IMMUNODIFFUSION STUDIES ON THE ANTIGENS OF MYXOVIRUS INFLUENZA A/PR8

by

Christine Margaret Johnson, B.Sc., M.Sc.

. . .

A thesis submitted to the Faculty of Medicine in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Department of Microbiology
and Immunology,
Faculty of Medicine,
University of Ottawa,
Ottawa.

September, 1967.

ACKNOWLEDGEMENTS

The candidate wishes to express her deepest gratitude to Dr. J.C.N. Westwood, Chairman of the Department of Microbiology and Immunology who directed this research, for his stimulating and critical supervision.

Special thanks are expressed to Dr. C.A. Mitchell for his support and fundamental advice, and to Dr. M. Beaulieu for his interest and encouragement during this research program.

The candidate also wishes to thank all the members of the Department of Microbiology and Immunology for their support, and especially Mr. Brian Uncles, Research Technician, for his valuable suggestions and generously given time during this research projet.

In addition, a special vote of thanks to the members of the Photography Department, the Medical Faculty Workshop and the Staff of the Animal House for their cooperation and assistance.

Finally, the candidate wishes to express special appreciation to her colleague S.A. Sattar, for his helpful advice and for his friendship.

The candidate was supported by a Scholarship from the University of Ottawa.

This work was made possible through a research grant from the Medical Research Council of Canada.

TABLE OF CONTENTS

INTRODUCTION

The multiplication of viruses in suitable host cells entails the synthesis of virus-specific materials, many of them antigenic, which are then assembled at the end of the eclipse phase, into infective progeny virus. Much of the antigenic material synthesized during this replication process does not, however, become incorporated into the progeny virus and may be extracted from infected cells as "soluble antigen", readily separable from the mature virus particle by centrifugation or filtration.

Until recently it was accepted that "soluble antigens" represented excess synthesis of virus structural components (Craigie 1932, Craigie & Wishart 1936) but recent work indicates that antigenic components may be synthesized which are not structural antigens and do not become incorporated into the virion (Westwood et al, 1965, Summers et al, 1965). The term "soluble antigen" would appear then to

cover two distinct classes of antigenic component, the one structural and the other non-structural. The significance of the latter class has not been determined. It is possible that they only represent by-products of virus synthesis with no functional significance, but the discovery that the replication of viruses of several groups entails the production of enzymes differing from those of the normal host cell indicates the development of a metabolic apparatus specifically concerned with virus synthesis (Luria, 1967). It is at least possible that the non-structural antigens are related to such an apparatus.

The significance of the structural soluble antigens has been investigated by immunofluorescent techniques to determine the site of development of the chemical constituents of the virus during the virus replication (Holterman, Hillis & Moffat, 1960, Moffat, Holterman & Hillis, 1960, and Hillis, Moffat & Holterman, 1960).

The relationship of the structural proteins to the final virus particle has also been extensively investigated. However, their significance has usually been related to the demonstration of characteristic biological activity such as infectivity, haemagglutination or in the case of myxoviruses, enzyme activity. It has been suggested

that the soluble antigens, and in particular, the non-structural antigens, may have a deeper significance relative to the mechanisms whereby cells are killed by the replication of viruses (Westwood, 1963).

Since the basis of virus disease lies in the death of individual cells, the mechanisms of cell death resulting from virus replication form a focal point in virus research. However, cell death may result from the occurrence within the cell, of a partial cycle of replication resulting in only the production of antigenic components of soluble antigen (eclipse phase). Therefore, since cellular damage and death is closely associated with the formation of soluble antigens, a knowledge of their characteristics is essential to an understanding of both the mechanisms of virus replication and the mechanisms of cell damage.

The general objectives of the present investigation ~~was~~ were to determine the relationship of the detectable soluble antigen components to both the antigens of the virus particle and those of the host cells, using influenza virus as the model. These studies were intended to form the basis for further extensive immunochemical investigations to assess the significance of the soluble antigens in the replication cycle of the virus.

The most sensitive method for the characterization of these soluble antigens is by means of immunological techniques (Van Regenmortel, 1966) and, in particular, micro-double diffusion analysis provides the additional advantage of being able to resolve individual components in highly complex antigenic systems. The technique was employed successfully by Westwood, Zwartow, Appleyard & Titmuss (1965) in the comparison of soluble and viral antigens of vaccinia. A modification of this technique (Johnson et al, 1964) was developed and used for the investigations here presented.

Virus - Host System

Influenza virus A/PR8 grown in chick embryos was selected as the basic virus-host system to be studied. This selection was made in order to minimize the technical difficulties of production of the materials to be investigated. The system has the following advantages:- (1) the growth and harvest of the virus is relatively quick and simple, (2) soluble antigens are produced in the chorioallantoic membranes which are also relatively simple to handle, (3) the methods of assaying viral activity are straightforward and (4) standard concentration procedures have been established (Harris, 1964).

In addition, the selection of influenza A/PR8 virus provided a system in which cell death was not necessarily

the final result of virus replication (Negroni & Tyrrell, 1959, Tyrrell, 1963). Therefore, a study of the antigenic characteristics of this virus and its soluble antigens could possibly reveal the mechanisms by which synthesis of viral proteins and nucleic acid may be apparently compatible with normal host cell synthesis.

LITERATURE REVIEW

Myxoviruses have a compound helical structure consisting of an inner core of ribonucleoprotein arranged helically and enclosed in a loose envelope from which a number of cylindrical spines project. (Horne, Watson, Wildy & Farnham, 1960). The influenza viruses are chemically complex structures with a nucleic acid content of less than 1% (Ada & Perry, 1954).

The virus envelope contains lipid (Hoyle, 1952) protein and carbohydrate (Frisch-Niggemeyer & Hoyle, 1956). Following treatment with lipid solvents such as ether (Hoyle, 1952, Schafer & Zillig, 1954) or detergents (Laver, 1963) the membrane disintegrates, releasing the internal ribonucleoprotein. This process is accompanied by the destruction of infectivity and in some cases, a loss of antigenic properties. (Laver, 1964).

The viral envelope contains the two biologically active fractions responsible for the haemagglutinating and neuraminidase activity of the virus. The internal antigens released by ether treatment, confer the type specificity of the myxovirus (Lennette & Horsfall, 1941; Lief, Fabiyi & Henle, 1958, Lief & Henle, 1959), whereas the envelope

antigens are strain specific and closely linked with the haemagglutinating component (Fabiyi, Lief & Henle, 1958).

The relationship of the lipids of the influenza virus to those of the host cell was studied by Kates et al (1961). They reported that the virus incorporates both lipid components present in the host cell before infection, (possibly nuclear membrane) and lipid components newly synthesized by the infected cells as a result of cell damage. They concluded that the virus did not specifically direct the lipid synthesis in the cell.

In addition to the specific virus structural components, it was found that normal host components were incorporated into the virus particle (Knight, 1944 & 1946). Attempts were made to establish whether the host antigens were incorporated as a structural component of the virus. In 1953, Smith, Belyavin and Sheffield demonstrated a cross-reaction in complement fixation tests between highly purified A/PR8 and B/Lee strains of influenza virus, which was possibly due to absorbed host contaminant. However, they found slight but significant immunological differences between the host membrane antigen and the viral "host component" suggesting that the latter was incorporated as an integral part of the virus particle (Smith et al, 1955).

Further information on the chemical structure of these host antigens has been provided by Harboe and co-workers (Harboe, 1963 a,b, Strandli et al, 1964, Haukenes et al, 1965 and Howe et al, 1967). The properties were found to include heat stability, resistance to the action of proteolytic enzymes and sensitivity to periodate, and it was shown to be a high molecular weight carbohydrate. Laver (1966) confirmed these findings. In addition, he found that the host antigen was bound covalently to the protein coat, and comprised approximately 5% of the dry weight of virus. Others, however, do not agree that the host antigen is incorporated as an integral part of the virus structure (Kroeger, 1962, Ananthanarayan, 1954 and Duc-Nguyen et al, 1966) and favour the host contaminant explanation.

The Virion

The chemical structure of the virus particle has been studied by a number of workers, A comparative study of the amino acid composition of protein components released from the influenza particles by treatment with ether (soluble antigen, haemagglutinin and membrane protein) revealed that the soluble antigen protein had a higher arginine content that the other proteins. (Hoyle & Davies, 1961). The association of this

histone-like protein with the ribonucleic acid of the virus was proposed as a fundamental part of the ribonucleoprotein core, however, the specificity of such an association has not been shown (Fazekas de Saint Groth, 1964).

Further studies on the disruption of influenza virus particles by detergent action, combined with terminal amino acid analyses suggested that they were composed of at least three and possibly five protein sub-units (Laver, 1962, 1963). The enzyme neuraminidase was isolated and the evidence suggested that the neuraminidase and haemagglutinin activities of the virus were located in separately bonded structures. Electrophoretic separation of sodium dodecyl sulfate disrupted particles permitted the recovery of three different protein fractions, one of which was capable of agglutinating red cells (Laver, 1964). The antigenic characteristics of these proteins was not investigated.

Soluble Antigens

The previous studies were concerned with the chemical identification of structural components of the virus particles. A correlation between the structural components and the soluble antigens was not determined.

The identification of virus structural antigens

with the soluble antigens present in infected cells must be based on antigenic, morphological, functional and chemical similarities between both the viral and infected cell components.

A direct correlation between the morphological structure of the influenza virus particle and its infectivity was made by Moore et al (1962).

Horne et al (1960) demonstrated the association of the strain specific (V) antigen with the surface projections of the virus particle. The haemagglutinating activity was also found to be related to these structures. However, the precise relationship of the viral haemagglutinating and neuraminidase activity with the surface projections of the virus is still controversial, since none of these studies correlate the three areas of chemical isolation, structural identification and biological activity. (Noll, Aoyagi & Orlando, 1962, Reginster, 1966 and Fazekas de Saint Groth, 1964).

Schafer (1957) and Rott, Waterson and Reda (1963) demonstrated that the nucleoprotein extracted from the virus particles was morphologically and antigenically identical with the soluble antigen present in infected cells. These results have been confirmed by a study of the immunodiffusion reactions of disintegrated influenza A1 and A2 virus particles

with specific antisera. (Styk and Hana, 1966).

One recent study, however, has provided tentative evidence for the existence of soluble non-structural virus specific proteins in cells infected with parainfluenza virus (de Vaux St. Cyr & Howe, 1966). Three 'new' precipitating antigens were found which differed from viral neuraminidase, haemagglutinin and normal cells. However, the possibility exists that these proteins might represent viral nucleoprotein, in which case they would be classified as structural antigens.

In conclusion, it is evident that while the nature of the lipid, protein, carbohydrate and ribonucleo-protein structural components of the influenza virus particles have been studied in great detail, information on the soluble antigens is scarce and has been obtained mainly by studies concerned with the role of the normal host antigens present in the virus particle. Therefore an investigation aimed at the immunological characterization of the influenza virus soluble antigens by the identification of structural and non-structural components would appear to be of value to an understanding of the mechanisms of virus replication, and its effect on the host cell.

PURPOSE

The purpose of these investigations was to detect and identify by means of the micro-immunodiffusion technique, both the antigens of the virus particle and the soluble antigens produced in chick embryos as a result of influenza virus replication.

In order to carry out these studies, it was necessary to equip and establish a new laboratory section. The technical facilities and procedures were developed as they were required.

MATERIALS AND METHODS

Virus Cultivation

Myxovirus influenza A/PR8 strain was grown in the allantoic cavities of 10-day chick embryos. An inoculum of 0.1 ml. of $10^{-5}EID_{50}$ was injected and the embryos incubated at 36°C. for 36 hours. Following 12 hours of refrigeration at 4°C. the allantoic fluid and chorioallantoic membranes were harvested. All materials were stored at -80°C.

Preparations of Antigens

Influenza A/PR8 soluble antigen (PR8/SA)

Extracts of PR8-infected chorioallantoic membranes were made by three cycles of freeze-thawing alternating with three cycles of homogenization at 8°C. in a Waring blendor. The resulting suspension was clarified at 2,000 r.p.m. for 20 min. and then centrifuged in the Spinco model L2 preparative ultra-centrifuge (35,000 g. for 90 minutes) to remove virus particles. The soluble antigen was tested for haemagglutinating activity and infectivity.

Virus concentrate (V-C)

PR8-infected allantoic fluid was concentrated by differential centrifugation. The fluid was first

clarified at 1,000 g. for 20 minutes and virus then deposited by spinning at 35,000 g. for 90 minutes. A 100-fold concentration was achieved in the 2 cycles. Fractions were designated according to cycle, i.e. V-C_1, V-C_2 and samples of supernatant fluid from high speed centrifugation were kept and designated SNF_1. SNF_2, etc.

Control soluble antigen

Preparations of uninfected chorioallantoic extracts (NE/SA) were made following the same procedure, as for the infected membranes.

Control AF concentrate (C_2N)

Uninfected allantoic fluid (AF) was subjected to two cycles of differential centrifugation (35,000 g. x 60 min.). The normal sedimentable material was 100 X concentrated.

Disruption of the Virus Particles

Heat Treatment

1 ml. samples of virus concentrate (V-C_2) were heated in a water bath at 45 C. or at 56 C. for 30 minutes, and stored at -80 C.

Ultrasonication

Small test tubes containing 1 ml. volumes of

virus concentrate ($V\text{-}C_2$) were sonicated for 60 seconds at maximum intensity, in the cup tip attachment of a Blackstone High Intensity Ultrasonic Probe (model BP-2, 200 watts). The samples were stored at -80°C. for testing.

Urea treatment

1 ml. of 8M urea was added to 1 ml. of virus concentrate and the mixture incubated at 4°C. overnight. The sample was then dialysed against running tap water for 4 hours. Samples were stored at -80°C.

Sodium Deoxycholate treatment

1.2 ml. of 10% sodium deoxycholate in distilled water was added to 10.8 ml. virus concentrate ($V\text{-}C_2$) and the mixture incubated at room temperature for 2 hours. Intact virus particles were sedimented by centrifugation at 35,000 g. for 60 minutes in the Spinco L2 ultra-centrifuge, using the 50 rotor with 2 ml. volume tube adaptors. The supernatant fluid was dialysed overnight against veronal buffered saline (VBS) at 8°C. This was the DOC/SNF fraction. The pellet was washed once in VBS and re-sedimented at 35,000 g. x 60 minutes. The pellet was then resuspended in the original volume of VBS and designated DOC/C_3. The wash - SNF was retained and all samples stored at -80° C. This method was adapted from

Laver (1963) and Mizutani & Mizutani (1965).

Sodium Dodecyl Sulfate treatment

0.2 ml. of an aqueous 10% solution of sodium dodecyl sulfate was added to 1.8 ml. of virus concentrate and the mixture incubated at room temperature for 2 hours. Following separation by ultra-centrifuging at 35,000 g. x 60 minutes, the SNF fraction appeared to be in two phases. Both phases were harvested separately (SDS/SNF upper and SDS/SNF lower) and both fractions were dialysed overnight against VBS at 8°C. The pellet was washed once in VBS, and re-centrifuged (35,000 g. x 60 min.) The pellet was resuspended in VBS (SDS/C_3) and stored at -80°C. (Method adapted from Laver, 1963).

Methanol-Chloroform treatment

The procedures described by Kates (1961) and Eckert (1966) were followed: 6.0 ml. of methanol-chloroform (2:1V/V) was added to 1.6 ml. virus concentrate, and the mixture was agitated gently on a Burrell shaker for 2 hours at room temperature. 1 volume of deionized water was added, followed by 1 volume of chloroform. The mixture was shaken for a few minutes and centrifuged. The supernatant aqueous-methanol phase was removed with a pipette and the volume reduced to 2 ml. by pervaporation. The chloroform residue

was evaporated at 37°C. to a volume of 1 ml. and stored at -80°C. for future tests.

Glacial acetic acid was added to the concentrated supernatant aqueous phase to a final concentration of 67% (4 ml. HAC) resulting in a clearing of the turbid suspension. A slight sediment formed and was removed by centrifugation. The supernatant fluid was dialysed successively against 0.1M acetic acid - 0.1M sodium acetic buffer at pH 4.5 (40 hours at 8°C.) and phosphate buffered saline at pH 7.4 (16 hours at 8°C.), and the final opaque solution adjusted to the original volume (1.6 ml.) with saline. This preparation was designated $CHCl_3$-Ac and stored at -80°C.

Pronase treatment

The procedure was described by Reginster (1966). 8 ml. of virus concentrate ($V\text{-}C_2$) was divided into four 2 ml. portions and centrifuged at 35,000 g. x 60 minutes. Three of the virus pellets were resuspended to the original concentration in phosphate buffer (pH 7.0, μ 0.01) containing 100 μg pronase per ml, and the fourth in phosphate buffer (pH 7.0, μ 0.01) to serve as a control. The mixtures were incubated at 37°C. for 6 hours, with intermittent agitation. A second cycle of high-speed

centrifugation (35,000 g. x 60 minutes) resulted in two fractions; PRO/SNF and PRO/C_3. The PRO/C_3 pellet was washed once in buffer, recentrifuged, and resuspended to the original volume with buffer. The control suspensions were designated SNF/buffer and C_3/buffer. All samples were stored at -80°C.

Antigen Assay Methods

Virus haemagglutination titrations

Twofold dilution series were made in Perspex trays using 0.2 ml. volumes in 0.85% saline. 0.2 ml. of 0.5% fowl red blood cells were added and the results read after 60 minutes at room temperature. HA titres were expressed as the reciprocal dilution of the 50% end point.

Virus infectivity titrations

Serial tenfold dilution series were made in phosphate-buffered saline (PBS) or 199 tissue culture medium. 0.1 ml. of each dilution was inoculated into the allantoic sac of four 10-day chick embryos. After 48-hour incubation at 35°C., the eggs were chilled, the allantoic fluids were harvested and tested for haemagglutination with 1% fowl red blood cells. The 50% infectious dose was calculated by the method of Reed and Muench.

Complement Fixation Tests

The microtitre system was used (Sever, 1962). Twofold serial dilutions of the test samples were set up in 0.025 ml volumes of veronal buffered saline, containing Ca^{++} and Mg^{++} . 0.050 ml. of 3MHD of guinea pig complement was added and 0.025 ml. of the optimal dilution of antiserum. Following overnight fixation at 4°C., sensitized sheep cells (0.05 ml.) were added and incubated at 37°C. for 30 minutes. The complement fixing titre was expressed as the reciprocal of the 50% end point.

Protein Estimations

The protein content was estimated by the method of Lowry et al (1951). Later tests were modified by substituting sodium citrate for sodium tartrate, to obtain a more stable solution (Leggett Bailey, 1962).

Electron Microscopy

Specimens were examined in a Phillips E/M 200 electron microscope, through the courtesy of Miss F. Doane of the E/M Unit at the School of Hygiene, University of Toronto. The magnification of the E/M 200 was calibrated with a commercially prepared grid with 54,864 lines/inch. The

magnification was then calculated from the formula:

$$Mp = \frac{1}{n} \cdot \frac{G(\text{lines/inch})}{2.54}$$

Mp = actual magnification on film

l = sum of distances between lines

n = number of lines (minimum of 10)

G = number of specimen lines/inch.

400 mesh copper grids, coated with formvar and stabilized with carbon, were used throughout. Specimens were mounted on the grids by several techniques which are described in detail in the Experimental section.

Normal Sera

Initial observations were made on preimmunization sera obtained from rabbits to be immunized with virus antigens. Following the observation of positive reactions in some of these sera, a further series of 24 sera was obtained from "normal" rabbits in a dealer's stock. These animals had not had any known contact with myxoviruses.

Sera from a number of other animal species were obtained from animals which had also no contact with a known source of myxovirus infection. These included guinea pigs, roosters, a goat and a sheep.

All sera used throughout these investigations were stored at -20°C. without the addition of preservatives.

Sera were inactivated at 56°C. for 30 minutes, as required.

New animals were routinely bled on arrival in our animal quarters and the sera tested immediately. Subsequent bleeds were obtained from all animals showing positive serum immunodiffusion reactions.

Serum was designated according to an abbreviation of the animal species, combined with the letter N-normal where it was required.

Antisera prepared in Rabbits and Guinea Pigs.

Parenteral immunization

Following an initial course of three injections of 0.5 ml. - 1.0 ml. antigen at ten day intervals, adult rabbits and guinea pigs were given monthly booster injections. Rabbits were immunized by the intramuscular route, whereas guinea pigs were immunized intraperitoneally. An equal volume of Freund's complete adjuvant was incorporated in most injections. The animals were bled 10 days after each booster injection by heart puncture (rabbit and guinea pigs), or by the marginal ear vein (rabbits). All sera were tested

before antiserum pools were made.

The antisera obtained by immunization with different antigen preparations were:

RaS/NE - rabbit antiserum to normal chorioallantoic membranes, (200-300 µg protein/ml. per injection).

RaS - rabbit Pan-Specific antiserum, produced by immunization with PR8-infected CAM extracts + virus concentrate ($V\text{-}C_2$), (2:1 vol/vol).

RaS/PR8 - rabbit antiserum to PR8-infected CAM extracts, (200-300 µg protein/ml. per injection), freed of virus particles by high-speed centrifugation.

RaS/C_2 - rabbit antiserum to virus concentrate (0.5 ml./injection).

GPaS - guinea pig complete antiserum.

Intranasal infection.

Selected rabbits or guinea pigs showing no detectable immunodiffusion reaction against virus infections (vide infra) were exposed to virus by the intranasal route. Approximately 0.25 ml. of influenza A/PR8-infected allantoic fluid was instilled into each nostril. This procedure did not cause the rabbit undue distress and an anaesthetic was

not used. Guinea pigs were lightly anaesthetized with ether. Convalescent sera were obtained 3-8 weeks following infection and at intervals thereafter.

RaS/IN - rabbit antiserum produced by induced virus infection.

GPaS/IN - guinea pig antiserum produced by induced PR8/virus infection.

Antisera prepared in Roosters.

Adult roosters (6 months) were immunized by intramuscular injections (0.5 - 1.0 ml.) of the complete antigens (PR8/SA+ V-C_2), infected and normal CAM extracts and virus-concentrate.

Freund's complete adjuvant was used only for the initial injection, followed by two weekly inoculations. Monthly booster injections were administered and the roosters were bled by the wing vein or by heart puncture ten days following each injection. The same antigen preparations as described above were used, and the antisera obtained, designated as follows:

RSaS - rooster antiserum to PR8/SA + V-C_2.

RSaS/PR8 - rooster antiserum to PR8-infected CAM extracts.

RSaS/C_2 - rooster antiserum to virus concentrate (V-C_2).

The roosters used for these procedures were obtained from the same poultry dealer who supplied the fertile hen's eggs for virus cultivation, and would therefore not show an antigenic response to their homologous antigens.

Antisera prepared in Rabbits by the Immunological Tolerance Technique.

Induction of tolerance to normal CAM in neonatal rabbits.

Because of the large quantities of normal egg extracts required for the tolerance induction schedule, only single litters (6-8) could be handled at one time. Two female rabbits (Dutch strain) were obtained locally and kept for breeding purposes. Following mating, they were housed in large monkey cages and provided with nesting boxes. Hay was supplied at all times and they were handled by the same individuals. Within 30 days of fertilization, 6 to 8 young rabbits were born in each litter. Within the first 12 hours of birth, usually 6-8 hours, each of the young rabbits was injected intraperitoneally with 1 ml. of normal CAM extract, containing 200-300 µg/ml. protein. Plastic disposable gloves were worn while handling the young and no disinfectant was used throughout these tests to avoid antagonizing the mother.

The initial injections were repeated every 12 hours for a total of 4 doses. Further intraperitoneal injections of normal CAM's were given at 3, 7, 10, 14, 21, 28, 55 and 60 days of age. (Gold & Freedman, 1965). The animals were bled by the marginal ear vein at 72 days of age to determine whether an immunological response had been obtained against normal CAM. The results included in section B of this thesis are from the first litter of a planned series of experiments. All the young survived the injection schedule.

Immunization of apparently tolerant rabbits.

The four animals which demonstrated the greatest degree of tolerance were immunized by intramuscular injections at 80, 84, 87 and 90 days of age, with PR8-infected CAM extracts. The animals were identified as TR-1, TR-3, TR-5 and TR-6. The two least tolerant rabbits were kept on the same immunization schedule, however NE/SA antigen was used. These were the control rabbits of this experimental litter (TR-C). The animals were then given injections at 21-25 day intervals and bled ten days following each inoculation.

Antiserum Titrations.

Haemagglutination Inhibition (HAI)

These tests were performed in Perspex

haemagglutination plates, according to the procedure given by Schmidt and Lennette (1965). Four haemagglutinating units of virus were used. The following procedures were used to inactivate non-specific inhibitors:

Heat -

The test sera was heated at 56°C. for 60 minutes.

Neuraminidase (RDE) -

1 ml. of serum was mixed with 1 ml. of RDE (General Biochemicals), diluted to contain 50 units/ml. and following incubation at 37°C for 15-16 hours, the mixture was heated at 56°C. for 30 minutes.

Potassium periodate -

Two volumes of freshly prepared M/90 KIO_4 was mixed with one volume of test serum and incubated at room temperature for 15 minutes. One volume of 3% glyerol (in saline) was then added. Following a second incubation of 15 minutes at room temperature, the mixture was heated at 56°C. for 30 minutes.

Trypsin (1:250) -

One volume of 0.8% trypsin (Difco) at pH 8.0 was added to two volumes of test serum. After 5 minutes incubation at room temperature, the mixture was heated at 56°C. for 30 minutes. (Sampaio & Isaacs, 1953).

Neutralization of Infectivity

Serial dilutions of serum in saline were mixed with equal volumes of virus, diluted in penicillin - streptomycin M199 tissue culture medium, to contain 10^2 EID_{50} per 0.2 ml., and the mixture incubated at room temperature for 60 minutes. Each mixture was inoculated in 0.2 ml. volumes into four 10-day embryonated eggs. The virus challenge dose was checked in parallel by a standard infectivity titration. After 48 hours incubation at 37°C, the allantoic fluid was harvested and tested for haemagglutination with fowl red blood cells in Perspex trays. 50% end points were calculated by the Reed-Muench method (1938).

Immunodiffusion Tests.

Gel diffusion (Ouchterlony method).

Agar gel precipitin reactions were carried out in 85 mm. plastic Petri dishes containing 15 ml. of 1% agarose in TRIS buffer (pH 7.4) and 0.5% sodium azide. The reactions were incubated in a humidity chamber placed in a constant temperature incubator at 22.5°C. for 2-5 days and then photographed. Non-precipitated material was removed by washing in saline for 3 days, and distilled water for 1 day. The reactions were then stained with Thiazine red R or Ponceau S, and differentiated with 5% acetic acid.

The agar was carefully transferred, by floating the gel out of the Petri dish in water, on to a 3½" x 4" lantern slide, covered with a piece of moist lens paper and allowed to dry at room temperature. These slides were stored as a permanent reaction record.

Micro-immunodiffusion test in agar.

The micro-double diffusion test in agar was performed according to the method described by Crowle (1958), using 2% Difco Noble agar or 1% agarose in the appropriate buffer with merthiolate (1: 10,000) or 0.1% sodium azide as preservative. The Perspex templates required in this procedure were specially made in our workshop department.

Micro-immunodiffusion test in cellulose acetate.

This method was developed for these investigations (Johnson et al, 1964) and the procedure is described in the next section, entitled Development of Cellulose Acetate Micro-Immunodiffusion.

Serum Fractionation.

Isolation of serum gamma globulin with DEAE.

The procedure described in Methods in Immunology (Campbell, Garvey, Cremer & Sussdorf, (1963)) was followed using 50 ml. of rabbit antiserum to influenza A/PR8

soluble antigen and virus concentrate (RaS). The fractions were FI and FII, which were tested separately and then pooled.

Ammonium sulfate fractionation of rabbit antiserum.

Rabbit antiserum (RaS, 100 ml.) was separated into euglobulin (Eug.) and pseudoglobulin (Ps) fractions by precipitation at 33% and 50% saturation with ammonium sulfate respectively, according to the procedure described by Cohen and Belyavin (1961). All fractionations were performed at room temperature. The ammonium sulfate was added slowly with gentle stirring, and the mixture allowed to stand for half an hour. The precipitates were centrifuged, washed with ammonium sulfate solutions at the appropriate concentration and dissolved in physiological saline (0.85%). Each fraction was then re-precipitated and re-dissolved in saline. The protein remaining soluble at 50% saturation with ammonium sulfate was the albumin fraction (Alb.). The euglobulin fraction was further separated into water-soluble and insoluble components by dialysis against distilled water.

All the protein fractions were dialysed against 0.85% saline at 4°C. to remove ammonium sulfate. The fractions were reduced to 75% of the original concentration and were stored at -20°C.

Isolation of serum gamma globulin using Rivanol.

Both normal and immune sera were fractionated. 20 ml. fresh Rivanol solution (0.4% in deionized water adjusted to pH 6.8 with Borax) were added to 5 ml. of test sera and refrigerated for one hour. The supernatant (G) was decanted from the gummy precipitate (A) and both portions were treated with sodium barbital (15% and saturated with diethylbarbituric acid) in molar excess over Rivanol, to convert the Rivanol into an insoluble yellow precipitate. 4 ml. of 15% sodium barbital was added to the supernatant fraction (G) and the resulting yellow precipitate removed by centrifugation. The clear, colourless solution containing mainly the gamma globulins was dialysed against saline at 4°C. overnight, and then concentrated to 40% of the original volume by dialysis against polyethylene glycol. The Rivanol precipitated fractions (A) were "derivanolized" by the action of 5 ml. 15% sodium barbital over a period of 12-18 hours at 4°C. A mechanical shaker was used to agitate the mixture.

The Rivanol salts were removed by centrifugation and the supernatant fluid (A) dialysed against saline at 4°C. The resulting fraction was concentrated to 2 ml. by dialysis against polyethylene glycol. All the fractions were stored at -20°C. This procedure was based on that

Isolation of serum gamma globulin using Rivanol.

Both normal and immune sera were fractionated. 20 ml. fresh Rivanol solution (0.4% in deionized water adjusted to pH 6.8 with Borax) were added to 5 ml. of test sera and refrigerated for one hour. The supernatant (G) was decanted from the gummy precipitate (A) and both portions were treated with sodium barbital (15% and saturated with diethylbarbituric acid) in molar excess over Rivanol, to convert the Rivanol into an insoluble yellow precipitate. 4 ml. of 15% sodium barbital was added to the supernatant fraction (G) and the resulting yellow precipitate removed by centrifugation. The clear, colourless solution containing mainly the gamma globulins was dialysed against saline at 4°C. overnight, and then concentrated to 40% of the original volume by dialysis against polyethylene glycol. The Rivanol precipitated fractions (A) were "derivanolized" by the action of 5 ml. 15% sodium barbital over a period of 12-18 hours at 4°C. A mechanical shaker was used to agitate the mixture.

The Rivanol salts were removed by centrifugation and the supernatant fluid (A) dialysed against saline at 4°C. The resulting fraction was concentrated to 2 ml. by dialysis against polyethylene glycol. All the fractions were stored at -20°C. This procedure was based on that

described by Menzel & Sherman (1964).

Cellulose Acetate Electrophoresis.

The Buchler electrophoresis constant current-constant voltage power supply (model 3-1014A) was used for all electrophoretic analyses and the procedures described in Volume II of Chromatographic and Electrophoretic Techniques were followed (Smith, 1960). S & S (Consolidated Laboratories) or Sepraphore III (Gelman) cellulose acetate papers were suspended in the appropriate buffers, and the samples applied with either a Gelman sample applicator or with lambda pipettes. Constant voltage was used for all runs and the average current recorded. After the run, the cellulose acetate strips were stained, using either Ponceau S or Thiazine red R.

Immunoelectrophoresis in Agar.

The procedures described in Immunodiffusion (Crowle, 1961) were followed. Glass slides (1" x 3", 2" x 3" or 3½" x 4") were cleaned and covered to a depth of 1 mm. with 1% agarose. When the agar had gelled, they were allowed to stabilize in a moisture chamber at room temperature for several hours. Wells and troughs were cut with the Buchler agar gel cutter and the agar removed from

the wells by suction. The slides were placed in the electrophoresis chamber and filter paper wicks used to connect the agar to the buffer. The system was allowed to stabilize for 10-15 minutes, and then the samples were placed in the wells with finely drawn-out Pasteur pipettes. Electrophoresis was carried out under constant voltage conditions appropriate for the sample. At the end of this period, the agar was removed from the troughs which were filled with antiserum.

The slides were incubated in moisture chambers at a constant temperature (22.5°C.) until precipitation lines developed. The reactions were washed for two days in several changes of saline to remove unprecipitated protein, followed by one day in distilled water to remove the salt. The reactions were photographed by direct enlargement onto photographic paper, then stained with Thiazine red R and differentiated with 5% acetic acid. The agar layer was then covered with strips of lens paper, wetted with 1% glycerine in water, and dried, slowly, at room temperature. The reactions were again photographed by direct enlargement.

The methods described above represent the basic techniques used throughout these experiments. In some cases, a single technique was applied to several different systems requiring changes in some of the test conditions (i.e. buffers used in electrophoresis). In these cases, such procedural details are included with the experimental results.

STANDARDIZATION OF PROTEIN ESTIMATION TECHNIQUES

INTRODUCTION

Throughout the course of this investigation it was anticipated that estimations of the protein content of a variety of samples would be required. Therefore, the necessary preliminary of testing and standardizing new equipment was combined with the selection and evaluation of techniques for such analyses.

The selection of techniques depends on the sample available, its physical characteristics and the protein content. Sample solutions derived from density gradient or column chromatography separations may be analysed both by direct measurement of absorption in the U.V. and by the Lowry micro-modification of the Folin phenol reaction (1951). However, if the fractions to be quantitated are obtained by electrophoresis or diffusion, both the detection and estimation may have to rely on protein staining and elution colorimetry. These three techniques were investigated and compared to determine their accuracy, sensitivity and limitations.

MATERIALS

Test Protein

Lab-Trol (Dade & Co.) was used as the test material. This proved to be a satisfactory standard material and we found Lab-Trol (LT 25xB) to have a convenient specific absorptivity of unity at 280 mμ.

Definition of absorptivity - $a = \frac{A}{bc}$ (Analyt. Chem., 1961)

where A = absorbance in absorbance units

b = pathlength in cm.

c = concentration in gram/litre.

Spectrophotometer

A Beckman DB spectrophotometer was used for all absorbance measurements.

Microburet

Distribution of μl volumes of protein and dye solutions were carried out using a syringe microburet, model No. S.B.2 (Micrometric Instrument Co., Cleveland, Ohio).

Dyes

Of the numerous dyes available for the staining of proteins, some have been recommended for use in immuno-electrophoresis and immunodiffusion studies. After a

preliminary study of the absorption spectra of a number of these the following three were selected for further study: Thiazine red R (Crowle, 1958); Ponceau S (Grunbaum et al, 1960); Fast green FCF. This last dye was chosen in preference to the stain lissamine green SF recommended by Brackenridge (1960, a,b,c) because of the confusion with the stain light green SF.

Only standardized dyes bearing the International Color Index numbers were used:

Thiazine red R	C.I. 14780
Ponceau S	C.I. 27195
Fast green FCF	C.I. 42053

All dyes used were made up from the dry powder state.

Statistical Procedures.

The results obtained were analysed by following the statistical procedures described by Mather (1951). The Coefficient of Variation, used to express error in many of the relationships studied, is the Standard Deviation expressed as a percentage of the mean. A Monroe Calculator (Monro-Matic, model 8N) was used for these calculations.

EXPERIMENTAL RESULTS

1. Protein Staining and Elution Colorimetry.

a) Properties of selected stains.

For staining proteins in cellulose acetate or agar films, Thiazine red R, Ponceau S and Fast green FCF are usually employed in acid solution, being dissolved in 3% trichloracetic acid (TCA) or in 1 - 3% acetic acid (HAC) according to usage. The acid used has the double function of precipitating or "fixing" the protein and enhancing the staining reaction. Various methods are recommended for subsequent elution of the stained protein for spectrophotometric estimation, and we have found that 2% Na_2CO_3 gives the best results (vide infra). The behaviour of the three dyes in each of these diluents was therefore studied.

Absorption Spectra.

The visible colour of the dye solutions and their absorption spectra depended in each case upon the diluent used. The colours and absorption peaks are given in Table 1 and the absorption spectra in Plate 1, figures 1, 2 and 3. The absorption peaks were determined manually, using a slit width of 0.1 mm., and were not taken from the less precise scan results.

TABLE 1

SUMMARY OF PRINCIPLE DYE CHARACTERISTICS

DYE	DILUENT	COLOUR OF SOLUTION	WAVE LENGTH OF MAXIMUM ABSORBANCE mμ	ABSORPTIVITY $a = \frac{A}{bc}$	COEFFICIENT OF VARIATION %
Ponceau S.	3% TCA	red	520	44.932	1.5
	3% AC	red	520	44.053	0.5
	2% Na_2CO_3	purple	520	32.5867	1.7
Thiazine Red R	3% TCA	orange	500	20.149	0.6
	1% AC	red	510	22.736	0.56
	2% Na_2CO_3	red	515	20.2914	0.3
Fast Green FCF	3% TCA	green	644	44.428	0.42
	3% AC	blue	644	98.66	2.22
	2% Na_2CO_3	blue	644	80.542	17.82

PLATE 1

Absorption spectra of three dyes in acetic acid, trichloracetic acid and sodium carbonate.

Figure 1 - Wavelengths of maximum absorbance of Ponceau S

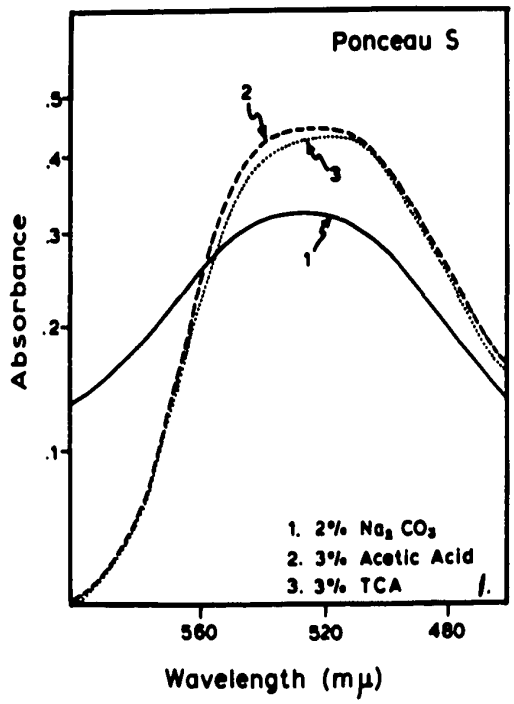
Figure 2 - Wavelengths of maximum absorbance of Thiazine red R

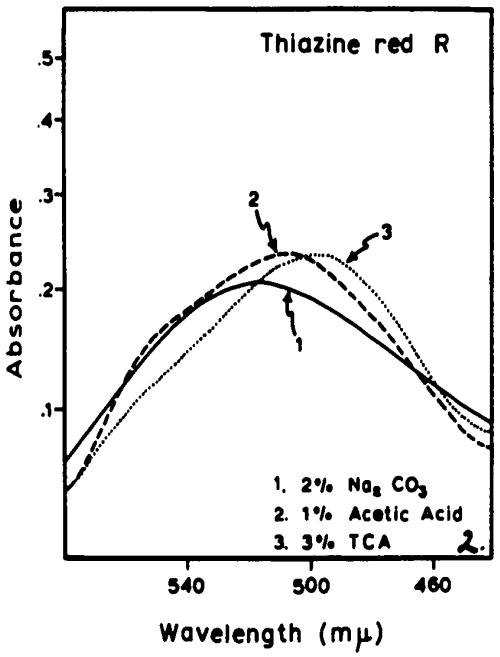
Figure 3 - Wavelengths of maximum absorbance of fast Green FCF

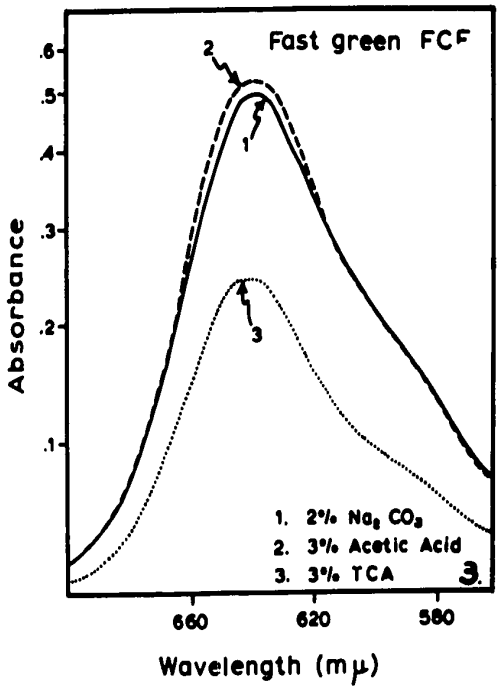
tri-

of

of


Ponceau S
Absorbance
.5
.4
.3
.2
.1
2
3
1
1. 2% Na_2CO_3
2. 3% Acetic Acid
3. 3% TCA
1.
560
520
480
Wavelength (mμ)


Thiazine red R
Absorbance
.5
.4
.3
.2
.1
2
3
1
1. 2% Na_2CO_3
2. 1% Acetic Acid
3. 3% TCA
2.
540
500
460
Wavelength (mμ)


Fast green FCF
Absorbance
.6
.5
.4
.3
.2
.1
2
1
3
1. 2% Na_2CO_3
2. 3% Acetic Acid
3. 3% TCA
3.
660
620
580
Wavelength (mμ)

Response curves.

To provide a reliable basis for protein determination, a dye should obey the Beer-Lambert law of linearity of relationship between concentration and degree of absorption. The three dyes were tested in this respect in a series of experiments and typical curves for each are given in Plate 2, figures 1, 2 and 3. Ponceau S and Fast green showed a linear relationship in all three diluents. Thiazine red R showed a linear relationship in HAC and Na_2CO_3, but a definite departure from linearity in TCA.

Statistical evaluation of results.

For the final evaluation of the three dyes, standard solutions were made up with maximum precision from the dried powders. From these, accurate dilutions were prepared in the respective diluents and absorptions measured for each at the appropriate wave length.

Using the formula $y = a + bx$, linear regressions were calculated from the data obtained.

y = optical density in absorbance units

x = concentration of dye in g/l

a = the deviation from the origin

b = regression coefficient.

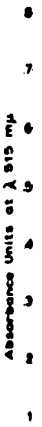
The evaluation of the results obtained is shown

Response Curves of Three Dyes in acetic acid, trichloracetic acid and sodium carbonate

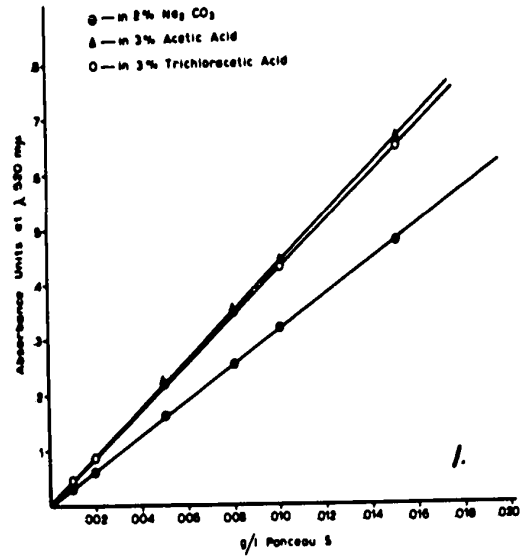
Figure 1 Ponceau S.

Figure 2 Thiazine red R

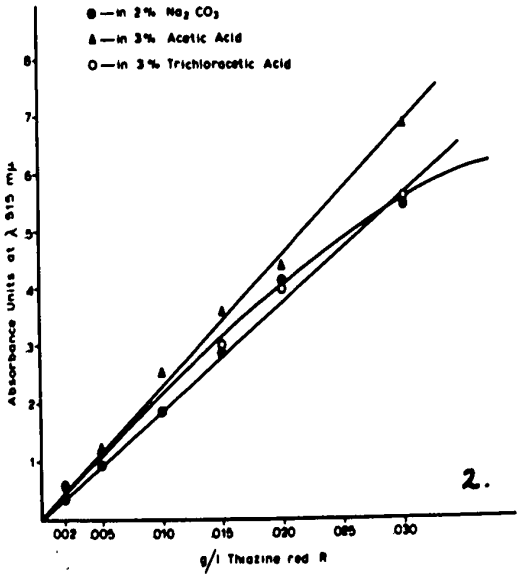
Figure 3 Fast Green FCF



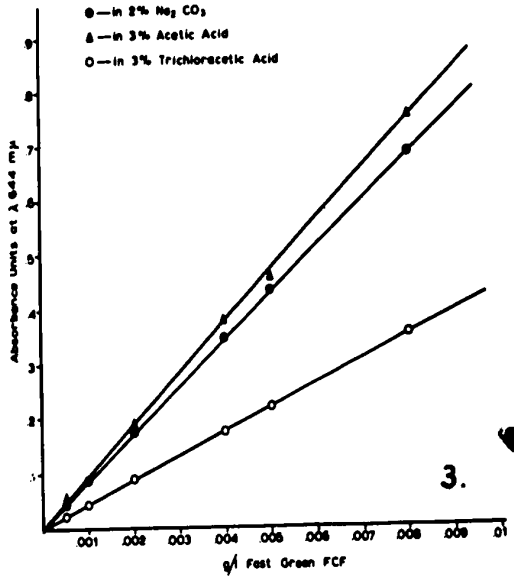
STANDARD CURVE OF PONCEAU S



STANDARD CURVE OF THIAZINE RED R



STANDARD CURVE OF FAST GREEN FCF



in Table 2.

Value of a.

Where the Beer-Lambert law is obeyed, the relationship of y to x should be linear with the curve passing through the origin, so that a = o within the limits of sampling error. It may be seen from the 5th column of Table 2 that the condition is satisfied by Ponceau S in all three diluents and by Thiazine red R in HAC and Na_2CO_3. The linear regression calculated for Thiazine red R in TCA, where there is a marked departure from linearity, shows a significant deviation from the value a = o. Similarly, the regressions calculated for Fast green FCF in both Na_2CO_3 and HAC show highly significant deviations from zero, indicating the non-linear response of this dye in these diluents.

Regression coefficient.

The regression coefficient, b, determines the slope of the curve relating the concentration to the optical density. It is numerically identical with absorptivity as defined, and is a characteristic constant for each dye-diluent system in which a linear response is obtained.

For each system the values determined for this constant, the error of its determination and its derived

fiducial limits are shown in columns 6-9 in Table 2.

Conclusion.

From these results Ponceau S was chosen for the study of protein staining and elution procedures.

The occurrence of the maximum absorbance at different wavelengths, depending on the diluent and the departure from linearity in the response of Thiazine red R, were considered to be disadvantages to the use of this dye. The excessive error obtained for the regression coefficient of Fast green FCF precluded the use of this dye for this type of analysis.

b) Evaluation of Elution Procedures.

Microlitre quantities of Lab Trol dilutions were applied to dry cellulose acetate paper (S&S, Consolidated Laboratories), using the microburet. The spots were allowed to dry at room temperature and then stained with Ponceau S in 3% TCA. Each spot was cut out, the stained protein eluted by one of the methods enumerated below and the optical density of the eluted dye measured in absorbance units at 520 mμ. Since the choice of procedure is governed by the effect of the eluent solution on cellulose acetate

TABLE 2

TABLE 2

COMPARISON OF

1	2	3	4	5
Dye	Diluent	Wavelength of Maximum Absorbance	a*	P that a = 0
Ponceau S.	3% TCA	520 mμ	.00254	.8 - .9
	2%Na_2CO_3	520 "	.00304	.8 - .7
	3 % AC	520 "	.0005	.8
Thiazine red R.	3 % TCA	500 "	.01397	.02 - .01
	2%Na_2CO_3	515 "	.0146	.3 - .2
	1 % AC	510 "	.0038	.2 - .1
Fast Green	3 % TCA	644 "	.00194	.7 - .5
	2%Na_2CO_3	644 "	2.46	.01 - .001
	3 % AC	644 "	1.72	< .001

* regression values for y = a + bx.

** SD_b - standard deviation of b.

NR not recorded.

NT Not tested.

*** Coefficient of Variation

DYE CHARACTERISTICS

6	7	8	9	10
Regression Coefficient b*	SD_b**	C. of V.***	Fiducial limits	Linearity
44.932	0.684	1.5%	46.290-43.564	yes
32.5867	0.559	1.7%	33.7047-31.4687	yes
44.053	0.216	0.5%	44.485-43.621	yes
20.1490	0.120	0.6%	20.389-19.909	no
20.2914	0.062	0.3%	20.3154-20.1674	yes
22.736	0.127	0.56%	22.890-22.582	yes
44.428	0.186	0.42%	44.8102-44.0569	yes
80.542	14.36	17.2%	NR	NT
98.66	2.19	2.22%	NR	NT

and on the stability of the dye, the following procedures were evaluated and the results are summarized in Table 3.

Chloroform & ethanol, and methylene chloride & ethanol.

Dissolving the cellulose acetate with mixtures of chloroform and ethanol (9:1) (Sherr, 1961) or methylene chloride and ethanol (8.8:1.2) (Markowitz and Isenberg, 1963) was not satisfactory since neither the stain nor the protein went into solution. The suspension which could be obtained by vigorous shaking was cloudy with irregular sized particles and was unsuitable for use in the spectrophotometer.

Sodium hydroxide.

N/10 NaOH elution, followed by addition of 0.1 ml. 40% acetic acid to adjust the pH (Smith, 1960) was effective but, unless the eluate was immediately separated, the NaOH caused rapid deterioration of the cellulose acetate paper. This resulted in cloudy solutions unfit for spectrophotometric evaluation.

6M & 8M urea.

Elution of stained protein with 6M or 8M urea solution was slow (3 hours minimum) and these suspensions also became cloudy due to deterioration of the cellulose

TABLE 3

TABLE 3

SUMMARY OF ELUTION PROCEDURES

Elution	Cellulose Acetate Paper	Protein	Dye	Comment
Chloroform and ethanol (9:1)	Dissolves C.A. within 15 minutes	Remains as a stained precipitate surface.	Remains bound to protein.	Not good for optical density readings.
Methylene chloride & ethanol 8.8:1.2	"	"	"	"
N/10 NaOH elutions + 40% acetic to return to acid pH	Deteriorates on standing and causes cloudy suspension.	Elutes Protein quickly.	Change of colour from blue in alkaline conditions back pH.	Not chosen because of effect on CA.

6M & 8M urea	Slowly deteriorates same as for N/10 NaOH.	Elutes very slowly - 3 hour minimum.	Slight decrease in dye intensity.	Not chosen because of effect on CA.
Na_2CO_3 in 50% methanol	Deteriorates due to the alcohol.	Elutes within 30 minutes	Slight shift to blue colour Less than caused by NaOH.	Alcohol poorly tolerated by CA.
2% Na_2CO_3 in water	Negligible effect.	Elutes in 30 minutes	Slight drop in intensity but Beer's law valid.	Chosen for elution procedure.

acetate. Though it is possible to centrifuge cloudy suspensions and take readings on the supernatant, this entails added manipulations.

Sodium carbonate in methanol.

Dilute sodium carbonate solution in 50% methanol (Ribeiro, 1961) was poorly tolerated by S&S cellulose acetate paper due to the alcohol.

Sodium carbonate.

2% sodium carbonate without alcohol was effective for protein elution and had no appreciable effect on the cellulose acetate over a 24 hour period. Therefore, 2% Na_2CO_3 was selected as the eluent of choice.

c) Dye-binding capacity of Protein

For the uptake of stain to provide a reliable basis for the estimation of protein, the dye-binding capacity of the protein must remain constant over the range of protein concentrations to be estimated. To investigate this relationship using Ponceau S, aliquots of Lab Trol dilutions were dispensed on cellulose acetate, and membranes stained according to recommended procedures. The intensity of the eluted dye was read in the spectrophotometer. The absorbance values obtained were converted to the equivalent weight of

Ponceau S (µg) by means of the Standard Curve for Ponceau S in 2% Na_2CO_3. The protein to dye relationship on a weight for weight basis was linear over the range of 150 µg. to 3.83 µg. protein per spot (fig.1) with a protein: dye ratio of 2.52/1. Statistical analysis showed that the lowest absorbance reading which gives an error of less than 10% Coefficient of Variation is 0.05 (fig.5 , page58). This represents 6.37 µg. of protein and is the smallest amount which can be estimated with acceptable error.

The lowest concentration of protein which can be estimated is dependent on the minimum amount of eluent which is required to elute the maximum amount of protein aliquot per spot. Since 1.5 ml. of 2% Na_2CO_3 solution is the minimum amount required to obtain a reading in the spectrophotometer, and a 20 µl aliquot of protein the largest size spot which can be eluted within 30 minutes, the lowest concentration of protein which can be determined by this elution technique is 6.37 µg protein per 20 µl solution or 0.319 g/l protein.

2. Direct Protein estimation at 280 mµ.

Protein estimations were standardized by measuring the ultra-violet absorption of dilutions of Lab Trol

FIGURE 1

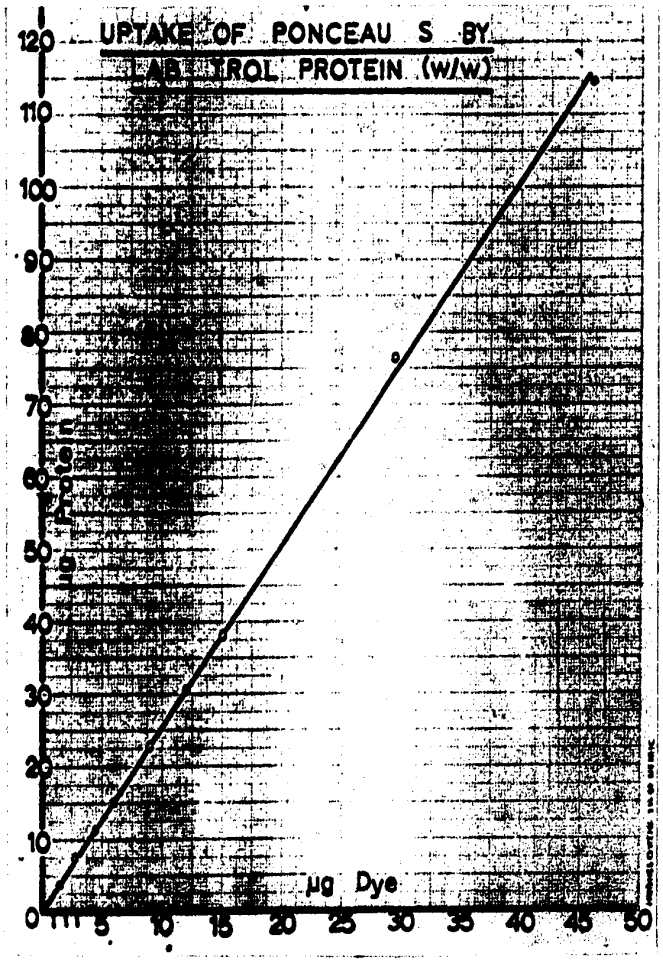
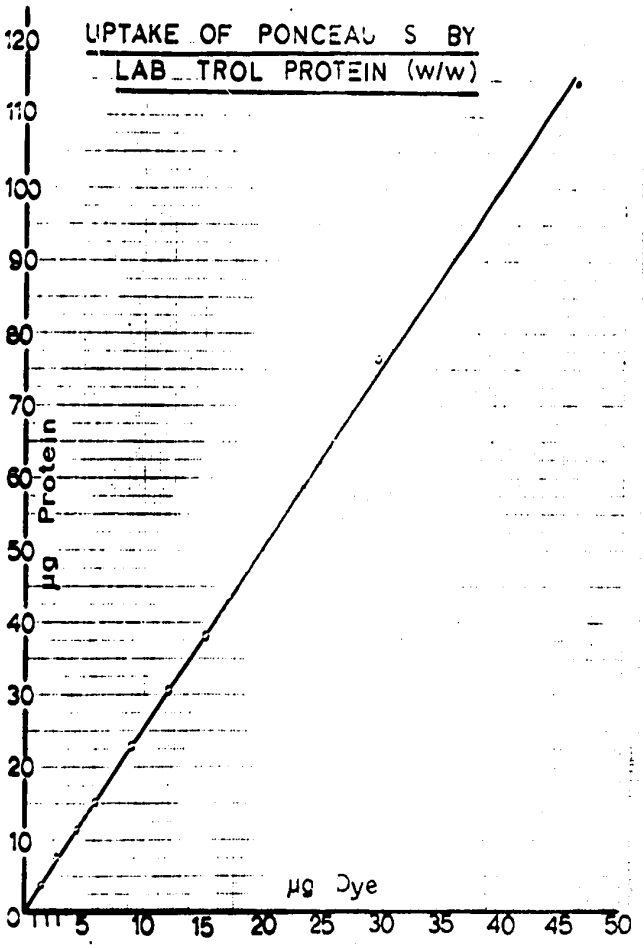

UPTAKE OF PONCEAU S BY
LAB. TROL PROTEIN (w/w)
120
110
100
90
80
70
60
50
40
30
20
10
0
µg Dye
5
10
15
20
25
30
35
40
45
50

FIGURE 1


UPTAKE OF PONCEAU S BY
LAB TROL PROTEIN (w/w)
120
110
100
90
80
70
60
50
40
30
20
10
0
µg Protein
µg Dye
5
10
15
20
25
30
35
40
45
50

at 280 mμ, by measuring the intensity of absorption of the blue colour produced by their reaction with the Folin-Ciocalteu reagent according to the method described by Lowry et al (1951). The combined results of three experiments in which the absorption at 280 mμ of various dilutions of Lab Trol was measured, are shown in Table 4 and figure 2. From these results the absorptivity of Lab Trol, calculated as the regression coefficient, was found to be 1.0043 ± S.D. 0.03.

3. Protein estimation using the Folin-Ciocalteu reagent.

A series of dilutions of Lab Trol were made containing from 0.017 to 0.7 g/l of protein. Protein estimations were carried out on duplicate samples from each dilution and read at = 500 mμ and = 700 mμ. 700 mμ was the wavelength of maximum absorbance and was used instead of 750 mμ, recommended by Lowry.

From each of the five dilutions containing from 0.017 - 0.28 g/l, 10 replicate determinations were made on each of two subsequent occasions. These tests were read at 700 mμ. The results of these experiments are shown in figure 3. The mean results and their error are presented in table 5.

The relationship of protein concentration to optical density is clearly not linear. Since the three series read at 700 mμ, constitute replicate determinations carried out on three separate occasions from the same material, a linear regression calculated from the results obtained over this portion of the curve forms a reasonable basis for the estimate of error. The mean Coefficient of Variation for the regression coefficient is 5% which represents 95% confidence limits of ± 10% of the mean value. All the values obtained for the three series lie within this figure. Therefore the standard Lab Trol curve shown in figure 3 was used for the estimation of protein content of unknown samples.

4. Error Entailed in the Use of the Microburet.

The syringe microburet was used in the making of replicate dilutions and the distribution of replicate samples for many of the experiments entailed in the present investigation.

The results obtained using this instrument were analysed and plotted as shown in figure 4. The actual values from which these calculations were made are included in the appendix.

TABLE 4

RESULTS OF DIRECT PROTEIN ESTIMATION

Lab-Trol* Dilution	No. of Samples	Absorbance Units	SD	Fiducial Limits	C. of V.**
µg/µl	N	λ 280 mµ			
.096	10	.0845	.005	.075-.085	5.9
.184	10	.184	.005	.178-.198	2.66
.191	10	.196	.008	.179-.212	4.16
.383	10	.383	.011	.361-.405	2.7
.460	10	.463	.004	.455-.471	0.86
.460	10	.454	.006	.443-.467	1.3
.5735	20	.575	.006	.563-.586	1.04
.574	9	.573	.008	.557-.589	1.39

* Regression of Absorbance units (y) on Lab Trol dilutions (x)

$$y = 1.0043x - 0.003$$

Probability that a=0 is 0.9 to 0.8, therefore,

$$y = 1.0043x$$

$$SD_b = 0.0287$$

Fiducial Limits of b=0.9756 to 1.0030, therefore, Absorptivity of Lab Trol=1 within the limits of sampling error.

