THE SENSITIVITY AND RELEVANCE OF SELECTED IMMUNOLOGICAL METHODS FOR THE ESTIMATION OF ANTIVIRAL ANTIBODY IN SERUM

by

GORDON C. INGLIS, B.Sc.

being a thesis submitted for the Degree of Doctor of Philosophy in the University of Glasgow

1980

To my mother

ACKNOWLEDGEMENTS

I wish to thank Dr. R.G. Sommerville, M.D., F.R.C.P.(Glasg), F.R.C.Path., for his overall supervision and guidance during the course of my project.

I am also indebted to Dr. Valerie Inglis, B.Sc., Ph.D., for her valuable advice and assistance.

I extend my thanks to Dr. Margaret Hutton, M.B., Ch.B. and Dr. Elizabeth Riddell for supplying the coded blood samples used in the forensic blind trial experiments.

I also express my gratitude to Miss Margaret Brough for her invaluable secretarial assistance in preparing the manuscript and acknowledge the warm fellowship of the laboratory staff at Belvidere Hospital, Glasgow during my 3 year contract.

Finally, I express my gratitude to my mother and my fiancée, Hilda, for their patience during the preparation of my thesis.

Abbreviations

Ab	:	Antibody
Ag	:	Antigen
¢	:	Coxsackievirus
cf	:	Compared with
CFT	:	Complement Fixation Test
CPE	:	Cytopathic Effect
CMV	:	Cytomegalovirus
EBV	:	Epstein - Barr Virus
EIA	:	Enzyme Immunoassay
ELISA	:	Enzyme - Linked Immunosorbent Assay
Flu	:	Influenza
HSV	:	Herpes Simplex Virus
IFA	:	Indirect Fluorescent Antibody
Ig G	:	Immunoglobulin G
Kc/sec	:	Kilocycles per second
N	:	No specific antiviral antibody detected
nt	:	Antibody detected in undiluted plasma sample
-	:	No result available
PBS	:	Phosphate Buffered Saline
r	:	Correlation coefficient
RSV	:	Respiratory Syncytial Virus
VAP	:	Virus Antibody Profile
w/v	:	Weight to volume ratio

CONTENTS

SUMMARY

The findings embodied in this thesis have been discussed in the following two sections.

A. Comparative Serology

The indirect fluorescent antibody test (IFA) and the enzyme-linked immunosorbent assay test (ELISA) have been investigated for their sensitivity in determining IgG antibody levels in human sera to Herpes simplex virus type 1, Coxsackievirus B_1 and Rotavirus. The results obtained have been correlated with those obtained from neutralisation or complement fixation tests in an attempt to evaluate the relevance of the antibodies detected by these serological procedures.

B. Virus Antibody Profiling

A novel technique for discriminating between human bloodstains has been developed and the suitability of the IFA and ELISA tests for use in this procedure has been investigated.

INTRODUCTION

Addressing a delegate conference at W.H.O. Geneva in March, 1979, the Director of Special Programmes relating to Immunology drew attention to the plethora of types of test available world-wide for measuring immunity to many different types of antigen. His address ranged over topics from immunity to parasites to immunity to bacteria and viruses. He concluded that, "the scientific community will sooner or later have to sit down and make some hard decisions about the significance and relevance of the various methods available for measuring immunity in different situations and to different immunogens. In particular, it is becoming increasingly imperative that we make decisions about two situations.

Firstly, we need to define a simple, reliable and if possible, rapid test which would indicate infection which is current and requires treatment, if such is available.

Secondly, we urgently need a single, simple and reliable test which would indicate in a particular disease situation and in a particular country, that a population group was immune to a particular antigen and that the immunity was protective. Only when these two tests have been defined can we begin to come to terms, on a global scale, with the problems of disease caused by infections of all descriptions from parasites to bacteria and viruses".

In view of the complexity of the problem, this may well be a pious hope, but the fact that WHO officials are suggesting a more concentrated focussing on the meaning and significance of immunity measurements sta rt s to reinforce the subject matter of this thesis,

relating to viruses. The problem is vast and complex - more with parasites than with viruses because of the larger size, organizational and antigenic complexity of the former.

Viruses vary in size from approximately 10 nm through 300 nm and vary considerably in antigenic complexity. To some extent it would be true to state that DNA viruses are more complex than are those containing RNA.

Before proceeding to discuss the possible methods of immunological approach to unravelling the significance of antibody measurement, I would like to give a brief resumé relating to the structure and composition of two viruses - one simple (picornavirus) and the other complicated by size and antigenic complexity (influenza virus). By doing so I shall be able to elaborate some of the relevant and complex factors which have a bearing on antibody measurement.

For brevity and relevance to my chosen subject, I shall discuss only the humoral response (as opposed to the secretory system) and I shall not consider the cellular aspects of immunity.

Perhaps the greatest amount of investigative work has been done on the relatively simple picornavirus which causes foot and mouth disease (FMDV). This belongs to a vast group of tiny (approximately 23 nm-diameter) RNA viruses which include the enteroviruses (polio, coxsackie and ECHO) all of which are acid stable, and the rhinoviruses plus FMDV with approximately 95 and seven distinct serotypes respectively. Both of these latter

groups are acid labile and have many subtypes amongst their conventional serotypes.

Most picornaviruses have four virus polypeptide subunits within the capsid (VP1 - 4), often in the ratio of 1:1:1:0.5 and some have a higher molecular weight peptide which has, curiously, been labelled V_0.

During the course of virus synthesis in infected cells various non capsid virus-specific proteins are produced. m-RNA is translated to large molecular weight protein products which are cleft during or subsequent to translation into a number of smaller proteins. Some of these latter become part of the virus capsid as V1-V4 polypeptides but others do not become part of the virus structure. The association of these with any specific viral function has not been proved but they are all antigenic.

Cowan and Graves (1966) concentrated fluids from BHK-21 infected cell cultures and obtained three precipitin bands in double immuno-diffusion tests when they used sera from convalescent animals. After a series of purification steps they were able to show that one band was due to intact virions (140S antigen); one to several protein subunits (12S), while the third was due to non capsid virus protein (VIA). Cowan and Graves (1966) were able to demonstrate the same three antigens in vesicles of animals which were infected with FMDV. Bradish et. al. (1960) showed that treatment of the purified 140S antigen with different acids resulted in the breakdown of the virion to RNA and 12S protein subunits each with diameter of 7 nm. Cowan (1968) further

purified the 12S component by DEAE cellulose chromatography and observed that the immunological relationship between the 140S and the 12S component derived from it was quite distinct. It appeared that the 12S component bore no obvious relation to the 140S antigen as previously suggested by Brown and Crick (1958), and by Ceglowski (1965). A similar apparent lack of relationship was shown for tobacco mosaic virus and turnip yellow mosaic virus by Rappaport et. al. (1965). Jerne (1960) considered the nature of antigenic determinants and concluded that conformational factors may play a role. The situation was resolved by Cowan (1968) who immunised guinea pigs with 12S antigen and demonstrated that the subsequently produced anti-12S antibody reacted also (in immunodiffusion tests) with the 140S antigen. Talbot and Brown (1972) subsequently demonstrated that the virus was composed of 60 subunits, each of which contained a molecule of VP1, VP2 and VP3. Trimers of these three subunits actually comprise the 12S antigen and this represents the 20 triangular faces of the icosahedral structure of the virus. The VP4 polypeptide had therefore to be located at the apposition of the icosahedral faces and this mathemetically satisfied the occurrence which was already established of one half of the molar concentration of VP4 polypeptide as compared with VP1-3.

Rowlands et. al. (1971) showed that trypsin reduced infectivity of FMDV and also decreased its ability to form neutralizing antibody. Burroughs, et. al. (1971) also demonstrated a similar finding but were able subsequently to show that this

came about because VP1 was cleft from the icosahedron by tryptic digestion. VP1 was therefore involved in adsorption of virus to susceptible cells and was the primary site of action of neutralizing antibody (Rowlands et. al. 1971).

The occurrence of RNA-free capsid has been reported for poliovirus by le Bouvier (1955, 1959) and by Mayer et. al. (1957), for coxsackie virus by Schmidt et. al. (1963), for several ECHOviruses by Forsgren (1968). These particles hold considerable interest because of possible relevance to structure and synthesis and also to diagnostic material, vaccines and the nature of the antibody response. Poliovirus has been studied in greatest detail and is known to contain two virus-sized particles - one RNA containing (D-antigen) and the other RNA-free (C antigen) (Mayer et. al., 1957). The D antigen may be converted to the C antigen by various denaturing procedures such as heating to 56°C or exposing to ultraviolet light (le Bouvier, 1959; Roizman, et. al, 1959). Katagiri et. al. (1967) suggested that the interaction between viral RNA and capsid protein produces a specific surface configuration which is changed when viral RNA is released, resulting in the D to C conversion. Such conversion results in the loss of VP4 but Breindl (1971) showed that, despite this loss and despite the suggestion that VP4 was an internal component, VP4 is actually a surface component and responsible for binding D-reactive antibodies. Kris and Mandel (1972) showed that surface conformation was extremely important by demonstrating in

electrophoretic experiments with poliovirus, that the interaction of a virion with even a single molecule of antibody which could only, after all, react with a single surface subunit (VP1-3, or VP4) resulted in a change of every subunit by co-operative or allosteric transition. Such action resulted in obvious changes in electrophoretic characteristics of the virion and consequently alterations of antigenic characteristics might easily arise.

Planterose, et. al. (1963) proved that the heat-labile complement fixing antigen could not be related to 140 or 12S antigens in FMDV. This led to Cowan and Graves (1966) demonstration of a third antigenically distinct antigen (VIA). This could not be a structural component of FMDV and if animals were immunized with purified but inactivated FMDV no antibody to this antigen could be demonstrated. Antibody was only present in the serum of actively infected animals during convalescence and consequently this antigen was called virus infection antigen (VIA). Cowan and Graves (1966) also showed that sera from animals infected with different serotypes of FMDV reacted with VIA antigen prepared from Type A virus. VIA was therefore not type specific and was also therefore potentially extremely useful for diagnosis. Cowan and Graves (1966) suggested that this VIA must be a virus-induced component and Polatnick, et. al (1967) showed that VIA was, in fact, viral RNA-dependent-RNA-polymerase. This was produced in large enough quantity during infection to be recognised as a foreign protein by the host and to generate an antibody response.

An example of a more complicated virus would be influenza.

Influenza virions vary in size and morphology but are generally spherical with a diameter of 80 to 120 nm. Freshly isolated strains are observed commonly to have a filamentous form of a similar diameter and up to 7 μm in length.(Choppin, Murphy and Tamm, 1960).

Unlike the relatively simple picornavirus, more complex viruses such as poxviruses, herpesviruses and also myxoviruses may contain significant amounts of lipid and carbohydrate in addition to the expected protein and nucleic acid constituents. The influenza virion is composed of 60-75% protein, 18-37% lipid, 5-7% nuclei acid carbohydrate and 0.8-1% RNA (Frommhagen, Knight and Freeman, 1959). Whereas the viral RNA of picornaviruses is a single stranded molecule, the RNA of influenza virus appears to consist of five or six smaller molecules giving a total molecular weight of approximately 2.5×10^6 daltons (Duesberg, 1968).

The virion consists of a helical nucleocapsid containing the viral RNA and protein, some of which is termed the internal soluble (S) or ribonucleoprotein (RNP) antigen (Kingsbury and Webster, 1969). The influenza viruses are divided into Types A, B and C, based on the immunological distinctiveness of the RNP antigen in complement fixation tests.

Surrounding the nucleocapsid is an envelope covered in "spikes". The envelope contains 3 distinct components - the haemagglutinin and the enzyme neuraminidase, which occur on morphologically different types of 'spikes' (Laver and Valentine, 1969) and a host

cell antigen (Harboe, 1963) which is primarily carbohydrate and appears to be attached to viral proteins.

Examination of purified influenza virus by electrophoresis on acrylamide gels has demonstrated seven distinct polypeptides. Three were carbohydrate free and four were glycoproteins (Compans, Klenk, Coliguiri and Choppin, 1970). Removal of the spikes from the virion by treatment with proteolytic enzymes also resulted in the loss of haemagglutinating and neuraminidase activity as well as the four glycoproteins. Thus the nucleocapsid contained 3 noncarbohydrate containing proteins, whereas the four glycoproteins were associated with the envelope.

The haemagglutinin subunit is approximately 140 $\mathring{A}$ long and composed of 2 distinct glycoproteins. Laver (1971) showed that these polypeptides have molecular weights of 60,000 and 12,000 daltons. A molecule of each is joined by disulphide bonds to form dimers and the haemagglutinin subunit in turn is composed of 2 dimers.

The neuraminidase subunit was also reported to consist of 2 polypeptides having molecular weights of approximately 58,000 daltons (Webster, 1970).

The proteins of the nucleocapsid are carbohydrate free but different investigators suggest different numbers of protein present. Three proteins were demonstrated by Compans et. al. (1970) whereas Skehel and Schild (1971) indicated 4 proteins. Schulze (1972) provided evidence that the nucleoprotein core of the virus was separated from the envelope proteins by a continuous layer of

lipid which protected the internal proteins from the effect of proteolytic enzymes.

Within the lipid coat the viral RNA was associated with VP2 to form the RNP which in turn was surrounded by a layer of protein VP3. Chow and Simpson (1971) suggest that the third nucleoprotein (VP1) was RNA-dependant-RNA-polymerase. Thus because influenza viruses have more structural components than FMDV, the influenza viruses are also antigenically more complex.

The haemagglutinin subunit is the means by which the virus attaches to specific glycoprotein receptor sites on the cell and is responsible for inducing antibody which neutralizes virus infectivity (Webster, Laver and Kilbourne, 1968). Schild (1970) demonstrated that sera from rabbits immunized with purified haemagglutinin had a high level of virus neutralizing and haemagglutination-inhibiting activity, but were free of antibody to neuraminidase or the internal RNP antigen.

The ability of ether or detergent disrupted virus as well as haemagglutinin subunits to induce the formation of neutralizing antibody is of considerable interest since these treatments abolish the pyrogenic activity of intact virus vaccines (Fazekas de St. Groth, Webster and Davenport, 1969) permitting the preparation of a more "desirable" product.

