization studies.

We observed wide variation of the phagocytic pickup of fluorescent plastic particles by individual WBC preparations without any obvious reasons for the different WBC reactions. With rat WBC, it was possible to follow release of the phagocytized fluorescent particles due to guinea pig serum lysis of the rat WBC.

WBC from mice actively immunized against poliovirus 1, poliovirus 2, and spent tissue culture fluid failed to permit viral identification by the in vitro phagolytic test procedure. Thus, with another species of animals and a rapid method of immunization (intravenous), we have not improved our possibility for developing this method of viral identification.

Antiglobulins tagged with FITC have been used to locate the serum globulin in normal and passively sensitized WBC; however, this method has definite limitations. One of the limitations is the precipitation reaction occurring if homologous serum is used to passively sensitize the cells or if the homologous serum has not been completely washed away from the WBC.

Intravenous injection of fluorescent plastic particles and of a fluorogenic compound failed to give a good in vivo method of tagging WBC. However, the injection of the 1.1 mμ ellipsoidal fluorescent plastic particles has been found to be a potential new and novel way of following RES clearance.

All of the data on the in vitro phagocytosis method for viral identification emphasizes the variability and difficulties to be encountered

in the use of such live cell methods. With sufficient work and proper conditions, this method may eventually be applied for viral identification; however, we are not optimistic that the variability can be overcome without considerable more research than was possible within the time limits of the current contract. Thus, with the encouragement and advice of our sponsors, we redirected our efforts during the latter part of the contract period to investigations of other methods, such as immunodiffusion and means of measuring soluble virus-antibody reactions.

## B. Synthetic Phospholipid Membranes

### 1. Introduction

The use of phospholipid bilayer membranes for immunological identification of virus is based on the following assumptions: (1) that an antigen-antibody-complement complex results in the formation of a phospholipase from one of the complement components; (2) that the phospholipase mentioned above will react with the phospholipids of the bilayer membrane and result in the transformation of these phospholipids into their lyso-derivatives (e.g., lecithin transformed into lysolecithin); (3) that the resulting lysolecithin or other lyso-compounds will alter the bilayer membranes, either by promoting rupture of the membranes or by altering their electrical characteristics.

Recent studies on phospholipid preparations have shown that it is possible to prepare bilayer phospholipid membranes 0.5 to 2.0 mm. in diameter which are relatively stable and which exhibit a high resistance to current flow.[15-17] (See Fig. 1.) Membranes as large as 10 mm. in diameter have been made.[15] Although these membranes are only about 60 Å thick, they offer a DC resistance of 0.2 to 4.0 meg $\Omega/cm^2$ and can withstand voltages up to approximately 200 mv. Thus, by monitoring the resistance and other electrical characteristics across apertures bearing these membranes, it is possible to detect changes in structure and composition of the membranes. Rupture of the membranes is, of course, accompanied by a rapid drop in resistance.

A number of investigations have shown that one of the components of complement may, in the presence of an antigen-antibody complex, be transformed into an enzyme, phospholipase.[18,19] There is evidence that a complement-derived phospholipase is responsible for the lysis of erythrocytes (for a brief review see Phillips and Middleton),[20] although some workers have questioned the role of phospholipase in complement-associated cell lysis.[20,21] Nevertheless, Klibansky and deVries[22] have shown an effect

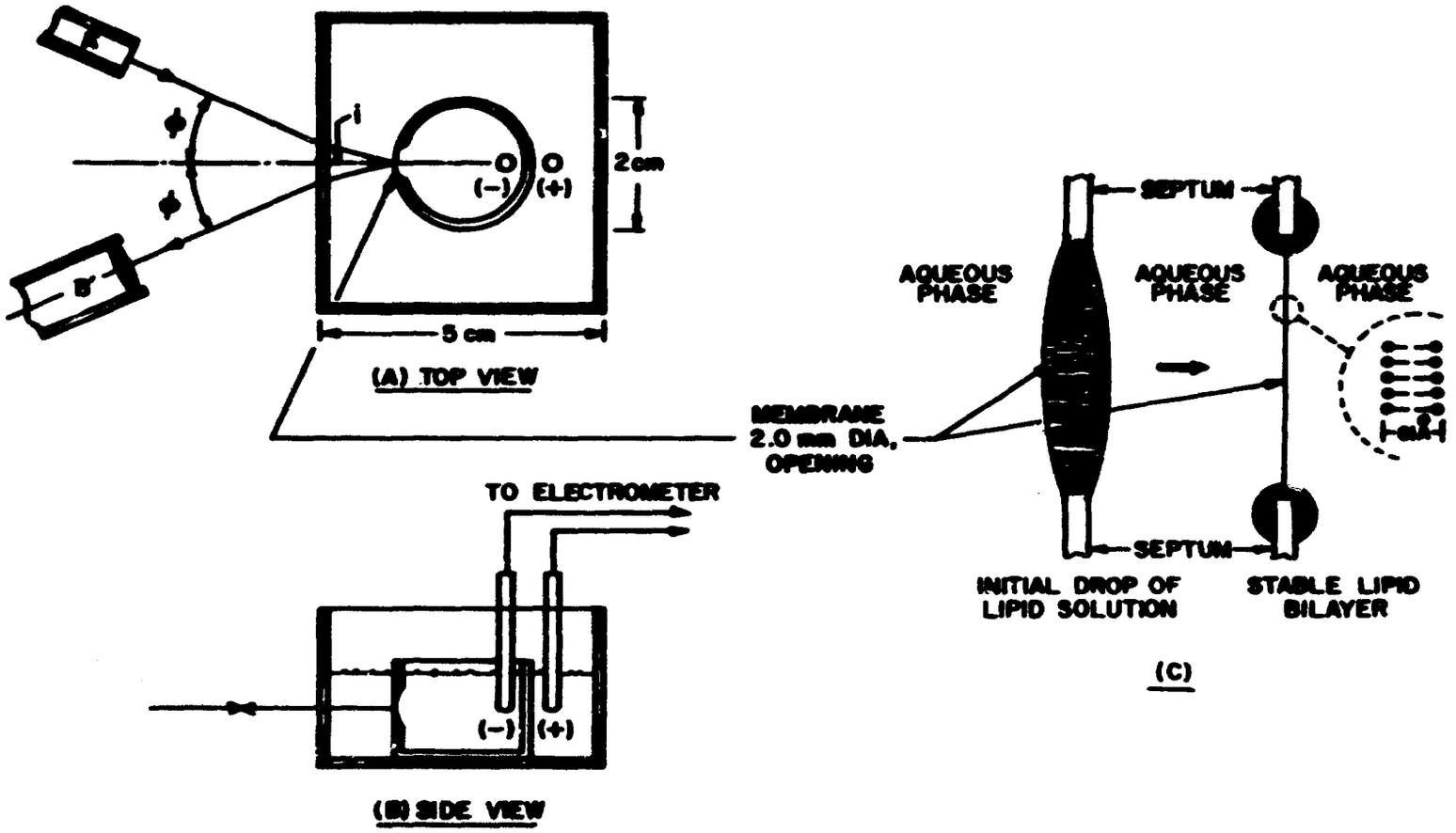


Fig. 1 - Phospholipid Membrane Device of Huang et al.[4]

(A) and (B): Top and side views of the 2-cm. diameter polyethylene cylindrical chamber contained within a lucite 5-cm. square chamber. Electrodes are identified by (-) and (+) with leads to an electrometer or other electrical device. At the left of the top view (A), are a light source, A', and a microscope, B', for observing membrane formation in the 2 mm. opening of the polyethylene cylinder. At the right (C) is an enlarged view of the 2-mm. opening showing it filled with a drop of lipid solution initially and after formation of a bilayer lipid membrane.

of lysolecithin in phospholipase A-treated blood on erythrocytes. Although the amount of lysolecithin formed was not adequate to produce hemolysis, it did cause RBC sphering and some loss of cholesterol from the membrane. These effects were reversed by albumin but not by fibrinogen, Y-globulin or hemoglobin.[22] Since lysolecithin is selectively bound by albumin,[23] it is probable that at least some of the lack of agreement among investigators on the role of phospholipase in cell lysis is due to a binding of the lysophosphatidyl compounds by albumin in solution or albumin bound to the cell surface. Keller,[24] for example, has pointed out that lysolecithin effects on cell membranes may be limited to the area of interaction of antigen and antibody. Therefore, if a phospholipase is in fact produced by complement-fixing antigen-antibody reactions, it is more likely that the phospholipase produced by this reaction would be capable of hydrolyzing phospholipids of a manufactured bilayer film if the antigen-antibody-complement complex were in contact with the film.

Previous studies on monolayered-phospholipids have shown that these monolayers are affected by phospholipase. In investigations by Hughes[25] on the effects of venom phospholipases acting on lecithin monolayers, it was found that very small quantities of phospholipase could be detected by the enzyme-induced lecithin-to-lysolecithin transformation. In these studies the lecithin-to-lysolecithin conversion was detected by changes in surface potential. Similar studies on the fall of surface potential of spread lecithin films have been reported by Colaccio and Rapport.[26] Enzymatically produced changes in monolayered phospholipid effected by the lecithin-to-lysolecithin conversion have also been studied by particle electrophoresis,[27,28] and by an isotope method.[29,30]

Although no one to date has reportedly utilized the bilayer phospholipid membrane of Mueller et al., and of Huang et al.,[15,16] for the detection of phospholipase activity, it is likely that changes in electrical resistance produced by the action of phospholipase would make this method one of the most sensitive ones available for phospholipase activity detection. Therefore, if the union of antigen plus antibody plus complement causes a complement-to-phospholipase transformation, this phospholipase might be best detected by its effect on the bilayer phospholipid membrane. Detection of the phospholipase would be facilitated if the antigen-antibody combination is in direct contact with the phospholipid membrane, as indicated above. Thus, one might anticipate that a single virus particle reacted with specific antibody and complement could cause localized changes and/or rupture of such a bilayer lipid membrane, if the immune complex were in contact with the membrane. The increased solubility of the enzymatically produced lyso-phosphatidyl compound would weaken the membrane structure. Test specificity (i.e., identification of the virus type) would be provided by the availability of highly specific antibody preparations against the various types of virus.

In view of the reasonable promise of success for virus identification with very small numbers of virus particles offered by the bilayer phospholipid membrane methods, the following study was initiated. The first part of the study was devoted to investigating the effects of a known phospholipase preparation on the electrical properties of these phospholipid membranes. The second part of the study was devoted to a study on the effects of antigen (virus)-antibody-complement complexes on these membranes.

## 2. Materials and Methods

a. Bilayer membrane cell device: A Lucite chamber fashioned from sheet Lucite was constructed for holding a polyethylene cup as shown in Fig. 2. This Lucite chamber was positioned in a Lucite constant temperature bath so that the level of the water in the bath was below the top of the Lucite chamber. Water in the constant temperature bath was held at 36.5°C by means of heated water passed from a Haake constant temperature circulator through a copper tubing coil in the bath (Fig. 3). Concrete blocks were used in a shock mounting to minimize the effect of vibrations on the membrane.

A portion of the wall of the polyethylene cup was milled from the inside as shown in Fig. 1-B and an approximately 1.0 mm. diameter hole formed at the thinned wall area with a heated needle guided with a Leitz micromanipulator. Care was taken to prevent the hot needle from actually touching the polyethylene. The best polyethylene cup units were prepared from trimmed screw cap polyethylene sample bottles (33 mm. high prior to trimming). These polyethylene cups were held in a piece of Lucite sheet cut to fit easily in the Lucite cell and drilled with a hole just large enough so that the polyethylene cup could be forced into it. This piece of Lucite served as a base for holding the polyethylene cup in place during each experiment.

Buffered saline was added to the Lucite cell and the polyethylene cup so that the level of buffer was about 1.5-2.0 mm. below the surface of the cup and about 7.0 mm. above the membrane opening in the polyethylene cup. The phospholipid mixture was added to the membrane opening by means of a small camel's hair or sable artist's paint brush after the electrodes had been positioned and the bath had reached the desired constant temperature of 36.5°C.

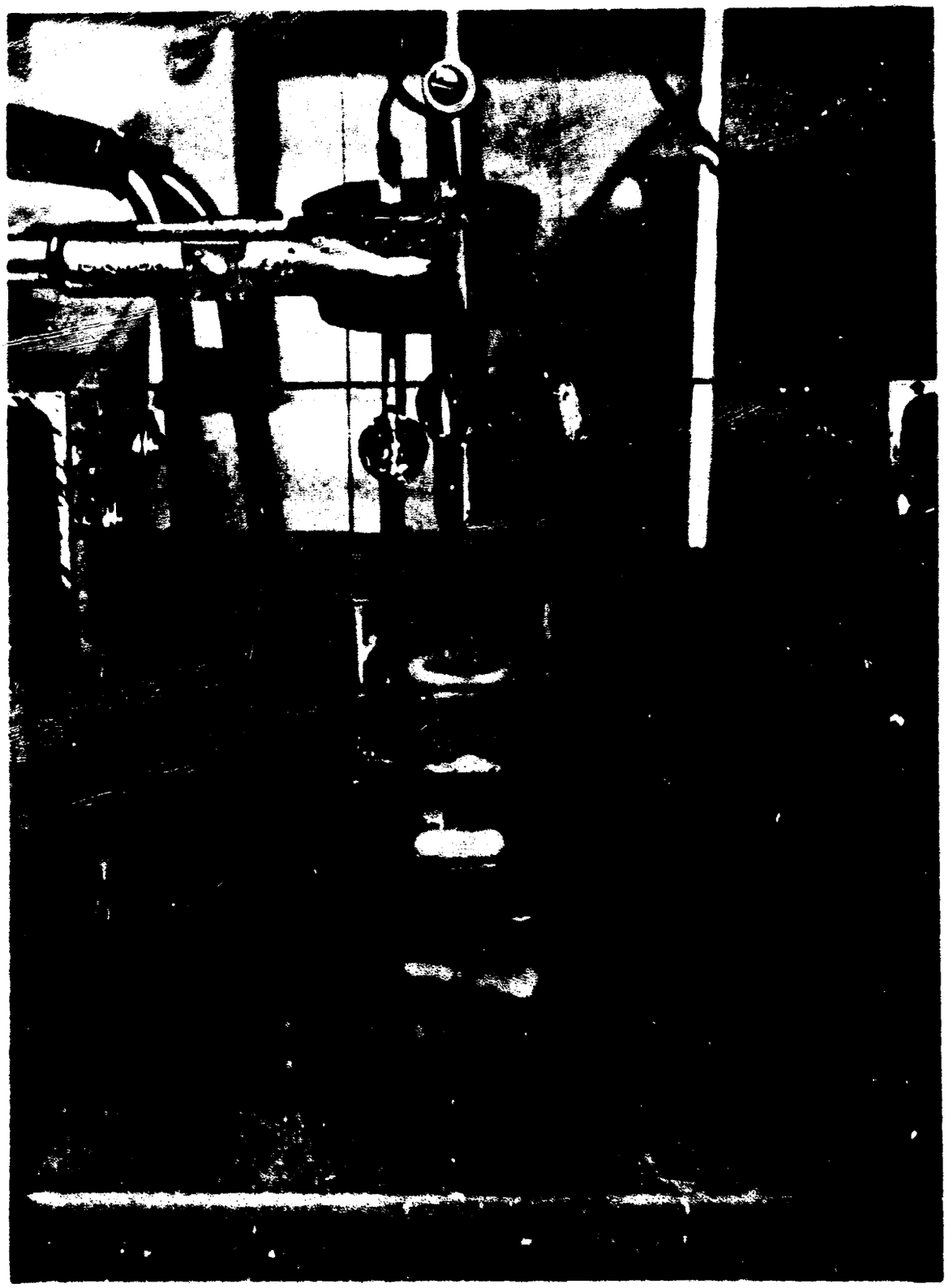

Fig. 2 - Lipid Bilayer Membrane Apparatus Showing Polyethylene Cup With 1 mm. Diameter Membrane and Electrodes

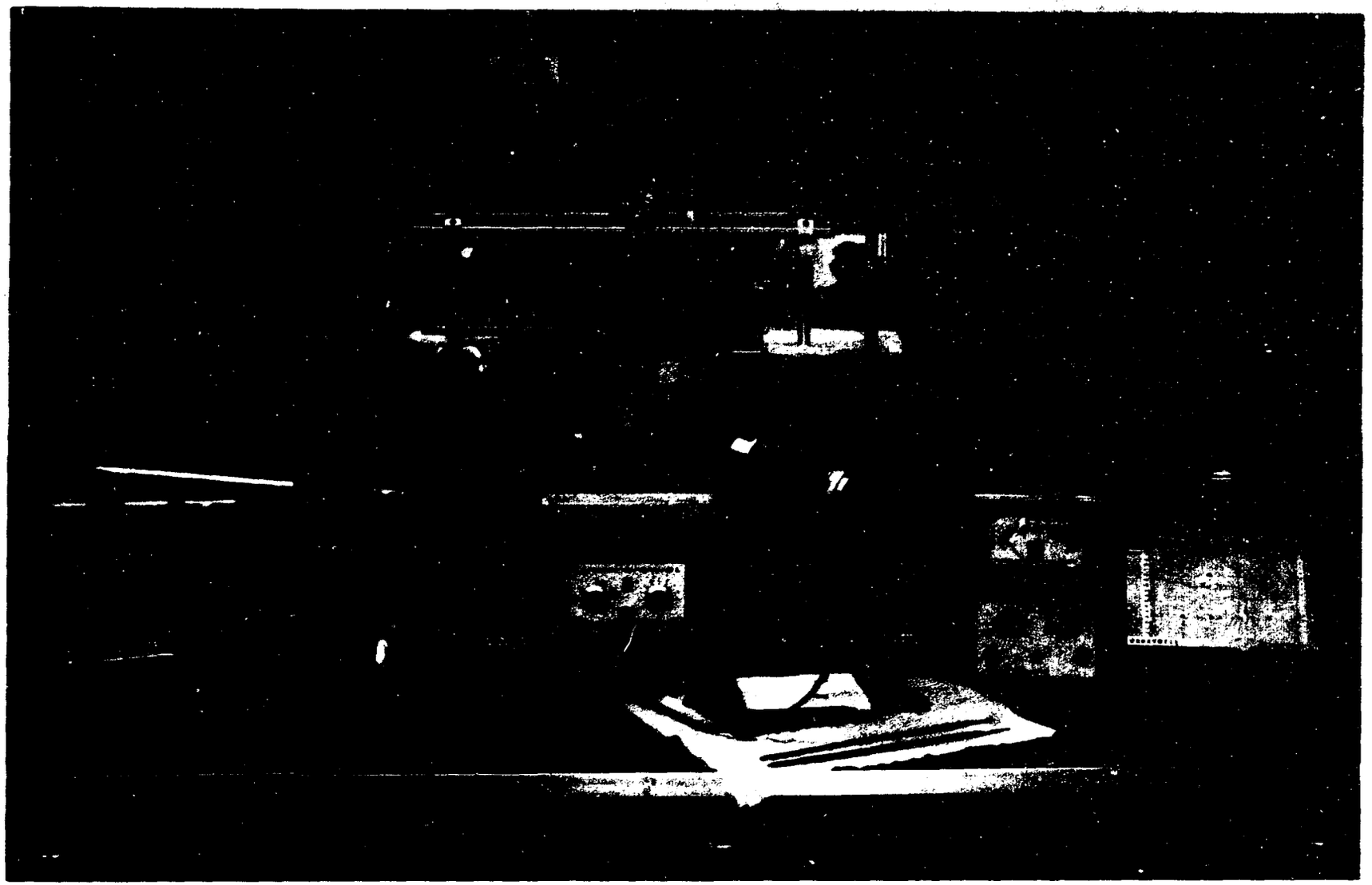

Fig. 3 - Lipid Bilayer Membrane Apparatus With Associated Equipment For Monitoring the Electrical Properties of the Membranes

b. Preparation and evaluation of egg phospholipid: The phospholipid used in the experiments reported here was prepared from fresh egg yolks by the method of Pangborn.[31] Lecithin prepared by this method was colorless and hence presumably relatively unoxidized. Lecithin preparations that we obtained commercially were, in contrast, brown in color. Since oxidation of the phospholipid affects the quality of the bilayer phospholipid membranes,[16,25] an attempt was made to use only relatively unoxidized phospholipid materials in this study. Three different batches of this lecithin were prepared and stored under nitrogen at -60°C. Portions of each batch were stored either solvent-free or dissolved in the n-tetradecane-chloroform-methanol solution of Huang et al.[16]

In some studies we added α-tocopherol to the phospholipid mixtures, since Wirth et al. [32] produced stable bilayer membranes for periods up to 13 hr. with 0.1 percent α-tocopherol in their lipid mixtures. Mueller et al. [15] also used α-tocopherol in their membrane preparations.