** Coefficient of Variation

FIGURE 2

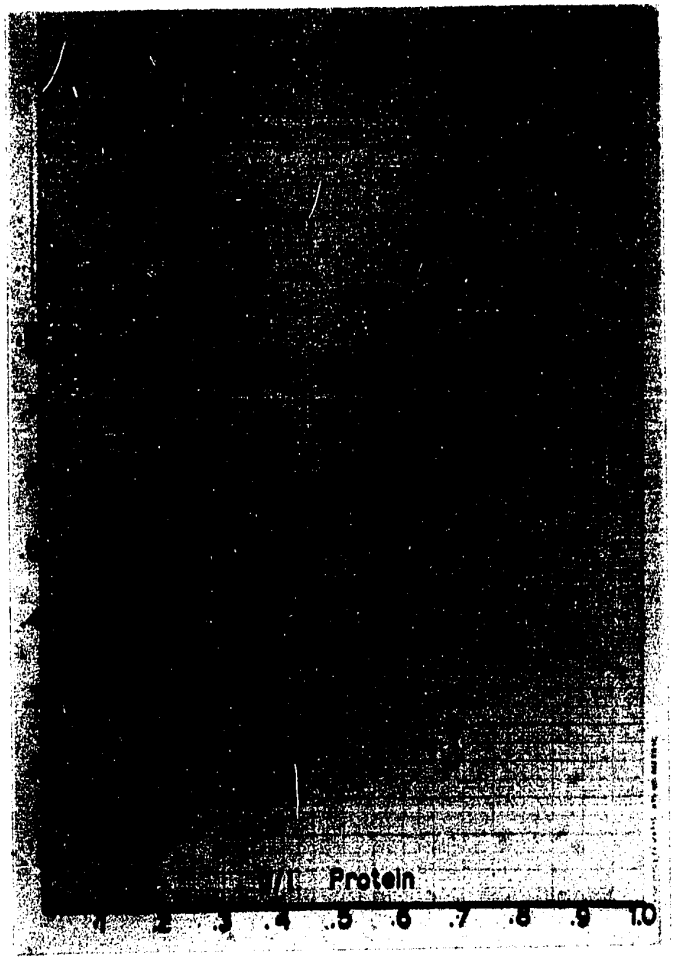


FIGURE 2

DIRECT PROTEIN ESTIMATION AT λ280 mμ

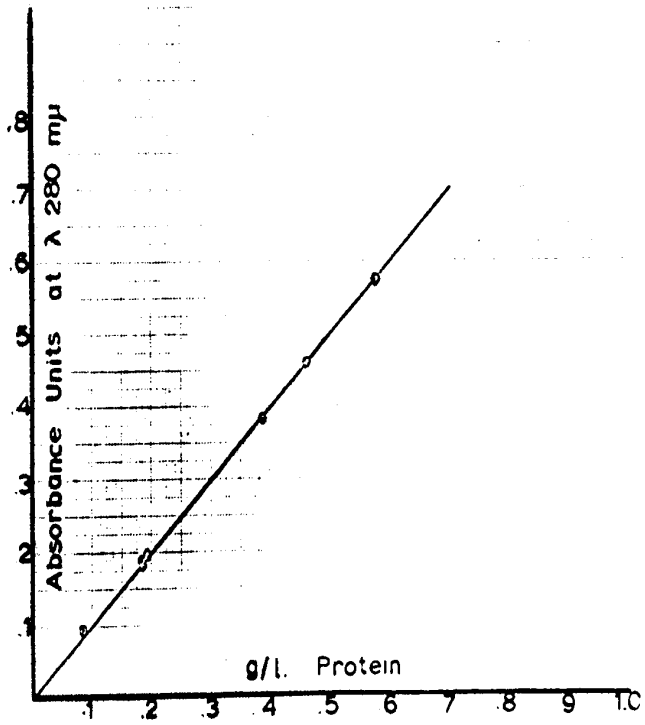


FIGURE 3

PROTEIN DETERMINATION
WITH FOLIN'S PHENOL REAGENT

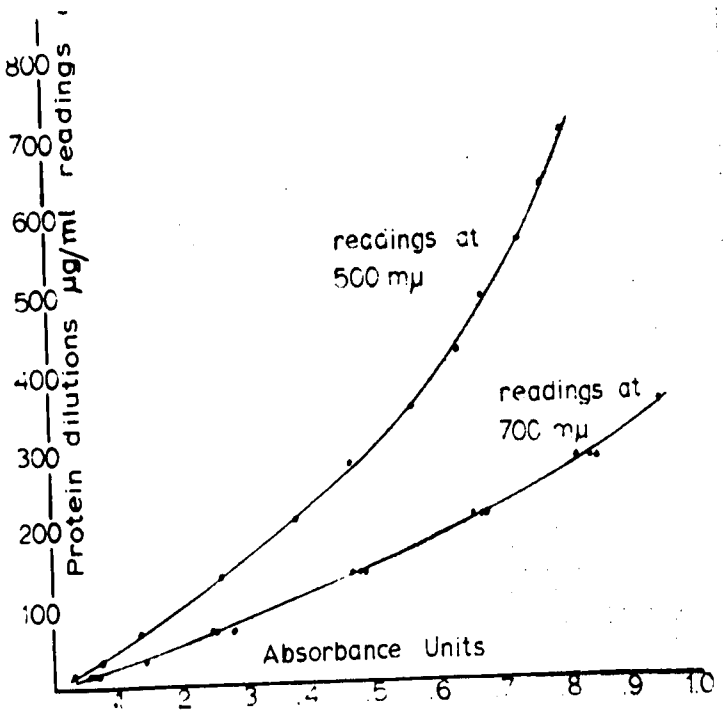


TABLE 5

RESULTS OF PROTEIN ESTIMATIONS WITH FOLIN'S PHENOL REAGENT

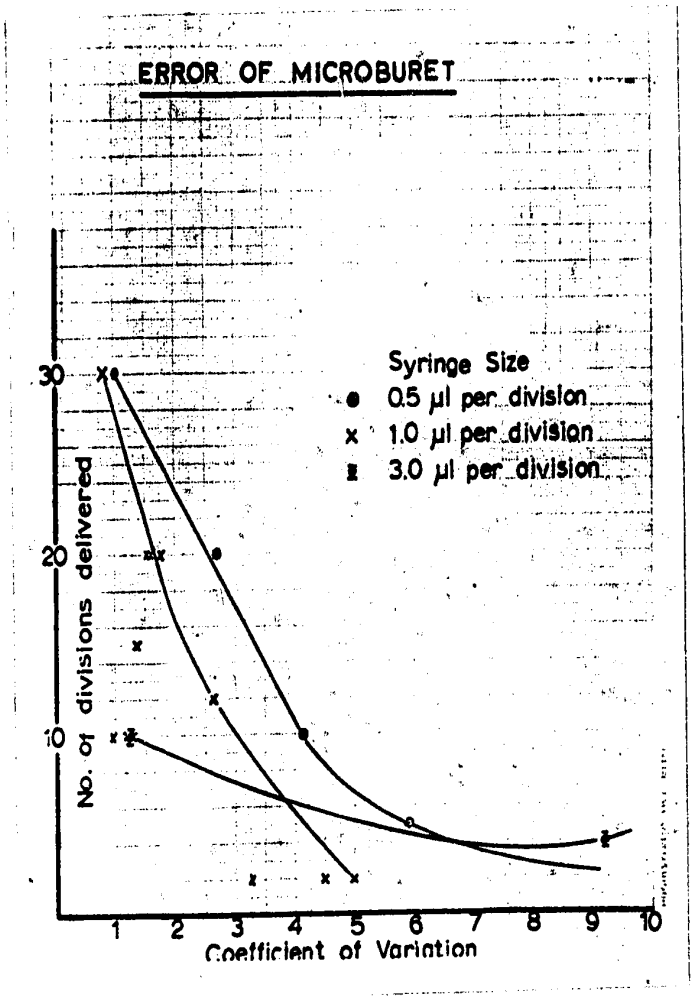
Experiment Number	Number of Samples of each Dilution	Mean Absorbance of Protein Dilutions (g/l) at 700 mμ					Regression line y = a + bx				F.L.*** of b
		.017	.070	.140	.210	.280	a	p* that a = 0	b	C.V** of b %	
1	2	.0625	.2445	.464	.653	.815	.037	.3	2.8647	4.33	3.1127 -2.616
	10	.057	.2522	.4772	.6607	.837	.034	.3-.2	2.9466	4.51	3.1127 -2680
2	C. of V** between samples	8.7%	2.2%	2.6%	3.4%	1.3%					
	10	.067	.281	.4885	.6929	.846	.053	.2-.1	2.945	4.9	3.139 -2.751
3	C. of V** between samples	4.5%	2.1%	2.2%	(N=6) 0.6%	0.8%					

* Probability

** Coefficient of Variation

*** Fiducial Limits

FIGURE 4


ERROR OF MICROBURET
Syringe Size
0.5 µl per division
1.0 µl per division
3.0 µl per division
No. of divisions delivered
30
20
10
1
2
3
4
5
6
7
8
9
10
Coefficient of Variation

From these results it can be stated that the microburet may be used for the distribution of replicate samples with an error not exceeding a Coefficient of Variation of 5%, provided that the optimal combination of syringe and sample volume is selected.

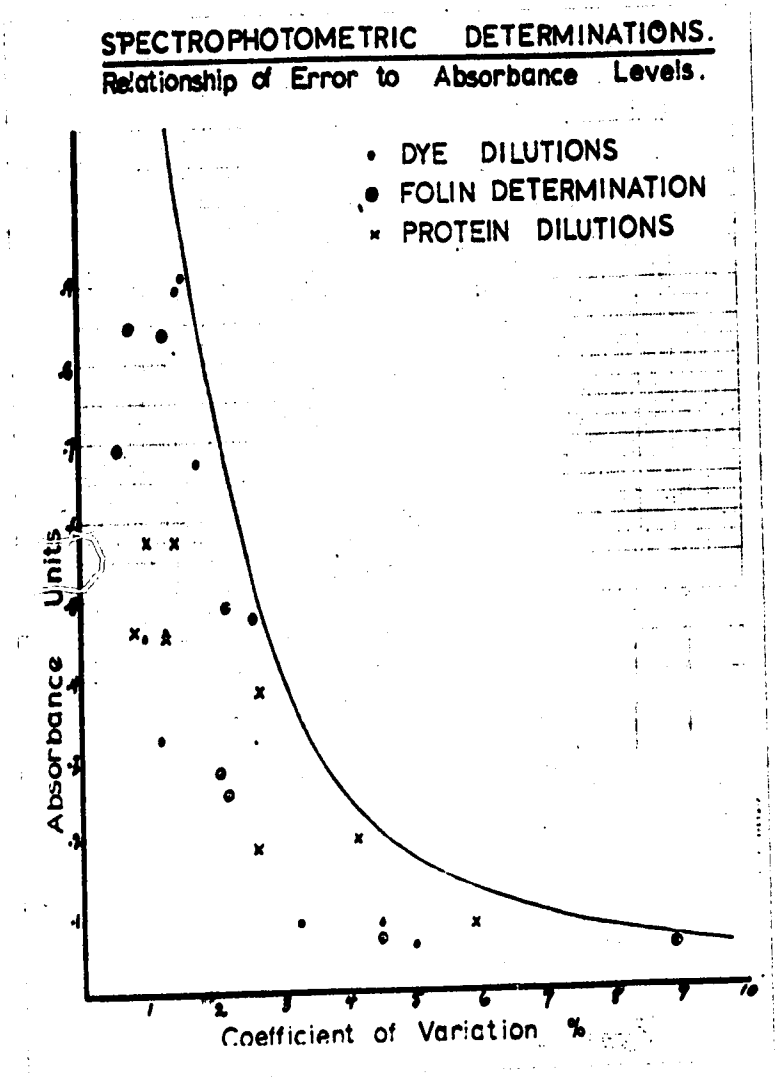
5. Error of Spectrophotometer Determinations.

In the present investigation spectrophotometry has constituted the final step in the determination of: a) the optical density of dye solutions at various wavelengths in the visible spectrum, b) the direct estimation of protein by absorption at 280 mμ in the ultra violet and c) the indirect estimation of protein using Folin-Ciocalteu reagent.

An analysis of the error variation observed in each type of investigation shows that the error is directly related to the optical density of the specimen examined. Therefore, estimates of error by all three methods were combined to give a single family of estimates, as shown in figure 5.

The line represents the maximum error which has been observed at all levels investigated and may be used to indicate the practical upper limit of error in the estimation of any unknown sample.

FIGURE 5


SPECTROPHOTOMETRIC DETERMINATIONS.
Relationship of Error to Absorbance Levels.
• DYE DILUTIONS
● FOLIN DETERMINATION
× PROTEIN DILUTIONS
Absorbance Units
Coefficient of Variation %

SUMMARY

There are inherent advantages in each of the three techniques for estimating the protein content of unknown solutions. A comparison of the degree of sensitivity of each method is shown in Table 6.

On the basis of these results, it is evident that all three techniques may be used for the estimation of protein with similar degrees of error. However, the reaction with Folin's phenol reagent using the technique of Lowry et al is the most sensitive.

TABLE 6

SENSITIVITY OF THREE PROCEDURES FOR ESTIMATION OF PROTEIN

PROCEDURE		AMOUNT OF SAMPLE REQUIRED FOR TESTING	UPPER LIMIT OF SENSITIVITY	LOWER LIMIT OF SENSITIVITY
Ultra-violet absorbance	280 mu.	1.5 ml.	0.80 g/l	0.050 g/l
Reaction with Folin's phenol reagent	500 mu.	0.2 ml.	0.70 g/l	0.07 g/l
	700 mu.	0.2 ml.	0.28 g/l	0.0175 g/l
Elution of dye-bound protein	520 mu.	between 0.5 ml. and 1.0 ml.	7.65 g/l	0.319 g/l

DEVELOPMENT OF THE MICRO-IMMUNODIFFUSION TECHNIQUE IN CELLULOSE ACETATE

INTRODUCTION

The value of the Ouchterlony double diffusion test for the comparative analysis of antigen-antibody precipitation patterns is now well established (Crowle, 1961). The advantage in the method lies in its resolution of many reacting constituents of a complex antigenic mixture at one time and simultaneously characterizing them with respect to another antigen mixture or known antigen. Ouchterlony (1958, 1962) originally described three types of patterns as a guide to the interpretation of the precipitating reactions. These patterns have been described as lines of identity, partial identity and non-identity. When two serologically identical antigens react with homologous antiserum, two identical lines will form and the lines will fuse at the proximal band tips signifying a reaction of identity. An antigen which is similar enough to another to precipitate some antibody in the antiserum against the homologous antigen will form a precipitation band. This band is arrested at its junction with the band which is formed by

the homologous antigen, however the latter band continues to grow forming a "spur" or "hook" which is the reaction of partial identity. When two serologically different antigens are compared using an antiserum, which contains antibodies to both antigens, each antigen-antibody system precipitates independently, so that the resulting bands of precipitation "cross over" in the reaction of non-identity.

The potential sensitivity and resolution of precipitin lines are the main factors to be considered in selecting an immunodiffusion technique for the analysis of complex antigen-antibody systems. In the micro modification, introduced by Wadsworth (1957) and developed by Crowle (1958), a continuous-flow diffusion system was achieved by using wells made in "Perspex" templates, which are placed on the surface of a thin layer of agar gel. Thus, relatively large volumes of reactants are constantly fed into a thin layer of agar, greatly increasing the sensitivity and resolution of the test while economizing on reagents. The successful application of this technique to the study of the antigens of vaccinia virus has been mentioned earlier.

However, the use of agar in the micro-techniques has certain disadvantages. Primarily there is the considerable risk of complete loss of the reaction due either to

the tearing of the fragile agar layer when the template is removed, or to its loosening and washing off during the staining procedures. Splitting and distortion of the agar layer during drying may also occur. Secondly, the test is relatively inflexible in that pH and electrolyte concentration of the agar cannot easily be varied. Thirdly, such pH and electrolyte concentration changes which may be studied, besides affecting the antigen-antibody reaction, may also cause undesirable complexing between the gel and the reagents. And finally, variations are encountered with different batches of agar, both as regards their mechanical stability and the reactions developing in them, (Crowle, 1961). For these reasons, a technique was developed which combined the advantages of the continuous-flow system with those offered by the use of cellulose acetate as a support medium for immunodiffusion (Johnson et al, 1964).

PROCEDURE

Cellulose acetate strips were cut transversely into pieces approximately 2.3 cm by 5.0 cm, and were soaked by floating them on the appropriate buffer then immersed for a minimum of 10 minutes or until required. Perspex templates of the type described by Crowle (1958, 1961) were used and care was always taken to keep the under surface of

each template smooth and polished. These materials are shown in Figure 6. To mount each template, a wet cellulose acetate strip was laid length-wise on a clean glass slide and the template placed at one end of the strip. The template was slipped into a central position along the wet surface, elminating air bubbles in the process. Excess buffer was blotted from the ends of the cellulose acetate strip which should extend beyond both ends of the template. Blotting was continued until the paper visible at the bottom of the wells just changed from a shiny to a matte appearance. The prepared slides were placed into small moisture chambers (square Petri dishes, containing damp filter paper). During the later stages of this study a modification of this procedure was introduced, as follows: the template was slipped along the wet surface to the centre and was then firmly pressed into position, while stainless steel chromatographic clips were applied as shown in figure 7. Blotting was then continued as before.

The test materials were applied with finely drawn-out glass pipettes, and all the wells of the individual templates filled as quickly as possible. A satisfactory preparation showed no leakage between the template, paper, and slide interfaces, and no change in the appearance of the

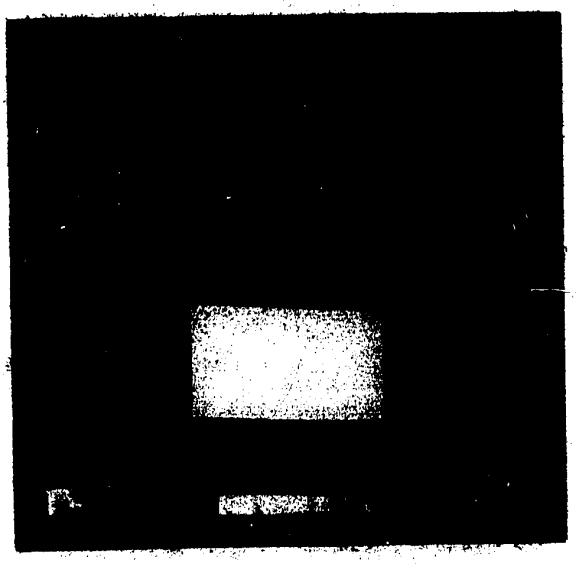


Figure 6 Photograph of the materials used for the preparation of a micro-immunodiffusion reaction chamber - glass microscope slide, perspex templates (2 patterns), a strip of cellulose acetate, two clamps and a pair of flat forceps.

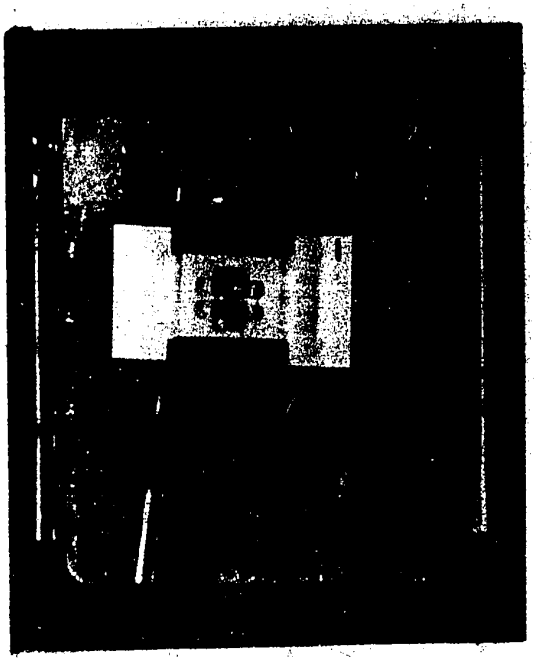


Figure 7 Photograph of the assembled reaction chamber placed on a plastic rack in the moisture chamber.

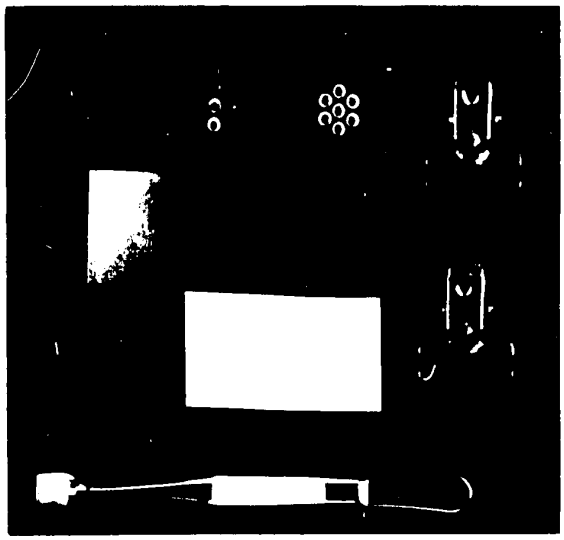


Figure 6 Photograph of the materials used for the preparation of a micro-immunodiffusion reaction chamber - glass microscope slide, perspex templates (2 patterns), a strip of cellulose acetate, two clamps and a pair of flat forceps.

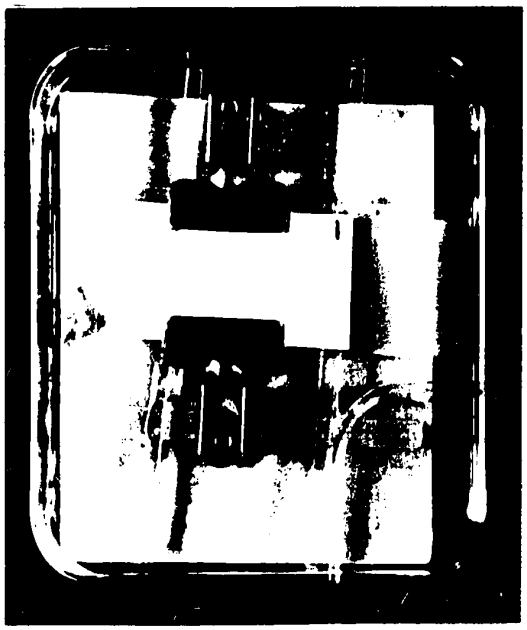


Figure 7 Photograph of the assembled reaction chamber placed on a plastic rack in the moisture chamber.

cellulose acetate at the bottom of the unfilled wells as the neighbouring wells were filled.

The individual moisture chambers were placed together into a larger container with a more efficient seal, which preserved a moist atmosphere throughout incubation. The time of incubation was 44 hours unless specified otherwise, at room temperature. When a constant temperature incubator was obtained, all reactions were incubated at 22.5°C.

After incubation, the templates were removed by holding the slides under running tap water. The strips were washed in physiological saline, at room temperature for a minimum of 4 hours and then for ten minutes in distilled water. Precipitin lines were stained by immersing the strips in 0.2% Ponceau S (CI 27195) in 3% trichloracetic acid or 0.1% Thiazine red R (CI 14780) in 1% acetic acid for a minimum of 30 minutes. The strips were differentiated in 5% acetic acid baths and then washed in distilled water to remove the acid. The strips were then blotted and dried at room temperature.

When the cellulose acetate strips were completely dry, they were cleared by immersion in Whitemor Oil No.120. The oil-soaked strip was mounted on a microscope

slide and a coverslip (No.2, 22mm. x 30mm.) placed over the reaction area. Excess oil was blotted with filter paper and the cellulose acetate trimmed to coverslip size. Clear varnish was used to seal the edges making a permanent mount. ~~as illustrated in figure 7.~~ The stained reactions were then enlarged and printed directly on photographic paper before final analysis of the results was made.

Comparison of Micro-Double Diffusion reactions in agar and in cellulose acetate.

In order to evaluate the technique using cellulose acetate, a direct comparison of the precipitin reactions of several antigen-antibody systems was made in agar and in cellulose acetate (CA). The micro-double diffusion agar precipitin technique described by Crowle (1958) was used for all the agar immunodiffusion tests.

The first test system was chosen to compare reactions of both 'H'-type and 'R'-type antibodies in agar and in cellulose acetate. The reactions are presented in Plate 3. 'H' or flocculating antibody, typified by horse antitoxins forms a precipitate with its antigen which is readily soluble in either antibody or antigen excess, whereas the 'R'-type antibody forms precipitates with antigen which are insoluble, or poorly so, in reactant excesses.

This latter type is typically produced by rabbits immunized with various antigens. At optimal proportions in each medium, the reactions between the toxoid (T) and the horse antitoxin (H-type ab.) show the characteristic high antigen resolution of the 'H' antibody. Included in the same pattern is the second reaction between the horse antitoxin (globulin) and rabbit anti-horse gamma globulin (R-type ab.). In both support media, the precipitin lines demonstrate characteristics of the R-antibody reaction in that they are more poorly resolved and form a dense but diffuse precipitate. The symmetry of the reaction patterns is similar in both agar and cellulose acetate, however better resolution of the lines is achieved in the cellulose acetate reaction. Furthermore, the reaction of partial identity between the reacting gamma globulin components common to both systems, is demonstrated quite clearly in cellulose acetate (arrowed).

A second system, comparing the reactions of 0.7% ovalbumen (A) with rabbit anti-ovalbumen (RaA) and with rabbit anti-horse albumin (RaHA) in agar (figures 1 & 2) and in cellulose acetate (figures 3 & 4) is shown in Plate 4. The duplicate reactions were stained with Thiazine red R (figures 1 & 3) and Ponceau S (figures 2 & 4) to compare the staining characteristics.

It can be seen from these reactions that: 1) a more complex system of reacting components is evident in the reactions in cellulose acetate (CA), 2) in the complex CA reaction, the better resolution of the precipitating components is achieved using Ponceau S (since a maximum of eight reacting complexes may be distinguished compared to the five shown by Thiazine red R), and 3) in the less complex agar reaction, Thiazine red R is the more sensitive stain, capable of revealing the presence of weak reactions with greater intensity than Ponceau S.

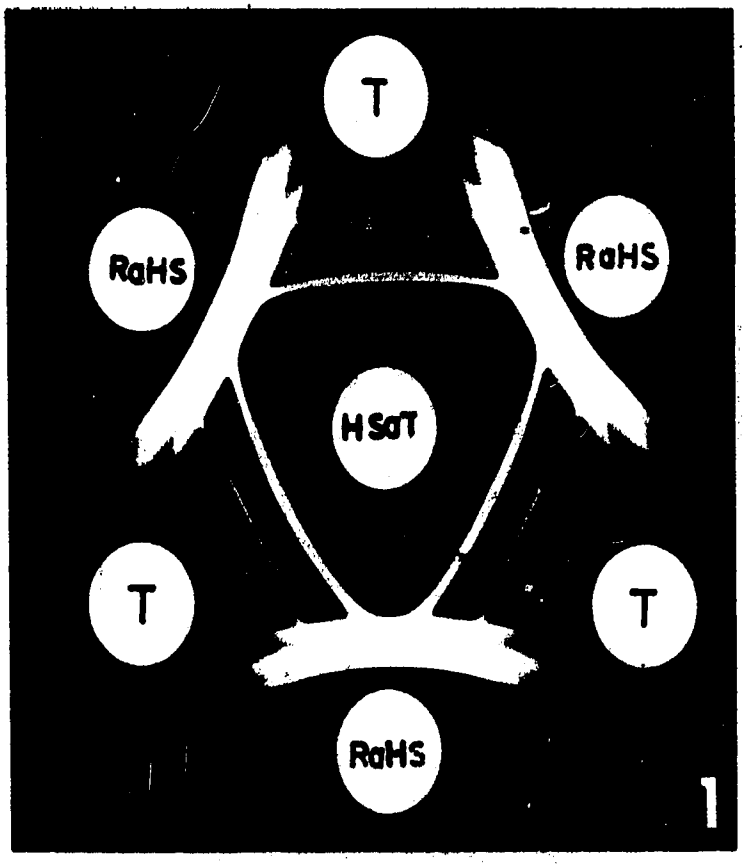
On the basis of these observations, all the preliminary immunodiffusion tests were stained by both Ponceau S and Thiazine red R. Once the reactions were characterized with respect to their intensity and complexity, the appropriate stain was chosen. In most cases, this was Thiazine red R.

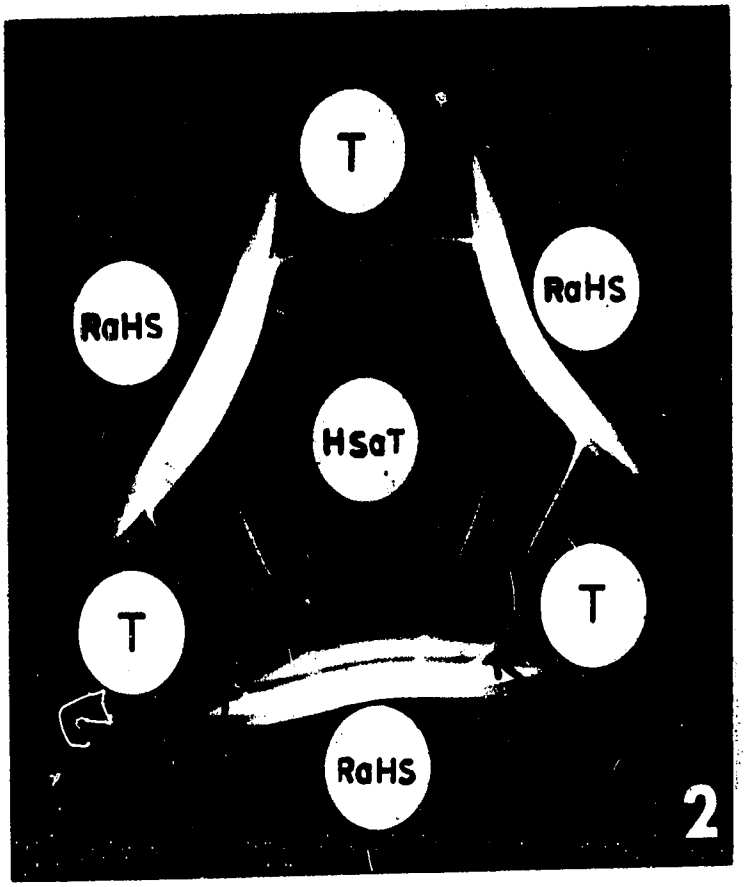
PLATE 3

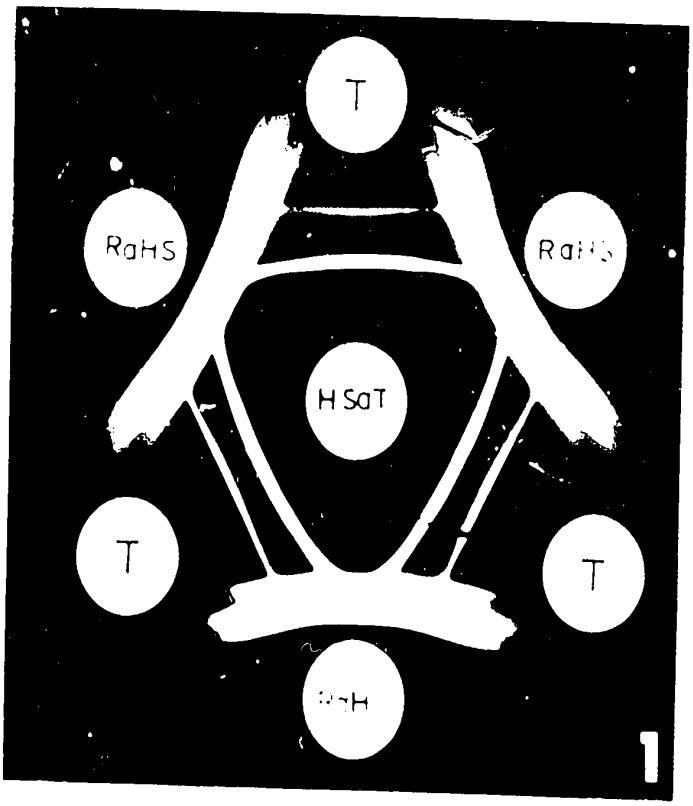
Comparison of Immunodiffusion Reactions between 'H'-type antibody (horse antitoxin, HSaT) and toxoid (T) and between 'R'-type antibody (rabbit anti-horse serum, RaHS) and horse serum (HSaT).

Figure 1 - Reactions in agar

Figure 2 - Reactions in cellulose acetate. Reaction of identity is indicated by arrows.


T
RaHS
RaHS
HSaT
T
T
RaHS
1


T
RaHS
RaHS
HSaT
T
T
RaHS
2


T
RaHS
HSaT
T
T
1

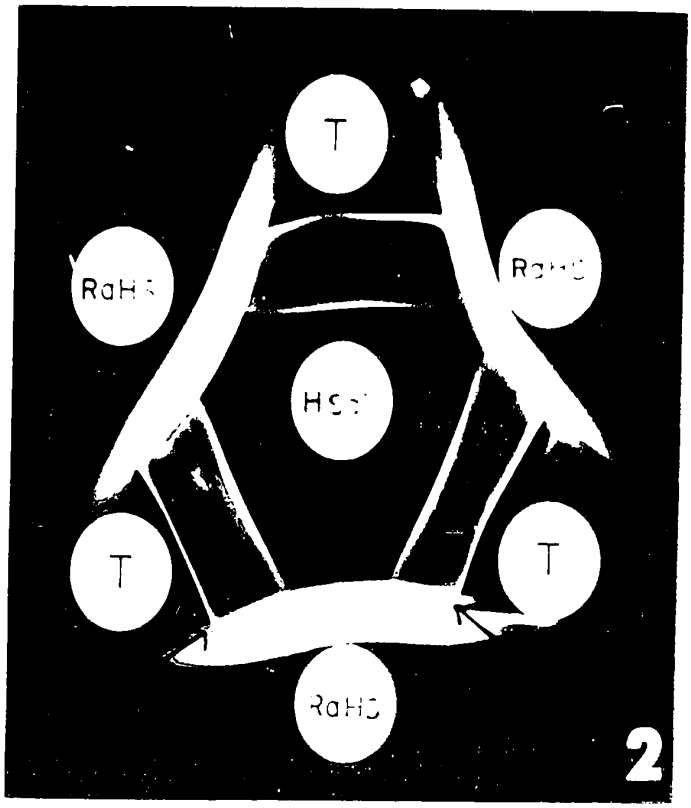

T
T
T
2

PLATE 4

Comparison of staining of Ponceau S and Thiazine R on Immunodiffusion Reactions in Agar and in Cellulose Acetate.

A - 0.7% ovalbumen
RaA - rabbit anti-ovalbumen serum
RaHA - rabbit anti-horse albumin serum.

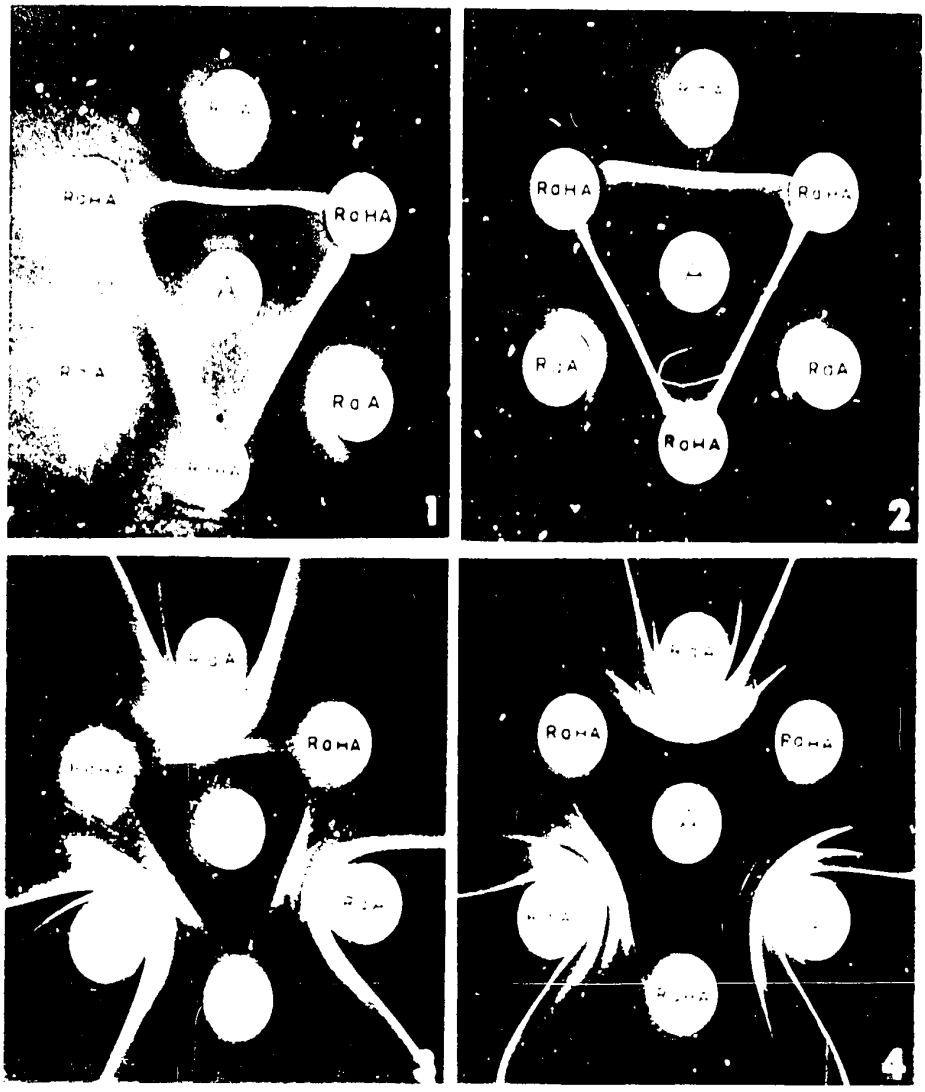
Figure 1 - Immunodiffusion reaction in agar stained with Thiazine red R.

Figure 2 - Immunodiffusion reaction in agar stained with Ponceau S.

Figure 3 - Immunodiffusion reaction in cellulose acetate stained with Thiazine red R.

Figure 4 - Immunodiffusion reaction in cellulose acetate stained with Ponceau S.

RcA
RcHA
RcHA
A
RcA
RcA
RcHA
1
RcA
RcHA
RcHA
A
RcA
RcA
RcHA
2
RcA
RcHA
RcHA
A
RcA
RcA
RcHA
3
RcA
RcHA
RcHA
A
RcA
RcA
RcHA
4


RoHA
RoHA
RoA
1
RoHA
RoHA
RoA
RoA
RoHA
2
RoHA
RoHA
4

Temperature of Incubation

The incubation temperature for immunodiffusion reactions should provide optimal conditions for the formation of the antigen-antibody complexes, and at the same time preserve a stable moist atmosphere which is necessary for the cellulose acetate immunodiffusion technique. Room temperature incubation (20°C-25°C.) was found to be best for these experiments. Incubation at refrigerator temperature resulted in the distortion or complete loss of reactions because the condensed water floated the template off the CA membrane and, at 37°C., the reactions were fuzzy, and the resolution of the precipitin lines was poor (Crowle, 1961).

Effect of Buffers and pH.

In cellulose acetate it was found that each antigen-antibody system required its own set of individual conditions for optimal reactions. The effect of TRIS and barbital buffers at different pH concentrations is illustrated in Plate 5 (figures 1, 2 & 3). From these reactions it can be seen that barbital buffer at pH 8.9 (fig. 3) provides the best conditions for the immunodiffusion analysis of this particular antigen-antibody system.

The optimal conditions for the influenza virus

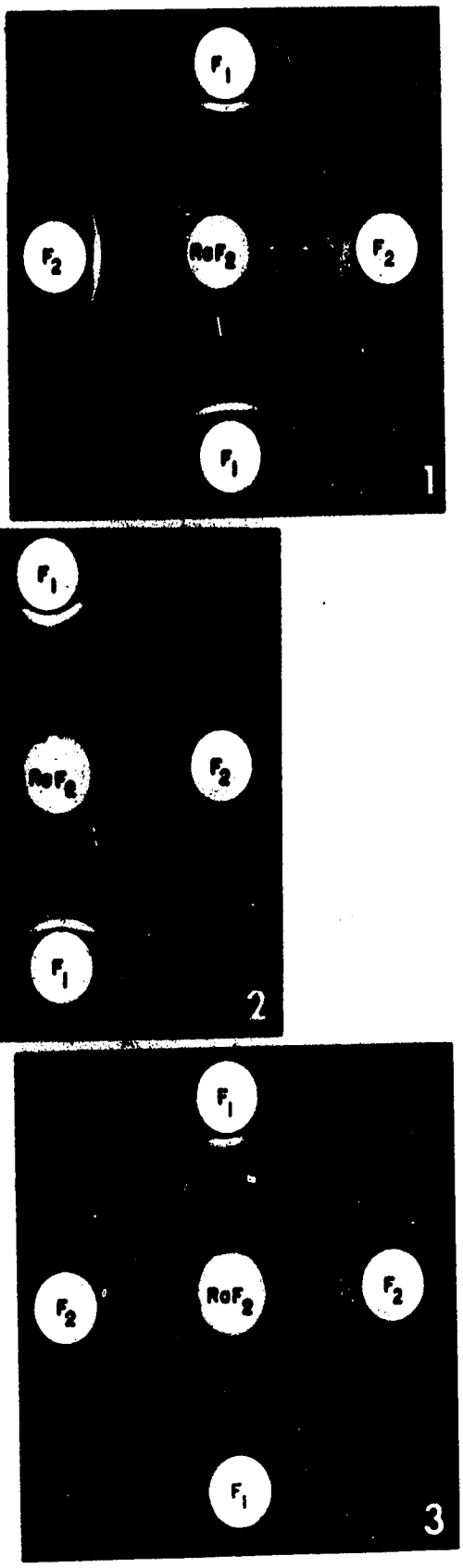
PLATE 5

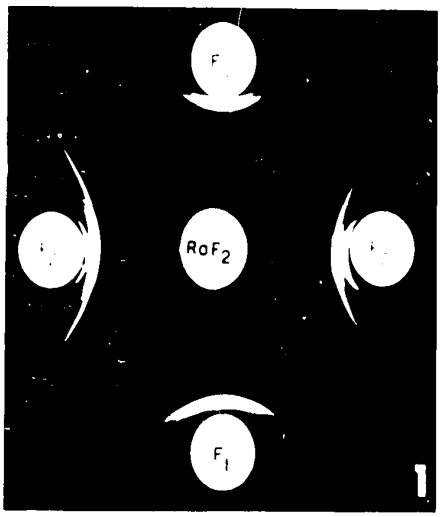
Sensitivity to changes in pH concentration illustrated by the immunodiffusion reactions between Rabbit anti-F_2 serum (RaF_2) and antigen preparations F_1 and F_2.

Figure 1 - TRIS buffer , pH 8.5 , showing the reaction is not contained within the boundary of the wells.

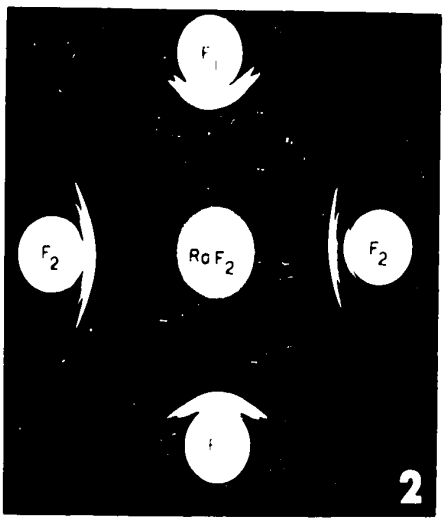
Figure 2 - Barbital buffer, pH 8.7 shows improvement in both resolution of lines and position of reaction.

Figure 3 - Barbital buffer, pH 8.9, shows reactions of identity in addition to good reaction resolution.

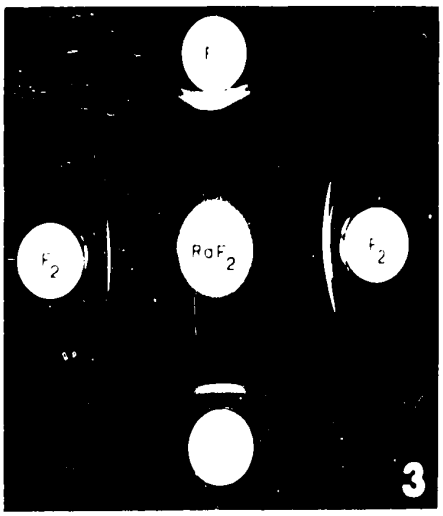

F1
F2
ReF3
F2
F1
1
F1
F2
ReF3
F2
F1
2
F1
F2
ReF3
F2
F1
3



1



2



3

concentrate, and soluble antigens were provided by TRIS or TRIS-maleate buffers at pH 7.4. TRIS buffer was used throughout these experimental procedures.

Summary

A micro-double diffusion system using cellulose acetate as the support media was developed and found to provide better separation and resolution of complex antigen-antibody reactions. This method had the additional advantage of providing a flexibility, lacking in the agar gel methods.

This micro-immunodiffusion technique in cellulose acetate was used as the standard method for immunodiffusion analyses throughout these investigations. In certain cases, the agar micro-immunodiffusion method was used to compare or clarify results obtained using cellulose acetate.

EXPERIMENTAL RESULTS

INFLUENZA VIRUS STRUCTURAL ANTIGENS

Concentration of Virus Particles.

Choice of method.

The use of adsorption onto and elution from chicken red blood cells, originally proposed by Hirst (1941) and McClelland & Hare (1941) has been the basis of most methods for the concentration and purification of influenza viruses. Other procedures have been recommended and include:- high-speed centrifugation (Elford & Andrews (1936), Stanley, 1944), methanol precipitation (Cox et al, 1947) and column adsorption with aluminium phosphate (Miller and Schlesinger, 1955, Frommhagen & Knight, 1959). Veeraraghavan & Sreevalsan (1961) compared these methods and a modification of a zinc hydroxide method (Newton & Bevis, 1959). Two of these procedures were chosen and evaluated:

1. Differential centrifugation, using the 21-batch rotor of the Spinco L-2-50 preparative ultra-centrifuge, and
2. R.B.C. adsorption and elution using human group 'O' R.B.C. (Sheffield, Smith & Belyavin, 1954), followed by differential centrifugation.

Following the R.B.C. elution cycles, both high speed deposits were pink, suggesting the contamination of the virus pellet with red cell debris. The presence of added contaminants is undesirable, especially in immuno-

logical analyses, therefore the adsorption-elution cycles were omitted. The flow sheet presented in figure 8 shows the final procedure adopted for the preparation of the virus concentrates. The haemagglutination and infectivity titres of samples from a typical harvest clearly show the efficiency of the procedure (Table 7).

Figure 8

Differential Centrifugation Procedure for the Concentration and Partial Purification of Influenza A/PR8 from Infected Allantoic Fluid.

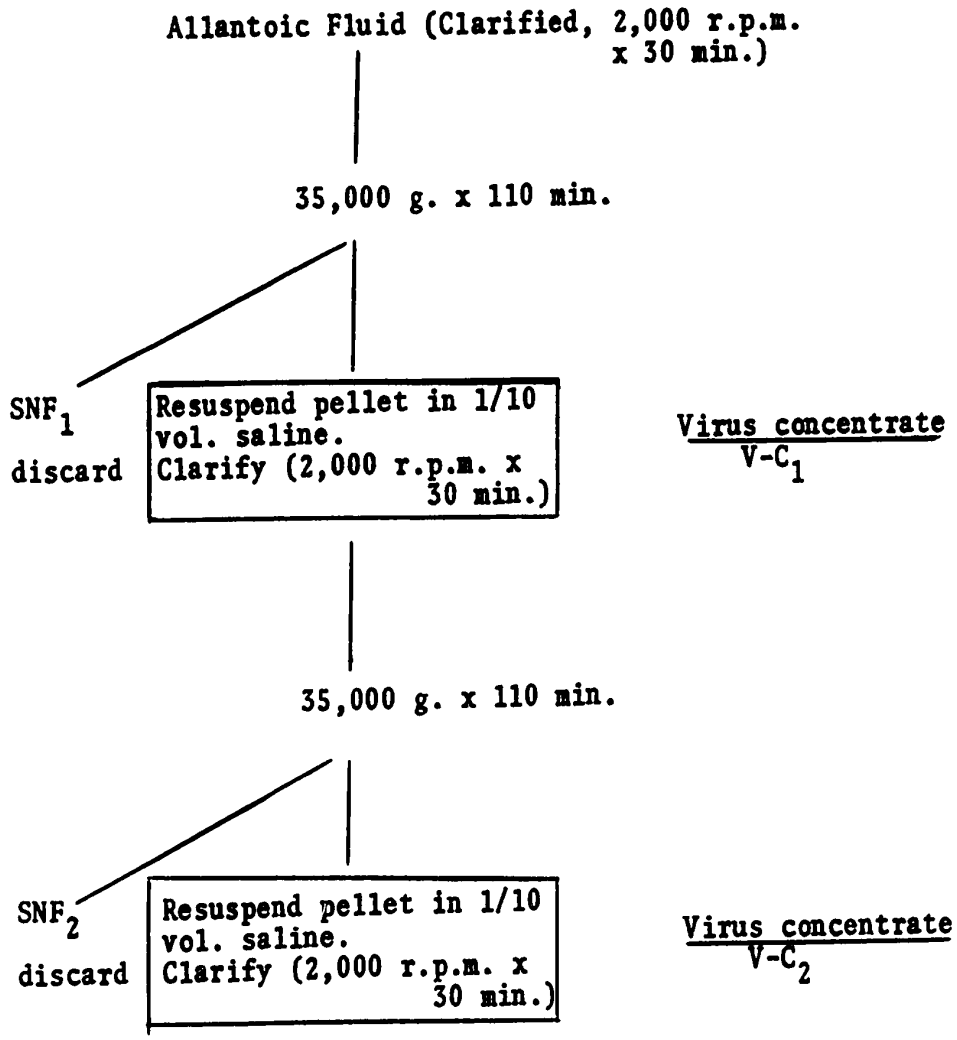


Table 7

Haemagglutination and Infectivity Titres following Differential Centrifugation and Concentration of Influenza A/PR8 Virus.

Virus Sample	Volume ml.	Concentration Factor	HA Titre.	Infectivity EID 50*
Alf.	1800	1	1,024	$10^{+8.5}$
SNF_1	-	-	64	$10^{+7.33}$
$V\text{-}C_1$	170	10	10,240	$10^{+9.77}$
SNF_2	-	-	128	$10^{+7.0}$
$V\text{-}C_2$	17	100	204,800	$10^{+10.33}$

(50% end points determined by Reed-Muench method).

Purity of the Virus Concentrate.

The purity of the virus concentrate was assessed by electron microscopy and by immunodiffusion reactions. Plate 6 shows an electron micrograph of a negatively stained preparation of untreated semi-purified concentrate. Since it was required that all impurities should be detectable, bovine serum albumin was not added to the concentrate, resulting in poor spreading of the specimen. The general appearance shown in this micrograph is typical of all the grids examined and shows a highly concentrated preparation of pleomorphic particles resembling influenza viruses, which are relatively free of gross contaminating debris. Non-viral matter (arrowed) is clearly detectable however, and constitutes a small proportion of the total material present. Such non-viral material is thought to consist of normal host components, possibly cell membrane which is closely attached to some of the virus particles, as a result of method of influenza virus release from the cell.

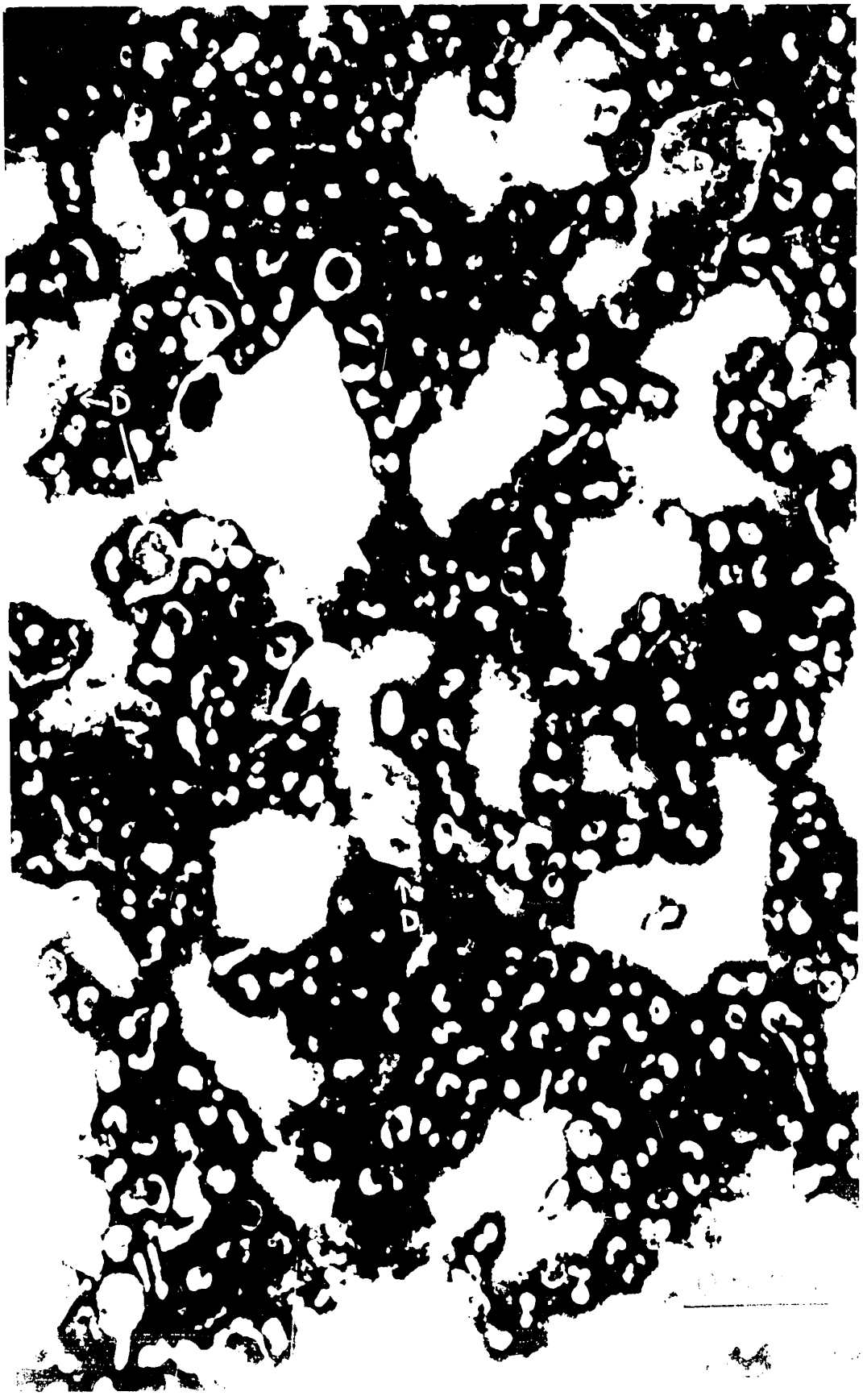
In order to assess the purity of the virus concentrate immunologically, antiserum to the virus concentrate was prepared by parenteral immunization of rabbits (RaS/C_2) and tested against virus concentrate ($V\text{-}C_2$),

PLATE 6

Electron microscopic examination of the purity of the virus-concentrate (V-C_3) by the negative staining technique (2%PTA). No BSA has been added to the concentrate. Non-viral debris is indicated (⟶D).

Electron microscopic examination of the purity of the virus-concentrate (V-C_3) by the negative staining technique (2% PTA). No BSA has been added to the concentrate. Non-viral debris is indicated (⟶D).

0.5 mμ


D
D

and normal embryo extracts (NE/SA). The immunodiffusion reactions are presented in Plate 7 (figures 1 & 2). It is clear that the rabbit antiserum contains specific antibodies which form two distinct precipitin lines with the antigen derived from host tissue (figure 2). These host antigens must have been present in the virus concentrate used for immunization. Therefore this reaction provides clear-cut evidence of the presence of host-cell antigens in the semi-purified virus concentrate. That some of the host antigen is non-viral debris was evident from the electron microscope examination, however, whether host component was also present as an integral part of virus structure was not determined by these tests.

PLATE 7

Detection of Host Components in preparations of semi-purified virus concentrate ($V\text{-}C_2$).

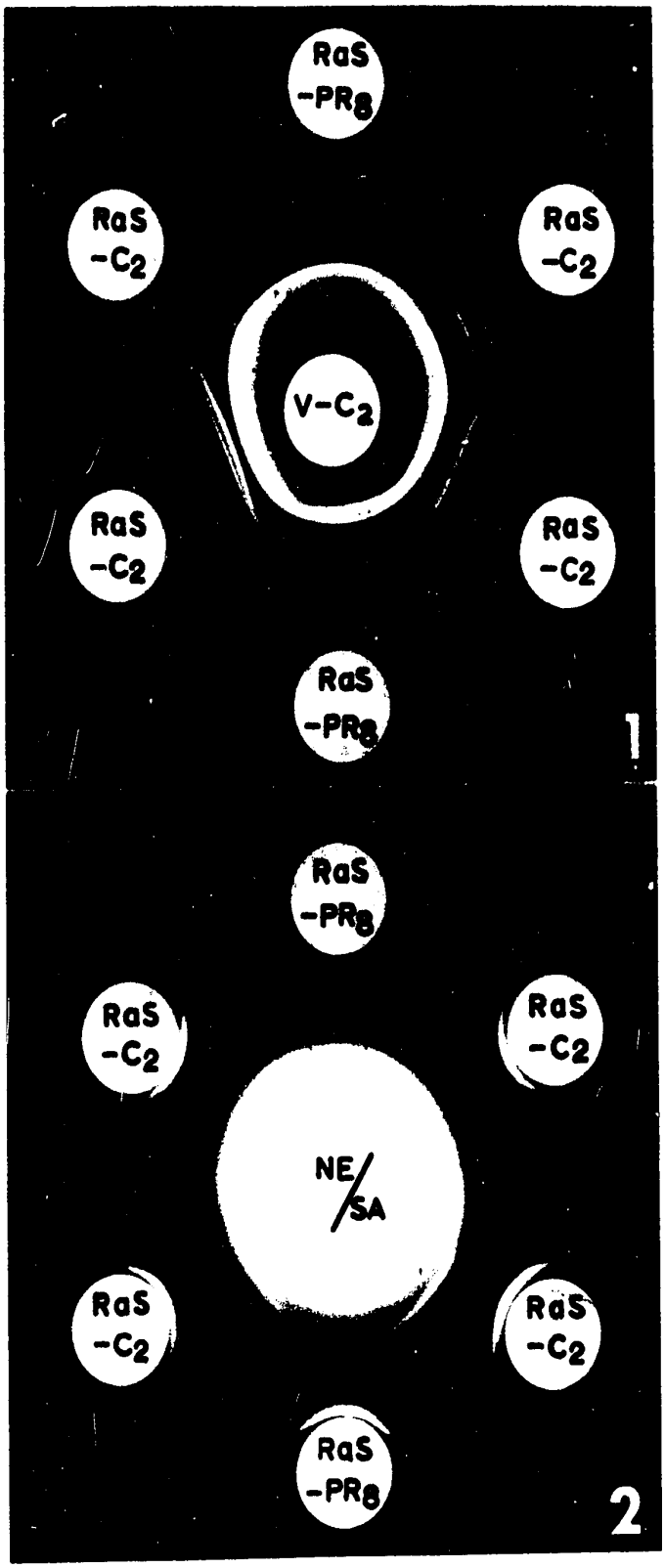
Figure 1 - Immunodiffusion reactions of rabbit anti-virus (RaS/C_2) and rabbit anti-PR8/SA (RaS/PR8) sera demonstrating the presence of at least five antigenic components detected by RaS/C_2 and three by RaS/PR8.

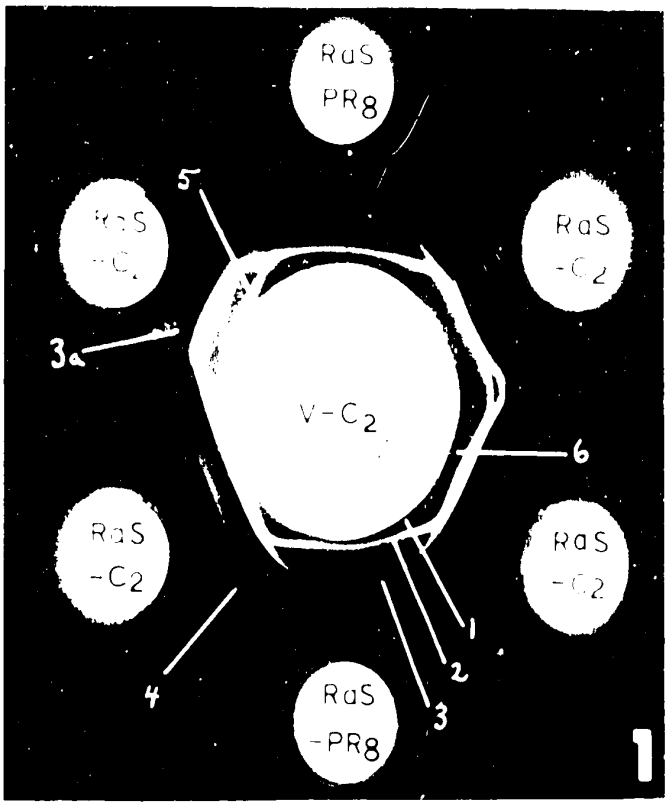
Figure 2 - Immunodiffusion reaction of the rabbit anti sera RaS/C_2 and RaS/PR8 with normal host antigens (NE/SA) demonstrating antibodies to host components in both RaS/C_2 and RaS/PR8 present as a result of the immune response to host components present in the immunizing antigens, $V\text{-}C_2$ and PR8/SA.

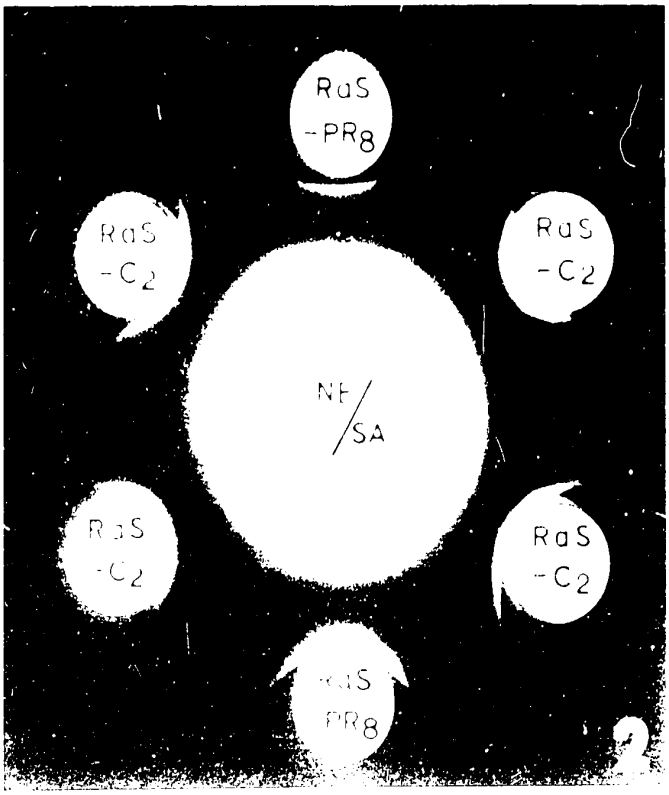
the

onents

zing




RaS
PR8
RaS
-C2
RaS
-C2
V-C2
RaS
-C2
RaS
-C2
RaS
-PR8
1
2
3
4
5
6
3a
1


RaS
-PR8
RaS
-C2
RaS
-C2
NF/SA
RaS
-C2
RaS
-C2
RaS
PR8

Studies on the Immunodiffusion Reactions of Semi-purified Virus Concentrate.

Diffusion of virus particles in cellulose acetate.

In preliminary immunodiffusion reactions, guinea pig antiserum to infected membrane extracts (GPaS/SA) was used to compare the soluble antigen and virus concentrate reactions in agar and in cellulose acetate. Normal guinea pig serum was included as control (GPNS). These reactions (Plate 8) showed precipitin lines which developed in cellulose acetate (figs. 3 & 4) with the concentrated virus particle preparation, but corresponding precipitin lines did not develop in agar (fig. 1 & 2). These reactions suggested the diffusion of components, possibly virus particles, which could not diffuse in agar (Polson, 1956). However, even the semi-purified virus concentrate contains soluble antigenic material including haemagglutinin which may be released as a result of virus disintegration. It was necessary, therefore, to establish whether the intact virus particle could in fact diffuse in cellulose acetate.