The other "spike" antigen on the virus surface is the enzyme neuraminidase which hydrolyses terminal sialic acid (N-acetyl neuraminic acid) from the viral haemagglutinin glycoprotein receptors on the cell surface permitting the eventual elution of the

virions (Gottschalk, 1966). Antibody to neuraminidase does not prevent the infection of susceptible cells but does cause an inhibition of the neuraminidase activity and a resultant failure of the virus to be released from the infected cells. It has been suggested by Schulman (1969) that although antibody to neuraminidase lacks true neutralizing capabilities, it may play a significant role in protection to the disease.

The true antigenic complexity of influenza virus is only partially suggested by the demonstration of a number of antigenically distinct components. Gradual antigenic variation of influenza A viruses may occur as a consequence of minor changes in the haemagglutinin or neuraminidase, or both.

Thus on encountering an invading virus, the individual's immune system is exposed to a multitude of viral components, the majority of which act as immunogens (i.e. substances capable of eliciting the formation of specific antibody molecules). The detection and measurement of such an immune response is the function of a branch of immunology called serology.

From the beginning of this century a bewildering array of serological techniques have been developed to aid in the determination of the antibody response to infection. The following literature survey serves to summarize some of the procedures involved and outlines their concommitant advantages and difficulties.

In addition to indicating the existence of a specific microbial infection in an individual and their immune status serology also

offers the possibility of differentiating one person from another by means of the specific antibodies which their serum contains.

Antibodies belonging to the IgG class can be found at relatively unchanged levels in the blood many years after exposure to an invading micro-organism (King, Werrett & Whitehead, 1976). Furthermore, IgG antibodies are stable in and readily extracted from dried blood (Werrett, King & Whitehead, 1976).

Exposure to various viruses during an individual's lifetime therefore leaves a measurable record of immunological experience which may enable an identification of an individual to be made from a sample of his blood either as a serum specimen or as a blood stain.

The possibility therefore exists that a biological "fingerprint"[2] may be obtained in the form of an "antibody profile" from a blood stain which could be used in forensic science for the identification of a suspect. It is important, therefore, that a single, sensitive, reliable serological technique should be found which would be applicable to this situation.

This thesis concentrates on examining two serological techniques, namely: the indirect fluorescent antibody test and enzyme-linked immunosorbent assay which show the greatest potential for fulfilling the aforementioned criteria in both clinical and forensic applications.

CHAPTER I

LITERATURE REVIEW

(a) Serological tests in virus disease diagnosis

The main function of a hospital virus diagnostic laboratory is to provide a diagnostic report from a pathological specimen which will enable the clinician to confirm his diagnosis and aid in prescribing the appropriate treatment. Many procedures have been devised over the years for virus antibody detection and the most suitable are generally those which are simple, inexpensive and capable of examining large numbers of serum samples quickly and efficiently.

The significance of the results, from the clinicians point of view, usually depends upon either detection of (a) specific antiviral antibodies at a particular predetermined level or (b) a four-fold or greater rise in titre of specific antiviral antibodies between two serum samples, taken 10-14 days apart, during the acute and convalescent phases of the illness.

To date, the most commonly employed serological tests for the detection and quantitation of antiviral antibodies have been the so-called "classical" reactions such as the complement fixation test, haemagglutination-inhibition and "in vitro" and "in vivo" neutralisation techniques. These procedures, although having been accepted by most virology laboratories are, however, still subject to numerous difficulties.

1) Complement Fixation Test (CFT)

Complement consists of 9 serum proteins that constitute about 10% of the globulins in man and other vertebrates. It forms an essential part of the immune response by co-operating in the lethal action (lysis)

of antibody on bacteria and other cells which are foreign to the human body.

The CFT (Casals, 1947) depends on the ability of antigen-antibody complexes to bind or "fix" the 9 components (C1-C9) of complement in an ordered sequence. When the first component C1q (one of three main sub fractions of C1, i.e. C1q, C1r, C1s) is activated by an immune complex, it acquires the ability to activate several molecules of the next component and so on producing a cascade effect with amplification. In this way the activation of one molecule of C1 can lead to the activation of thousands of the later components, culminating in the binding and activation of many C8 and C9 proteins which have the ability to disrupt the cell membrane on which they are fixed.

The ability of complement to cause the lysis of suitably sensitized red blood cells provides the indicator system for the CFT which is best considered in two stages.

Stage 1 : Serial dilutions of antiserum are prepared in a suitable buffer, e.g. Veronal buffer and incubated at $4^{o}C$ overnight with carefully measured amounts of virus antigen and complement. If the appropriate Ab-Ag complexes are formed, complement is "fixed", i.e. prevented from taking part in the second stage of the test.

Stage 2 : To detect the presence or absence of free complement, indicator cells consisting usually of sheep red blood cells sensitized by coating with rabbit antibodies to sheep erythrocytes (haemolysin) are added and incubated at $37^{o}C$ for a further 30 minutes.

Result : A. Haemolysis of the red cells indicates that free complement has persisted and therefore that an effective Ag-Ab reaction has not occurred in Stage 1, i.e. the antiserum did not contain antibodies to the viral Ag under investigation.

B. The absence of haemolysis indicates that complement has been fixed and therefore that an Ag-Ab reaction has occurred in Stage 1. The amount of specific antiviral antibody existing in the serum sample is indicated by determining the final dilution of antiserum in the titration to give less than 50% lysis of the red blood cells.

The complement fixation test is the most widely used of the classical serological reactions, however, it suffers from several fundamental problems.

Due to the large number of reactants involved in the assay, accurate standardization of all the test reactants is of prime importance for a reliable CFT. Since the test consists of 5 variables, 3 of which, i.e. antigen, complement and haemolysin, are assayed for use on the basis of a fourth variable - sheep erythrocytes, the utmost accuracy in measurement of the reactants is required especially when small volumes of reagents, e.g. 0.025 ml, are used, as in the microtitre method developed by Sever (1962). With the 5th reagent, the serum under investigation, being yet another variable in the system, the test is obviously open to an overall error greater than the expected error due to each of the variables.

For this reason, a number of controls must be incorporated into the CFT to monitor the reliability of each component of the system, e.g.

1) Sensitized erythrocytes alone must show no lysis during their incubation in Veronal buffer.

2) Sensitized erythrocytes + complement must show complete lysis at the end of the assay procedure.

3) Antigen + complement + erythrocytes must show complete lysis.

4) Antibody + complement + erythrocytes must show complete lysis.

These last two controls monitor the phenomenon known as anticomplementarity, i.e. that either reactant - Ag or Ab does not inactivate or "fix" complement without the other. Such a difficulty is often experienced with antigenic material which has been obtained from infected tissue, e.g. virus, rickettsia and chlamydia propagation. This problem can often be overcome by purifying the antigens via extraction with acetone or benzene (Espana and Hammon, 1948), by removal of non-specific protein by precipitants such as fluorocarbons or by high speed centrifugation (Lennette and Schmidt, 1964). Undiluted or slightly diluted antisera are also frequently anticomplementary usually owing to some denatured and aggregated immunoglobulins. In some patients, repeated bleedings will consistently yield sera which are anticomplementary whereas in others the sera may be anticomplementary on some occasions but not all (Lighter, 1953). Often this problem can be overcome by following a minor modification of the method described by Taran (1946). The serum is incubated with guinea pig complement at 4^{o}C overnight, then at 37^{o}C for 30 minutes before being inactivated at 56-60^{o}C for 30 minutes.

Finally, anticomplementary reactions of sera may be due to extraneous factors namely bacterial or chemical contamination. Alcohol, anticoagulants (such as citrate, oxalate and heparin) & preservatives can all give rise to anticomplementary properties in the serum but by far the commonest cause of anticomplementary activity is bacterial contamination of the specimen(Lennette, et. al. 1964). Improvements in sensitivity and specificity of the CFT would also be desirable but it has been suggested by Schubert, Stanford and Tiffany (1951) that these two factors have an inverse relationship.

By examining the relative sensitivities and the relative specificities of the 5 complement fixation techniques for the Rickettsioses, Schubert, et. al. concluded that the most sensitive test was the least specific; the most specific test was the least sensitive and the other three tests were placed between these two extremes.

Ideally, highly sensitive tests for the determination of low levels of specific antibody in sera are required when dealing with serological surveys to determine the extent to which immunity to a particular organism is present in a population. However, when faced with a situation where increased sensitivity leads to decreased specificity resulting in false positive or negative results (Schubert, et. al. 1951), the value of such a test in the virus diagnostic laboratory is questionable.

Recently, however, Zissis and Clinet (1974), recognizing the technical difficulties encountered in the CF reaction, described a modified test which involves a change in the haemolytic system. By using a 0.2% red blood cell suspension, instead of the usual 2% concentration, with a 1/80 - 1/100 dilution of complement and a 1/1000 - 1/2000 dilution of haemolysin, they claim four basic improvements

a) a stable reading which can be taken at different times depending on incubation temperature, b) excellent reproducibility, c) smaller quantity of antigen required, d) increased sensitivity.

2) Haemagglutination -Inhibition (HAI)

Haemagglutination, i.e. the capability of agglutinating red blood cells was first discovered by Hirst (1941) for influenza virus. Since then many viruses, from the very small ones such as foot and mouth disease virus (a picornavirus) to large ones such as poxviruses have been found to cause haemagglutination of erythrocytes.

Subsequently, specific antiviral antibody was shown to prevent or inhibit haemagglutination if the virus was premixed with the antiserum. This phenomenon provides the basis for the haemagglutination-inhibition test, the mechanism for which is very simple, i.e. attachment of antibody molecules to the virion hinders adsorption to the red blood cells.

The HAI test is potentially superior to the CFT in terms of simplicity. HAI merely consists of :-

1) The addition of a standardized amount of virus to dilutions of the serum under investigation.

2) After incubation for 1 hour at 37^{o}C, a suitable dilution of erythrocytes of the appropriate animal species is added and left at room temperature for approximately 30 minutes to settle out either as (a) an agglutinated layer of red blood cells indicating the absence of specific antiviral antibody in the specimen or (b) as a button indicating the presence of specific antiviral antibodies in the antiserum.

HAI, therefore, contains far fewer variable components than the CFT but even so it has two main difficulties.

Firstly, the extreme specificity of the HAI test can be a problem.

In the case of influenza viruses (ortho-myxoviruses), immunological subtypes are distinguished by major antigenic differences of the external antigens, i.e. the haemagglutinin and neuraminidase. The haemagglutinin in particular undergoes antigenic changes, related to a minor change in its amino acid sequence, every few years, but usually only a single subtype is prevalent at any one time. In the HAI test, therefore, the haemagglutinin used as Ag must be of the same subtype as that on the prevalent infecting influenza virions. If a different haemagglutinin were to be employed in the HAI system from that on the virion infecting the patient and eliciting the formation of specific anti-influenza antibodies, a false negative HAI test would result. Ideally, then, the haemagglutinin used as Ag in the HAI tests should be broad in scope and monitored each season to confirm that it is compatible with the influenza strain currently prevalent in the community.

This ideal is seldom achieved, however, and usually clinical HAI tests call for the use of several types of haemagglutinin to assure the detection of the appropriate viral antibody. The CFT, on the other hand, involves the group specific internal or nucleocapsid antigen which does not undergo antigenic changes and thus is a simpler, less time consuming and more reliable assay to perform even allowing for all its concomitant difficulties.

The second difficulty arises from the presence of non-specific inhibitors of haemagglutination which are present in all patient's sera and will obscure the specific inhibition caused by antiviral antibodies.

From the very first application of the HAI test in Virology, for the detection of influenza antibodies (Hirst, Rickard, Whitman & Horsfall, 1942), it was recognised that the reliability of the method was affected by the action of serum constituents, other than specific antibodies (Smith and Westwood, 1950; Francis, 1947), which could inhibit influenza virus haemagglutination.

Within 9 years, three types of non-specific inhibitor to myxoviruses had been discovered (Isaacs and Bozzo, 1951).

McCrea (1946) described an inhibitor found in normal rabbit sera, Francis (1947) described an inhibitor of normal human sera and furthermore, characterised it as a mucopolysaccharide. Ginsberg and Horsfall (1949) described an inhibitor in human, guinea pig, mouse and rabbit sera which inhibited haemagglutination and prevented infection with influenza virus. The presence of this inhibitor was later substantiated by Chu (1951) with his work on normal mouse sera.

HAI testing can only be undertaken if the effects of these non-specific inhibitors of virus haemagglutination are eliminated. This can sometimes be achieved quite easily depending upon the type of inhibitor involved. Some of them are heat labile, e.g. Chu inhibitors, and can be destroyed by simply heating the sera at 56^{o}C for 30 minutes. Heat stable inhibitors, on the other hand, e.g. Francis inhibitor, pose

much more of a problem. With an increase in the understanding of the interaction of orthomyxoviruses with cell surfaces, however, chemicals have been found which can neutralize these non-specific substances.

Orthomyxoviruses attach to receptors on the membrane of red blood cells via the haemagglutinating spikes of the virion, envelope. The erythrocyte receptors are mucoproteins with terminal N-acetylneuraminic acid (NANA) residues (see diagram A). These receptors can be inactivated by periodate which oxidises glycol groups of sugars or by neuraminidase, which splits off N-acetylneuraminic acid.