Qualitative examinations of our lecithin preparations were done with Eastman Chromagram No. K301R2 silica gel thin-layer chromatography sheets which had been activated by heating for 1 hr. at 100°C. Lecithin samples (10 mg.) dissolved in 0.5 ml. of chloroform were spotted onto the chromatography sheets and the sheets developed with a chloroform/methanol/water (65-25-4) mixture in a chromagram developing apparatus for about 2 hr. The sheets were then sprayed with a solution of rhodamine-B in ethanol and the phospholipid spots observed under ultraviolet light. The results of the TLC tests showed that lecithin was present as a major fraction of the total phospholipid in our extracted yolk preparations.

Since the phospholipid bilayer membranes were used to detect immunological reactions, some consideration was given to ionic strength, pH and divalent cation composition of the saline solution used in the cell unit. Three buffered salines were used, all with 0.85 percent NaCl in distilled-deionized water: pH 7.2, 0.01 M $NaH_2PO_4$-$Na_2HPO_4$ (PBS); pH 7.2, 0.05 ionic strength tris; and pH 7.4-7.5 veronal buffer (VBS). The best results in regard to membrane formation were obtained with the veronal buffered saline, which is also the buffered saline recommended for complement fixation tests.[33] It is probable that the $Ca^{++}$ and $Mg^{++}$ ions present in VBS serve to stabilize the phospholipid bilayer membranes.

c. Proteolipid: "Ox" brain proteolipid was prepared by the method of Mueller et al. (1963)[15] and by their later method (1964).[34] After preparation, the lipids were placed in chloroform-methanol (2 gm. proteolipid per 48 ml. chloroform and 32 ml. methanol) and stored at 4°C. n-Tetradecane was added to constitute 18 percent of the final mixture by volume just prior to use in membrane preparation.

d. Preparation of bilayer phospholipid membranes: Bilayer phospholipid membranes approximately 60 Å thick and about 1.0 mm. in diameter were produced by a modification of the method of Huang et al.[16] Initial studies were carried out with the polyethylene cell units shown in Fig. 2. To prepare phospholipid membranes, phospholipid-containing mixtures added to the aperture in each polyethylene unit and bilayer membranes formed as shown in Fig. 1-C. The phospholipid mixture used was the one recommended by Huang et al.:[16] 2.0 percent lecithin; 18.0 percent n-tetradecane; 48.0 percent chloroform; and 32.0 percent methanol.

e. Optical evaluation of membranes: A Unitron stereoscopic microscope (40X) plus a Leitz microscope lamp as a light source were used to observe the membrane during the course of the experiments. Electrical measurements were not made until the lecithin solution pulled away from the center of the hole to give a "black" bilayer membrane.

f. DC electrical measurements: Measurement of current flow through the phospholipid membranes was done with Ag-AgCl + saturated KCl glass electrodes connected to a DC voltage source (1-1/2 v. Eveready battery) via a Beckman Helipot potentiometer and a Keithley model 220 electrometer as shown in Fig. 4. The electrometer in turn was connected to a Honeywell Electronik 19 recorder in order to obtain a continuous record of current changes. The voltages applied were kept low (approximately 50 mv.) in order to minimize membrane breakdown due to the voltage.

g. AC electrical measurements: Changes in voltage drop across the membrane window, due either to breakage of the phospholipd membrane or to changes in the electrical characteristics of the membrane, were measured with the equipment shown in Fig. 5. A 200-cycle, alternating-current signal was continuously fed across the membrane from a Hewlett-Packard 241A Oscillator. A $10^6$ Ω resistance was interposed in the circuit as shown. All lines were shielded and individual pieces of equipment, including the water bath, were grounded to reduce 60 cycle AC noise.

Silver/silver chloride + saturated KCl glass electrodes were positioned in the inner and outer chambers (i.e., on either side of the membrane window) as for the DC studies described above. A Columbia Research Laboratories model 4000 cathode follower (approximately unity gain) and a Hewlett-Packard model 130 A oscilloscope were used as shown. When no membrane was present in the window, there was a small voltage drop across the electrodes of approximately 2 mv. in most experiments. This voltage drop was subtracted from measured voltages with membranes in the membrane window.

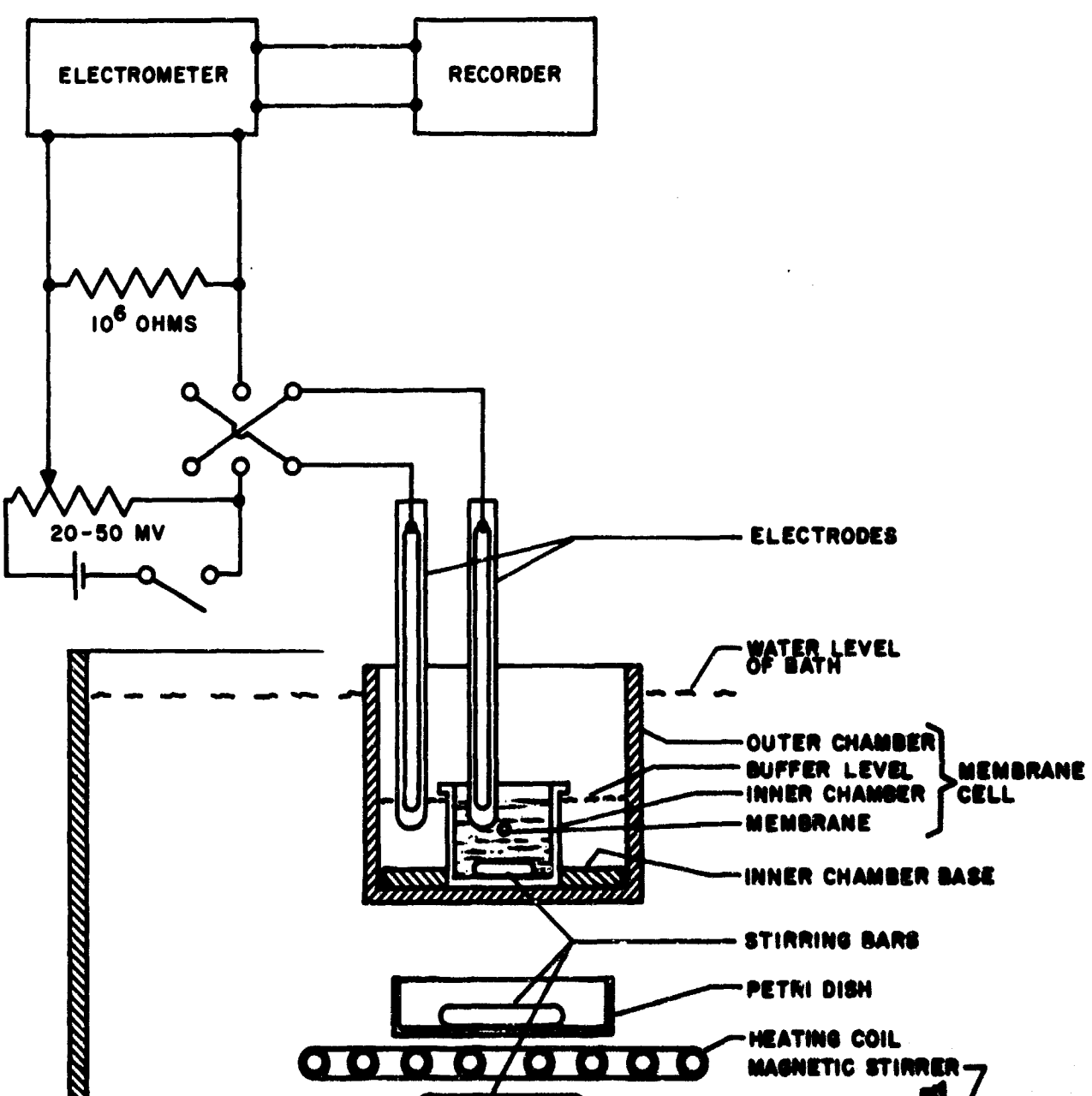


Fig. 4 - Membrane Cell Device

This figure shows an abbreviated view of the Lucite-and-polyethylene membrane cell device positioned in a Lucite constant temperature bath, which in turn is positioned over a magnetic stirrer unit. Asbestos-wick glass electrodes containing saturated KCl and a Ag-AgCl strip are shown in their normal operating positions (one inside the polyethylene cup and one in the medium surrounding the cup). The voltage source is a 1-1/2 v. battery connected to a Beckman Helipot potentiometer (voltage divider); the electrometer is a Keithly 220 DC VTVM plus a decade shunt; and the recorder is a Honeywell Electronik 19 unit.

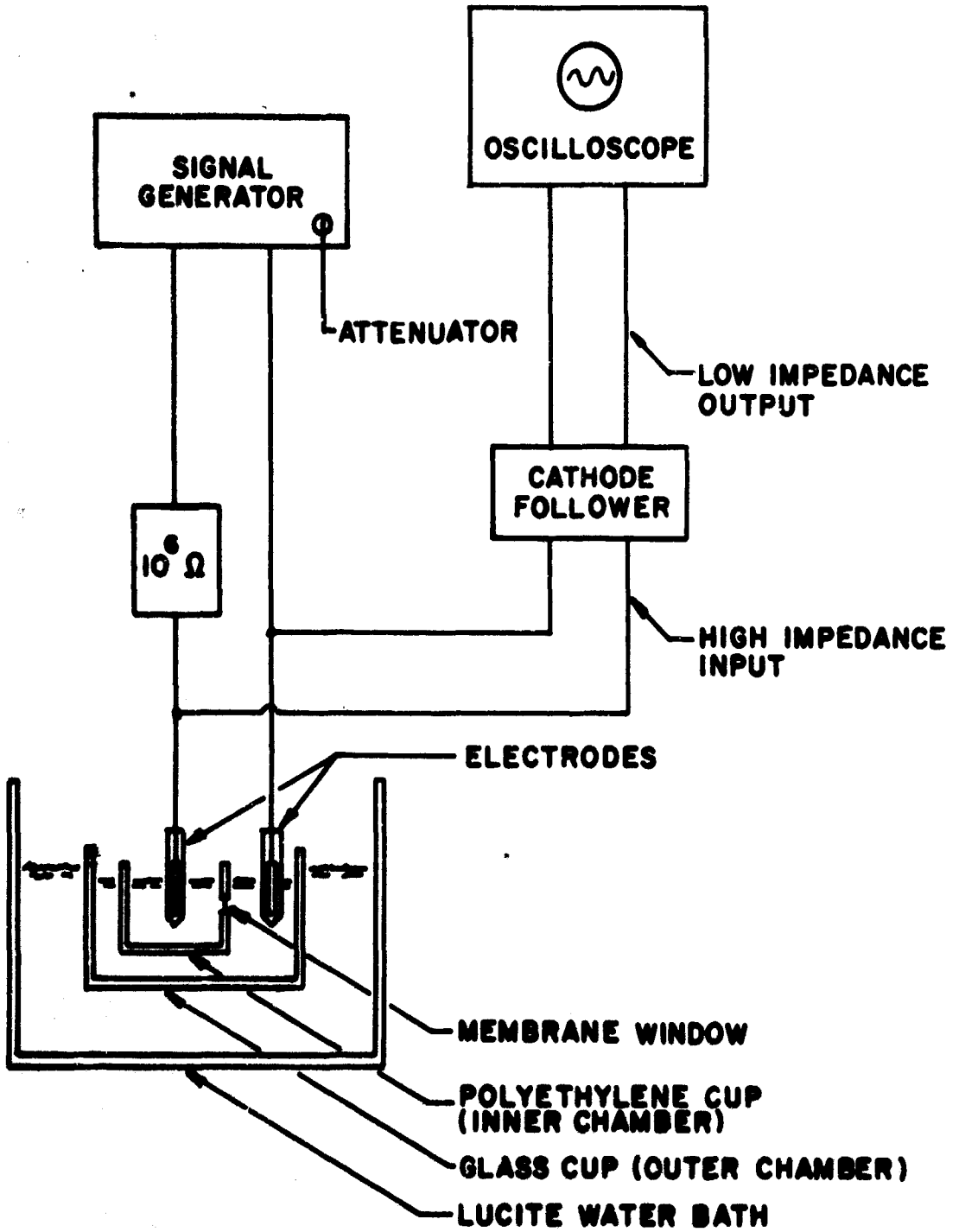


Fig. 5 - Diagram of Equipment Arrangement Used to Measure Membrane Impedance

3. Experiments

a. Membrane stability: Phospholipid membranes were produced with our lecithin preparations when veronal buffered complement-fixation saline was used for the cell unit. Much longer lasting membranes were produced with this VBS as compared with phosphate or tris-buffered saline (see Table 10, p. 58).

One problem which frequently occurred during the experimental runs was the appearance of a small bubble on the membrane. This bubble produced some disturbances in the electrical measurements. Another problem which occurred with all of the buffered salines was that membranes frequently ruptured within 5 min. after their application. The results summarized in Table 10 include only those membranes which lasted more than 5 min., although they also include data from membranes which were accidentally ruptured from unavoidable vibrations.

In order to produce a membrane, it was often necessary to make a large number of attempts with phospholipid mixtures applied by the sable brush. Frequently no membrane at all was formed or, if formed, it lasted only a few seconds. Table 11 summarizes the results obtained in a series of experiments run from 4 March to 6 May 1966, with lecithin and proteolipid mixtures. The table contains data for membranes which lasted for more than a few seconds but does not include the total number of brush attempts (i.e., it does not include "membranes" which lasted for very brief intervals or failures to form any membrane at all).

TABLE 11

PHOSPHOLIPID (LECITHIN OR PROTEOLIPID) BILAYER MEMBRANE STABILITY

(Bilayer membranes produced from a mixture of purified egg lecithin or bovine brain proteolipid in n-tetradecane, chloroform and methanol during the period from 4 March to 6 May 1966.)

| | Membranes | | |
|---|---|---|---|
| | Total No. Made | No. Lasting 5 Min. or Longer | % Lasting 5 Min. or Longer |
| Lecithin | 297 | 87 | 29.3 |
| Proteolipid | 144 | 51 | 35.4 |

TABLE 10

LIFE AND DC RESISTANCE OF PHOSPHOLIPID BILAYER MEMBRANES FORMED IN VARIOUS BUFFERED SALINES

| Buffered Salines | No. of Runs | Avg. Time of Run (min.) | ± S.D. | Avg. Resistance x $10^6$ Ohms | ± S.D. | Avg. Resistance Breakage x $10^6$ Ohms | ± S.D. |
|---|---|---|---|---|---|---|---|
| Phosphate Buffer pH 7.2 | 36 | 20.27 | 10.7 | 29.01 | 16.73 | 24.36 | 16.53 |
| Tris Buffer pH 7.2 | 11 | 16.86 | 11.5 | 16.5 | 7.78 | 15.9 | 16.3 |
| Veronal Buffer pH 7.4 - 7.5 | 24 | 40.04 | 34.6 | 20.4 | 22.6 | 15.3 | 12.3 |

The earlier experiments with egg lecithin for the production of membranes gave a higher percentage of successful membranes -- ones lasting over 5 min. -- than the later experiments (see Table 12). Although lecithin preparations were kept at freezer temperature, approximately -60°C, until the day of use, prolonged storage may have allowed a detrimental alteration of the lipid. In regard to the brain proteolipid material, however, storage at refrigerator temperature (approximately 4°C) for about one month did not seem to change significantly its ability to form membranes.

TABLE 12

LECITHIN BILAYER MEMBRANES: EFFECT OF STORAGE OF LECITHIN ON MEMBRANE STABILITY

| | Membranes | | |
|---|---|---|---|
| | Total No. Made | No. Lasting 5 Min. or Longer | % Lasting 5 Min. or Longer |
| 4 March to 29 March | 154 | 85 | 55.2 |
| 30 March to 6 April | 143 | 2 | 1.4 |

b. DC studies: Both lecithin and proteolipid membranes were tested against immunological reactants (antigen-antibody-complement) as well as against snake venom phospholipase. The substances used in these tests are summarized in Table 13. None of these materials, in membranes tested by the DC voltage method, showed any consistent effects on the membranes. Unfortunately, there was so much variability in control membrane lifetime (duration prior to breakage) that good evaluation of immunologic reactant effects on membranes was not possible.

c. AC studies: In studies on the effects of these immunological reactants on the electrical properties of membranes subjected to AC current, there was again too much variability in membrane reaction prior to the addition of immunological reactants to evaluate the results. No rapid, transient electrical effects (less than 0.1-0.5 sec. in duration) were observed, nor was there any consistent long-term effect (several seconds to several minutes or longer). Toro-Goyco et al. [35] reported transient effects (reductions in impedance across their phospholipid bilayer membranes lasting about 1/10 sec.) with antigen-antibody reaction as well as with enzyme-substrate interactions in studies on these membranes with 200 cycle alternating current. Hence, in our AC studies we have looked for both transient and long-term effects.

TABLE 13

SUBSTANCES (IMMUNOLOGICAL REACTANTS AND VENOMS)
TESTED FOR THEIR EFFECTS ON
PHOSPHOLIPID MEMBRANES

Human serum
Anti-human serum
Human serum albumin (HSA)
Anti-HSA antiserum
Heat-denatured HSA complex (CA)
Purified frog yolk protein (YP)
Anti-YP antiserum
Anti-poliovirus immune globulin
Complement (guinea pig serum, reconstituted lyophilized)
Control rabbit serum
Naja flava venom
Naja naja venom
Naja naja venom (heated 100°C, 15 min.)

There was some suggestion of a lower membrane resistance after the addition of various reactants. However, resistance drops often occurred with each one of the immunological reactants alone, thereby making any effects by immune complexes meaningless for purposes of immune reaction identification.

d. Venom studies: Naja naja venom was prepared as solutions of heated (100°C, 15 min., pH 6) or unheated material which were diluted with VBS to an equilvalent venom concentration of 0.5 mg/ml prior to addition to the membrane-containing polyethylene cup. Approximately 0.05 ml. of venom solution was added to the solution within the cup (about 2 ml. volume); drops in resistance followed the addition of Naja naja and Naja flava venoms. It is interesting that the phospholipases in the snake venoms did not seem to promote actual rupture of the phospholipid membranes, although again variability in lifespans of untreated phospholipid membranes made difficult the evaluation of venom effects.