Influenza virus breakdown occurs during storage at 4°C. For this reason, all samples were stored at -80°C. However, this breakdown could not be completely avoided because repeated freezing and thawing results in the release from the virus particle of soluble antigenic components

PLATE 8

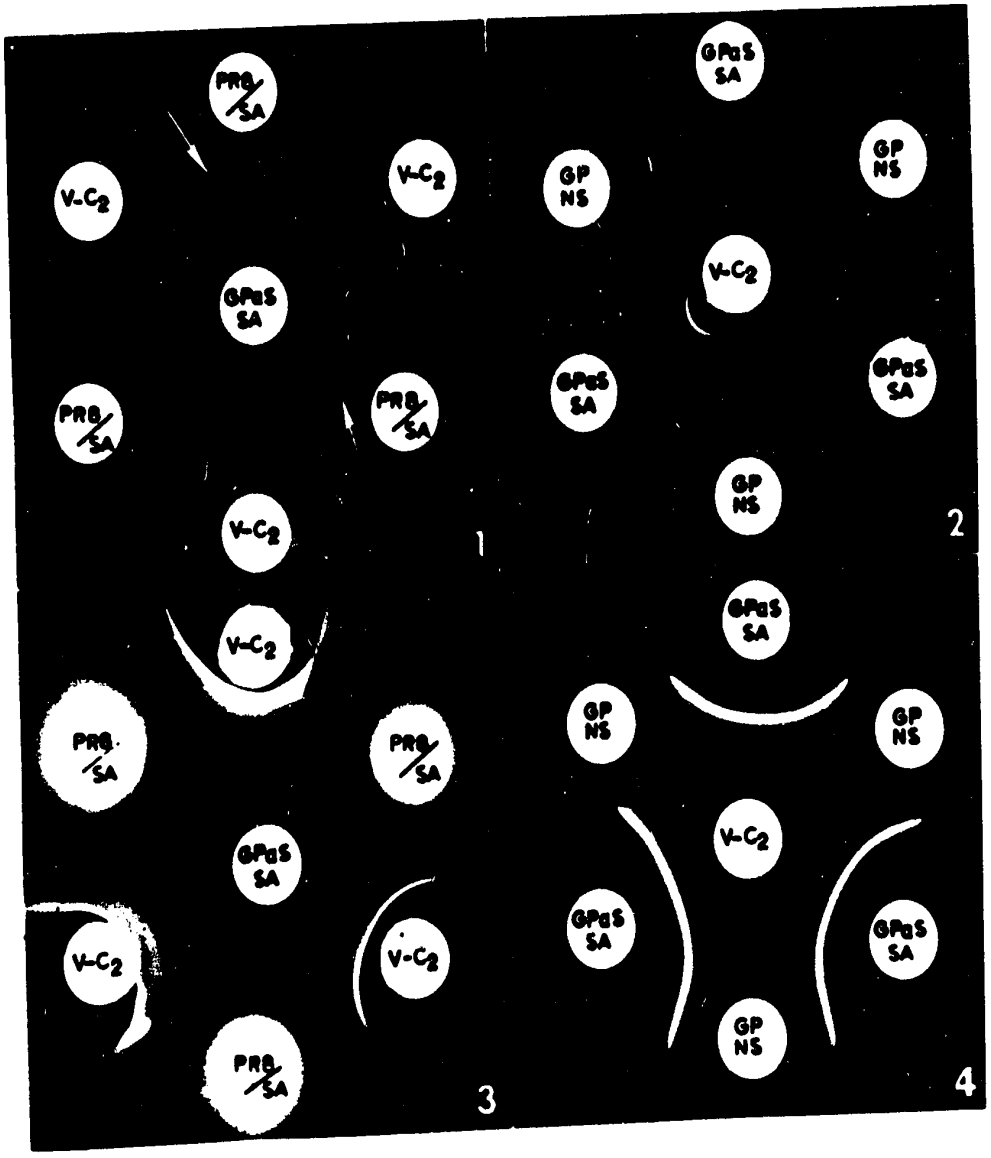
COMPARISON of Immunodiffusion Reactions of Virus Concentrate ($V\text{-}C_2$) and PR8 soluble antigen (PR8/SA) in Agar and Cellulose Acetate

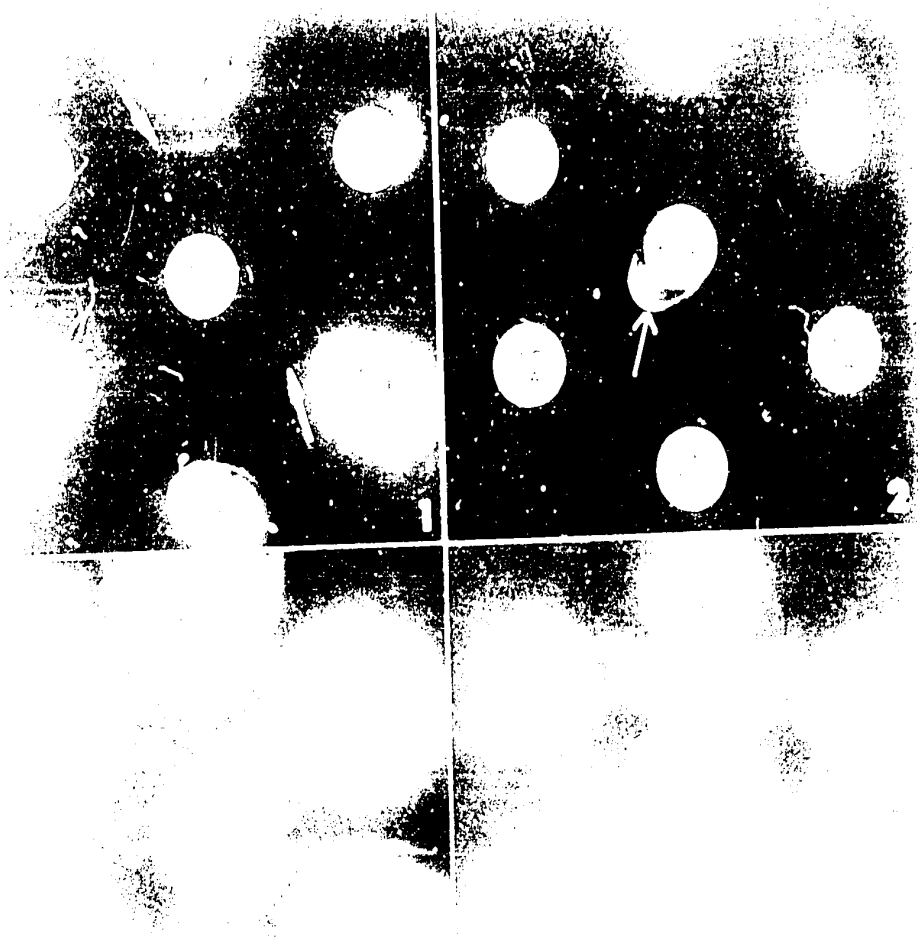
GPaS/SA - guinea pig anti PR8/SA serum
GPNS - guinea pig normal serum

Figure 1 - Reactions in agar showing faint zone of precipitation ($\rightarrow$) between PR8/SA and guinea pig antiserum. No reaction is evident with $V\text{-}C_2$.

Figure 2 - Reactions in agar showing precipitin reactions between $V\text{-}C_2$ and both immune (GPaS) and normal (GPNS) guinea pig sera. The precipitin line indicated ($\longrightarrow$) has formed in the area where a slight leak occurred between the agar and template interface.

Figures 3 & 4 - Reactions with normal and immune sera in cellulose acetate showing intense precipitin reactions with virus concentrate ($V\text{-}C_2$) a faint reaction with PR8/SA. There is no reaction evident between virus concentrate ($V\text{-}C_2$) and the normal guinea pig serum (GPNS).


PRB/SA
V-C2
GPaS SA
GP NS
1
2
3
4


1
2

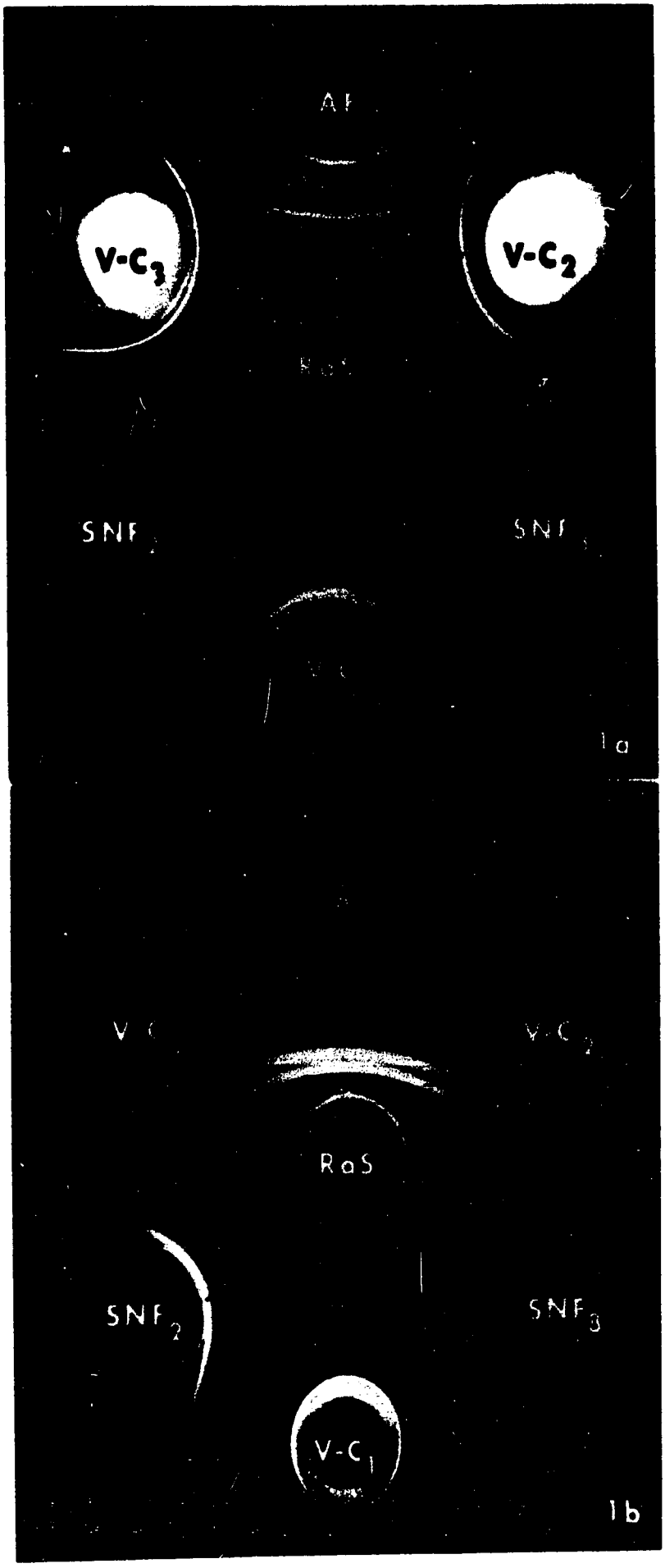
detectable by immunodiffusion. Therefore, a third differential centrifugation cycle was carried out immediately before testing by immunodiffusion in cellulose acetate. Because the purification and test procedures required several days for their completion, some of the samples had to be again stored at -80°C. The number of freeze-thaw cycles to which each preparation had been subjected at the time of the tests in cellulose acetate and in agar was therefore recorded.

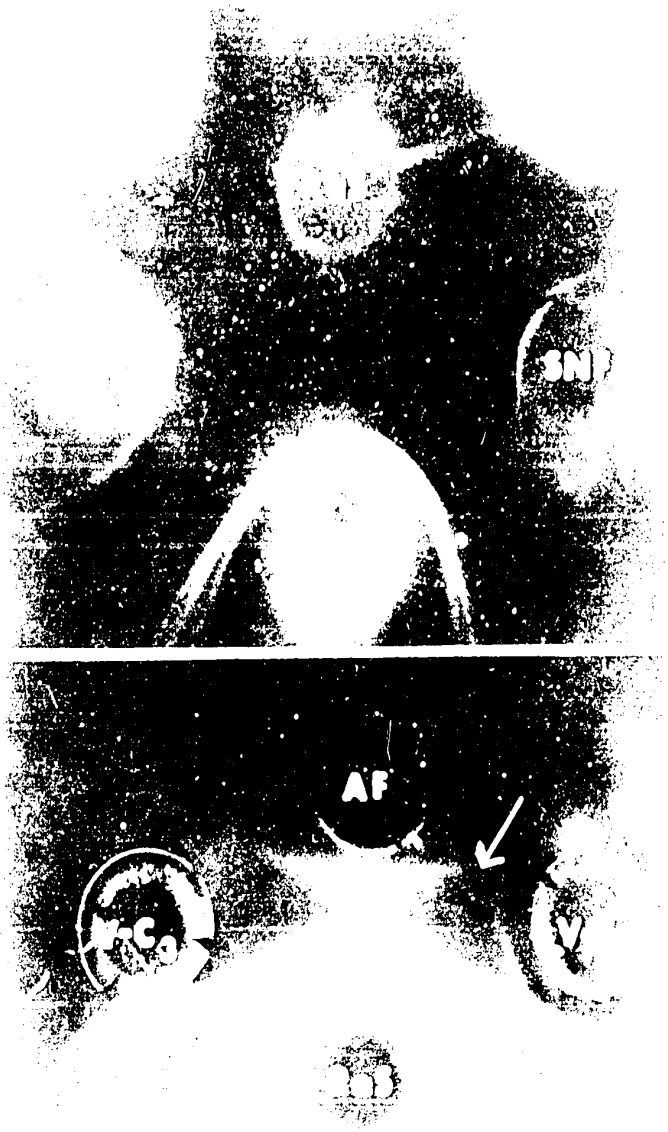
A fresh batch of virus concentrate V-C_2, prepared according to the standard procedure, was thawed and centrifuged a third time at 35,000 g. for 90 minutes. The supernatant fluid (SNF_3) was decanted and replaced with an equal volume of sterile physiological saline into which the virus pellet was resuspended (V-C_3). These preparations were tested immediately by immunodiffusion in cellulose acetate against antiserum from parenterally immunized rabbits (RaS) but the duplicate test in agar had to be performed at a later date.

In cellulose acetate (Plate 9, figure 1a) there are strong reactions associated with the three virus concentrate fractions, but in agar (figure 1b) there is no reaction associated with the final virus concentrate V-C_3, and only faint reactions with the other two fractions, V-C_1

PLATE 9

Figure 1a and 1b - Comparative immunodiffusion reactions in cellulose acetate (a) and agar (b), demonstrating the diffusion of virus particles in cellulose acetate only. Host antigens present in the allantoic fluid (AF) react strongly with the rabbit pan-specific serum (RaS). The presence of host antigen in V-C_2 (figure b) is shown by the reaction of identity (arrowed). No host antigens are detectable in V-C_3 or SNF_3 in agar.


V-C3
V-C2
SNF2
SNF3
1a
V-C2
RaS
SNF2
SNF3
V-C1
1b


3N
AF
TC
V

and V-C_2. The reaction with SNF_3 is negligible. The absence of reactions in agar associated with the V-C_3 sample was expected since influenza virus particles are over the critical size (70 mμ) for diffusion in this medium. By contrast the strong reactions given by V-C_3, as well as by V-C_1 and V-C_2 in cellulose acetate shows conclusively that the virus particle does diffuse in the membrane.

The degree of purification of the virus preparations is indicated in both the cellulose acetate and agar reactions (figures 1a & 1b) by the decreasing intensity of the lines produced by the precipitation of the egg antigens present in the allantoic fluid (AF), and in the supernatant fluids (SNF_2 and SNF_3). In the agar reaction (figure 1b), the occurrence of precipitin lines with the V-C_1 and V-C_2 fractions was due to the breakdown of virus by repeated freezing and thawing. These samples had been subjected to three freeze-thaw cycles. However, in the case of V-C_3, which had been through only one freeze-thaw cycle, no detectable breakdown had apparently occurred, as shown by the absence of precipitin line formation.

The effect of the combination of agar gel and cellulose acetate as a matrix for immunodiffusion was investigated in order to:

1. confirm the inability of virus particles to diffuse in 1% agar gel,
2. determine the concentration of agar gel which would permit diffusion of virus particles, and
3. investigate the possibility that agar might provide a stabilizing effect on the free flowing systems of diffusion in cellulose acetate membranes.

To prepare the combination matrix, the cellulose acetate was soaked by floating on the appropriate dilution of melted agar contained in a Petri dish on a warming tray at 45°C. The templates were mounted in the manner already described, and clips were used to hold the templates in place, with the exception of the plain agar reaction chambers.

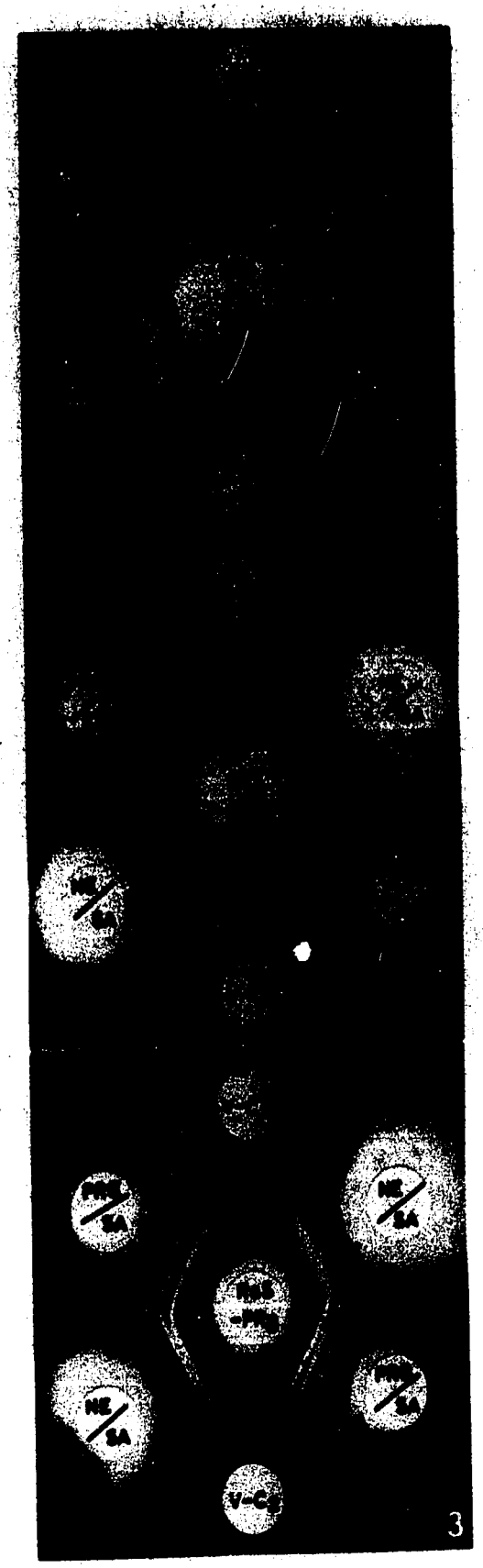
The reactions of virus concentrate ($V\text{-}C_2$) infected soluble antigen (PR8/SA) and normal membrane extracts (NE/SA) against pan-specific antiserum (RaS) were compared under these conditions.

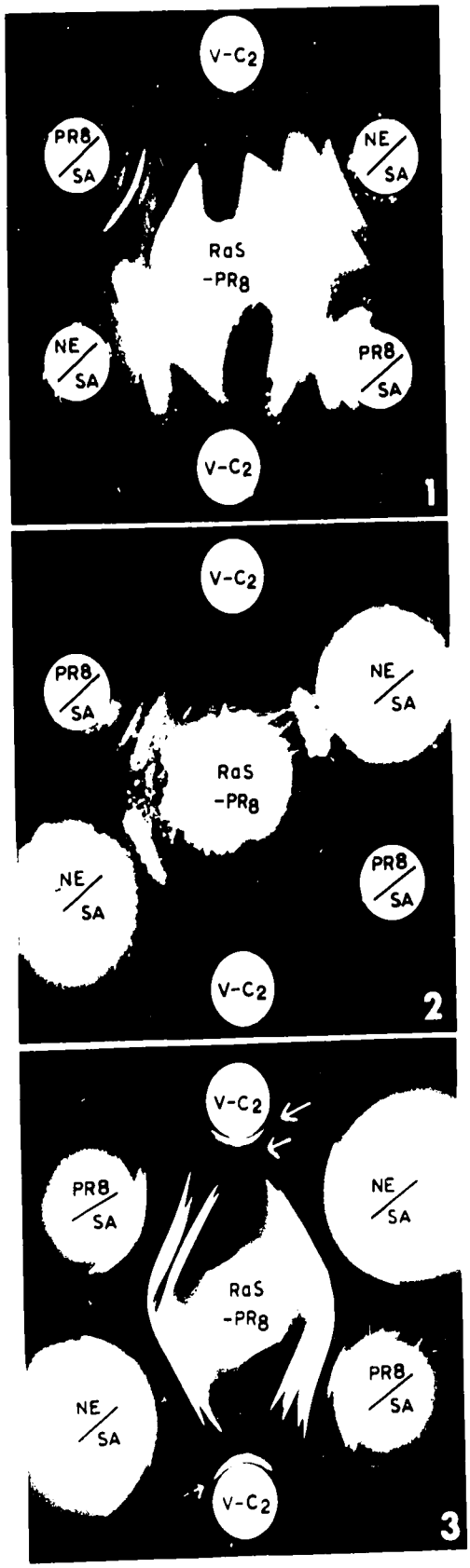
In Plate 10 the results of direct comparison of the reactions in agar and in cellulose acetate alone

PLATE 10

Figures 1, 2 & 3 - Comparison of a standard immunodiffusion reaction in 1% agarose (figure 1), 1% agarose + cellulose acetate (figure 2) and in cellulose acetate (figure 3). Precipitin reactions with V-C_2 have only occurred in cellulose acetate.

RaS/PR8	-	rabbit(anti-PR8 soluble antigen) antiserum.
V-C_2	-	virus concentrate
PR8/SA - PR8		soluble antigen
NE/SA	-	normal host membrane extract.


3


V-C2
PR8/SA
NE/SA
RaS-PR8
NE/SA
PR8/SA
V-C2
1
V-C2
PR8/SA
NE/SA
RaS-PR8
NE/SA
PR8/SA
V-C2
2
V-C2
PR8/SA
NE/SA
RaS-PR8
NE/SA
PR8/SA
V-C2
3

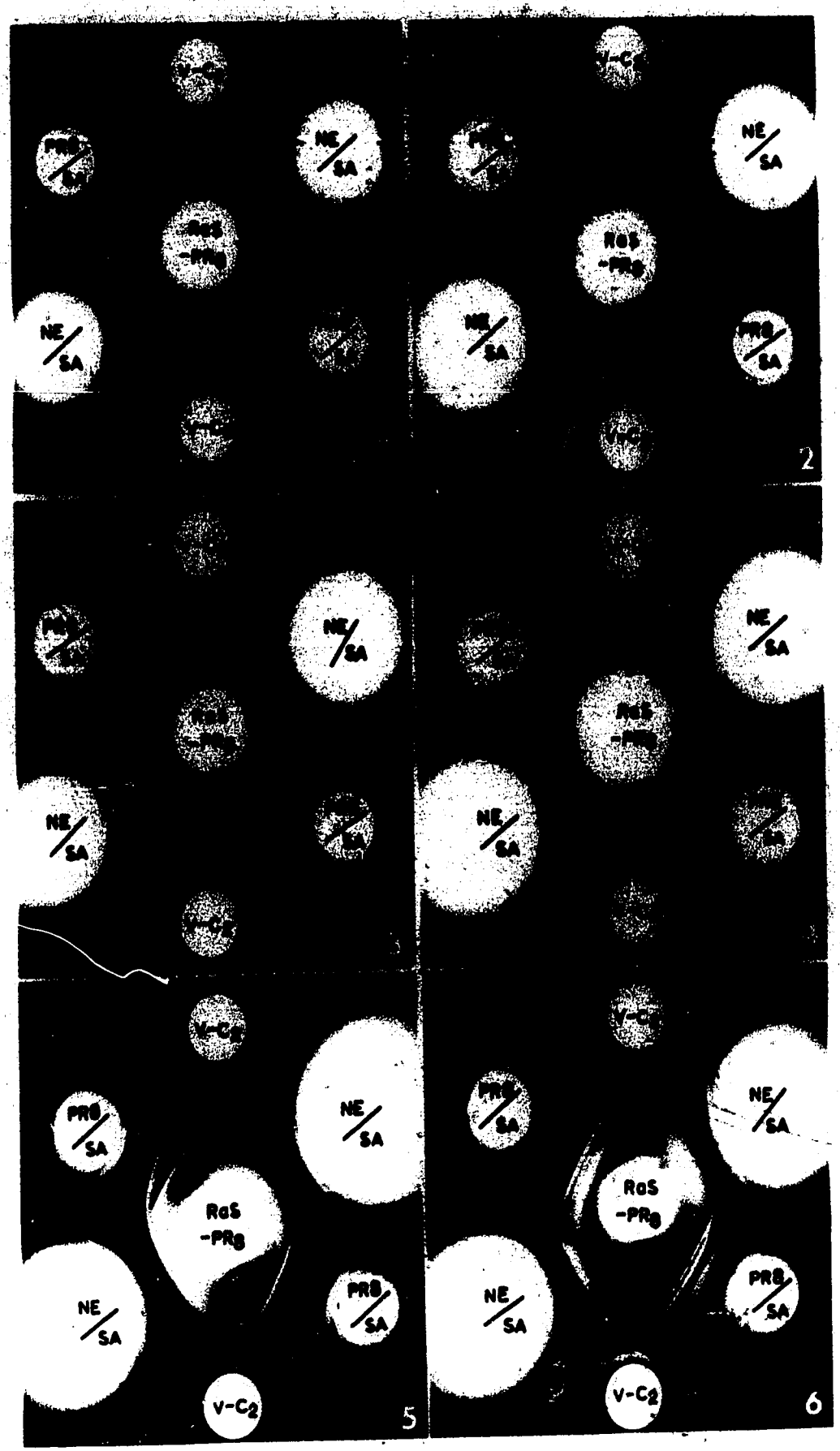
(figures 1 & 3) and in combination (figure 2) are shown. The reaction occurring in cellulose acetate membrane offers the best detail and resolution necessary for a detailed analysis. There are no reactions with virus antigen (V-C) in the presence of 1% agar (figs. 1 & 2) and the precipitin line resulting from virus-antibody reaction, though weak, is readily visible in cellulose acetate (fig. 3). It is apparent that the many reacting components of the soluble antigens and their reactions of partial and complete identity with NESA are more clearly resolved by the cellulose acetate reaction. The combination of agar and cellulose acetate (fig. 2) appeared to be quite unsuccessful. Possibly the gel interacts with the cellulose acetate in such a way as to effectively fill the membrane pores, while the membrane matrix reduces the porosity of the gel. Also, since clips were used, this blocking effect would be augmented by the added pressure.

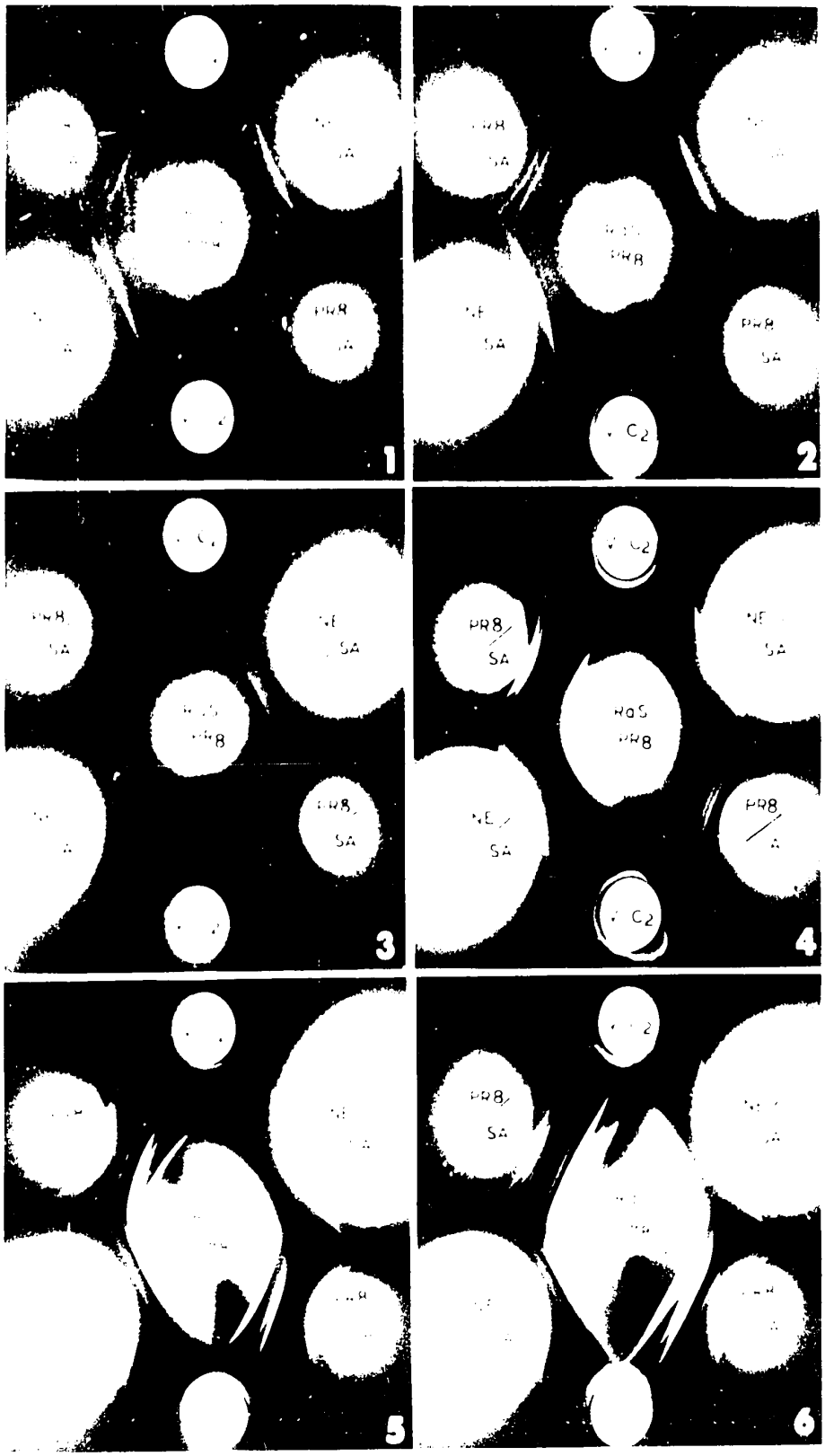
The reactions presented in the Plate 11 demonstrate the effect of decreasing concentrations of agar in the combination matrix. The reactions of the normal and infected soluble antigens increased in resolution and complexity as the concentration of agar decreased. At agar concentrations of 0.8% (fig.1) there were no virus precipitin lines, however such a reaction was present at an agar

PLATE 11

Immunodiffusion Reactions demonstrating the Concentration of agarose which permits diffusion of Influenza Virus particles.

Figure 1 - 6, the comparison of a standard reacting system diffusing in cellulose acetate combined with decreasing concentrations of agarose in TRIS buffer (pH7.4): 0.8% (figure 1), 0.6% (figure 2) 0.4% (figure 3), 0.2% (figure 4), 0.1% (figure 5) and 0.05% (figure 6). It can be seen that the V-C_2 reaction is detected at concentrations of 0.6% and below. In addition the number of reacting components detected in both soluble extracts (PR8/SA & NE/SA) increases with decreasing concentrations of agarose.


NE/SA
RaS-PRg
PRB/SA
V-C2
2
5
6


PR8
SA
1
PR8
SA
2
PR8
SA
3
PR8/
SA
4
PR8/
SA
5
PR8/
SA
6

concentration of 0.6% (figure 2). The limiting concentration of agar for the diffusion of influenza virus particles was too low to permit solid gel formation, thus precluding the use of agar in the immunodiffusion studies of influenza A/PR8 virus particles.

Reaction of virus concentrate with serum from normal rabbits.

During the preliminary tests on the rabbit antiserum to normal embryo(NE) and PR8-infected embryo extracts (SA), control immunodiffusion reactions were set up using normal rabbit sera obtained prior to immunization. The results of one of these control immunodiffusion reactions is presented in Plate 12 (figure 1a). Distinct precipitin lines formed by a reaction between the rabbit serum (NRS) and the virus concentrate (V-C_2) can be clearly seen, however, there is no reaction between the rabbit serum and either the normal chick embryo extract (NE) or the soluble antigen preparation (SA).

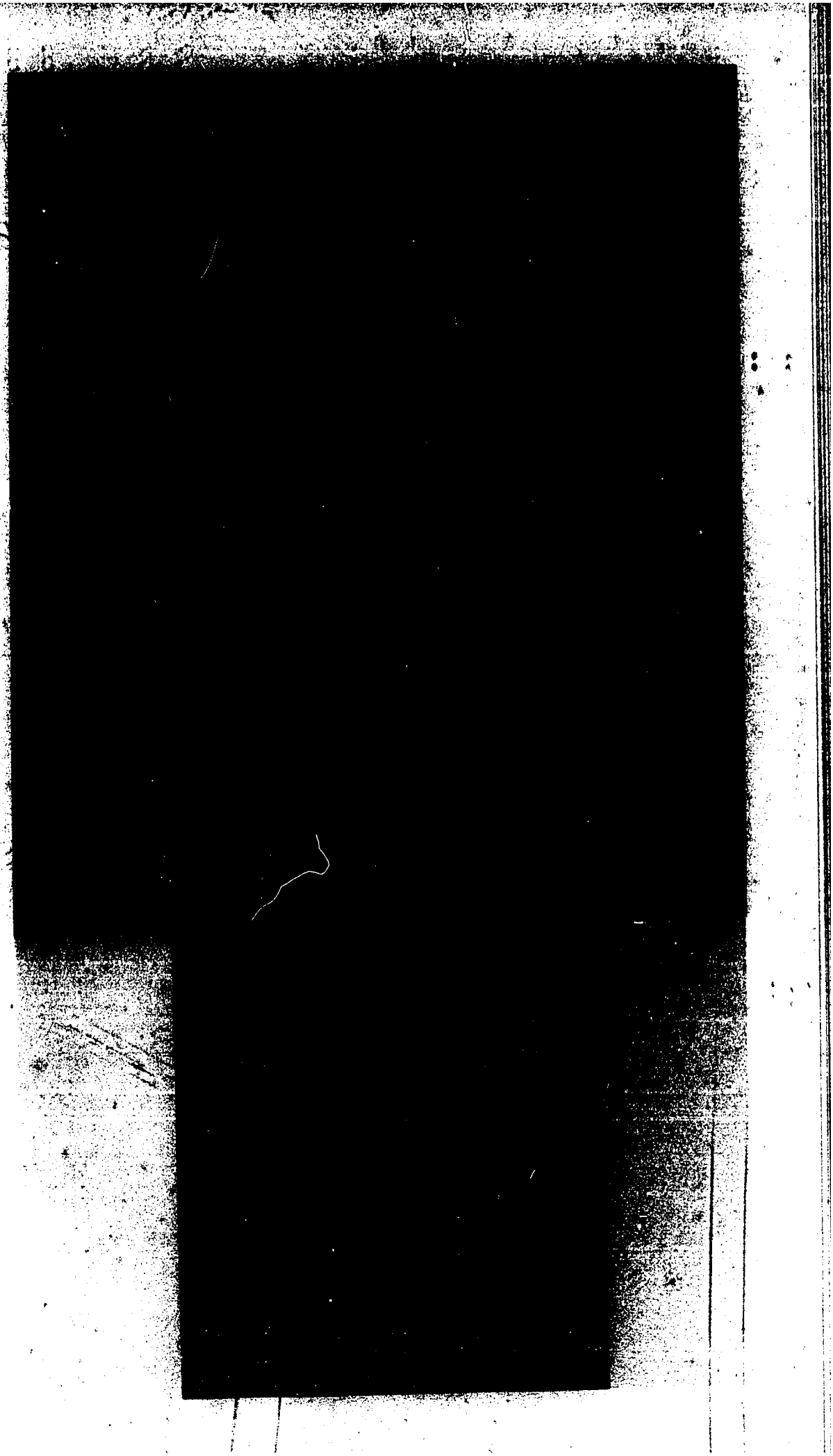
Furthermore, it was found (figure 2b) that the precipitin reaction was specific for the viral particles present in the concentrate and was not due to the normal sedimentable material prepared from uninfected allantoic fluid (C_2N). Comparisons using agar as diffusing medium

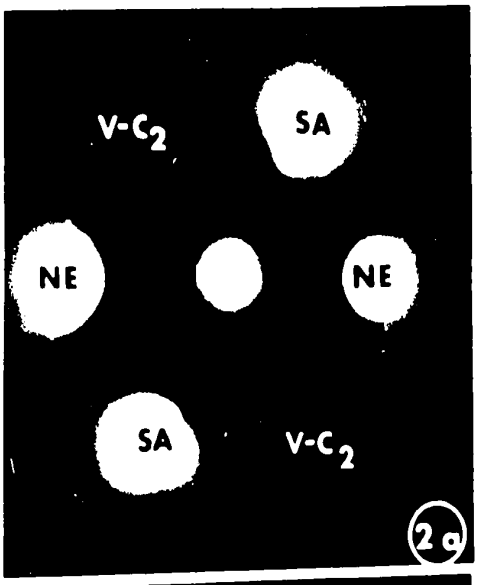
PLATE 12

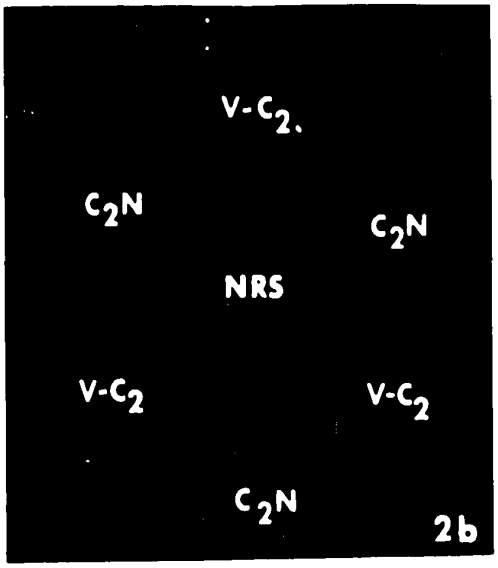
Figure 2a and 2b - Immunodiffusion precipitin reactions in cellulose acetate between "normal" rabbit sera (NRS) and influenza A/PR8 concentrate ($\underline{V\text{-}C_2}$) showing a, the absence of reaction with normal chick embryo extract (NE) or soluble antigen (SA), b, the absence of reaction with a preparation of normal sedimentable material ($\underline{C_2N}$).

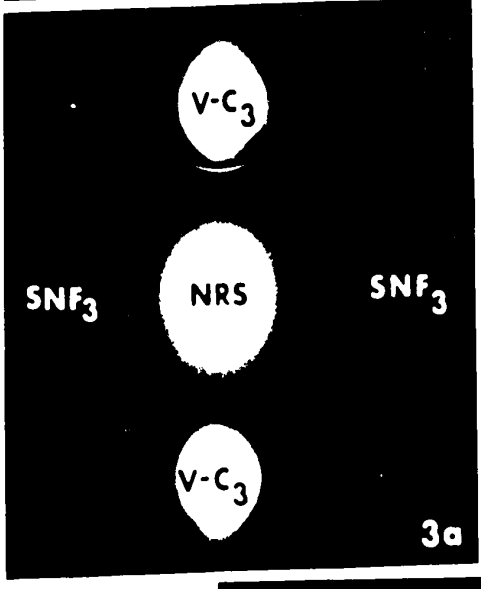
Figure 3a and 3b - Comparative immunodiffusion reactions in cellulose acetate (a) and agar (b) demonstrating the presence of a reaction between "normal" rabbit serum (NRS) and PR8 virus concentrate ($\underline{V\text{-}C_3}$) in cellulose acetate only.

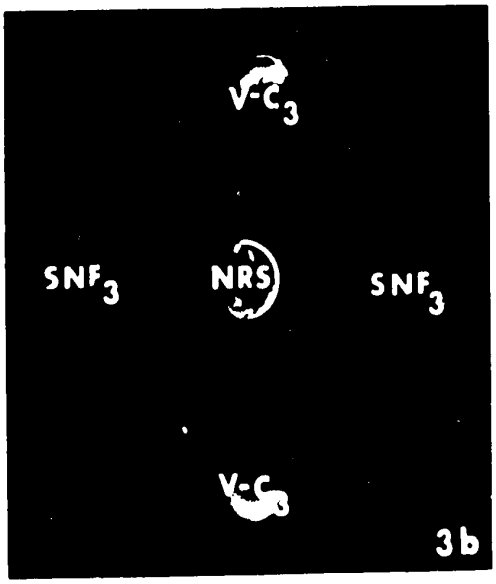
Figure 4 - Immunodiffusion reaction in cellulose acetate between post-infection rabbit serum (RaS/IN) and sequential virus preparations.

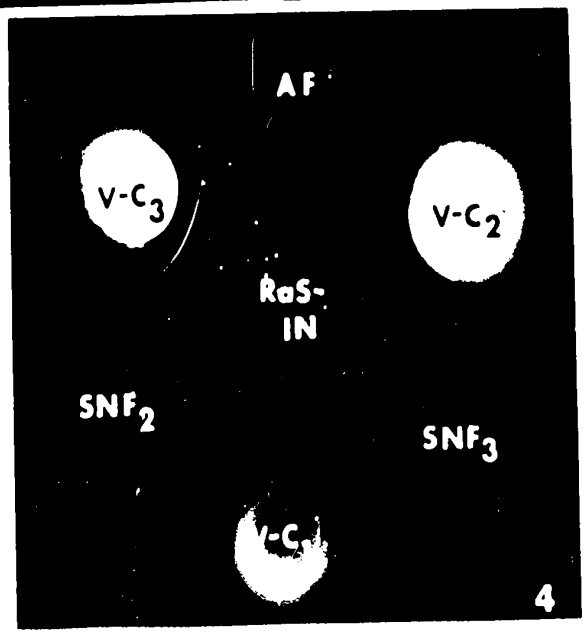



V-C2
SA
NE
NE
SA
V-C2
2a


V-C2
C2N
C2N
NRS
V-C2
V-C2
C2N
2b


V-C3
SNF3
NRS
SNF3
V-C3
3a


V-C3
SNF3
NRS
SNF3
V-C3
3b


AF
V-C3
V-C2
RaS-
IN
SNF2
SNF3
4

(figures 3a & 3b) demonstrated that the reaction was associated with the intact virus particle only since it did not occur in agar. As a result of these observations, further investigations were required to determine whether the reaction could be due to specific antibody present in the "normal" serum, or if it was due to the presence of influenza virus haemagglutination inhibitors.

Susceptibility of rabbits to infection with influenza A/PR8 virus.

In order to determine whether the reaction could be attributed to the presence of antibody (or a non-specific inhibitor) an attempt was made to demonstrate whether rabbits were in fact, susceptible to intranasal infection with influenza A/PR8 virus. Susceptibility could be indicated by the appearance of specific antibodies, Lief et al (1958), which could then be compared with the normal component by means of the immunodiffusion test.

Rabbits were exposed intranasally to influenza A/PR8 virus, as described in the previous section. The results of haemagglutination inhibition titrations on both heat-inactivated and periodate-treated NRS, normal and convalescent sera are shown in Table 8. No change in the NRS HAI titre occurred following periodate treatment, which

is recommended for the removal of most non-specific haemagglutination inhibitors (Cohen et al, 1963). In view of the precipitin lines evident in the immunodiffusion reaction, the titre of 8, though very low, was regarded as significant. The drop in titre of convalescent serum was probably caused by the periodate treatment, since such an effect has been reported by Harboe and Reenaas (1959), Geft & Polyak (1963), Cohen and Dorman (1965) and Andersen, Abele and Vannier (1966).

The development of antibodies to the influenza virus particle (V antigen) was shown by the presence of two precipitin lines in the immunodiffusion reaction pattern in cellulose acetate (Plate 12, figure 4). In control tests there were no reactions detectable between the post-infection antiserum and uninfected chick embryo preparations.

The development of antibodies directed against the soluble antigen components of the virus particle (S-antigen) was indicated by the precipitin lines numbered 1 and 3, which developed between the intranasally immunized rabbit serum (RaS/IN) and only the soluble antigen preparations (SA_1 & SA_2), (Plate 13, figure 5).

Table 8

Haemagglutination-inhibition of Influenza A/PR8 Virus with Normal and Immune Rabbit Sera.

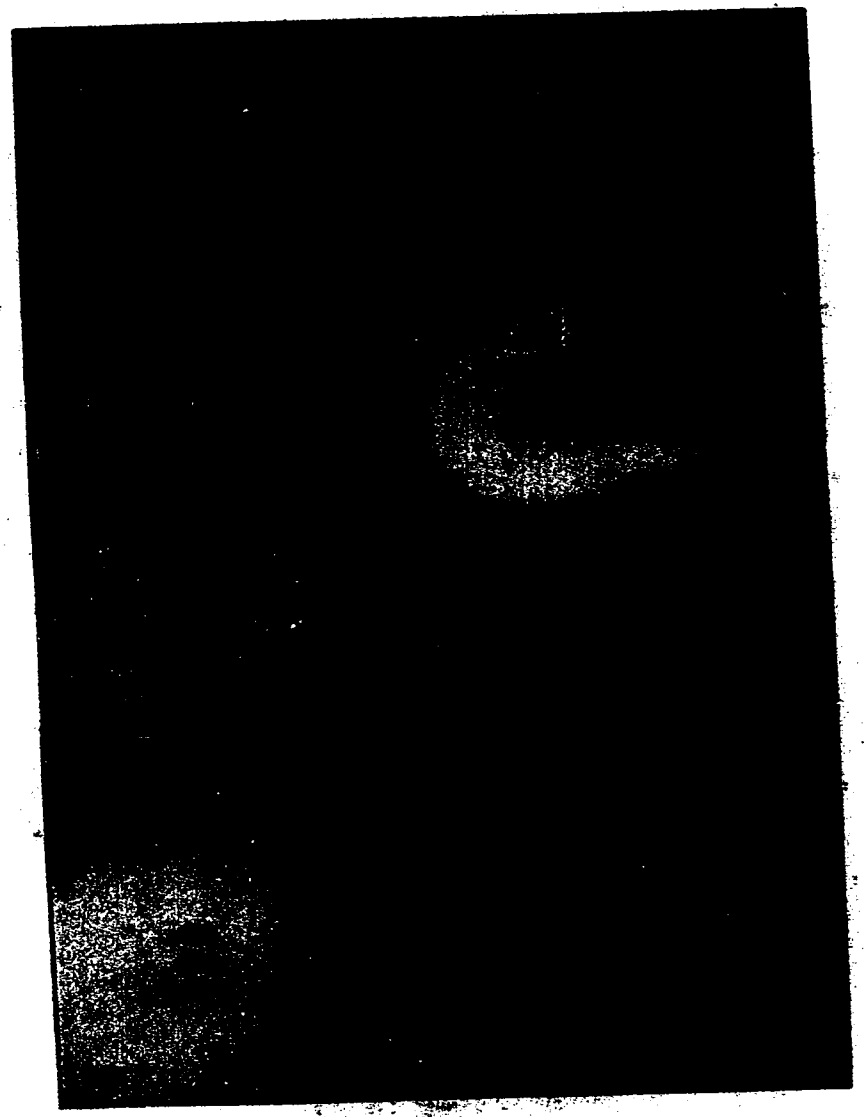
	HAI Titre*	
	Serum heated 56° for 30 min.	Serum treated with periodate
"Normal" Rabbit serum (NRS)	8	8
RS (Control)	0	0
RaS/IN (8 weeks)	512	128
RaS/IN (16 weeks)	256	128
RaS/IN (32 weeks)	256	128

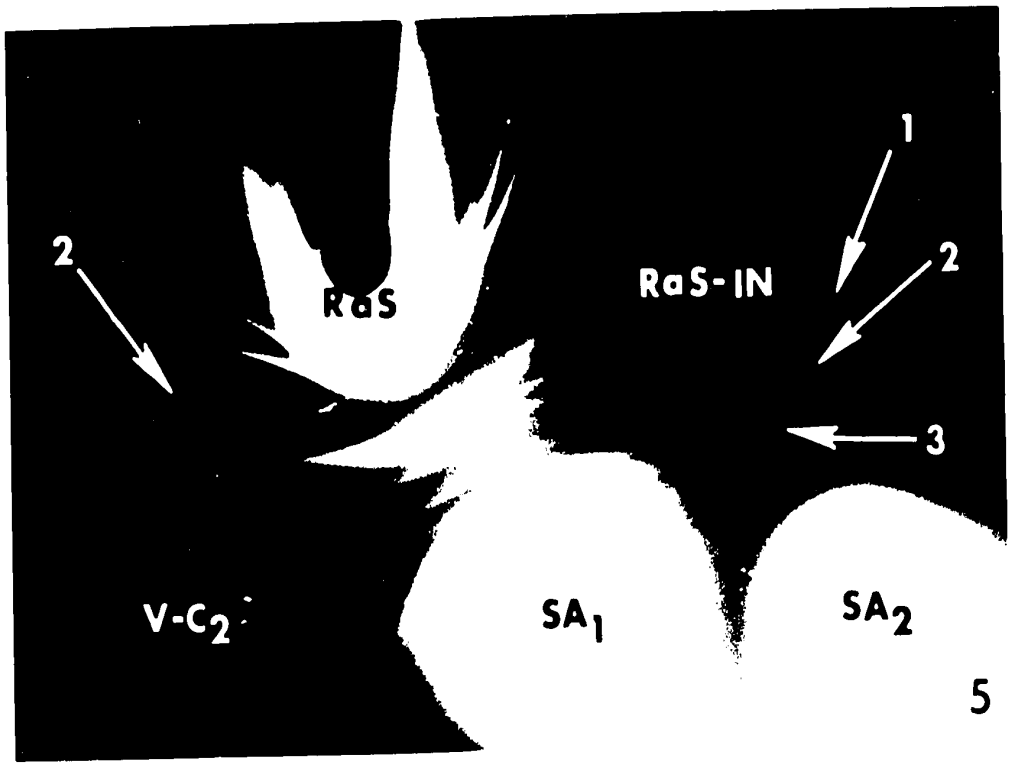
*Titres are expressed as the reciprocal of the last serum dilution showing inhibition of haemagglutination.

PLATE 13

Figure 5 - Immunodiffusion reactions in cellulose acetate comparing the precipitin reactions of serum from parentally immunized (RaS) and intranasally infected (RaS/IN) rabbits developed by both virus($V\text{-}C_2$) and soluble antigen (SA_1 & A_2) preparations.

Reactions of identity are demonstrated by fusion of precipitin lines (1, 2 & 3). The reactions of identity between both sera and both soluble antigen preparations (lines 1 & 3) indicate the presence, in both antisera, of antibody to soluble antigen. The reaction of identity (2) demonstrates the presence of viral antibody in both antisera.





5

The relationship between the reacting components in post-infection sera (RaS/IN) and the sera of parenterally immunized rabbits (RaS) was investigated, and is demonstrated by comparing their reactions with viral (V) and soluble (SA) antigens, Plate 13 (figure 5). There are three main precipitating components (arrowed) present in the post-intranasal serum (RaS/IN). These components (1, 2 and 3) show reactions of identity with those precipitating antibodies produced by parenteral immunization (RaS). One of these components (2) reacts with both the viral ($V\text{-}C_2$) and soluble (SA_1 and SA_2) antigen preparations, and is therefore identified as being viral (V) antibody. Since, as previously stated, there is no reaction between RaS/IN and normal egg preparations, and the reacting components (1 and 3), shared by the two different antisera react only with components in the soluble (SA) antigen preparations, these are identified as being S-antibodies.

Lief et al (1958) reported that the presence of S-antibodies in convalescent serum was the result of active influenza infection. It was also shown that S-antibodies were not produced following intranasal immunization with either ultraviolet-inactivated influenza virus or with normal allantoic fluid. Therefore, it was concluded that rabbits were susceptible to intranasal

infection with influenza A/PR8 virus, even though there may be no evident clinical symptoms of disease.

Immunodiffusion analysis of normal and immune sera.

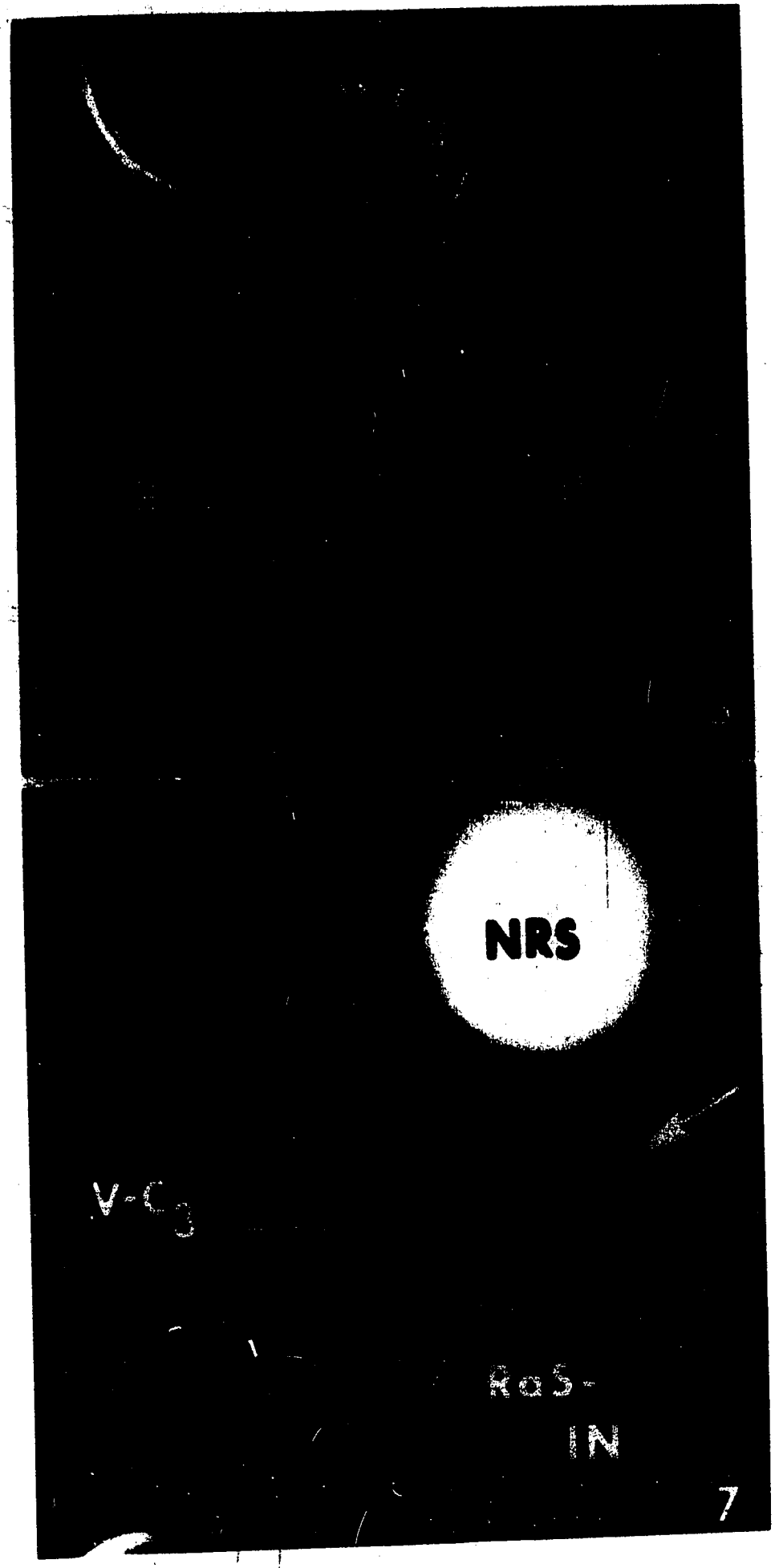
In order to demonstrate the identity of the precipitins in the normal reacting serum, several tests were necessary. A comparison between the reacting components of the parenterally immunized antisera (RaS) and the normal reacting serum (NRS) was made using virus antigen V-C_2, and the reaction is shown in Plate 14 (fig.6). Several precipitin lines can be seen between each antiserum and the virus antigen. Three of these precipitin lines (arrowed) are shared by both antisera and are interpreted as 'reactions of identity' between components in the "normal" reacting serum (NRS) and the specific influenza A/PR8 antisera (RaS), which indicate the presence of identical antibodies in both (Crowle, 1961).

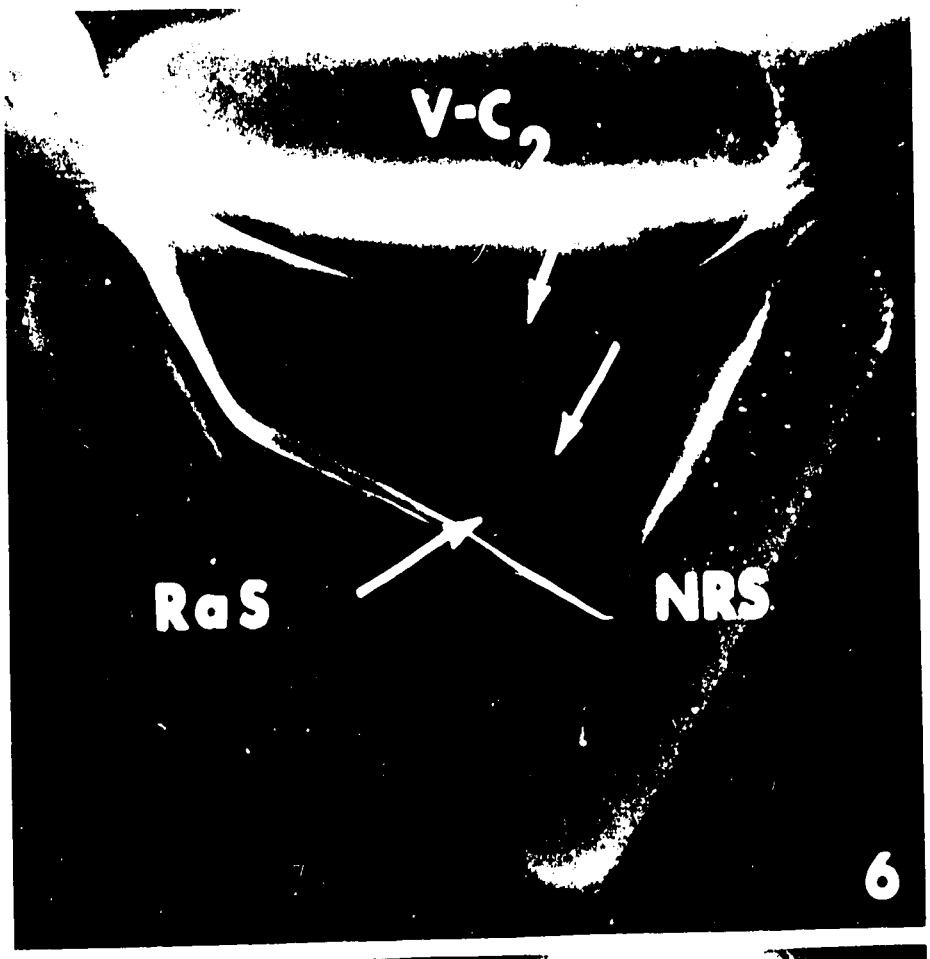
The reacting components in NRS and the post-infection antisera (RaS/IN) were then compared using the virus antigen preparations V-C_3. The resulting reaction of identity is presented in Plate 14 (figure 7). The linkage between the precipitin lines (arrowed) demonstrates the identity of the normal reacting component with that precipitating component present in the serum, as a result

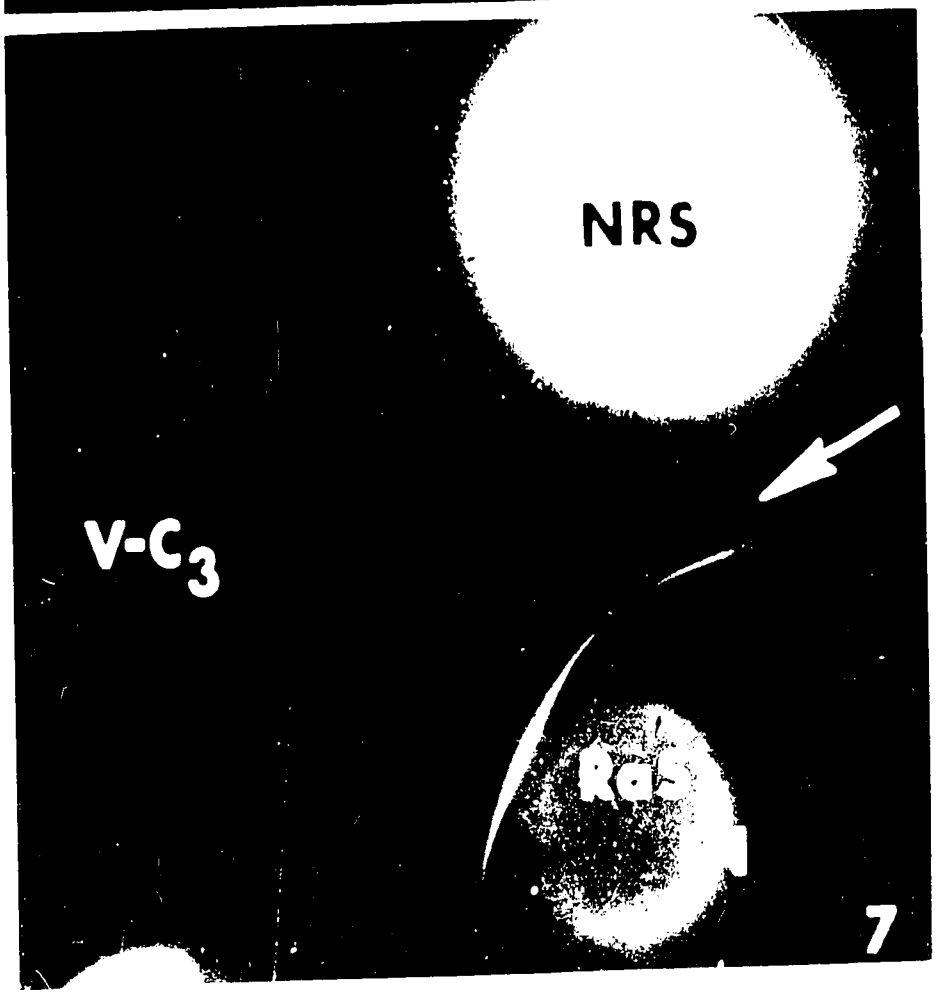
PLATE 14

Figure 6 - Immunodiffusion reaction in cellulose acetate comparing the reactions between virus antigen ($\underline{V\text{-}C_2}$) and both "normal" rabbit serum (NRS) and specific antiserum from parenterally immunized rabbits (RaS). The reactions of identity are indicated by arrows.

Figure 7 - Immunodiffusion reaction in cellulose acetate comparing the reactions of virus antigen ($\underline{V\text{-}C_3}$) with both antiserum produced by intranasal infection (RaS/IN) and normal rabbit serum (NRS). The reaction of identity is indicated by an arrow.


NRS
V-C3
Ra5-
IN
7


V-C2
RaS
NRS
6


NRS
V-C3
Ra
7

of intranasal infection.

In conclusion, it has been demonstrated by immunodiffusion reactions that rabbits are susceptible to induced influenza A infection since such infection gives rise to specific serum antibodies, which are identical to some of the specific antibodies produced after a course of parenteral immunization with influenza A/PR8 preparations. Further analysis has shown that in both cases these specific antibodies showed reactions of identity with the reacting components present in the serum of normal rabbits. Thus, the occurrence of natural influenza A/PR8, or closely related influenza A strain infections amongst rabbit populations, should be considered as a logical explanation for the presence of the normal serum reacting component.

Species distribution of Influenza PR8 antibodies in the serum of normal animals.

A limited survey of different normal animal sera, which were readily available, was undertaken in order to evaluate these conclusions. The presence of antiviral activity was determined by the immunodiffusion reaction in cellulose acetate using semi-purified virus concentrate V-C_2 as the antigen. The results are tabulated in Table 9.

This was a preliminary screening and no attempt was made to correlate the results of the immunodiffusion studies with the presence or absence of non-specific haemagglutination inhibitor, nor was an attempt made to analyze the reacting components with respect to the original normal serum, NRS.

These results have formed the basis of a new enquiry into the epidemiology of myxovirus infections, which is presently being investigated by a colleague in this department.

Table 9

Reactions of animal sera tested by the immunodiffusion technique against PR8 virus concentrate.

Animal	Total tested	Virus specific reactivity	
		No. positive	No. negative
Rabbit	30	6	24
Guinea pig	7	2	5
Goat	1	1	-
Sheep	1	1	-
Rooster	5	2	3

Characterization of the Influenza Virus Precipitins Present in the Normal Serum of Rabbits.

It was established that a precipitating component in the "normal" rabbit serum (NRS) showed a reaction of identity with specific precipitins produced in rabbits both by induced infection and by parenteral immunization. The presence of such viral precipitins could have important implications with respect to the epidemiology of human influenza. Whether these precipitins were in fact immunoglobulins or not was open to question because there have been reports that non-specific haemagglutination inhibitors might be produced as a result of influenza infection (Cohen and Dorman, 1965, Geft and Polyak, 1963). Since certain of these non-specific inhibitors were reported to be capable of flocculating influenza virus particles (Belyavin, 1957, Biddle and Stevenson, 1966) and since Styk and Hana (1966) reported the formation of two precipitin lines in an agar micro-immunodiffusion reaction between a beta inhibitor preparation and ether-treated influenza A1 virus, it was necessary to identify the reacting component as either a non-specific inhibitor of the type classified by Cohen et al, 1963, or an immunoglobulin.

Characterization of influenza virus precipitins obtained from a new stock of rabbits.

The studies to this point had been made on normal serum prebleed samples from rabbits, which had subsequently been immunized. Only a few ml. remained, which were kept for standard reference. Therefore, a new source of "normal" rabbit serum containing the precipitating component was required. Consequently, the preliminary studies were concerned with (1) screening sera from new rabbits for the presence of influenza virus precipitins, and (2) identifying detectable precipitins with those present in the original NRS.

Figure 1, in Plate 15, shows the results of several immunodiffusion reactions demonstrating the presence of precipitins in normal rabbit sera. On the basis of these reactions, we selected several sera for further analysis (Serum 503 (figure 1) was one of those chosen).