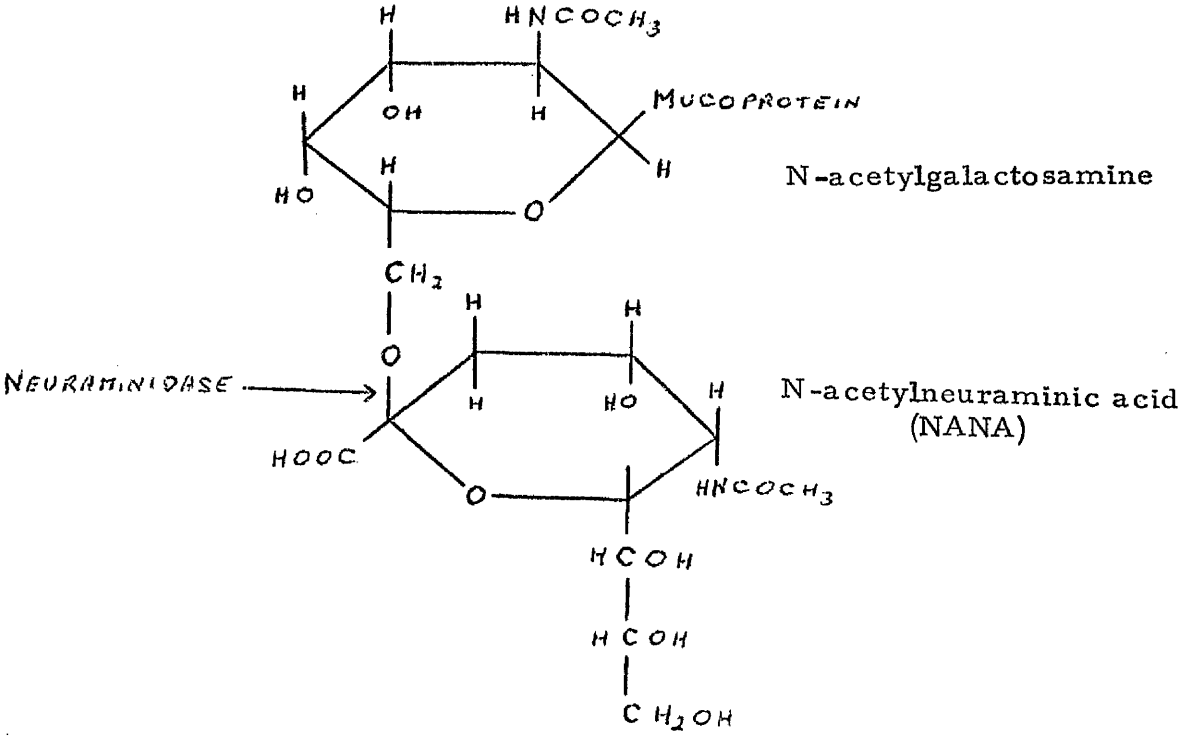


Diagram A: Erythrocyte Receptor

Sialic acid-containing mucoproteins with terminal NANA residues, e.g. the Francis inhibitor which binds to the virions haemagglutinating spikes and competitively inhibits haemagglutination, can be inactivated by removing the terminal NANA residues. Pre-treatment of sera for use in HAI tests with neuraminidase or potassium periodate therefore minimises the effect of these non-specific inhibitors of haemagglutination. Neuraminidase is obtained from culture filtrates of Vibrio cholera and is commonly referred to as receptor destroying enzyme (R.D.E.).

The result of non-specific inhibition of haemagglutination is important from several aspects. In the case of children, their sera often contain a high level of non-specific inhibitors and virtually no antibody for certain virus strains (Hilleman and Werner, 1953). Also, inhibitors may prevent agglutination at much higher dilutions than the specific antibodies so that a true diagnostic rise in antibody titre may be masked and may not be detectable until the sera are treated with RDE.

Removal of non-specific inhibitors from serum is therefore vitally important if basic antibody levels to the various strains of influenza virus are to be reliably determined. Problems of non-specific inhibition are by no means restricted to the orthomyxoviruses, however.

In the case of the arthropod borne viruses (Togaviruses), the situation is complex. The virus haemagglutinin to, for example, St. Louis encephalitis virus (SLE) is derived from infected mouse brains. Mouse brains, however, are also the source of non-specific inhibitors which are generally lipoprotein in nature (Chanock and Sabin, 1953). This non-specific inhibitor is very difficult to remove from the

haemagglutinin suspension, possibly because it is bound to the haemagglutinin *in vivo*. The test is further complicated by the fact that inhibitor to SLE is also found in normal sera which does not contain specific antibodies to SLE. A similar situation arises when Japanese B encephalitis virus is tested by HAI. The normal inhibitor in serum against this haemagglutinin can be removed with chloraform (Sabin and Buescher, 1950), however, similar treatment of serum in the SLE test removed only 75% of the inhibitor, not enough to permit its use in HAI tests for specific antibody.

Precipitation by an appropriate amount of acetone with subsequent washings of the precipitate by acetone is required to yield a fraction that is sufficiently devoid of inhibitor for a reliable HAI result (Casals and Brown, 1954).

The sensitivity and reliability of the HAI test can be increased by the pretreatment of sera to remove heat labile and heat stable non-specific inhibitors, but such procedures add appreciably to the time involved in performing the tests.

3. Neutralisation Tests

The neutralisation test is the most sensitive of the "classical" immunological reactions. It is followed in sensitivity by haemagglutination-inhibition and finally complement fixation. These differences parallel the amount of antigen required for each test, e.g. with influenza virus about 10^3 viral particles are required for neutralisation tests, at least 10^7 for haemagglutination-inhibition and an even greater number for complement fixation (Davis, Dulbecco, Eisen, Ginsberg & Wood, 1973).

For these reasons, the neutralising antibodies usually have higher titres (hence are detected more easily) than haemagglutination-inhibiting or complement fixing antibodies.

The neutralisation test is based on the principle that when a specific immune serum is added to its corresponding virus, the virus is rendered non-infective or "neutralised".

Various methods for detecting neutralising antibodies have been developed over the years. Which technique is used is dependant upon the virus in question since different viruses produce observable infective activity in different test systems.

Demonstration of infectivity, therefore, requires the use of living tissues, e.g. (a) in vivo systems involving various animals such as the white mouse, (b) in ovo systems involving embryonated hen's eggs, (c) in vitro systems involving various types of tissue culture cell monolayers.

Problems of standardisation were encountered in the past when in vivo and in ovo tests were compared.

For example, a mixture of serum and virus which appeared to be completely neutralised as judged by tests in one host system, were often highly infective in another (Tyrrell and Horsfall, 1953). In the case of influenza virus many mixtures of virus and immune sera which are non-infective in the mouse are infective in the chick embryo. This is dependant largely upon the fact that the amount of un-neutralised virus required to infect the mouse is much greater than that required to infect the chick embryo. The use of the chick embryo, therefore, largely superseded the rabbit or mouse as an experimental host for investigations of viruses such as vaccinia, variola and herpes simplex.

The main procedure for the determination of the neutralising titre of sera in tests involving chick embryos is based on the ability of the virus under investigation, e. g. vaccinia, variola, to produce plaques or pocklike lesions on the chorioallantoic membrane (Burnet and Lush, 1939). Plaques are zones of cell lysis caused by virus replication disseminating from a single infected cell.

Pock formation, like that of a plaque, begins with the infection of a single cell. Since the chorioallantoic membrane is complex, however, and contains several different cell types and blood vessels, the response to the local infection is also complex; it involves both cell proliferation and cell death, accompanied by oedema and haemorrhage. Pocks, therefore, appear as opaque areas, usually white and often haemorrhagic on the transparent chorioallantoic membrane. The neutralising capacity of the serum is therefore related to the quantitative reduction in the number of virus particles capable of producing lesions, which is indicated by the number of plaques produced.

This procedure, however, is time consuming and is subject to a number of inaccuracies (Burnet and Faris, 1942) introduced by non-specific lesions, usually of the ulcer type caused by small haemorrhages of the membrane, variable distribution of inocula on membranes and personal interpretations in the definition of lesions.

Quantitative studies of herpes simplex virus and antibody by the pock counting method are further complicated by the low virulence for the chick embryo of freshly recovered strains (Kilbourne, 1951).

A single concentration of virus capable of producing a reasonable number of pocks and a single concentration of serum are usually employed in estimating the amount of neutralising antibody.

Differing amounts of antibodies are estimated on the basis of the extent of the reduction in the number of pocks which develop (Burnet, et. al. 1939) but it has been reported (Shaffer and Enders, 1939) that a tenfold change in antibody concentration may not alter the pock count.

With the use of tissue culture technology, however, these problems have largely been overcome.

Two main methods of determining the levels of neutralising antibodies in sera have been developed.

a) Determination of a 50% titre of neutralising antibody by a 50% endpoint method.

This titration can be performed in two ways.

1) using a constant inoculum of virus to infect the assay system and dilutions of serum to investigate the neutralising titre of the antiserum, i.e. "constant virus-varying serum titration".

2) using one particular concentration of serum and dilutions of virus inoculum, i.e. "constant serum-varying virus titration".

The first procedure is the most commonly employed since it is statistically more accurate than the constant serum-varying virus titration (Tyrrell, et. al. 1953).

The adoption of this particular procedure for use in neutralisation tests followed a controversy which raged in the 1940's over the human immune response to herpes simplex virus.

Burnet and Lush (1939) had devised a method for accurately measuring herpetic antibody in which mixtures of undiluted serum and one or more dilutions of egg passaged herpes simplex virus were inoculated onto the chorioallantoic membranes of chick embryos, i.e. the constant serum-varying virus titration.

The results were expressed as the percentage reduction of membranal lesions or pocks, in comparison with the number of lesions developing on virus control membranes. The degree of viral neutralisation, determined in this manner, was thought to be directly related to the amounts of antibody in the serum. The actual findings indicated that human sera either contained large amounts of herpetic antibody or none at all. Burnet and Lush, therefore, concluded that the human immune response to infection with herpes simplex virus was, "all or nothing". This finding was subsequently endorsed by Hazard (1949) and Dudgeon (1950). Rose (1952), however, in a series of experiments, tested human sera for herpetic antibody by two types of neutralisation test. In one, the principle was to employ constant amounts of undiluted serum and either one or several concentrations of virus, while in the other, the concentration of virus was held constant and the amount of serum was varied. Significant variations in the neutralising capacity of sera containing herpetic antibody were not detected when the undiluted sera were tested against single or multiple

concentrations of virus; these same sera, however, showed considerable differences in their ability to neutralise virus when they were serially diluted and tested against a single concentration of virus.

Constant virus-varying serum titrations were, therefore, generally adopted and the neutralisation titre of the serum was calculated as the serum dilution at which 50% of the test units were protected. In this case the test units can be of mice, chick embryos or tissue culture tubes.

b) Plaque reduction test.

This method is the most sensitive of the neutralisation assays. The procedure involves incubating an inoculum of about 100 plaque forming units with a serial dilution of the serum under test. Each mixture is then added to a monolayer culture, which is overlaid with agar and incubated. The endpoint is an 80% reduction of the number of plaques as against a similarly infected control culture containing no antiserum. This assay is more time consuming and more elaborate than the first method, but even so, it represents a reliable method for quantitating neutralising antibodies to viruses which do not replicate suitably in ordinary cell culture systems but do propagate under conditions favourable to plaque formation (Lennette & Schmidt, 1964).

Since their original description, the classical immunological reactions have been capable of determining overall serum antibody levels to the majority of the commonly infecting viruses. However difficulties have been encountered because these tests depend upon an indicator system and many extraneous factors, such as aggregated immunoglobulin (anti-complementary serum) or non-specific inhibitors can seriously affect the interpretation of these test systems unless stringent precautions are employed to exclude these potential difficulties. Other problems are associated with these procedures. The sensitivity of the complement fixation test tends to be rather low and both complement fixation and neutralisation are fairly time consuming. These objections to the use of the classical reactions in the diagnosis of virus disease have gained greater importance with the potential advent of antiviral chemotherapy. Research has therefore turned its attention to developing techniques which are sensitive, reliable and able to provide a rapid virus diagnosis within a few hours of the patient's admission to hospital. Some of these techniques, such as electron microscopy and fluorescence microscopy are now in routine use. Other techniques such as enzyme-linked assays and gas chromatography require further assessment and development.

Two of these procedures (fluorescence microscopy and Enzyme-linked Immunosorbent Assay) have been evaluated in terms of their sensitivity and relevance in the course of this project and therefore will be dealt with in detail in subsequent chapters of the literature review.

Relevance is an aspect of serology which has been little investigated. Most studies have been concerned with intact virions and rather less attention has been given to the importance of isolated subunits, incomplete virions and to the various "soluble" and other virus-associated antigens. From immunization studies it has long been known that immunization with inactivated (but not physically or chemically denatured) virions produces antibodies only for the surface components of the virus. These antibodies have neutralizing and often haemagglutination - inhibiting activities against the virions as well as complement fixing and precipitating activities with antigens of the viral coat. Viruses which multiply in the host cells lead to the formation of antibodies against all the viral antigens produced in the course of their replication including neutralizing or haemagglutination-inhibiting antibodies for surface antigens and complement fixing or precipitating antibodies for both surface and internal antigens. Immunization with internal components of the virions produces complement fixing and precipitating antibodies against the component antigens (Davis, et. al. 1973). Therefore, the antibodies which react in the various immunological assays are probably not identical.

The significance of the antibody response to a virus may be considered from many different points of view dependent upon the interests of the investigator. In the diagnosis of a virus infection it is obviously important that the serological test

involved should detect specific antiviral antibodies formed by the host in response to the disease. However, it has been reported by some investigators that in a small percentage of cases, different antiviral antibody titres to a specific virus infection can be detected from the same serum sample by different serological techniques.

In 1972, Norrby and Gollmar investigated the appearance and persistence of antibodies produced after infection with measles virus. Two serological tests were studied, namely the haemagglutination-inhibition test (HAI) and the haemolysis-inhibition test (HLI). According to Norrby, previous studies of paramyxoviruses indicated that the haemolysin activity of these viruses becomes expressed efficiently only when the haemolysin was associated with the haemagglutinin structures. As a consequence of this, antibodies which inhibited haemagglutination also inhibited haemolysis. However, the reverse need not be true if a haemagglutinin not associated with haemolysin is used as test antigen. Therefore, these two serological tests for determining antibody titres to measles virus sometimes give conflicting results. When Norrby, et. al. investigated 15 late convalescent measles sera by HAI and HLI, one serum was found to contain a high titre of HLI antibodies and virtually no HAI antibodies. Similar characteristics were displayed by 2 of 15 serum samples collected from patients with multiple sclerosis. These findings prompted Salmi, Norrby and Panelius (1972) to investigate the reliability of other serological techniques to measure measles antibodies in the serum and cerebrospinal fluid from patients

suffering from subacute sclerosing panencephalitis (SSPE) or multiple sclerosis (MS). Matched serum and cerebrospinal fluid samples from 8 cases of SSPE and 15 cases of MS were investigated for measles antibody by neutralization, HLI, HAI, CF and immunodiffusion tests. Salmi, et. al. found a high degree of correlation between titres of neutralizing and HLI antibodies but a less strict correlation between tests of HLI and HAI antibodies. Serum from 2 cases of MS and one case of SSPE contained high titres of HLI and neutralizing antibodies in the presence of only low titres of HAI antibodies. The HLI technique was therefore of greater value than the HAI test in the determination of measles antibody. Salmi, et. al. also found that the major fraction of antibodies detected in the CF and immunodiffusion tests reacted with measles n ucleocapsid and furthermore there was a tendency for nucleocapsid CF antibody titres (as compared to neutralization and HLI antibody titres) to be higher in samples from patients with SSPE than from cases of MS.