To test the lecithinase activity of our venom preparations the solutions described above were added to suspensions of mouse RBC in VBS

and incubated at 37°C for 30-, 60-, and 120-min. periods. Both heated and unheated Naja naja venom solutions were tested and found to hemolyze RBC, thus indicating the presence of active lecithinase in these preparations.

e. Saline solutions: Lecithin and proteolipid membranes were tested in a number of solutions to determine whether or not buffered solutions were preferable to unbuffered saline for improved membrane stability. Isotonic saline (0.15 M and 0.85 percent NaCl), as well as phosphate buffered saline (PBS) and veronal buffered saline (VBS) were used. The latter solution, VBS, was selected for most of the studies since it has been widely used for complement fixation and, therefore, should be more suitable for the investigation of effects of immune reactions involving complement on phospholipid membranes. VBS contains both $Ca^{++}$ and $Mg^{++}$ ions, which are known to be involved in reactions of complement with immune complexes. The results of the studies reported here showed that VBS is a satisfactory medium for bilayer phospholipid membrane formation relative to unbuffered or to phosphate buffered salines.

f. Effect of additives: A series of experiments were carried out to determine whether either cholesterol or α-tocopherol, when added to the lecithin-tetradecane-chloroform-methanol mixture, would increase membrane stability. No increase in membrane stability resulted from the addition of these materials to the lecithin-containing solutions. This failure to increase membrane stability may have been influenced by the relatively long storage of the lecithin prior to the addition of the cholesterol or α-tocopherol.

## 4. Discussion

If immunological reactions do in fact result in a production of phospholipase from one of the components of complement, this phospholipase could provide the basis for a very sensitive detection of immunological reactions, since it should be possible to detect the resulting phospholipase from its enzymatic effects on phospholipids of bilayer synthetic membranes. We therefore carried out the studies described here in the hope of finding changes in the electrical properties of phospholipid bilayer membranes, since these membranes should provide a sensitive means of detecting enzymatic effects on the phospholipids through changes in electrical properties.

The results of the studies reported here do not show any consistent evidence that it is possible to measure immunological reactions with the system employed. The negative findings do not, of course, mean that this system cannot be used for measuring immunological reactions.

It is interesting that Toro-Goyco et al. [35] have reported that insulin-anti-insulin reactions can be detected with a system similar to one of the types (alternating current) used here. J. del Castillo and co-workers[36] have reported success on the use of ox brain phospholipid-cholesterol mixtures for the preparation of membranes for the detection of both substrate-enzyme interactions and also antigen-antibody reactions. However, as we learned from Dr. Thompson,[37] all problems are not solved and often the results cannot be repeated. Still we believe that the original results were correct; the difficulty is that there are so many variables it is not possible to control them all at this time.

A serious problem which occurs with phospholipid materials is that they are readily oxidized, particularly the unsaturated fatty acid chains in the β-position (on the second carbon of the glycerol portion of the molecule). Huang et al. [16] have called attention to the detrimental effects of oxygen on phospholipid membranes made from purified lecithin and Mueller et al. [34] have likewise indicated that exposure to light and air causes a deterioration of their proteolipid phospholipid preparation. Our difficulties encountered in preparing stable membranes, especially with purified lecithin, may very well have been due to the oxidation of the phospholipids.

Variability in response of our own phospholipid preparations in regard to erratic duration of membrane survival has made it virtually impossible to evaluate the effects of either venom phospholipases or immunological reactants on these membranes. There were frequent indications that these substances were influencing the electrical properties of the membranes, but no quantitative or other reliable estimate of the effects can be made. Thus membranes often ruptured after the addition of antibody + antigen + complement (in that order), but rupture was also frequently effected by any one of the reactants alone, or even without the addition of any other material.

## 5. Conclusions

Before any meaningful evaluation of the phospholipid bilayer membrane system for identifying immunological reactions can be performed, it will be necessary to devote a substantial effort to improving the entire system. Although some of the additional effort will have to be directed at the actual physical setup of the bath, polyethylene cup, etc., as well as to the electrical measuring system, a major effort will have to be spent on methods for obtaining (and maintaining) a stable and satisfactory phospholipid material for membrane manufacture. As far as the possible

utilization of the lipid bilayer membrane for the identification of virus is concerned, however, there is little justification for further immunological studies with it until the entire bilayer system is substantially improved.

## C. Particle Electrophoresis

### 1. Introduction

Charged microscopic particles suspended in an electrolyte readily undergo migration when placed in an electric field. The rate of migration is a function of the charge on the surface of the particle, the strength of the electric field, the viscosity of the suspending medium and certain other factors. It has been shown that the addition of substances which change the surface charge of the particles also changes the migration rates of the particles. Fresh red blood cells, white blood cells, tanned red blood cells, and other particles falling within the size range easily seen with a microscope have been extensively studied by the particle electrophoresis method.[38]

Since the reaction of an antibody with its homologous antigen produces a neutralization of charges, we then expected that particles coated with antibody and those coated first with antibody and then reacted with antigen would possess different migration rates in an electric field. If this reaction of virus with its antibody-coated latex particle can be shown to be specific and sensitive, then by the use of this phenomenon and certain additional techniques antibody-coated particles should be useful for the identification of virus particles.

In order to use electrophoretic mobility measurements for the identification of virus we anticipate that it will be necessary to prepare a series of particles each coated with a different viral antibody. The particles could be color coded so that each would represent a single antibody coating. Addition of an unknown virus to a mixture of these different colored particles should result in a decrease in the electrophoretic migration rate of only those particles bearing the specific viral antibody. Determination of which color of particles reacted with the virus should permit an identification of the viral agent. The use of an AC particle electrophoresis apparatus of the type described by Sher and Schwan,[39] along with a color photographic process to record which color of particle showed the reduced migration rate, should complete the rapid identification process.

Before attempting the construction of an AC particle electrophoresis apparatus similar to that of Sher and Schwan to test this identification method, we chose to establish with more certainty our original premise that a relatively few virus particles are sufficient to produce a neutralization of the surface charge of an antibody-coated particle and thereby produce a marked decrease in its electrophoretic mobility. Thus we have continued work with the electrophoresis apparatus (Fig. 6) used in our earlier studies and have attempted (1) to establish the quantitative relationship between virus and antibody requirements, (2) to determine the specificity of viral attachment and (3) to confirm that the change in electrophoretic migration rate of a particle coated with antigen and then reacted with its antibody[40] is like the change observed when the antibody-coated particle is allowed to react with antigen.

In our search for antibody particles with the best combination of properties for use in virus identification, we have studied the preparation and reactivity of antibody-coated (1) latex particles in the 6 - 14 micron size range, and (2) tanned sheep red blood cells (RBC). In these studies the particles were coated with antipolioglobulin and they were allowed to react with poliovirus.

## 2. Antibody-Coated Latex Particles

### a. Materials and methods:

(1) Apparatus: Electrophoretic migration rates were made in a cell electrophoresis apparatus[41] (see Fig. 6). Silver/silver chloride electrodes located 190 mm. apart were employed along with an applied voltage of 120 v. The electrophoresis chamber was enclosed in a lucite box through which water at 30°C was circulated continuously. The DC power supply was connected to a reversing switch which permitted reversal of the polarity of the cell between each of the measurements. A reticule in the microscope eyepiece made it possible to time the movement of the particles over a 0.10 mm. course. The electrophoresis cell was 0.4094 mm. in average thickness, and all measurements were made in the middle of the chamber so as to minimize the interactions of the charged particles with the interior surfaces of the vessel. The migration rates were calculated on the basis of the following formula:

$$\frac{d \times D}{t \times V} = \text{electrophoretic mobility in microns/sec/v/cm}$$

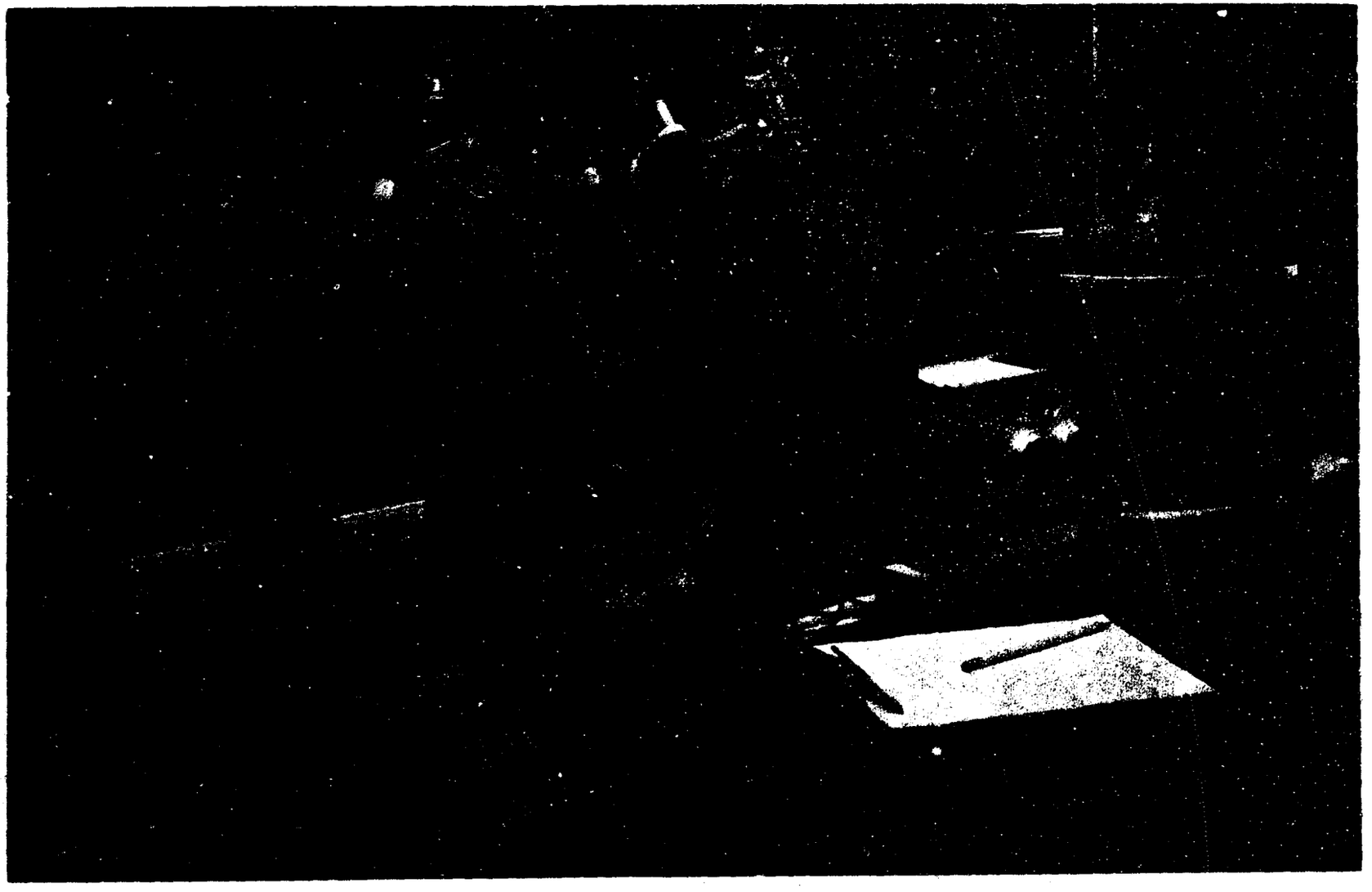

Fig. 6 - Apparatus for Particle Electrophoresis

where d = distance the particle travels in microns
D = distance between electrodes in centimeters
t = time for migration in seconds
V = total voltage applied

The antipoliovirus purified γ-globulin was prepared in this laboratory by multiple injection of rabbits with poliovirus type 2 over a period of four weeks, followed by an application of the ammonium sulfate precipitation technique for the preparation of the purified globulin. Titration of this antiglobulin showed it to possess a potency of 1/100 - 1/1000 as determined by virus neutralization tests. Normal rabbit globulin fractionated by this same procedure was utilized in some of the control procedures. The 6 - 14 micron styrene-divinylbenzene copolymer latex particles were obtained from the Dow Chemical Company.

(2) Preparation of antibody-coated latex particles: In an effort to obtain latex particles with the antibody chemically bound to the surface, the surface of the latex particles was nitrated, reduced, diazotized and coupled with the appropriate antibody or control globulin. The procedure is a modification of the procedure of Gyenes and Sehon[42] and is described briefly here.

Two hundred milligrams of 6 - 14 micron spheres, obtained by centrifugation of 2 ml. of 10 percent styrene-divinylbenzene copolymer latex, was suspended in 1 ml. of concentrated sulfuric acid and mixed with 0.5 ml. of concentrated nitric acid. The mixture was next placed in a water bath at 37°C for 2 hr. and then poured onto about 10 g. of ice. The resulting particles were washed twice with water by centrifuging and separating the supernatant. The washed particles were placed in a solution of 0.5 g. of sodium hydrosulfite in 8 ml. of 2N sodium hydroxide and heated on a steam bath for three days. The resulting latex particles contain a layer of aromatic amino groups on their surfaces. The particles were then washed twice in water and added to 4.0 ml. of distilled water to which six drops of concentrated hydrochloric acid had been added. The suspension was cooled to 0°C during the addition of 1 ml. of 10 percent potassium nitrite solution. The mixture was shaken occasionally and kept at 0° for 1 hr. At this time the particles were washed twice with ice water and then used immediately for the coupling with the various protein solutions. The cold diazotized particles were now suspended in 10 ml. of cold phosphate buffered saline, pH 7.2, and divided into two parts. To 5.0 ml. of this suspension was added 0.2 ml. of antipoliovirus-globulin (1/100 - 1/1000). To the remainder of the suspension was added 0.12 ml. of normal rabbit serum. Both were allowed to remain at 5°C with occasional shaking for 48 hr. At the end of this time the supernatant layers were tested for the presence of excess protein and the tests were both positive. This was

considered to be an indication that sufficient protein was present for reaction with the diazonium groups on the surface of the particles.

In the procedure of Gyenes[42] the entire quantity of polystyrene was dissolved in the nitration mixture and then the nitro groups were reduced with sodium hydrosulfite. In his procedure titration showed that the polymer contained on the average two amino residues per five styrene residues. In our procedure there was an attempt to avoid destruction of the spherical shape of the latex particles. This meant that with our milder nitration procedure the chemical modifications are restricted to the surface of the particles and therefore the weight of the changed material is small relative to the mass of the particles themselves. Evidence of the success in diazotization of the particles and their subsequent coupling with protein is given below:

(a) On warming the diazotized particles to 30° there was some evolution of gas.

(b) Reaction of the particles with an alcoholic solution of β-naphthol resulted in the darkening of the color of the particles but no fluorescence. For comparison benzenediazonium chloride was coupled with β-naphthol to produce a similar colored dye; this also did not fluoresce in the long wavelength ultraviolet.

(c) Fluorescent antihuman serum (0.1 ml. in 3 ml. of buffer) was reacted with 0.1 g. of diazotized particles. Although the resulting particles did not fluoresce, the fluorescence of the buffered solution fell to zero thereby indicating the removal of the fluorescein-labeled antibody from the solution. The lack of fluorescence on the particles was presumed to be due to fluorescence quenching.

b. Experiments: In an effort to determine the effect of poliovirus on the electrophoretic migration rate of antibody-coated latex particles, the experiment shown in Table 14 was conducted. The latex particles in the sample number 1 are more highly charged as a result of the amino groups on their surfaces and they migrate at a rapid rate. Samples 2 and 4 show the reduced migration rates caused by the coupling of the diazotized amino groups with globulins; the observed electrophoretic migration of 3.021 microns/sec/v/cm for the particles coated with antipoliovirus 2 and 2.977 for the particles coated with the normal rabbit serum are nearly identical. The incubation of samples 2 and 4 with tissue culture medium prior to the measurements was to compensate for the presence of tissue culture medium in which the poliovirus was suspended (samples 3 and 5).

TABLE 14

ELECTROPHORETIC MIGRATION RATES OF [illegible] LATEX PARTICLES[a/]

| Sample No. | 1st Treatment[b/] | 2nd Treatment[b/] | 3rd Treatment[c/] | μ/sec/v/cm[d/] |
|---|---|---|---|---|
| 1 | None | None | None | 4.440 |
| 2 | Diazotization | Antipoliovirus 2 Globulin | Tissue Culture Medium | 3.021 |
| 3 | Diazotization | Antipoliovirus 2 Globulin | Poliovirus 2[e/] | 2.706 |
| 4 | Diazotization | Normal Rabbit Serum | Tissue Culture Medium | 2.977 |
| 5 | Diazotization | Normal Rabbit Serum | Poliovirus 2 | 2.708 |
| 6 | Diazotization | Antipoliovirus 2 Globulin | 0.2 M Sucrose in 0.02 M Phosphate Buffer | 2.61[illegible] |

a/ Styrene-divinylbenzene copolymer latex particles whose surface had been nitrated and reduced (i.e., covered with a layer of aromatic amino groups but no protein).

b/ Treatments 1 and 2 were conducted at 0 - 5°C. Treatment 2 included overnight contact of the protein with the diazotized particles.

c/ Treatment 3 was conducted at 37°C for 1 hr.

d/ All electrophoretic measurements were made at 30°C with the particles suspended in 0.2 M sucrose in 0.02 M phosphate buffer.

e/ One milliliter of poliovirus 2 containing $1.56 \times 10^7$ particles was reacted with 0.1 ml. of latex particles suspension containing $4 \times 10^6$ particles.

The use of normal rabbit serum in samples 4 and 5 was designed to answer the question of whether the viral neutralization reaction was specific for its homologous antibody. Sample 6 was intended to show the stability of the antibody-coated particles at 37°C in the medium used for the electrophoresis experiment.

c. Conclusions and recommendations: In the particle electrophoresis experiments reported above we described our preparation of polyaminostyrene-coated latex particles in the 6 - 14 μ size range and a procedure for coating them with viral antibody. Experiments on the identification of antigens with these particles indicated that (1) there was not enough antibody on the surface of the plastic particles to make them responsive to viral antigen, (2) the widespread differences in the particle size and electrophoretic mobilities caused inaccuracies in the migration rate data, and (3) there was difficulty in keeping the particles in the microscope's field of view because of their high density as compared to a buffered 10 percent sugar solution used as the suspending medium.

However, when about four poliovirus particles are added to one antipoliovirus-coated latex particle there is an easily detectable reduction in migration rate of that latex particle. However, the reaction of the normal rabbit serum-coated latex particles with the same ratio of poliovirus particles produced the same reduction in migration rate. This indicates that the action of the poliovirus on the latex particle may be non-specific.

The drop in migration rate for the antibody particles incubated with the sucrose phosphate buffer (sample 6) is not understood, but may be due to the presence of unreacted diazonium chloride groups on the surface of these particles which are destroyed on warming.

On the basis of these experiments it is not possible for us to recommend the particle electrophoresis approach for the rapid detection of a relatively few virus particles. The observation that particles coated with normal rabbit serum reacted to poliovirus in the same manner and to the same degree as particles coated with antipoliovirus serum is disturbing but may not present insurmountable difficulties. In the first place we are not absolutely sure that there was any antibody on the surface of the particles which were used in the experiment. In the second place, it may be possible to desensitise the particles with tissue culture fluid or other material prior to the treatment with the virus. Desensitization procedures are used routinely for the preparation of the sensitised particles for use in the latex fixation tests.[45]

## 8. Antibody Coated Tanned Red Blood Cells

a. Materials and methods: In the present extension of the particle electrophoresis study we have investigated the sensitization of formalin-fixed, tanned sheep erythrocytes with both non-viral and viral antibodies, and we have investigated the possibility of using the electrophoretic mobilities of these sensitized particles as a means for identification of non-viral and viral antigens. Procedures for coating tanned RBC with antibody have been reported by a number of authors.[44,45]

Formalin-fixed sheep erythrocytes were obtained from Difco Laboratories under the name of Bacto Formocells. These were then tanned with 1:2000 tannic acid and reacted with the appropriate antiserum or purified γ-globulin from immune animals by the procedure suggested by Difco Laboratories.