The identity of the normal serum precipitin with a precipitating component present in specific influenza A/PR8 rabbit antiserum is demonstrated by the reaction shown in Plate 15, figure 2. The linkage between the normal serum reaction and a specific precipitating component in the antiserum is clear. The antigen used in this reaction was a fully infective V-C_2 preparation.

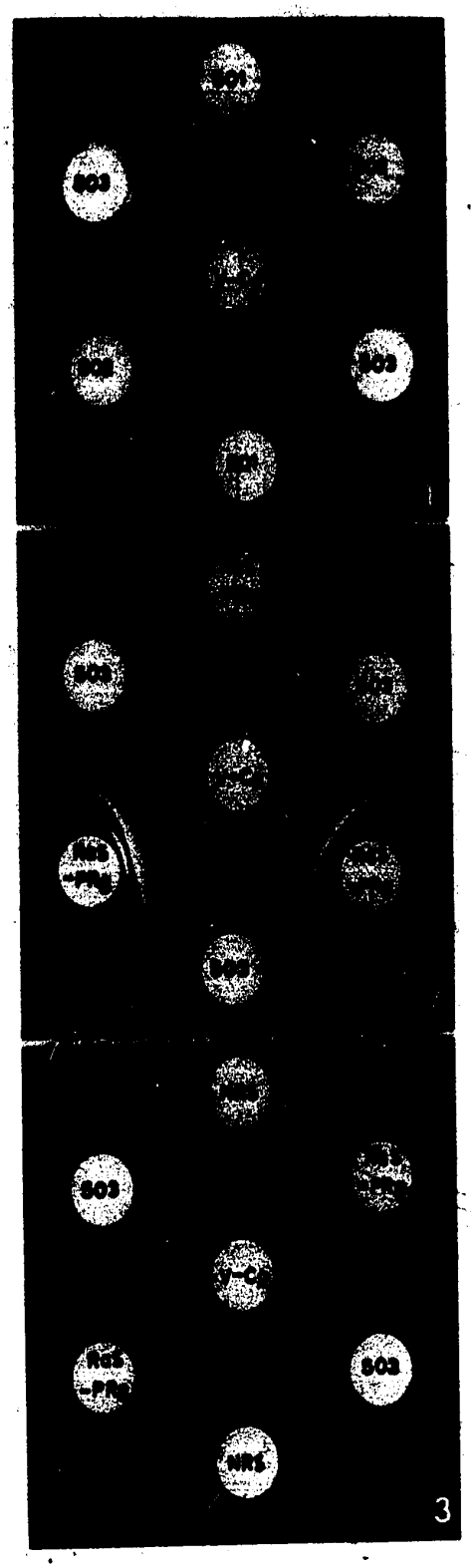
PLATE 15

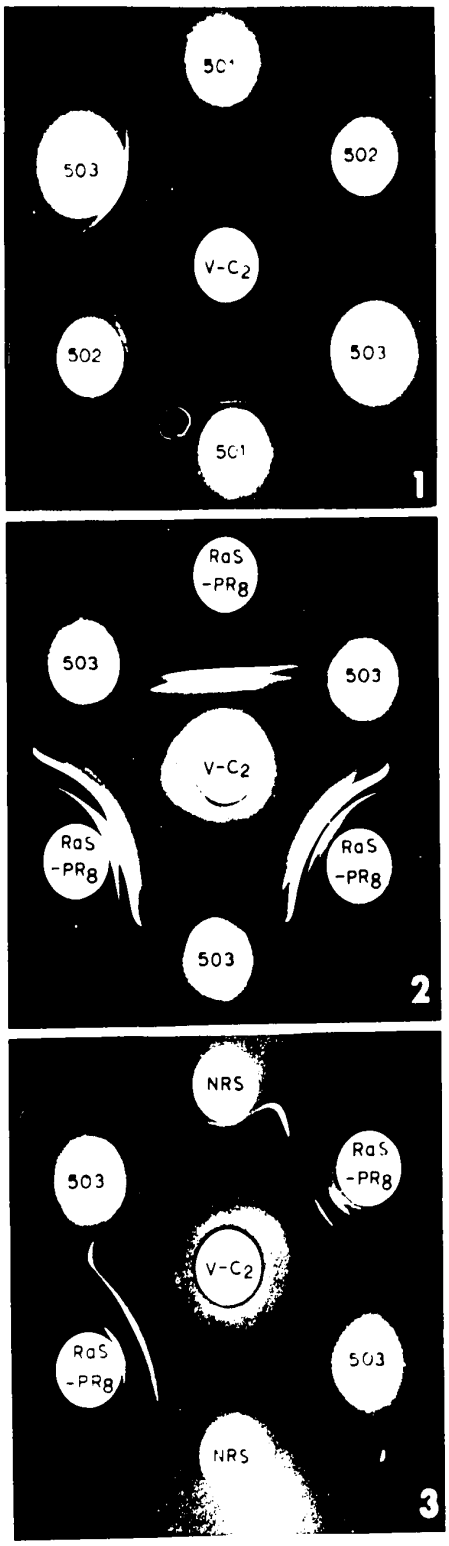
Identification of the normal Reacting Component in selected 'normal' rabbit sera.

Figure 1 - Immunodiffusion reactions of three 'normal' sera (50', 502 & 503) with virus concentrate ($V\text{-}C_2$) indicating precipitating component.

Figure 2 - Identification of the precipitating component in 'normal' rabbit serum (503) with antibodies present in rabbit anti-PR8 serum. Reactions of identity can be clearly seen.

Figure 3 - Identification of the NRS 'normal' antibody with the precipitating component of the 'normal' rabbit serum (503) by means of reaction of identity with rabbit anti-PR8 serum (RaS/PR8).


3


501
503
502
V-C2
502
503
501
1
RaS
-PR8
503
503
V-C2
RaS
-PR8
RaS
-PR8
503
2
NRS
503
RaS
-PR8
V-C2
RaS
-PR8
503
NRS
3

When an attempt was made to identify these precipitins with those present in the original NRS, a new problem was encountered. It was found that the NRS precipitating reactions, both of the original and the new sera were not strong enough to fuse across to form either linkages or intersections (reaction of non-identity). Since these studies were concerned with the identification of the precipitins in the new sera, no attempts were made to concentrate and possibly alter by the concentration procedure, the components detectable in whole serum. Therefore, an indirect immunodiffusion reaction was used to identify weakly reacting components in the different sera. A strongly reacting antiserum, known to contain the same precipitating components as are present in one of the weakly reacting sera, was used to form a reacting bridge between the new precipitating reaction and the original. If the same precipitins are present in the test reaction, the precipitin line will link between the three reactions. This type of reaction was found to be of value throughout the study of weak reactions, and was termed the "indirect" reaction of identity. Figure 3 in Plate 15, illustrates the indirect reaction of identity obtained between the original NRS and the serum 503. On the basis of this type of reaction, the presence of the NRS component was identified in

three out of the six rabbit sera originally screened.

An adequate supply of "normal" serum had been obtained from each of these animals during the first week of their arrival, and was stored separately at -20°C. Subsequent bleeds from these animals were tested routinely to determine whether an increase in the precipitating component could be detected. Such an increase would most probably be due to the development of antibody as a result of cross-infection with influenza A/PR8, or related myxoviruses, because our rabbits were housed in an area where there were no precautions against cross-infection. Therefore, only the serum obtained from the positive rabbits within the first 10 days of arrival was used for the chemical identification of the active component.

Results of serum treatment for the removal of non-specific inhibitors of haemagglutination.

The normal serum of a variety of animals may contain non-specific inhibitors of haemagglutination, and the term non-specific inhibitor is employed to distinguish this type of haemagglutination inhibition from that of specific antibody. Three main classes of inhibitor, α, β and γ have been recognized and classified on the basis of their biological activity. Their characteristics have been summarized by Cohen et al (1963).

In order to determine whether the immunodiffusion precipitation reaction shown by the "normal" rabbit serum was due to non-specific components, possibly haemagglutination inhibitors, the influenza haemagglutination inhibition characteristics of the sera were investigated by comparing the effect of the different serum treatments recommended for the removal of these inhibitors, using the standard haemagglutination inhibition test. Treated sera were also tested in the immunodiffusion reaction to compare the effect of such serum treatments on the precipitation reaction with V-C antigen.

The methods of serum treatment included:- heating at 56°C. for 60 minutes, treatments with neuraminidase,

TABLE 10

The Effect of Serum Treatments
on Influenza Virus Haemagglutination Inhibition
by Normal Rabbit Serum

Serum Treatment	Indicator Virus	HAI titre** Rabbit Sera 503	505	506
Untreated serum	influenza virus (A/PR8)	512	512	512
	influenza virus (A/PR8) heat inactivated*	2048	2048	2048
Heat$_{56^{\circ}}$ for 60'	influenza virus (A/PR8) unheated	512	512	512
RDE		128	64	64
KIO_4		8	8	64
Trypsin		1024	512	512

*Influenza A/PR8 virus heated at 56° for 60'

**Titres are expressed as the reciprocal of the last serum dilution showing inhibition of haemagglution.

trypsin, and potassium periodate. Both live and heat-inactivated influenza A/PR8 virus was used as the indicator virus. The results are shown in Table 10.

There was no complete inactivation ~~of activity~~ as a result of these treatments. Since there have been several reports concerning the marked reduction of antibody titres as a result of periodate treatment (Harboe and Reenaas, 1959, Cohen and Dorman, 1965, and Andersen, Abele and Vannier, 1966), the reduction in the titre of the NRS sera by the periodate was inconclusive. In a survey of normal sera from a variety of animals, Ananthanarayan and Paniker (1960) found several inconsistencies in the effects of serum treatments, and in a number of instances complete inactivation of activity was not achieved. Their interpretation, however, did not give any consideration to the possible existence of natural antibody. The technique for the removal of inhibitors was considered to be faulty.

The presence of α-inhibitor in the normal serum is indicated by the increase in titre which is obtained when heat-inactivated indicator virus is used. But since the serum titre is unchanged by treatments known to inactive α-inhibitor, it cannot be the only active component present in the serum. The presence of β-inhibitor is not demonstrated because heat

inactivation and trypsinization failed to destroy the inhibition activity.

The results of these tests and their comparison with the characteristics of influenza virus haemagglutination inhibitors is summarized in Table 11. The results of "in ovo" virus neutralization titrations failed to demonstrate the presence of virus neutralizing inhibitor (γ) or antibody.

The effect of the different serum treatments on the immunodiffusion reaction is illustrated in Plate 16. The immunodiffusion precipitation titre of the normal rabbit serum against V-C_2 was 1:2 (figure 1). Since serum treatments resulted in diluted mixtures, the KIO_4-treated samples (1:4 dilution) could not be expected to react, however, the samples from the other procedures (RDE - 1:2, trypsin - 2:3) did react visibly with virus concentrate (figure 2).

The effect of potassium periodate on both NRS and immune serum reactions was tested and the resulting immunodiffusion pattern is presented in figure 3. There is clearly a very marked reduction in the intensity of the antigen-antibody reaction, suggesting the destruction of specific antibody activity by periodate treatment.

TABLE 11

Comparison of the Characteristics of the non-specific Haemagglutination Inhibitors of Influenza Virus with the Properties of Rabbit 'NRS'

Characteristic	Non-Specific Inhibitors			NRS	
	α	β	γ	HAI	Precipitation
Destruction by Heat	-	+	-	-	-
Inhibition of:					
Living Virus	-	+	+	+	+
Heated Virus (56°C x 30')	+	+	+	+	+
Inactivation by:					
RDE	+	-	-	-	-
Trypsin	+	+	±	-	-
Periodate	+	-	+	-	NR*

* Not recorded because serum dilution by the treatment was outside the range of sensitivity.

PLATE 16

Immunodiffusion reactions of 'normal' sera following treatments for the removal of non-specific inhibitors of haemagglutination by influenza virus.

Figure 1 - Precipitin reactions of normal serum (RS) dilutions (1/2, 1/3 & 1/4) showing a reaction at a 1/2 dilution and a faint reaction barely detectable at a dilution of 1/3.

Figure 2 - Immunodiffusion reactions of normal sera following different treatments.

RS-nT - untreated normal serum control, undiluted

RS-H - serum heated at $56^{o}C$ for 60 min., undiluted

RS-RDE - serum treated by receptor destroying enzyme, diluted 1/2 by treatment

RS-KIO_4 - serum treated by KIO_4, final dilution after treatment of 1/4

RST' - serum treated with trypsin, final dilution of 2/3.

Figure 3 - Comparison of the effect of KIO_4 on the precipitin reactions of NRS and immune serum (RaS). NRS' and RaS' are KIO_4-treated sera at final dilutions of 1/4.

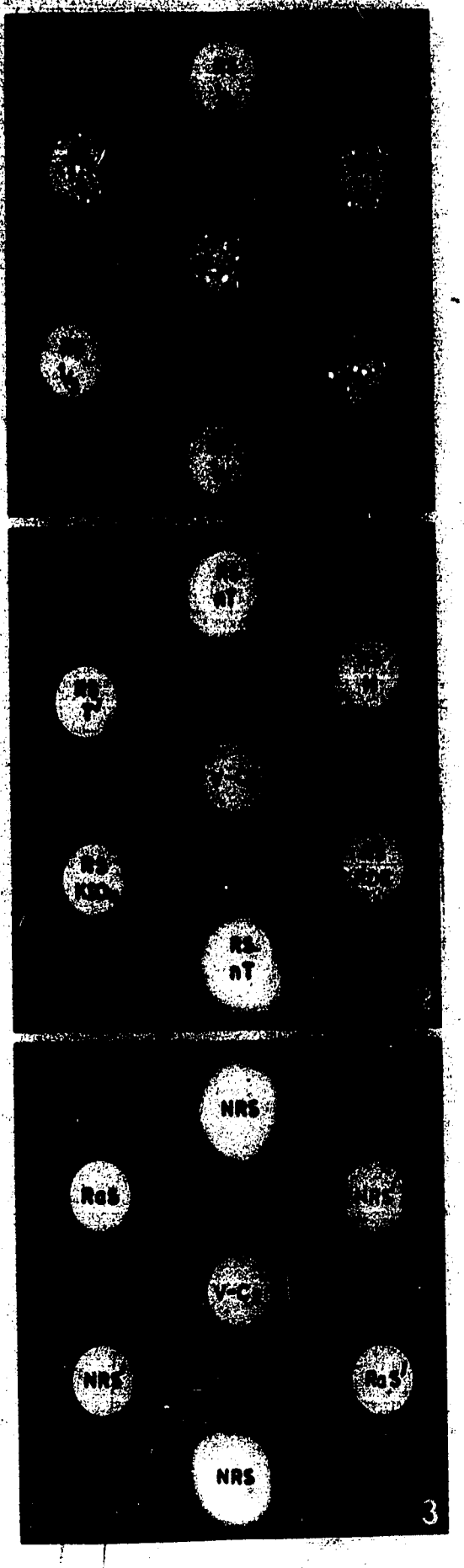
t

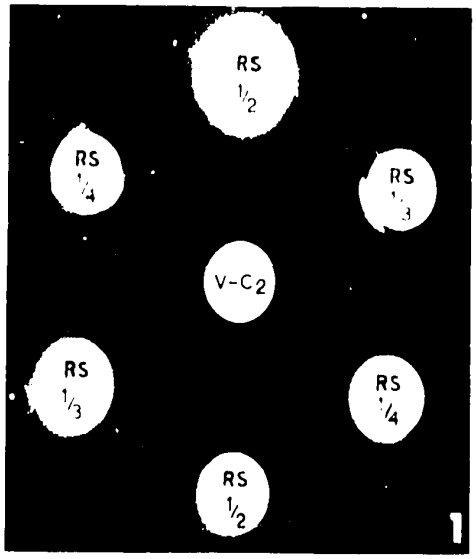
inal

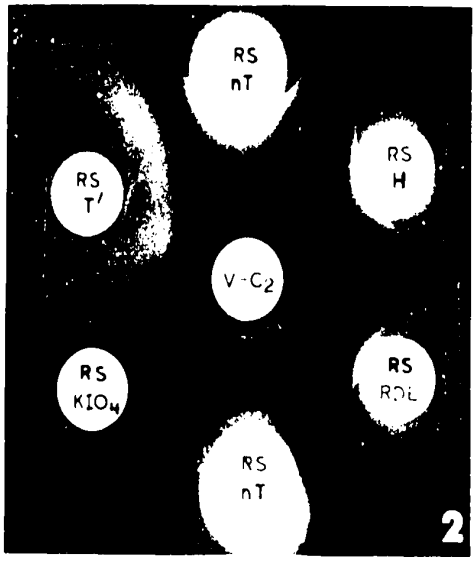
-

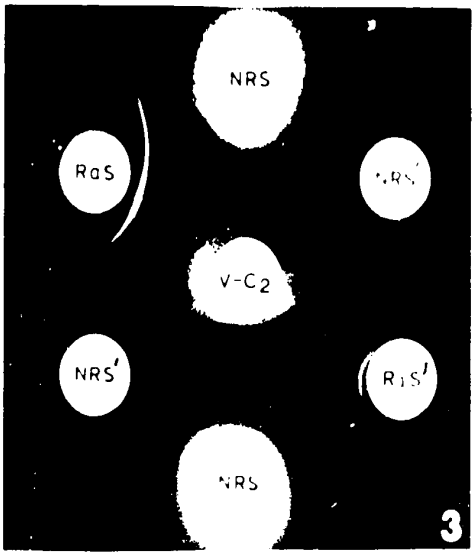
RaS).

1




RS
1/2
RS
1/4
RS
1/3
V-C2
RS
1/3
RS
1/4
RS
1/2
1


RS
nT
RS
T'
RS
H
V-C2
RS
KIO4
RS
nT
2


NRS
RaS
V-C2
NRS'
NRS
3

It was concluded that the precipitin reaction between the virus particle and the normal serum component was not due to the presence of alpha or beta-type inhibitors. Biddle and Stevenson (1966) reported that gamma inhibitory activity showed a marked ability to flocculate influenza A/PR8 virus, and that this ability was destroyed by treatment with periodate. Because of the dilutions of the serum, the immunodiffusion reaction could not be used to distinguish γ inhibitor from antibody, and further tests were required.

Identification of the active component by serum fractionation procedures.

There have been reports of the association of inhibitor activity with different globulin fractions, and others have specifically noted its absence from γglobulin. In 1965, Cohen, Biddle and Newland showed that the distribution of inhibitory activity varies with the method of fractionation. They concluded that, by "salting out" with $(NH_4)_2SO_4$, 'HA' inhibitor was found in several fractions, but using ion-exchange chromatography with column electrophoresis horse serum gamma inhibitor was confined to the alpha globulin component. Styk and Hana (1962) found that beta-inhibitor activity is connected with the β_2-macro globulin (gM globulin). However, β-inhibitor was not

detected as a component in the NRS reaction.

Since the virus precipitating capability of the "normal" rabbit serum was due either to the presence of specific antibody or to a gamma-type inhibitor, an attempt was made to identify the serum fraction which carried the activity and thus determine the nature of the active component.

Because location of inhibitory activity in the serum fraction varied according to technique (Cohen et al, 1965), several fractionation procedures were chosen, each relying on different physicochemical properties for their selective action. Details of the procedures have been described under Methods.

Rivanol treatment.

Treatment of serum with rivanol (2-ethoxy-6,9 diamino acridine lactate) has been highly recommended for the isolation of gamma globulin (Horejsi and Smetana, 1956, and Stansty & Horejsi, 1961). The effect is due to a stoichiometric interaction caused by electrostatic forces, thus, on addition of rivanol to the serum, serum proteins are selectively precipitated in combination with the rivanol, while gamma globulins remain in the supernatant fluid. The distribution of serum proteins between the soluble and

insoluble fractions varies with the experimental conditions, pH, ionic strength and dye concentration. The rivanol protein precipitate may be dissociated into a highly insoluble rivanol precipitate with the accompanying release of protein into solution. This method had the added advantage of being easily applied to small amounts of serum samples (2-5 ml.), and was chosen for the preliminary fractionation of the normal serum samples.

DEAE - cellulose adsorption.

Anionic adsorption of non-gamma globulin components from whole serum was developed by Peterson & Sober (1956) and modified by Stanworth (1960). Because of the scarcity of the available normal reacting serum and the complications involved in its characterization, influenza A/PR8 antiserum was fractionated. The resulting fractions were then used to identify the normal reacting component by means of the "reaction of identity" in the immunodiffusion tests. The adsorption procedure is described in the section on Methods.

Ammonium sulfate fractionation.

Fractional precipitation of serum proteins by "salting-out" with ammonium sulfate (Cohn et al, 1940, Kendal, 1937) was used to prepare influenza A/PR8 antiserum

fractions which were then characterized by electrophoresis and immunoelectrophoresis. These fractions were then used to identify the normal reacting component using the immunodiffusion "reaction of identity".

i. Analysis of the serum fractions to locate inhibitory and precipitating activity.

The results of the serological tests on the serum fractions are presented in Tables 12 and 13.

The quantity of the 'G' fraction obtained by rivanol treatment was limited, and in order to have enough material for the immunodiffusion analyses, HAI determinations were not made. The results in Table 12 are recorded as positive or negative because the fractionation procedure was not quantitative and no further significance could be attached to single observations based on one or two experiments.

On the other hand, the fractionation of antiserum by DEAE or ammonium sulfate was quantitative since each fraction was adjusted to the same final volume as the original serum. For this reason the haemagglutination inhibition due to the antibody activity of each fraction served as an index for the comparison of the two procedures. The recovery of HAI antibody activity in both DEAE fractions

TABLE 12

Haemagglutination Inhibition Characteristics
of Normal Reacting Serum
Fractions obtained with Rivanol.

Serum Fractions	Serum Treatment			
	Untreated	Heat 56°C/60	RDE	KIO_4
503 whole serum	+	+	+	±
503 "A"	+	+	+	+
506 whole serum	+	+	+	+
506 "A"	+	+	+	-
RaS-immune control:				
"A"	+	+	+	+
"G"	+	NT	+	NT

NT - not tested

TABLE 13

HAI and Neutralizing Titres of the Anti-PR8 Serum Fractions obtained by DEAE and $(NH_4)_2SO_4$ Fractionation

	Serum Fraction	Haemagglutination Inhibition* Untreated serum	56°C/60°	RDE	KIO_4	Neutralization**
DEAE	RaS whole serum	8192	8192	8192	8192	
	Fraction 1	2048	2048	2048	2048	NT
	Fraction 11	2048	2048	4096	2048	NT
$(NH_4)_2SO_4$	RaS Untreated	4096	4096		2048	3000
	Albumin	<8	<8		<8	<10
	Pseudoglobulin	1024	1024		512	1380
	Euglobulin (water soluble)	128	128		64	360
	Euglobulin (water insoluble)	64	64		32	160

NT - not tested

**Neutralization titres are expressed as the reciprocal of the 50% end point calculated by the Reed-Meunch method.

*HAI titres are expressed as the reciprocal of the last serum dilution showing inhibition of haemagglutination.

I & II was good. Ammonium sulfate fractionation resulted in the complete elimination of HAI activity from the albumin fraction, however, compared to the DEAE fractions, there was considerably less antibody activity present in the three globulin fractions. The neutralization of egg infectivity (EID_{50} - Table 13) demonstrated the presence of neutralizing antibody in the ammonium sulfate globulin fractions, and not in the albumin fraction.

The precipitating activity of the fractions was investigated by the cellulose acetate micro-immunodiffusion technique. The reactions shown in Plate 17 (figures 1 and 3) demonstrate the presence of virus precipitating activity in both DEAE fractions (FI & FII) and in all the ammonium sulfate fractions, except the albumin. The absence of precipitating activity in the albumin fraction is demonstrated conclusively by the absence of any inflection in the precipitin lines developed by its neighbouring reactions. Parallel reactions with soluble antigen preparations (figures 2 & 4) confirmed this distribution of antibody activity.

The immunodiffusion analyses of the rivanol-treated fractions were less clear cut. It was found, by means of reactions with rivanol-treated specific antiserum, that there was a considerable loss in precipitating ability.

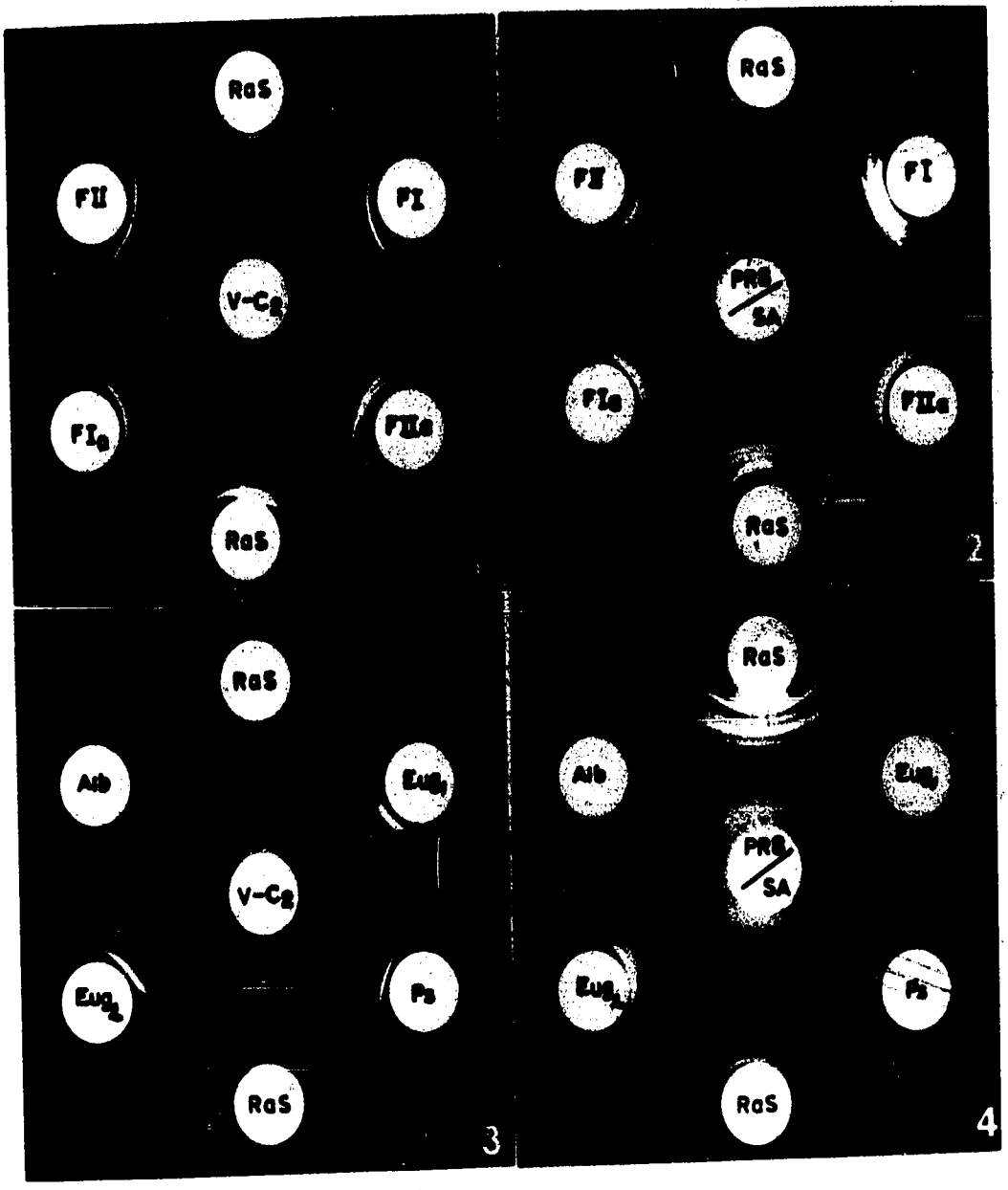
PLATE 17

Immunodiffusion Reactions of Immune Serum Fractions obtained by DEAE and $(NH_4)_2SO_4$ Fractionation Procedures.

Figures 1 & 2 - Comparative reactions of untreated antiserum (RaS) and DEAE fractions with virus concentrate V-C_2 (figure 1) and PR8 soluble antigen (figure 2).
FI & FII - first and second fractions after adsorption of sera by DEAE
FIa & FIIa - samples from first washing of DEAE.

Figures 3 & 4 - Comparative reactions of untreated antiserum (RaS) and fractions obtained by $(NH_4)_2SO_4$ treatment with virus concentrate V-C_2 (figure 3) and PR8 soluble antigen (figure 4).
No reaction can be detected between the albumin (Alb) fraction and either V-C_2 or PR8/SA antigens.

Eug 1 - euglobulin water-insoluble fraction
Eug 2 - euglobulin water-soluble fraction
Ps - pseudoglobulin fraction


RaS
FII
FI
V-C2
FIa
FIIa
RaS
2
RaS
FII
FI
PRL/SA
FIa
FIIa
RaS
RaS
Alb
Eug1
V-C2
Eug2
Ps
RaS
3
RaS
Alb
Eug1
PRL/SA
Eug2
Ps
RaS
4

1
2
3
4

The two reactions presented in Plate 18 demonstrate this finding in the case of antiserum fractions reacting with viral antigen ($V\text{-}C_2$) (figure 1) and normal serum fractions in an 'indirect reaction of identity' with antiserum (figure 2). The distortion of the reaction pattern was thought to be due to the differences in the viscosity of the A and G samples, and was a considerable disadvantage. However, precipitating activity could be detected in both the 'G' ('gamma' globulin) and the 'A' (recovered serum proteins) rivanol fractions of the 'normal' reacting serum.

In summary, biological activity as determined by HAI titrations, neutralization, immunodiffusion precipitation reactions was found to be absent only in the case of the ammonium sulfate 'albumin' fraction.

ii. Analysis of the serum fractions.

The analysis of the rivanol and ammonium sulfate fractions was made to identify the electrophoretic components and to compare the characteristics with those of rabbit serum fractions obtained from Hyland Laboratories. (Plate 19). These reactions showed that:

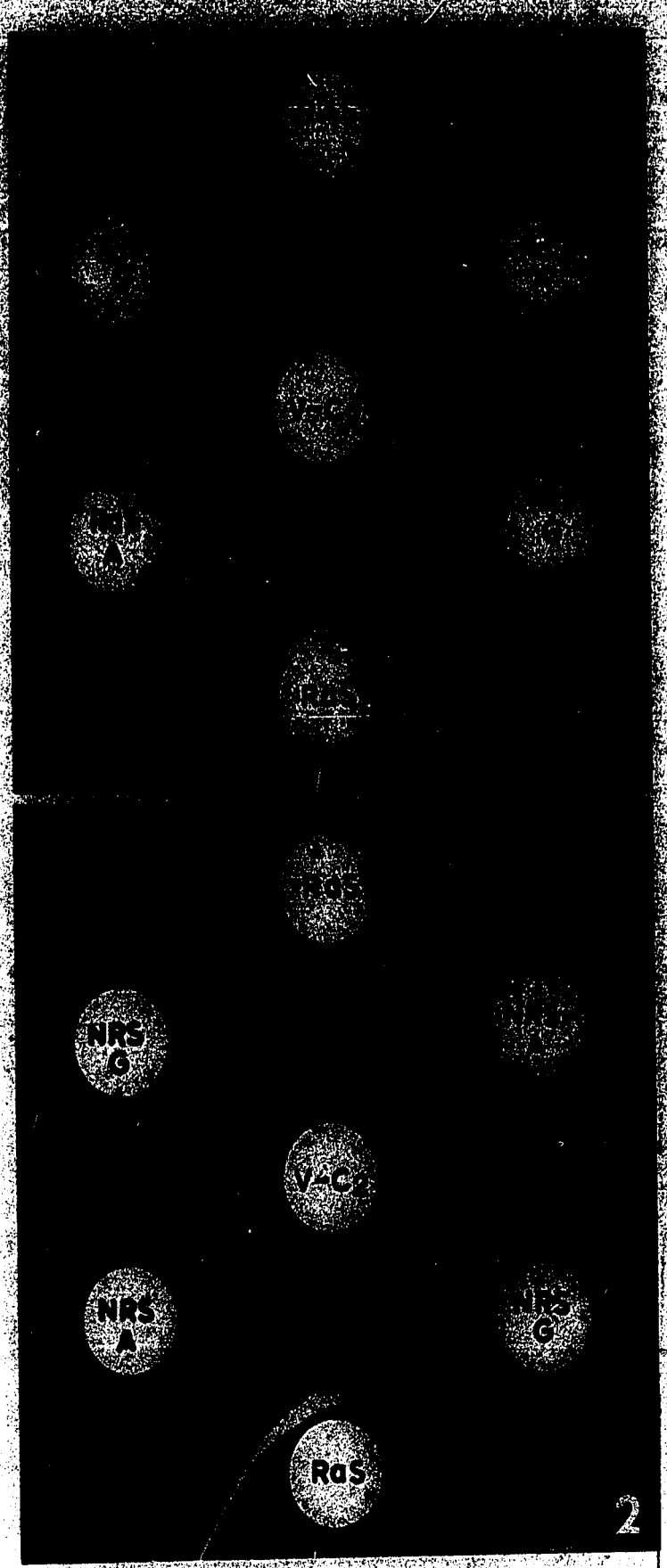
1) gamma globulin was present in all fractions with the exception of the ammonium sulfate albumin "fraction", and successful isolation of gamma globulin had only been

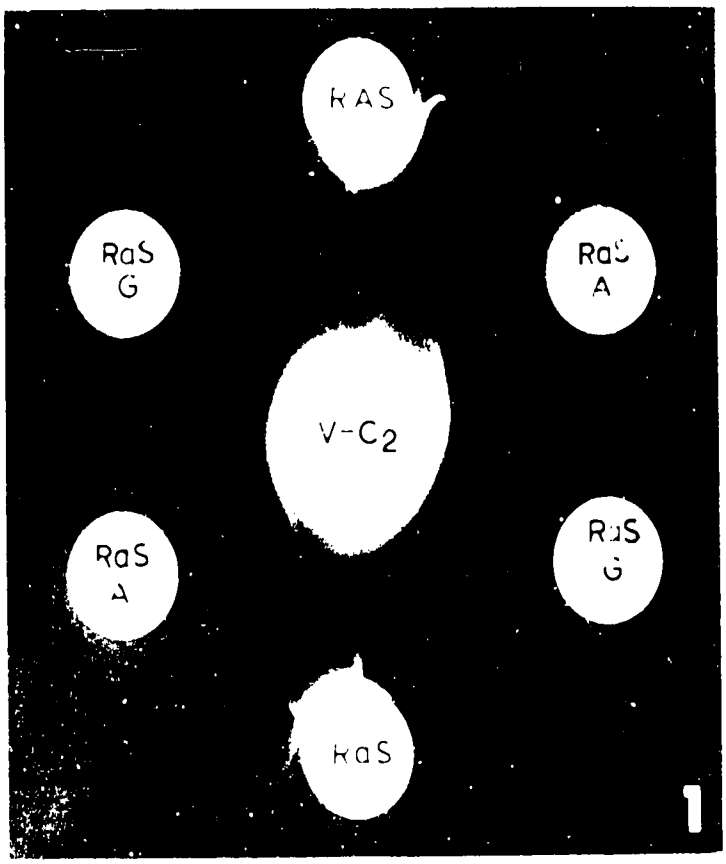
PLATE 18

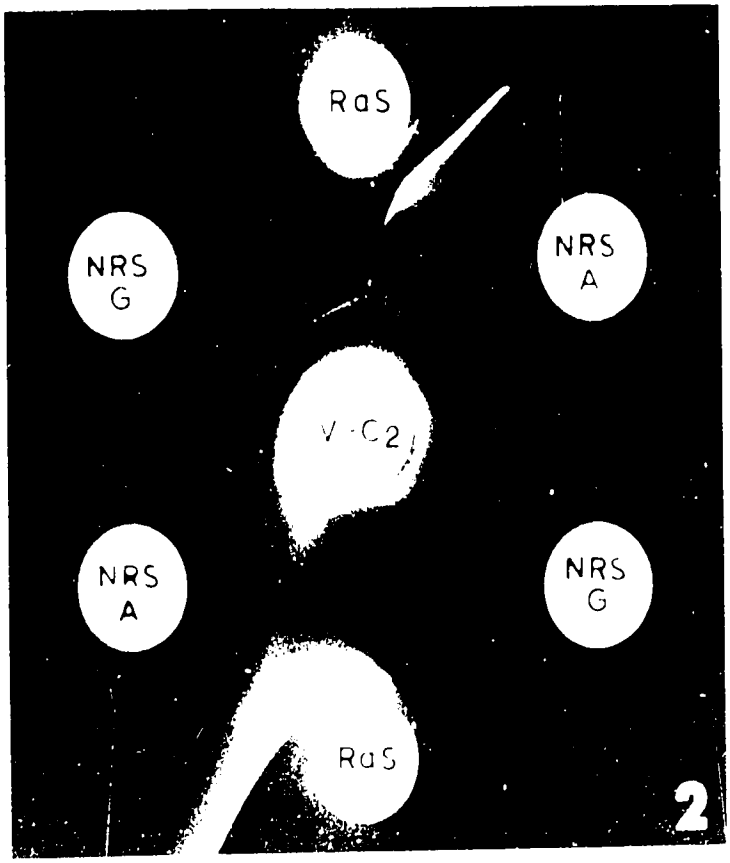
Immunodiffusion Reactions of both Normal and Immune Serum Fractions obtained by Rivanol treatment.

Figure 1 - Comparison of the reactions of V-C_2 with untreated antiserum (RaS) and fractions A, (precipitated by rivanol) and G (not precipitated by rivanol) of rabbit antiserum (RaS). Both A and G fractions show reactions of identity with the RaS reaction.

Figure 2 - Comparison of the reactions of V-C_2 with untreated antiserum (RaS) and NRS fractions 'A' and 'G', demonstrating the presence of 'normal' precipitins in both NRS fractions which show faint reactions of identity with the RaS reaction.


NRS
G
V4C
NRS
A
RaS
2


RAS
RaS G
RaS A
V-C2
RaS A
RaS G
RaS
1


RaS
NRS G
NRS A
V-C2
NRS A
NRS G
RaS
2

achieved by ammonium sulfate fractionation,

2) the rivanol 'G' fraction showed a component which migrated in the beta region in addition to the gamma globulin, and

3) all the components were detected in the pseudoglobulin fraction, though the albumin content was minimal.

A summary of the immunoelectrophoretic characteristics of the serum fractions and their biological activity is presented in Table 14.

These results suggested that the virus activity was due to a component (or components) with either beta or gamma globulin characteristics, which was present in the rivanol G fraction. Further investigations were made to determine the identity of the reacting component in the normal reacting serum by means of the immunodiffusion "reaction of identity", with the reactions of the immune serum fractions.

iii. Immunodiffusion analysis to determine the identity of the virus precipitating component in "normal" rabbit serum.

The preliminary immunodiffusion reactions were required to establish the identity of the components in the antiserum fractions with those in the original untreated antiserum. The reaction shown in Plate 20 (figure 1)

TABLE 14

SUMMARY OF THE CHARACTERISTICS OF NORMAL AND IMMUNE SERA

Serum	Treatment	Fractions	Electrophoretic Characteristics				Serological Activity		
			Albumin	α	β	γ	HAI*	N**	P***
Immune Serum	DEAE cellulose adsorption	FI	-	+	+	+	+	NT	+
Immune Serum	$(NH_4)_2SO_4$ precipitation	Albumin	+	+	+	-	-	-	-
		Pseudo-globulin	+ (slight)	+	+	+	+	+	+
		Euglobulin	-	-	-	+	+	+	+
'Normal' Serum	Rivanol precipitation	'A'	+	+	+	+	+	NT	+
		'G'	-	-	+	+	+	NT	+

* Haemagglutination Inhibition

** N - neutralization of infectivity

*** P - Precipitation reaction

NT - not tested

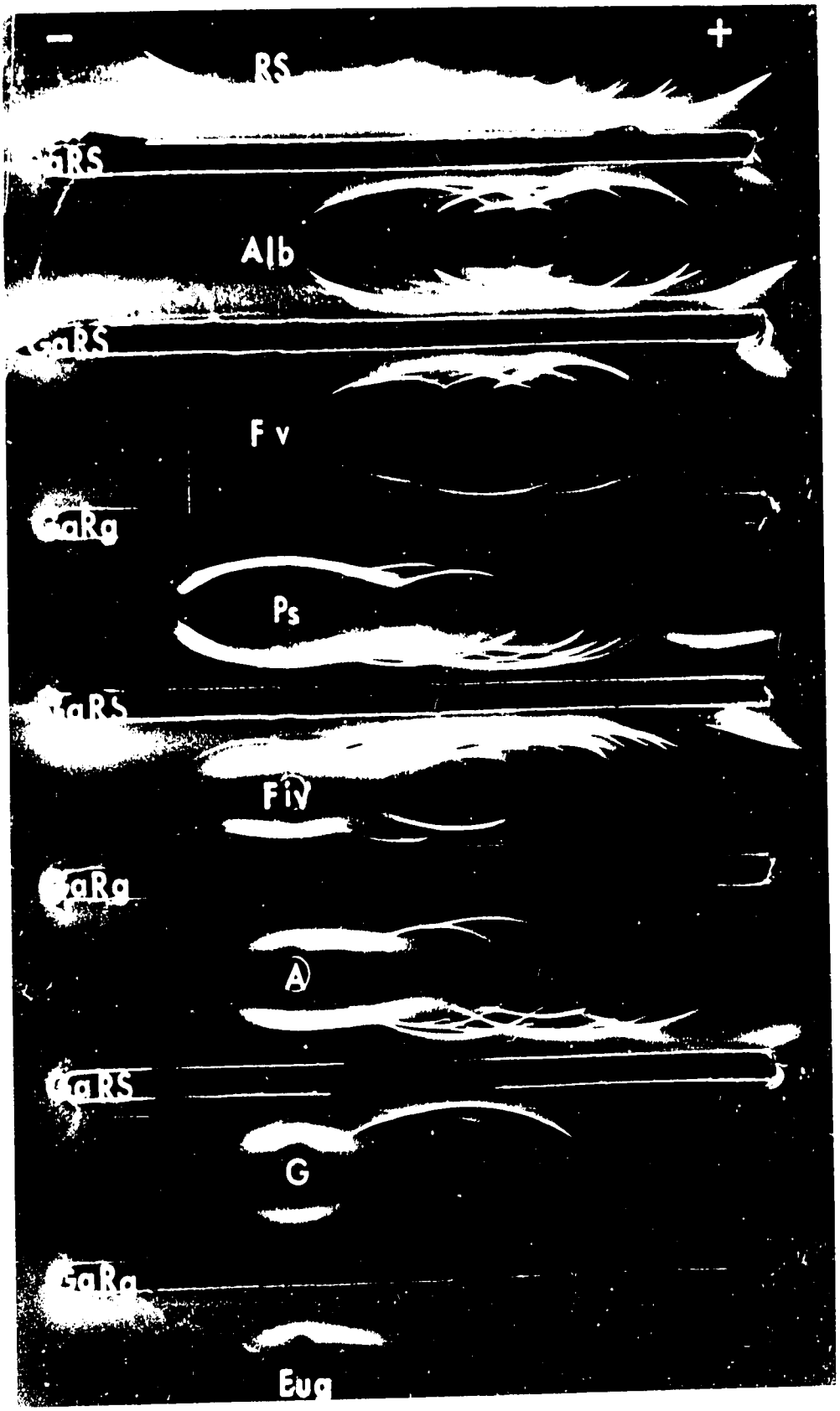
PLATE 19

Electrophoretic characteristics of Serum fractions determined by Immunoelectrophoresis in agar, using goat antisera to rabbit serum (GaRS) and to rabbit globulins (γ, β and γ (Fc$_\gamma$)) to detect the serum proteins.

The slow-moving globulin components are present in all fractions except the albumin (Alb) obtained by $(NH_4)_2SO_4$ and the commercial IV albumin fraction (Wand). Only γ globulins are detected in the fraction (Neg.) while a component with mobility characteristics of β globulin can be seen in rivanol C fraction. The fiv commercially prepared globulin fraction, pseudoglobulin (Ps) and the rivanol 'C' fraction contain serum proteins with the immunoelectrophoretic characteristics of albumin and γ, and γ globulins.

Electrophoretic characteristics of Serum fractions determined by Immunoelectrophoresis in agar, using goat antisera to rabbit serum (GaRS) and to rabbit globulins α, β and γ (GaRg) to detect the serum proteins.

The slow-moving γ globulin components are present in all fractions except the albumin (Alb) obtained by $(NH_4)_2SO_4$ and the commercial FV albumin fraction (Hyland). Only γ globulins are detected in the fraction (Eug.) while a component with mobility characteristics of β globulin can be seen in rivanol G fraction. The Fiv commercially prepared globulin fraction, pseudoglobulin (Ps) and the rivanol 'A' fraction contain serum proteins with the immunoelectrophoretic characteristics of albumin and α, β and γ globulins.


−
+
RS
aRS
Alb
aRS
F v
aRa
Ps
aRS
F v
aRa
A
aRS
G
aRa
Eua

illustrates the identity of the components and the distribution of the specific anti-viral precipitating activity in the three ammonium sulfate fractions of the influenza A/PR8 antiserum. No precipitins can be detected in the albumin fraction. It can be seen that the specific antibody detectable in the euglobulin fractions is also present in the pseudoglobulin fraction and the untreated antiserum. This suggests that the specificity of the antibody in the serum fraction has not been altered by the procedure.

The second reaction illustrated in Plate 20 (fig. 2) is a further demonstration of the relationship between the precipitin components in both the pseudoglobulin and the euglobulin fractions, and specific rabbit antiserum produced both by parenteral immunization (RaS/PR8) by intranasal infection (RaS/IN).

The linkages between the precipitin lines clearly show the identity of the reacting antibodies, and demonstrate, within the limitations of the immunodiffusion reaction, that the ammonium sulfate fractionation procedure has not altered the specificity of the antibodies.

The relationship between the V-C_2 reactions of the "normal" reacting serum and the antiserum fractions was investigated, Plate 21 (figures 1 & 2). A clear reaction

PLATE 20

Identification of Antibodies present in Immune Serum Fractions prepared by ammonium sulfate fractionation.

Figure 1 - The reactions of V-C_2 with the euglobulin fractions (Eug.) showing reactions of identity with the untreated rabbit antiserum (RaS) and pseudoglobulin (Ps) fractions which demonstrate the presence of virus precipitins in the γ globulin and the pseudoglobulin antiserum fractions.

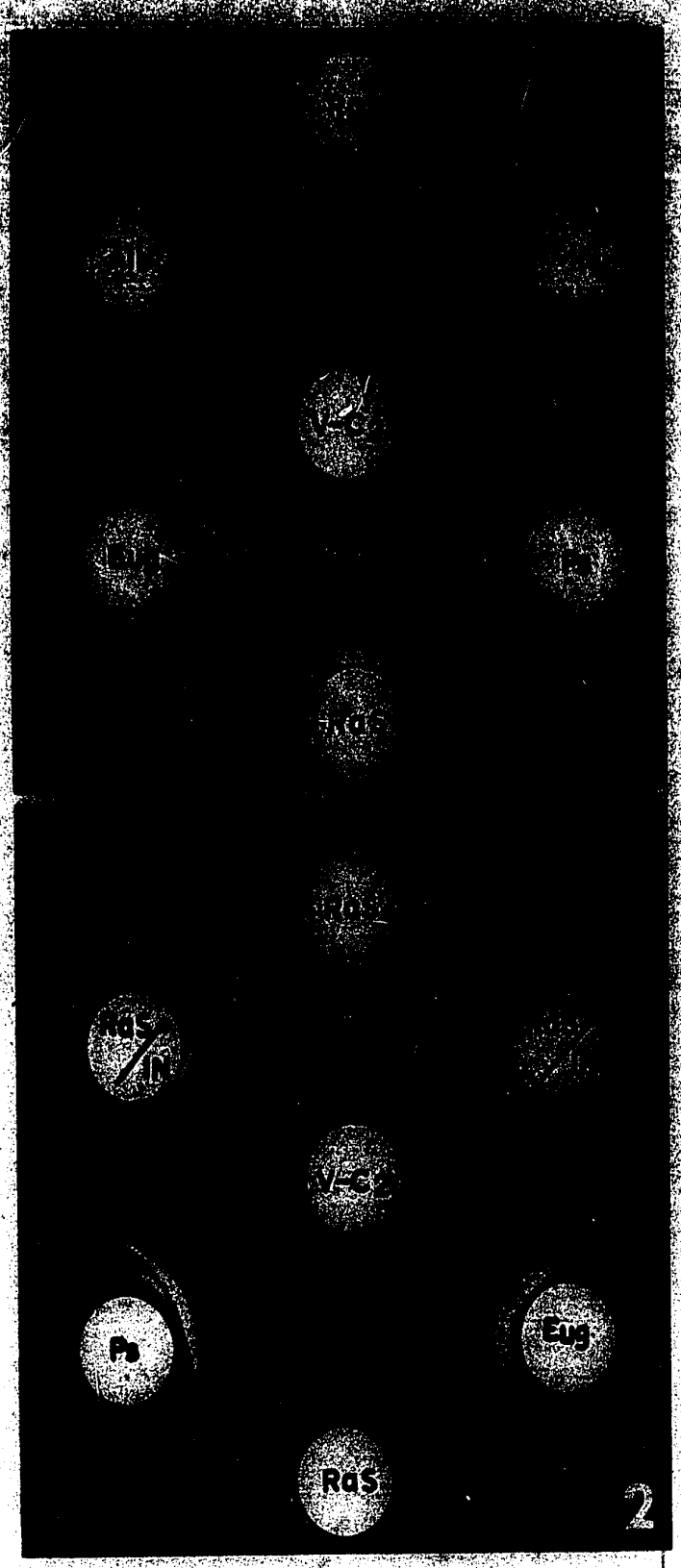
Figure 2 - The identification of the precipitins in the euglobulin (Eug.) fraction with influenza A antibodies in the specific rabbit convalescent antiserum (RaS/IN) is demonstrated by the reaction of identity (⟷) between their reactions with V-C_2.

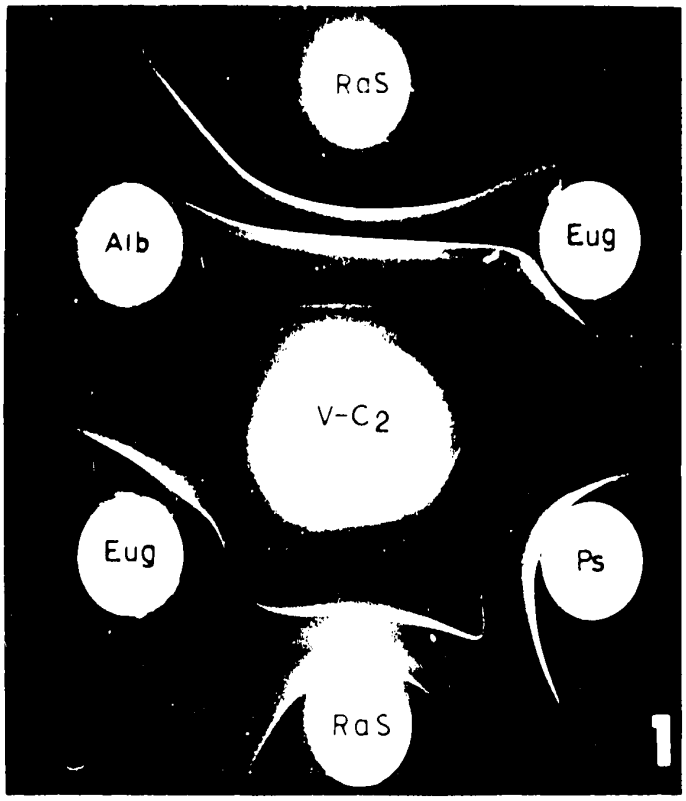
n

e

ns.

ity




RaS
Alb
Eug
V-C2
Eug
Ps
RaS
1

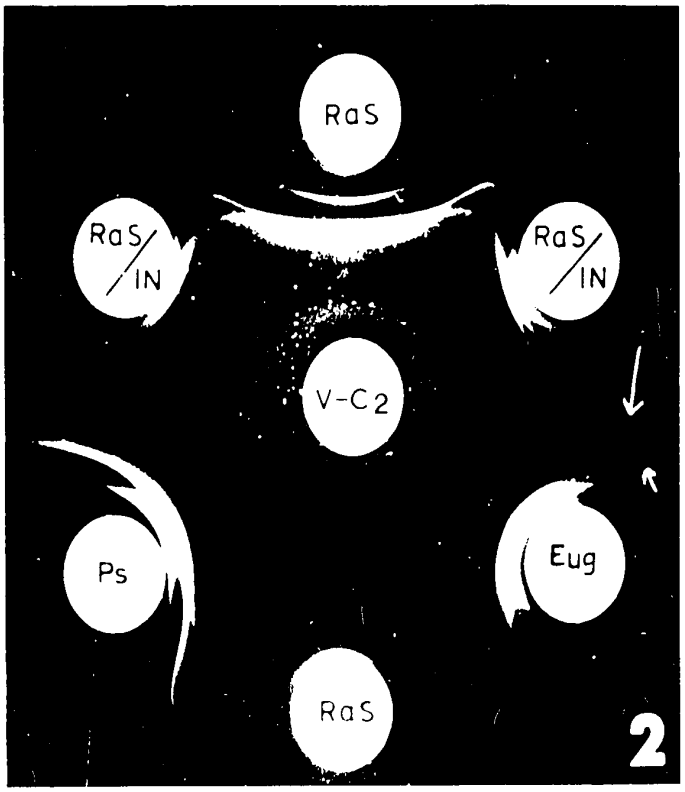

RaS
RaS/IN
RaS/IN
V-C2
Ps
Eug
RaS
2

PLATE 21

Comparison of Immunodiffusion Reactions between NRS and Antiserum Fractions and the Antigen V-C_2.

Figure 1 - The reactions between V-C_2 and NRS (normal reacting serum) show reactions of identity with components present in both antiserum (Pad) the pseudoglobulin fraction (Pf). The relationship between NRS and the euglobulin (Eug.) component is not evident.

Figure 2 - The normal precipitating component shows direct reactions of identity with components present in untreated (Rad) and X-irradiated (Rx) antiserum fractions. The reaction of identity is further emphasized by the reaction of non-identity, indicated by the arrow.

PLATE XI

Comparison of Immunodiffusion Reactions between NRS and Antiserum Fractions and the Antigen V-C_2.

Figure 1 - The reactions between V-C_2 and NRS (normal reacting serum) show reactions of identity with components present in both untreated (RaS) the pseudoglobulin fraction (Ps). The relationship between NRS and the euglobulin (Eug.) components is not evident.

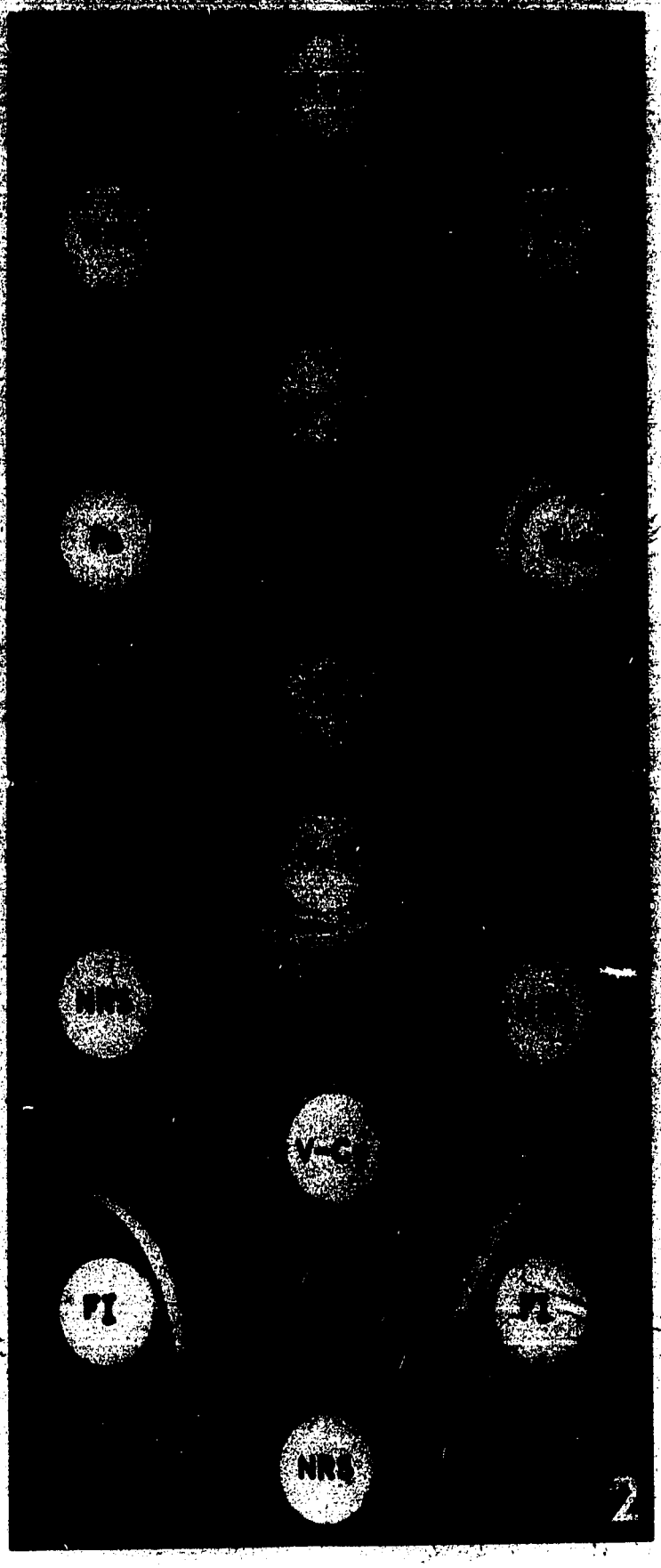
Figure 2 - The normal precipitating component shows clear reactions of identity with components present in untreated (RaS) and FI and FII antiserum fractions. The reaction of identity is further emphasized by the reaction of non-identity, indicated by the arrow.

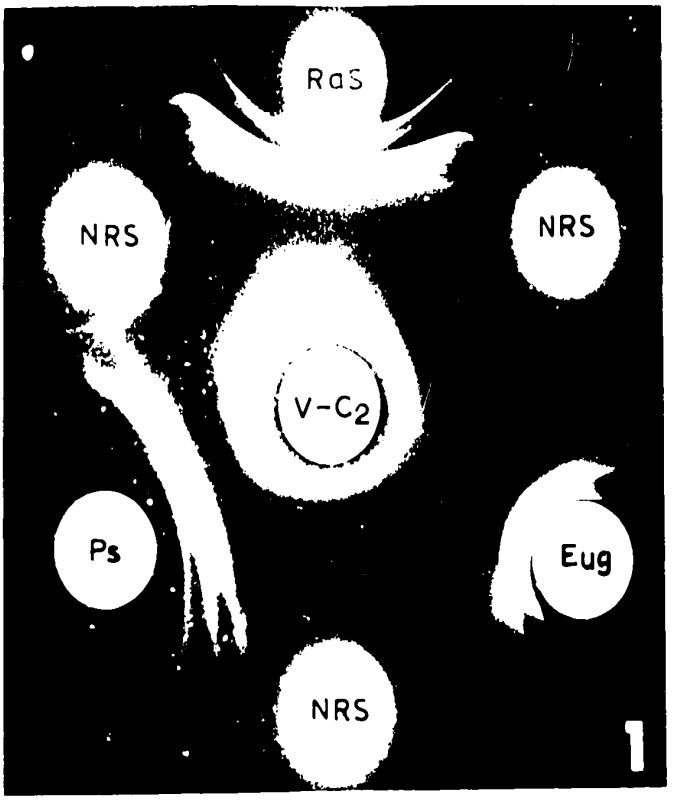
PLATE #1

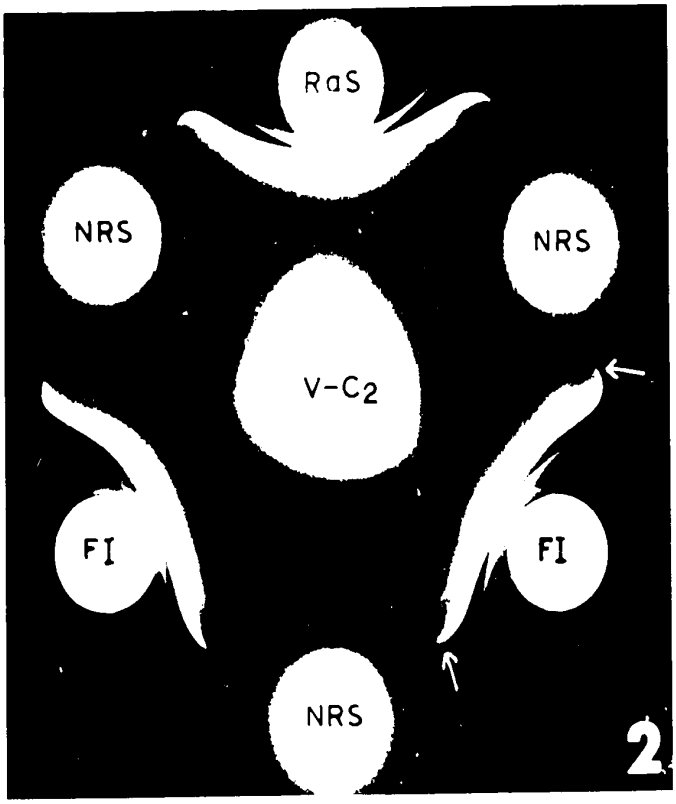
Comparison of Immunodiffusion Reactions between NRS and Antiserum Fractions and the Antigen V-C_2.

Figure 1 - The reactions between V-C_2 and NRS (normal reacting serum) show reactions of identity with components present in both untreated (RaS) the pseudoglobulin fraction (Ps). The relationship between NRS and the euglobulin (Eug.) components is not evident.

Figure 2 - The normal precipitating component shows clear reactions of identity with components present in untreated (RaS) and FI and FII antiserum fractions. The reaction of identity is further emphasized by the reaction of non-identity, indicated by the arrow.


NRS
2


RaS
NRS
NRS
V-C2
Ps
Eug
NRS
1


RaS
NRS
NRS
V-C2
FI
FI
NRS
2

of identity between the "normal" reacting serum (NRS) and both the untreated antiserum and the pseudoglobulin fraction was demonstrated (figure 1). The relationship between the NRS reaction and the euglobulin fraction was inconclusive.

In the reactions with V-C_2 shown in figure 2, it can be seen that one of the precipitating components is common to all the sera (figure 2). The reaction of identity was further emphasized by the crossing lines (arrowed), representing components present in the FI fractions and absent from the NRS serum.

From these analyses the normal serum component could be identified as an α, β, or γ globulin, known to be present in the pseudoglobulin and DEAE FI and FII fractions.

In the next series of tests, the immunodiffusion reactions of rivanol-fractionated normal serum were compared with both immune and untreated normal serum. The results of these tests are shown in Plate 22 (figs. 1 & 2). It can be seen that the "normal" precipitating component is present in the 'A' fraction, which contained all the electrophoretic components, albumin, and the three globulins (figure 1).

In the second pattern (fig.2) the reactions of identity between the normal serum (NRS), untreated antiserum

(RaS) and the 'G' fraction (NRS'G'), shows that the "normal" precipitating component is present in the 'G' fraction. Since the rivanol 'G' fraction contained β and γ globulins, the normal active component was identified as a serum component showing β or γ globulin characteristics.

The reaction shown in figure 3 (Plate 22) demonstrates the identification of the normal serum reaction component with the precipitin component present both in the euglobulin (Eug.) and the pseudoglobulin (Ps) fraction. The reaction of identity between the precipitating components is quite clear, despite the poor resolution of the normal serum precipitin line.

Since these normal serum precipitins demonstrate a reaction of identity with the precipitating antibodies present in the euglobulin fraction, which were characterized as gamma globulin, it was concluded that the virus precipitating component in the "normal" rabbit sera was in fact antibody, which is present probably as a result of a previous natural infection with with influenza A or a related myxovirus. This conclusion is supported by the fact that rabbits were shown to be susceptible to infection with influenza A/PR8.