Recent work by Armstrong, Fraser and Shirodaria (1979) confirms Salmi, et. al.'s findings that human sera contain higher titres of haemolysin inhibiting antibodies than HAI antibodies. The haemolysin of measles virus has been shown to be functionally and antigenically sensitive to acetone (Armstrong, et. al. 1979), therefore, absorption of human sera with acetone treated, measles virus infected Vero cells removes antibodies to all measles structural antigens except haemolysin. This procedure thus provides a simple and useful method for the preparation of monospecific antisera to measles virus haemolysin.

Studies of another paramyxovirus, mumps virus (Henle, Harris and Henle, 1948) demonstrated that human CF antibodies against S antigen (mumps nucleocapsids) appeared earlier and disappeared more rapidly than those against V antigen (envelope). In the diagnosis of current or recent mumps infection, measurement of CF antibodies against S or V antigen would therefore be equally relevant but in the determination of the immune status of an individual before administration of mumps vaccine, only the absence of CF antibody to V antigen would be deemed to be relevant.

In the case of Rubella virus, antibodies can be detected by HAI, neutralisation, CF, Radial haemolysis or Radioimmunoassay tests. However, for the determination of immunity to Rubella virus, HAI antibody detection is more relevant than CF antibody. CF antibody levels rise within 4 weeks of infection and then decline to a lower level in approximately 6 months after infection with Rubella. HAI antibodies tend to persist at higher levels. HAI antibodies to Rubella also parallel neutralizing antibodies and the presence of either antibody can be interpreted as reflecting protective immunity (Schluederberg, Horstmann, Andiman and Randolf, 1978).

In a recent publication from this laboratory, Macfarlane (1972) showed that tissue culture grown viruses inoculated into guinea pigs produced antisera which gave conflicting results when tested by neutralisation, complement fixation and the indirect fluorescent antibody tests. In some cases high levels of neutralizing antibodies were detected in guinea pig antisera to respiratory syncytial virus (RSV) whereas low levels of antibody were detected by CF or IFA.

In the case of guinea pig antisera to herpes simplex virus type 1, high levels of antibodies were detected by neutralisation and IFA tests but none by CF. Discrepancies have also been reported between CF and ELISA test procedures in determining antibody levels to herpes simplex virus (Gilman and Docherty, 1977). In a survey of 30 human serum samples, one specimen produced a high CF result and an abnormally low antibody level by ELISA. Another serum with a low CF titre showed a high titre when tested by ELISA. Gilman and Do cherty speculated that these differences reflected the type of immunoglobulin detected by the two test systems since CF detects IgM and three of the four subclasses of IgG indiscriminately whereas ELISA (because of the nature of the antibody-enzyme conjugate) was primarily directed at IgG (all subclasses).

Griffiths, Buie and Heath (1978), described similar discrepancies between results obtained from CF and two IFA assays for CMV antibodies in human sera. Since the normal IFA test, in the hands of an inexperienced operator, can be complicated by non-specific binding of IgG to Fc receptors which are produced when CMV infects fibroblast cell cultures (Keller, Peitchel, Goldman and Goldman, 1976) an alternative staining procedure, the anti-complement immunofluorescence test (ACIF) (see Chapter 1, section C) which is usually unaffected by non-specific Fc binding was also employed in their serological survey. Of 406 sera investigated, 5 sera (1.2%) gave results which were consistently discordant. Three were seropositive in both immunofluorescence assays but negative in the CF test. Griffiths, et. al. suggested that this was due to the

greater sensitivity of the immunofluorescence assays over the CF test. The fourth serum sample was positive in the ACIF test, but negative in the other 2 assays. The authors suggested that this result indicated a slight superiority of the ACIF test over the normal IFA test for detecting low titre antibodies. However, since this same serum sample showed anticomplementary activity to a titre of 4 in the CF test it was possible that the ACIF test may also have been affected by this activity. The fifth serum sample had a CF titre of 8 but was negative in both of the immunofluorescence assays. This difference was consistently obtained and suggested the possibility that the CF test may occasionally detect specific antibodies of a different nature from those detected by the immunofluorescence assays.

This last observation of Griffiths, et. al. was substantiated by Booth, Hannington, Aziz and Stern (1979) who reported frequent discrepancies between ELISA results and CF antibody titres when examining 90 human sera for CMV antibodies. According to Booth, et. al. these discrepancies could not be satisfactorily explained by poor reproducibility of either test or by the variable presence of nonspecific inhibitors or by the presence in the antigen preparations of the nonspecific, intracytoplasmic Fc receptor that develops in CMV-infected cells. The simplest explanation appeared to be that human sera contained variable mixtures of CMV IgG antibodies, only some of which readily fix complement, whereas the ELISA technique, based on a globulin-antiglobulin reaction mechanism had a much wider recognition range for antibody.

All the previous reports have dealt with discrepancies which have arisen between serological tests based on different indicator systems, such as CF, HAI, IFA, etc. The main purpose of my project was to examine two tests based on the same globulin-antiglobulin reaction mechanism, namely IFA and ELISA and investigate the correlation of antibody titres to three viruses (Herpes simplex type 1, Coxsackie virus B_1 and Rotavirus), which were detected in human sera.

The relevance of the antibodies being detected by these two tests was also investigated by examining their correlation with neutralisation tests for HSV1 and Coxsackie B_1 (suggesting the existence of protective antibody in the sera).

Since human Rotavirus can only be grown in a human kidney tissue culture cell system, the IFA technique has relied heavily upon the detection of antibodies in human sera directed against a close antigenically related calf Rotavirus which can be cultivated in Primary Calf Kidney cells. The relevance of the IFA and ELISA Rotavirus antibody titre results was, therefore, investigated by correlating them with the antibody titres obtained with the same human sera in a CF test which used a human Rotavirus from a faecal extract as antigen.

b) Forensic Serology

Introduction

The two main situations in which blood group serology has a forensic application are:-

1) In the solution of problems of doubtful paternity. (Polesky & Kraus, 1977)

2) In the identification of stains from body fluids, especially blood but also seminal fluid, saliva, etc. (Dodd, 1972), as to type and possible source.

The second aspect (blood stains) is the subject of this review because the presence of blood as a dried stain can yield valuable information to help convict or acquit a suspect.

Diagnostic Test for Blood

The first test applied to a sample received for serological analysis must confirm that the stain on the material is blood and not red dye, red paint, etc. This is usually established by examining the stain for the presence of peroxidase. Various substrates (phenolphthalein or leucomalachite green) can be used with hydrogen peroxide to confirm the presence of peroxidase, producing free O_2 which in turn oxidises a hydrogen donor to a coloured compound. In some cases, plant peroxidases may be a contaminant but animal and plant peroxidases can be differentiated on the basis of their thermostability, i.e. plant peroxidases are rapidly inactivated at $100^{\circ}C$ whereas animal peroxidases are relatively stable for up to 5 minutes at $100^{\circ}C$ (Culliford, 1971).

Precipitin Reaction

Immunoprecipitation is then used to assess whether the blood

is human or animal in origin.

Precipitating serum antibodies were originally described in 1897 when Kraus produced precipitates with cholera antisera mixed with Vibrio cholera culture filtrates.

Species specific precipitating antisera are used as follows:-

If a dilute extract of human blood from a stain is carefully layered on top of an antiserum raised in a rabbit against human blood, a positive reaction is indicated by the development of a precipitin line at the interface of the two solutions. See diagram below.

A : Saline extract of human bloodstain

C : Precipitate

B : Antihuman antiserum

The precipitin reaction can also be visualized in agar gels. In the double diffusion method of Ouchterlony (1962) antigen and antibody placed in wells cut in agar gel, diffuse towards each other and will precipitate to form an opaque line in the region where they meet in optimal proportions if the antibodies are specific for the antigens.

A more sophisticated development of these relatively simple techniques was introduced in 1953 by Grabar and Williams by combining the principle of precipitation in gels with the separation of protein fractions on the basis of their varying electrophoretic mobilities. In 1964, Culliford demonstrated the sensitivity of crossed over electrophoresis for the detection of blood species in forensic serology.

Blood Grouping

The determination of groups, e.g. A, B, O or M, N and factors such as red blood cell enzymes in blood stains has developed since agglutination between Vibrio cholera and the red cells of various animal species was first described by Bordet (1896). These initial findings led to the discovery by Landsteiner (1899) that agglutination was a biological phenomenon which permitted the classification of human blood into distinct groups. Landsteiner's theory was simply that, "Never is a given agglutinogen and its own agglutinin present in one and the same blood." This raised the question of the individuality of fresh and dried blood.

The most widely used red cell antigen system is the ABO system which classifies blood according to genetically determined types of mucopolysaccharides on the surface of erythrocytes. The proportion of people in each blood group remains fairly constant within a particular community. Wide variations, however, can be encountered from country to country. See Table A.

	O	A	B	AB
United States (average of the States of New York and Ohio-mixed races)	38	41	15	6
United Kingdom (mainly Anglo-Saxon)	47	42	8	3
Punjab (typical of all non-Dravidian Indians)	42	23	34	1

Table A. Distribution of Blood Groups (in percentages) (Kind & Overman, 1972)

In liquid blood samples, the presence or absence of a particular antigen is established by observing whether or not the erythrocytes are agglutinated by an antiserum containing known specific antibodies

to the mucopolysaccharides on the surface of the erythrocytes. Thus an antiserum containing antibodies to group A red blood cell antigens will agglutinate only red cells of the group A type.

In dried bloodstains, however, the erythrocytes may rupture, therefore, direct agglutination tests are not possible but the mucopolysaccharide antigens are relatively stable in the dry state. The ABO antigens survive for many years and retain the capability of combining with specific antibodies (Culliford, 1971).

The formation of visible antigen/antibody complexes provides the basis of all the methods employed in the detection of erythrocyte antigens in dried bloodstains and several novel methods have now been developed for their detection.

1. Absorption - inhibition

This method has been in use for several decades. Aliquots of animal antisera to one of the ABO system blood groups are added to portions of stain or extracts of stain and after a suitable period of absorption, the residual sera are tested with red cell suspensions of appropriate group (e.g. group A erythrocytes are added to anti-A sera). The degree of agglutination produced is compared with controls containing the same amounts of specific antiserum and antigens. A substantial reduction in the ability of a serum, added to stained material, to agglutinate the red cell suspension indicates the presence of the appropriate antigen in the stain.

This method is relatively insensitive compared with more recent techniques and in practise has proved to be satisfactory only for grouping in the ABO system (Culliford, 1971). According to Nickolls and Pereira (1962) the method suffers from fundamental disadvantages:-

a) When dealing with very small or faint blood stains, or with weak antigens, although a certain amount of absorption may have taken place, the fall in titre of the antisera may be too small to be of any significance or may even be so slight that it is unrecognisable.

b) There is a possibility that in certain circumstances the absorbed antibody is eluted out again, thus vitiating the result.

c) Any foreign material present in the stain, since it cannot always be removed prior to testing, may affect either the antiserum by itself, causing an absorption or the red cell suspension by causing lysis or agglutination : any of these effects renders the test valueless.

Techniques have also been investigated in which immune reactions are obtained directly from the antibody absorbed to the antigen in the stain.

In 1956, Coombs, Bedford and Rouillard described a method of showing the presence of A and B group antigens in human epidermal cells by what is essentially an additive process. The epidermal cells were treated with undiluted human anti-A or anti-B group sera. After one hour, the antiserum was removed, the epidermal cells were washed and the appropriate red cell suspension was added to the epidermal cells. A positive reaction was determined microscopically by observing the red cells clustering round the epidermal cells and adhering to them.

See diagram B.

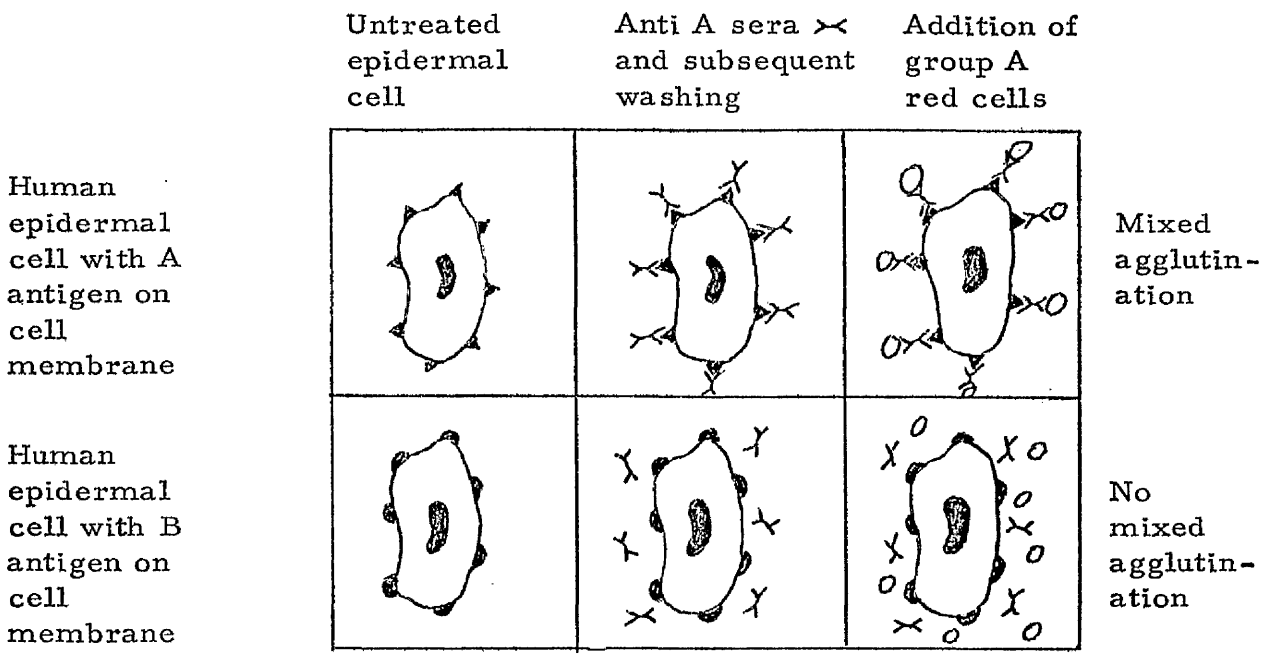


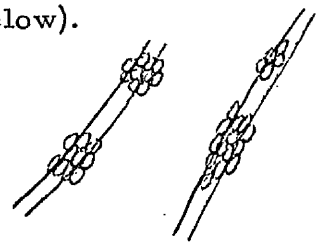
Diagram B Mixed erythrocyte-epidermal cell agglutination reaction for detection of the A antigen on epidermal cells of human skin

This approach leads to greater sensitivity (Culliford, 1971). A positive reaction can be detected when a small proportion of the antibody content of the group A or group B antiserum is absorbed to the epidermal cells.