Frog yolk protein (FYP) and anti-FYP antiserum (rabbit) were prepared by Dr. Ringle on another research study, and the former was dissolved in 0.8 molar sodium chloride solution prior to its use in the reaction with cells which had been sensitized with the anti-FYP.

The preparation of poliovirus 2, poliovirus 2 immune rabbit serum and ammonium sulfate-precipitated antipoliovirus 2 γ-globulin were described in an earlier report.[1] The preparation and assay of Semliki Forest Virus (SFV) and anti-SFV serum were described earlier in this report.

Purified antihuman γ-globulin, Pentex Brand, was obtained from Gallard-Schlesinger Chemical Manufacturing Company. Fraction II purified human plasma γ-globulin was obtained from Cutter Laboratories.

The apparatus employed for making the electrophoresis measurements was described above. All measurements were made at 30°C with 120 v. applied between the two silver/silver chloride electrodes. The current flowing in the system was checked for each sample introduced into the apparatus to make certain that there were no hidden air bubbles in the system; the current ranged from 0.40 to 0.45 ma. Phosphate buffer (0.02 M) containing 68 g. of sucrose per liter was employed because it restricted the current flow to levels where heating effects were not a problem and because the specific gravity was high enough to retard the settling out of the RBC. The polarity of the applied direct current was reversed after each measurement of migration time. Each rate calculation given in the tables represents data from at least 40 migration time measurements.

After runs with live virus the apparatus was filled with 10 percent formalin and allowed to stand overnight before cleaning with warm Alconox solution.

b. Experimental results: In the first of the current series of experiments with the particle electrophoresis method for identification of antigens, a study was conducted in which antifrog yolk protein serum sensitized RBC were compared for their electrophoretic migration rates with those which have also been incubated with frog yolk protein solution. As may be seen from Table 15, Samples I and II, the "immune" reaction of the anti-FYP with the FYP had an insignificant effect on the migration rate.

In order to determine the possible effect of FYP on the migration of RBC sensitized with non-homologous antisera, RBC which had been sensitized with antihuman serum (rabbit) -- not antihuman γ-globulins described in later experiments -- were tested alone, in the presence of FYP and also in the presence of human serum (Samples III, IV and V). The FYP had a greater effect on the migration rate of the antihuman sensitized cells (Sample IV) than on the cells sensitized with the homologous antiserum (Sample II). From this we concluded that the minor changes in migration rate observed in this experiment are not due to specific immune reactions.

The variability of the results obtained in this first experiment suggested that no antibody may have become attached to the surface of the tanned RBC during the sensitization procedure. To determine if the antipoliovirus 2 γ-globulin sensitized cells really possessed a layer of antibody on their surfaces, they were tested on a spot plate with poliovirus 2, but they did not agglutinate. This result was not altogether unexpected since agglutination of antibody sensitized RBC has been reported to be much more difficult than the agglutination of antigen sensitized RBC; Richter et al. have reported that sensitization of RBC with γ-globulin fractions of antisera gave cells which would not agglutinate on the addition of either small or large quantities of antigen.[16]

An alternative test was conducted to determine if any of the sensitizing globulins had become attached to the surface of the cells during the tannic acid sensitization procedure. Since both the antihuman γ-globulin and the antipoliovirus 2 γ-globulin used for RBC sensitization were of rabbit origin, they were known to contain normal rabbit globulins (antigen) in addition to antiglobulins; reaction of cells sensitized with these materials with antirabbit serum of goat origin was therefore expected to agglutinate the cells (Table 16). The positive agglutination reactions obtained established the presence of rabbit globulins on the surface of the sensitized cells; hopefully some of these were the desired antiglobulins which would be detectable by electrophoretic methods.

TABLE 15

[redacted] OF RABBIT [redacted] PROTEIN (FYP) ON THE ELECTRO-OSMOTIC [redacted] OF ANTI-FYP AND ANTI-HUMAN SERUM SENSITIZED [redacted] BLOOD CELLS (EBC)

| Number | Substance Used to Sensitize the Normal Sheep EBC* | Treatment of Sensitized Cells** | Migration | |
|---|---|---|---|---|
| | | | Time (sec.) | Rate (μ/sec/v/cm) |
| I | Anti-FYP serum (rabbit) | None | 4.62 | 3.42 |
| II | Anti-FYP serum (rabbit) | Frog yolk protein*** | 4.45 | 3.57 |
| III | Anti-human serum (rabbit) | None | 4.92 | 3.23 |
| IV | Anti-human serum (rabbit) | Frog yolk protein | 5.78 | 2.70 |
| V | Anti-human serum (rabbit) | Human serum | 5.34 | 2.97 |

* Difco Bacto-Formocells (stabilized sheep erythrocytes) were tanned and reacted with the antisera shown.

** A 2 per cent suspension of sensitized cells was incubated 1 hr. at 30° with an equal volume of antigen.

*** In the case of FYP the cells were washed first with 0.4 M NaCl to prevent precipitation of the lipoprotein on the cells.

TABLE 16

HEMAGGLUTINATION OF SENSITIZED RBC SHOWING THAT ADDITION OF RABBIT SERUM COMPONENTS OCCURRED DURING THE SENSITIZATION PROCESS

| | Difco Bacto-Formocells Sensitized With: | Source of Globulin | Conc. of Antirabbit Serum (Goat) Added 1:1 | 1:10 | 1:100 | 1:1000 |
|---|---|---|---|---|---|---|
| I | Antihuman γ-Globulin | Rabbit | +++ | ++++ | ++++ | +++ |
| II | Antipoliovirus 2 γ-Globulin | Rabbit | ++ | +++ | ++ | ± |

In a third experiment the formalin-fixed, tanned sheep erythrocytes were sensitized with a purified γ-globulin fraction from antihuman serum of rabbit origin. We had reasoned that immune serum contains as much as 85 percent of albumins and other non-antibody materials, and that the use of a purer antibody to sensitize the RBC would increase our chances of obtaining a change in migration rate on the addition of antigen. As may be seen in Table 17, sample pairs I and II and also III and IV, the addition of antigen produces an easily observed drop in migration rate of the sensitized particles. Sample V shows the migration rate of the unsensitized and untreated formalin fixed and tanned RBC and Sample VI shows the effect of the purified γ-globulin on the migration of the unsensitized but tanned cells; in this latter case incubation of the tanned cells with the γ-globulin probably produced antigen-sensitized cells with the migration rate shown.

Since we had demonstrated that anti-HSG sensitized cells gave a lowered migration rate when they were contacted with the homologous antigen, it became important to learn how the quantity of antigen affected this response. As may be seen in Table 18 anti-HSG sensitized RBC were challenged with various dilutions of HSG. It was not possible to calculate the equivalence of the reactants since we had no information on the quantity of antibody on the surface of the sensitized RBC. It will be seen that the 0.1 percent solution (see Sample 2) contained sufficient antigen to react with the antibody on the cells, since the addition of a tenfold excess of antigen (Sample 1) gave no further decrease in mobility rates. The possibility that "antigen excess," a situation which prevents some precipitin reactions, may be reducing the amount of the effect by solubilizing antibody on the particle surfaces is of interest from an academic viewpoint.

TABLE 17

EFFECT OF PURIFIED HUMAN GAMMA GLOBULIN (HGG) ON ELECTROPHORETIC MOBILITY OF ANTI-HGG SENSITIZED AND UNSENSITIZED RBC

| Sample | Substance Used to Sensitize the Tanned Sheep RBC* | Treatment of Cells** | Migration Time (sec.) | Migration Rate (μ/sec/v/cm) |
|---|---|---|---|---|
| I | Anti-HGG | None | 3.93 | 4.03 |
| II | Anti-HGG | Fraction II HGG | 10.39 | 1.53 |
| III*** | Anti-HGG | None | 3.98 | 4.01 |
| IV*** | Anti-HGG | Fraction II HGG | 7.64 | 2.07 |
| V | None | None | 4.18 | 3.78 |
| VI | None | Fraction II HGG | 6.02 | 2.63 |

* Difco Bacto-Formocells were tanned and reacted with the purified globulin shown.

** The cells were incubated with the globulin or buffer for 1 hr. at 30°C.

*** Samples Nos. III and IV represent an attempt to reproduce the results obtained in the earlier experiments I and II.

TABLE 18

THE EFFECT OF DIFFERENT CONCENTRATIONS OF HUMAN GAMMA GLOBULIN (HGG) ON THE ELECTROPHORETIC MOBILITY OF ANTI-HGG SENSITIZED RBC'S

| Sample | Conc. of Human Gamma Globulin added (wt/vol %)* | Migration Time (sec.) | Migration Rate (μ/sec/v/cm) |
|---|---|---|---|
| 1 | 1.0 | 5.474 | 2.89 |
| 2 | 0.1 | 5.615 | 2.82 |
| 3 | 0.01 | 4.002 | 3.96 |
| 4 | 0.001 | 3.558 | 4.45 |
| 5 | 0.0001 | 3.344 | 4.74 |

* In each case, 0.9 ml. of the purified HGG fraction II solutions was mixed with 0.2 ml. of a 0.1 percent suspension of anti-HGG sensitized sheep RBC and incubated for 2 hr. at 37°.

The effect of poliovirus 2 on the electrophoretic mobility of antipoliovirus 2 γ-globulin sensitized red blood cells is presented in Table 19 (p.76). Trials 1 and 2 were conducted so that it would be possible to avoid any antigen excess and to gain some idea about the numbers of virus particles which would be required to produce a measurable change in migration rates. Use of more than 10 viable particles per RBC were not investigated since it was apparent that larger multiplicities of virus particles would not be available in the identification of 100 particles. Lower concentrations of virus particles were chosen partly because there may be a number of non-viable but antigenically active particles for each viable virus particle present. The results of the two trials seem to show that poliovirus 2 cannot be identified in this manner.

The effect of Semliki Forest Virus on the electrophoretic mobility of anti-SFV serum sensitized red blood cells is presented in Table 20. Viral multiplicities of 0.03 to 30.0 per RBC were investigated, and the resulting slight changes in migration rates are too small and erratic to form the basis of an identification based upon this procedure.

TABLE 20

EFFECT OF SEMLIKI FOREST VIRUS (SFV) ON THE ELECTROPHORETIC MIGRATION OF ANTI-SFV SERUM SENSITIZED RBC

| Sample | Treatment Given to Anti-SFV Serum Sensitized Sheep RBC* | No. of Virus Particles per RBC | Migration | |
|---|---|---|---|---|
| | | | Time (sec.) | Rate (μ/sec/v/cm) |
| I | $1.8 \times 10^8$ SFV | 30.0 | 4.27 | 3.71 |
| II | $1.8 \times 10^7$ SFV | 3.0 | 3.90 | 4.06 |
| III | $1.8 \times 10^6$ SFV | 0.3 | 3.71 | 4.27 |
| IV | $1.8 \times 10^5$ SFV | 0.03 | 4.10 | 3.83 |
| V | 0.02 M Phosphate buffer, pH 7.2 + 0.2 M sucrose | None | 3.82 | 4.14 |

* A suspension containing about $6 \times 10^6$ anti-SFV serum sensitized tanned sheep RBC was incubated for 1 hr. at 37°C with the quantity of virus shown.

TABLE 19

EFFECT OF POLIOVIRUS-2 ON THE ELECTROPHORETIC MOBILITY OF ANTI-POLIOVIRUS-2 GAMMA GLOBULIN SENSITIZED RBC

| Sample | Treatment Given to Anti-poliovirus-2 Gamma Globulin Sensitized Cells* | No. of Virus Particles per RBC | Migration | |
|---|---|---|---|---|
| | | | Time (sec.) | Rate (μ/sec/v/cm) |
| **Trial 1**** | | | | |
| I-I | 0.02 M Phosphate buffer + 0.2 M sucrose | None | 4.26 | 3.72 |
| I-II | Tissue culture fluid (used Medium 199) | None | 4.22 | 3.75 |
| I-III | $10^4$ Poliovirus-2 | 0.000075 | 4.06 | 3.90 |
| I-IV | $10^5$ Poliovirus-2 | 0.00075 | 4.12 | 3.84 |
| I-V | $10^6$ Poliovirus-2 | 0.0075 | 4.16 | 3.81 |
| I-VI | $10^7$ Poliovirus-2 | 0.075 | 4.98 | 3.18 |
| **Trial 2** | | | | |
| II-I | $10^7$ Poliovirus-2 | 2.0 | 3.60 | 4.40 |
| II-II | $10^7$ Poliovirus-2 | 10.0 | 3.35 | 4.72 |
| II-III | Tissue culture fluid (used Medium 199) | None | 3.52 | 4.50 |
| II-IV | 0.02 M Phosphate buffer + 0.2 M sucrose | None | 3.18 | 4.98 |

* Incubation was done at 37° for 2 hr.

** Trials I and II were done at different times and are not completely comparable.

c. Discussion: The objective of this study was to determine if virus could be identified by measurement of their effect on the electrophoretic mobility of antibody sensitized RBC. Rather than limit our virus sample size to 100 particles for these studies, we elected to use larger quantities and to determine the approximate sensitivity of the method by controlling the ratio of virus particles to the sensitized RBC. Use of the larger quantities permitted us to employ the particle electrophoresis apparatus which was already on hand, rather than build a micro AC particle electrophoresis apparatus as we had suggested earlier.

As mentioned above, the sensitization of the RBC with the non-viral and viral antibodies posed some problems. The two primary methods under consideration were (1) the use of the "reversed BDB technique[47] in which bis(diazotized benzidine) was coupled first to the RBC and then to the antibody, and (2) the tannic acid technique.[46] In view of the proof that globulins were attached to the RBC during the sensitization reaction (cf. Table 16), we used this method exclusively for the sensitization of the cells. Recently available information about the possible dissociation of γ-globulin suggests that several molecular species are important in the immune reaction;[48] one might therefore presume that a whole antiserum would be better for sensitization of cells than highly purified materials. However, the failure of the particle electrophoresis method to identify RBC which had been sensitized with whole antisera and the success in identification of cells sensitized with purified antiglobulins, leads us to believe that a further improvement in sensitized cell sensitivity might be achieved by the use of purified antibody for the sensitization of the cells. This view is further substantiated by the observation of M. Richter et al.,[46] that unless RBC are sensitized with purified antibodies, they will not agglutinate on the addition of homologous antigen. It is unfortunate that time did not permit the production of the purified Semliki Forest Virus and poliovirus 2 antibodies for use in RBC sensitization and particle electrophoresis studies.

d. Conclusions: The present study has shown that it is possible to identify some non-viral antigens quickly by means of the particle electrophoresis method provided the RBC have been sensitized with the purified antiglobulins instead of with the whole antisera. Viruses have not yet been identified by this method possibly because the sensitized RBC did not possess a sufficient quantity of antibody. Further experimentation on the identification of virus with RBC sensitized with purified antibodies of virus would be of a great deal of scientific interest but would probably not change our present view that the particle electrophoresis

method using antibody coated tanned RBC for the identification of virus is not very promising. On the other hand, use of antigen-coated particles for the detection of antibodies appears more promising than the reverse, and may be worthy of further study.

## D. Hydrogen Overvoltage

### 1. Introduction

In an earlier study[1] we had suggested that it might be possible to coat a platinum electrode with antibody and then to determine the change in hydrogen overvoltage which occurs when an antigen is brought into contact with that coated electrode. This procedure, if successful, would provide a direct electrical measure of the immune reaction and would therefore be an important technique which might be applied to viral identification. The initial idea on the use of hydrogen overvoltage measurements resulted from the observations of Juda et al.[49] that (1) $10^{-8}$ eq/liter of egg albumin produced hydrogen overvoltage measurements of about 250 mv. greater than that of the solution without the albumin and that (2) experiments with various substances indicated that larger molecules produced the greater overvoltages. A few very preliminary experiments were conducted with this approach and they are referred to briefly in our Final Report.[1] At that time we ventured the opinion that this approach did not appear too promising due to the non-specific nature of the electrode-poisoning phenomena.

More recently we have talked with Dr. David N. Kramer of the Edgewood Arsenal Research Laboratories, and have learned from his personal experience with the hydrogen overvoltage procedure of several modifications which might increase our chance for success in the detection of the immune reaction by this technique. One of his important suggestions was that the protein should be attached to the platinized electrode by electrodeposition prior to the overvoltage measurements. In order to avoid the possibility of overlooking a good method for the monitoring of the immune reaction by electrical methods, we have reconsidered the matter and are presenting a description of our experiments, the results obtained, and a re-evaluation of the procedure for virus identification.

## 2. Materials and Methods

The apparatus employed for these overvoltage studies is shown in Fig. 7. Two electrode chambers were employed in an effort to avoid possible contamination of the reference electrode. Experience with the method showed that it was necessary to pass a stream of hydrogen gas over the working cathode as well as over the reference electrode in order to obtain stable overvoltage measurements. Although Juda et al.[49] used both static and flowing systems, they did not describe the construction or operation of their equipment; we found that more reproducible data were obtained when a magnetic stirrer was used in the working electrode chamber.

The liquid medium used for these experiments consisted either of 0.1 M veronal buffered saline (VBS), pH 7.5, or 0.1 N sulfuric acid, pH 1.8. The former was selected because it is excellent for obtaining the precipitin reaction.[50] The sulfuric acid was used because it is the solution previously employed when the high sensitivity to large molecules containing nitrogen was observed.[49]

Three platinized platinum electrodes were employed in this study. They were made from 18 gauge platinum wire and had an area of approximately 1 $cm^2$ each. They were platinized by placing them in a solution containing 3 percent chloroplatinic acid and 0.03 percent lead acetate and then applying 1-1/2 v. of alternating polarity between them until they were lightly coated with a gray deposit of platinum.

In order to scan the effect of various current densities at the working electrode on the over-potential, the applied voltage was increased from 0 - 3 v. over a period of 2-1/2 min. by means of a 4 rpm gear reduction motor which drove a 10-turn, 1,000 ohm Helipot potentiometer.

The difference in potential between the reference electrode and the working cathode was determined with a Leeds and Northrup Model 7401 pH meter set to measure 0 - 1,400 mv. and to feed the Y-axis of an Electro Instruments, Inc., XY-Recorder Model 320. The current from the working electrodes was fed through a milliammeter and either a 10,100 or 1,000 ohm shunt which was selected on the basis of current density range desired and the conductivities of the solutions. The milliammeter was used to calibrate the currents flowing to the X-axis of the recorder.

The electrodes were cleaned between experiments by washing in 0.1 N sulfuric acid followed by passage of more than 200 ma. between electrodes with frequent reversal of polarity. Following this, the electrodes were washed in distilled water. No test was developed to show that the electrodes treated in this manner were free of protein contamination.