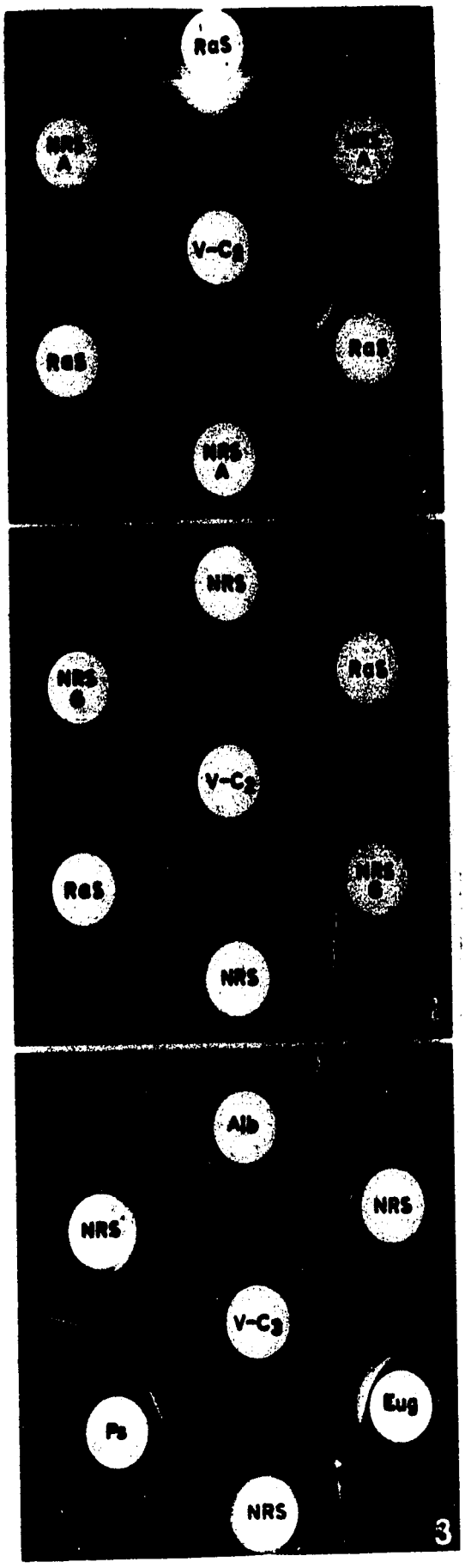
PLATE 22

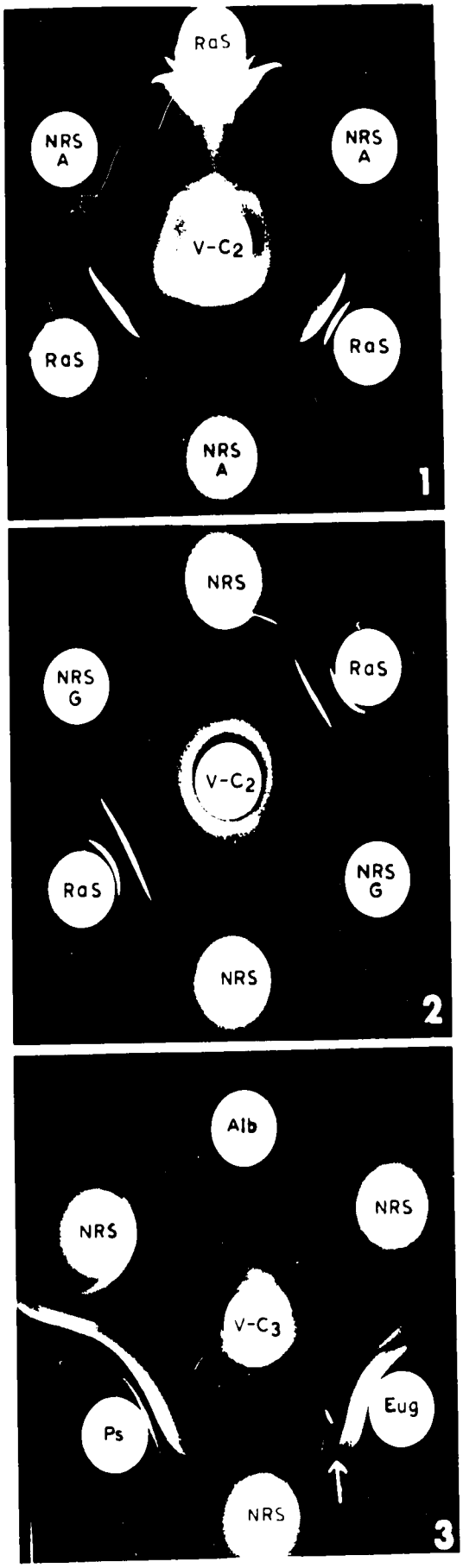
Identification of the NRS Reacting Component.

Figure 1 - Reactions of identity between the NRS 'A' fractions (rivanol treated) and untreated rabbit antiserum demonstrate presence of precipitating component in 'A' fraction.

Figure 2 - The presence of the NRS component in the NRS 'G' fraction is detected by the reactions of identity between the untreated NRS and rabbit antiserum (RaS) with the NRS (G) fraction.

Figure 3 - The reacting component in untreated NRS, is identified by the reaction of identity (Arrowed) with the reaction of the euglobulin (Eug.) fraction and virus concentrate ($V\text{-}C_3$). The precipitating component is also present in the pseudoglobulin fraction (Ps).


RaS
V-Cg
RaS
RaS
NRS
NRS
RaS
V-Cg
RaS
NRS
Alb
NRS
NRS
V-Cg
Eug
Pa
NRS
3


RaS
NRS A
NRS A
V-C2
RaS
RaS
NRS A
1
NRS
NRS G
RaS
V-C2
RaS
NRS G
NRS
2
Alb
NRS
NRS
V-C3
Eug
Ps
NRS
3

Summary.

The investigation of the antigenic structure of influenza A/PR8 virus has so far been concerned with reactions which occurred because of the diffusion of the virus particle in cellulose acetate.

It was found that the control prebleed serum from non-immunized rabbits precipitated the virus (V-C) antigen in the cellulose acetate immunodiffusion reaction. Because non-specific inhibitors of haemagglutination by influenza viruses are found in the serum of a wide variety of animals, including rabbits, and could be responsible for the reaction manifested by the "normal" serum, further studies were made to identify the reacting component. It was found that the normal precipitating component showed a reaction of identity with specific antibodies contained in the gamma globulin fraction of immune serum. The presence of these "normal" antibodies might be an indication of previous infection with the same or related viruses.

In view of this finding, it was realized that, even if rabbit prebleed serum did not demonstrate anti-viral activity, there was always the possibility of cross-infection with other rabbits. Therefore, antiserum produced by immunization with normal membrane extracts might show the

presence of viral antibodies, acquired as the result of natural infection. Such a reaction might be erroneously interpreted as indicating the presence of normal host component showing antiviral activity. Furthermore, the use of specific rabbit antisera to distinguish between the soluble antigens was limited, since natural infection would result in the presence of viral antibodies. All preliminary analyses of the virus antigens were limited to the identification of structural antigens, by means of reactions of identity between the known antigen-antiserum reaction and the test antigen-antiserum reaction. By the process of elimination, those antigens which could not be identified as structural antigens could then be characterized as being non-structural soluble antigens with either host or virus specificity.

Therefore, the next step in these investigations was to detect and identify the antigenic constituents of the virus particles, the structural antigens.

Antigenic Characterization of Influenza Virus Structural Components.

Influenza viruses are chemically complex structures possessing at least four biologically active components:- haemagglutinin, neuraminidase, internal and external antigens. Following the introduction of the negative staining technique (Brenner & Horne, 1959), classical descriptions of the morphology of influenza virus were made by Horne et al (1960). Since then morphological studies have been made to demonstrate, 1) the relationship between virus structure and infectivity (Moore et al, 1962, Barry, Horne & Waterson, 1962, Morgan, Hare & Rose, 1962), 2) the association of the haemagglutinin with the surface structure of the particle (Riffkind et al, 1960, and Lafferty & Oerteles, 1963), 3) the structure of the internal ribonucleoprotein (Hoyle, Horne & Waterson, 1961) and 4) the effect of Vitamin A on the morphology of the virus particle (Blough, 1963).

Another approach to the problem of understanding the architecture of the influenza viruses has been to correlate the effect of various chemical agents on the disruption of virus particles, with the production of smaller biologically active components responsible for haemagglutinin and neuraminidase activity (Hoyle, Horne & Waterson, 1961, Laver, 1963, and Reginster 1965, Blough, 1963).

The physical-chemical studies of Laver (1964), have resulted in the correlation of the chemistry of some of the polypeptide chains of influenza particles with two of the biological properties of the virus, haemagglutinin and neuraminidase activity.

The purpose of this present study was to detect as many as possible of the antigenic components of the influenza A/PR8 virus particle, using the immunodiffusion reaction. These tests would require antigen preparations containing 'unaltered' virus structural components, obtained by the disruption of intact particles.

Effect of selected treatments on Virus Morphology.

The first experiments undertaken were to study the effects of a variety of agents on the structure influenza virus particle by examination with the electron microscope. The reagents for virus degradation were selected on the basis of their characteristic mode of action, as well as for their historical significance in the study of influenza viruses. These treatments were as follows:-

1. Mechanical disruption - by ultrasonication.
2. Effect of distilled H_2O at different pH levels.
3. Chemical treatments -
   a) Lipid solvents - ether

- chloroform

b) surface active agents -

- sodium deoxycholate
- saponin
- Tween 80

c) protein denaturants -

- urea
- guanidine hydrochloride

4. Enzymatic degradation with trypsin.

All the specimens were examined in a Phillips E/M 200 electron microscope at the School of Hygiene, University of Toronto.

Morphology of untreated influenza A/PR8 virus.

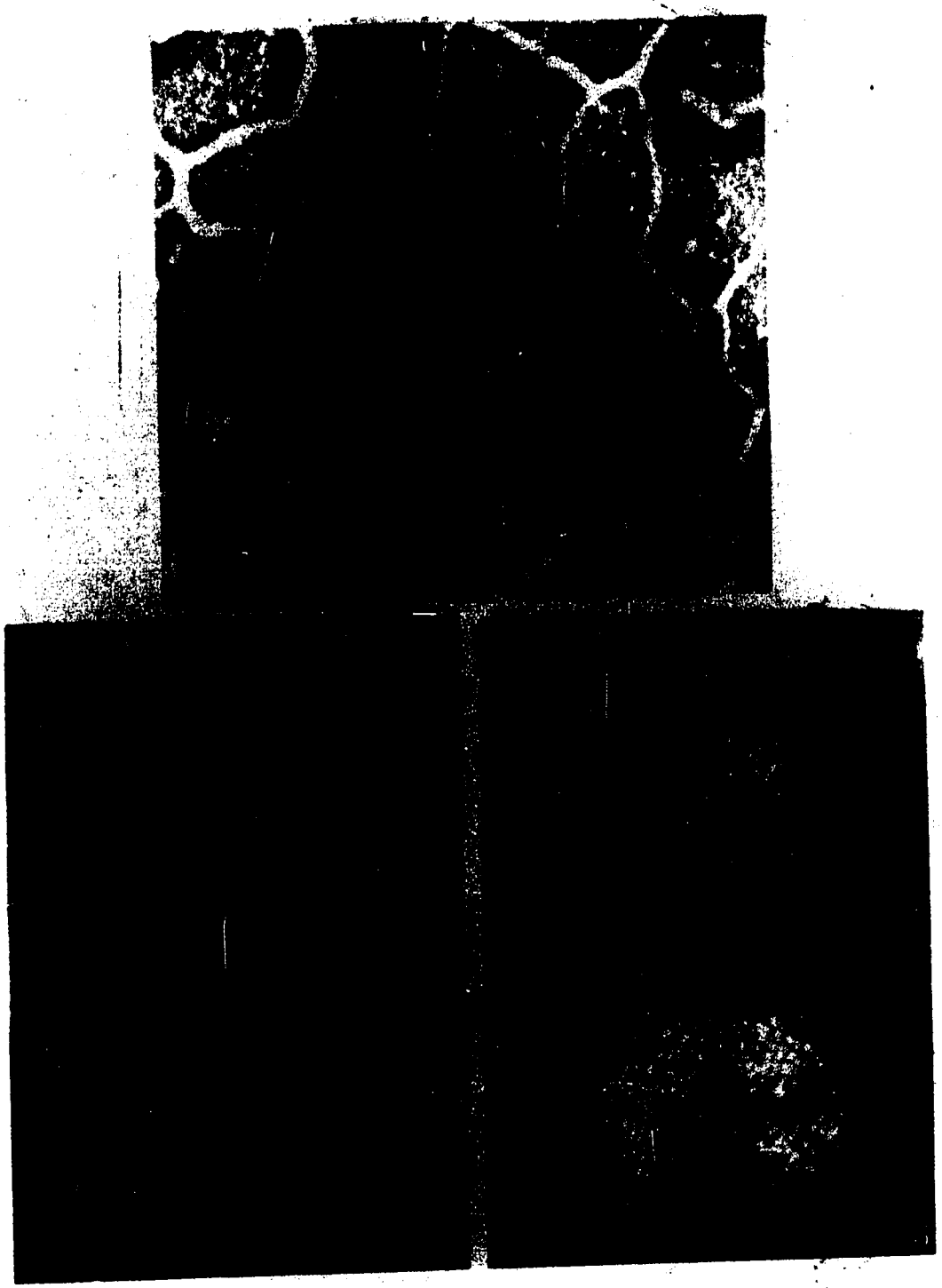
The morphology of the untreated virus particles contained in a 1:10 dilution of the virus concentrate ($V\text{-}C_2$) was studied using the negative staining surface-spreading technique of Parsons (~~1960~~, 1963 a & b ~~1966~~). Pleomorphic intact particles, varying in size from 80 mμ - 250 mμ and with the characteristic surface projections, are shown in Plate 23 (figure 1). Measured at higher magnification (figures 2 & 3) these surface projections are approximately 10-15 mμ in length and appear to arise out of the membrane surface of the viral particle. No internal structural details are evident in these latter electron micrographs. The morphology of these particles is consistent with the characteristic

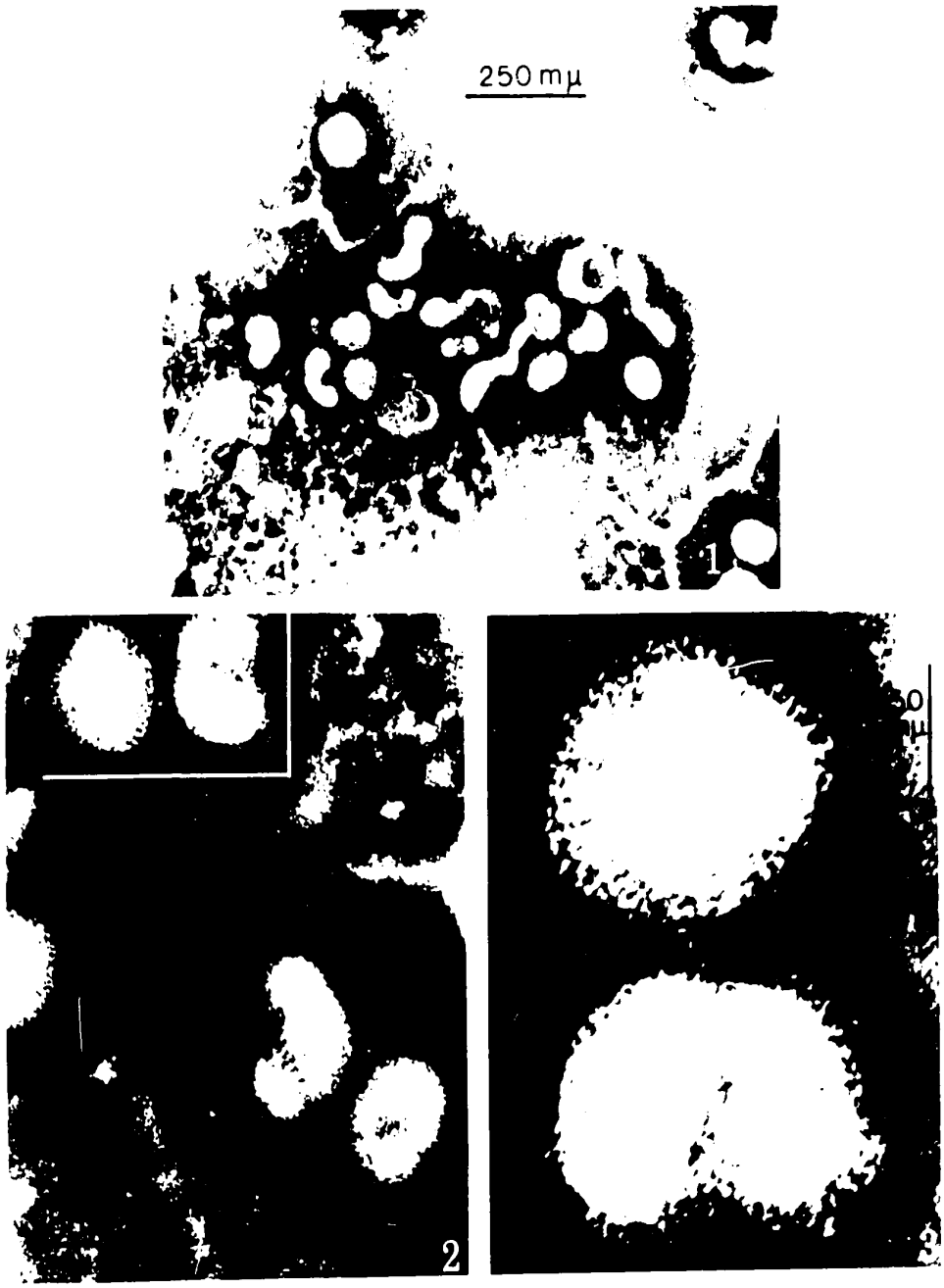
PLATE 23

Morphology of Untreated Influenza A/PR8 virus.

Figure 1 - The appearance of virus particles in a 1/10 dilution of virus concentrate prepared for electron microscopy by the surface-spreading technique.

Figures 2 & 3 - The characteristic structure of influenza particles revealed by higher magnification of the surface-spread preparations.




250 mμ
1
2
3

structure of influenza A viruses (Horne et al 1960, 1961) and demonstrates that the influenza virus preparation used for this study was a highly concentrated preparation consisting mainly of structurally intact particles.

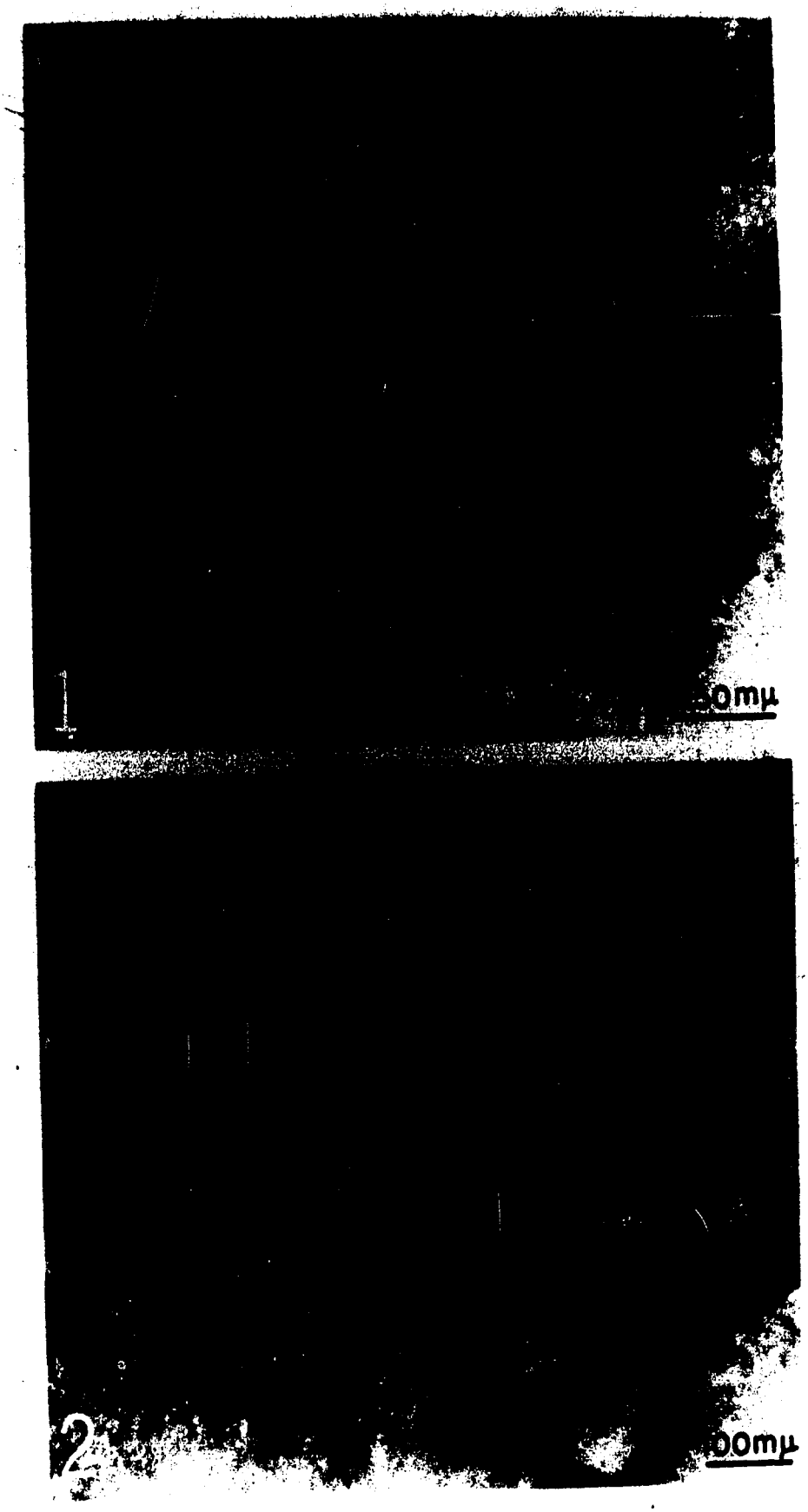
Mechanical disruption.

The specimen grids of the ultrasonicated virus were prepared in the standard manner:- one drop of virus suspension placed on the carbon-stabilized formvar grid plus one drop of 2% phosphotungstic acid (pH 6.5) without bovine serum albumin, and after several seconds, drained and air dried.

The extent of virus disruption following 60 second sonication is shown in Plate 24 (figures 1 & 2). A large number of the virus membranes appear to have ruptured at a point of stress, permitting the escape of internal components and resulting in the less dense appearance of the internal structure. The increase in the amount of debris is quite evident and many structures throughout the general background appear to be membrane fragments with the projections intact. A few of these fragments appear in the typical "rosette" symmetry (figure 1) (Hoyle et al, 1961) but not all. ~~The presence of~~ Structural components showing the helical symmetry (Hoyle et al, 1961) of the internal ribonucleoprotein could not be seen.

Morphology of Influenza Virus A/PR8 following Ultrasonication.

Figures 1 & 2 - The disruption of influenza A particles following ultrasonication for 60 seconds. Nearly all the particles show ruptured envelopes and fragments of envelopes with projections can be seen (⟶M).


0mμ
2
00mμ

It was possible that ultrasonication caused the immediate release and disruption of the internal nucleoprotein and that this would serve as an explanation for the profound loss in virus infectivity which results. This treatment would appear to offer good possibilities for the preparation of structural antigen.

Effect of H^+ concentration.

This effect was investigated with a view to determining the correct pH conditions for immunodiffusion and immuno-electrophoretic reactions. One in ten dilutions of the suspension of virus concentrate were made using distilled water adjusted with suitable amounts of N/10 HCl or N/10 NaOH to pH 5.5, 7.2 and 8.4, and allowed to stand at room temperature for ten minutes. Grids were prepared and negatively stained with 2% PTA. The effect on virus structure, as shown in Plate 25 (figures 1-5) was considerable, especially at the alkaline pH. At pH 5.5 (figures 1 & 2) the particles do not appear to have lost their characteristic appearance and typical pleomorphic forms can be seen. At a neutral pH (figure 3) the superficial morphology of the particles was not greatly different from the original untreated, however, internally they appeared to be less dense. Also, the long pleomorphic forms were not evident.

The effect of the alkaline distilled water is illustrated by figures 4 and 5. Virus particles of average size (100 mμ) and structural morphology can be seen, however large structures showing the membrane characteristics of influenza virus were predominant (figure 4). At the higher magnification (figure 5) the particles appeared to be coalescing as a result of the fusion of the surface membrane, to form one giant virus particle. There is some indication of the presence of the internal component within the giant particle (arrowed - figures 4 & 5).

This membrane phenomenon is of interest because it is analogous to the mechanism of myxovirus-cell penetration proposed by Hoyle (1962) Dale & Choppin (1962) and Silverstein & Marcus (1964), where the virus and cell membranes fuse permitting the release of the internal virus constituents into the cell. Similarly syncytial formation by infected cells is known to be affected by pH changes in the medium. All such effects could be the result of altered charge densities at the surface of the structures.

Further investigations combining this treatment with various ionic concentrations and correlating the effects with the biological properties of the virus would be of interest. That these influenza particles do coalesce in this

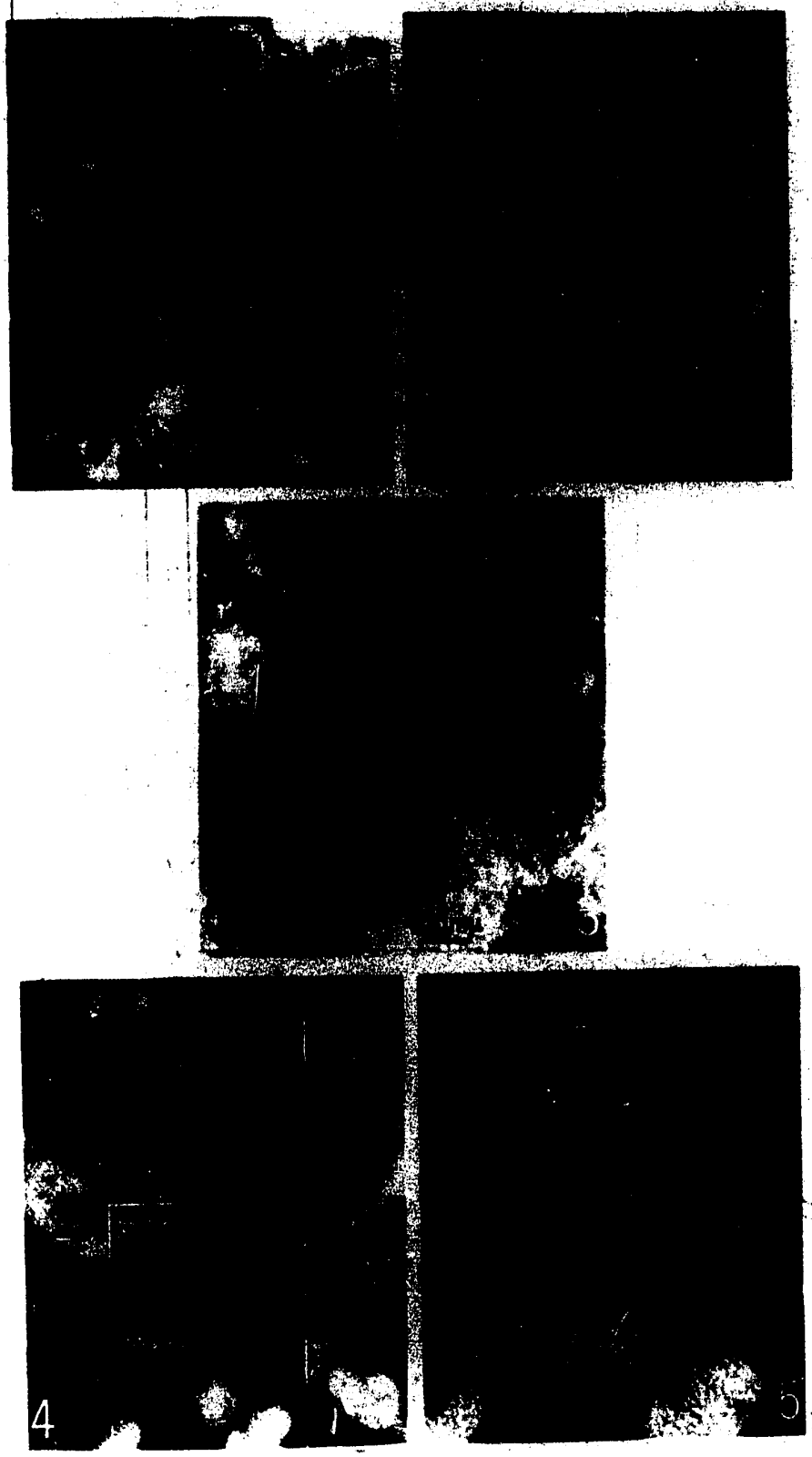
PLATE 25

Effect of pH concentration on the morphology of influenza A/PR8 virus.

Figures 1 & 2 - The appearance of influenza particles after 10 min. at pH 5.5

Figure 3 - Influenza virus particles after 10 min. at pH 7.4

Figures 4 & 5 - Influenza virus particles after 10 min. at pH 8.5. Internal component is indicated by (I).

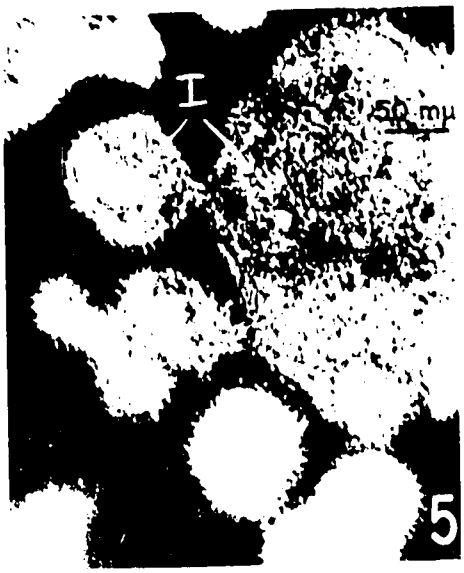

4
5


0mμ
1


100 mμ
2


100mμ


100mμ


I
50 mμ
5

manner, might partially explain the unsuccessful attempts at paper electrophoresis where the virus was found to remain at the origin (Ruttkay-Nedecky & Ivanicova (1965) and personal observations). Such coalesced material probably consists of particles larger than the membrane pore size, and thus the virus is trapped at the origin.

Lipid solvents.

Ether.

When myxoviruses are treated with ether, lipid in the outer membrane is dissolved, releasing the ribonucleoprotein which then breaks into short lengths. The remaining pieces of outer membrane curl up to form small rosette- shaped particles (Hoyle et al, 1961). The lipid of the cell membrane is believed to be essential to virus infectivity since ether treatment destroys this property. The usual method of ether treatment consists of adding ½ volume of ether to the virus suspension, shaking and then incubating at 37°C. for two hours, with intermittent shaking. The ether is then allowed to evaporate at 37°C. overnight.

This method was slightly modified in an attempt to achieve partial disruption, and thus to study the process of disruption. Ether was added (1:1 vol/vol) and incubated at room temperature for 30 minutes with periodic shaking. The ether was pipetted off and the remainder quickly evaporated by slight agitation. Grids were prepared,

negatively stained and examined. The results of this treatment are shown in figures 1 & 2 of Plate 26. There appears to be a distinct boundary developing between the base of the projections and the internal dense components. This is most evident in the enlarged area which shows the entire surface lifting away from the inner components. The nucleoprotein appears to be loosely wound around an electron-dense core. Rosette-shaped structures were not apparent, but the process of formation could be implied by the irregularities seen in the membrane as it detached from the intact particle. Finally, the length of the projections seemed to be increased slightly, however, this was an illusion caused by a shrinkage of the membranes away from the base of the spikes (A).

Chloroform.

Chloroform may be used to produce influenza soluble antigen from infected membranes for routine use in the complement fixation test. Such treatments almost completely removed haemagglutinin and caused a marked reduction in virus antigen. Since chloroform breaks down the virus particles, it was selected for comparison with the effect of ether treatment. Chloroform was added (2% vol/vol) to the semi-purified virus concentrate. The suspension was well shaken several times during incubation

for 20 minutes at room temperature. The aqueous phase was then removed and grids were prepared and negatively stained.

The effect on the surface projections and the membranes is clear in figures 3 & 4 of Plate 26. The membrane appears as a dense layer into which the spikes are embedded.

In both figures 3 & 4, ruptures in the membrane (R) allow it to separate away from the particle, revealing the internal structural components wound into a loosely packed core. Rosette-shaped structures (C) are evident and are probably released membrane similar to those seen after ether treatment (Hoyle et al, 1961). The morphology of the spikes shows that structural definition is diminished. The thickening of the membrane at the base appears to foreshorten the spikes slightly. In the upper right corner of figure 5, the final stages of degradation of what was probably a complete outer membrane is illustrated by the apparent fusion of the structures into a blurred image.

Of further interest is the appearance of the small particles which are scattered throughout the background of figures 3 & 4, and which are orientated as a circular pattern in the lower left corner of figure 5. The possibility that these are particles derived from the fragmentation of the spikes is suggested by the slightly beaded appearance (F)

PLATE 26

Morphology of Influenza A/PR8 following Ether Treatment and Chloroform Treatment.

Figures 1 & 2 - The effect of ether on the appearance of influenza virus particles.

I - internal nucleoprotein.

A - particles appearing elongated because underlying membrane is not evident.

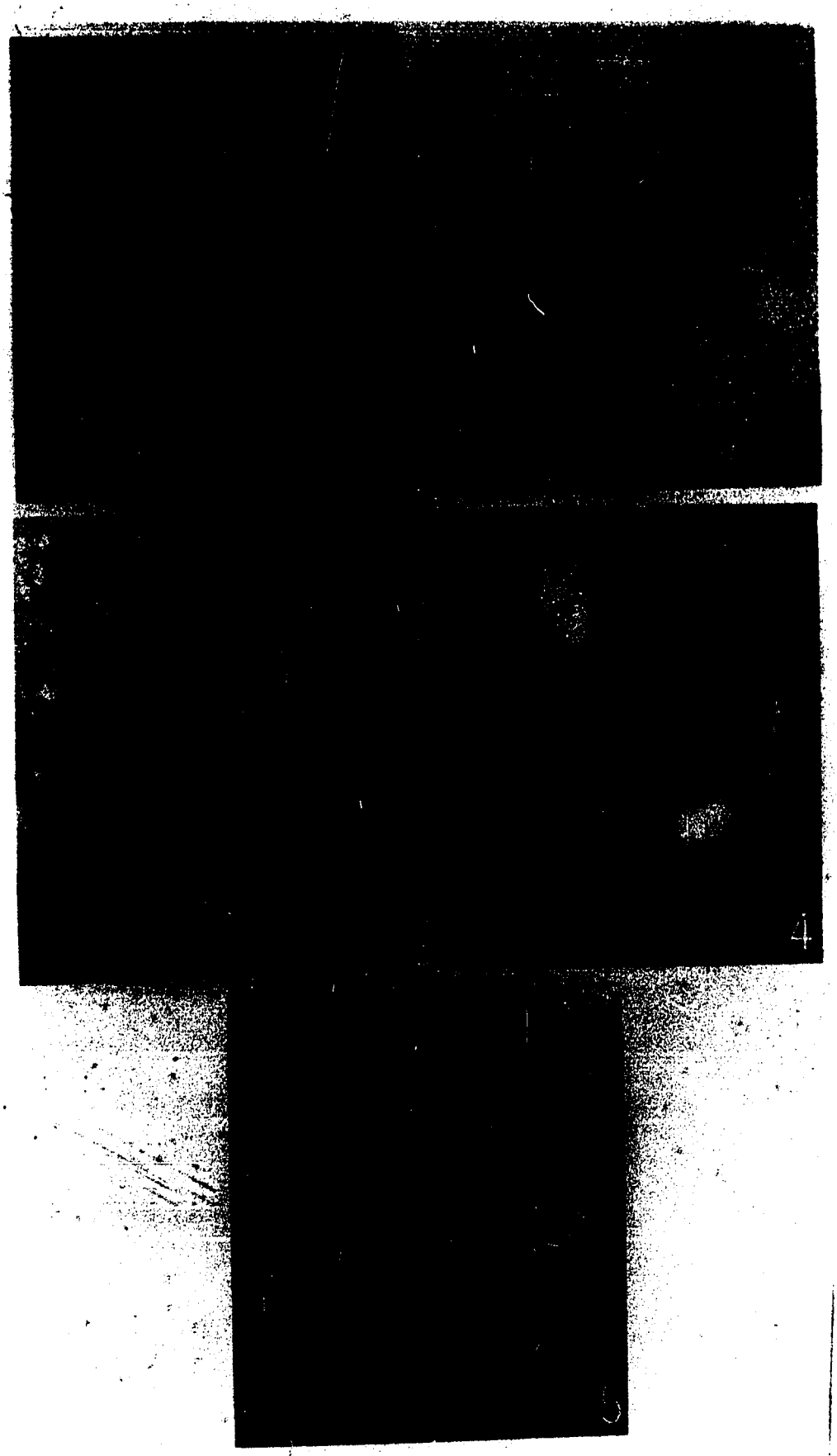
Figures 3, 4 & 5 The effect of chloroform on the appearance of influenza virus particles.

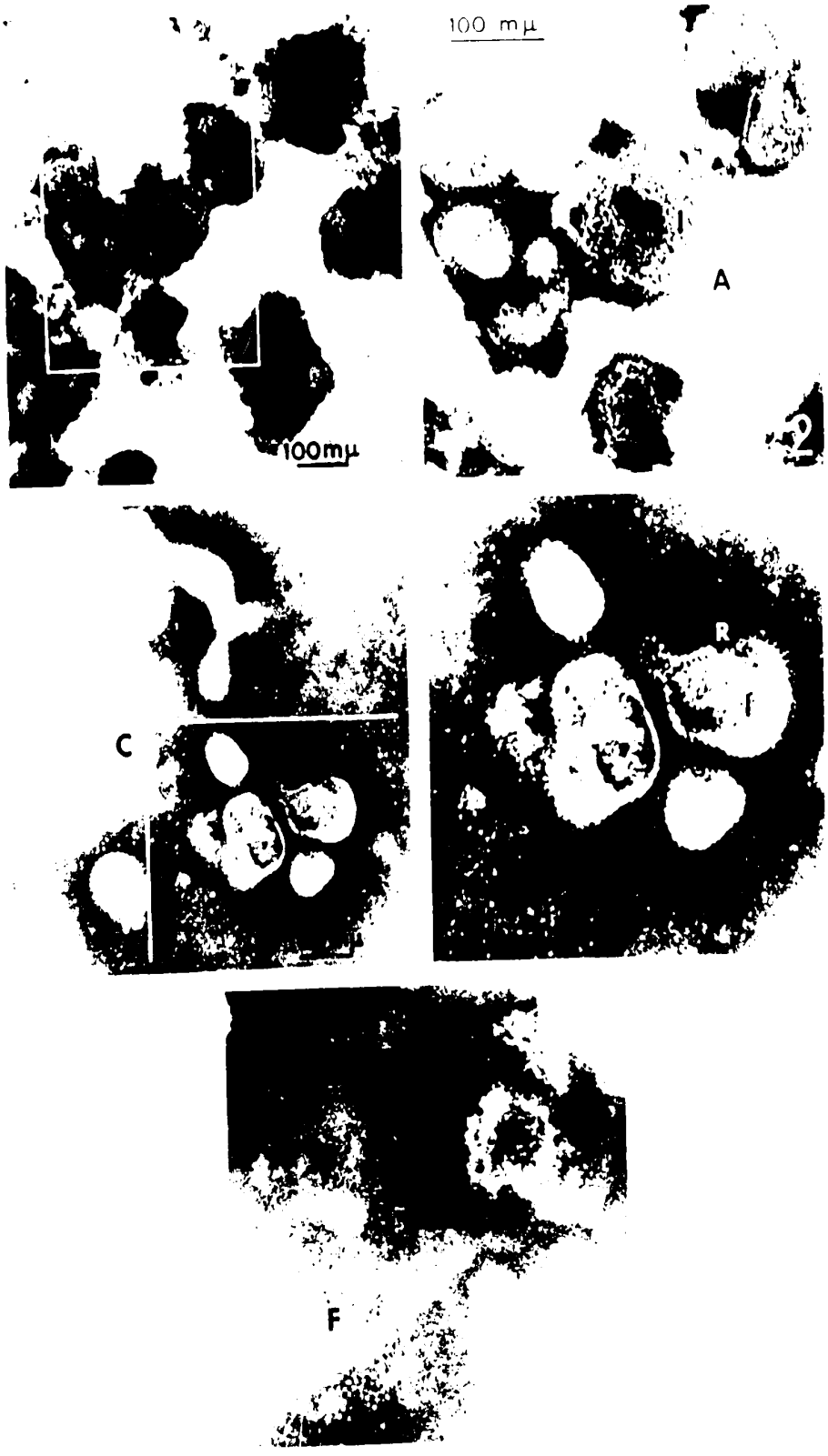
I - internal nucleoprotein.

C - fragment of virus envelope (rosette).

R - rupture in envelope allowing release of internal component.

F - fragments of surface projections.




100 mμ
100mμ
A
2
C
R
I
F

of many of the spikes and the approximate size of the beads. Similar structures in the form of beaded threads have been demonstrated in association with tissue cultures of mumps, NDV, Sendai and influenza viruses (Horne et al, 1960) and were thought to be molecules of mucoprotein inhibitor. The particles appearing in figures 3,4 & 5 are not connected, but appear to be distinct and separate entities, with a uniform size of approximately 5 mμ. Without further electron microscope studies combined with a study of the antigenic properties of these structures, further interpretations are not justified.

Surface active agents.

Detergents and saponins are powerful hemolytic agents (Springer, 1963) and cause profound alterations in cell surface structures (Newton, 1958). For this reason, the detergents, Tween 80 (de Thé & O'Connor, 1966), sodium deoxycholate and sodium lauryl sulfate (Laver, 1963) have been used for the disruption of influenza viruses. In order to compare the effects, three of the four most commonly used surface active agents were selected for testing. Unfortunately sodium lauryl (dodecyl) sulfate was not available at the time of the E/M studies and therefore could not be included.

These tests were designed to obtain evidence of structural changes withing the shortest time, therefore the

final concentration of these agents was arbitrarily selected as 0.5% and the time of incubation was kept to a minimum.

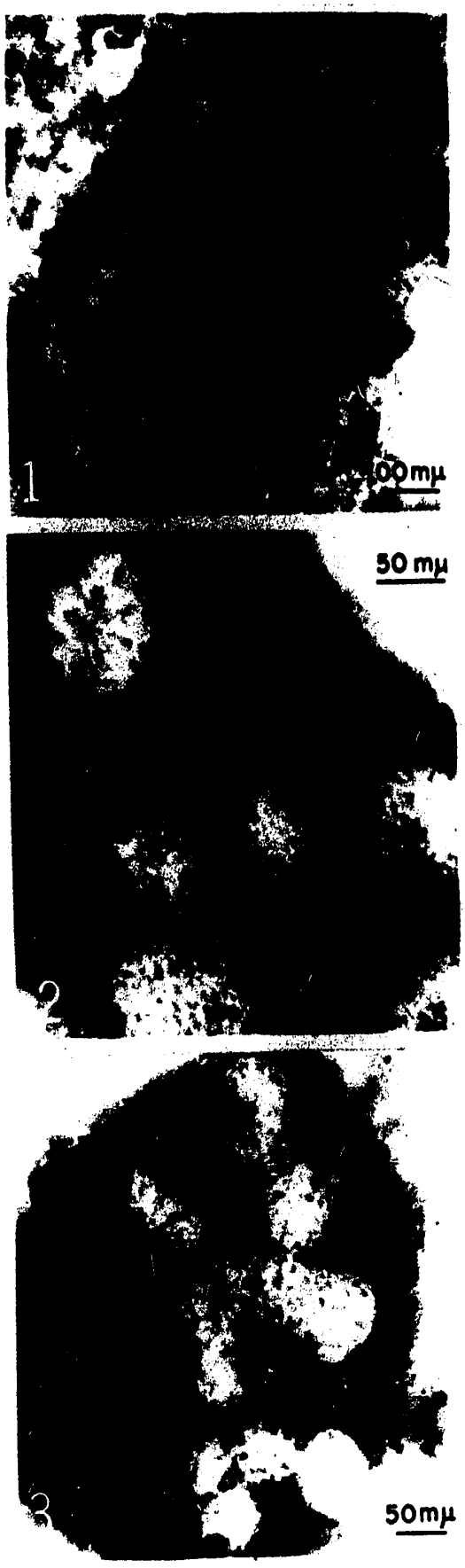
The effect of the surface active agents was studied at intervals of 1, 2 and 4 minutes, and the protein denaturants at 2, 5 and 10 minutes. Grids were prepared directly from the test medium during the treatments. At the appropriate intervals, the carbon-stabilized formvar grids were floated, coated side down, on the surface of the suspension to pick up a drop of test material. The excess fluid was drained with a piece of filter paper and the specimen was washed with distilled water, and negatively stained immediately with 2% PTA. Bovine albumin was not used to improve spreading of these preparations.

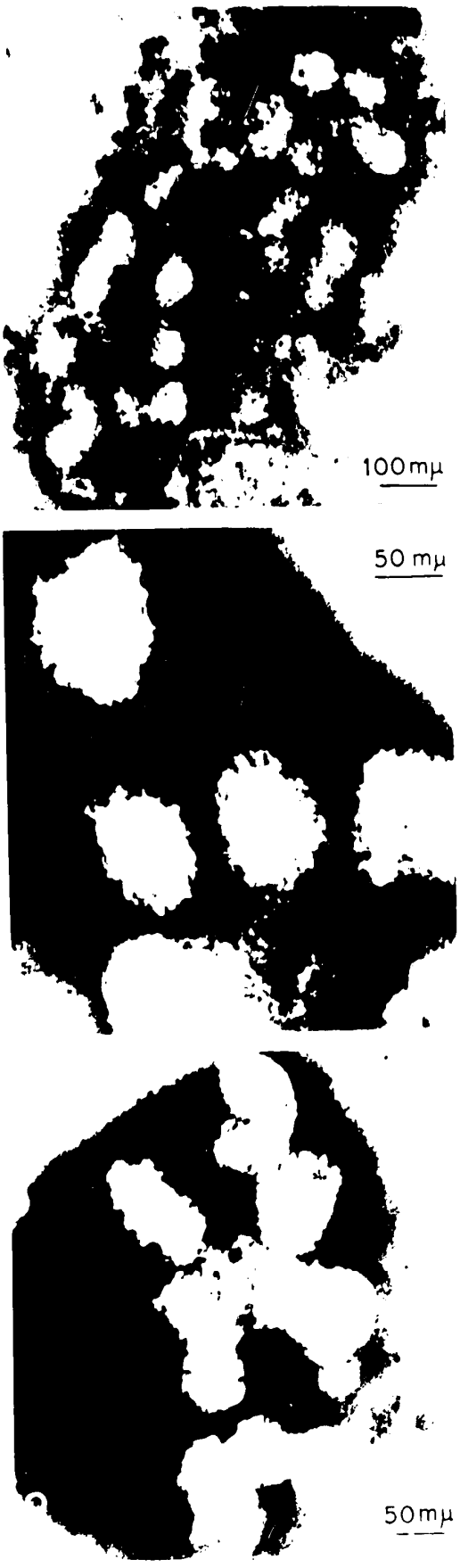
Tween 80.

The effect of 0.5% of the non-ionic detergent Tween 80 (polyethylinozide-sorbitanmonooleate) is shown in Plate 27. Figures 1, 2 & 3 represent treatments of 1, 2 and 4 minutes respectively. Within the first minute, the typical virus morphology has been obliterated. The particles have fused together, and a complete loss of surface structural integrity with no disruption of the membrane is evident. It is apparent that the detergent was too concentrated.

PLATE 27

The effect of Tween 80 on morphology of influenza virus particles after 1 minute (figure 1) 2 minutes (figure 2) and 4 minutes (figure 3).


1
50mμ
50 mμ
2
3
50mμ


100mμ
50 mμ
50mμ

Saponin.

0.5% saponin has a marked effect on the surface projections of the virus particle (figures 1-5, Plate 28). The particles appear clumped together, apparently by some interaction between the projections. There appears to be a fusion of structural components across the mid-section of these projections (figure 3, arrowed) which may then curl away from the internal components, as illustrated in figures 1 & 2. Thickening of the membrane at the base of the projections is not apparent and in figures 3 and 5, the projections have apparently disappeared in several areas revealing a smooth surface underneath.

After two and four minutes, there was very little debris or fragmented structural components. Rather than disrupting the virus particle into separate components, saponin has acted mainly on the envelop and surface projections, resulting in the loss of structural detail and the agglutination of the particles. Such an effect would not be of value in the production of structural antigens.

Sodium deoxycholate.

The disruption of virus particles with the release of internal component (figure 1) and fragments of the surface components (P) (figures 2 & 3) is illustrated

PLATE 28

The Effect of saponin on the morphology of influenza virus particles after 1 minute (figures 1 & 2), 2 minutes (figure 3) and 4 minutes (figures 4 &5).

R - Rupture of the envelope.

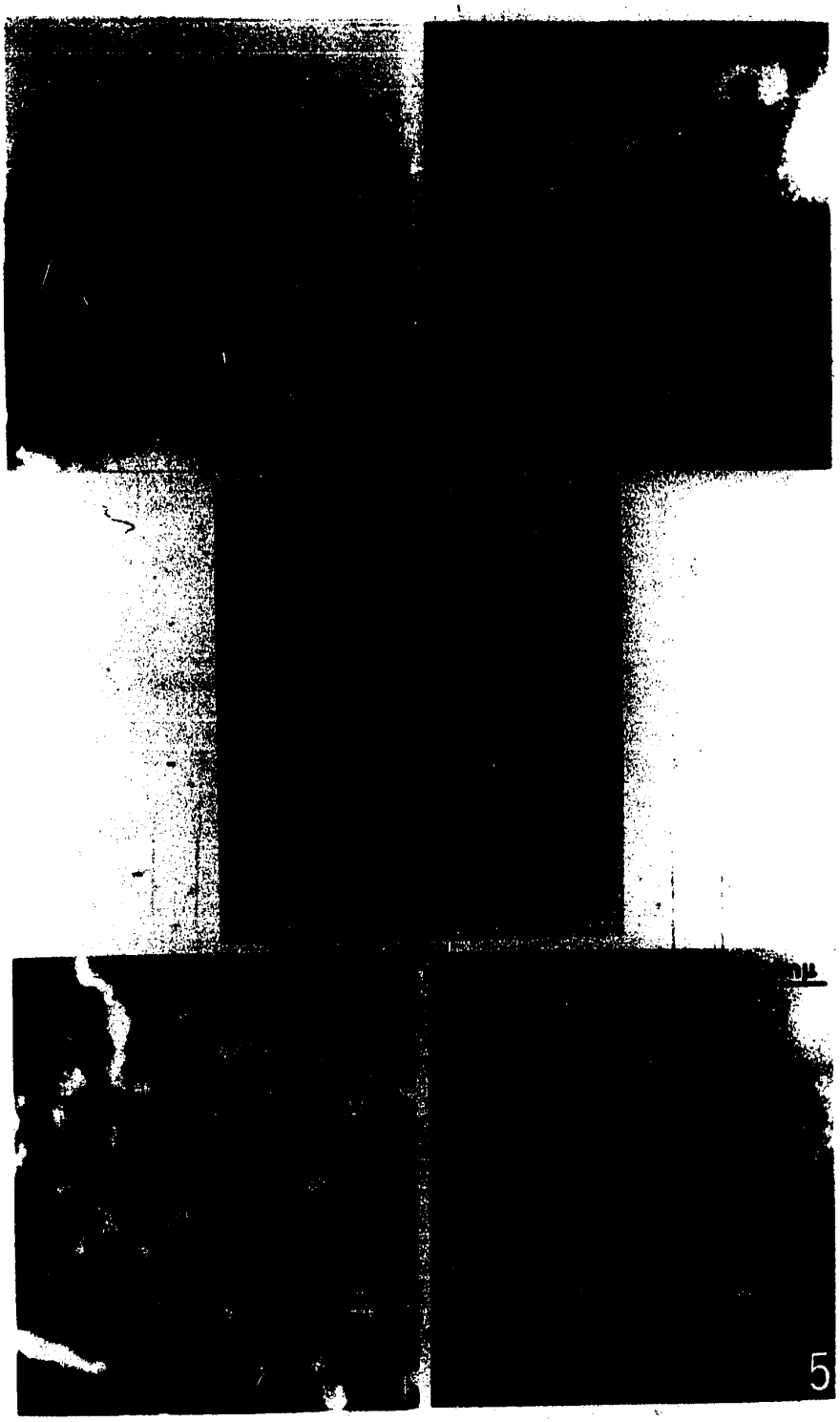
B - Formation of bridges between midsection of surface projections (figure 3).

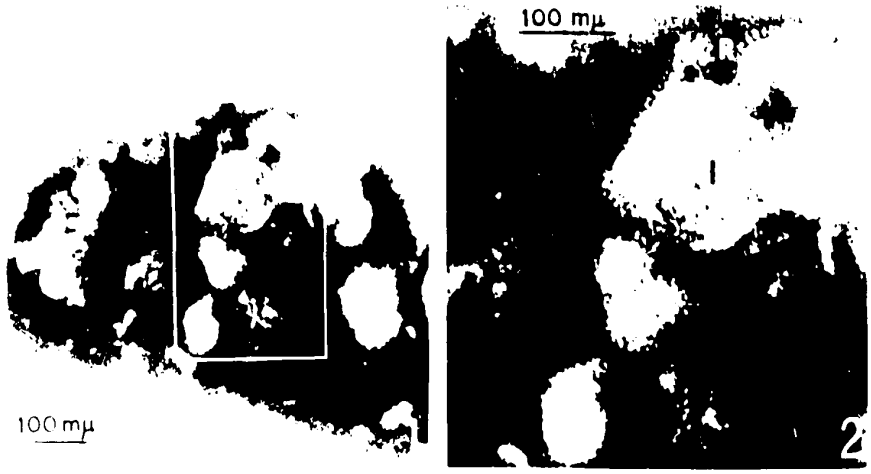
I - Internal nucleoprotein.

enza

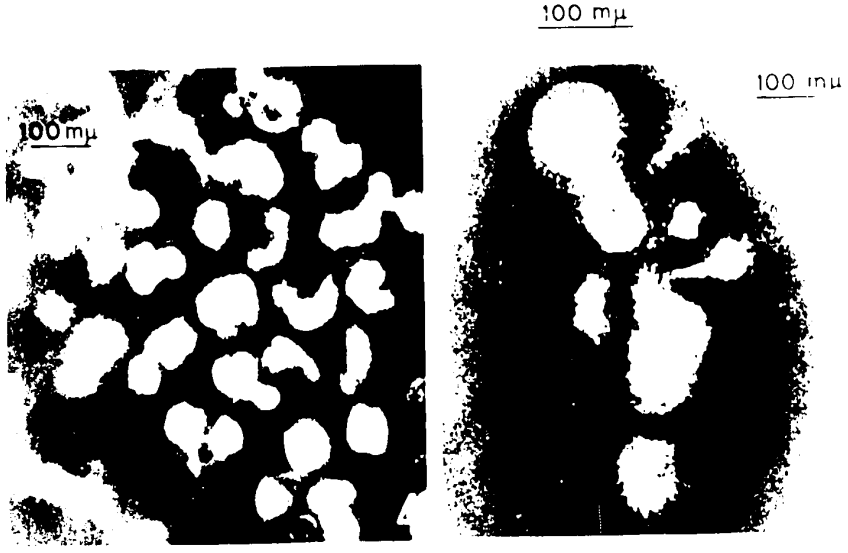
).

ns




100 mμ
100 mμ
2


B


100 mμ
100 mμ
100 mμ

in Plate 29.

It would appear, from figure 3, that the surface projections become detached from the underlying membrane, with subsequent formation of rosettes (figures 4 & 5). The fate of the internal nucleoprotein is not immediately apparent, however close inspection of the area in the lower left corner of figure 2 reveals a cluster of small particles, probably units of the surface projections, and in the area above, small fragments, which resemble the internal nucleoprotein structures described by Horne et al (1960).

The effect of sodium deoxycholate on the influenza virus particle shows the value of its use in the production of sub-units for analytical procedures. In addition, the treatment does not appear to drastically alter the structure of the sub-units morphologically as in the case of Tween 80 and saponin.

Protein denaturation.

Denaturation of proteins is the transition from a highly ordered to a less ordered state, and is accompanied by a loss of the biological activity. Since proteins were probably denatured by the preceeding disruption procedures, ultrasonication, the action of acids, bases, organic solvents and detergents (Karlson, 1963, Haurowitz, 1963), it was of

PLATE 29

The effect of Sodium deoxycholate on the morphology of influenza virus particles following

1 minute (figure 1), 2 minutes (figures 2 & 3) and 4 minutes (figures 4 & 5).

I - internal nucleoprotein.

P - fragments of virus envelope with projections.

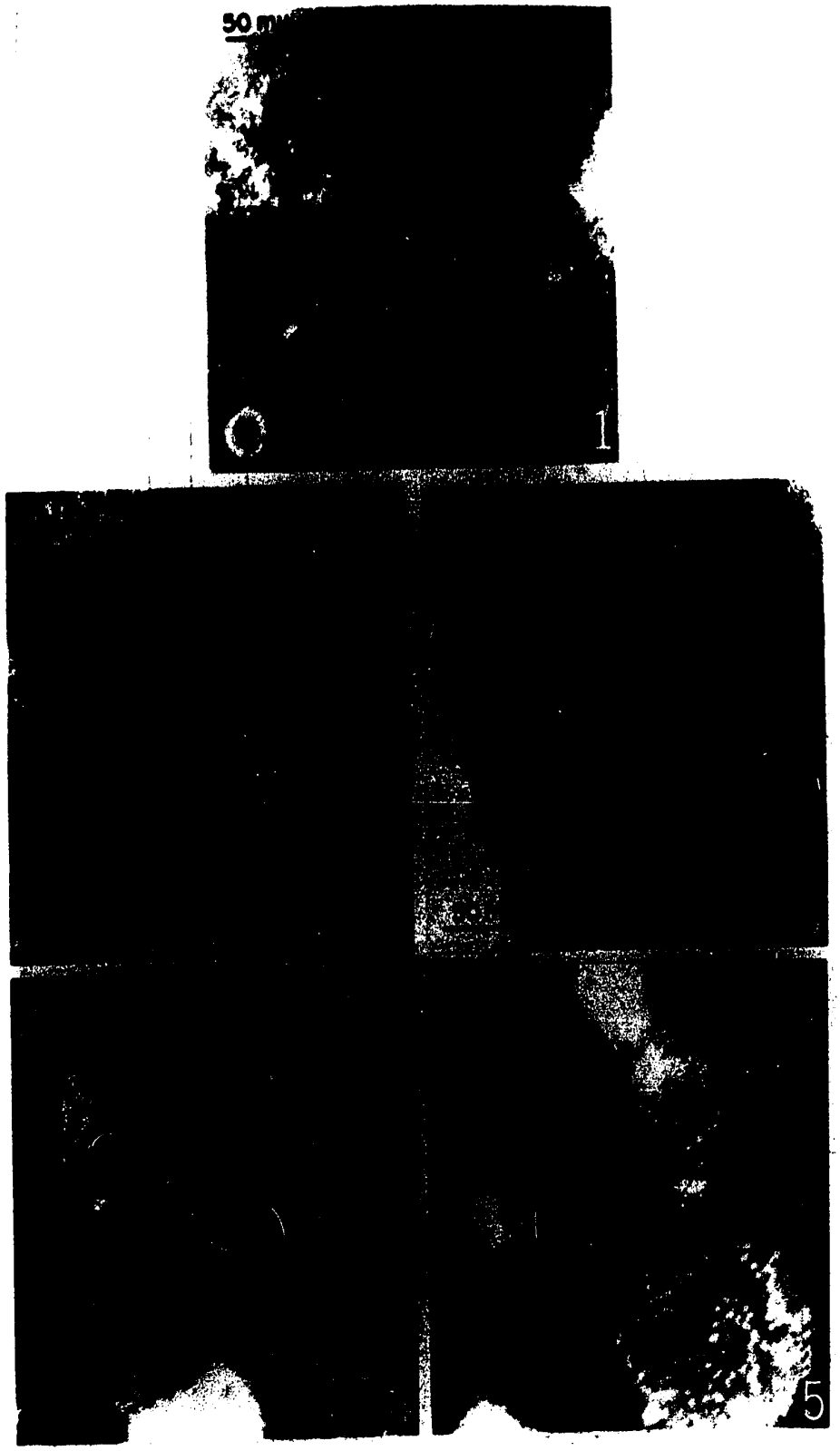
C - rosettes formed from surface projections.

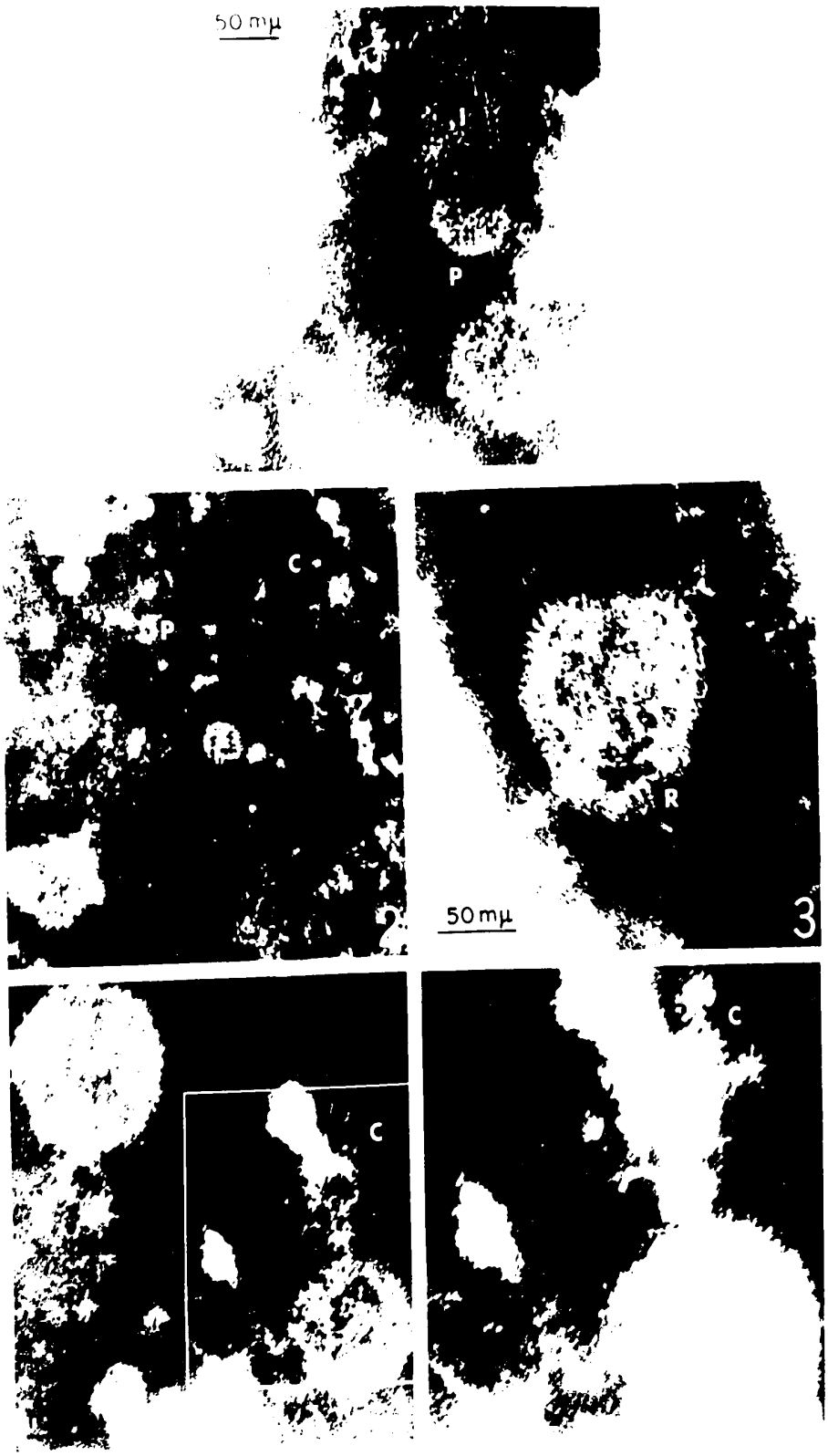
gy'

& 3)

pe

ace




50 mμ
P
C
P
R
50 mμ
3
C
C

interest to investigate the effects of the protein denaturants urea and guanidine hydrochloride on the virus morphology. A bacteriostatic effect of 6% urea, due possibly to cell surface activity was established in 1906 and reviewed in 1944 (Weinstein and McDonald, 1945). At high concentrations (6-8M urea and 4M guanidine HCl) both reagents are known to dissociate proteins into subunits. (Edelman & Poulik, 1961, Whitney & Tanford, 1962, and Kolthoff et al, 1957).