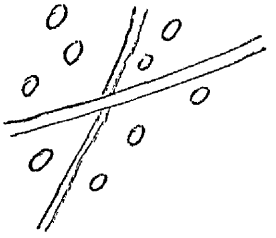
Coombs and Dodd (1961) modified this procedure slightly to allow its use for typing blood stained material. The new technique (called mixed agglutination) is used to group dried bloodstains of the A B O system. It works in practice as follows:-

Bloodstained fibres are totally submerged in various specific antisera (anti-A, anti-B, etc.) and after a period to allow absorption of specific antibodies to the antigens in the blood stain, the unbound antibody is washed away. The fibres are then submerged in indicator cell suspensions of appropriate blood group (A, B, etc.)

and incubated. During this period agglutination of red cells specific for the antibody absorbed to the fibres will occur. When viewed microscopically, the fibres will be seen to be coated with a layer of red cells of the same antigenic group as the stain. (See figure below).



Positive reaction
Erythrocytes agglutinated and coating the bloodstained fibres



Negative reaction
Erythrocytes floating free in suspension

Mixed agglutination has proved to be very satisfactory for the A B O system and can be used on extremely small stains - even a few bloodstained fibres (Culliford, 1971). Even so, the sensitivity of this technique has been increased by the introduction of the absorption-elution procedure which works like this:-

After absorption of bloodstain antigen to specific antiserum (anti-A or anti-B, etc.) and subsequent removal of unbound antiserum by washing, as in the mixed agglutination procedure, the antibody attached to the agglutinated erythrocytes on the bloodstained fibres is eluted by raising the temperature of the reactant solution to 56°C. The eluate is then tested with indicator red blood cells of an appropriate group and agglutination indicates the presence of an antigen of the same type as that of the indicator erythrocytes.

Absorption-elution is capable of A B O grouping on stains as

small as 1 mm diameter (Culliford, 1971). It can also be used for typing other red cell antigens such as MN and Rhesus (Rh) in dried bloodstains because it is more sensitive than mixed agglutination. One proposed reason for its greater sensitivity is that in mixed agglutination, the immune reaction only takes place at the antigenic sites exposed on the surface of the bloodstained fibres, whereas in absorption-elution, the submerged sites are also ultimately involved. Culliford, 1971, has suggested that red cells agglutinate more readily with each other than with debris adhering to fibres.

Classification of a human bloodstain is not restricted to analysis of its red cell antigenic group. Many other systems have been developed to characterise the blood further, an example being characterization of the red cell's polymorphic enzyme systems.

Polymorphic forms of human red cell phosphoglucomutase (PGM) were first described by Spencer, Hopkinson & Harris in 1964. They discovered two alleles at one locus, the heterozygate and the two homozygates giving three commonly occurring variants, PGM 1, PGM 2-1 and PGM 2. These variants can be detected and characterized by electrophoresis. Other enzymes which can be detected by such methods include Adenylate kinase, 6 phosphogluconate dehydrogenase, glucose 6-phosphate dehydrogenase, adenosine deaminase and pseudocholinesterase (Culliford, 1971).

Polymorphic protein systems are also frequently employed in characterising blood stains, e.g. polyacrylamide gel electrophoresis is used to identify various haemoglobin (Hb) variants such as HbA, HbS, HbC, HbD and Hb E, which may exist in blood.

All these systems are based on the existence of genetically controlled polymorphisms. Using the information gained by blood grouping and relating it to the frequency of occurrence of particular combinations of groups within a defined population several bloodstains may be distinguished and this may reduce the population size from which the blood could have originated.

Non-genetic markers

The results of experiments on the identification of anti-parasitic antibodies in bloodstains (antibody profiling) by King (1974) introduced the new concept to forensic serology of the use of non-genetic markers for the characterization of individuals. Using the indirect fluorescent antibody technique to determine the blood antibody content to 5 different microbial antigens, King obtained a discriminating power equal to that of the A B O blood grouping method.

King & Whitehead (1975) subsequently showed that this technique could be used with a high degree of confidence to identify the age range of the donor of a bloodstain. Thus, antibody profiling was able to discriminate between blood samples and also to provide useful information regarding age of donor.

Hoste, Brocteur, Donea & Andre (1977) investigated the use of the hepatitis B virus to distinguish between stains. This antigen was shown to be very stable in the dried state for at least 6 months. However, in vivo, the persistence of HBs antigen varied greatly from one individual to another. In a healthy carrier or in a subject with chronic hepatitis, HBs antigen may persist from 1 month to 6-7 years following infection whereas in cases of acute infection the antigen

may not be detectable after one or two weeks.

If HBs antigen was found in a bloodstain, it may not always be possible to associate the stain with the suspect especially if the individual suffered from acute hepatitis and more than 2 weeks had elapsed between the deposition of the bloodstain and the collection of a blood sample.

This problem is not relevant when dealing with antibodies as markers in blood and stains. The half life of soluble antibodies in dried blood has been shown to be approximately 6 weeks (Werrett, King & Whitehead, 1976). Antibody in vivo, persists at a basal level throughout life (King, Werrett & Whitehead, 1976). Antibody profiling, therefore, offers more scope for development.

In the 2 years between conception and the start of this project a little further investigative work had been performed by King, et.al. (1975) who had used it successfully to discriminate between bloodstains from adults and children using 5 microbial antigens. In 1976, Werrett and King showed that the presence of specific allergen-associated antibodies of the IgE type could be detected and quantitated in bloodstains by a radioimmunoassay technique known as the radioallergosorbent test (RAST). This work was particularly attractive since allergies show geographical specificity. Thus allergy to ragweed affects a large number of the inhabitants of the Northern United States of America but is almost unknown in Europe.

The concept of antibody profiling, therefore, offered the possibility of differentiating between bloodstains and yielding qualitative information which could prove useful in a discriminatory manner.

The second section of this thesis records the results of virus antibody profiling trials and attempts to analyse the significance of these results.

c) FLUORESCENT ANTIBODY TECHNIQUE

Principle of fluorescence

According to Udenfriend (1962), 'An oversimplified definition of fluorescence is that it is the immediate emission of light from a molecule or atom following the absorption of radiation.'

The energy changes associated with fluorescence can be understood by taking a simple diatomic molecule as a model system and looking at its potential energy as a function of nuclear separation (Fothergill, 1964), see Figure C. The principle is similar for more complicated molecules. The normal, or ground, state of the molecule when in equilibrium with its surroundings at room temperature, is represented by curve G (Fig. C). Curve E_1 corresponds to the first excited state of the molecule. The horizontal lines associated with each curve depict the various energy levels which the molecule can show depending on the vibrational states of the molecule.

Transition 'A', due to the absorption of a quantum of light depicts the increase in the potential energy of the molecule from the ground state G to one of the higher levels of the excited state E_1.

The vibrational energy of the excited molecule is then usually lost through collisions with other molecules until the lowest vibrational level of the excited state is eventually reached. If this level is maintained for approximately 10^{-8} seconds, it becomes possible for the molecule to lose a quantum of radiation (transition F) thereby returning to one of the higher vibrational levels of the ground state.

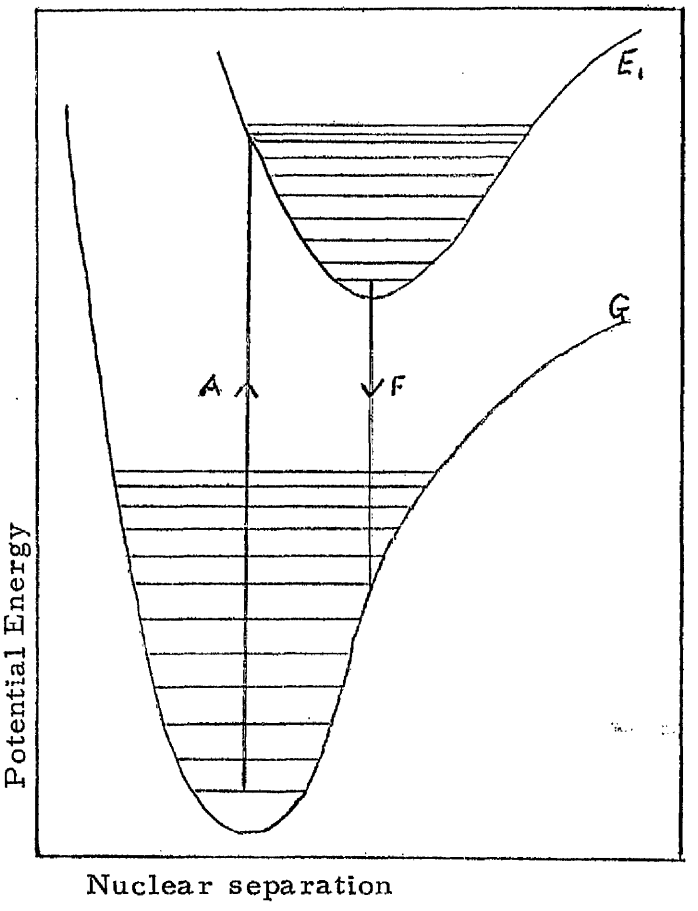


Figure C. Energy diagram of a simple diatomic molecule showing absorption of radiation and emission of fluorescence (Fothergill, 1964)

From Figure C, it can be seen that the energy difference of A is greater than that of F. Planck's Law states that, "the energy of radiation is directly proportional to its frequency and inversely proportional to its wavelength." It, therefore, follows that the radiation of transition 'A' will be of higher frequency and shorter wavelength than transition F. In other words, the fluorescence emitted by the molecule will be of longer wavelength than the radiation required to excite it (Stoke's Law) e.g. the peak absorbance level of fluorescein is 490 nm (violet) but its fluorescent emission peak is at 525 nm (yellow-green).

Application in Microscopy

The immunofluorescent technique has its beginnings in a paper published by Marrack (1934) in which he was able to demonstrate that typhoid bacteria agglutinated by antibody labelled with a red dye could be visualised microscopically as distinct red clumps. For the first time, it had become possible to demonstrate an antigen-antibody interaction directly, without resorting to the use of secondary phenomena such as the complement fixation, haemagglutination or precipitation reactions.

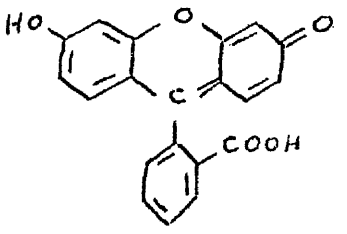
In attempting to improve this technique, 7 years later, Coons, Creech & Jones (1941) employed a fluorescent dye called anthracene, attached to pneumococcal antibody to identify pneumococcal bacteria microscopically. Fluorescent dyes (fluorochromes) have since gained favour for use as labels because they can be seen at much lower concentrations than ordinary dyes (Nairn, 1964) and therefore, greatly increase the sensitivity of the procedure (Udenfriend, 1962). The major disadvantage of anthracene as a fluorescent marker, however,

lies in the fact that it produces a bright blue fluorescence, very similar to the blue-green autofluorescence of ordinary mammalian tissue when exposed to ultraviolet (u.v.) light in a microscopic preparation.

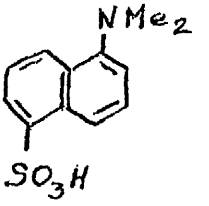
The following year, however, Coons, Creech, Jones and Berliner (1942) improved the test by substituting the anthracene with fluorescein isocyanate which fluoresces with a brilliant apple green colour, sufficiently different from the blue-green autofluorescence of tissue to permit its use in tracing antigens with labelled antibody in situ, in histologic preparations. The production of this conjugate was a tedious affair, however, since fluorescein isocyanate only couples to protein in the presence of phosgene gas.

Other fluorescent markers followed (see Figure D). Weber (1952) introduced 1-dimethylaminonaphthalein-5-sulphonic acid (DANS) which has an intense yellow-green fluorescence; Chadwick, McEntegart & Nairn (1958a) overcame the difficulties of colour contrast by utilising the orange fluorescent label, lissamine rhodamine B (RB 200); but the fluorochrome fluorescein isothiocyanate (FITC), (Riggs, Seiwald, Burckhalter, Downs & Metcalf, 1958) has been the most commonly used due to its simple conjugation procedure to link it with antibody. The immunofluorescence technique, therefore, was originally devised and developed as a method for tracing antigens by labelled antibodies but it has subsequently been used for the detection and characterisation of specific serum antibodies by studying their reactions with known antigens.

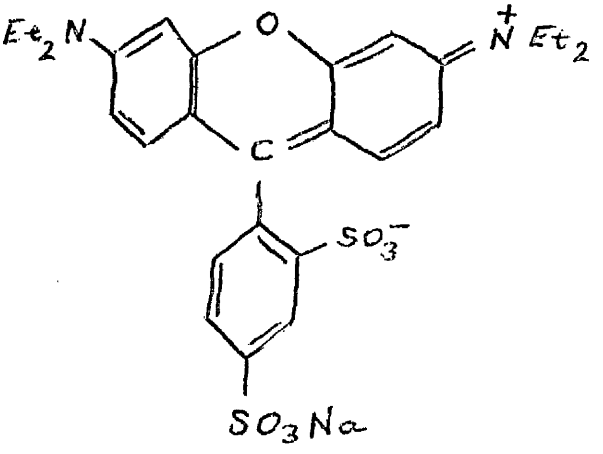
In short, the fluorescent antibody method, introduced by Coons, et. al (1941) and subsequently applied to Virology, has combined the sensitivity and specificity of immunology with the precision of



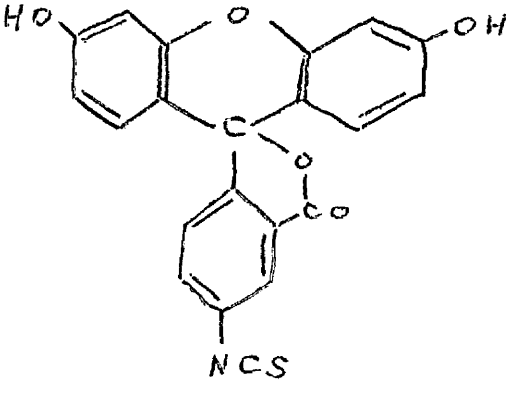
Fluorescein



1-Dimethylaminonaphthalene-5-sulphonic acid (DANS)



Lissamine Rhodamine B
(RB 200)



Fluorescein Isothiocyanate
(FITC)

Figure D: Fluorochrome molecules

microscopy to:-

a) yield new information on the virus multiplication cycle in infected host cells and

b) greatly decrease the assay time taken to measure the immune response to viral infections in routine serology.