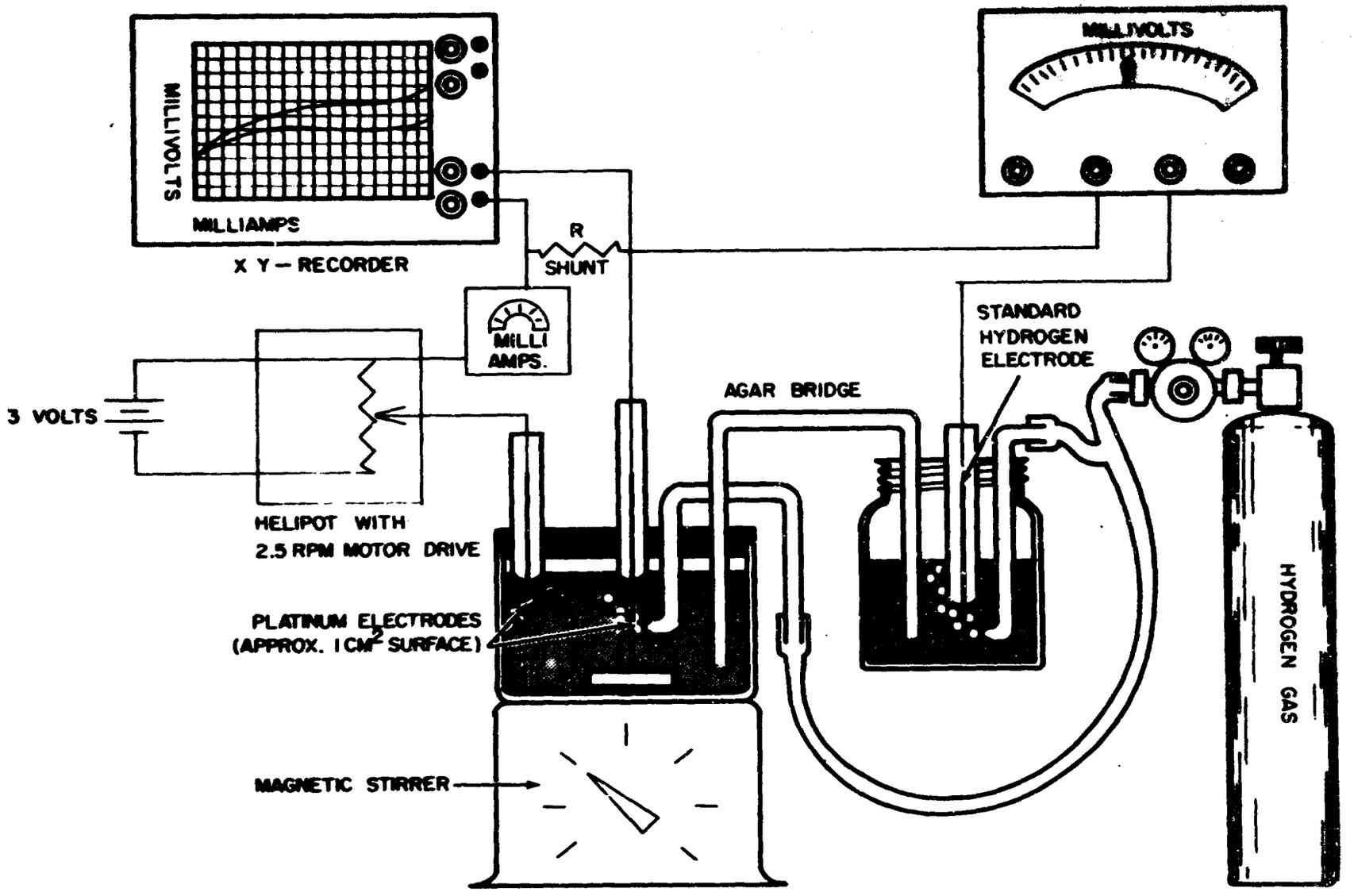


Fig. 7 - Apparatus for Measurement of Hydrogen Overvoltages

3. Experimental Results

One experiment conducted at a pH of 7.5 using veronal buffered saline (VBS) as the solution for the working electrode, the agar bridge and the hydrogen reference electrode, is presented in Fig. 8. Examination of the legend will show that there are three overvoltage curves obtained by gradually increasing the current applied to the working electrodes and measuring the difference in potential between the reference hydrogen electrode and the working electrode at which hydrogen is evolved. Curve I represents a scan of the overvoltage when only VBS is present. Curve II represents the overpotential obtained when 0.1 ml. of antihuman serum albumin was added to the 25 ml. of VBS in the working electrode chamber. Curve III represents the overvoltage measurement obtained when 0.1 ml. of 1 percent human serum albumin was added to the solution used for making Curve II. Under these conditions this system responded to neither the presence of protein nor to the immune reaction which should have taken place under these conditions.

The next experiment was similar to the last except that the measurements were made using 25 ml. of 0.1 N $H_2SO_4$ at a pH of 1.8. As may be seen from Fig. 9, Curve I is the operpotential curve obtained for sulfuric acid alone. Prior to making Curve II, 0.5 ml. of antihuman serum albumin was added to the working electrode chamber and an effort to electroplate the platinized platinum electrode with the antibody by passage of an electric current through the solution for 5 min. was made; it was assumed that in the acid solution the amphoteric Y-globulins would migrate to the cathode and be deposited upon it; Curve II was obtained with the "antibody plated" platinum electrode. After making this curve 0.5 ml. of a 1 percent solution of human serum albumin (HSA) was added to the solution remaining in the working electrode chamber and the overvoltage was then measured again, Curve III. In spite of the observed increases in overvoltage, it is doubtful that an antigen-antibody reaction occurred under the conditions of this experiment. Kabat et al.[50] have reported that the precipitin reactions are known to occur in the range of pH 7.5 to 4.6 and immune complexes are known to dissociate at lower pH.[51] It is more likely that each of the two additions of protein to the working electrode solutions caused an increase in the overvoltage.

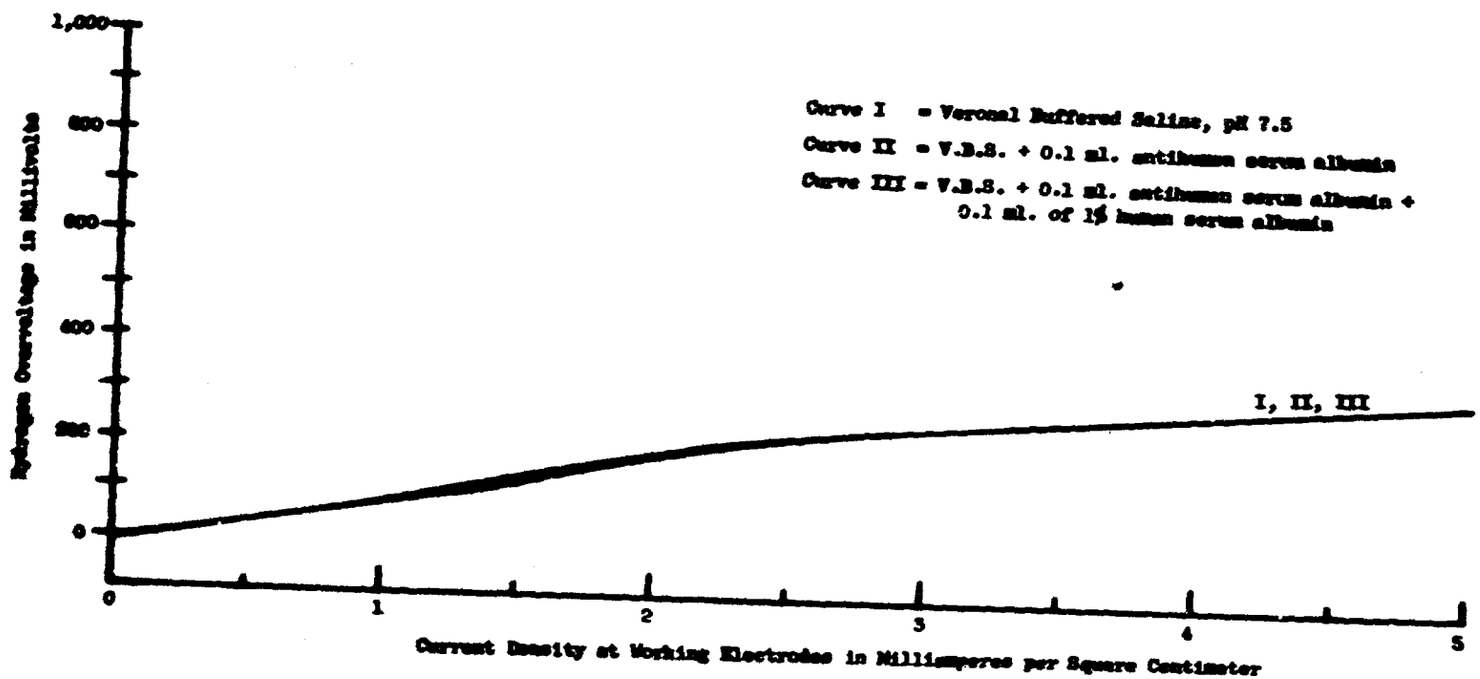


Fig. 8 - Effect of Anti-HSA and HSA on Overvoltage Measurements at pH 7.5

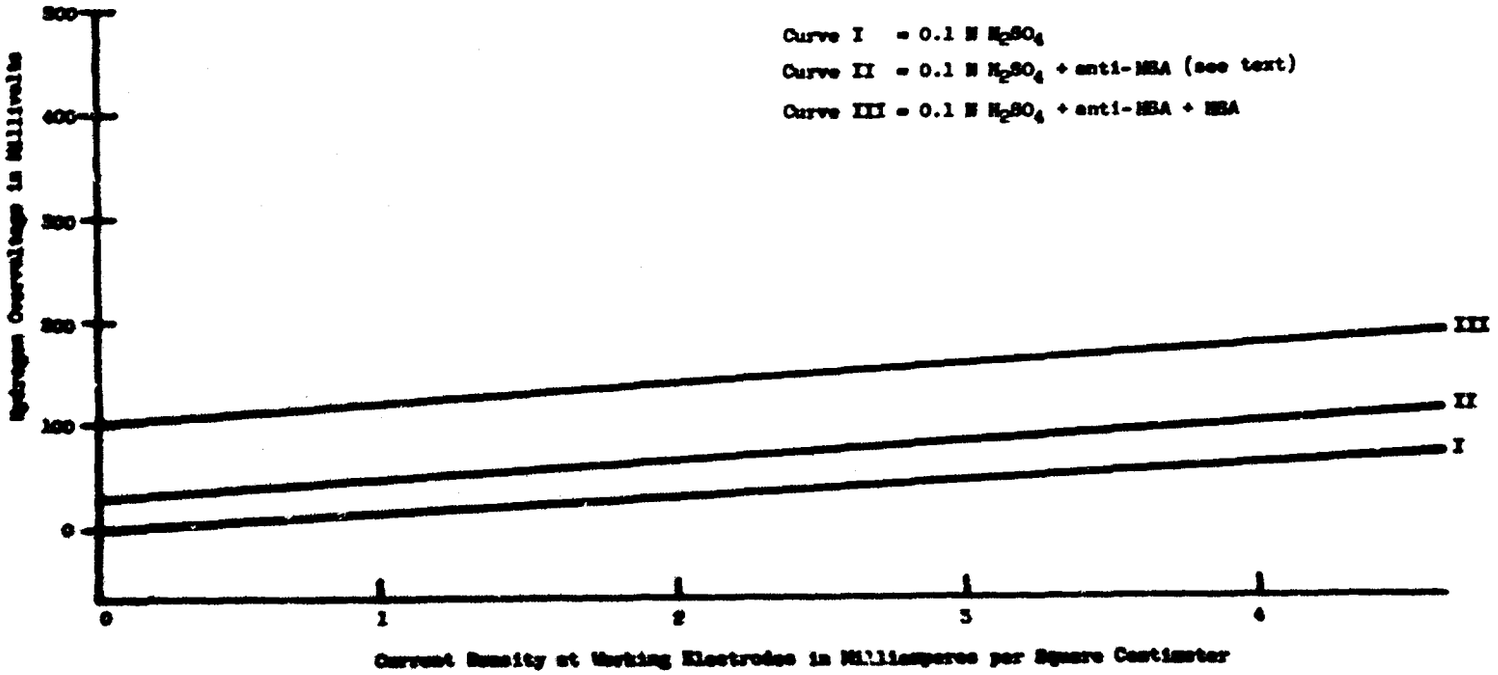


Fig. 9 - Effect of Anti-HSA and HSA on Overvoltage Measurements at pH 1.8

## 4. Conclusions

The present study has confirmed the observation that hydrogen overvoltage measurements offer a sensitive method for the detection of the presence of low concentrations of protein. Although the ultimate sensitivity of the method for detection of globulins was not established, no difficulty was encountered in detecting 0.5 mg. of protein in 25 ml. of 0.1 sulfuric acid. Hydrogen overvoltage experiments conducted at pH 7.5 were unable to detect either the presence of protein or the antigen-antibody reaction. We are not sure that additional effort to apply this method to the detection viral antigen is justified at this time. However, it would be very interesting to determine if there is a pH intermediate between 7.5 and 1.8 where the antigen-antibody reaction will occur and where the protein and/or antigen-antibody reaction can be detected by the hydrogen overvoltage method.

# E. Immunodiffusion

## 1. Introduction

Since the early work of Oudin[52] and Ochterlony,[53] the immunodiffusion techniques for evaluating antigen-antibody reactions have been applied to an ever-increasing number of systems. Ringle and Herndon (from Midwest Research Institute) developed a method for the immunological analysis of microliter and submicroliter volumes of reactants.[54] During our earlier work[55] three antigen-antibody systems were investigated by such a double diffusion procedure. No precipitin bands were observed when poliovirus 2 was reacted with antiserum to poliovirus 2. With the Southern Bean Mosaic Virus one band appeared when it was reacted with its antiserum. Also, two precipitin bands were formed in double diffusion tests between SP-10 phage of B. subtilis, and an antiserum prepared against the SP-10 phage. It was concluded that for some viruses no precipitin bands at all were obtained and for others the methods were at best capable of detecting $10^4$ or $10^6$ viral particles. No additional work was conducted then even though we speculated that the use of fluorescent antibody might improve the possibility of detecting small quantities of virus.

Several potential advantages of microimmunodiffusion tests are worthy of mention: (1) The procedure may be done rapidly (Ringle and Herndon[54] obtained precipitin bands in a matter of hours). (2) This antigen-antibody reaction may be done with non-viable material; therefore, disrupted and non-living material as well as viable cultures can be used for immunological analysis. (3) The method offers the specificity so essential to an identification system.

In our attempts to develop an in vitro phagocytic method employing sensitized WBC for the identification of virus, we encountered a number of problems which were insurmountable in the time remaining for their investigation (see earlier section on Phagocytosis in vitro). Following consultation with the sponsor, we resumed our investigation of microimmunodiffusion and fluorescent-labeled antibody reactions with the hope that sufficient sensitivity could be obtained to permit us to identify not 100 or fewer, but $10^4$ or fewer viral particles. The following sections indicate the methods, experiments, and conclusions derived from our new studies on immunodiffusion for viral identification.

## 2. Materials and Methods

The antisera and antigens for these tests were those utilized and obtained from the experiments and animals for the phagocytic studies described earlier in the report. In some of the immunodiffusion tests these reagents were specially treated or purified and this will be described in the experimental section.

For the actual immunodiffusion tests, glass microtubes (of 3 mm. or smaller diameter) were utilized for the reactions. For double diffusion tests 1.0 percent Specially Purified Difco Agar was added to the capillary tube and the antiserum and antigen added at opposite ends of the agar plug, care being taken to be sure to prevent air bubbles between the agar and the reagent. After addition of the serum and antigen each end of the capillary tube was sealed and it was mounted horizontally on a carrier rack. For single diffusion tests 3.0 percent Specially Purified Difco Agar was liquefied, cooled to 45°C, mixed with the appropriate amount of antiserum and the serum-agar mixture added to the capillary tubes. After the agar containing the antiserum had hardened, the appropriate antigen dilution was added to the tube, again being sure that no air bubbles were between the agar and the antigen layer. The tubes were then sealed and handled as were the double diffusion tests. The diffusion tests were first incubated at 37°C, then placed at 4°C. These periods varied depending upon the tests. Observations were made for precipitin band development by a number of methods. These included macroscopic observations, microscopic observations, dark field techniques, "black light" observations and fluorescent microscopy.

## 3. Experiments

Double diffusion in agar was tested by reacting a rabbit antiserum to Semliki Forest Virus (SFV) and SFV which had been grown in chick embryo tissue cultures. Undiluted antiserum was used and it was tested

against serial ten-fold viral dilutions; the highest virus concentration contained $10^6$ or $10^7$ $TCID_{50}$. These tests were performed in melting point capillary tubes with 1.6 - 1.8 mm. I.D. The 1.0 percent purified agar plug was about 9 mm. long and was added to the tube by capillary action. Undiluted antiserum was added on one side of the plug and the appropriate virus dilution on the other side of the plug. The ends of the capillary tubes were sealed with parafilm to prevent evaporation. Incubation was at 37°C for 1-1/2 days and then in the refrigerator (5°C) for two weeks. No precipitin or reaction bands were seen in any capillary using visual or dark field microscopic observation.

Since no precipitin bands were found in the SFV study (cited above), we elected to do our next diffusion studies with FITC-labeled antisera known to be capable of forming bands in agar. Two such series of reactions were carried out -- the first with antirabbit globulin (goat origin) and the second with antirabbit globulin (sheep origin). Normal rabbit serum was used as the antigen.

In capillary tube tests using double diffusion between undiluted FITC-labled globulin (goat origin) and normal rabbit serum diluted from 1 - 10 through 1 - 1,000,000 no bands were seen by visual, microscopic, dark-field, or black light observation after overnight incubation at 37°C and three additional days at 5°C. At the same time and with the same reagents, single diffusion tests were set up in capillaries. Agar containing 1 - 50 FITC-labled antirabbit globulin was prepared by mixing 1 - 10 anti-globulin with agar at about 45°C. This was drawn into the tube by capillary action, allowed to solidify and then overlaid with diluted normal rabbit serum as antigen. Dilutions of normal rabbit serum from 1 - 10 through 1 - 1,000,000 were used. No precipitin bands in the agar containing the antiserum were observed; however, the interface between the agar and liquid (normal serum dilution) became somewhat hazy yet no true precipitin occurred. The antirabbit globulin used in this test developed a fair amount of insoluble precipitate upon standing and this may have adversely influenced the test.

With a good quality of antirabbit globulin of sheep origin single and double diffusion tests were set and definite precipitin bands were observed. These tests were made in 3 mm. (I.D.) glass tubing. The agar plugs were made with 1 percent purified agar and were 10 - 15 mm. in length. For the double diffusion tests, undiluted FITC-labeled antiglobulin was placed on one side of the plugs and undiluted through 1 - 1,000,000 dilutions of fresh rabbit serum placed on the opposite side of the agar. The tubes were sealed, first with Vaseline, and then by melted paraffin. For single diffusion tests a 15 - 16 mm. agar plug of a 1 - 4 or 1 - 10 FITC-labeled antirabbit globulin was positioned in the tube and overlaid with buffered

saline (as a control) or the diluted rabbit serum (the antigen). The reactions were allowed to take place at 37°C for 18 hr. and then at 5°C. Precipitin bands were observed in both single and double diffusion tests. The band formation was faster in the single diffusion tests and approximately 5 hr. after the test was started, bands were observed both by diffuse light and with the fluorescent microscope. Undiluted, 1 - 10, 1 - 100 and 1 - 1,000 dilutions of normal rabbit sera produced bands in this short time; however, the zone of precipitin formation was only at the interface and had not moved into the agar to any appreciable degree. When these tests had been incubated for 21 hr. at 37°C and then 7 hr. at 5°C, bands had formed in the tubes containing more dilute antigen (normal rabbit serum) and multiple bands appeared in the tubes. Some of the bands had moved into the agar plug containing the FITC-labeled antiglobulin. By fluorescence microscopy the reactions were easy to read and the band formation was definitely visible at one ten-fold greater dilution of antigen than was visible by the ordinary methods used for observing immunodiffusion, including ordinary diffuse light and dark field observation. Thus, the fluorescent antibody enhanced the sensitivity of these immunodiffusion tests by a factor of 10.

In double diffusion tests using the same reagents as the tests cited in the previous paragraph, bands were observed but required four to five days to develop. The bands were also easier to read by the fluorescent technique; however, no greater sensitivity was observed.