In these tests, 2% urea and 1% guanidine HCl were arbitrarily selected to investigate the effect of relatively weak concentrations on the protein subunits of the complex virus particle. The grids were prepared, as described for the surface active agents, at intervals of 2, 5 and 10 minutes.

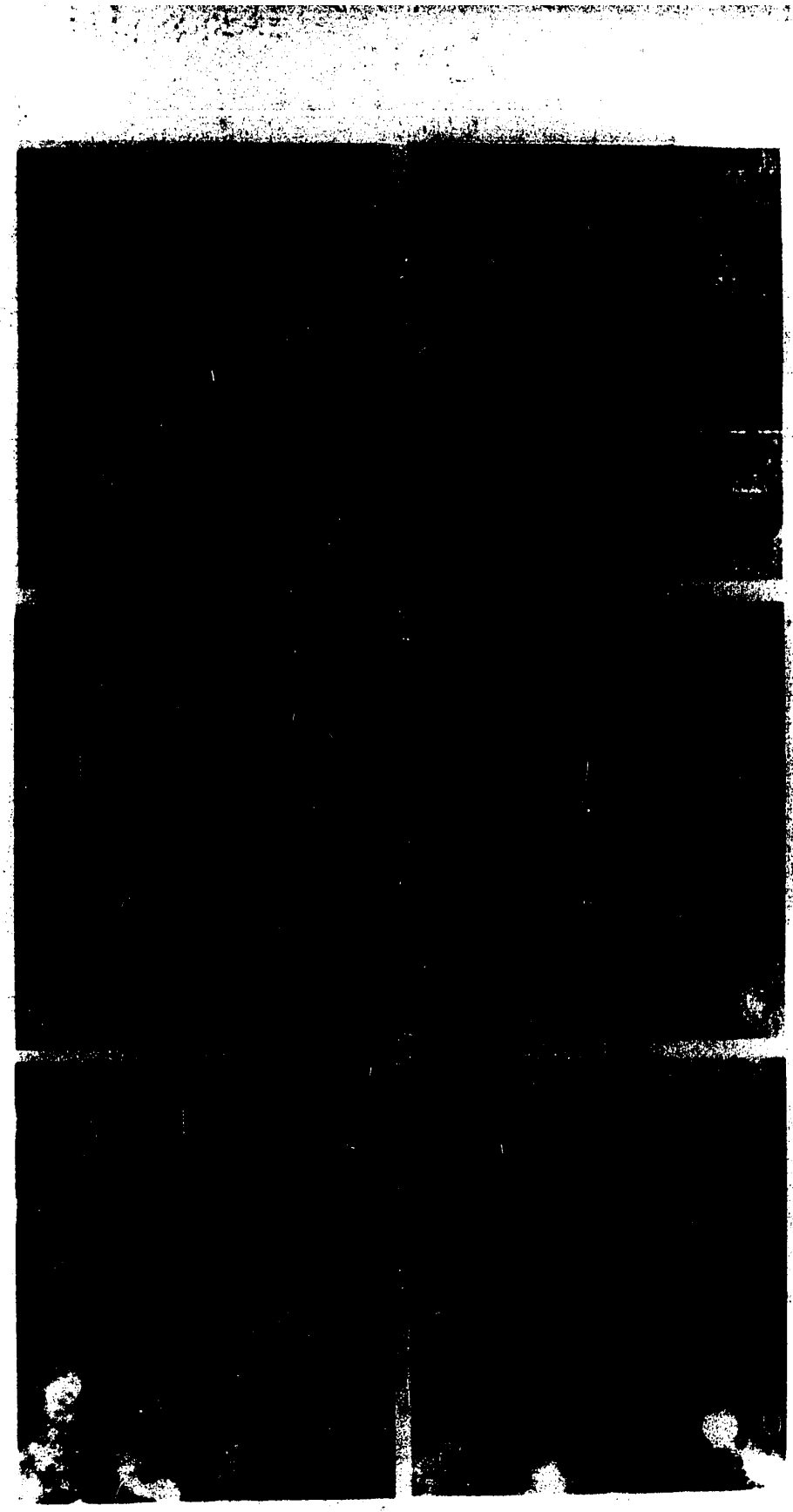
Urea.

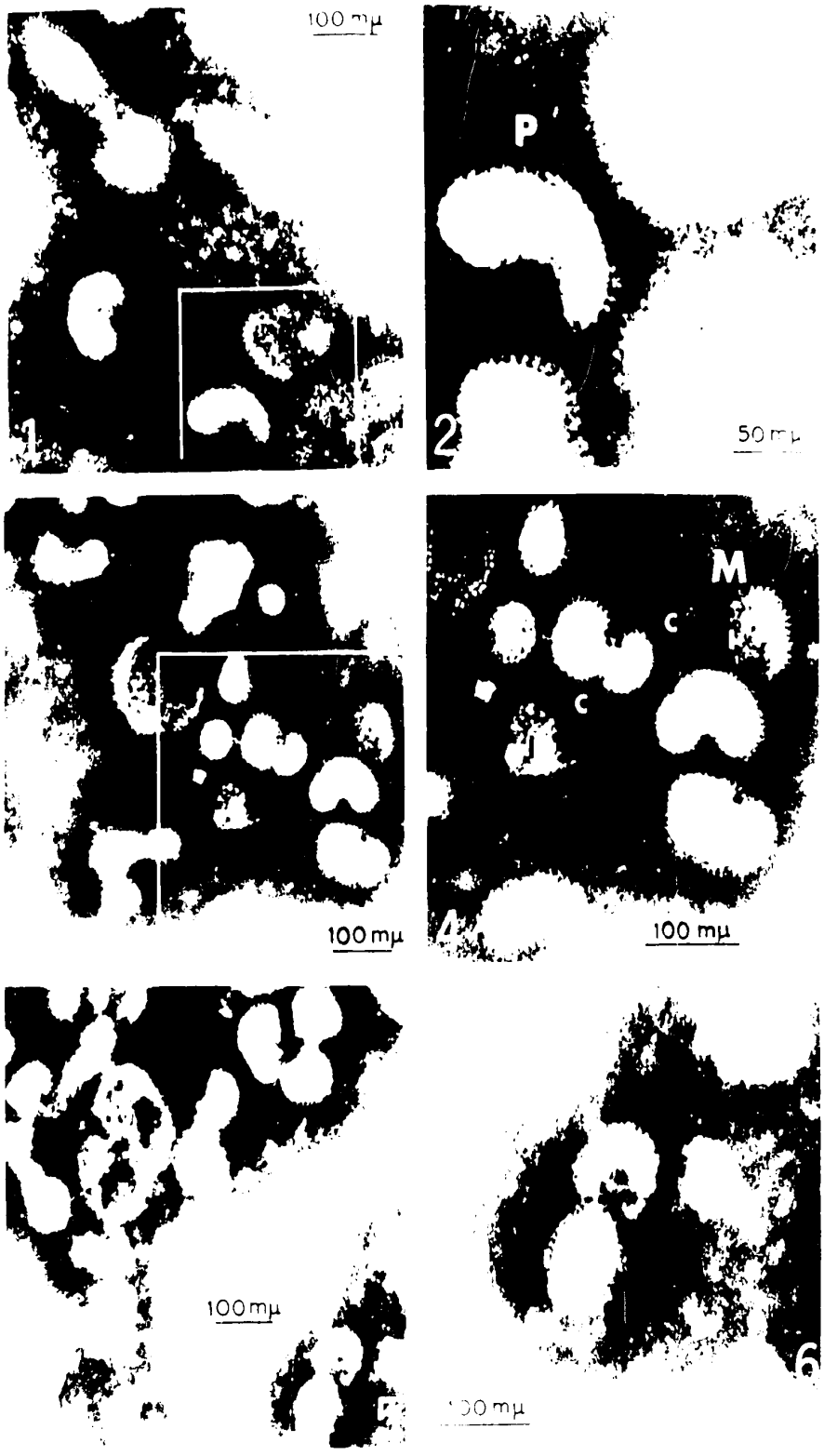
The effect of 2% urea on the virus particles is illustrated in Plate 30 (figures 1-6). Even after two minutes, several particles can be seen in the process of disruption. The structure of the internal component of these particles is evident in both figure 1 and, in more detail, in figure 2. It is also evident that the length of the surface projection (P) of the particles appears to have doubled (20 mμ) (figure 2). There appeared to be some

PLATE 30

The effect of urea on the morphology of influenza A virus after treatment for 2 minutes (figures1 & 2) 5 minutes (figures 3 & 4) and 10 minutes (figures 5 & 6)

P - surface projections which appear to be elongated.
C - 'rosettes' formed from envelope fragments.
I - internal nucleoprotein.
M - fragment of membrane associated with internal nucleoprotein.




100 mμ
1
P
2
50 mμ
M
C
C
100 mμ
100 mμ
100 mμ
6

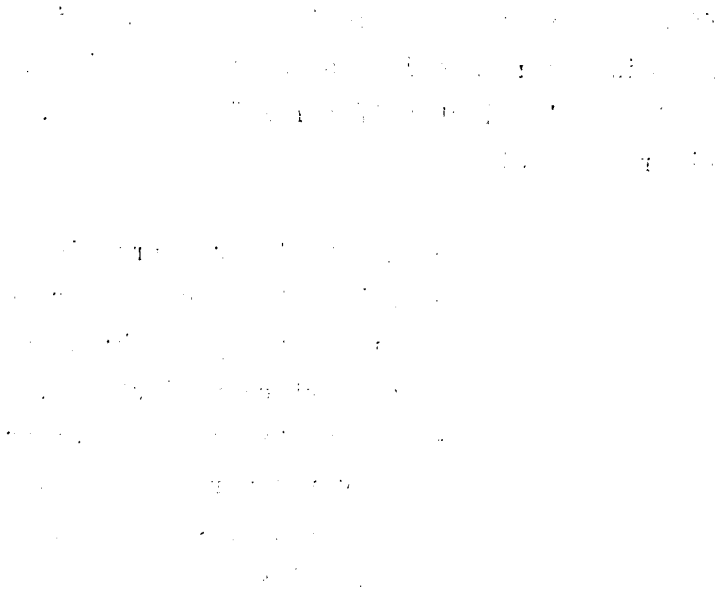
aggregation of particles which became more pronounced after 10 minutes (figure 5). The internal structure of the partially disrupted particle in Figure 4 (upper right) is quite similar to the helical structures described by Horne et al (1960) and Hoyle et al (1961). A fragment of membrane complete with surface projections has remained and appears to be closely associated (M) with internal components. Rosette formation is also evident following urea treatment (C). Small particles similar to those appearing after chloroform treatment can be seen, and their number seems to increase with the time of treatment with urea. It can also be seen that there is a loss of definition, especially of the finer structural details of the membrane and projections, which seem to be detaching from the internal component.

Guanidine hydrochloride (HCI).

After 5 minutes in 1% guanidine HCl, the appearance of virus structure was similar to that observed following urea treatment (Plate 31). The disruption of two filamentous particles can be seen in the upper right corner of figure 3.

The disruption of the membrane with partial release of the internal nucleoprotein is shown in figure 4, and several small sub-units (S) appear to have detached from the projection on the upper right. The increase in length

PLATE 31



Effect of treatment of influenza A virus with guanidine hydrochloride for 5 minutes (figures 1 & 2) and 10 minutes (figures 3 & 4) and trypsin (figures 5 & 6)

I - internal nucleoprotein

S - sub-units which appear to originate by the breakdown of surface projections.

F - indendations along surface of virus particle, possibly points of fusion of virus particles.

5

6


M
2
100 mμ
R
S
4
50 mμ

of the projections is also evident.

The action of dilute urea and guanidine HCl on the virus particle was not one of complete destruction, in fact, in every field examined there were intact virus particles. These appeared normal and would probably be infectious since Buckland and Tyrrell (1963) reported the action of 3M urea for 1 hour was required for complete influenza virus inactivation. In addition, since a concentration of 0.8M guanidine HCl has no denaturing effect on native serum albumin (Kolthoff et al, 1957), the action of this reagent at lower concentration could not be expected to achieve complete denaturation of virus protein. However, these studies demonstrate that some parts of the virus surface are susceptible to the action of dilute urea and guanidine HCl, and the nature of this action, on the basis of a morphological study, apparently differs from the action of the surface active agents, especially with regard to the elongation of the spikes.

The identity of the small particles is of interest and it is hoped that further work combining their isolation and purification with morphological, immunological and biological identification can soon be undertaken.

Trypsin.

Cleeland & Sugg (1964) reported that the 1-hour

trypsinization (250 µg/ml) at alkaline pH of influenza A/PR8, caused the complete inactivation of haemagglutinins without causing an appreciable decrease in the HAI antibody combining reaction. In addition, they found that non-infectious, non-haemagglutinating material was capable of inhibiting the neutralization of whole virus by specific antibody.

The results of treatment of the virus concentrate with 0.25% trypsin (pH 8.0) for 10 minutes at 37°C. are shown in Plate 31, figures 5 & 6. Pleomorphism is very pronounced, and there appear to be indentations (I) formed along the surface of the particles, showing a thickening of the layer at the base of the projections. Whether these are the sites of fusion or future rupture is not known. These patches are most evident in the long (800 mµ) virus particle in figure 5. The surface also seems to be bare in several areas and there is no structural evidence of the internal component. Some small particles appear in the background. Ruptures in the membrane are evident (upper right corner, figure 5), and there is aggregation of virus particles. However, this has been shown to result at alkaline pH. The state of aggregation shown in figure 6 is hard to interpret. There is a resemblance to the type of membrane curling which results in formation of rosettes. However at alkaline pH

the virus particles fuse into a giant viral structure (Plate 25, figures 5 & 6). The surface structures are clear but appear to be losing the definition, possibly indicating the enzymatic degradation of protein units.

Summary of Results of E/M Studies.

These studies were intended to give a preliminary indication of the action of a variety of reagents on the structure of influenza virus particles. It was not possible to interpret the results conclusively, but in some cases speculations could be made based on the observed effects. As a guide to the selection of reagents for virus disruption and production of structural antigens, these results have demonstrated that since each reagent appeared to have its own characteristic effect on the particle, each set of structural antigens may vary according to the reagent selected. Several reagents, however, did appear better suited for preliminary studies in which the most complete disruption with the least amount of structural damage is required. Therefore, techniques involving the use of urea, chloroform and sodium deoxycholate were selected. The enzyme pronase was substituted for trypsin since the pH conditions required were neutral. The anionic detergent sodium dodecyl sulfate was substituted for the non-ionic Tween 80, which appeared

to have the most destructive effect of those tested.

The results of the antigenic analyses of these preparations are described in the following section.

The effect of Disruption Procedures on Influenza Virus.

The breakdown of virus particles into structural components may be achieved by physical and chemical methods. In the preceeding experiments, attempts were made to evaluate the morphological effects of several disrupting agents with a view to selecting the most efficient procedure. The purpose of the present study was to evaluate the effect of the selected virus disruption procedures on the biological activity and antigenic structure of the virus particle. Unfortunately, the results of these procedures could not be evaluated by examination in the electron microscope.

The procedures used have been described in "Materials and Methods", and the resulting fractions were tested for haemagglutinating activity and infectivity. The effect of the treatments on the virus structural components was determined by the micro-immunodiffusion technique in cellulose acetate.

Storage at -20°C. (C_2St).

Samples of virus concentrate were tested following storage at -20°C. for 12 months with intermittent freeze-thaw cycles. There was complete loss of infectivity. There was a two-fold decrease in the haemagglutinating activity.

The immunodiffusion reaction of a freshly prepared concentrate (V-C_2) was compared in a parallel test with that of the stored concentrate, as shown in Plate 32 (figures 1& 2). The antiserum used was prepared in rabbits against virus concentrate (RaS-C_2) and contained host and virus antibodies.

In addition to the three precipitating components common to both reactions (1, 2 & 3) it can be seen that a new precipitin line (4, figure 2) may be detected following the storage breakdown of the virus. It is also evident that there are reactions of identity between the immune serum (1 & 3, figures 1 & 2) and the normal serum (RS) which supports the previous finding of antibody to influenza in the sera of normal rabbits. In both reactions, line 2 may be due to a reaction with host (H) antigenic components, either associated with the virus particle or present as debris. It was concluded that four virus antigenic components could be detected as a result of storage inactivation.

Heat.

Samples of virus concentrate were heated for 30 minutes at 45°C. (C_2-45) and at 56°C. (C_2-56). Infectivity was almost completely destroyed by 56°C., and there was a

100-fold reduction in the haemagglutinating activity. There was a 30% loss of infectivity as a result of heating at 45°C. The immunodiffusion reactions are shown in figure 3 (Plate 32) in a comparative pattern including unheated virus concentrate ($V\text{-}C_2$), using rabbit antiserum to soluble antigens (RaS/PR8). One antigenic component is detected in both heated preparations and links in a reaction of identity with the $V\text{-}C_2$ (arrow). Several weak reaction lines which appear in the control virus reaction are not evident after heat treatment. The decreased intensity of the reactions ($V\text{-}C_2 > C_245 > C_256$) suggests a progressive loss of antigenic activity. These reactions suggest that there are two heat-stable components present in the virus particle, one resistant to 56°C. and the other to 45°C.

Ultrasonication.

After 60 second ultrasonication of the virus particles, there was an almost complete loss of infectivity (99%). The immunodiffusion reaction of the sonicated virus concentrate (C_2S) was compared with an untreated $V\text{-}C_2$ preparation (Plate 32, figure 4). In this reaction, it can be seen that the non-precipitated protein in the area of four of the heavy reaction lines has not been completely washed, and has obscured the specific reaction. However, three components could be detected following ultrasonication

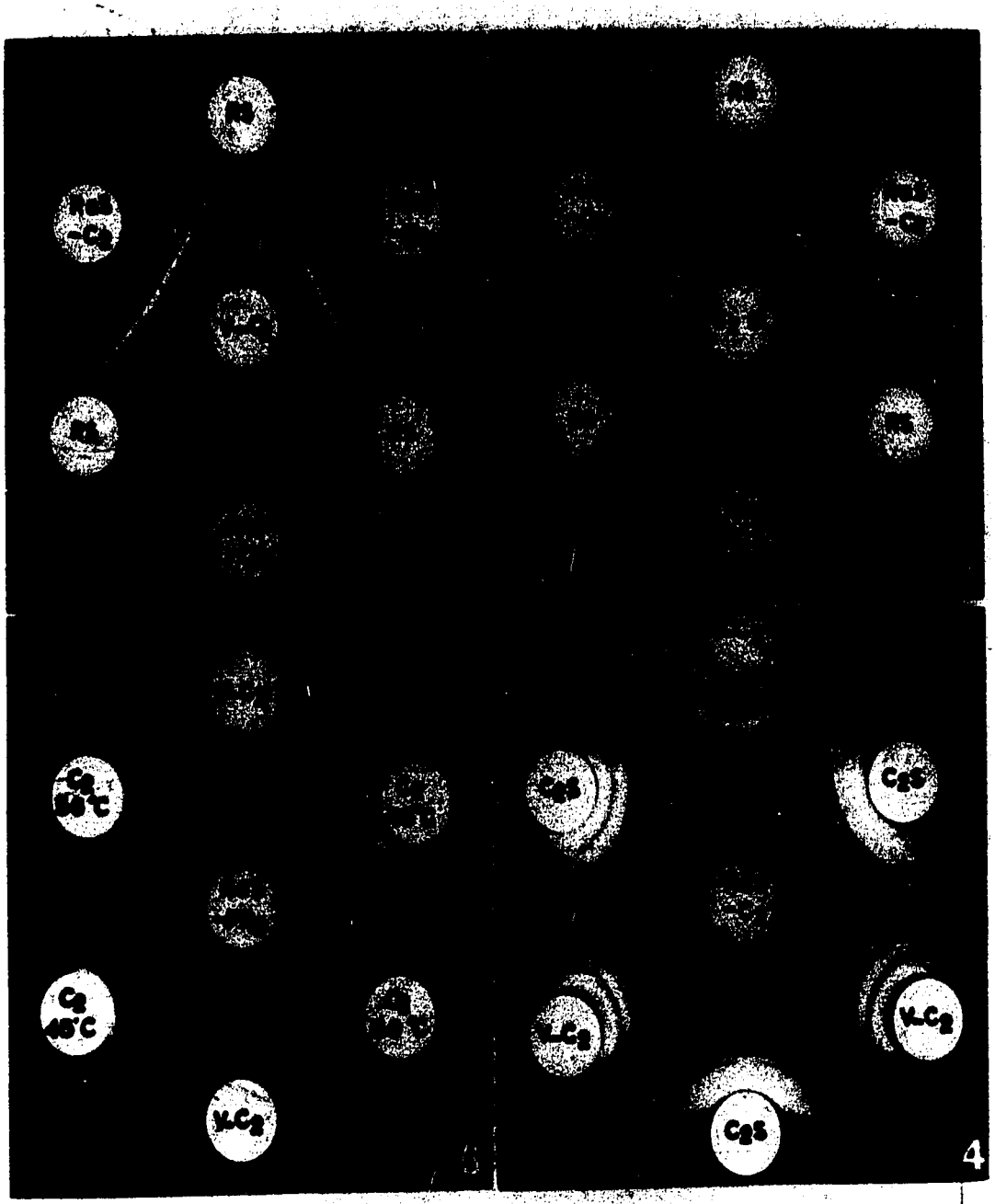
PLATE 32

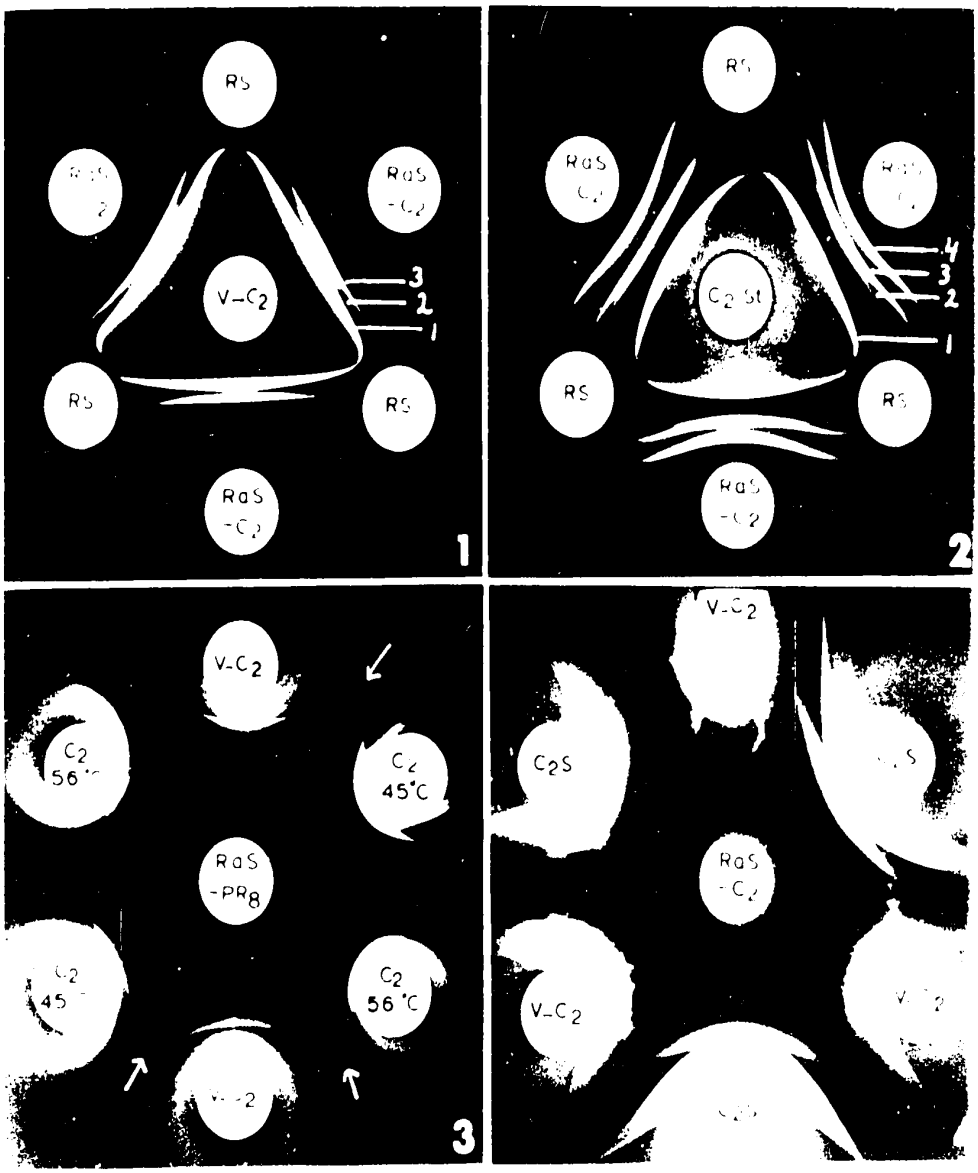
Results of Disruption of Virus Particle detected by Immunodiffusion reactions using specific antisera.

Figures 1 & 2 - The effect of storage on the breakdown of the virus particles detected by rabbit antiserum to virus concentrate (RaS/C_2)
$V\text{-}C_2$ - virus concentrate
C_2St - virus concentrate stored over a period of 12 months at 20°C with intermittent freeze - thaw cycles.

Figure 3 - The effect of heat on the antigens of the influenza virus particle. Reactions of identity are indicated with arrows.
C_2 - 45°C - virus concentrate heated at 45°C for 30 minutes
C_2 - 56°C - virus concentrate heated at 56°C for 30 minutes.
RaS/PR8 - rabbit antiserum & virus soluble antigens

Figure 4 - The effect of 60 second sonication on influenza A virus particles (C_2S) compared to untreated ($V\text{-}C_2$) using rabbit anti-virus serum (RaS/C_2)


C2S
4


RS
RaS
-C2
V-C2
3
2
1
1
RS
RaS
-C2
C2 St
4
3
2
1
2
V-C2
C2
56°C
C2
45°C
RaS
-PR8
C2
56°C
V-C2
3
V-C2
C2S
RaS
-C2
V-C2

and these showed reactions of identity with the V-C_2 reaction.

Urea.

Virus concentrate treated with 8M urea (C_2U) was tested with pan-specific antiserum (RaS), and the resulting reactions are shown in Plate 33 (figures 1 & 2). These comparative reactions show a) the complete loss of detectable antigenic components as a result of prolonged treatment (48 hours) (figure 1) and b) the presence of at least 2, possibly 4, precipitating components which have formed diffuse bands as compared to the control (C_2) (fig.2) suggesting a loss of antigenic specificity as a result of the denaturation of the proteins. A reaction of identity can be seen between one of the untreated V-C antigens and one of the released antigens (1).

Sodium deoxycholate (DOC).

Two fractions were obtained following virus treatment with 10% sodium deoxycholate: the pellet (DOC/C_3) of "intact" particles remaining and the supernatant (DOC/SNF) containing the disrupted components. Infectious virus was present in each fraction, however there was a 4 log drop in the DOC/C_3 titre and a 7 log drop in the DOC/SNF. Approximately 16% of the haemagglutinating activity was recovered

in the DOC/SNF fraction and less than 0.5% was present in the DOC/C_3. The results of the immunodiffusion reactions are shown in Plate 33 (figures 3 & 4). In the first comparison three components (1, 2 & 3) can be seen in the reaction of the untreated control virus concentrate (V-C_2). There is no reaction detectable between the DOC/C_3 and the pan-specific antiserum (RaS), nor is the presence of minimal amounts of antigen evident since there is no deflection of precipitin lines in the vicinity of the DOC/C_3 well. At least three, and possibly four, components can be counted in the DOC/SNF fraction which represent disrupted virus antigens. One of the precipitin lines shows a reaction of identity with a virus particle component (line 1). In a second reaction, comparing only the DOC fractions, at least five, possibly six, components can be counted in the DOC/SNF. In this reaction the supernatant fluid from the washed pellet DOC/C_3 was included, and the presence of viral components is indicated by the linkage with two of the DOC/SNF reactions. Again, the pan-specific antiserum (RaS) did not detect any antigens in the DOC/C_3 resuspended pellet.

Sodium dodecyl sulfate (SDS).

Treatment of virus concentrate with 10% sodium dodecyl sulfate (Laver, 1963) resulted in three fractions,

PLATE 33

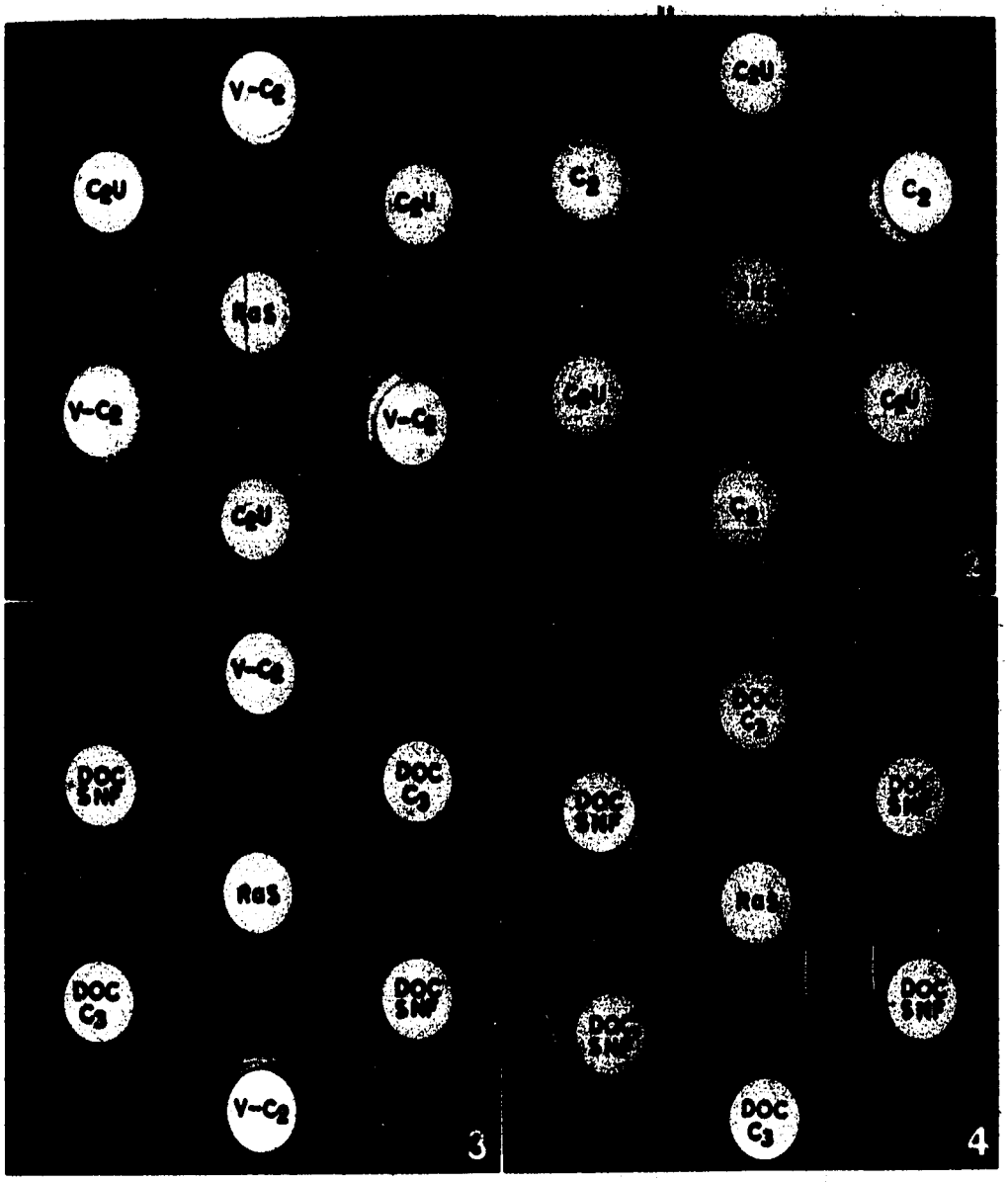
Results of the Disruption of Virus Particles detected by Immunodiffusion reactions using specific antisera.

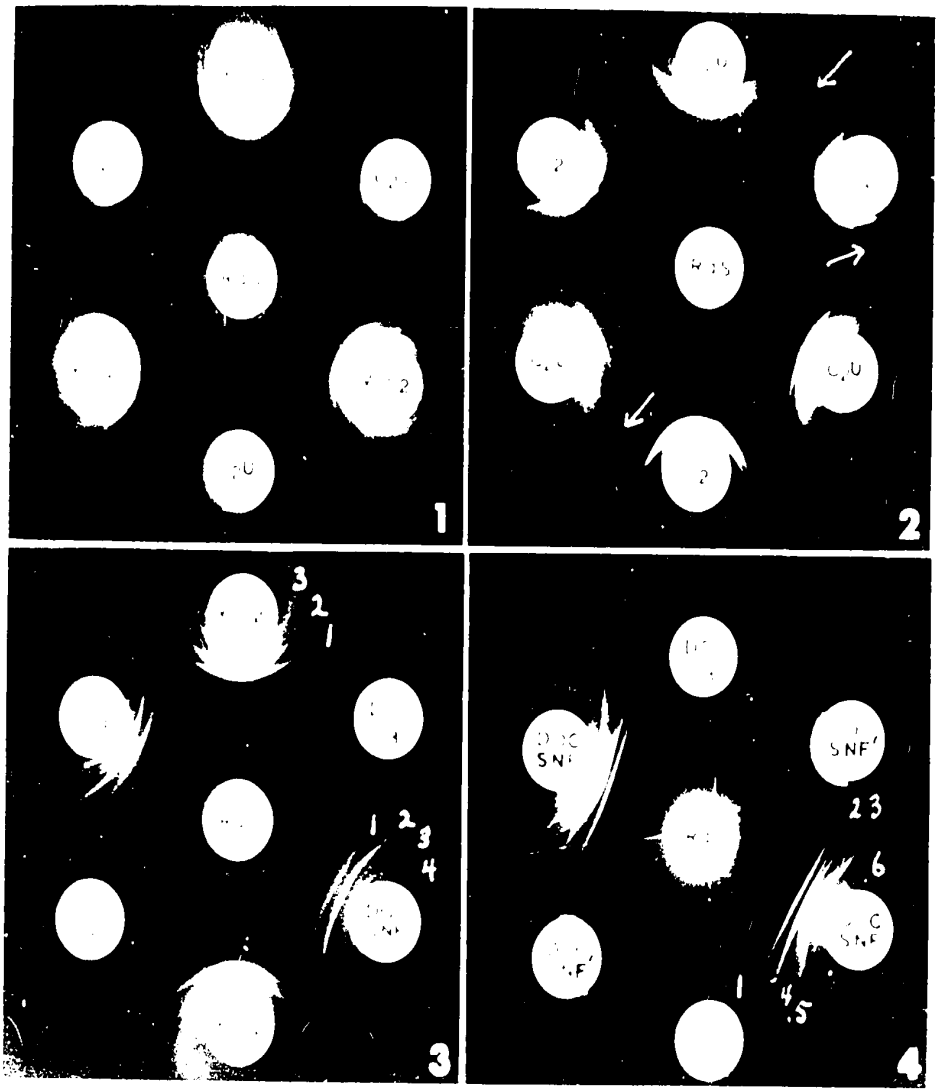
Figure 1 - Effect of prolonged (48 hrs) incubation of virus concentrate in the presence of 4 M urea (C_2U) compared to untreated virus concentrate ($V\text{-}C_2$)

Figure 2 - Effect of 2 hour treatment of virus concentrate with 4 M urea (C_2U) compared to control virus (C_2). Reactions of identity are arrowed. Rabbit pan-specific antiserum (RaS) was used to detect virus antigens in both reactions.

Figure 3 - Comparison of the antigens of untreated virus concentrate ($V\text{-}C_2$) with virus fractions obtained by treatment with sodium deoxychlate (DOC/SNF) and (DOC/C_3)

Figure 4 - Comparison of the antigens present in the DOC-treated virus fractions, DOC-SNF, DOC/C_3 and the SNF from the first DOC/C_3 wash, fraction DOC/SNF'. Rabbit pan-specific antiserum (RaS) was used to detect virus antigens in both reactions.


V-C2
C4U
C3
Ras
DOC C3
DOC SNF
3
4


1
2
3
4

SDS/C_3, and two supernatant fractions SDS/SNF-upper and SDS/SNF-lower. Infectious virus was not demonstrated in the SDS/SNF fractions and the titre of the SDS-C_3 sample was $10^{-2.5}$ representing nearly a 100% (8 log) loss of infectivity. The reactions shown in figure 1 of Plate 34 show that antigenic activity in the SDS/C_3 pellet was not detectable by the pan-specific antiserum (RaS). The reactions shown in figure 2 show the presence of four antigenic components in both the SNF-lower and SNF-upper fraction (three in the latter), however, the precipitin lines lack good definition and evidence of identity with the untreated virus controls is poor. These fractions were pooled since both showed antigenic activity, and the four antigens were still detectable. There is a suggestion of a linkage between one line (1) and a virus particle component. The diffuse reaction shown by the virus control in the lower right well of the pattern was thought to be due to the interference of residual SDS in the SNF fraction, which could be detected by hemolytic titrations, following 19 hours dialysis at 4°C. This problem had not occurred using the sodium deoxycholate surface active agent.

Methanol-chloroform (M-C).

The precipitate obtained following lipid extraction (Kates, 1961) was dissolved in acetic acid

(Eckert, 1966) and dialysed successively against acetate buffer (pH 4.5) and phosphate buffered saline (pH 7.4) at 4°C (see 'Materials and Methods'). According to the results described by Eckert approximately 15% haemagglutinating activity was recovered in this acetic acid fraction. In the immunodiffusion reaction shown in Plate 34 (figure 3), antigenic components in the acetic acid fraction were not detected using the pan-specific antiserum. Further comparisons were not made since the reactions of the other fractions were also negative. Infectivity was completely destroyed by this fractionation procedure.

Pronase (PRO).

Since the treatment of influenza virus with pronase results in shaving off the outer layer of the virus envelope (Reginster, 1965), leaving the smooth internal structures of the same size, it was expected that the antigenic activity would be divided into two fractions after high speed centrifugation. These fractions were obtained by the procedure described in Methods, and designated PRO/SNF and PRO/C_3. The control preparations were C_2C and SNF/C. No haemagglutinating activity was present in the PRO/SNF fraction, and 9% of the original activity was sedimented in the PRO/C_3. There was no significant change in the HA or infectivity of the virus control

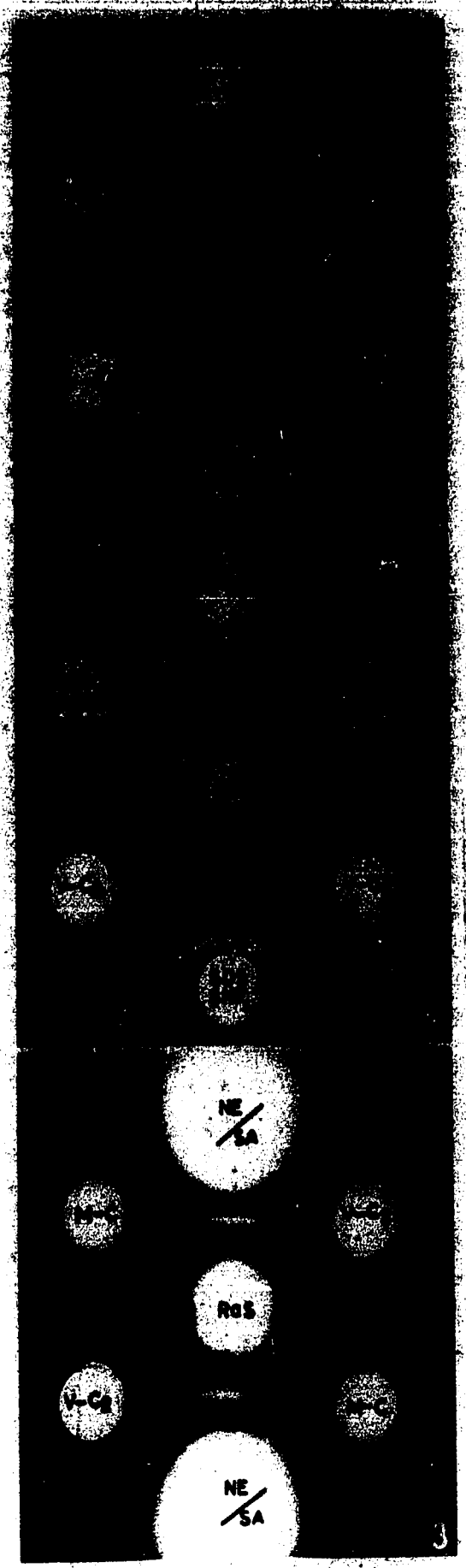
PLATE 34

Results of the Disruption of Virus particles detected by Immunodiffusion Reactions using specific antiserum (RaS)

Figure 1 - Comparison of the antigenic reaction of sodium dodecyl sulfate-sedimentable fraction (SDS/C_3) with untreated virus concentrate ($V\text{-}C_2$). SDS/C_3' = SNF from first washing of SDS/C_3 pellet.

Figure 2 - Comparison of immunodiffusion reaction of sodium dodecyl sulfate non-sedimentable fraction (SDS/SNF) with untreated virus concentrate ($V\text{-}C_2$)

Figure 3 - Analysis of virus particle antigens obtained by methanol-chloroform treatment followed by acetic acid (M-C). No reaction is detectable between the M-C fraction and the pan-specific antiserum (RaS).


NE/SA
RaS
NE/SA

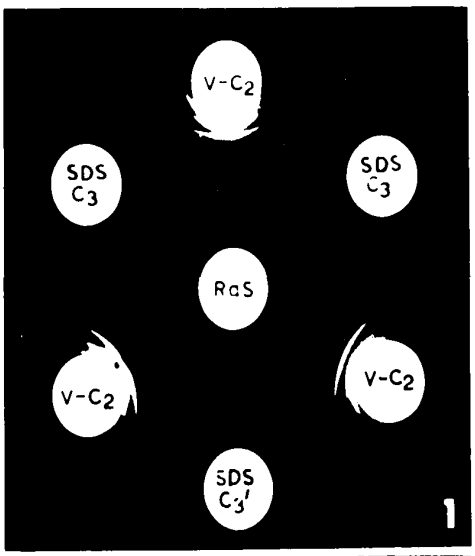
ected
serum

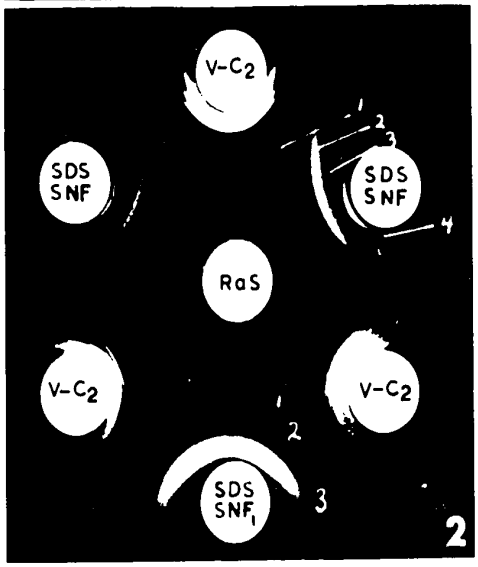
on
able
irus
rom

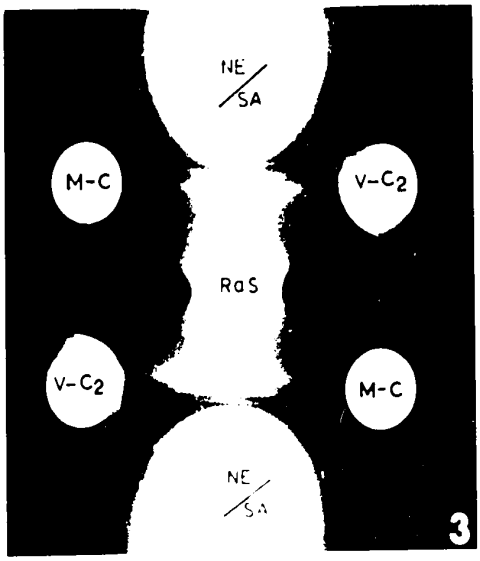
tion
men-
eated

s

an-







preparation. The results of infectivity titrations showed that no infectious virus was present in the SNF fraction and the titre of the PRO/C_3 fraction had dropped from the original $10^{10.33}EID_{50}$/0.1ml. to $10^{6.5}EID_{50}$/0.1 ml. In the immunodiffusion reactions of these fractions, shown in Plate 35, it can be seen that three antigenic components (1, 2 & 3) can be detected in the PRO/C_3 fraction. Two of these (2 & 3) appear to link with a component of the untreated virus control (C_2C).

The PRO/SNF fraction also contains three antigenic components detectable by RaS/C_2. Of these components, two show diffuse reactions of partial identity with the control C_2C reaction (a & b). These diffuse reactions might indicate a loss of antigenic specificity as a result of enzyme degradation of the antigens, especially since no precautions were taken to inactivate the pronase activity at the time of the virus treatment.

PLATE 35

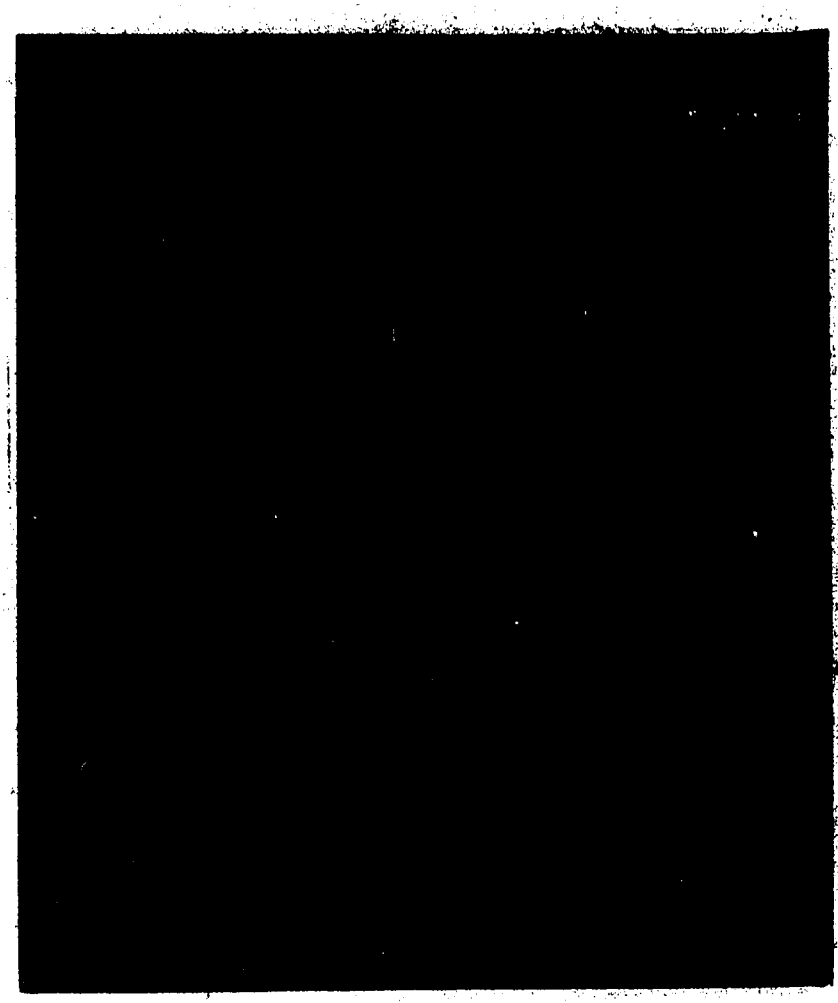
The effect of treatment with pronase, on the virus particle. There is a reaction of identity (2 & 3) between the control virus concentrate (C_2C) and the enzyme-treated virus pellet (PRO/C_3).

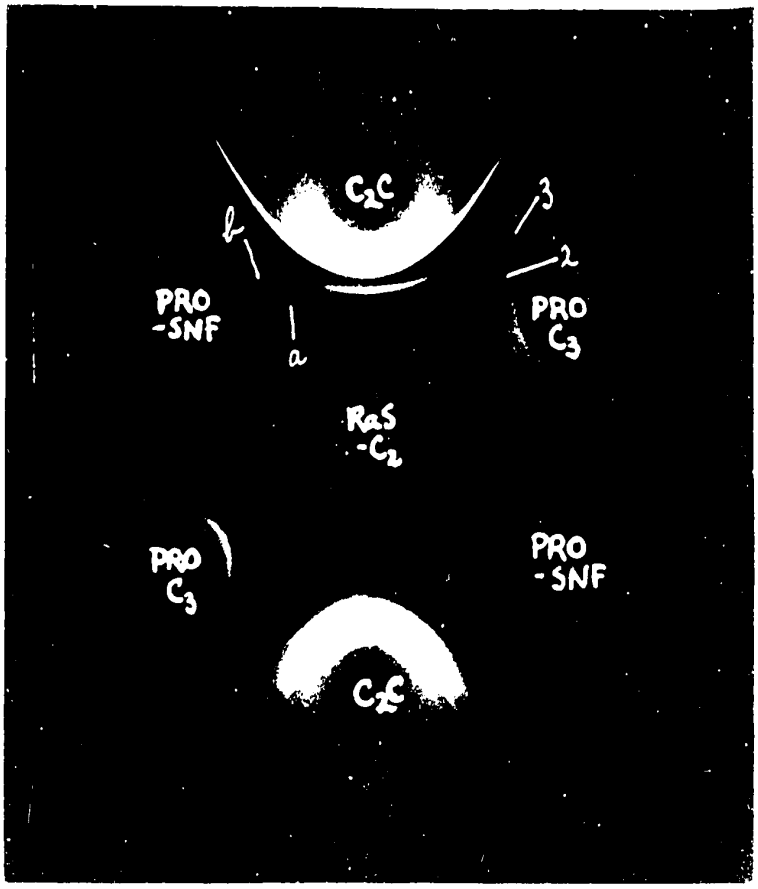
A reaction of partial identity (a&b) can be seen between the reaction of the C_2C and the supernatant fluid after enzyme treatment (PRO/SNF).

rus

btween

a




C2C
3
2
1
b
a
PRO
-SNF
PRO
C3
RaS
-C2
PRO
C3
PRO
-SNF
C2C

Conclusions.

The release of virus particle antigens, following the treatment of virus concentrate ($V\text{-}C_2$) by eight different procedures, was investigated using the micro-immunodiffusion technique in cellulose acetate. The efficiency of the disruption of the virus particle was evaluated on the basis of: a) the number of antigenic components detected following the treatment, b) the intensity and resolution of the reactions of the released antigens with specific antisera, c) the relationship of the reactions of released components to the reactions of untreated virus particles, and d) the presence of biological activity, such as haemagglutination or infectivity, in the fractions. A brief summary of the results is shown in Table 15. It was concluded that treatment of the virus particles by the methanol-chloroform procedure was not satisfactory. Treatment with urea appeared to show promise, however further tests were discontinued since the antigenic activity was lost after 48 hours, and a new batch of $V\text{-}C_2$ was not available. Samples of stored virus (C_2St) were not available for further analysis, however, these results indicated that at least one antigen is released following breakdown of the particle at -20°C. Further antigenic studies on the effect of other temperatures (i.e. 4°C. and

TABLE 15

SUMMARY OF RESULTS OF VIRUS DISRUPTION PROCEDURES

Virus Concentrate Samples after Treatment	Maximum Number of Antigens Detected	Quality of Immunodiffusion Reaction	Haemagglutination Titration % Change	Infectivity EID_{50}/0.1 ml. Before	Infectivity EID_{50}/0.1 ml. After
Storage	4	good	no change	$10^{10.7}$	0
Heat 45 C	2	weak	-30	$10^{10.5}$	$10^{9.0}$
Heat 56 C	1	weak	-90	$10^{10.5}$	0
Ultrasonication	3	good	no change	$10^{10.3}$	$10^{7.2}$
Urea	4	diffuse	N.T.*	N.T.	N.T.
DOC $-C_3$	0	no reaction	-99.5	$10^{10.5}$	$10^{4.5}$
DOC -SNF	6	good	-84	$10^{10.5}$	$10^{4.2}$
SDS C_3	0	no reaction	no HA.	$10^{10.5}$	$10^{2.5}$
SDS SNF	4	diffuse	N.T.	n.s**	0
M-C (HAc fraction)	0	no reaction	no HA.	N.T.	N.T.
Pronase C_3	3	fair	-91	$10^{10.3}$	$10^{6.0}$
Pronase SNF	3	one good, two diffuse	0	n.s.	$10^{0.5}$

* N.T. - not tested ** n.s. - no sample available

37°C) on the release of viral antigens are planned.

It can be seen from these results that the most efficient disruption of the virus particle was achieved by treatments with sodium deoxycholate, sodium dodecyl sulfate, pronase or sonication. The next step in the investigation was the analysis of the antigens released by these procedures in order to identify the virus structural antigens.

Identification of Influenza Virus Structural Components.

The identification of the virus structural antigens was complicated by host components present in the virus concentrate either as integral component or as a non-specific host debris (Plate 7, page 82). Since it was not possible to determine the nature of the association between the host antigens and the virus particle without more elaborate virus purification procedures, these antigens were provisionally classified as "structural antigens" with virus or host (antigenic) specificity. The first step in the analysis of such antigenic components released by the disruption of the virus particles was to identify the host components (HS) and the virus specific (VS) components. For this purpose specific antisera were prepared as follows: 1) rabbit antiserum specific for virus antigens was produced by induced infection with influenza A/PR8 (RaS/IN), and contained no antibodies to host (egg) antigens; 2) rabbit antiserum was produced by parenteral immunization with a V-C_2 preparation, and contained antibodies to both host and virus antigens (RaS-C_2); 3) rooster antiserum was produced by parenteral immunization with V-C_2 and contained antibodies to virus structural antigens, and showed no reaction with any host antigen preparations (RSaS-C_2). The serological characteristics of these antisera are presented in the

following section, Table 16.

The reactions are presented in Plate 36 (figs. 1-6). It can be seen that: 1) in each reaction the greatest number of precipitating components are detected by the RaS/C_2 antiserum, and 2) in all the reactions, except in figure 6, at least one of the components in the RaS/C_2 reaction complex is not detected by the virus specific antisera, RSaS/C_2 and RaS/IN. These components were provisionally identified as the host strutural antigens.

The reactions of identity which formed between the reactions of the other reacting components served to identify the virus specific structural antigens, released by the disruption of the intact virus. These virus structural antigens were designated the 'VS' antigens (Virus Structural).

In the immunodiffusion reactions of both the sonicated virus (C_2S, fig. 1) and the heated virus preparations (C_256^o, figure 2), the presence of both 'HS' and 'VS' antigens could be detected. The loss of antigenic activity by heating is evident by the decrease in the intensity of the reaction. The presence of the two 'VS' antigens, one strong reaction and one very faint (2), was demonstrated by the rooster antiserum (RSaS-C_2).

In the reaction with sonicated virus (figure 1) the two strong lines developed by the RaS-C_2 antiserum were identified as 'VS' reactions, by reactions of identity with the RaS/IN antiserum, and the weak precipitin line developing between the two was provisionally identified as the host (HS) reaction line, since a comparable component was not detected by the other antisera.

In figure 3, the reactions of the DOC/SNF antigens, at least four antigens are detected by the rabbit antiserum (RaS-C_2). The three 'VS' components are identified by the reaction of identity with the specific rabbit antisera (RaS/IN). The remaining component is previsionally identified as a host (H) antigen.

The rooster antiserum also indicates the presence of three virus antigens (VS) and these reactions can be seen to fuse into faint reactions of identity with the former RaS/IN reactions. Therefore, three of the DOC/SNF antigens are identified as virus structural antigens.

In the reactions of the SDS/SNF fractions (fig. 4) it can be seen that four antigens are detected by the rabbit anti- C_2 serum (RaS/C_2).

Three of these reactions link with the reaction

of the rabbit convalescent serum, and are therefore identified as 'Vg' components. Three antigens are also detected by the rooster antisera, and one of these can be seen to link with the RaS/IN reaction.

A diffuse band of precipitate (B) can be seen in the reactions of rabbit antiserum (RaS/IN) with SDS/SNF and this band shows reactions of partial identity with the leading precipitin reactions of both the rooster ($RSaS/C_2$) and rabbit (RaS/C_2) antisera, suggesting that: 1) a new component not detectable in any of the other virus antigens has been revealed by the specific rabbit antiserum (RaS/IN), or 2) the residual detergent (SDS) contained in the SDS/SNF antigen fraction has denatured some of the serum proteins non-specifically, thus forming the diffuse ring of precipitate. It is believed that the latter explanation is correct for two reasons: a) the precipitin line is not characteristic of reactions with the rabbit convalescent serum, with respect to the position or intensity, and b) the band appears to be the same width and intensity throughout, which is not a characteristic of specific ag-ab reactions. Therefore, it was concluded that SDS fractionation produced four antigens detectable by immunodiffusion reactions with specific antisera. One of these antigens is host specific, and the other three are virus structural

antigens.

Following DOC and SDS treatment, the antigenic activity was detected only in the supernatant fluid after high speed centrifugation (35,000 g. x 60 mins.) It was evident these treatments caused virus disruption with the release of slower sedimenting viral components. Immunodiffusion analysis revealed that these fractions contain four antigenically distinct sub-units which could be characterized as virus structural or host components, by means of reactions with specific antisera.

Enzymatic treatment with pronase, however, resulted in a different separation of components, as is shown in the reactions presented in figures 5 and 6. Four precipitin lines can be seen in the reaction of PRO/SNF with RaS-C_2 (figure 5), of which only one reaction line can be positively identified as virus specific, by its reaction of identity with RaS/IN and RSaS/C_2. The identity of the other three lines cannot be established with certainty, however since comparable antigens are not detected by either the specific rabbit or rooster antisera, they are provisionally identified as host antigens (H).

In the immunodiffusion reactions of the pronase-treated virus fraction (PRO/C_3), which was sedimented at

35,000 g. x 60 minutes (figure 6), one antigenic component is detected by the rabbit antiserum (RaS/C_2) and identified as a 'VS' antigen by its reaction of identity with the specific RaS/IN antiserum. A second VS component is detected by the specific rabbit (RaS/IN) and rooster (RSaS/C_2) antisera. The pronase-C_3 fraction apparently contains two antigens, only one of which is detected by the rabbit anti- C_2 serum. This finding is contrary to all the previous results which have shown that the rabbit antiserum (RaS/C_2) was capable of detecting more antigenic components than the rooster anti- C_2 serum. No explanation for this reaction can be given without further investigation.

These immunodiffusion reactions established the identity of the 'VS' structural components detected in each fraction. The comparison of these structural components (VS) using the specific rooster antiserum (RSaS-C_2) is shown in Plate 37 (figures 1-3).

It can be seen in the three reactions, that at least two, in some cases four, virus structural antigens (VS) are detected by the rooster antiserum (RSaS/C_2). In figures 1 & 2, no reactions of identity have developed between the antigens of the different fractions.

PLATE 36

Identification of Host and Virus Specific Antigens using the following specific antisera:

RaS/C_2 - rabbit antiserum prepared against virus concentrate (V-C_2).

RaS/IN - rabbit antiserum prepared by induced intranasal infection with influenza A/PR8.

R&aS/C_2 - rooster antiserum prepared against virus concentrate (V-C_2).

Figure 1 - The immunodiffusion reaction of virus particles antigens released by 60 second sonication (C2S).

Figure 2 - The reaction of viral antigens heated at 56 C for 30 minutes ($C_2$56 C).

Figure 3 - The reaction of viral antigens present in the supernatant fluid fraction following sodium deoxycholate treatment (DOC/SNF). Four components can be counted, three of which are virus specific.

Figure 4 - The reactions of viral antigens present in the supernatant fluid fraction following sodium dodecyl sulfate treatment (SDS/SNF). The band of non-specific precipitation (B) is due to the presence of SDS detergent in the fraction. Four components can be counted, three of which are virus specific.

Figure 5 - The reactions of viral antigens present in the supernatant fluid after pronase treatment (PRO/SNF). Three antigenic components can be counted, one of which is virus specific.

Figure 6 - The reactions of the sedimentable viral antigens following treatment with pronase (PRO/C_2). Two virus specific antigens are detected by the rooster antiserum and the convalescent antiserum of the rabbit. One of these is also detected by the rabbit anti-C_2 serum.

sing

st

duced
enza A/PR8.

nst

rticles
m (C2S).