Conjugation

Ideally, the conjugation of fluorochromes to individual serum proteins should produce conjugates with a high fluorescence emission, while retaining the biological characteristics of the native protein molecules. It is now fairly well established that conjugation of a small number of fluorochrome molecules to a single molecule of serum protein has little effect upon the antigenicity of the protein (Fothergill, 1964).

The fluorescent brightness of the conjugate depends upon the number of fluorochrome molecules which are chemically bound to the protein molecules. The labelling of a single protein molecule with a large number of fluorochrome molecules, however, need not produce brighter fluorescence. Coons and Kaplan (1950) compared the brightness of staining of fluorescein isocyanate conjugates of different degrees of labelling and found that the fluorescence intensity of the stained preparation increased at first with increasing ratio of fluorochrome to protein and then decreased.

The critical factor in obtaining maximum fluorescence, therefore, depends upon the ratio of fluorochrome molecules per molecule of protein.

The molecular ratio of fluorochrome to protein in a purified conjugate can be calculated quite simply by measuring the fluorochrome and protein concentrations and substituting the values in the equation:-

$$\frac{\text{Moles fluorochrome}}{\text{Moles protein}} = x \frac{\text{O.D.}}{C_p}$$

where O.D. = optical density at λmax for the fluorochrome component

C_p = concentration of protein in mg/ml. (conjugate is usually diluted about 1:100 in phosphate-buffered saline to yield a protein concentration of approximately 0.5 mg/ml)

x = factor derived from molecular weights and extinction coefficients (the value of x is 1.9 for FITC, 1.4 for RB200 and 25 for DANS conjugates).

A further factor which influences the fluorescence of conjugates is the pH of the surrounding environment. The fluorescence intensity of fluorescein conjugates varies markedly with pH, with a maximum brightness around pH 8.0. RB200 conjugates show little variation around pH 7.0 but their fluorescence emission is approximately doubled at pH 4 and pH 10.5. DANS conjugates, however, appear to produce fairly constant fluorescence over the range pH 1.6 to 14 (Weber, 1952).

The criteria influencing the choice of a suitable fluorochrome for use in immunofluorescence have been listed by Chadwick, et. al. (1958b).

1. It should have chemical groups which will covalently bond to protein molecules, with any unreacted fluorescent material being easily removed. (Creech and Jones, 1941, showed that the most probable site of conjugation between globulin and FITC or RB 200 occurred through the E-amino groups of lysine).

2. It should retain a high fluorescence efficiency when conjugated to the protein. (The fluorescence emission of RB 200 changes from yellow to orange when conjugated to protein; fluorescein on the other hand has the same colour whether conjugated or not).

3. The colour of its fluorescence emission should contrast sufficiently with the background autofluorescence of the infected tissue, thus permitting unambiguous interpretations to be made.

4. The conjugate should be stable and retain its immunological characteristics under normal storage conditions.

5. Its conjugation to protein should be a simple, short procedure.

Conjugate preparation

The labelling of specific classes of antiviral antibodies, e.g. IgG, IgA with fluorochromes demands a pure anti-globulin raised in an animal immunised with a highly purified antigen. Viral antigens propagated in tissue culture cells must be freed from any contaminating cell debris before inoculation into a laboratory animal, to avoid raising antibodies to the "foreign" tissue culture cell components as well as the viral antigen. This is quite critical if the resulting globulin is to be used to detect virus in the same tissue culture system.

MacFarlane, McLure & Sommerville (1971) have reported a procedure which can be applied to many virus types and results in the removal of more than 90% of the contaminating protein from the

virus suspension. MacFarlane, et. al (1971) prepared a 60% suspension of barium sulphate in demineralized water by mixing equimolar quantities of barium chloride and sodium sulphate. The resulting white precipitate was harvested by centrifugation at 500 g for 5 minutes and added to the virus suspension as a paste in the ratio of 6.5 parts barium sulphate : 10 parts virus suspension. This mixture was shaken on a reciprocating shaker at 37°C for 60 minutes. The barium sulphate with attached virus was then deposited by centrifugation (500 g, 5 minutes) and the clear supernatant was discarded. Virus was eluted from the barium sulphate by resuspending the deposit in 5.0 ml 0.25M sodium citrate and shaking for 60 minutes at 4°C on the reciprocating shaker. Virus free barium sulphate was then sedimented by centrifugation (500 g, 5 minutes) and the resulting purified virus suspension in sodium citrate was aspirated off.

For the indirect staining technique, an anti-species globulin is required for conjugation which must also be free from non-specific antibodies. The required globulin fraction can be separated from the other components of immune serum by various methods such as ammonium sulphate precipitation of serum IgG (Stelos, 1967) or sodium sulphate precipitation (Sommerville, 1967).

The conjugation of a fluorochrome such as FITC to purified immunoglobulin is achieved by slowly adding 3 mg FITC to every 1 ml of immunoglobulin in 0.5 M carbonate-bicarbonate buffer pH 9.0 and stirring at 4°C whilst the fluorochrome is added over a period of about 15 minutes. Stirring is continued overnight at 4°C. The

resulting conjugate can be further purified and unreacted fluorescent material removed by passage through a column of G-25 Sephadex, pH 7.2 which retains free fluorescein and other molecules up to 5,000 molecular weight, while the conjugated globulin passes straight through as a homologous band.

Poor quality conjugates producing a high level of non-specific fluorescence are usually attributable to:-

a) inadequate purification of the immunising antigen

b) poor fractionation of the immunoglobulin from the resulting antiserum

c) overconjugation using too high a ratio of fluorochrome to protein

d) negatively charged conjugated proteins.

With FITC conjugates, Goldstein, Slizys & Chase (1961) found that the higher the ratio of fluorescein to protein was, the more negatively charged the protein became. These highly charged molecules could then absorb non-specifically to tissue cells and obscure true specific staining. Their removal from the conjugate can be effected by absorption with acetone dried tissue powder such as human liver or placenta or by passage through DEAE cellulose.

Nowadays, however, commercially prepared and relatively purified conjugates to a large number of viruses and antispecies globulins are available.

Microscopy

Light source

The fluorescent antibody test requires equipment capable of producing light in the blue to ultraviolet wavelengths to excite the fluorochrome molecules in the specimen. Two suitable light sources are in common use:-

a) the mercury vapour lamp which emits a wide spectrum of light extending from the ultraviolet range to the infra-red

b) the quartz-iodine lamp. This lamp does not emit ultraviolet light but short wave visible blue-violet light which makes it unsuitable for examining poorly fluorescent material.

Primary filters

Various filters are located in the lamp housing. These filters reduce the amount of heat reaching the specimen and exclude the unwanted infra-red and visible wavelengths thus permitting only ultra-violet and blue light to illuminate the specimen.

Barrier filters

Filters are also placed between the objective lens and the eye of the microscopist. These filters serve two functions:

a) to prevent harmful u.v. light from reaching the observer's eye

b) to obtain suitable colour contrasts in the specimen between natural tissue autofluorescence and specific fluorochrome fluorescence.

Darkground illumination

The production of a bright specimen image against a dark background can be achieved in two ways:-

a) by the use of a dark ground condenser which produces a "cone" of light such that no light will enter the objective lens unless it has been diverted by an object (or emitted by an object) on the microscope stage.

b) by means of ring condensers forming an integral part of the objective lens, i.e. incident light illumination. Here the light passes down the outer ring of the objective lens and only light

which has been reflected (or emitted) from the specimen enters the central lens of the objective.

The instrument used in the course of this project was a Leitz Wezlar Ortholux 11 binocular microscope equipped with incident light darkground objectives and a 50W super pressure mercury lamp.

Fluorescent antibody techniques

There are four ways in which the fluorescent antibody test can be performed depending upon whether antigen or antibody is to be detected. See Figure E.

a) Direct test

Antigen present in tissue cells and fixed on a microscope slide can be visualised by reacting it with a fluorochrome labelled antibody. This procedure requires a conjugate made specifically for the identification of each particular antigen. Visualisation of different types of antigens therefore requires a large variety of individually labelled sera. This situation can be avoided by using a double layer method.

b) Indirect test (Weller & Coons, 1954)

Here the antigen is first reacted with specific antiserum and then, after removal of all unbound serum, exposed to fluorescently labelled antiglobulin antibody. In this way, many different unlabelled human antisera can be used with only a single preparation of fluorescent rabbit anti-human globulin.

This procedure is routinely used in serology to investigate and titrate unknown sera for the presence of antibodies to specific antigens.

A further advantage of this method relates to the fact that the

number of fluorochrome molecules per molecule of antigen is greater than in the direct test. The result is a brighter fluorescent image and a more sensitive assay technique.

c) Sandwich test

This is a double layer procedure to visualise specific antibody. Antibody containing cells, e.g. in a preparation of lymphoid tissue, are first of all exposed to a solution of homologous antigen. After removal of surplus unbound antigen, a second layer of fluorescence labelled antibody is applied to locate those cells which had specifically bound the antigen.

d) Complement test

The final method involves fluorescence labelled antibody to complement (usually C4). It was introduced in 1958 by Goldwasser and Shepard for poliovirus type 1 antibody detection. Complement binds or "fixes" to antibody in antigen-antibody reactions. The presence of such immune complexes could therefore be visualised by introducing an anti-complement conjugate into the test system.

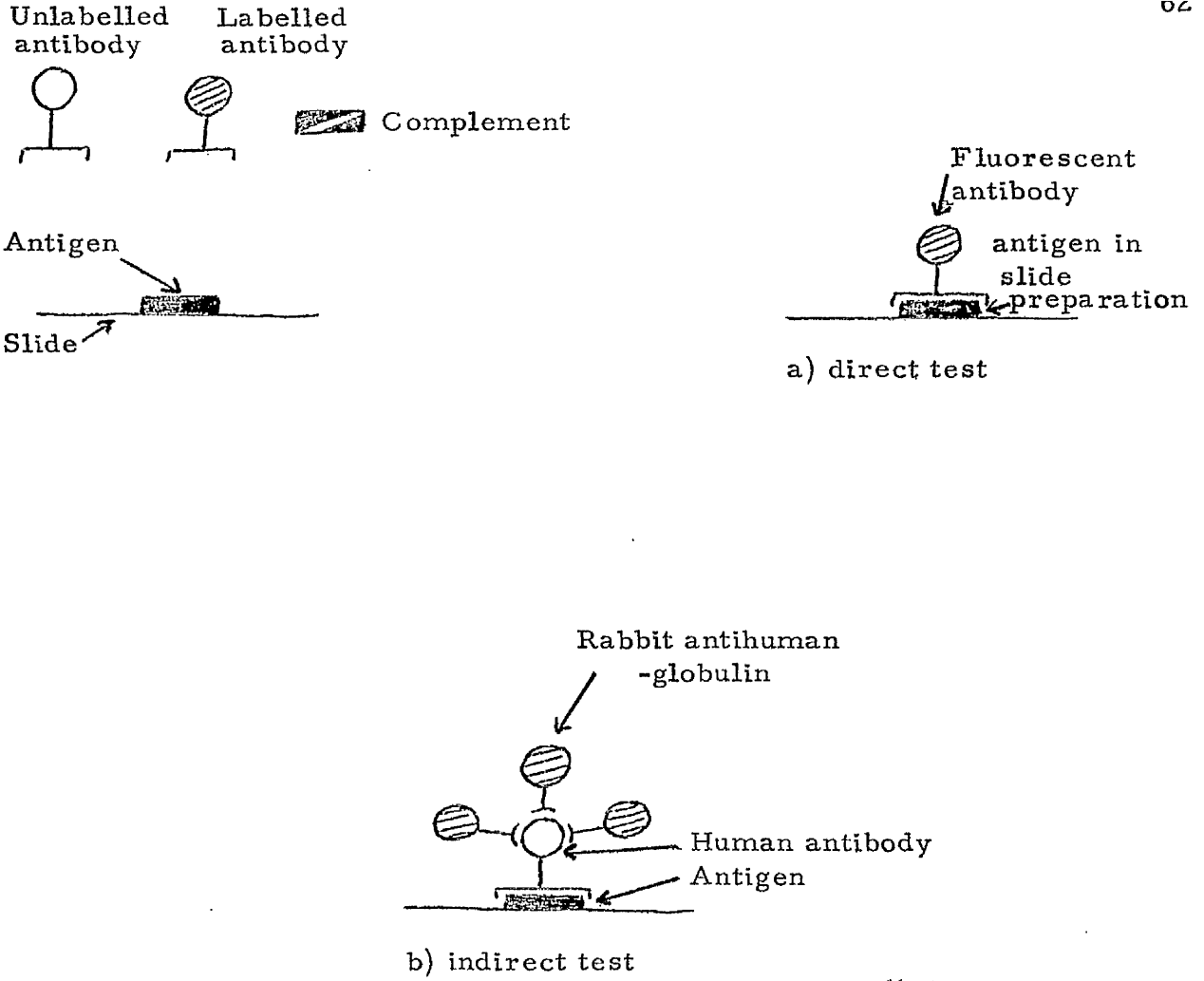


Figure E: Fluorescent antibody techniques

d) ENZYME IMMUNOASSAY (EIA)

Introduction

Enzyme labelled antibodies have been used for the detection and localization of immune reactants in histological preparations since the original description by Nakane, Sri Ram and Pierce in 1966. Similar enzyme preparations have since been used for the visualisation of precipitin lines in immunodiffusion and immunoelectrophoresis, e.g. enzyme linked immunoelectrodiffusion assay (ELIEDA) (Pinon & Dropsy, 1977).

The use of enzymes to label antigens, haptens and antibodies in immunological tests and quantitative immunoassays was investigated independently by two groups of investigators (Engvall & Perlmann, 1971 and 1972; Van Weemen & Schuurs, 1971, 1972 and 1974).