FITC-labeled poliovirus 1 antiserum was prepared by a modification of the method of Spendlove[9] and freed of unconjugated FITC by two passages through a Sephadex G-25 column.[56] This conjugated antiserum was utilized in single and double diffusion studies in 1.6 - 1.8 mm. (I.D.) capillary tubes. Undiluted FITC-labeled poliovirus 1 antiserum was tested by double diffusion against $10^6$ to $10^2$ $TCID_{50}$ of poliovirus 1 (grown in LLC-$MK_2$ cell line) and no precipitin bands were formed following overnight 37°C incubation and seven days at 5°C. These reactions were observed by fluorescent techniques as well as by the usual methods for observing diffusion reactions. Single diffusion tests between the FITC-labeled poliovirus 1 antiserum and poliovirus 1 were also negative. For the single diffusion studies, the agar layer contained a final dilution of 1 - 4 of the FITC-labeled antiserum.

In the double diffusion tests with antirabbit globulin cited earlier in this report, the agar zone between the antigen and antibody was 10 - 15 mm. long and 3 mm. in diameter and four to five days were required for band formation. An experiment was performed to determine if the bands would form faster if the agar zone were shorter and of smaller diameter. Capillary tubing of 1.6 - 1.8 mm. diameter was used and the

agar plugs were only 2 mm. long. When FITC-labeled antirabbit globulin and normal rabbit serum were placed on opposite sides of such 2 mm. plugs, the precipitin bands formed much faster. Within several hours there was suggestive evidence of band formation and by 24 hr. very good clear-cut bands were observed by all methods of observations. However, these were observed most easily with the fluorescent microscope. Two distinct bands were observed when the 1 - 1000 dilution of normal rabbit serum (the antigen) was reacted with a 1 - 4 dilution of FITC-labeled antirabbit globulin. No bands were observed when the antigen was diluted to 1 - 10,000. These results confirmed our belief that a double diffusion test with FITC-labeled antisera in a small agar plug would become positive more quickly than in a long plug. However, the double diffusion test appears to be slower with fluorescein conjugated antiserum than a double diffusion test with non-conjugated antiserum. This is probably directly related to the fact that the FITC-labeled antibody does not diffuse as fast an non-conjugated serum.

Based on the test procedure just described, additional fluorescent microimmunodiffusion tests were performed using two different viral systems, namely, poliovirus 1 and Semliki Forest Virus. Neither of these tests resulted in a positive viral identification, further substantiating our earlier work. However, details of these tests are presented in the following paragraphs in order to demonstrate the type of reagents used and to illustrate some interesting corollary observations.

FITC-labeled poliovirus 1 antibody was prepared by a modification of the method of Spendlove[9] and freed of unconjugated FITC by two passages through a Sephadex G-25 column.[56] The FITC-labeled antibody was then fractionated by two passages over a Sephadex G-200 (molecular sieve) column. At each of these passages only selected fractions were pooled so that the final FITC-labeled antibody fraction probably contained only the 7-S antibody component. This labeled antibody neutralized 100 $TCID_{50}$ of poliovirus 1 when diluted 1 - 32 and tested in LLC-$MK_2$ monkey kidney tissue cultures.

Rhodamine isothiocyanate-labeled purified poliovirus 1 was prepared for use in the immunodiffusion and molecular sieve studies. This will be described later in the report.

Double diffusion tests were performed using the two reagents just described; namely, a purified FITC-labeled poliovirus 1 antibody and a rhodamine isothiocyanate-labeled purified poliovirus 1 antigen. Two dilutions of the antibody were used, 1 - 4 and 1 - 40, and these were each tested against five ten-fold dilutions of the purified rhodamine-labeled virus. These dilutions contained $10^2$ - $10^5$ $TCID_{50}$ of poliovirus 1. Thus,

tubes containing antigen excess or antibody excess were used in the double diffusion tests. Capillary tubing (1.6 - 1.8 mm.) and 2 mm. plugs of agar were used for these tests, thereby giving the optimum conditions observed in the antiglobulin-globulin tests described above.

In these double diffusion tests with labeled antisera and labeled virus no precipitin bands were observed following 2 hr. at 37°C, overnight at 5°C and two weeks at 5°C. Observations were made macroscopically and microscopically including use of the fluorescent microscope with a variety of filters. Thus, even with reagents with two different fluorescent labels, we still failed to obtain a positive precipitin band with poliovirus 1 and its antiserum.

In order to check viral immunodiffusion with the Semliki Forest Virus system using a fluorescent-labeled antiserum, rabbit antiserum was labeled by FITC by the modification of the method of Spendlove[9] and freed of non-conjugated FITC by two passages over Sephadex G-25.[56] This antiserum had been prepared from rabbits immunized with SFV grown in chick embryo tissue culture containing calf serum; therefore, it contained antibody to the calf serum (and possibly chick embryo tissue) as well as neutralizing antibody to SFV.

Double diffusion reactions were set up using the FITC-labeled antibody to SFV in tests against three different antigens; (1) SFV mouse brain suspension, (2) SFV from chick embryo tissue cultures, and (3) normal calf serum. The FITC-labeled antiserum was equivalent to a 1 - 5 dilution of unlabeled antiserum and was used without further dilutions in the immunodiffusion tests. Each of the three antigens was tested in 1 - 10 through 1 - 10,000 dilutions. The reactions were carried out in the small 1.6 - 1.8 mm. capillary tubes and the agar plugs between the antibody and antigens were 2 mm. in length thus providing optimum conditions based on a known precipitating system. The antigen-antibody reactions were allowed to incubate at 37°C for 1/2 hr., observed and then placed at 5°C. Additional observations were made after 18 hr. at 5°C and up to two weeks at 5°C.

In the reactions between the SFV antiserum and the SFV mouse brain suspension no precipitin bands or zones were observed; therefore, the results for SFV identification were negative. However, reactions were observed between the SFV antisera and (1) normal calf serum, and (2) the SFV from chick embryo tissue cultures. We attributed both of the positive reactions to the calf serum antibody present in the labeled rabbit antiserum. In fact, the reactions suggested that the quantity of calf serum was directly related to the dilutions used in the test. The optimum band formation was obtained with a 1 - 1000 dilution of normal calf serum and

a 1 - 10 dilution for the SFV chick embryo tissue antigen. Since the SFV chick embryo tissue antigen contained 1/50th or less of calf serum, these results appear to correlate exactly with the calf serum level. The precipitin reactions with the calf serum antigen and the SFV from chick embryo tissue cultures were positive after 1-1/2 hr. at 37°C but bands were not distinct even after 18 hr. at 5°C; however, by the fourth day distinct bands were observed in the higher dilutions of antigen for the two positive reactions. These were much easier to see by fluorescence microscopy than by other means of observation.

This experiment emphasizes the importance of controlling all the antigens in an immunologic system, since the reaction to the calf serum in the tissue culture SFV antigen could have been interpreted as a positive viral identification had we not tested the SFV in the form of mouse brain extracts.

Since all of our poliovirus and SFV tests by immunodiffusion had been negative, we felt that it would be desirable to test an antiserum prepared in some other laboratory; therefore, a bivalent antiserum was obtained from the School of Aerospace Medicine. The antiserum received was a rabbit serum against both poliovirus 1 and poliovirus 3, and had been made using antigens supplied by the CDC (Communicable Disease Laboratory, Atlanta, Georgia).

Details of our immunodiffusion tests with the bivalent antiserum will not be given but the rabbit antiserum was tested against both types 1 and 2 polioviruses with completely negative results. We utilized both FITC-labeled antiserum (prepared at MRI) and non-labeled serum against a graded series of dilutions for polioviruses 1 and 2; thus we should have gotten a positive reaction with type 1 and a negative reaction with type 2 poliovirus. However, by both double diffusion and single diffusion tests, we failed to get precipitins or band formation. The immunodiffusion tests were read by all our usual methods including fluorescent microscopy.

## 4. Conclusions

These data on immunodiffusion suggest that fluorescent-labeled antibody may improve the sensitivity of the procedure; however, this may only be true in tests in which a true precipitate is formed. Both the SFV and poliovirus 1 studies indicate that with virus-viral antibody reactions the complex formed is "soluble" or at least much less prone to form a true precipitate than is observed in antigen-antibody reactions between bacterial antigens and antibody or between globulins and their antibodies. It is obvious that additional research is necessary to develop a highly sensitive immunodiffusion test for viruses particularly at lower levels of virus.

One possible method of reducing the solubility of "soluble" viral antigen-antibody reaction products in the FITC antibody virus identification procedure would involve the use of ammonium sulfate or other protein precipitants, but this has not been studied. We had originally hoped that use of FITC-labeled antiserum would enable us to visualize fluorescence bands of the soluble viral antigen-antibody conjugates in our double diffusion tubes.

FITC-labeled antiserum appears to diffuse through agar much more slowly than unlabeled antiserum as judged by the location of the fluorescent precipitin bands. Whether the migration rates of the labeled and unlabeled sera would be more similar in a non-ionic gel has not yet been established. This property of slower diffusion of the FITC-bagged antibody must be borne in mind when new methods of viral identification such as described in the next section of this report are considered.

Most of the immunodiffusion studies reported were done using 2 mm. agar plugs in 1.6 - 1.8 mm. capillary tubes and 0.05 ml. of the antigen and/or antibody in the individual tests. As reported by Ringle and Herndon[54] these immunodiffusion tests can be performed in smaller capillaries and with smaller quantities by the use of the micromanipulator, thus increasing the sensitivity of the tests. This will be of greater value for known precipitating systems such as those described in the next paragraph.

We feel that extension of the immunodiffusion studies for viral identification is not advisable until some newer methods become available. However, the application of some of these modified techniques to bacterial (and possibly fungal) antigens and to hypersensitivity studies may prove quite beneficial. This is particularly true for the fluorescent antibody-antigen reactions since they appear to be more sensitive, more rapid, and easier to read. By proper design of a slit, it should be possible to use the automatic scanning mechanism available for the Aminco-Bowman Spectrophotofluorometer to measure these fluorescent band formations. Such a technique offers a great potential for bacterial identification, diagnosis and possibly for detection. It would also offer the other advantage of microimmunodiffusion; namely, that it is very rapid.

The application of rhodamine-labeling to viruses has many applications and should be fully investigated as a means of tagging viruses. This will be discussed more fully in the next section.

## F. Studies of "Soluble" Virus-Antibody Complexes

### 1. Introduction

As stated in the previous section, we feel that it may be necessary to measure "soluble" antigen-antibody complexes in order to identify viruses within the limitations imposed upon the project. We know that poliovirus and poliovirus antiserum combine to form a neutralized complex, since we can readily test the complex in tissue cultures and prove that the virus is tied up in the complex. Yet, we do not see a visible precipitate in these neutralized mixtures. Also, we know that complexes of antibody and as little as 10 - 100 $TCID_{50}$ can exist, since the neutralized complex fails to infect appropriate tissue cultures. Similar statements can be made for most virus-antibody systems. Thus, we feel that some mechanism for detecting the virus-antibody complex must exist which is independent of tissue culture or animal test methods. In fact, the work of Giron, Hellman and Schmidt at Brooks Air Force Base bears strongly on this point.[57,58] In their studies FITC-labeled poliovirus antibody was found to be retained on columns of DEAE Sephadex. When poliovirus was passed through the column containing the tagged antisera it was complexed and retained in the matrix of the ion-exchange column. The virus could be released by the low pH dissociation methods applicable to antigen-antibody complexes.

The complexes of virus and antibody (soluble ?) must be different from (1) unbound antibody, and (2) unbound virus; otherwise they would not be "neutralized." This difference can be due to a number of chemical, physical, and biological properties. No attempt to review the literature on antigen-antibody complexes will be included in this report; however, we believe that antigen-antibody complexes may have different charges and may have different molecular size than either of the two components from which the complexes are derived. If the charge or ionic status of the complex is altered from that of the antigen (virus), then an ion-exchange material should theoretically separate the complex from the unbound components. The DEAE Sephadex A-25 used by Giron et al. (cited above) is an ion-exchange medium and thereby might accomplish the separation. Additional studies on such media appeared desirable. Also we felt that such studies should be supplemented with experiments employing molecular sieves such as Sephadex (various sieve sizes available) and agarose materials (Sepharose,[59] Bio-Gel-A,[60] etc.). Such materials of various pore sizes separate molecules of differing molecular weight by physical means and constitute one of the least (if not the least) damaging of the separation methods available. Molecular sieves are available for fractionation of substances with molecular weights of less than 1,000 up to 150,000,000. Columns prepared

from such molecular sieves permit the passage of the larger molecules first, and a proportionate delay or complete retention of smaller molecules. These gel filtrations are functionally related to the pore size of the gel bed (generally spherical beaded agar or agarose materials).

Figure 10 illustrates the range of separations achieved by Bio-Gel-A (one of newest molecular sieves and only recently available). It is apparent that enzymes, globulins, and viruses could readily be separated by passage through such columns. Steeve and Ackers reported on the use of a wide range of molecules for the calibration of agar gels[61] and the same authors reported the separation of Tobacco Mosaic Virus and Southern Bean Mosaic Virus by agar gel filtration.[62]

In theory, the molecular sieves should permit the separation of an antigen or an antibody, or both from a soluble antigen-antibody complex. This assumes that the antigen-antibody complex has a different molecular weight (size) from the individual antigen and antibody components. Figure 11 is a theoretical elution curve for a virus, viral antibody and virus-antibody complex. No report of such a virus-antibody study could be found in the literature; therefore we felt that we should attempt preliminary studies aimed at using such methods for viral identification. Our theoretical considerations were bolstered by the report of Boyns and Hardwicke.[63] These two British scientists demonstrated a similar phenomenon with soluble antigen-antibody complexes of rabbit antibody to bovine serum albumin. They separated and purified the complex from the other serum fractions by means of Sephadex G-200 columns. They also demonstrated that the soluble antigen-antibody complexes were still active when injected into animals. In their experiment they worked under the condition of antigen excess while in our work we are more concerned with the antibody excess situation.

Based on the above discussion and the publication of Boyns and Hardwicke, we did some preliminary studies aimed at molecular separation of the virus antigen-antibody complex. Since the Aminco-Bowman Spectrophotofluorometer was available and had such an exceedingly good sensitivity, we utilized it to measure protein and the fluorescence due to the fluorescent antibody used in our studies.

The following sections describe the materials and methods, experiments, and conclusions and recommendations for the studies of "soluble" virus-antibody complexes.

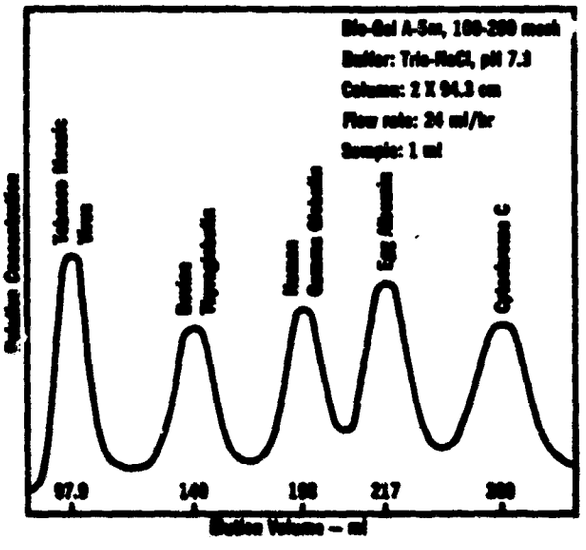


Fig. 10 - Separation of Complex Virus-Protein Mixture by Gell Diffusion As Presented in the Bio-Gel A Literature.[60]

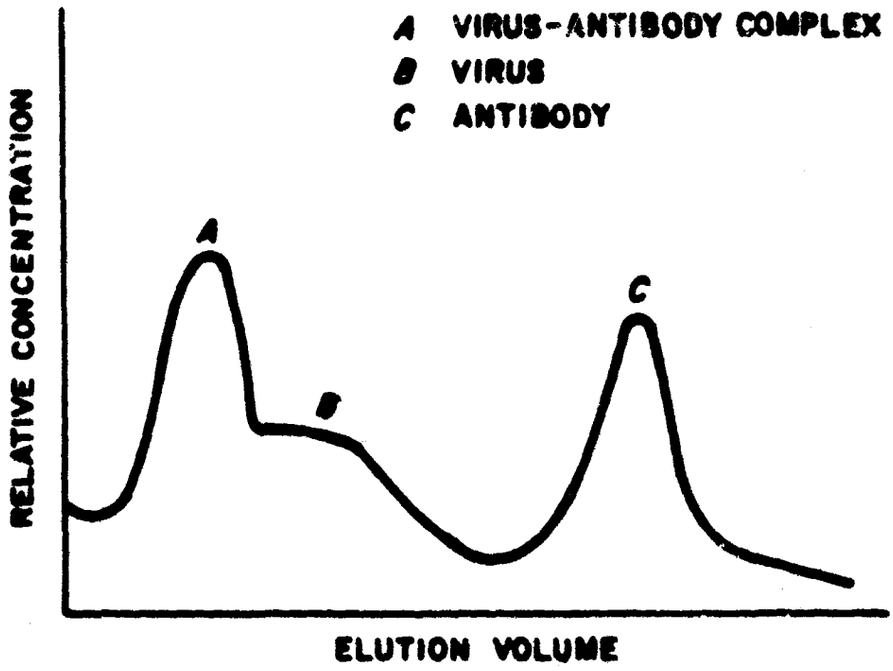


Fig. 11 - A Theoretical Curve Showing Expected Separation of a Virus-Antibody Mixture When the Antibody is in Slight Excess.

## 1. Materials and Methods

The materials and methods varied considerably from experiment to experiment depending upon the goal of the study; therefore, many of the details are described in the experimental section which follows.

In general the preparation of the columns of the ion-exchange and gel filtration compounds followed those given by the manufacturers.[64,65]

Protein and fluorescent compounds were assayed by means of the Aminco-Bowman Spectrophotofluorometer. Excitation and emission wavelengths were varied as needed. In general protein and nucleic acids were measured at 280 mμ and 260 mμ. Fluorescein was assayed at 495 mμ and rhodamine at 365 mμ. Virus titers and antibody levels were determined in LLC-$MK_2$ monkey kidney cell cultures using medium 199[66] for culture maintenance.

## 2. Experiments

The experiments performed during this study are all preliminary in nature and only provide a base upon which to extend and explore the general phenomenon of separation of virus-antibody complexes. Much of this work has been of a preparative nature and some of reagents were also used in the studies on microimmunodiffusion summarized earlier in this report.

a. Preparative experiments: An FITC-labeled normal rabbit serum was fractionated using a Sephadex G-200 column. Sephadex G-200 is the agar gel for separating compounds with molecular weights of 5,000 to 800,000. By following the fluorescence emitted following ultraviolet excitation at 260 mμ, 280 mμ, and 495 mμ, we were able to demonstrate fractions with various protein-fluorescence ratios. These appeared to follow the distribution of the various proteins found in normal serum. Thus, we were able to fractionate a fluorescein-labeled antibody in the same manner as has been reported for unlabeled sera.[67,68]

After the above procedure had been shown to be feasible, a FITC-tagged poliovirus 1 rabbit antiserum was fractionated by passage through a Sephadex G-200 column. The fractions were again evaluated by their fluorescence at 260 mμ, 280 mμ, and at 495 mμ. The first two for protein and 495 mμ for the fluorescein label; each fraction was also checked for the development of a precipitate with saturated ammonium sulfate to determine which fractions were globulins, albumin, etc. Four fractions were selected as being the best antibody (7S); these were pooled and rerun over another G-200 Sephadex column. Again the four best fractions were pooled and this purified FITC-tagged poliovirus antibody was

tested for neutralizing antibody and used for immunodiffusion and molecular sieve studies for soluble antigen-antibody conjugates. This purified labeled antibody in a 1 - 32 dilution was capable of neutralizing approximately 100 $TCID_{50}$ of poliovirus 1.

b. Preparation of purified rhodamine-tagged poliovirus 1: Brunhilde poliovirus 1 was purified by passage through a column of DEAE Sephadex A-25 by the method of Giron and Hellman.[57] Our results paralleled those of Giron. A virus pool was prepared from the three fractions having the highest virus titer and low protein content. Approximately 99 percent of the virus was recovered in this pool. It contained a higher ratio of 260 - 280 mμ fluorescence than subsequent less pure fractions coming off the Sephadex A-25 column. Therefore, the ratio of RNA to protein was higher than for non-purified virus. This was expected on the basis of the work of Schwerdt and Schaffer[69] for purified poliovirus.