56 C

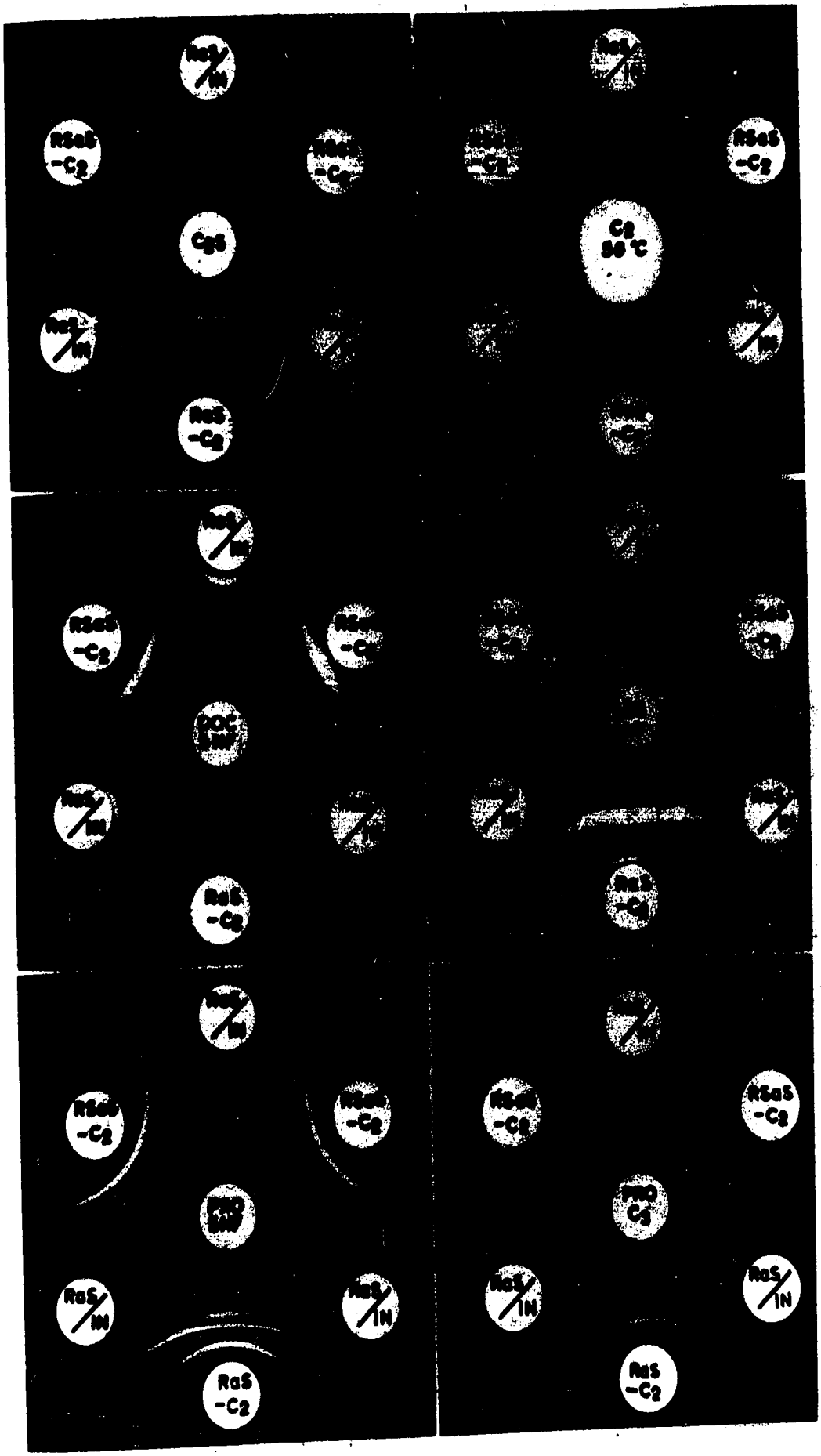
n the
lium

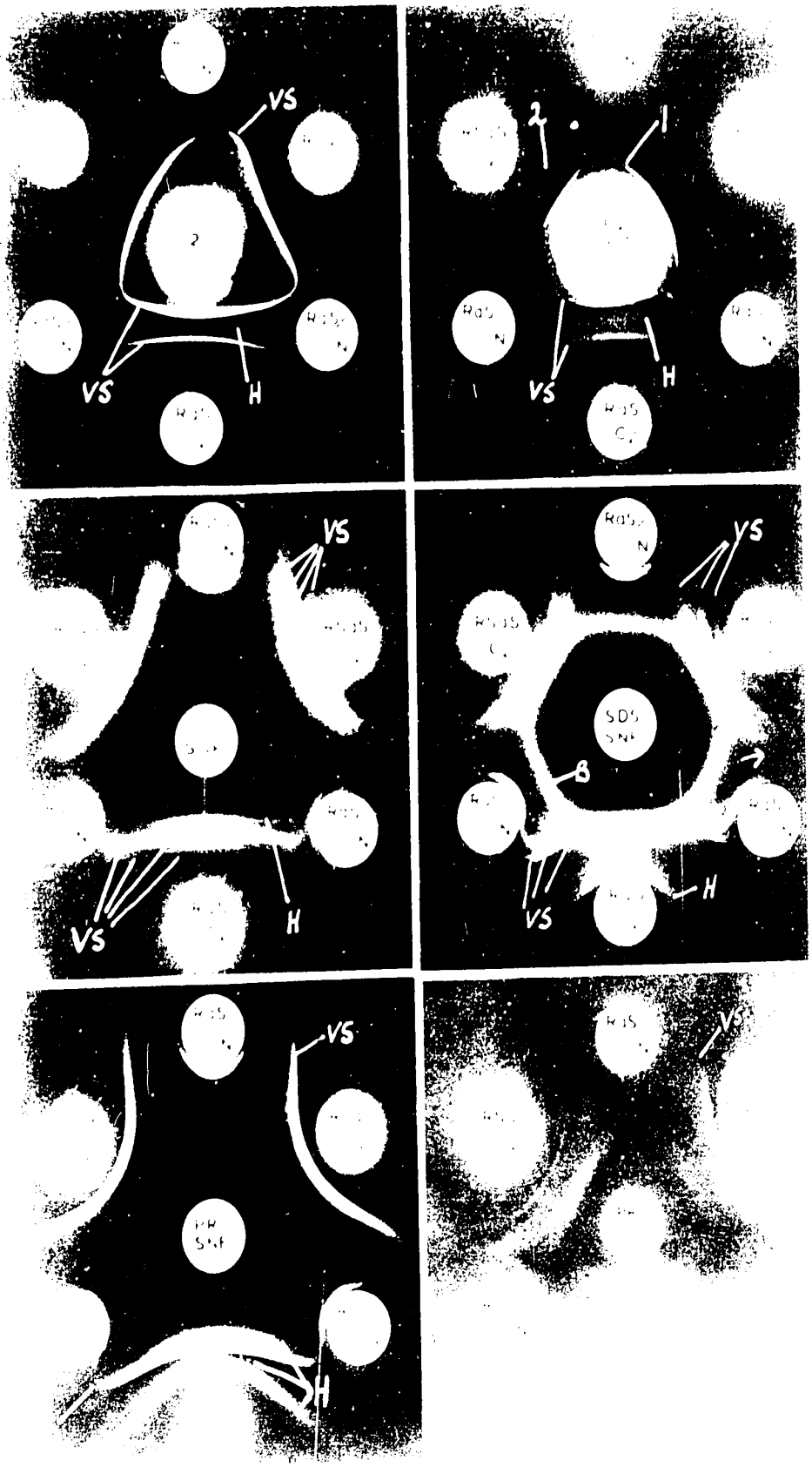
h

in
sodium
e
s
the
d,

in
tment
an be

antigens
).
by
ent
is
m.




VS
VS
H
2
1
VS
H
VS
H
VS
H
VS
H
VS

In figure 2, three, and possibly four, components can be counted in the DOC/SNF reaction, and two components in the SDS/SNF reaction. There is a reaction of non-identity (arrowed) between one of the DOC antigens (1) and one of the SDS antigens (1), however it is not clear whether the DOC components, (1) or (2), show reactions of partial or complete identity with the SDS components (2).

These reactions suggest that 1) there is one antigenic component which is present in both the DOC and SNF fractions, and 2) disruption of the virus particle with DOC and SDS results in fractions containing virus structural antigens with different antigenic characteristics, both as regards specificity and diffusion characteristics.

In figure 3, it can be seen that the two antigenic components detected in the pronase/SNF reaction show reactions of identity with two of the four components present in the DOC/SNF. One of these PRO/SNF antigens is also present in the control SNF_3/C obtained by washing an untreated virus concentrate (see pronase procedure, Materials and Methods, page 17). This linkage suggests that the antigenic specificity of the reacting component has not been altered as a result of either pronase or sodium deoxycholate treatments.

PLATE 37

Comparison of the Immunodiffusion reactions of Virus Structural Antigens using specific Rooster antiserum ($RSaS/C_2$)

Figure 1 - A reaction comparing the DOC/SNF antigens with control virus concentrate (C_2), sonicated virus preparations (C_2S) and virus antigens heated at 56 C. for 30 min. ($C_2$56 C.).

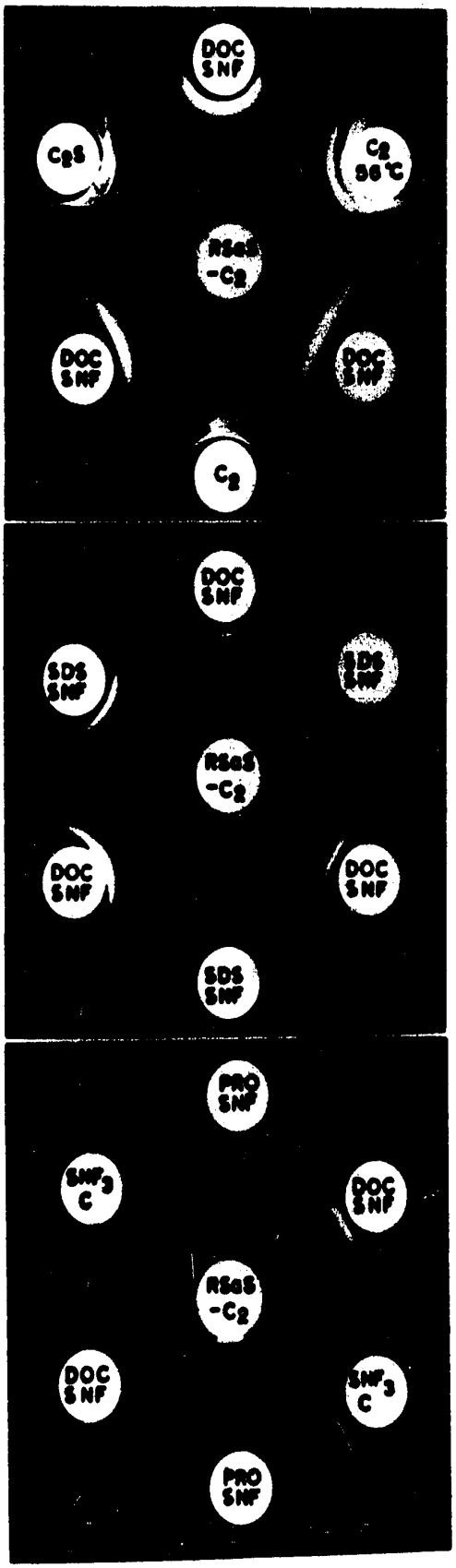
Figure 2 - The reaction comparing DOC/SNF antigens with sodium dodecyl sulfate (SDS/SNF) antigens. Two, possible three, structural components can be counted in each fraction. DOC/SNF, line 1, appears to cross over SDS/SNF line 1 in a reaction of non-identity, and may link with SDS/SNF line 2, in a reaction of identity (arrow).

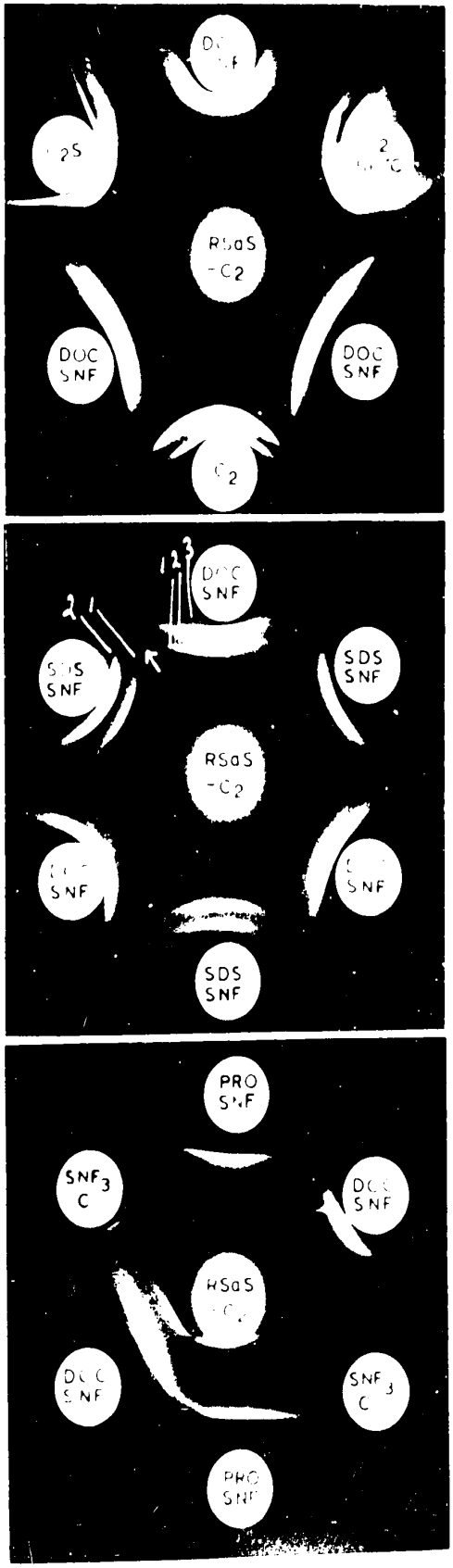
Figure 3 - The comparison of DOC/SNF antigens with PRO/SNF antigens and the control antigens in SNF_3/C. Two reacting components are detectable in both DOC and PRO fractions and these show reactions of identity with each other.

(RSaS/C_2)

s
tated
ons

tigens.
ts'
P,
le 1
ink




RSaS
DOC
SNF
DOC
SNF
SDS
SNF
SDS
SNF
PRO
SNF
DOC
SNF
SNF3
C
SNF3
C
PRO
SNF

Summary and Conclusions.

1. These studies have been concerned with the production and analysis of the influenza A/PR8 virus structural antigens.

2. Electron microscope studies on the effect of several reagents on the morphology of the virus particle were of value in the selection of the final treatments.

3. Four disruption procedures have produced fractions containing both host and virus structural antigens. These antigens could be detected readily with specific antisera and were satisfactory preparations for use in the identification of other structural antigens, especially those in extracts of influenza A/PR8-infected chorioallantoic membrane.

4. Disruption of the virus particles with sodium deoxycholate (DOC) resulted in a supernatant fraction which consisted of five antigenically distinct components of which at least three could be identified as virus specific structural antigens. One of the five components showed a reaction of identity with the untreated virus particle, demonstrating its relationship with an antigenic structure, possibly on the surface of the particle. One of the

components released by sodium deoxycholate showed a faint reaction of non-identity with a component released by sodium dodecyl sulfate. Two of the components could be identified with two antigens present in the pronase-supernatant fraction.

5. Disruption of the virus particles with sodium dodecyl sulfate (SDS) does not appear to be as satisfactory as with DOC, primarily because the presence of residual SDS interferes with the antigen-antibody reaction. Four detectable antigenic components were released by SDS treatment and three of these were identified as virus specific structural antigens. However, the relationship between these antigens was not clearly established. A difference in antigenic specificity was demonstrated by one component in each fraction, but linkages or intersections could not be detected between the reactions of the other components.

6. Four antigenic components were detected in the non-sedimenting PRO/SNF fraction, of which only one could be identified as virus specific structural antigen. Since haemagglutinating activity could not be demonstrated in the PRO/SNF fraction, this virus antigenic component was probably not haemagglutinin.

7. Two virus specific structural antigens were detected in the sedimenting fraction PRO/C_3. No host antigen was detected in this fraction.

8. Since at least two of the structural antigens produced by these three procedures did not show identical specificities, each of the four fractions would be of value for the identification of structural antigens in the soluble antigen.

Therefore, the next step in the investigation was to analyze the 'soluble' antigens extracted from influenza A/PR8-infected chorioallantoic membranes.

INFLUENZA VIRUS SOLUBLE ANTIGENS

INFLUENZA VIRUS SOLUBLE ANTIGENS

Identification of Influenza Virus Soluble Antigens.

The "soluble antigen" of a virus is historically accepted as being that antigenic complex of viral specificity, extractable from infected cells and separable from the virus particle by centrifugation or filtration. Sometimes a single component of the complex dominates serological reactivity and, in the case of the myxoviruses, it is the group-specific RNP antigen which dominates the complement fixation reaction. Hence in diagnostic serology myxoviruses react either as type-specific virus (V) antigen of the lipoprotein envelope, or as group-specific soluble (S) antigen which is identical with the RNP of the virus core.

It is clear that this apparent simplicity represents a gross over-simplification of the antigenic constitution of this group. As already shown in the previous section, the virus particle antigens were in fact a complex of at least 5 components and, although there have been no extensive studies of "soluble antigens", it must be presumed that this too is antigenically complex. In order to form the basis for an experimental approach to the problem of the constitution of "soluble antigens", the following hypothesis was put forward:-

A saline extract of infected cells, after centrifugation at 35,000 g. for 60 minutes to remove virus particles and gross cell debris, will contain the following antigenic components: 1) normal host antigens identical with those obtainable from non-infected cells (host or 'H' antigens); 2) viral antigens identical with those demonstrable in the virus particle and hence to be regarded as structural antigens (viral or 'VS' antigens); 3) antigens present only in infected cells and hence arising as a result of virus replication but not demonstrable as structural component of the virus particle (virus non-structural or 'VnS' antigens); 4) modified host antigens whose reactivity has been altered by the infective process, and which may or may not be demonstrable in the virus particle. Antigens of this group which might be regarded as having dual specificity would be labelled 'VH' antigens.

Experimental approach.

Normal and PR8-soluble antigens were prepared from saline extracts of normal and infected chick embryos according to the methods described. The protein content was estimated by the Lowry modification of the Folin-phenol method (Legget Bailey, 1962). A standard pool of each preparation was made and contained 244 µg/ml. (NE/SA) and

249 µg/ml. (PR8/SA). No haemagglutination activity could be demonstrated. Infectivity titrations show that infective virus was present at titres varying from 10^{-2} to 10^{-3} EID_{50}/0.1 ml. Neither preparation was modified or fractionated in any way so as to avoid possible changes in the antigenic composition. All samples were stored at -20°C.

The first step in the investigation was to produce a reagent capable of detecting antigens of all the groups described. Antisera of the broadest relevant specificity were therefore prepared by parenteral hyper-immunization of rabbits, using as antigen a crude saline extract of PR8 virus-infected chorioallantoic membrane to which had been added an appropriate volume of virus particle concentrate. Suitable antisera were pooled to provide the 'pan-specific' antiserum pool (RaS). A second group of rabbits was immunized with similar material prepared from uninfected chick embryos (RaS/NE). Antisera were also prepared to virus concentrate (RaS/C_2) and to extracts of infected membranes (RaS/PR8).

In addition, three independent immunization procedures were used in an attempt to produce antisera specific for viral antigens foreign to the host cells. These procedures were: 1) intranasal infection of rabbits

and guinea pigs to produce convalescent serum pools, 2) production of antisera in genetically homologous roosters in order to suppress the response to host components, and 3) induction of tolerance to normal host antigens.

The serological properties of the antisera obtained by the conventional immunization procedures are shown in Table 16.

Control rooster serum was obtained following a course of immunization with NE/SA. No antigenic response was evident and no precipitation was detected in the immunodiffusion reaction (Plate 38, figure 1). In further control reactions, rooster anti-PR8 serum and convalescent guinea pig (GPaS/IN) and rabbit (RaS/IN) antisera showed no precipitation when tested against normal membrane extracts (NE/SA) (figure 2).

Reactions of soluble antigen with pan-specific antiserum.

In Plate 39, the reactions of the following three antigens are compared against the pan-specific antiserum:

1) normal embryo soluble antigens (NE/SA).
2) PR8-infected soluble antigens (PR8/SA).
3) semi-purified virus concentrate ($V\text{-}C_2$).

TABLE 16

Serological Properties of Antisera used to Analyse Soluble Antigens.

ANTISERUM	NEUT*	HAI*	CFT* NE/SA	CFT* PR/SA
RaSNE	<10	< 8	64	128
RaS	3,000	4,096	64	128
RaS/C_2	>30,000	2,048	16	512
RaS/IN	500	256	8	12
RSaS/SA	2,000	1,024	NT	NT
RSaS/C_2	>4,000	2,048	NT	NT

NT - not tested

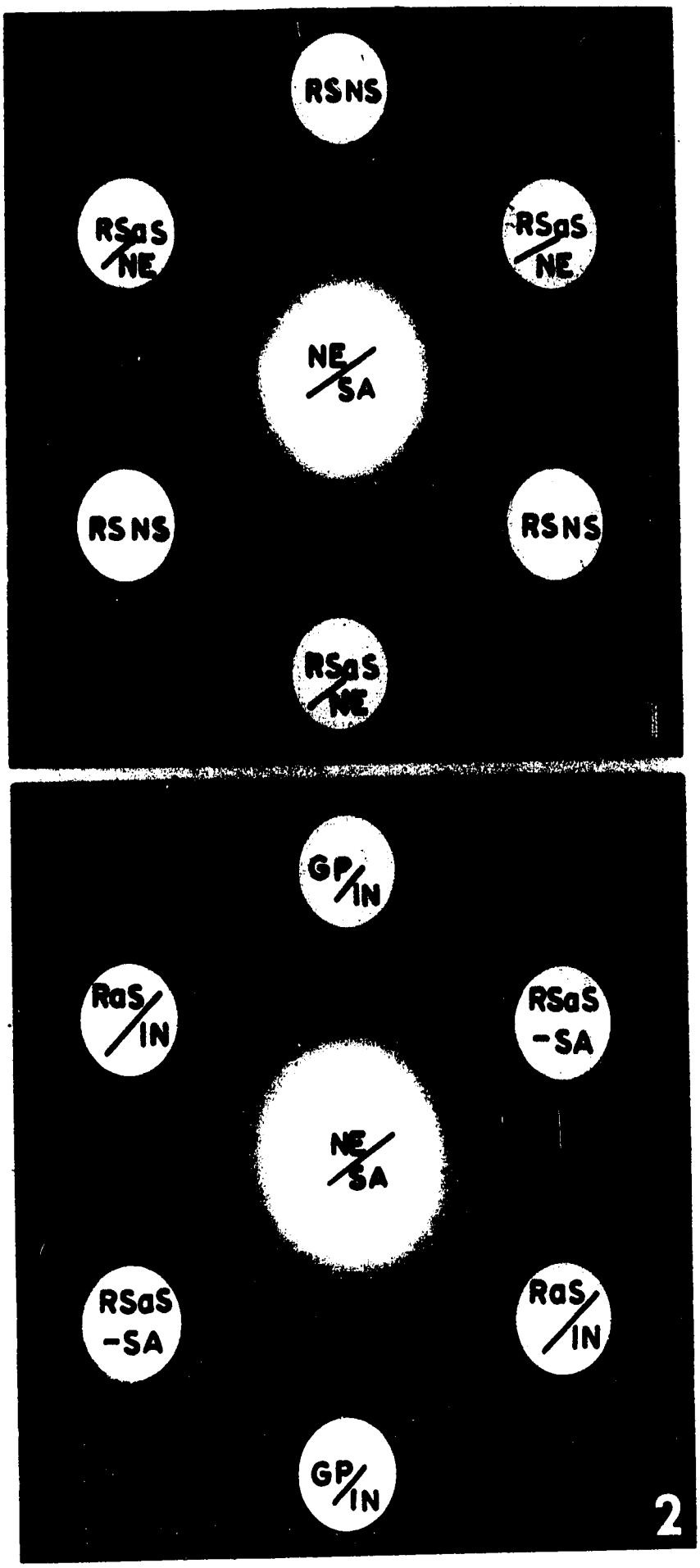
*Titres are expressed as the reciprocal of the endpoint dilution.

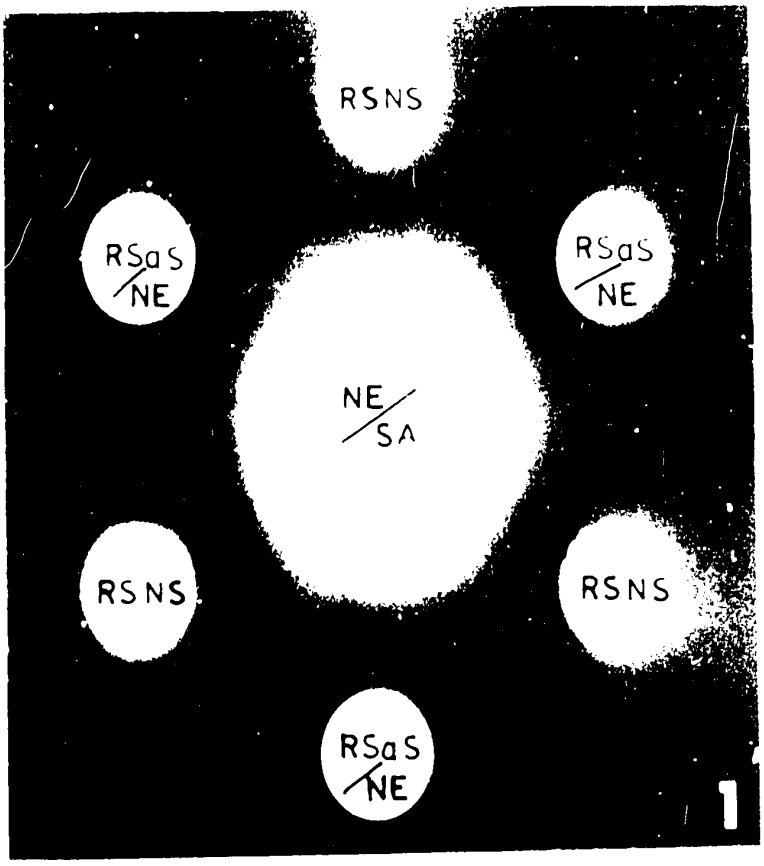
PLATE 38

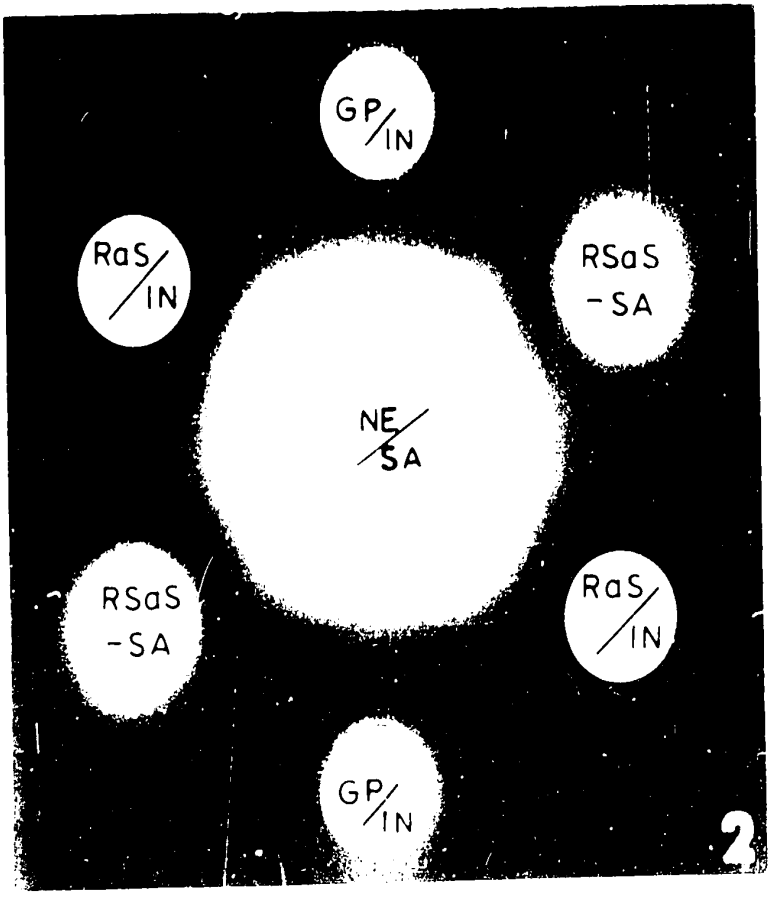
The Control Immunodiffusion Reactions of the specific antisera to Viral Antigens in PR8/SA.

Figure 1 - No precipitin reaction is detectable between the normal rooster serum (RSNS) and the serum from roosters immunized with host antigens (RSaS/NE).

Figure 2 - No precipitin reactions are detectable between the host antigens (NE/SA) and both guinea pig and rabbit antisera from animals immunized by intranasal infection (RaS/IN & GPaS/IN) and rooster anti-PR8 soluble antigen serum (RSaS/SA).


RSNS
RSaS/NE
RSaS/NE
NE/SA
RSNS
RSNS
RSaS/NE
GP/IN
RaS/IN
RSaS-SA
NE/SA
RSaS-SA
RaS/IN
GP/IN
2


RSNS
RSaS/NE
RSaS/NE
NE/SA
RSNS
RSNS
RSaS/NE
1


GP/IN
RaS/IN
RSaS-SA
NE/SA
RSaS-SA
RaS/IN
GP/IN
2

These reactions emphasize the complexity of the reacting systems. It may be seen that:

(a) at least twelve components are detectable in the normal antigen NE/SA, and

(b) at least seventeen components are detectable in the infected antigen, PR8/SA;

(c) none of the components of either soluble antigens show a reaction of identity with the virus particle antigens ($V\text{-}C_2$);

(d) at least four components show reactions of identity indicating their presence in both normal and infected soluble antigens;

(e) a group of at least three components (V-SA arrowed) are present in the PR8/SA reaction but are not detectable in the NE/SA. These presumptive viral antigens do not appear to link with any $V\text{-}C_2$ components (see (c) above);

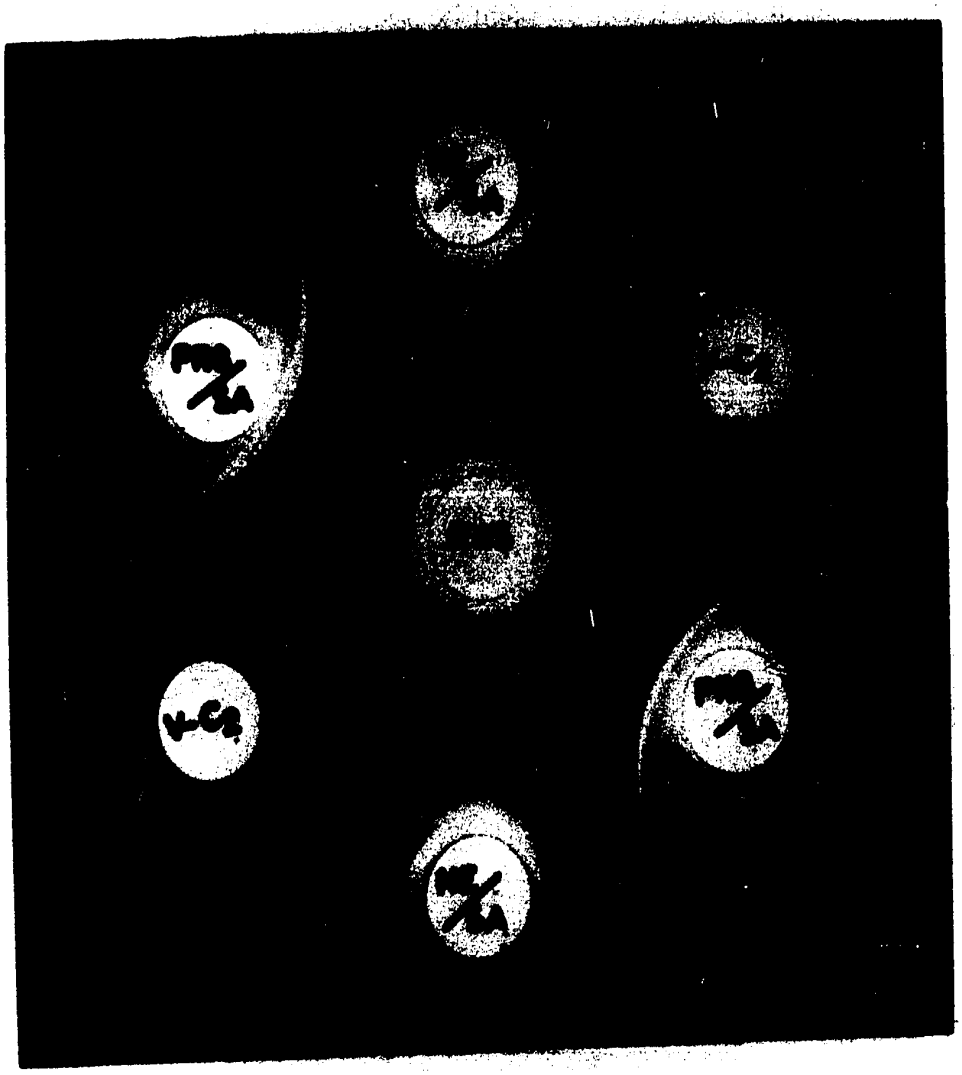
(f) one component (1, arrowed) of PR8/SA gives clear reactions of non-identity with $V\text{-}C_2$ and NE/SA indicating an antigen peculiar to the virus-infected extract. This antigen is part of a PR8/SA precipitating complex which gives two clear linkages with normal host antigens;

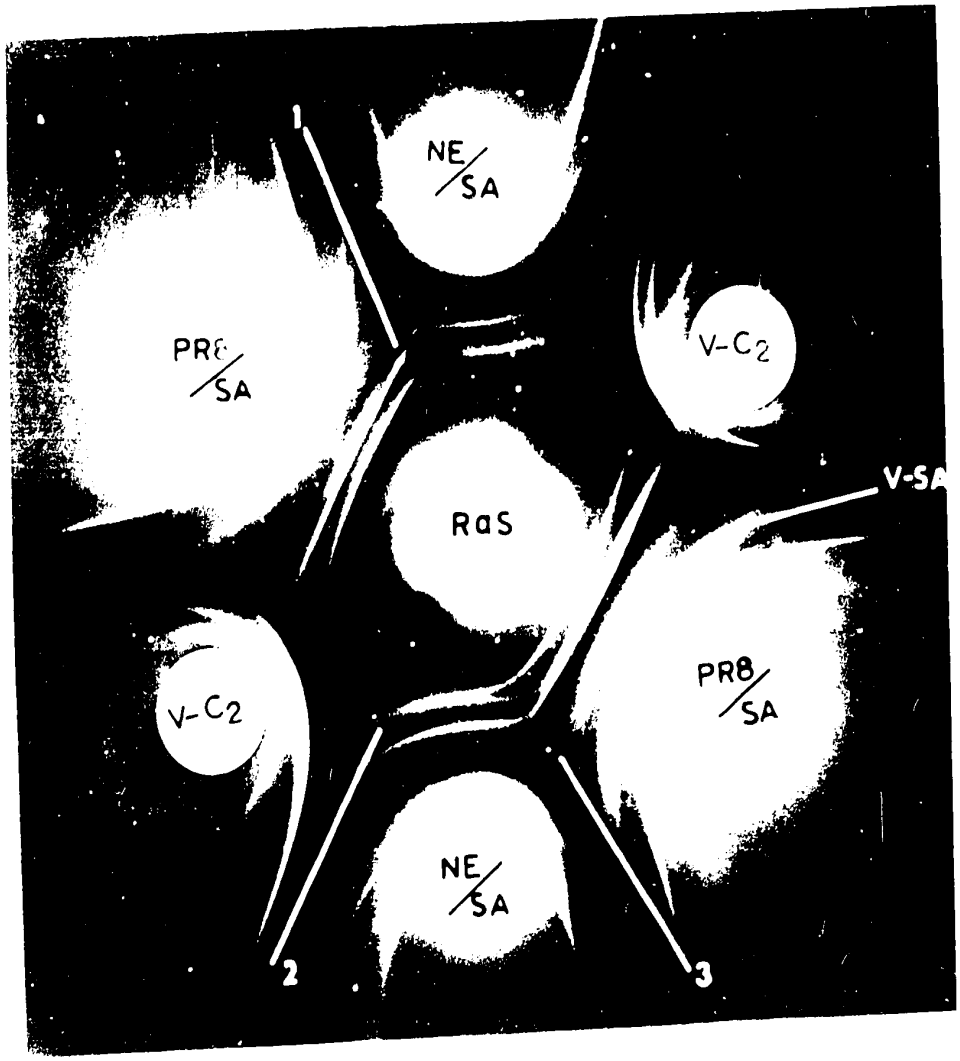
(g) one line in the NE/SA (2, arrowed) normal host pattern splits to give separate reactions of identity

PLATE 39

The reaction comparing the antigens in the virus concentrate ($V\text{-}C_2$), PR8-soluble antigen (PR8/SA) and normal host extract (NE/SA) using the rabbit pan-specific antiserum (RaS). A virus specific component (1) and host altered components (2) & (3) are detected. Also there are antigens detectable PR8/SA but not evident in the NE/SA reaction (V-SA).

con-




1
NE/SA
PR8/SA
V-C2
V-SA
RaS
V-C2
PR8/SA
NE/SA
2
3

with two distinct components of PR8/SA;

(h) one NE/SA line fuses into a broad band of precipitate in the PR8/SA pattern (3, arrowed).

Conclusions.

1. The twelve NE/SA components (a) are by definition host components (H).

2. The PR8/SA component (f) is identified provisionally as a non-structural virus antigen (VnS). This identification could, however, be in error since the V-C_2 antigen was mainly intact virus particles, which would exclude the internal components from the reaction.

3. Reactions (g) and (h) above could be accounted for by changes in the antigenic specificity of the host components involved or by alteration of their diffusion characteristics. These antigens would then correspond to the VH group.

4. The group of components labelled 'V-SA' (e) are presumptively viral antigens but the reaction does not indicate whether they are structural or non-structural.

5. No structural 'VS' antigens can be definitely identified in the PR8/SA.

It has been noted (a and b) that the complexity of the PR8/SA reaction is greater than that of the NE/SA.

It may also be seen that the intensity of the reaction pattern is greater with the infected extract.

The same difference was noted when these two preparations were compared in reactions with the antiserum prepared against extracts of uninfected membranes. This difference was consistently found when using pan-specific antisera, and was accompanied by an apparently greater antigenicity on the part of the infected as opposed to the normal extract.

This finding suggests that the process of virus replication permits the extraction of a greater quantity and variety of normal host components from infected cells than occurs with normal cells. This in turn implies changes in intracellular host constituents, which may be of importance with respect to cell damage resulting from virus infection.

Reactions of soluble antigen against specific sera.

When normal and infected extracts were tested using antisera prepared against soluble antigens freed from virus and gross debris by ultra-centrifugation, less complex patterns were obtained.

In the reaction shown in Plate 40, figure 1,

seven host components are detectable in the NE/SA reaction (H1-H7) and all but H4 of these are also present in the PR8/SA reaction. Figure 2 shows the reactions against RaS/PR8. In the NE/SA pattern six host components (H1-H6) are detectable. Of these H1 splits into 3 components (labelled V1,2,3) in the PR8/SA pattern, while H2 and H3 combine to a single line, labelled V4. No specifically virus antigens could be detected in these reactions.

When rooster antiserum was used, however, virus-specific components were clearly revealed (figure 3), in which at least two components are suggested. The reaction suggests that these antigens do not link with the strongly reacting structural components demonstrated in the V-C_3 reaction.

From later studies (Plate 44), it could be inferred that at least one of these components carried host antigenic specificity in addition to its viral component, and is therefore represented by one of the reaction lines in the reactions of figure 2.

The designation 'HV' was applied to components with this dual type of antigenic specificity.

PLATE 40

The comparison of the immunodiffusion reactions of Host (NE/SA) and PR8-soluble antigens (PR8/SA) with rabbit anti-host serum (RaS/NE) figure 1) and rabbit-PR8-soluble antigens (RaS-PR8, figure 2) and rooster anti-PR8-soluble antigens (RSaS/SA, figure 3).

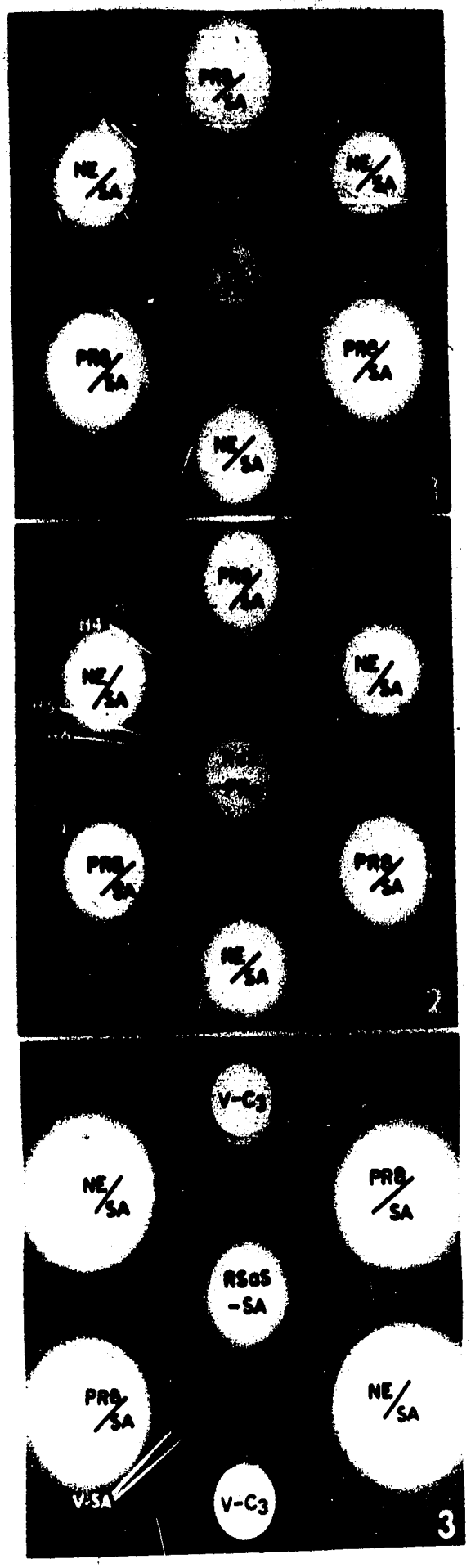
Figure 1 - All the reacting components must be host-specific and are labelled H1 - H7.

Figure 2 - The components associated with the host antigen reaction are labelled 'H' and those associated with the infected antigen extracts are labelled 'V'. The reactions of identity between these antigens serve to identify their relationships.

Figure 3 - The components detected by the rooster antisera are virus-specific antigens (V-SA).

ntigen
iated
belled
hese
ships.

A).



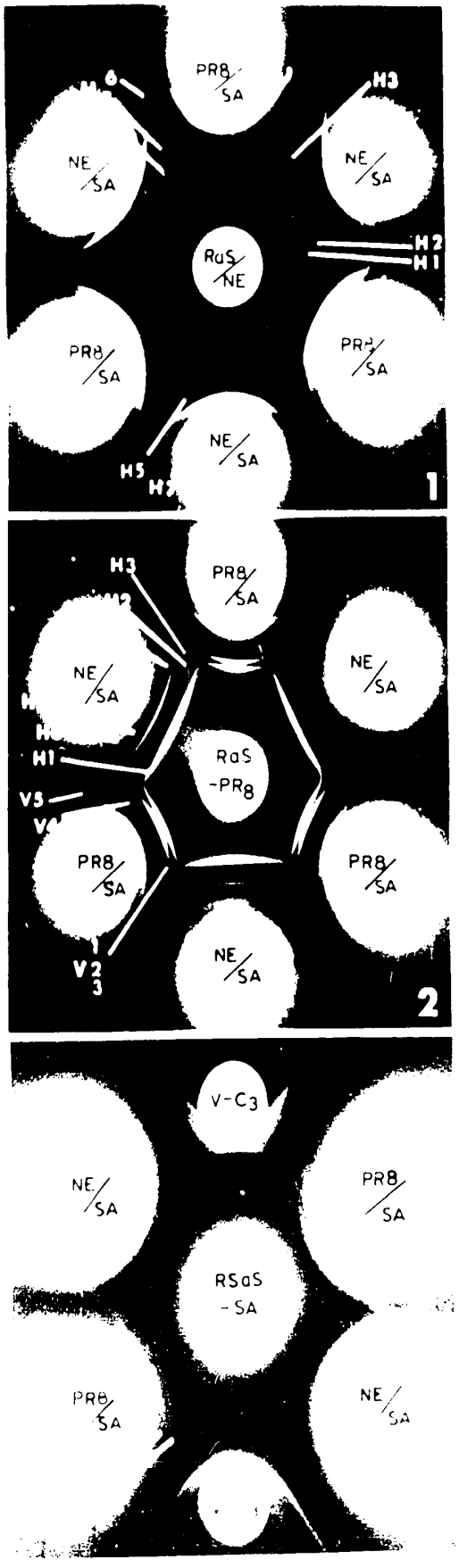
t-

r

t-

antigen
ociated
labelled
these
onships.

-SA).



Summary.

These reactions show the presence in the infected material of: seven host antigens (H), of which at least one probably carried virus specificity and is an 'HV' component. In addition, three components of the infected pattern fuse to a single line in the NE/SA pattern, and two components of the latter fuse to form a single line in the former. This suggests that four of the 'H' components have undergone some alteration as a result of infection.

Investigations using antisera from 'tolerant' rabbits.

New-born animals exposed to foreign antigens within 12 hours of birth tend to accept such antigens as "self", and fail to react to them by the production of antibodies. This suppression of the normal immune response has been termed "Immune tolerance" (Billingham, Brent and Medawar, 1953) and has been used to distinguish abnormal antigens present in normal host extracts (Gold and Freedman, 1965).

In the hope that rabbits, rendered tolerant to chick embryo antigens, would respond immunologically to only those components of PR8 soluble antigen which carried viral specificity, litters of neonatal rabbits were

inoculated with NE/SA preparations as described in Materials and Methods. Some animals of each litter were then immunized with extracts of virus-infected embryos. In the remaining animals tolerance was maintained by continued inoculations of uninfected membrane extracts.

The level of immune tolerance to NE/SA and the production of PR8-specific antibodies was determined by: 1) complement fixation tests, 2) Ouchterlony gel-diffusion reactions (1958), 3) cellulose acetate micro-immunodiffusion reactions.

HAI and neutralization tests were performed on the immune sera.

The results of the complement fixation reactions (Table 17) showed the presence in the tolerant sera (T)* low levels of complement fixing antibody against both NE and PR8 soluble antigens. Following three immunizing doses with PR8+ C_2 antigen in four test animals (A), there was an increase in the CF antibody titres to both NE/SA and to PR8/SA.

The titres of control tolerant sera showed that only partial tolerance was achieved in respect to normal chick embryo antigens. The antiviral response was best

---

* For the sake of brevity the sera obtained from tolerant rabbits will be referred to as "tolerant sera".

shown by the HAI and neutralization titres attained as shown in Table 18.

The comparison of the immunodiffusion reactions of the tolerant sera by the Ouchterlony and the micro-immunodiffusion techniques is shown in Plates 41 and 42, figures 1-8. An almost complete state of immune tolerance was suggested by the less sensitive Ouchterlony method (figs. 1, 2, 5, 6) whereas the micro-immunodiffusion reactions showed that tolerance was not in fact complete and supports the results of the CF titrations (figs. 3, 4, 7, 8). The greater intensity of the response to the PR8-soluble antigen is indicative of the greater antigenicity of the latter, as compared with the normal extract. This supports the earlier conclusion regarding the release of normal host components by virus infection.

A more detailed analysis of the immune responses of the immunized tolerant rabbits (TR) and the tolerant control rabbits (TR-C) was made on sera obtained 42 days after the start of immunization (70 + 42 days). The immunodiffusion reactions developed with NE/SA (Plate 43, figure 1) show that antibody production to host antigen was suppressed to the extent that only one strongly reacting host (H) antibody was detected which was common to all the sera. In addition,

TABLE 17

Results of Complement Fixation Titrations of Sera from 'Tolerant' Rabbits.

Rabbits.	Complement Fixation Titres NE/SA		PR8/SA	
	T*	A**	T*	A**
TR-C 2	32	64	32	64
TR-C 4	16	64	32	64
TR 1	32	64	16	128
TR 3	16	64	32	128
TR 5	<8	64	<8	128
TR 6	16	48	32	128

* T - Serum from 'tolerant' rabbits before immunization.

** A - Serum following immunization with PR8/SA (TR rabbits) or with NE/SA (TR-C rabbits).

TABLE 18

Results of HAI and Neutralization Titrations of Antisera produced by 'Tolerant' Rabbits.

Rabbit	HAI		Neutralization	
	T*	A**	T*	A**
***TC-2	0	0	NT	NT
TC-4	0	0	NT	NT
TR-1	0	512	<8	6309
TR-3	0	512	<8	6309
TR-5	0	256	<8	NT
TR-6	0	512	<8	5012

NT - not tested

* T - Tolerant sera prior to immunization with PR8/SA

** A - Immune serum following injections of PR8/SA antigen (TR- rabbits)

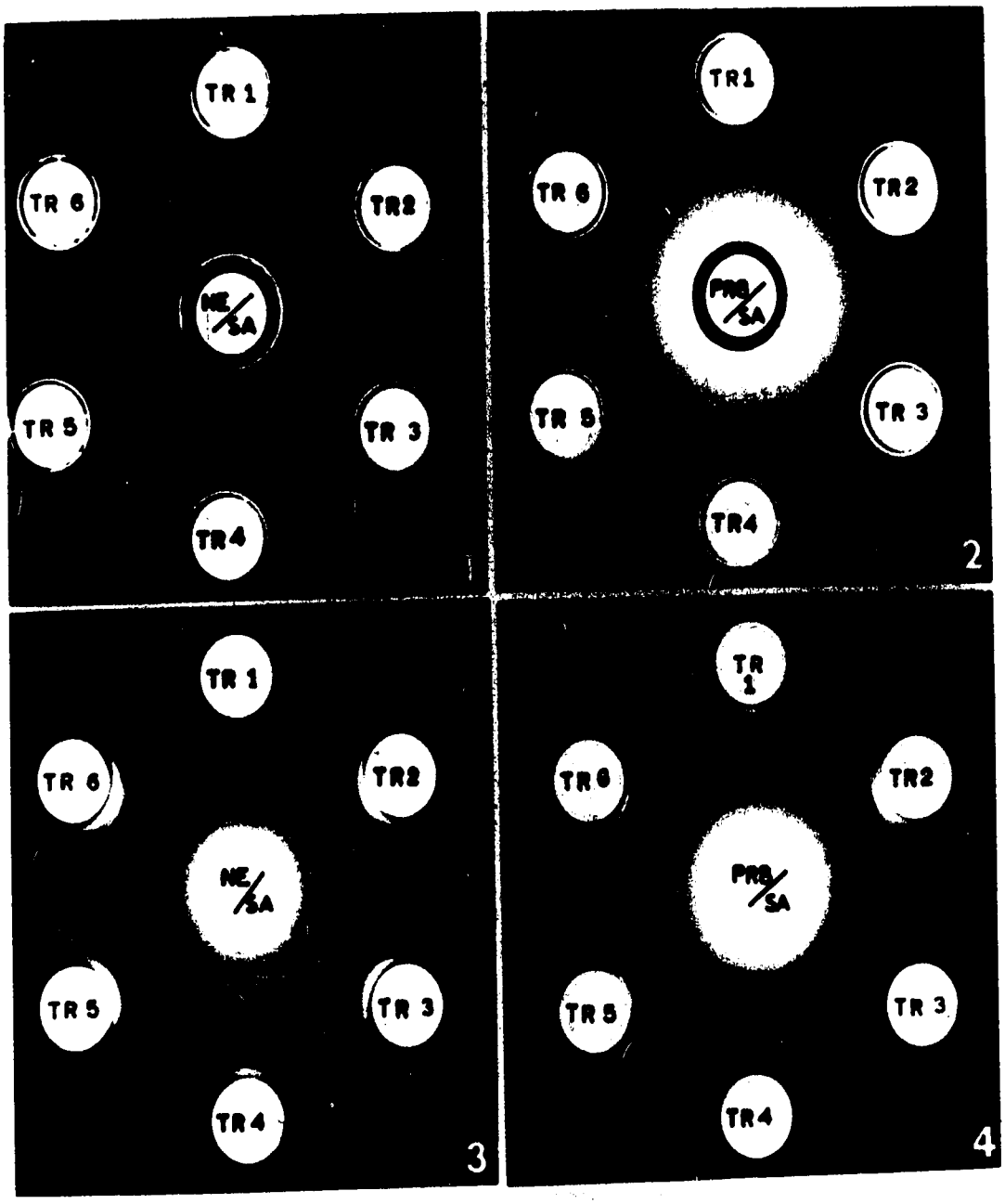
***TC - Control rabbits with tolerance maintained by injections with NE/SA.

PLATE 41

The comparison of the sensitivity of the Ouchterlony gel diffusion and cellulose acetate micro-immunodiffusion techniques. The determination of the level of tolerance of rabbits to host antigens (NE/SA).

Figures 1 & 2 - The Ouchterlony technique of gel diffusion. The reactions of sera from 'tolerant' rabbits (TR-1 to TR-6) to host antigens (figure 1, NE/SA) and to PR8-soluble antigens (figure 2, PR8/SA). A faint zone of precipitate can be seen in the TR-2 reaction with NE/SA (arrowed, figure 1).

Figures 3 & 4 - The cellulose acetate micro-immuno-diffusion technique. The reactions of all the TR rabbit sera with both NE/SA and PR8/SA show that specific host antibodies are present in the 'tolerant' sera indicating a state of partial tolerance.


TR 1
TR 6
TR2
NE/SA
TR 5
TR 3
TR4
1
TR1
TR 6
TR2
PR8/SA
TR 5
TR 3
TR4
2
TR 1
TR 6
TR2
NE/SA
TR 5
TR 3
TR 4
3
TR 1
TR 6
TR2
PR8/SA
TR 5
TR 3
TR4
4

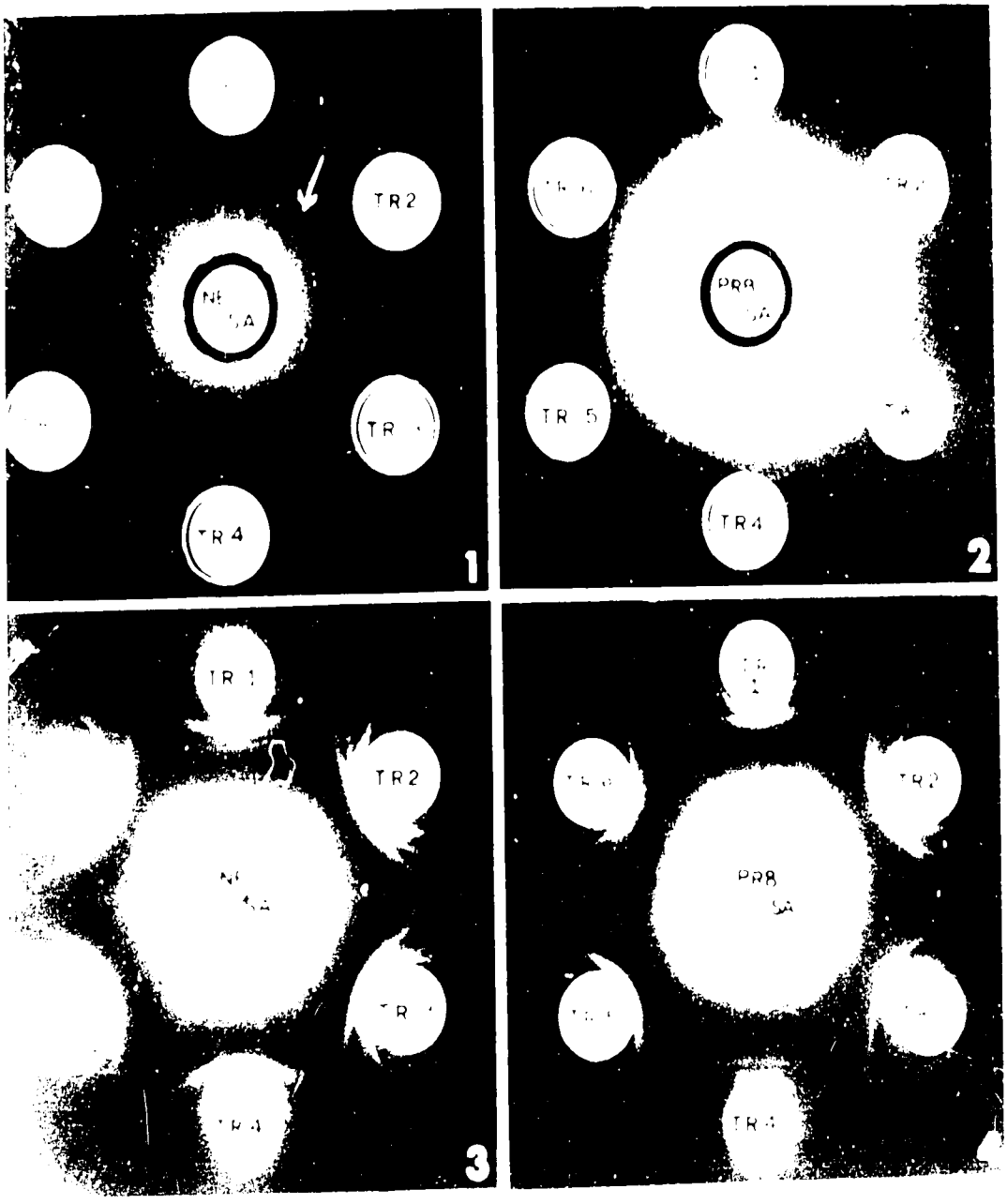
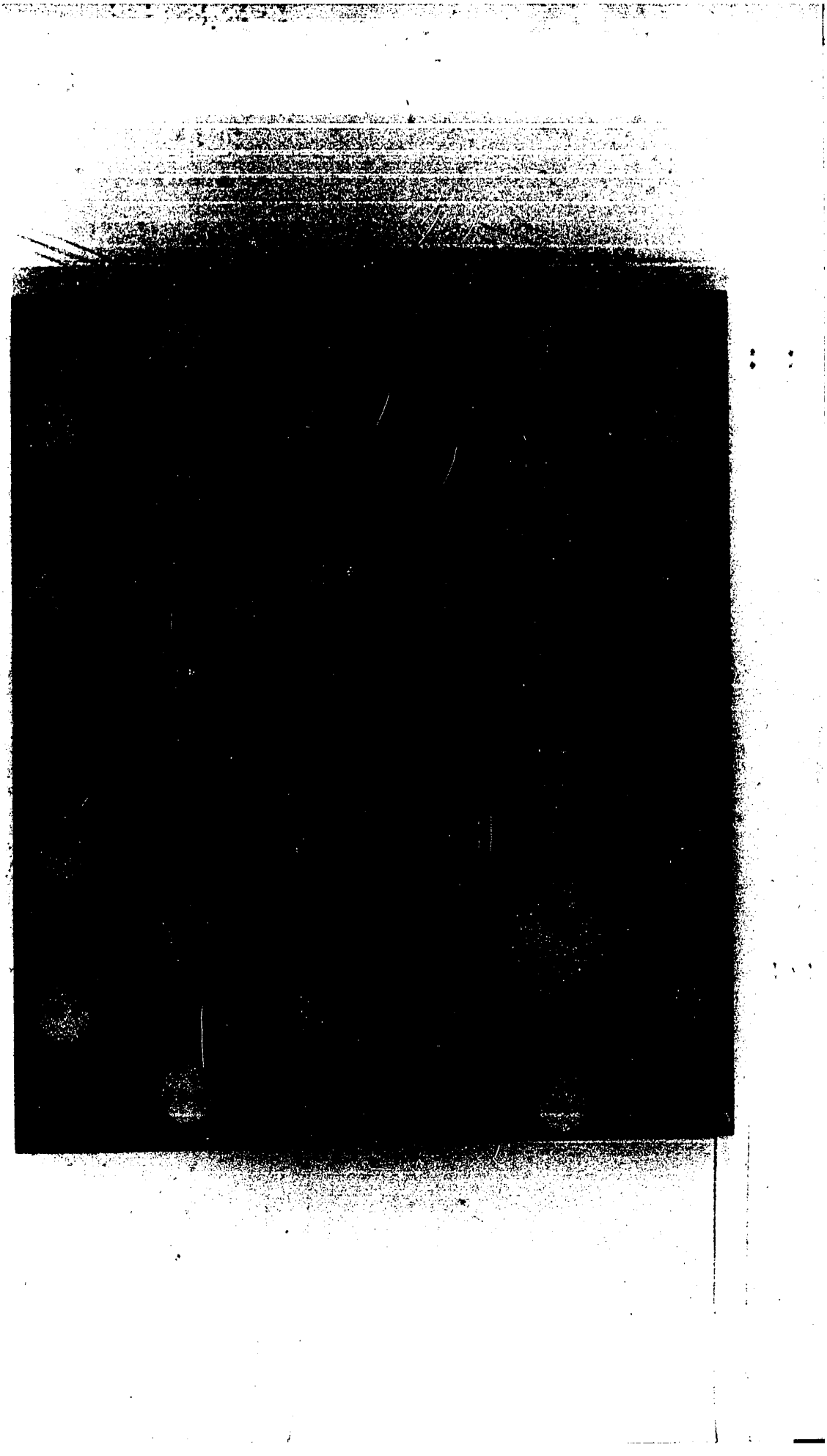

TR2
TR4
1
PR8
TR 5
TR4
2
TR 1
TR2
3
PR8

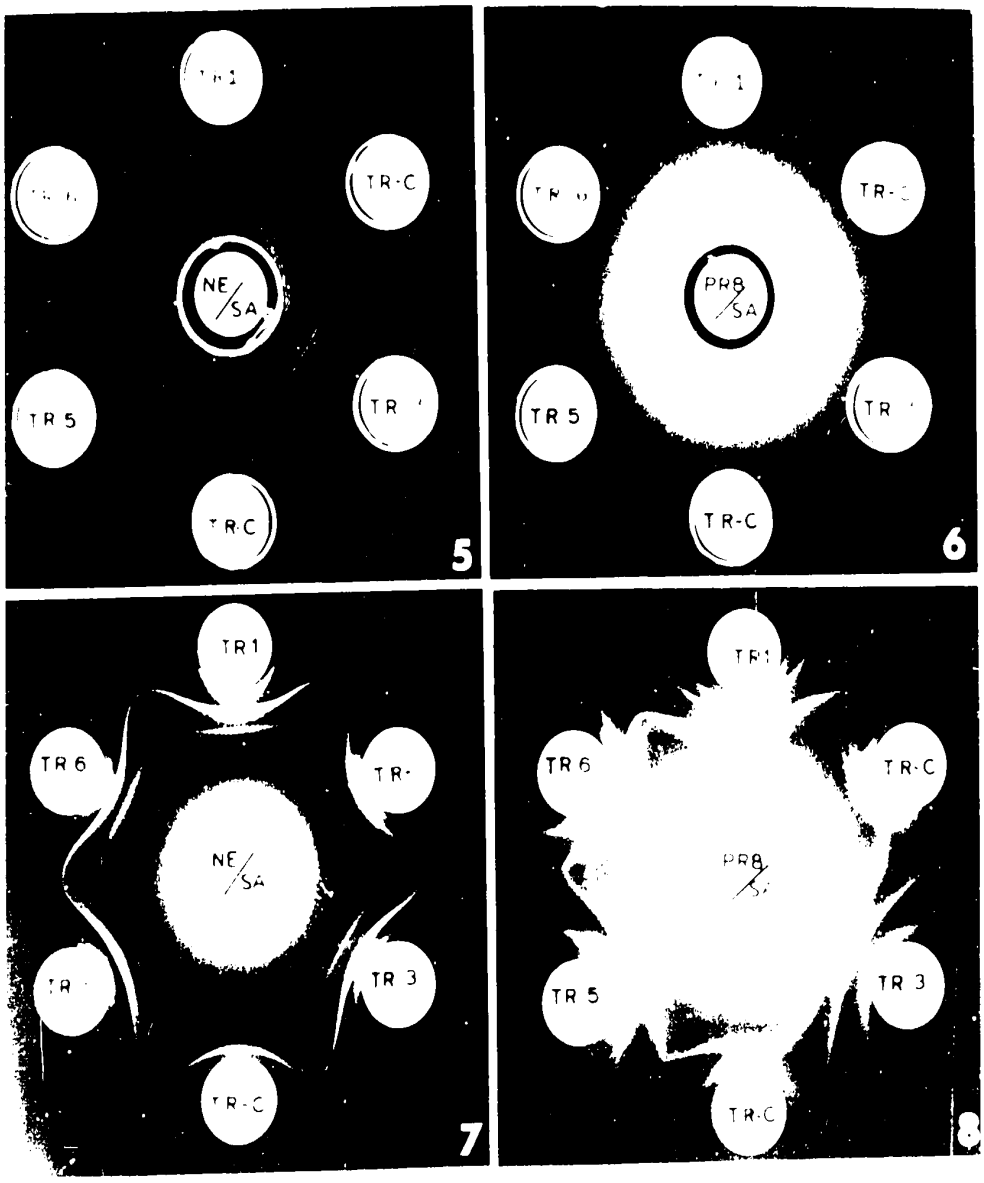
PLATE 42

Comparison of the sensitivity of the Ouchterlony gel diffusion and cellulose acetate micro-immunodiffusion techniques. The evaluation of the immune response of partially tolerant rabbits to PR8-soluble antigens at the 23rd day.