In 1971, Engvall and Perlmann devised an enzyme immunoassay for the quantitation of rabbit immunoglobulin G. This assay consisted of a modified version of the radioimmunosorbent technique (RIST) of Wide and Porath (1966). In RIST, use is made of radioactively labelled antigen and insolubilized antibodies, covalently coupled to cellulose or Sephadex. The binding of labelled antigen is competitively inhibited by unlabelled antigen in standard solutions or unknown samples. Engvall and Perlmann, however, discovered that labelling of the antigen (rabbit IgG) with the enzyme alkaline phosphatase from calf intestinal mucosa by use of glutaraldehyde (Avrameus, 1969), resulted in a more stable labelled antigen which required simpler equipment for its detection and measurement than the complex scintillation counter required for radioactively labelled compounds.

Nomenclature

Various names are given to the assays which involve enzymes as labels:

E.I.A. "Enzyme-immunoassay" is generally applied to every method in which the extent of binding of enzyme-labelled antigen, hapten or antibody to its immune reactant is measured.

E.M.I.T. "Enzyme-multiplied immunoassay technique" is a trade name of the Syva Corporation, U.S.A. and is used exclusively for a special group of assays, the homogeneous enzyme immunoassays. (Bastiani, 1978). A diagrammatic outline of this type of assay is given in Figure F.

This assay has been applied to the detection of drugs of abuse, e.g. morphine, barbiturates; for the detection of cardiovascular drugs, e.g. digoxin, quinidine; and for the assay of hormones such as cortisol, oestriol and thyroxine T4 (Bastiani, 1978).

In the homogeneous enzyme immunoassay enzymes such as lysozyme, malate dehydrogenase or glucose-6-phosphate dehydrogenase are used to label hapten in such a way that the enzyme retains its activity. On reaction of the labelled hapten with antibody, however, the activity of the enzyme label in the hapten antibody complex is inhibited. In the assay, the unknown

sample is mixed with the labelled hapten and with specific antibody to the hapten. If hapten is present in the unknown sample, it competes with the labelled hapten for the limited amount of antibody with the result that there is less antibody available to inhibit the enzyme activity of the labelled hapten.

C.E.L.I.A. "Competitive enzyme-linked immunosorbent assay" is a term given to a particular type of EIA developed by Yorde, Sasse, Wang, Hussa & Garancis (1976). In this test, the binding of antibody to antigen linked to agarose is competitively inhibited by the free antigen to be measured. The amount of first antibody bound to the immunosorbent (agarose) is measured by an enzymatic technique in which a heterologous bridging agent and a soluble antibody-enzyme immune complex are applied in sequence.

E.L.I.S.A. "Enzyme-linked immunosorbent assay". This term is usually applied to antibody assays and is perhaps the most useful term since it identifies the heterogeneous enzyme assay yet at the same time clearly differentiates it from the tests which employ antibody-peroxidase conjugates for staining reactions in microscopy.
A diagrammatic representation of the indirect method of ELISA for the assay of antibody is given in Figure G. This procedure was evaluated and utilised during the course of my project.

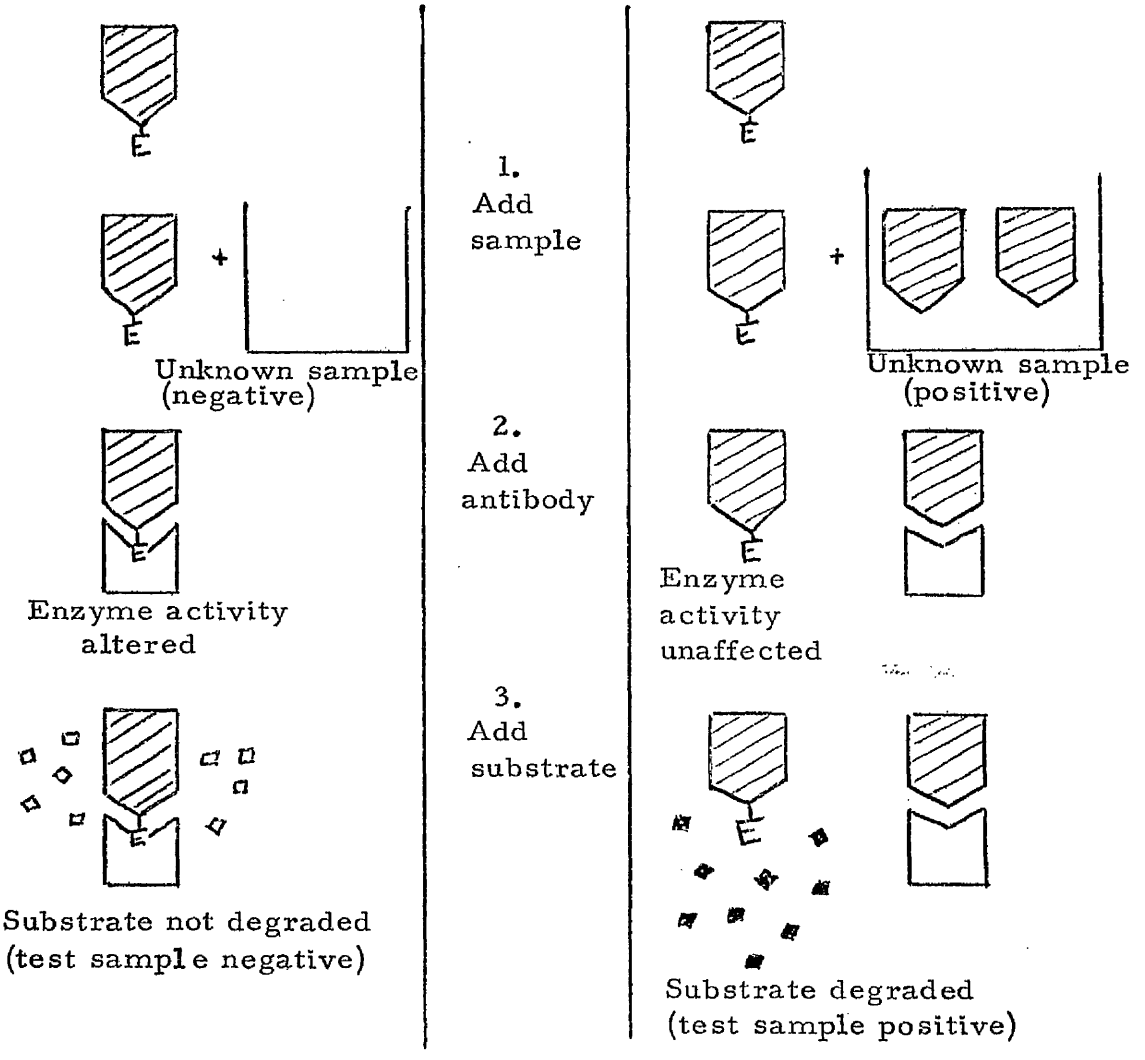
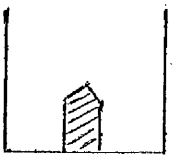


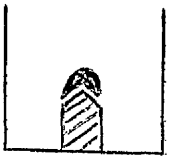
Figure F: The homogeneous enzyme immunoassay
(Voller, Bartlett and Bidwell, 1978)

1. Antigen absorbed to plate



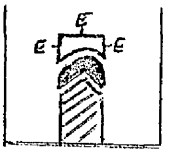
Wash

2. Add serum. Any specific antibody attaches to antigen



Wash

3. Add enzyme labelled antiglobulin which attaches to antibody



Wash

4. Add substrate

amount hydrolysed =

amount of antibody present

Figure G: The indirect method of ELISA for assay of antibody

(Voller, Bartlett and Bidwell, 1978)

In the same year, 1971, Van Weemen and Schuurs succeeded in developing a similar enzyme immunoassay for the detection of human chorionic gonadotrophin (HCG). In this case the antigen HCG was conjugated with the enzyme horse radish peroxidase by use of gluteraldehyde.

In the following year, 1972, Van Weemen and Schuurs applied the enzyme immunoassay technique to the determination of haptens. They used oestradiol and oestriol, coupled through succinyl bridges to horse radish peroxidase and antibodies against these steroid hormones.

By 1974, Van Weemen and Schuurs had successfully labelled antibodies with horse radish peroxidase to measure human chorionic gonadotrophin. This followed Engvall and Perlmann's experiments in 1972 when they labelled sheep anti-rabbit IgG with the enzyme alkaline phosphatase for the quantitation of specific antibodies in human serum albumin coated tubes. It is this latter application of enzymes, for the detection of specific antibodies, which will be the main subject of this review.

Enzyme immunoassays can be classified as either homogeneous or heterogeneous (Rubenstein, Schneider & Ullman, 1972) thus:-

a) Assays in which the label in the labelled reagent behaves differently depending on whether or not it is bound to its specific counterpart in the immune reaction, and which therefore do not require a physical separation of the reactants into two fractions, are called "homogeneous enzyme-immunoassays"
(Rubenstein, et. al., 1972).

b) Assays in which the label behaves more or less identically irrespective of whether or not it is bound to its specific counterpart in the immune reaction, and which therefore require a separation of the reactants (as in fluorescence or radioimmunoassay) into two fractions, are called "heterogeneous enzyme immunoassays".

The separation of the bound and free components of the heterogeneous assay has been attempted in various ways, as follows:-

Several enzyme immunoassays have been described in which the immune complex formed was precipitated out of solution with a second antibody (double antibody technique), e.g. α-foetoprotein (Belanger, Hamel, Dufour & Pouliot, 1976), (Kirkpatrick, Wepsic & Nakamura, 1977) and cortisol (Comoglio & Celada, 1976). In one particular study by Lauer and Erlanger (1974) the antigen and antibody concentrations used were so high that the immune complexes precipitated directly, i.e. without the addition of second antibody. In this case the bound and free fractions could therefore be separated by low speed centrifugation.

Most of the other heterogeneous enzyme-immunoassays, however, are performed with a solid phase which is prepared prior to use in the assay.

Two different principles of solid phase support have been described:-

a) Particulate material suspended in the test medium

1) Cellulose powder was used in the previously described pioneering experiments of Engvall and Perlmann (1971) and Van Weemen and Schuurs (1971, 1972, 1974). It was also the material of choice for the detection of total oestrogens and

human placental lactogen developed by Bosch, van Hell, Brands, Van Weemen and Schuurs, (1975) and subsequently investigated by Barbour (1976). Cellulose has also been used successfully by Maiolini & Masseyef (1975) for the assay of rat and human ∝ -foetoprotein.

ii) Agarose has been used in the assay of human IgG by Kato, Hamaguchi, Fukui & Ishikawa (1975) and in the assay of human choriogonadotrophin by Yarde, Sasse, Wang, Hussa & Garancis (1976).

Both cellulose and agarose have antigen or (second) antibody bound to them by means of the method developed by Axen, Porath & Ernback (1967). This procedure involves two stages. Firstly, a "reactive intermediate" is formed by treating the polysaccharide (cellulose or agarose) for a short period with aqueous cyanogen halide solution under alkaline conditions. Secondly, this "reactive intermediate" is coupled with protein or peptide in a neutral or preferably slightly alkaline aqueous medium.

The use of cyanogen halides for this purpose gives a high yield of bound polypeptide or protein which retains a substantial part of its activity.

(iii) Plastic discs, as solid phase support for horse antibody to hepatitis B surface antigen (HB_sAg) wereused by Halbert & Anken (1977) in an enzyme-labelled "sandwich" assay for the detection of HB_sAg.

iv) Gilman and Docherty (1977) described a plastic bead solid phase system for the detection of herpes simplex antibody by ELISA. This bead system has certain advantages over the other procedures previously described, e.g.

a) The beads can be obtained with uniform diameters, therefore they have uniform surface areas.

b) They each contact the base of a container at only one small point.

c) They can be totally immersed in reagent solutions.

For these reasons spherical beads give highly reproducible results in ELISA procedures (Smith & Gehle, 1977). Processing and transferring the beads during the ELISA test, however, left much to be desired.

The use of forceps to transfer each bead from one reagent in the assay to the next was tedious, time consuming and subject to inadvertent small variations inevitable with manual handling.

Smith, et. al. (1977) therefore improved this technique using ferromagnetic spheres coated with an outer layer of polycarbonate to which the viral antigens were absorbed.

A specially designed magnetic transfer device consisting of 24, 1/4 x 1/4 inch cylindrical magnets in a 4 x 6 assay was then used to transfer the beads (24 at a time) from one assay reagent to another.

b) The inner wall of a solid phase container.

i) For many tests polystyrene tubes have been used, e.g. in the detection of serum anti-DNA antibody in Systemic Lupus Erythematosus (Pesce, Mendoza, Boreisha, Gaizutis & Pollack, 1974) or for the assay of antibodies to plasmodium vivax and p. falciparum (Voller, Huldt, Thors & Engvall, 1975).

ii) The wells of microtitration plates also provide suitable solid phase surfaces onto which antigen or antibody can be absorbed. Plates have been used successfully, for example the assay of Chagas' disease, i.e. American trypanosomiasis caused by Trypanosoma cruzi (Voller, Draper, Bidwell & Bartlett, 1975).
Ruitenberg and van Knapen (1977) subsequently applied the ELISA to the detection of antibodies to both helminths (Trichinella spiralis, Toxocara canis) and protozoa (Trypanosoma species and Toxoplasma gondii) using both tubes and plates and tentatively concluded that plates resulted in an assay of higher sensitivity than the tube tests.

In the heterogeneous enzyme immunoassay, one can choose for a particular system and within limits in which of the two phases the enzyme label will be determined. The use of the liquid phase, or supernatant, for this purpose, however, has several disadvantages: the necessity of transferring the liquid to a second test tube and the possibility of interference of the enzyme reaction by constituents of the assay fluid.

If, on the other hand, the label is determined in the solid phase, the procedure for washing the solid phase should be given adequate attention. An incomplete washing of the unbound assay components from the immunosorbent will affect the accuracy and precision of the eventual assay.

In the indirect method of ELISA, the antigen is immobilised on to the solid phase. Test sera are then incubated with the solid phase and any antibody in the test sera becomes attached to the antigen on the solid phase. After washing to remove unreacted serum components, an antiglobulin enzyme conjugate is added and incubated. This will become attached to any antibody already fixed to the antigen. Washing again removes unreacted material and finally the enzyme substrate is added. The degree of colour change will be a measure of the amount of the conjugate fixed. This in turn is proportional to the antibody level in the test sample.

Choice of enzymes

Enzymes make admirably suitable labels because their catalytic properties allow them to act as amplifiers. So much so in fact, that many enzyme molecules can catalyze the formation of more than 10^5 product molecules per minute (Wisdom, 1976). No single enzyme has yet been found that is ideal for use as a label in every EIA. Instead, the enzyme which is most suitable for a particular assay system has to be evaluated with care.