The purified poliovirus 1 was labeled with rhodamine-β-isothiocyanate (Mann Research Laboratories) following basically the same modified procedure of Spendlove[9] that we had used for FITC-tagging of antibody. The rhodamine isothiocyanate was suspended in 0.01 M phosphate buffer, pH 7.1 and added to the purified poliovirus. (Final concentration = 1 mg. rhodamine/ml. The pH was adjusted to 9.1 with 0.04 N NaOH and the mixture allowed to stand 0.5 hr. at room temperature. It was immediately fractionated by passage over a column of Sephadex G-25. The unbound rhodamine remained on the column. No visual rhodamine was apparent in the first 10 fractions, but by black light rhodamine fluorescence was detected in all 10 fractions. In fractions 11 and 12 gross amounts of rhodamine were apparent even by observations under ordinary light. Assay of the fractions for fluorescence was performed by excitation at 365 mμ. The protein, nucleic acid and rhodamine curves were proportionate for the first three fractions and the levels of each gradually increased. However, from the fifth through the tenth fractions the protein increased much faster than did the rhodamine level. This we interpreted as indicative of less pure virus, and borne out when viral assays in tissue culture were carried out. A pool the first four fractions contained 60 percent of the virus treated with rhodamine. The pool of fractions five to eight contained only 23 percent of the virus. Thus, we believe that the virus in the pool of fractions one to four was viable and labeled with rhodamine.

This pool of rhodamine-labeled poliovirus 1 was subsequently used in several immunodiffusion studies. Although it has not been used as such, it should be a good fluorescent reagent for gel filtration studies of virus-antibody mixtures.

c. Column tests of virus-antibody mixtures: Sephadex G-200 was used to study the elution pattern of two mixtures: (1) Poliovirus 1 and FITC-labeled poliovirus antiserum, and (2) Poliovirus 1 and FITC-labeled normal rabbit serum. The mixtures had been prepared the day before and allowed to react overnight at 5°C. This was done in order to bring about the maximal neutralization of the poliovirus in mixture (1). Poliovirus ($10^3$ - $10^4$ $TCID_{50}$) was used in each mixture. Fluorescence was measured for 14 sequential fractions coming off the column following application of each mixture. Figures 12a and 12b are graphs of the assay results. The graph in Fig. 12a plots the relative fluorescence for the samples at the various wavelengths listed on the right- and lefthand ordinates. Figure 12b plots the ratios of the 280 mμ to 260 mμ fluorescence for the various samples. The higher the ratio the more protein to nucleic acid is in the sample. Three differences were noted between the fractions coming from the two mixtures. Fluorescence of mixture (1) attributable to the FITC on the poliovirus antiserum followed a flatter curve than the fluorescence from mixture (2) attributable to FITC on the normal serum. The protein curve (280 mμ) was much different in the fractions from mixture (1) than from mixture (2). Much less protein was eluted in any one fraction of mixture (1) than in mixture (2) and the mixture of poliovirus and antiserum did not have a sharp peak of protein release. By far the most noticeable difference between the two mixtures was the comparison of the 280 mμ to 260 mμ fluorescence in the fractions collected from the two mixtures. A very striking increase in the ratio of 280 mμ to 260 mμ fluorescence occurred in the poliovirus-poliovirus antiserum mixture. The increased 280 mμ to 260 mμ ratio possibly indicates greater protein to virus ratio in these fractions.

Since the work of Giron et al., cited earlier, indicates that FITC-labeled sera do not move to any extent when placed on a column of DEAE Sephadex A-25, we decided to see if poliovirus neutralized by a poliovirus antisera might move through such a column and thus be separated from the unbound FITC-labeled serum. To test this hypothesis, we prepared mixtures of (1) poliovirus 1 ($10^6$ $TCID_{50}$) and poliovirus antiserum (FITC-labeled), and (2) spent tissue culture medium and poliovirus antiserum (FITC-labeled). These two mixtures were incubated 1 hr. in a waterbath at 37°C to permit virus neutralization. They were then fractionated on a DEAE-Sephadex A-25 column and the fractions assayed on the Aminco-Bowman with 280, 260, and 495 mμ excitation. Again, differences were observed in the curves for the two mixtures, but we are not sure how to interpret the results. Figures 13a, 13b, and 13c are plots of the relative intensity in the various fractions. Some protein came off the column quicker in the spent tissue culture-antibody mixture than in the virus-antibody mixture.

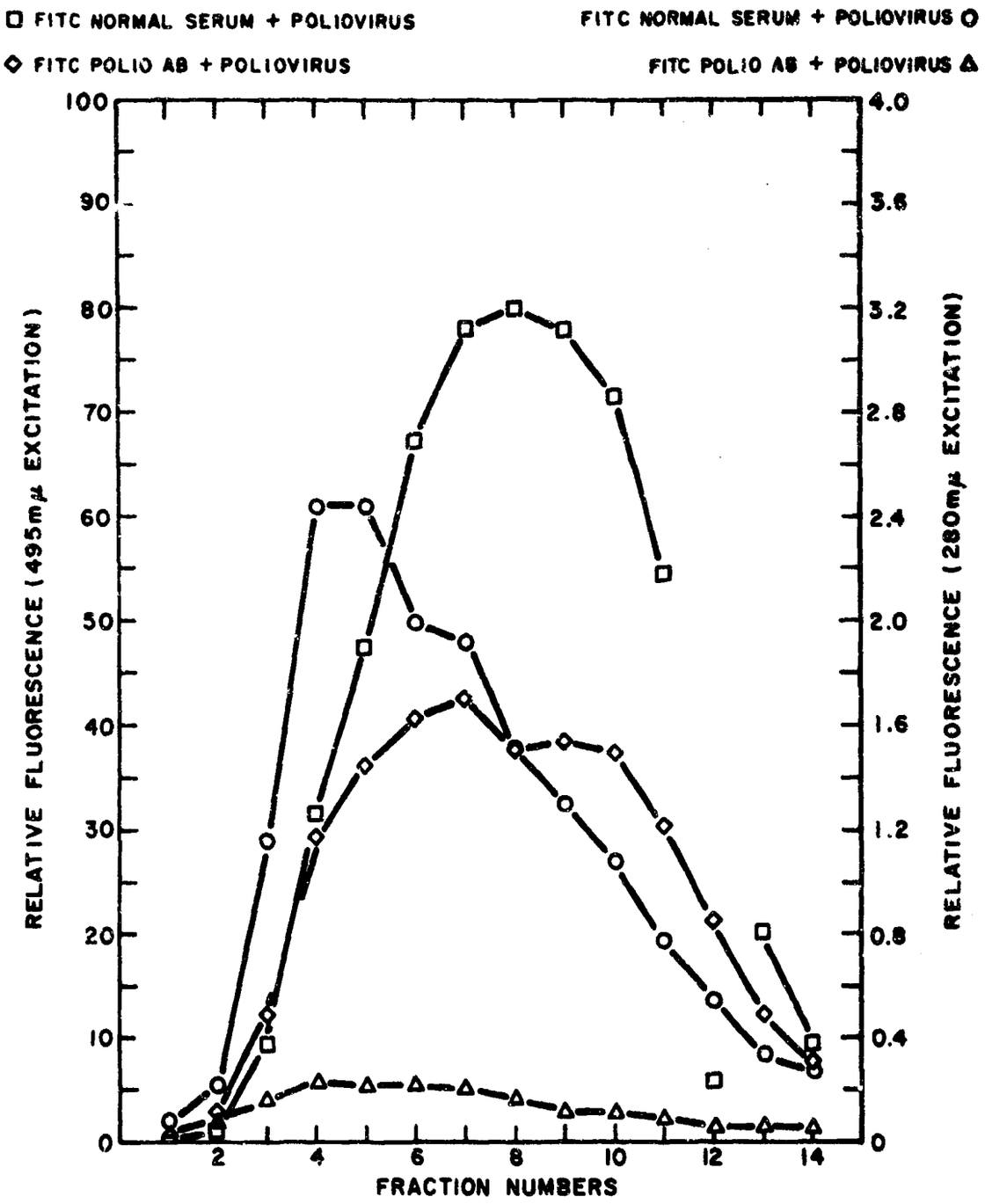


Fig. 12a - Trial Separation of "Soluble" Antigen-Antibody Complexes on Sephadex G-200

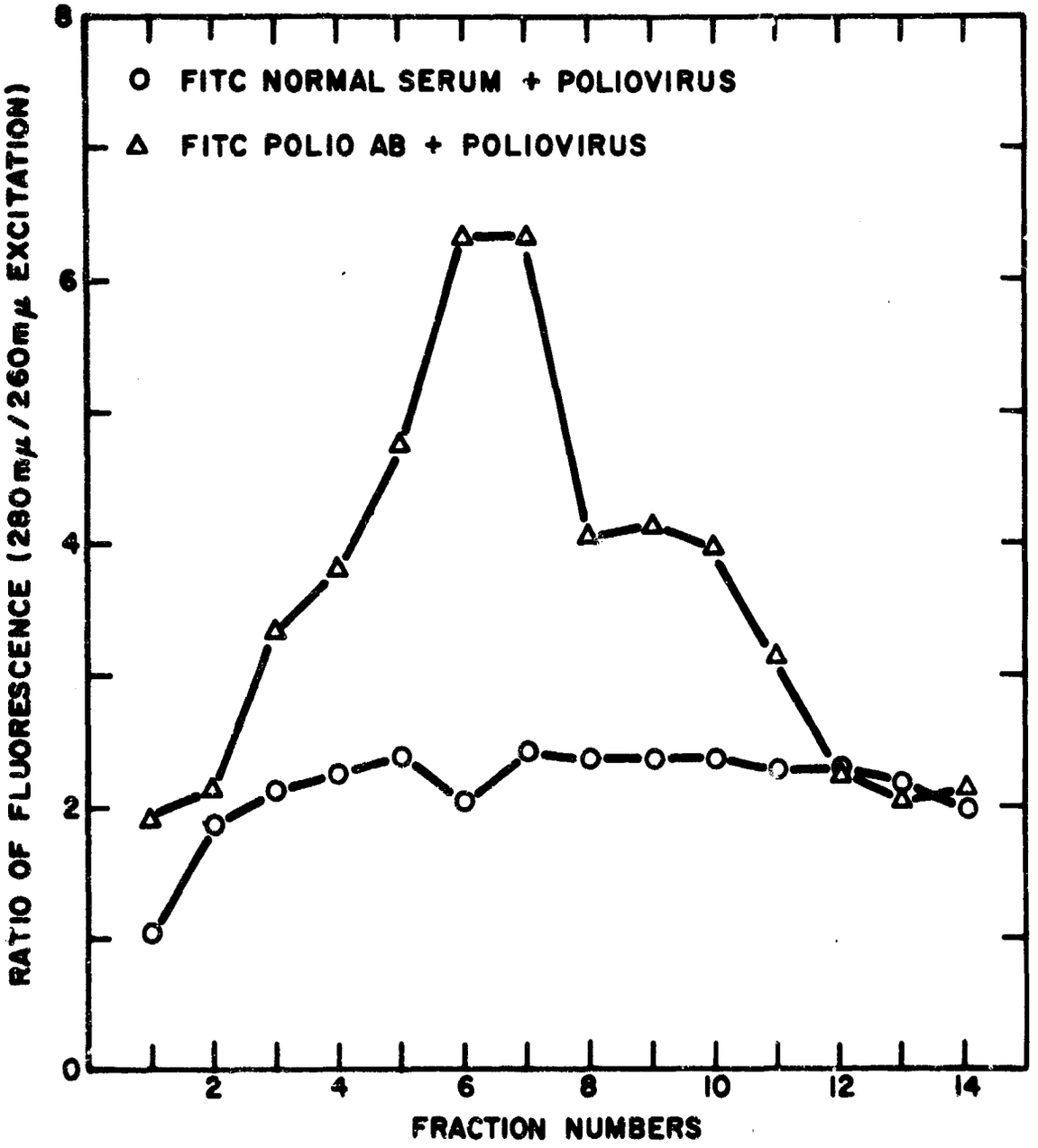


Fig. 12b - Trial Separation of "Soluble" Antigen-Antibody Complexes on Sephadex G-200

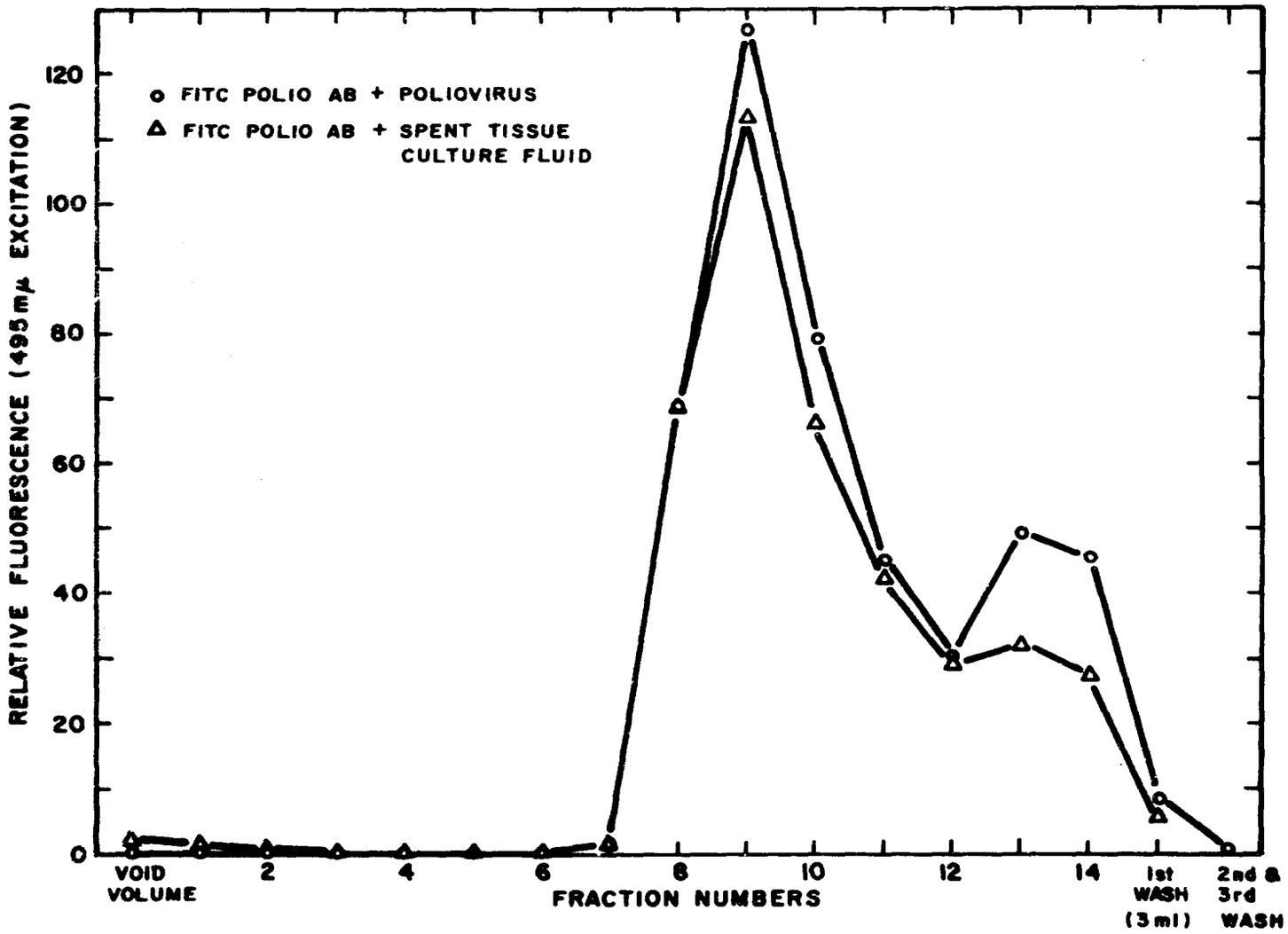


Fig. 13a - Trial Separation of "Soluble" Antigen-Antibody Complexes on DEAE Sephadex A-25

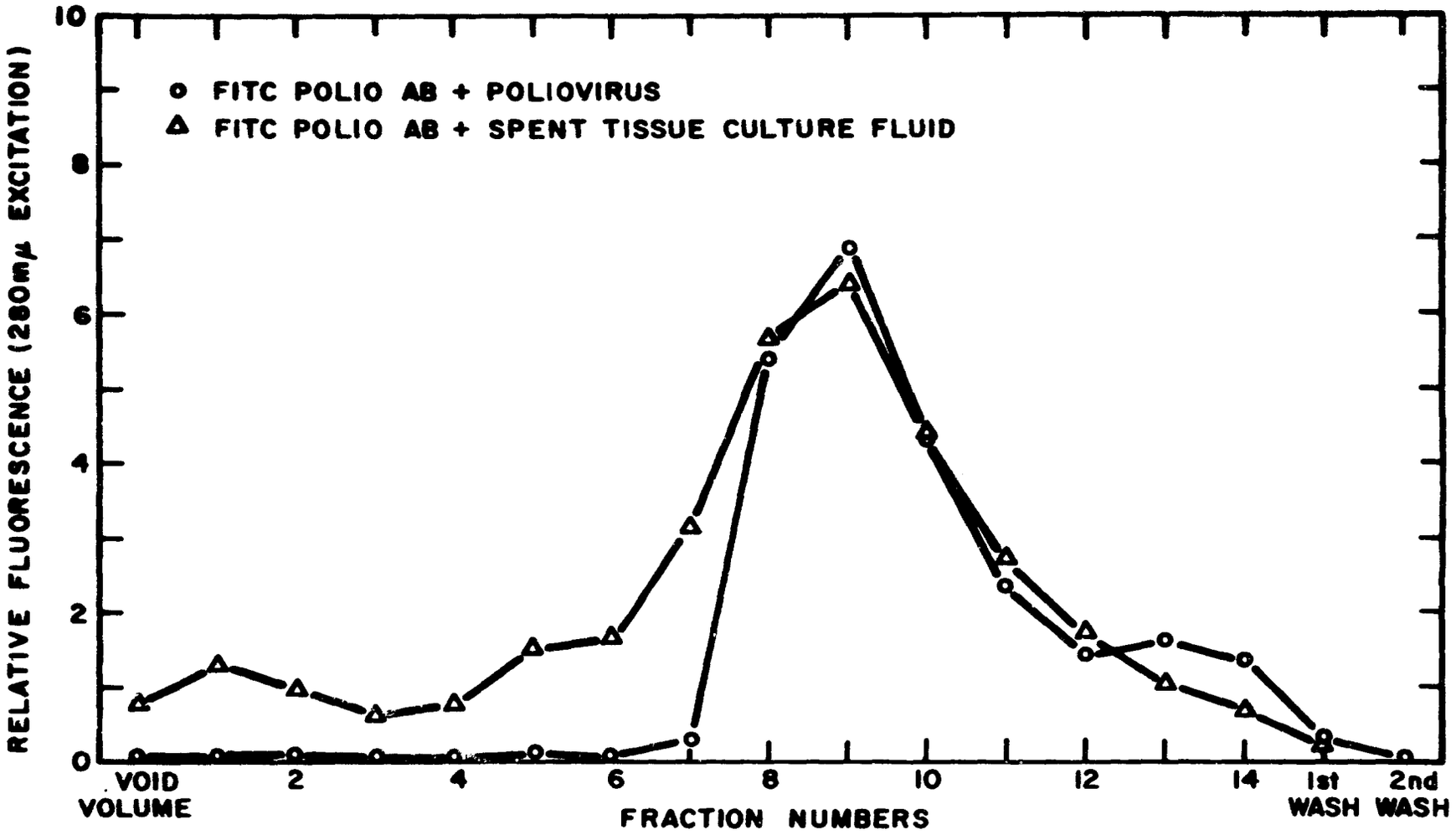


Fig. 13b - Trial Separation of "Soluble" Antigen-Antibody Complexes on DEAE Sephadex A-25