Figures 5 & 6 - In the Ouchterlony gel diffusion reaction one component is evident in the pattern with NE/SA (figure 5). Sera from the tolerant control rabbits (TR-C) show a less intense reaction compared to the immune animal sera.
In the reaction with PR8/SA, (figure 6) two reacting components are visible with both control (TR-C) and immune sera reactions (TR-1,3,5 &6).

Figures 7 & 8 - In the parallel cellulose acetate reactions there are at least three components detected in the NE/SA reaction (figure 7) and up to seven lines may be counted in the reactions of the sera from the immunized 'tolerant' rabbits with PR8/SA (figure8).




TR-C
NE/SA
TR 5
TR-C
5
PRB/SA
TR 5
TR-C
6
TR1
TR 6
NE/SA
TR 3
TR-C
7
TR1
TR 6
TR-C
PRB/
TR 5
TR 3
TR-C

the control sera indicate two, possibly three, further components, one of which is also present in TR-1 and TR-3. The sera of the immunized rabbits show the presence of anti-host antibodies which are not evident in the control reactions. These have been labelled 'VH'.

In the second reaction (figure 2), the same sera were tested against PR8/SA. At least seven reacting components can be counted in the reactions with the immunized sera among which the host antibodies (H1 & H2) and the 'HV' component could be detected. Five precipitin lines which did not appear to show reactions of identity with the host components were provisionally identified as virus-specific ag-ab reactions (V1-V5). The relationship of the virus and host components is illustrated by the following reactions:

1. The V1 component detected by the immune sera, shows reactions of non-identity with the TR-C4 and TR-C2 anti host reactions. This indicates the presence, in the immune sera, of presumably virus specific antibodies and is the first evidence of the presence of virus specific antigens in the infected soluble antigen preparations.

2. The V2 component is closely associated with the HV-1 reaction complex. However, the virus specificity of this component is indicated by the reaction of partial

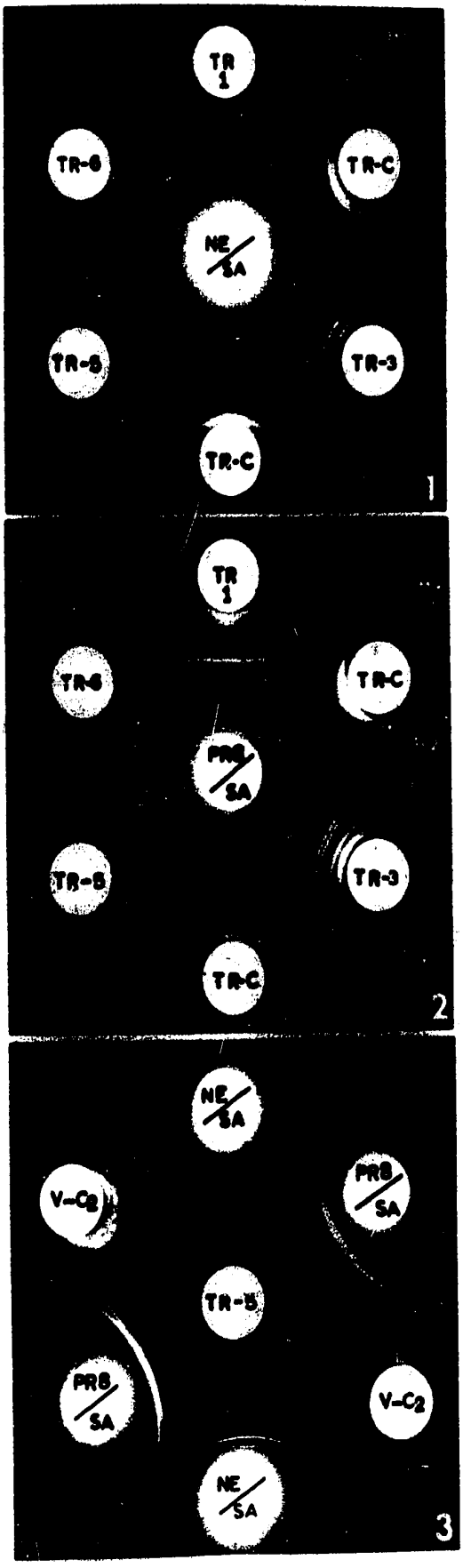
PLATE 43

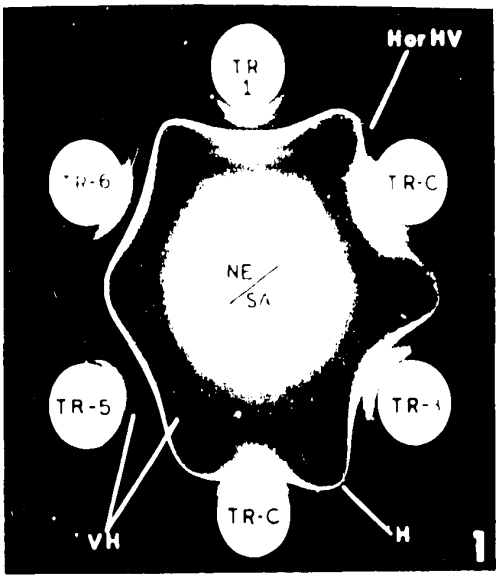
The Analysis of the Immune Response of Partially Tolerant Rabbits (day 40).

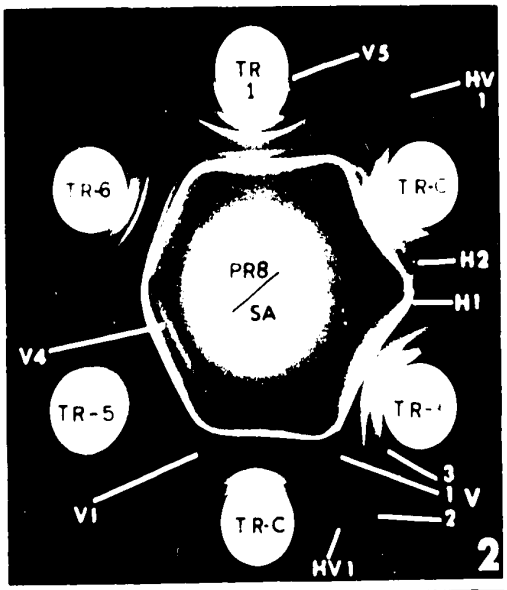
Figure 1 - The immunodiffusion reactions between host antigens (NE/SA) and sera from immunized 'tolerant' rabbits (TR-1, TR-3, TR-5 & TR-6) and the control tolerant sera (TR-C 2 & TR-C 4) demonstrating components with host (H) and dual host-virus specificity (VH).

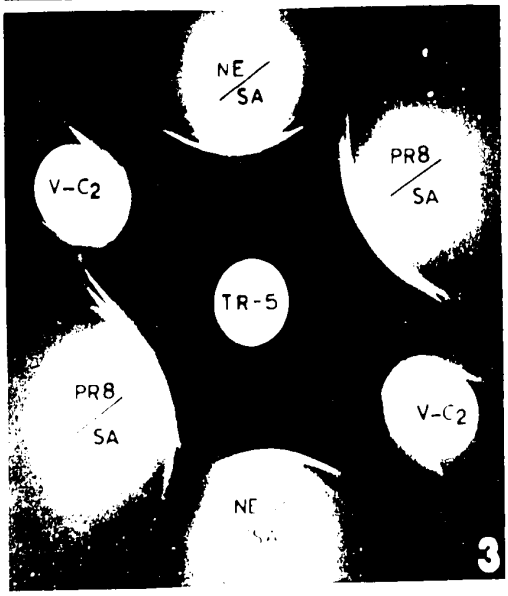
Figure 2 - The immunodiffusion reactions between the PR8-soluble antigens (PR8/SA) and sera from both the immunized 'tolerant' rabbits (TR-1 to 6) and the control tolerant sera (TR-C 2&4). At least three host components (H) are detectable in the control tolerant reactions. Five virus-associated components (V1-V5) are detectable in the immune reactions which are not detec table in the control reactions.

Figure 3 - The comparison of the reactions of virus concentrate ($V\text{-}C_2$), PR8-soluble antigens (PR8/SA) and host antigens (NE/SA) with sera from an immunized 'tolerant' rabbit (TR-5) The reaction of identity indicates the presence of components with dual specificity (HV). At least four virus-specific (V) components can be detected in the PR8/SA which do no appear in the NE/SA reaction.


TR 1
TR-6
TR-C
NE/SA
TR-5
TR-3
TR-C
1
TR 1
TR-6
TR-C
PR8/SA
TR-5
TR-3
TR-C
2
NE/SA
V-C2
PR8/SA
TR-5
PR8/SA
V-C2
NE/SA
3


TR 1
Hor HV
TR-6
TR-C
NE/SA
TR-5
TR-4
TR-C
VH
H
1


TR 1
V5
HV 1
TR-6
TR-C
PR8/SA
H2
H1
V4
TR-5
TR-4
3
V
1
2
V1
TR-C
HV1
2


NE/SA
PR8/SA
V-C2
TR-5
PR8/SA
V-C2
NE
3

identity between the control pattern (TRC-4) and the immune patterns of TR-5 and TR-3 (arrowed, V2 and HV1).

3. The V3, V4 and V5 components are not apparent in the control host reactions, and were therefore provisionally identified as virus-associated antigen-antibody reactions.

The reaction shown in figure 3 (Plate 43) shows the direct comparison of the normal and infected soluble antigens using one of the tolerant rabbit antisera (TR-5). The presence of the 'HV' component is evident, and three virus components are apparent in the PR8/SA reaction, which are not detectable in the NE/SA reaction.

Thus, the immunization of animals rendered partially tolerant to host antigens has resulted in the detection of <u>five virus antigens</u> present in the PR8-infected soluble antigen extracts. The identification of two of these antigens as virus-specific is based on the presumption that antibodies produced in the 'tolerant' rabbits, which do not show host specificity when compared with the control tolerant antisera, must have been stimulated by virus-specific antigens different from the host antigens. This is, however, an oversimplification of the 'tolerant immune reaction', since it has been shown that antigens closely related to the tolerance-inducing antigen may either (a) cause a breakdown

of tolerance or (b) maintain the tolerant state (Cinader et al, 1967 & St.Rose et al, 1967). From the results of the previous analyses, it is evident that the virus specific antigens must be closely related to the host antigens, therefore it is possible that the increased antigenicity, characteristic of the infected soluble antigen, may have caused an increased response to host and/or host-virus antigens. Without comparative reactions between sera hyperimmune to both host and infected antigens, and the tolerant antisera, further identification of these PR8 virus antigens cannot be made.

Identification of the structural antigens in the PR8-infected soluble antigen.

Virus soluble antigens include two distinct classes of viral components: 1) structural antigens which are identical to the antigens of the virus particle, and 2) non-structural antigens which are not incorporated into the virus particle, nor are they present in the normal host preparations.

In the present investigation, the following three groups of PR8 antigens have been detected: 1) host specific antigens (H), 2) virus specific antigens (V) and 3) antigens showing both host and virus specificity (VH).

The reactions of PR8/SA and NE/SA with specific antisera were first compared. The antibodies in each antiserum could be characterized according to structural, non-structural or host specificity on the basis of the properties of the immunizing antigens. These characteristics have been tabulated in Table 19. Both rabbit and rooster anti-virus (RaS/C_2 and RSaS/C_2) sera could be classified as anti-structural sera, and of these only RSaS/C_2 was free from anti-host antibody. Since host antigens may be incorporated into the virus as structural components and/or contaminant, the normal host antigens were included in the analysis in an attempt to identify the host component.

A complex analysis is shown in Plate 44. This is a 12-hour reaction in which the precipitin lines are not fully developed. It can be seen that:

(a) host antigens (H) have been detected by the reaction of the NE/SA in the central well with the following antisera, RaS/C_2, RaS/NE and RaS/PR8. No anti-host reactions can be seen with RaS/IN, RSaS/C_2 or RSaS/SA;

(b) RaS/C_2 gives an anti-host reaction against NE/SA, RSaS/SA and PR8/SA. In this reaction the rooster serum proteins are detected as normal host antigens.

TABLE 19

Immunodiffusion Reactions of the Specific Antisera Used for the Identification of the Soluble Antigens.

Immunized Animal	Immunization Material	Antiserum Abbreviation	Reactions with antigens present in PR8 soluble extracts		
			Structural Ag.	Non-structural Ag.	Host Ag.
Rabbit	Host CAM extracts	RaS/NE	?	?	+
Rabbit	PR8-soluble antigen	RaS/PR8	+	+	+
Rabbit	PR8-Virus concentrate	RaS/C_2	+	-	+
Rabbit	Intranasal injection	RaS/1N	+	+	-
Rooster	PR8-Virus concentrate	RSaS/C_2	+	-	-
Rooster	PR8-soluble antigen	RSaS/PR8	+	+	-

(c) virus specific antigens (V) are detected by the RaS/IN, RSaS/C_2 and RSaS/SA antisera. Both the RaS/IN and RSaS/SA reactions show identity with the ag-ab reactions of the RSaS/C_2. Therefore the antigen is identified as structural.

(d) the same structural antigen in PR8/SA shows a comparable reaction of identity between the RSaS/SA and the RaS/C_2 reactions.

(e) the structural antigen detected by the RaS/C_2 shows a reaction of non-identity (arrowed) with host antigen which is detected by both RaS/NE and RaS/C_2. The structural antigen is therefore clearly a virus specific structural component (VS) which is present in the PR8-infected soluble antigen.

(f) fully developed 44-hour reactions showed that this structural component did not carry host specificity.

In the second reaction, the specific rabbit anti-virus serum RaS/C_2 was used to analyze the relationships between the virus particle antigens (VS and HS) and both the normal host and infected soluble antigens (Plate 45). In figure 1, no reactions of identity can be seen between the host antigens (NE/SA) and the virus particle concentrate (V-C_2),

PLATE 44

The immunodiffusion reaction after a short, 12-hour incubation period, demonstrating the identification of host (H) and virus (V) antigenic components using specific antisera.

RaS-NE - rabbit anti-host serum.

RaS-PR8 - rabbit anti-PR8 soluble antigen serum.

RaS/IN - rabbit antiserum induced by intranasal infection with influenza A/PR8.

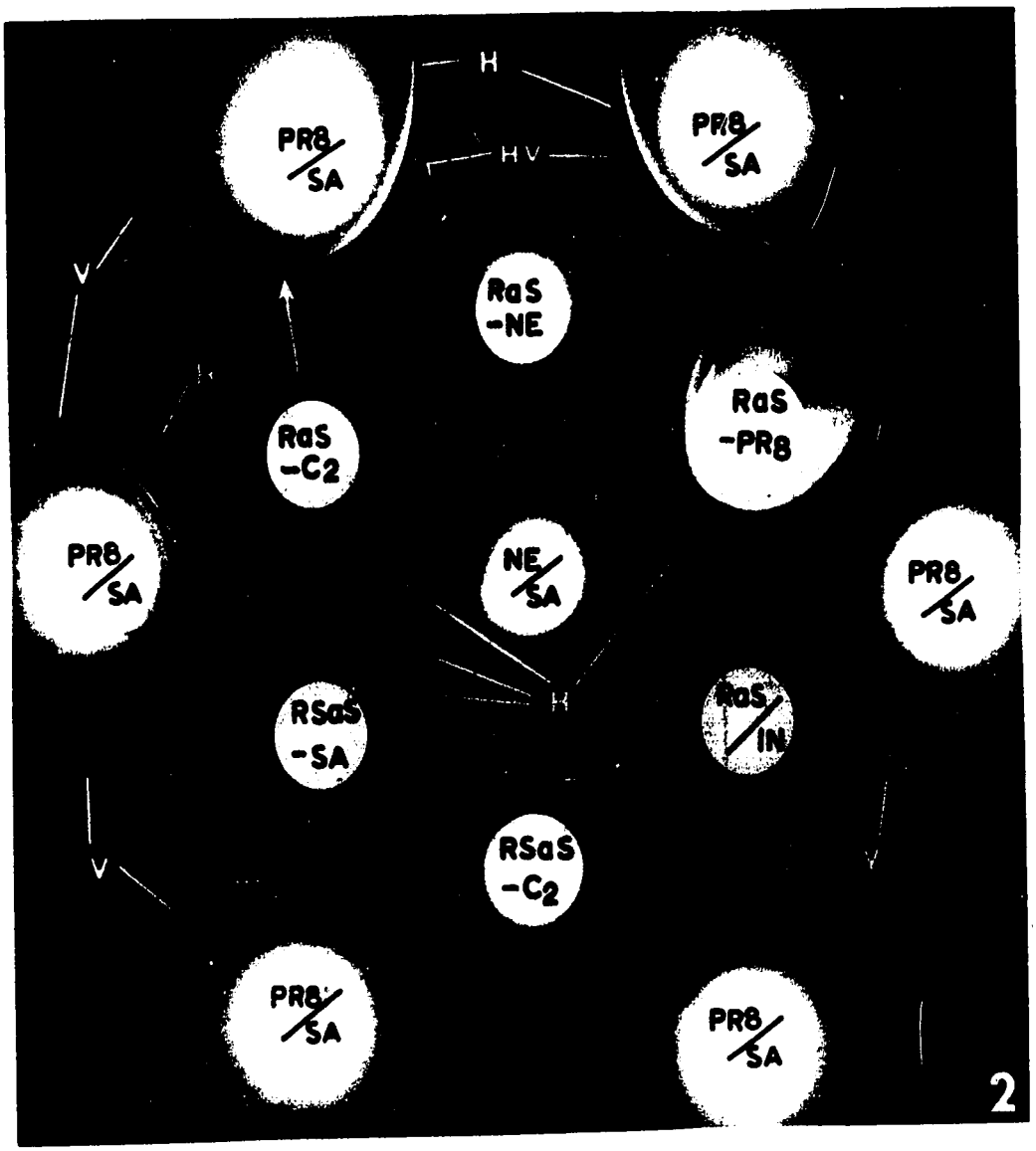
RSaS-C_2 - rooster anti-virus serum.

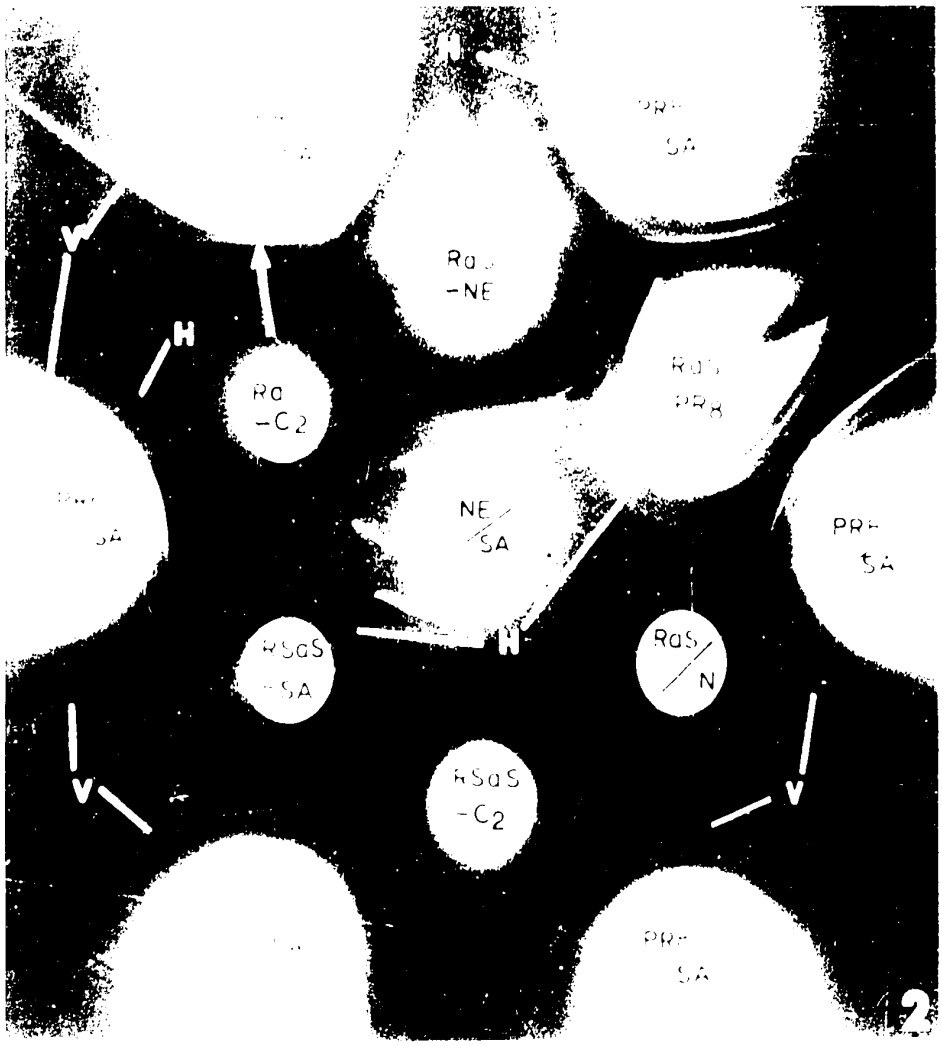
RSaS-SA - rooster anti-PR8/SA serum.

RaS-C_2 - rabbit anti-virus serum.

NE/SA - uninfected host CAM antigens.

PR8/SA - PR8-soluble antigens from infected CAM.


H
HV
V
PR8/SA
PR8/SA
RaS-NE
RaS-PR8
RaS-C2
PR8/SA
NE/SA
PR8/SA
H
RaS/IN
RSaS-SA
RSaS-C2
V
PR8/SA
PR8/SA
2


SA
Ra
-NE
H
Ra
-C2
NE
SA
PRE
SA
RaS
N
RSaS
-C2
V
V
V
SA
12

but two components (V) of the PR8/SA reaction can be identified with structural antigens in the virus concentrate ($V\text{-}C_2$).

In figure 2, the antigens released by the sodium deoxycholate (DOC/SNF) and sodium dodecyl sulfate (SDS/SNF) procedures are compared with infected and normal host antigens. It can be seen that:

(a) one of the five DOC/SNF structural antigens is identical to the structural antigen detected in the PR8/SA by the RaS/C_2 serum;

(b) this antigen does not show any host specificity;

(c) an unclear reaction of identity is demonstrated between the PR8/SA structural antigen and the antigens in the SDS/SNF fraction. This lack of clarity is believed to be due either to a loss in antigenic specificity as a result of the SDS treatment or the presence of residual detergent, which could interfere with the ag-ab reaction or a combination of both.

In figure 3, it is evident that:

(a) one of the structural antigens of the pronase

PLATE 45

The identification of the Virus Structural Antigens in PR8-soluble antigen using the specific rabbit antiserum, antiserum RaS-C_2.

Figure 1 - The detection of two 'V' antigenic components in PR8/SA. The reactions of identity are arrowed.

Figure 2 - The identification of one virus structural antigenic component (VsC) in PR8/SA by the reaction of identity with one antigenic component in the sodium deoxycholate-released virus antigens (DOC/SNF) and possible in the sodium dodecyl sulfate-released viral antigens (SDS/SNF).

Figure 3 - The comparison of the reactions of the pronase-released virus antigens (PRO/SNF) with the structural antigens of PR8/SA. The arrow VsC-2 indicates a virus structural component not present in PR8/SA. The VsC-1a line indicates a virus structural component which shows a reaction of partial identity with the PR8/SA structural antigen.

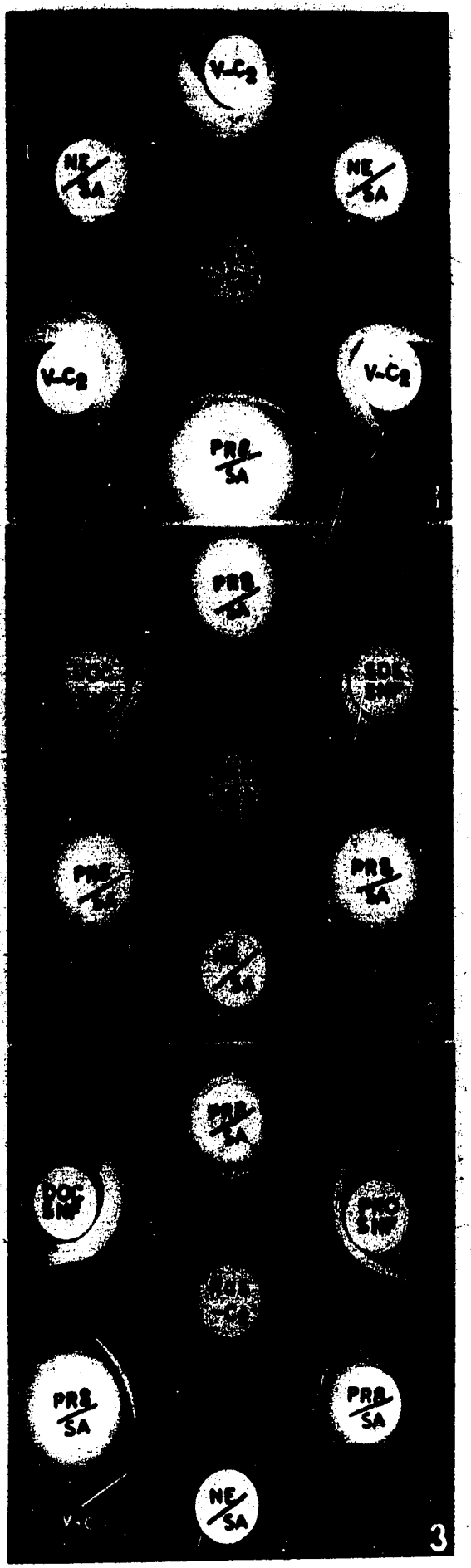
tiserum,

onents
re

ral
the

leased
n
al

F)
The
l
sC-1a
nent
tity



serum,

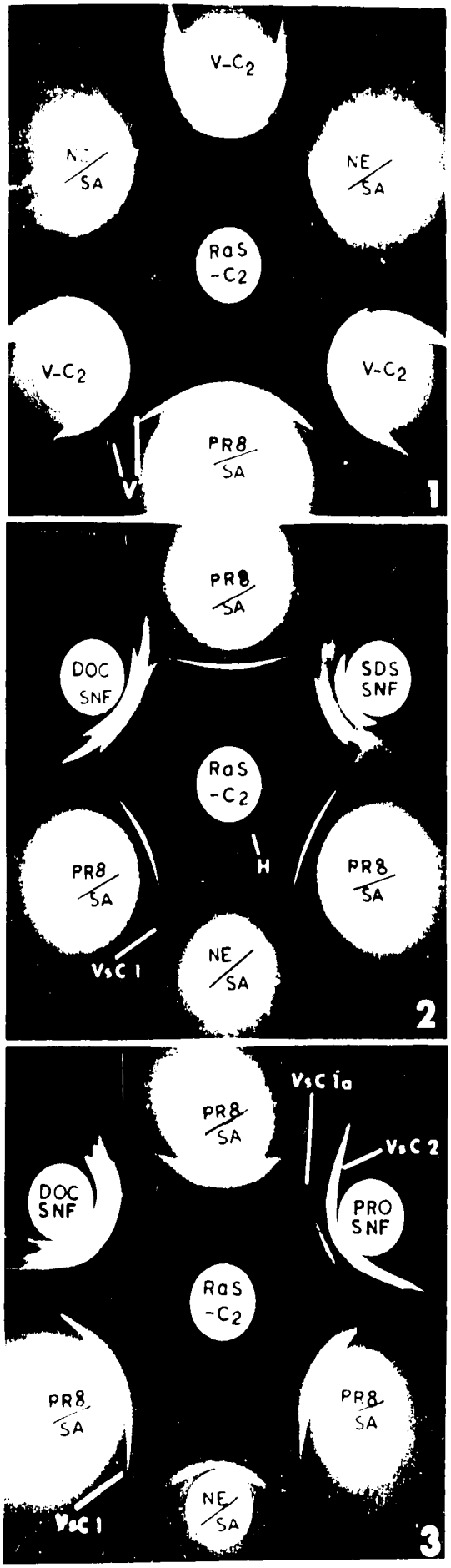
ents

l
e

ased

he

-la
nt
ty



fraction (PRO/SNF) (VsC_2) is not present in the PR8/SA, and

(b) the second structural antigen detected in the PRO/SNF fraction (VsC-1a) links into the virus structural component of PR8/SA, suggesting that the latter is a complex antigen;

(c) The RaS/C_2 serum has detected normal host antigens in the NE/SA, however no host specificity can be associated with the virus structural antigen present in PR8/SA.

To summarize, the virus structural component in the PR8/SA is barely identifiable as such in the comparison with 'intact' virus (figure 1), but is clearly shown to be a major component of the virus by extraction with DOC (figure 2). Use of the pronase extract suggests that the component is an antigenic complex (figure 3).

SUMMARY OF RESULTS

1. A highly sensitive micro-immunodiffusion technique was developed which permits better separation and resolution of complex antigen-antibody reactions than the micro-gel diffusion method. It was established that intact influenza virus particles could diffuse in the cellulose acetate and thus participate in immunodiffusion reactions.

2. Normal rabbit sera were found to precipitate influenza virus particles in the immunodiffusion reactions. Rabbits were found to be susceptible to infection with influenza A/PR8, and the antibodies produced as a result of infection showed reactions of identity with the virus precipitins in the normal rabbit sera. The precipitating component in the 'normal' rabbit sera was identified as a gamma globulin by serum fractionation studies. It was concluded that the virus precipitins in normal rabbit sera were antibody, probably present as a result of previous infection with influenza A/PR8, or a closely related myxovirus.

3. Studies on the morphology of influenza virus particles by electron microscopy revealed that it was possible to distinguish differences in the disruptive effects of several reagents on the structure of the influenza virus particle. On the basis of these E/M studies, the following agents for the disruption of the virus particle were selected: ultrasonication, surface-active detergents (sodium deoxycholate and sodium dodecyl sulfate), urea, chloroform and pronase.

4. The most efficient virus disrupting agent was sodium deoxycholate. Five virus particle antigens were detected by the immunodiffusion reaction, of which at least three were identified as virus specific structural antigens and one was identified as a host antigen. Disruption procedures using sodium dodecyl sulfate or pronase result in the release of virus antigens with different characteristics compared to the deoxycholate produced antigens, and further chemical and immunological analyses should include antigens obtained by all three procedures.

5. The influenza virus soluble antigens consist of three classes of antigens which can be identified as host specific (H), virus specific (V), and both host and virus specific (VH). Using antisera to viral antigens produced

in rabbits rendered partially tolerant to host antigens, five virus specific soluble antigens were provisionally identified.

6. One of the virus soluble antigens was identified as a virus specific structural antigen by means of the reaction of identity with the structural antigens released by DOC treatment.

GENERAL DISCUSSION

The immunological analysis of complex antigenic systems is dependent on the sensitivity of the technique used to detect the antigen-antibody reactions, the nature and specificity of the antisera used to detect the antigens, and finally the nature of the antigens themselves.

In these investigations a highly sensitive cellulose acetate micro-immunodiffusion technique was developed which offered better preparation and resolution of complex ag-ab reactions than other methods, using gel as the support media for diffusion. The antisera and antigens used in these investigations were mixtures of reactants of varying 'avidity' and of differing specificity. In such cases it is well known that the resulting bands of precipitate are likely to represent a range of ag-ab reactions and in any given test a complex of several antigenically distinct components of similar diffusing characteristics, may form a single band.

We were concerned in these investigations with

the detection and identification of as many antigenic components as possible. Under certain circumstances a single ag-ab complex may form more than one precipitin band in double immunodiffusion reactions and consequently one cannot be certain whether every line appearing in any one reaction represents a separate antigenic component. However, this question can be decided only at the stage of chemical isolation of individual components. At the present stage of the investigation, it is necessary to regard each precipitin line as representing the presence of individual antigenic components.

In all immunological analyses one must be sure of the reagents used, especially the analytical immune sera. In this context it was necessary to investigate the significance of the immunodiffusion reaction of the virus concentrate with the normal rabbit sera (NRS). On the basis of immunological, serological and chemical fractionation studies, it was concluded that rabbits were apparently susceptible to induced infection with influenza A/PR8 and that the antibodies could be detected in the 'normal' sera as a result of some previous experience with a myxovirus infection.

A variety of animals, including sheep, goats,

guinea pigs and fowl are also believed to be susceptible to influenza infection, as a result of preliminary screening test.

The presence of such 'natural' antibodies could vitiate the interpretation of immunological analyses of the antigens of influenza virus. For instance, recent observations have been reported which we are unable to confirm. Howe et al (1967) showed that the immunization of rabbits with normal chick-embryo tissue components resulted in the detection of antibodies capable of inhibiting influenza virus haemagglutination reactions. Clearly, such conclusions require the greatest caution unless all possibility of cross-infection has been avoided by rigid isolation procedures.

The framework of this investigation has been built around the hypothesis which predicted the presence of four classes of influenza soluble antigen components: host, host-altered by infection, virus structural and virus non-structural. The results obtained by immunodiffusion analysis support the hypothesis. The identification of the soluble antigens showing virus specificity as either structural or non-structural antigens was limited by the close antigenic similarity which was apparent between the host and virus soluble antigens. However, one of the virus soluble components

was clearly a structural antigen which was demonstrable in the virus particle antigens.

Disruptive procedures were found to release five detectable antigenic components from the virus particle, but at this stage of the investigation it is not possible to say how these might be related to the five virus components which Laver obtained by electrophoretic separation (1963).

It is clear that this work is only a foundation for the next stage of the investigation which must be concerned with fractionation, isolation and precise identification of the different classes of antigenic components which have been shown to be present.

REFERENCES

ADA, G.L. and Perry, B.T., 1954. The nucleic acid content of influenza virus. Aust. J. Exper. Biol., 32, 453-468.

ANALYTICAL CHEMISTRY, 1961. Spectrometry nomenclature. Anal. Chem., 33, 1968.

ANANTHANARAYAN, B.A. and Paniker, C.K.J., 1960. Non-specific inhibitors of influenza viruses in normal sera. Bull-Wld. Hlth.Org., 22, 409-419.

ANANTHANARAYAN, R., 1954. The fabric of virus elementary bodies. Brit.J. Exp. Path., 35, 381-388.

ANDERSEN, B.R., Abele, D.C., and Vannier, W.E., 1966. Effects of mild periodate oxidation on antibodies. J. Immunol., 97, 913-924.

BARRY, R.D., Horne, R.W., & Waterson, A.P., 1962. The incomplete forms of influenza virus. Z. Naturforsch., 17b, 749-750.

BELYAVIN, G., 1957. The influenza virus flocculation reaction as a method of antigenic typing. J. Hyg.(Camb.), 55, 281-289.

BIDDLE, F., and Stevenson, J.P., 1966. Flocculation of influenza viruses by horse serum inhibitor. Nature, 209, 1223-1225.

BILLINGHAM, R.E., Brent, L. and Medawar, P.B., 1953. Actively acquired tolerance of foreign cells. Nature, 172, 603-606.

BLOUGH, H.A., 1963. The effect of vitamin A alcohol on the morphology of myxoviruses. 1. The production and comparison of artificially produced filamentous virus. Virology, 19, 349-358.

BRACKENRIDGE, C.J., 1960a. Optimal staining conditions for the quantitative analysis of human serum protein fractions by cellulose acetate electrophoresis. Anal. Chem., 32, 1353-1356.

BRACKENRIDGE, C.J., 1960b. Optimal fractionation conditions for the quantitative analysis of human serum protein fractions by cellulose acetate electrophoresis. Anal. Chem., 32, 1357-1359.

BRACKENRIDGE, C.J., 1960c. Variable dye uptake in the quantitative analysis of abnormal globulins by cellulose acetate electrophoresis. Anal. Chem., 32, 1359-1360.

BRENNER, S. & Horne, R.W., 1959. A negative staining method for high resolution electron microscopy of viruses. Biochem. et Biophys. Acta., 34, 103-110.

BUCKLAND, F.E. & Tyrrell, D.A.J., 1963. A comparative study of virus haemagglutinins. The stability of haemagglutinins and red cell receptors to certain physical and chemical treatments. J. gen. Microbiol. 32, 241-253.

CAMPBELL, D.H., Garvey, J.S., Cremer, N.E. and Sussdorf, D.H., 1964. Methods in Immunology. W.A. Benjamin Inc., N.Y. and Amsterdam.

CINADER, B., St. Rose, J.E.M., and Yoshimura, M., 1967. The effect of cross-reacting antigens on the tolerant state. J. Exp. Med., 125, 1057-1073.

CLEELAND, R., and Sugg, J.Y., 1964. Serologic and antigenic properties of type A influenza virus after trypsin treatment. J. Immunol., 93, 414-419.

COHEN, A. and Belyavin, G., 1961. The influenza virus haemagglutination inhibitors of normal rabbit serum.
1. Separation of the inhibitory components. Virology, 13, 58-67.

COHEN, A., Biddle, F., and Newland, S.E., 1965. Analysis of horse serum inhibitors of A_2 influenza virus haemagglutination. Brit. J. Exp. Path. 46, 497-513.

COHEN, A. and Dorman, J., 1965. Non-specific inhibitors of A_2 influenza viruses in human sera. Acta Virol. 9, 519-525.

COHEN, A., Newland, S.E. and Biddle, F., 1963. Inhibition of influenza virus haemagglutination: A difference in behaviour of sera from a single species. Virology, 20, 518-529.

COHN , E.J., McMeekin, T.L., Oncley, J.L., Newell, J.M. and Hughes, W.L., 1940. Preparation and properties of serum and plasma proteins.
1. Size and charge of proteins separating upon equilibration across membranes with ammonium sulfate solutions of controlled

pH, ionic strength and temperature. J. Am. Chem. Soc., 62, 3386-3393.

COX, H.R., Van der Scheer, J., Aiston, S. and Bohnel, E., 1947. Purification and concentration of influenza virus by means of alcohol precipitation. J. Immunol., 56, 149-166.

CRAIGIE, J., 1932. Nature of vaccinia flocculation reaction, and observations on elementary bodies of vaccinia. Brit. J. Exp. Path., 13, 259-268.

CRAIGIE, J. and Wishart, F.O., 1936. Studies on the soluble precipitable substances of vaccinia: 1. The dissociation in vitro of soluble precipitable substances from elementary bodies of vaccinia. J. Exp. Med. 64, 803-818.

CROWLE, A.J., 1958. A simplified micro double-diffusion agar precipitin technique. J. Lab. Clin. Med., 52, 784-787.

CROWLE, A.J., 1961., in Immunodiffusion. Academic Press, N.Y. and London.

DALES, S. and Choppin, P.W., 1962. Attachment and penetration of influenza virus. Virology, 18, 489-493.

DUC-NGUYEN, H., Rose, H.M., and Morgan, C., 1966. The electron microscope study of changes at the surface of influenza-infected cells, as revealed by Ferritin-conjugated antibodies. Virology, 28, 404-412.

ECKERT, E.A., 1966. Envelope protein(s) derived from influenza virus. J. Bact., 91, 1907-1910.

EDELMAN, G.M. and Poulik, M.D., 1961. Studies on structural units of the globulins. J. Exp. Med., 113, 861-884.

ELFORD, W.J. and Andrewes, C.H., 1936. Centrifugation studies: viruses of vaccinia, influenza and Rous sarcoma. Brit. J. exp. Path., 17, 422-430.

FABIYI, A., Lief, F.S. and Henle, W., 1958. Antigenic analysis of influenza viruses by complement fixation.
II. The production of antisera to strain-specific V antigens in guinea pigs.
J. Immunol., 81, 467-477.

FAZEKAS de St. Groth, S., 1964. P.168. Cellular biology of myxovirus infections - Ciba Symp. Ed. by Wolsterholme, G.E.W. & Knight, J. J. & A. Churchill (Publ.), 104 Gloucester Place, London, W.1.

FRISCH-NIGGEMEYER, W. and Hoyle, L., 1956. The nucleic acid and carbohydrate content of influenza virus A and of virus fractions produced by ether disintegration. J. Hyg. 54, 201-212.

FROMMHAGEN, L.H. and Knight, C.A., 1959. Column purification of influenza virus. Virology, 8, 198-208.

GEFT, R.A. and Polyak, R.Y.A., 1963. Removal of thermostable inhibitors against A_2 influenza viruses from immune sera by Rivanol. Acta Virol. 7, 91.

GOLD, P. and Freedman, S.O., 1965. Demonstration of tumor-specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. J. Exp. Med., 121, 439-462.

GRUNBAUM, B.W., Kirk, P.L. & Atchley, W.A., 1960. Micro-electrophoresis on cellulose acetate membranes. Anal. Chem., 32, 1361-1362.

HARBOE, A., 1963a. The influenza virus haemagglutination inhibition by antibody to host material. Acta Pathol. Microbiol. Scand., 57, 317-330.

HARBOE, A., 1963b. The normal allantoic antigen which neutralizes the influenza virus HI-antibody to host material. Acta Pathol. Microbiol. Scand., 57, 488-492.

HARBOE, A. and Reenaas, R., 1959. The course of antibody development to influenza A_2 strains. Acta Pathol. Microbiol. Scand., 46, 266-272.

HARRIS, R.J.C., 1964. Techniques in experimental virology. Academic Press, N.Y.

HAUKENES, G., Harboe, A., and Mortensson - Egmund K., 1965. A uronic and sialic acid free chick allantoic mucopolysaccharide sulfate which combines with influenza HI-antibody to host material. Acta Pathol. Microbiol. Scand., 64, 534-542.

HAUROWITZ, F., 1963. The chemistry and function of proteins. Academic Press, N.Y. and London.

HILLIS, W.D., Moffat, M.A.J. & Holtermann, O.A., 1960. Development of soluble (S) and viral (V) antigens of influenza A virus in tissue culture as studied by the fluorescent antibody technique.
3. Studies on the abortive cycle of replication in Hela cells. Acta Pathol. Microbiol. Scand., 50, 419-429.

HIRST, G.K., 1941. Agglutination of red cells by allantoic fluid of chick embryos infected with influenza virus. Science, 94, 22-23.

HOLTERMANN, O.A., Hillis, W.D. & Moffat, M.J., 1960. The development of soluble (S) and viral (V) antigens of influenza A virus in tissue culture as studied by the fluorescent antibody technique.
1. Studies employing a low multiplicity of infection in beef embryo kidney cells. Acta Pathol. Microbiol. Scand., 50, 398-408.

HOREJSI, J. and Smetana R., 1956. Isolation of gamma globulin from blood serum by Rivanol. Acta Med. Scand., 155, 65-70.

HORNE, R.W., Waterson, A.P., Wildy, P. & Farnham, A.E., 1960. The structure and composition of myxoviruses. I. Electron microscope studies of the structure of myxovirus particles by negative staining techniques. Virology, 11, 79-98.

HORNE, R.W. and Wildy, P., 1963. Virus structure revealed by negative staining. Adv. Virus Res. 10, 101-170.

HOWE, C., Lee, L.T., Harboe, A., and Haukenes, G., 1967. Immunological study of influenza virus and associated host tissue components. J. Immunol. 98 (3), 543-557.

HOYLE, L., 1952. Structure of the influenza virus. The relation between biological activity and chemical structure of virus fractions. J. Hyg. Camb., 50, 229-245.

HOYLE, L. and Davies, S., 1961. Amino acid composition of the protein components of influenza virus A. Virology, 13, 53-57.

HOYLE, L., Horne, R.W. and Waterson, A.P., 1961. The structure and composition of the myxoviruses.
II. Components released from influenza virus particle by ether. Virology, 13, 448-459.

HOYLE, L., Horne, R.W. and Waterson, A.P., 1962. The structure and composition of the myxoviruses.
III. The interaction of influenza virus particles with cytoplasmic particles derived from normal chorioallantoic membrane cells. Virology, 17, 533-542.

JOHNSON, C.M., Westwood, J.C.N. and Beaulieu, M., 1964. A continuous-flow micro double-diffusion technique with cellulose acetate. Nature (Lond.) 204, 1321-1322.

KARLSON, P., 1963. Introduction to modern biochemistry. Academic Press, N.Y. and London.

KATES, M., Allison, A.C., Tyrrell, D.A.J., and James, A.T., 1961. Lipids of influenza virus and their relation to those of the host cell. Biochem. Biophys. Acta. 52, 455-466.

KENDALL, F.E., 1937. Studies on serum proteins; identification of single serum globulin by immunological means. Its distribution in sera of normal individuals and of patients with cirrhosis of liver and with chronic glomerulonephritis. J. Clin. Invest. 16, 921-931.

KNIGHT, C.A., 1944. A sedimentable component of allantoic fluid and its relationship to influenza viruses. J. Exp . Med. 80, 83-100.

KNIGHT, C.A., 1946. Precipitin reactions of highly purified influenza viruses and related materials. J. Exp . Med. 83, 281-294.

KOLTHOFF, I.M., Anastasi, A., Stricks, W., Tan, B.H. and Deshmukh, G.S., 1957. Reactivity of sulfhydryl and disulfide upon denaturation of proteins.
1. Sulfhydryl in native serum albumin and upon denaturation in guanidine hydrochloride solution. J. Am. Chem. Soc., 79, 5102-5110.

KROEGER, A.V., 1962. Evidence for the absence of anaphylactogenic host protein in highly purified PR8 influenza virus. J. Immun., 89, 136-144.

LEGGETT, Bailey J., 1962. P.293. Techniques in protein chemistry. Elsevier Publishing Company, New York.

LENNETTE, E.H. and Horsfall, F.L. Jr., 1941. Studies on influenza virus: complement fixing antigen of influenza A and swine influenza viruses. J. Eap. Med., 73, 581-599.

LIEF, F.S., Fabiyi, A. and Henle, W., 1958. Antigenic analyses of influenza viruses by complement fixation.
I. The production of antibodies to the soluble antigen in guinea pigs.
J. Immunol., 80, 53-65.

LIEF, F.S. and Henle, W., 1959. Methods and procedures for use of complement fixation technique in type and strain specific diagnosis of influenza. Bull Wld. Hlth Org., 20, 411-420.

LOWRY, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265-275.

LURIA, S.E. and Darnell, J.E., 1967. General virology, 2nd edition. John Wiley & Sons Inc.

LAFFERTY, K.J. and Oertelis, S., 1963. The interaction between virus and antibody. III. Examination of virus antibody complexes with the electron microscope. Virology, 21, 91-99.

LAVER, W.G., 1962. The structure of influenza viruses. 1. N-terminal amino acid analyses. Virology, 18, 19-32.

LAVER, W.G., 1963. The structure of influenza viruses. 3. The disruption of the virus particle and separation of neuraminidase activity. Virology, 20, 251-262.

LAVER, W.G., 1964. Structural studies on the protein sub-units from three strains of influenza virus. J. Mol. Biol., 9, 109-124.

LAVER, W.G. and Webster, R.G., 1966. The structure of influenza viruses. IV. Chemical studies of the host antigen. Virology, 30, 104-115.

MARKOWITZ, A.S. and Isenberg, J., 1963. Ultra-micro precipitin procedure on cellulose acetate. Biochem. Biophys. Res. Comm., 12, 56-61.

MATHER, K., 1951. Statistical analysis in biology. Methuen and Co.Ltd., Publ., London.

McCLELLAND, L. and Hare, R., 1941. Adsorption of influenza virus by red cells and a new in vitro method of measuring antibodies for influenza virus. Canad. J. Pub. Health. 32, 530-538.

MENZEL, A.E.O., and Sherman, W.B., 1964. The antibody mechanism of Ragweed allergy. Electro-phoretic and chemical studies.

III. An improved method of fractionation with Rivanol and its application to ragweed reagenic serums. Int. Arch. Allergy, 24, 127-146.

MILLER, H.K. and Schlesinger, R.W., 1955. Differentiation and purification of influenza viruses by adsorption on aluminum phosphate. J. Immunol, 75, 155-175.

MIZUTANI, H. and Mizutani, H., 1965. Small haemagglutinin of influenza virus and its responses to antibodies and inhibitor. Virology, 26, 761-763.

MOFFAT, M.A.J., Holtermann, O.A. and Hillis, W.D., 1960. The development of soluble (S) and viral (V) antigens of influenza A in tissue culture is studied by fluorescent antibody technique.
2. Studies employing a high multiplicity of infection in beef embryo kidney cells. Acta Path. et Micro. Scand., 50, 409-418.

MOORE, D.H., Davies, M.C., Levine, S. and Englert, M.E., 1962. Correlation of structure with infectivity of influenza virus. Virology, 17, 470-479.

MORGAN, C., Rifkind, R.A. and Rose, H.M., 1962. The use of Ferritin-conjugated antibodies in electron microscope studies of influenza and vaccinia viruses. Cold Spring Harbor Symps., 27, 57-65.

NEGRONI, G. and Tyrrell, D.A.J., 1959. Morphological observations on tissue cultures of epithelial cells infected with influenza A viruses. J. Path Bact., 77, 497-504.

NEWTON, B.A., 1958. Surface-active bactericides in The Strategy of Chemotherapy. The 8th Symp. of Soc. Gen. Micro. Cambridge University Press.

NEWTON, N. and Bevis, R.E., 1959. Purification of animal viruses with $Zn(OH)_2$. Virology, 8, 344-351.

NOLL, H., Aoyagi, T. and Orlando, J., 1962. The structural relationship of sialidase to the influenza virus surface. Virology, 18, 154-157.

OUCHTERLONY, O., 1958. Diffusion-in-gel methods for immunological analysis. Progr. Allergy, 5, 1-78.

OUCHTERLONY, O., 1962. Diffusion-in-gel methods for immunological analysis. Progr. Allergy, 6, 30-154.

PARSONS, D.F., 1963a. Negative staining of thinly spread cells and associated virus. J. Cell. Biol., 16, 620-626.

PARSONS, D.F., 1963b. Morphology of K virus and its relation to the Papova group of viruses. Virology, 20, 385-388.

PETERSON, E.A. and Sober, H.A., 1956. Chromatography of proteins.
1. Cellulose ion-encharge adsorbents.
J. Am. Chem. Soc., 78, 751-755.

POLSON, A., 1956. Separation of small infective components of MEF_1, poliomyelitis and horse sickness viruses, by migration into agar gel. Biochim. Biophys. Acta, 19, 53-57.

REED, L.J. and Muench, H., 1938. A simple method of estimating fifty per cent. endpoints. Am. J. Hyg., 27, 493-497.

REGINSTER, M., 1965. Modification of influenza virus morphology by Streptomyces albus G caseinase C. Acta virol. 9, 462-463.

REGINSTER, M., 1966. Effects of pronase on influenza virus. Acta virol., 10, 111-116.

RIBEIRO, L.P., Mitidieri, E. & Affonso, O.R., 1961. Paper electrophoresis, pp. 59-61. Publ. by Elsevier Publishing Company, London.

RIFKIND, R.A., Hsu, K.C., Morgan, C., Seegal, B.C., Knox, A.W. and Rose, H.M., 1960. Use of Ferritin-conjugated antibody to localize antigen by electron microscope. Nature (Lond.) 187, 1094-1095.

ROTT, R. and Schafer, W., 1964. Ciba Fdn. Symp: Cellular Biology of Myxovirus Infections. P.30. Ed. by Wolstenholme, G.E.W. and Knight, J. J. & A. Churchill Ltd., 104 Gloucester Place, London.

RUTTKAY-Nedecky, G. and Ivanicova, S., 1965. Electrophoretic control of influenza A_2 virus purification and demonstration of an additional virus-specific electrophoretic component in the purified virus preparations. Acta virol. 9, 508-518.

SAMPAIO, A.A. de C. and Isaacs, A., 1953. The action of trypsin on normal serum inhibitors of influenza virus haemagglutination. Brit. J. Exper. Path., 34, 152-158.

SCHAFER, W., 1957. The nature of viruses - ciba symp. P.91. J. & A. Churchill, 104 Gloucester Place, London, W.1.

SCHAFER, W. and Zillig, W., 1954. Über den aufbau des virus - Elementarteilchens der Klassischen Geflügelpest.
1. Gewinnung, physikalisch - chemische und biologische Eigenschaften einiger Spaltprodukte. Z. Naturf 9b, 799.

SCHMIDT, N.J. and Lennette,E.H., 1965. P.1211. Viral and Rickettsial infections of Man. Ed. by Horsfall, F.L. and Tamm Igor. 4th ed. J.B. Lippencott Co. Publ.

SEVER, J.L., 1962. Applications of a microtechnique to viral serological investigations. J. Immun. 88.

SHEFFIELD, F.W., Smith, Wilson, and Belyavin, G., 1954. Purification of influenza virus by red cell adsorption and elution. Brit. J. Exp. Path., 35, 214-222.

SHERR, George H., 1961. Cellulose acetate electrophoresis in microbiology and immunology. Trans. N.Y. Acad. Sci., 23, 519-530.

SILVERSTEIN, S.C. and Marcus, P.I., 1964. Early stages of Newcastle disease virus - hela cell interaction: an electron microscope study. Virology, 23, 370-80.

SMITH, Ivor, 1960. Chromatographic and Electrophoretic Techniques. Vol.II. Zone Electrophoresis. Interscience Publishers Inc., New York.

SMITH, W., Belyavin, G. and Sheffield, F.W., 1953. A host protein component of influenza viruses. Nature, 172, 669-670.

SMITH, W., Belyavin, G. and Sheffield, F.W., 1955. The host tissue component of influenza viruses. Proc. Roy. Soc. B.143, 504-522.

SPRINGER, G.F., 1963. Enzymatic and nonenzymatic alterations of erythrocyte surface antigens. Bact. Rev., 27, 191-227.

STANLEY, W., 1944. Evaluation of methods for concentration and purification of influenza virus. J. Exp. Med., 79, 255-266.

STANWORTH, D.R., 1960. A rapid method of preparing pure serum gamma globulin. Nature, 188, 156-1960.

STASTNY, M. and Horejsi, J., 1961. The interaction of acridine dyes with blood plasma proteins. Clin. Chem. Acta, 6, 782-793.

STRANDLI, O.K., Mortensson - Egmund K. and Harboe, A., 1964. Purification of the normal allantoic antigen which reacts with influenza virus HI antibody to host material. Acta Pathol. Microbiol. Scand., 60, 265-270.

ST. ROSE, J.E.M. and Cinader, B., 1967. The effect of tolerance on the specificity of the antibody response and on immunogenicity. J. Exp. Med. 125, 1031-1055.

STYK, L. and Hana, B., 1962. Characterization of antibodies, cofactor and nonspecific viral inhibitors on Sephadex G-200. Acta virol., 6, 479.

STYK, L. and Hana, B., 1966. Immunodiffusion studies on the reaction of influenza virus with specific antibody and non-specific serum beta inhibitor. Acta virol., 10, 281-290.

SUMMERS, D.F., Maigel, J.V., Jr., and Darnell, J.E. Jr., 1965. Evidence for virus-specific noncapsid proteins in poliovirus-infected hela cells. Proc. Nat. Acad. Sci. U.S.A., 54, 505-513.

de-THÉ, Guy, and O'Connor, T.E., 1966. Structure of murine leukemia virus after disruption with Tween-ether and comparison with two myxoviruses. Virology, 28, 713-728.

TYRRELL, D.A.J., 1963. Mechanisms of cell infection. III. Virus release, in Mechanisms of Virus Infection, p.225-254. Ed. by Wilson Smith. Academic Press, New York.

VAN REGENMORTEL, M.H.V., 1966. Plant virus serology. Adv. in virus res., 12, 207-271.

de VAUX ST. CYR, C. and Howe, C., 1966. Immunochemical study of parainfluenza virus (type 2) in amnion cells. J. Bact., 91, 1911-1916.

VEERAROGHAVAN, N. and Sreevalsan, T., 1961. Evaluation of some methods of concentration and purification of influenza virus. Bull Wld. Hlth. Org., 24, 695-702.

WADSWORTH, C., 1957. A slide microtechnique for the analysis of immune precipitates in gel. Int. Arch. Allergy, 10, 355-360.

WEINSTEIN, L. and McDonald, A., 1945. The effect of urea, urethane and other carbamates on bacterial growth. Science, 101, 44.

WESTWOOD, J.C.N., 1963. Virus pathogenicity in Mechanisms of Virus Infection. P.255-307. Ed. Wilson Smith, Academic Press, New York.

WESTWOOD, J.C.N., Zwartouw, H.T., Appleyard, G. and Titmuss, D.H.J., 1965. Comparison of the soluble antigens and virus particle antigens of vaccinia virus. J. Gen. Microbiol., 38, 47-53.

WHITNEY, P.L. and Tanford, Charles, 1962. Solubility of amino acids in aqueous urea solutions and its implications for the denaturation of proteins by urea. J. Biol. Chem., 237 PC 1735-1737.

APPENDIX

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

COMBINED RESULTS FOR ERROR IN USE OF MICROBURET

yringe Size	No. of Divisions Per Sample	Solution Dispensed	Total Diluent	Final Concn µg/µl	No. of Samples	(max)	O.D. Absorbance Units	Sd	C. of V. %
µl. er division	2	.2% Ponceau S.	2 ml. TCA	.002	20	520	.089	.004	4.5
			2 ml. AC	.002	20	520	.091	.003	3.27
			2 ml. Na_2CO_3	.002	20	520	.060	.003	5.0
	10	.2% Ponceau S.	2 ml. TCA	.01	20	520	.453	.0045	.97
			2 ml. AC	.01	20	520	.459	.0063	1.3
			2 ml. Na_2CO_3	.01	20	520	.324	.004	1.2
	12	Lab Trol	5 ml. Saline	.184	10	280	.184	.005	2.66
	15	Lab Trol	2 ml. Saline	.574	9	280	.573	.008	1.39
	20	.2% Ponceau S.	2 ml. TCA	.02	20	520	.895	.0138	1.54
			2 ml. AC	.02	20	520	.912	.015	1.60
			2 ml. Na_2CO_3	.02	20	520	.674	.012	1.78
	30	Lab Trol	5 ml. Saline	.460	10	280	.463	.004	.86
.5 µl. er division	5	Lab Trol	2 ml. Saline	.096	10	280	.0845	.005	5.9
	10	Lab Trol	2 ml. Saline	.191	10	280	.196	.008	4.16
	20	Lab Trol	2 ml. Saline	.3835	10	280	.383	.011	2.7
	30	Lab Trol	2 ml. Saline	.5735	20	280	.575	.006	1.04
µl. er division	4	Lab Trol	5 ml. Saline	.184	10	280	.186	.017	9.2
	10	Lab Trol	5 ml. Saline	.460	10	280	.454	.006	1.3

KEY TO ABBREVIATIONS

AF or AlF	Allantoic fluid
C_2N	Normal sedimentable material from uninfected allantoic fluid.
DOC	Sodium deoxycholate.
GS	Goat serum.
GPS	Guinea pig serum
GPaS	Guinea pig antiserum to both influenza A/PR8 and egg antigens.
GPaS/IN	Guinea pig antiserum prepared by intranasal infection with influenza A/PR8 virus.
NE/SA	Saline extract of chorioallantoic membranes from un-infected chick embryos (12 days).
NRS	"Normal" rabbit serum.
PBS	Phosphate buffered saline.
PR8/SA	Saline extract of chorioallantoic membranes from chick embryos infected with influenza A/PR8.
PRO	Pronase.
RS	Rabbit serum
RaS	Rabbit antiserum prepared against the complete spectrum of viral, soluble and egg antigens- 'pan specific'.
RaS/C_2	Rabbit antiserum to virus concentrate V-C_2

RaS/IN	Rabbit antiserum prepared by intranasal infection with influenza A/PR8 virus.
RaS/NE	Rabbit antiserum to uninfected chick embryo CA membrane extracts.
RaS/PR8	Rabbit antiserum to extracts of influenza A/PR8 infected chick embryo CA membranes.
RSS	Rooster serum
RSaS	Rooster antiserum to pooled PR8/SA and $V\text{-}C_2$ antigens.
$RSaS/C_2$	Rooster antiserum to virus concentrate $V\text{-}C_2$.
RSaS/NE	Rooster serum following a series of injections with extracts of uninfected chick embryo CA membranes.
RSaS/PR8	Rooster antiserum to extracts of influenza A/PR8 infected chick embryo CA membranes.
SA	Soluble antigen.
SDS	Sodium dodecyl sulfate.
SNF_1, SNF_2, SNF_3	Supernatant fluid, from $V\text{-}C_1$, $V\text{-}C_2$ and $V\text{-}C_3$ respectively, containing residual haemagglutinin and soluble antigen.
VBS	Veronal buffered saline.
V	virus
$V\text{-}C_1$, $V\text{-}C_2$	Semipurified PR8 virus concentrates, 10x and 100x respectively.
$V\text{-}C_3$	Special preparation of PR8 virus concentrate (100x) containing minimal amounts of free haemagglutinin and soluble antigen.

GLOSSARY

Analytical Antiserum. Immune serum which has been prepared against a specific antigen or group of antigens, (i.e. virus concentrate or chick embryo extract) and which is then used to detect those antigens in samples obtained by various fractionation or disruption procedures, using the immunodiffusion reaction.

'H' antibody. Flocculating type of antibody most commonly produced by the horse injected with protein antigens, which forms specific precipitates with its antigen which dissolve readily in either antigen or antibody excess.

Host Antigen (H). A collective term to include all antigens detectable in the saline extracts of normal uninfected chick embryos. There is no relationship to the 'H' antibody (see above).

Immunologic. Tolerance. Any impressed diminution in immunologic responses below those which normally occur after antigenic excitation.*

*Chase, M.W. Immunologic Tolerance. Ann. Rev. Microbiol. 13: 349, 1959.

Following a series of injections of a group of protein antigens (NE/SA) in the neonatal rabbit, when no circulating antibody to NE/SA can be demonstrated after further routine immunization procedures, such animals are regarded as being immunologically tolerant.

Indirect Reaction of Identity. When two reacting systems are too weak to form a reaction of identity in an immunodiffusion test, it is possible to show the relationship by interpolating a strongly reacting related system between the two. A linkage formed between each of the weak systems and the same precipitin band of the known strong reaction demonstrates the presence of the same component in both weak systems. The reaction is termed the indirect reaction of identity.

Non-Specific Inhibitor. Substances of either protein, lipid or carbohydrate (lipo-polysaccharide) nature which may be present in the serum of non-immunized animals and which inhibit the haemagglutination of fowl R.B.C.s by myxoviruses. Such non-specific inhibitors may be inactivated by several different serum treatments.

Non-Structural Antigen. Any antigen which may be detected following virus replication which is not identical to the antigens of the host nor to the antigens incorporated into the mature virus particle, i.e. structural antigens.

Pan-Specific Antiserum. A term used here to describe antiserum prepared against the complete spectrum of influenza A/PR8 antigens, host, structural and non-structural.

Partial Tolerance. Following a series of injections of a multiple antigen in the neonatal rabbit, when there is a suppression of antibody to only a portion of the antigenic components after further routine immunization procedures, such animals are regarded as being tolerant to part of the antigenic stimuli and thus in a state of partial tolerance.

'R' Antibody. Precipitating type of antibody produced by rabbits which forms a precipitate with its antigen practically insoluble in antibody excess and often not readily soluble in antigen excess.

Reaction of Identity. The reaction formed between two immunologically identical antigens reacting with an antibody in a comparative immunodiffusion test. The bands formed by each component reacting independently, tend to bend toward each other at the tip and finally fuse or coalesce.

Reaction of Non-Identity. The pattern formed when two unrelated components react in an immunodiffusion test. Precipitin bands form independently which cross over each other indifferently.

Reaction of Partial Identity. The pattern produced when partially related antigens are compared using a single antiserum (or vice versa), their bands extending beyond the point of crossing being acutely curved and distinctly fainter than they were before the point of crossing. In other cases the formation of a spur, an extension of a precipitin band linkage, also depicts a reaction of partial identity.

'S' Antigen. A soluble (S) antigen found in infected tissues which has been shown to be identical to the ribonucleoprotein (RNP) antigen released from infectious influenza virus particles by ether treatment.

Soluble Antigen. (SA) The saline extract of chorioallantoic membranes from either normal or infected 12 day chick embryos, which has been centrifuged to remove virus particles

Structural Antigens. Those antigenic components incorporated into the mature virus particle and which may be detected by disruption of the virus particle.

'V' Antigen. The strain specific antigen of myxoviruses which is detected by complement fixation and virus neutralization tests and which is identical, or closely associated, with the strain specific haemagglutinin.