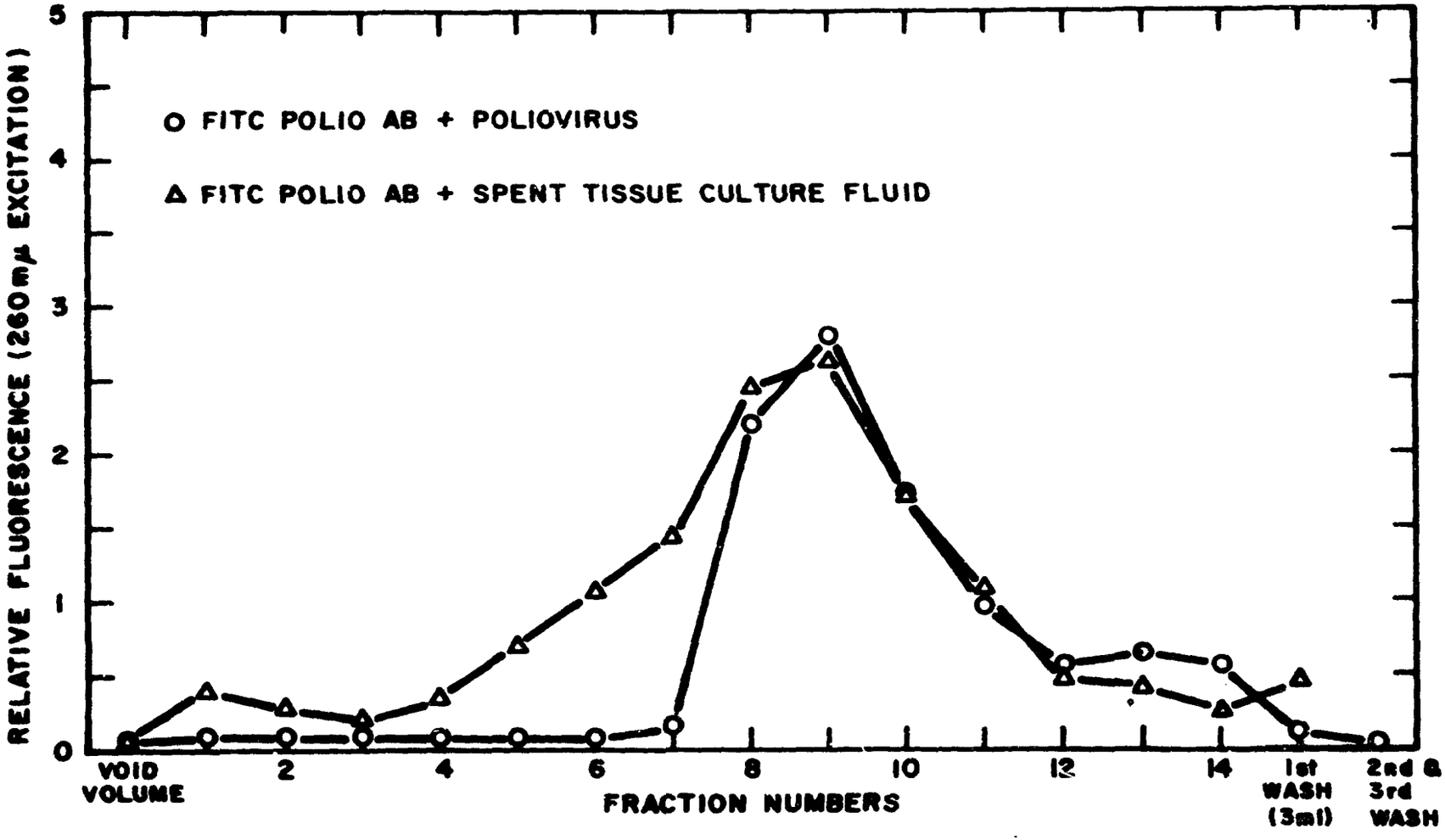


Fig. 13c - Trial Separation of "Soluble" Antigen-Antibody Complexes on DEAE Sephadex A-25

Also, when the poliovirus-poliovirus antibody mixture was fractionated, there was an extra small peak of fluorescence due to FITC that moved off the column after the main fluorescein peak. This peak did not occur with the mixture of spent tissue culture medium and poliovirus antiserum (FITC-labeled).

It should be pointed out that the elution of the FITC-labeled poliovirus antibody from DEAE Sephadex A-25 in this experiment is in contrast to that of Giron[57] in which his tagged antiserum held its position on DEAE Sephadex A-25. This difference may have been due to the methods of column preparation, the buffers used, the incubation of the mixtures of antibody, or other factors.

Sepharose 4B is an agarose gel filtration medium which is used for fractionation of substances with molecular weights of 300,000 to 2,000,000. Columns of Sepharose B were used to fractionate two mixtures: (1) purified poliovirus 1 (not labeled) mixed with purified FITC-labeled poliovirus 1 antiserum, and (2) M/15 phosphate buffer mixed with purified FITC-labeled poliovirus 1 antiserum. The two purified materials, poliovirus 1 and the purified FITC-labeled poliovirus antiserum, were those described previously in this section. The mixtures were prepared and incubated in a 37°C waterbath for 1 hr. (for neutralization to occur) before they were added to the column. Approximately $10^{6.5}$ $TCID_{50}$ of poliovirus 1 were used in each mixture. Fractions were collected from each mixture and assayed (as described before) for protein and fluorescence due to the FITC. It had been hoped that the mixture of poliovirus and poliovirus antibody would move off the column at a different rate than the mixture of FITC-polio antibody and buffer. Within the limits of assay the protein and fluorescence in both mixtures followed similar elution patterns; therefore the data did not permit us to distinguish or measure the "soluble" virus-antibody complex. The possibility must be considered that agarose columns may be capable of breaking up the virus-antibody complex. If such separation did occur we should have seen a change in our protein elution patterns but we did not.

The purified agar used in our immunodiffusion studies behaves both as a molecular sieve and as an ion exchange substance. Since during ion exchange an ion replacement takes place, we felt it would be desirable to try immunodiffusion with a non-ionic exchange material. We performed two very preliminary experiments attempting to use the non-ionic Sepharose 4B as the medium for immunodiffusion in capillary tubes and for viral identification reactions. Due to the beaded nature of the material, Sepharose 4B was difficult to use in the capillary tubes normally employed for micro-immunodiffusion. One attempt to react FITC-labeled poliovirus 1 antiserum with poliovirus 1 failed to work. However, in 3 mm. glass columns,

Sepharose 4B gave a suggestion that it might be used. We applied a 1 + 4 dilution of FITC-labeled antirabbit globulin to the Sepharose 4B and immediately followed this with diluted normal rabbit serum. As soon as the rabbit serum had been added to the column of Sepharose the ends of the tube were sealed with paraffin and the column observed for fluorescence using the "black light." Within 15 min. an area of precipitation (band ?) formed at the top of the column. This precipitation became more pronounced by 2 hr. The precipitate was poorly visible by ordinary light but readily seen with the "black light" and with the ultraviolet microscope. This latter study with a known precipitating system suggested to us that microcolumns of Sepharose or other gel filtration medium might be valuable once we know how to measure or separate the soluble virus-antibody complexes.

## 3. Conclusions

Although these experiments on "soluble" virus-antibody complexes were all of a preliminary nature, the data were sufficiently encouraging to suggest that these methods merit considerably more investigation. The different patterns of elution of protein and of fluorescence antibody in the studies with DEAE Sephadex A-25 and Sephadex G-200 are not completely understood but a soluble complex may have been demonstrated, particularly in those fractions in which the protein to nucleic acid ratios were high.

The use of fluorescent-labeled antibody and its altered diffusion rate may have complicated the studies, yet it provided a good tool with which to study the complexes. The FITC-labeled antibody was active in standard neutralization tests; therefore, such tests should be more regularly used in future investigations.

Our preliminary attempt at rhodamine labeling of poliovirus was very encouraging. We believe that such virus may be easily made and that by minor procedural changes, high concentrations of purified-labeled virus can be obtained. Such labeled virus will permit a fluorescent label to be used on each of the components of the soluble complexes. This will permit better evaluation of the relative positions and concentration of both virus and antibody in the various fractions derived from gel filtration studies. The tissue culture infectivity of the rhodamine-tagged virus will permit us to test fractions for both fluorescence and viable virus. Tissue culture tests will be necessary to prove that the virus-antibody complex has been broken or is still intact.

We believe that the rhodamine label (and possibly other fluorescent labels) may be added to an unknown sample and this labeled sample,

then used for viral identification by such methods as used by Schmidt and Giron.[70] The latter utilized a radioactive label to follow the retention of poliovirus on FITC-labeled poliovirus antiserum positioned on DEAE Sephadex A-25. Fluorescent-tagged virus should have several advantages over radioactive virus for gel filtration studies.

Future "soluble" virus-antibody studies must include investigations of complexes made in antigen excess and in antibody excess in order to define the parameters of the column fractionations. In such studies we feel that it is imperative to use tissue cultures for neutralization tests and virus titers both before and after the column experiments.

The effect of complement on the gel filtration of "soluble" complexes is an unknown and should be evaluated. The use of complement might permit an indirect assay procedure to be developed. The indirect assay of the complexes by means of labeled-antiglobulin sera should also be investigated.

These gel filtration studies lend themselves to the use of radioactive labels on either one or both virus and antibody, and radioactive tags might be worth investigating. As previously mentioned, we believe the fluorescent-tagged reagents are easier to use (and possibly get); however, radioactive tagging provides a highly sensitive tool.

These "soluble" complex studies are extremely encouraging. By the application of the correct molecular sieve, labels, and methods of assay, it may be possible to adapt such methods to extremely small amounts of virus and antibody. We believe that the utilization of more highly purified antisera and possibly labeled and/or purified virus may provide us a system for viral identification that can then be developed for use with routine samples. This is particularly true if a preparative procedure on the viral sample such as the DEAE-25 column method (as developed at Brooks Air Force Base) can be applied before the antibody reaction is attempted.

In addition to viral identification by soluble complex assays, we believe the above techniques and principles will have great potential for other systems such as for bacterial and fungal identification and hypersensitivity studies.

## III. GENERAL CONCLUSIONS AND RECOMMENDATIONS

In the experimental section of this report we have discussed briefly our findings concerning the applicability of each of the six identification methods for the identification of either 100 or 10,000 virus particles in 6 hr. In this section we are attempting to relate each of these experimental approaches to the project objectives and to each other.

With the phagocytosis in vitro method we were able, on several occasions, to achieve positive identification of 250 - 1,000 $TCID_{50}$ of poliovirus (both types 1 and 2) and Semliki Forest Virus in less than 6 hr. Although these identifications were performed and confirmed by different operators, extreme difficulty was encountered in repeating the preparation of the sensitized WBC which possessed the high sensitivity to viral antigens and which responded in the identification tests. In spite of our failures, we are of the opinion that basically this approach to virus identification can be made to work if someone will study it for a period of two or three years. Although we could not assign a high priority to support of this approach, we do believe that it can become an effective virus identification method for as little as 250 virus particles.

Our experience with the phospholipid bilayer membrane method for the identification of virus showed us clearly that the preparation of sensitive membranes is an art instead of a science. As J. del Castillo[36] pointed out in his paper not all of the ox-brain phospholipd extracts produce films which show the impedance changes necessary for the recognition of antigen by antibody. As he explains it, the variation is in the individual ox brain employed in making the extracts rather than in the technique for processing the phospholipid. These findings plus our experimental studies add up to the conclusion that no one knows what makes a phospholipid membrane reactive or inactive. Here again basic research will be needed to explain the biochemistry of "reactive" ox brains and to reduce this approach to a well defined and reliable system. We feel that the potential applicability of such a system is adequate to justify continued studies of this approach.

The particle electrophoresis method for the identification of virus was tried with Semliki Forest Virus antiserum-coated red blood cells and with poliovirus 2 antiserum-coated red blood cells. Results of these studies were negative even though both high and low concentrations of virus were used to challenge the antiserum-coated RBC. The failure of the method cannot be blamed on the absence of antibodies on the particles as their presence was demonstrated in agglutination tests. The studies with antihuman-Y-globulin- (rabbit serum) coated red blood cells showed that

challenge with human serum gave sufficient reduction (50 - 60 percent) of the electrophoretic migration rate of the sensitized cells to permit the identification of the presence of the human γ-globulin. In conclusion, our experiments with the use of particle electrophoresis for the identification of virus were unsuccessful. We are not recommending additional studies along this line since it is unlikely that the use of highly purified 7S antibody in place of the ammonium sulfate precipitated antipoliovirus 2 antiserum would give enough of a boost in sensitivity to make the method successful. On the other hand, the method may be useful for the detection of bacterial or other non-viral antigens; in addition, we have reason to believe that antigen-coated red blood cells (possibly viral or non-viral antigens) would be useful by particle electrophoresis for the detection of antibodies.

Hydrogen overvoltage measurements were made to determine the ability of antiserum plated on the working cathode to detect the presence of antigens. Our results confirmed the observation that small quantities of protein in solution are readily detected by this method at pH of 1.8; however, at or near neutral pH we were unable to detect either the presence of protein or the antigen-antibody reaction. Further studies on this approach for virus identification are not recommended.

The immunodiffusion studies were aimed at finding a method to make them more sensitive and to enable the visualization of the zone of viral antigen-antibody reaction when such a conjugate is "soluble." Experiments with polioviruses 1 and 2 showed that the double diffusion method no precipitin band was obtained when it was allowed to incubate with its antibody. The use of fluorescent antipoliovirus 1 and fluorescent antipoliovirus 2 reacted with poliovirus 1 or 2 show that there is no fluorescence band formed to indicate the location of the conjugates. Also, no band formation was observed in the Semliki Forest Virus system. There is, however, ample proof that the fluorescent antibody is able to neutralize the virus. An important observation in this study was that the tagged antiserum diffused much more slowly through agar than the unlabeled antiserum. Another was that with a precipitating antigen-antibody system it is possible to detect the zone of reactance more quickly and at a tenfold greater dilution when the fluorescent-labeled antibody is used in place of the unlabeled antibody and the immunodiffusion tubes are examined with the aid of the fluorescent microscope. The higher sensitivity was observed in single diffusion tests. We are not now recommending additional studies of the immunodiffusion procedure for virus identification but we do believe that the method holds great promise in the identification of bacterial or other non-viral antigens. Also the method should provide additional sensitivity for antibody assays.

Studies on the identification of virus through the separation and detection of the soluble virus-antibody conjugates are still of a very preliminary nature and it is too soon to conclude what the ultimate application of these procedures to this problem will be. However, we have been highly encouraged with our experiments on the fluorescence labeling of virus and the retention of the immunological properties and viability of the labeled virus.

Studies on the use of molecular sieves (Sephadex G-200 and Bio-Gel-A) and an ion-exchange resin (DEAE Sephadex A-25) have shown that these materials are useful for the fractionation or purification of antisera, fluorescent-labeled antisera, virus preparations, and we believe also, soluble virus-antibody conjugates. In our studies we have observed the protein and nucleic-acid fluorescence peaks of the various separated fractions and also the fluorescence of the labels as measures of the separations achieved. In our opinion, studies on the development of methods for the separation and identification of soluble virus-antibody conjugates through the use of fluorescent tags on either the antibody or the virus or both, combined with the use of ion-exchange resins and molecular sieves, is a most promising approach to virus identification and we are now recommending that a concerted effort be made to continue the preliminary studies which are reported here. The recommended studies should include the attachment of a number of different fluorescence labels to different viruses for use in the identification process, quantitative studies to determine the minimum-labeled virus which can be detected by their fluorescence and studies to optimize the labeling, separation, and identification procedures. We anticipate that these fluorescence labeling and gel filtration techniques will find wide application in studies of non-viral antigens (bacterial, fungal, etc.) antibodies and sensitization reactions.

Our demonstration that the fluorescent labels are quickly and easily attached to virus suggests that perhaps fluorescent-labeled virus could be used in place of the isotopically labeled virus in the rapid identification procedure of Schmidt and Giron[70] employing immobilized antibodies. We believe that this substitution would result in a simplification of the identification procedure and the elimination of the necessity for handling radioactive materials.

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13. ABSTRACT

Six unique methods for the rapid identification of virus have been devised and investigated to determine their potential suitability for use in the fabrication of a new virus identification system. In the phagocytosis in vitro studies we prepared immune WBC and observed their response when challenged with virus. Impedance measurements on phospholipid bilayer membranes in buffer solutions have been used to detect the immune reaction. The effects of virus challenge on the electrophoretic migration rate of antibody coated plastic particles and tanned sheep red blood cells have been measured. Hydrogen overvoltage measurements have been performed on solutions containing antibody and antigen-antibody complexes. Fluorescent tagged antibody has been employed in the single and double immunodiffusion techniques in an effort to visualize the "soluble" virus-antibody conjugates. Virus has been tagged with rhodamine to permit its use with immobilized antibody for a viral identification process. Molecular sieves and ion exchange resins have been employed in studies for the separation and detection of "soluble" virus-antibody complexes. From these studies we have concluded that the in vitro phagocytosis approach is likely to lead to a very sensitive virus identification system if methods for obtaining uniformly sensitive WBC can be developed. The phospholipid bilayer membrane approach also suffers from the difficulty of being difficult to control or reproduce. Fundamental research on both of these approaches is recommended although immediate success is not anticipated. The

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| | ROLE | WT | ROLE | WT | ROLE | WT |
| Semliki Forest Virus | | | | | | |
| Poliovirus 1 and 2 virus identification procedures | | | | | | |
| Immunodiffusion | | | | | | |
| Phospholipids | | | | | | |
| Immune lysis | | | | | | |
| Hydrogen overvoltage | | | | | | |
| Particle electrophoresis | | | | | | |
| Fluorescence methods | | | | | | |
| Fluorescent tagged antibody and virus | | | | | | |
| Molecular sieves | | | | | | |
| Soluble antigen-antibody complexes | | | | | | |
| Lipid bilayer membranes | | | | | | |
| Gel diffusion | | | | | | |

use of fluorescent antibodies in combination with the immunodiffusion process is not expected to be valuable for the identification of virus; however, this approach for the detection of insoluble antigen-antibody conjugates is expected to yield at least a tenfold increase in sensitivity over the usual immunodiffusion methods. Our results with the "soluble" virus-antibody complexes are of a preliminary nature but they are most encouraging. We are recommending that a high priority be given to an extension of these studies and that the new studies include specifically: 1. The labeling of viruses with a multiplicity of fluorescent tags. 2. The use of molecular sieves for the separation of "soluble" virus-antibody complexes. 3. Quantitative estimates of the minimum level of virus which can be identified by the combined use of fluorescent-tagged virus and/or antibody and gel filtration techniques. In addition, studies should be conducted to determine the suitability of fluorescent tagging of virus to replace isotopically-tagged virus in the identification system based on the use of immobilized antibodies.