The main criteria which are important for the choice of an enzyme, for use in an immunoassay, can be summarized as follows:

a) Available cheaply in highest purity

b) High specific activity (i.e. turnover number)

Note: the turnover number is the number of substrate molecules converted to product per enzyme site per unit of time.

Another way of formulating this criterion could be the enzyme activity expressed in International Units per mole of enzyme.

c) Stable under assay and storage conditions

d) Soluble

e) Assay method which is simple, sensitive, rapid and cheap

f) Absent from biological fluids or present in minute amounts which will not appreciably increase the amount of substrate degraded.

g) Substrate, inhibitors and disturbing factors absent from biological fluids

h) Capable of retaining activity while undergoing appropriate linkage reactions

i) Stable at reaction temperature .

Somewhat surprisingly, many enzymes have been described in the literature that can satisfy most of these conditions. The following enzymes have been used as labels in heterogeneous EIA : horse-radish peroxidase; alkaline phosphatase; β- D-galactosidase; glucose oxidase; glucoamylase; carbonic anhydrase and acetylcholinesterase (Schuurs and van Weemen, 1977).

The first two enzymes, namely, horse-radish peroxidase and alkaline phosphatase are the most widely employed in ELISA. At a recent meeting on "ELISA for Infectious Agents" (National Institutes of Health, Bethesda, Maryland, U.S.A., September 9-10, 1976) it was concluded that horse-radish peroxidase and alkaline phosphatase were about equal in performance, but that horse-radish peroxidase was to be preferred because of availability and the brightness of the colour developed.

Objections are now being raised, however, to the use of horse-radish peroxidase since the majority of its most commonly used substrates such as 5-amino-salicylic acid and o-phenylene diamine are potentially carcinogenic.

Characteristics of enzyme-immunoassays

Investigators working in ELISA research usually outline the advantages of ELISA as follows:-

1. Specific assays of wide applicability.

ELISA has been investigated in almost every branch of biological science, e.g.

Endocrinology:	Assay of human chorionic gonadotrophin (Van Weemen & Schuurs, 1971).
	Determination of oestradiol and oestriol (Van Weemen & Schuurs, 1972).
Immunology:	Quantitative assay of immunoglobulin G (Engvall & Perlmann, 1971).
	Measurement of serum anti-DNA antibody in systemic lupus erythematosus. (Pesce, et. al., 1974).
Haematology:	Assay of Factor VIII related antigen. (Bartlett, Dormandy, Hawkey, Stableforth & Voller, 1976).
Virology:	Assay of Rubella antibodies. (Voller & Bidwell, 1975).
	Detection of antibodies to measles and cytomegalovirus infections (Voller & Bidwell, 1976).

Detection of hepatitis B surface antigen.
(Walters, Kuijpers, Kacaki & Schuurs, 1976).

Bacteriology: Assay of antibodies to salmonella O antigens
(Carlsson, Lindberg & Hammarström, 1972).

Parasitology: Detection of toxoplasma antibody.
(Voller, Bidwell, Bartlett, Fleck, Perkins
& Oladehin, 1976).

Agriculture: Detection of 7 plant viruses including arabis
mosaic virus (AMV), plum pox virus (PPV)
and raspberry ringspot virus (RRV).
(Clark & Adams, 1977).

Biochemistry: Assay for a pregnancy associated α-macroglobulin
(Stimson & Sinclair, 1974).

Epidemiology: Study of human Rotavirus Types 1 and 2.
(Yolken, Wyatt, Zissis, Brandt, Rodriquez,
Kim, Parrott, Urrutia, Mata, Greenberg,
Kapikian & Chanock, 1978).

2. Sensitivity of assays. Direct comparisons between sensitivities of assays are difficult for a number of reasons (Schuurs, et. al. 1977), i.e.

a) Sensitivities are dependent on the affinity of Ag-Ab reactions and can therefore be influenced by the quality of the antisera.

b) Sensitivities depend on the nature and molecular weight of antigen or hapten, so that sensitivity comparisons between different antigens or haptens may be of questionable value.

c) Assay conditions, such as type of sample fluid, reagent concentrations, incubation period, incubation temperature, are of the utmost importance.

For these reasons, only broad generalisations can be made. The sensitivity (i.e. the least amount of antibody detectable) varies widely as reported by different investigators, but levels as low as 1 pg/l of specific antibody are reported to have been measured accurately (Wisdom, 1976). ELISA is, however, generally accepted to be more sensitive than agglutination, quantitative precipitation and immunofluorescence (Wisdom, 1976). Other important advantages of the ELISA related to sensitivity are (a) the small amount of sample required, making it admirably suitable for detecting antibody in the sera of infants or in circumstances where only limited amounts of sample are available, such as in forensic serology. Voller, Draper, Bidwell & Bartlett (1975) report that as little as 10 µl of serum is sufficient for several assays of antibody to different antigens.
(b) the greater sensitivity allows shortening of the time interval between successive samples of serum for detecting a rising antibody titre in viral infections, from 10-14 days to as little as 2-3 days (Leinikki & Passila, 1977).

3. Equipment required is relatively cheap and widely available.
In its simplest form, ELISA requires only the special grade of solid phase support and reagents. Some investigators report reliable results being obtained from simple visual determination of the serum titration and points, e.g. detection of antibody to Rubella virus (Gravell, Dorsett, Gutenson & Ley, 1977) and assay of antibody to Herpes simplex virus (Smith, et. al. 1977).

However, to ensure objectivity, the results are best measured spectrophotometrically. The equipment used has ranged from simple spectrophotometers to complex specially designed ELISA readers, (e.g. Multiskan plate reader, Flow Laboratories, Irvine, Scotland).

4. Reagents are relatively cheap and have a long shelf life. The reagents involved, such as the antigen and conjugate can be prepared by the investigator, thereby reducing the cost per test even more. The enzymes used are generally very stable and do not suffer from the disadvantage of a limited half life such as the commonest Radioimmunoassay reagent I^{125} which has a half life of only 2 months. In the lyophilised state, shelf lives, for alkaline phosphatase conjugated anti-human IgG, of over 7 years have been reported to date (Institute Virion Ltd.).

5. Manipulations are simple.

ELISA tests do not require highly trained personnel. Reliable results are easily obtained provided the test is performed with reasonable care and accuracy.

6. Assays can be very rapid.

Most assays can be performed in as little as 5 hours (Voller, Bidwell, & Bartlett, 1976).

7. Potential for automation.

Automated titration systems, plate washers and ELISA readers greatly increase the number of serological specimens which can be processed in a working day.

8. No radiation hazards.

ELISA is not affected by the strict regulatory safety legislation governing radioactive isotope storage, manipulation and disposal, although biohazards remain as in any test system using unknown serum samples.

NOTE

The following chapter (Chapter II) records the procedures involved in performing the various experiments which were undertaken in the course of this project.

The reader is advised, however, that the explanation of the results tabulated in Chapter III is confined to the discussion section (Chapter IV, pages 252 through 286).

CHAPTER II

EXPERIMENTAL DATA (1)

MATERIALS & METHODS

a) Indirect Fluorescent Antibody Technique

Introduction

The indirect fluorescent antibody technique demands extreme purity of virus and virus antigenic material. The following is therefore a summary of culture techniques which were employed in propagating the viruses used in this project.

Tissue culture techniques

I. General Requirements

(a) Glassware: All glassware for tissue culture must be scrupulously cleaned to remove grease and dirt which would otherwise prevent culture cells from adhering to the glass surfaces. The containers must also be sterilised to kill bacteria, yeasts, fungi, etc. which would contaminate the culture cells during the course of their propagation. The following washing protocol was therefore adopted for the preparation of all glassware.

1. Immerse in 1% chloros for 1 hour
2. Immerse in 0.1 N HCl for 1 hour
3. Immerse in 0.2% pyroneg for 1 hour
4. Rinse 6 times in tap water, emptying between rinses
5. Rinse 3 times in deionized water
6. Dry in hot air oven ($160^{\circ}C$)
7. Autoclave at $121^{\circ}C$ for 20 minutes (15 lbs/sq. in.)

Note: Steps 1 -5 of this procedure may also be performed in a programmable automatic glassware washing machine.

(b) Reagents: Tissue culture cells require i) the correct physical conditions of pH and osmotic pressure for survival and growth, ii) materials which they cannot synthesise, including amino acids, carbohydrates, vitamins and inorganic ions.

The essential components of tissue culture media have now been characterized. The pH and osmotic pressure of the cells environment is dictated by the combination of salts and carbohydrate used but cells will only survive for a brief time in an isotonic balanced salt and carbohydrate solution alone. For prolonged survival essential amino acids, vitamins, and proteins are required. Usually the serum proteins are supplied by a 1% solution of foetal bovine serum (F.B.S.). Eagle (1955) developed and described a widely used medium but others with slightly different compositions, e.g. Morgan, Morton and Parkers Medium No. 199, are also in common use.

(c) Antibiotics: Antibiotics are added to tissue culture media only as a safeguard against possible contamination of the tissue culture cells by bacteria or fungi during handling. The antibiotics are commonly penicillin (20-50 units/ml), streptomycin (50μg/ml) and nystatin (20μg/ml).

Mycoplasma contamination may pose very serious problems in continuous cell lines but some control of this contamination may be achieved by kanamycin (100-200μg/ml), although prolonged use is not advised.

(d) Glutamine: Glutamine has an important but as yet undefined role in cell metabolism. It is commonly added to tissue culture media separately to give a final concentration of 2 mM (Paul, 1975).

(e) Trypsin: Trypsin is a proteolytic enzyme but can be used at a final concentration of 0.025% w/v to remove cells from glass surfaces.

(f) Glucose-Versene: The disodium salt of ethylenediaminetetra acetic acid, is a chelating agent which binds calcium and magnesium ions (Versene).

A mixture of versene (200μg/ml) and glucose (100μg/ml) in Dulbecco A PBS has been found to be useful in facilitating the removal of tissue culture cell monolayers from glass. This solution is sterilised by autoclaving at 115^{o}C for 10 minutes and stored at room temperature.

II. Tissue Culture Cells

5 types of tissue culture cell were used:

a) Primary monkey kidney

b) Vero

c) Calf kidney

d) Lymphoblastoid cells carrying EBV genome

e) Human embryo lung

a) Primary Monkey Kidney Cells

These were obtained from Gibco Biocult Ltd. as 120 sq. cm. monolayers. On receipt of these cells, the maintenance medium

(see Appendix) was replaced with 10 ml of a 1 : 1 mixture of trypsin and versene as previously described and incubated at 37°C for 5 minutes to remove the cells from the sides of the bottle. The resulting suspension of cells was centrifuged at 500 g for 10 minutes. The supernatant was discarded, the cells resuspended in 30 ml of Growth Medium (see Appendix) and inoculated into 3 x 40 sq. cm. surface area bottles as 10 ml of cell suspension.

Incubation at 37°C for 4 days allowed formation of a confluent monolayer. The growth medium was then replaced by 10 ml of maintenance medium and further incubated at 37°C until required for use (usually within 4 days).

b) Vero cell line

This is a continuous cell "line" derived from the kidney of the African Green Monkey, Cercopithecus aethiops. The cells were propagated firstly by stripping a 40 sq. cm. confluent monolayer of Vero cells off the glass surface in the following manner. 10 ml of trypsin - versene solution was left on the monolayer at room temperature for 10 - 15 seconds. The trypsin - versene solution was then discarded and the monolayer of cells (now opaque)was easily removed by gentle agitation after the addition of some 30 ml of Growth Medium (see appendix). 10 ml of the resulting single cell suspension was inoculated into each of 3 x 40 sq. cm. bottles and incubated for 2 days at 37°C to allow a monolayer of cells to form. The growth medium was then replaced by 10 ml of maintenance medium and incubated at 37°C until required for use (usually within 4 days).

c) Calf Kidney Cells

These cells were used exclusively for the propagation of calf rotavirus.

A freshly dissected sterile kidney from a recently killed calf (within 5 hours) was obtained from the local abbatoir. The outer layers of fat and fascia were removing using sterile scissors and forceps. Small pieces of the cortex were then removed and minced with the scissors in a sterile petri dish (9 cm) before being transferred to a 500 ml bottle containing 100 ml sterile PBS.

The tissue was washed free from blood in several changes of sterile PBS, continuing until the PBS supernatant remained clear. The clear PBS was then removed and 200 ml of a 0.25% trypsin solution (Gibco Biocult Ltd.) was added. 100 ml of trypsin containing approximately half of the kidney tissue was immediately transferred to another 500 ml bottle. A sterile magnetic bar was introduced into each 500 ml bottle and magnetic agitation proceeded for 10 minutes at room temperature.

Single and damaged cells were discarded with the trypsin solution after 10 minutes and 100 ml of fresh 0.25% trypsin solution was added to each bottle. Magnetic agitation was continued at room temperature for 30 minutes before the supernatant fluid containing individual kidney cells was removed, stored at 4^{o}C and replaced with 100 ml of fresh 0.25% trypsin. The process was then repeated.

The stored trypsin/cell suspensions were centrifuged at 500 g for 5 minutes to sediment the cells. The supernatant was discarded

and 10 ml of Growth Medium (see Appendix) was used to resuspend the cells. 5 ml aliquots of cells were used to inoculate two 40 sq. cm. surface area bottles.

This procedure was repeated until the required number of bottles had been inoculated. The cells were incubated horizontally at 37°C and the growth medium (10 ml) was changed on the next 2 consecutive days.

When a monolayer had formed (usually by the 3rd or 4th day) the growth medium was replaced with 10 ml maintenance medium and incubated at 37°C until required for use (usually within the next 4 days).

d) Epstein-Barr Virus Carrier Cultures

This type of cell culture consists of lymphoblastoid cells, a proportion of which carry the EBV genome. The experiments used EB_3 cells grown in suspension at 37°C in an atmosphere of 10% CO_2. The medium (see Appendix) was changed every 3-4 days, i.e. the suspended cells were sedimented by low speed centrifugation (500 g, 5 minutes) then resuspended in fresh growth medium and further incubated at 37°C in 10% CO_2.

Doubling of cell number occurs in approximately 7 days and cultures were "split" when the cell density reached 8×10^5 cells/ml. This was achieved by adding an extra volume of growth medium and transferring half the suspension to each of 2 fresh bottles.

To allow expression of the EB_3 virus carried by the cells, the growth medium is replaced by a medium deficient in the amino acid arginine. The following protocol was adopted for the preparation of

the cells expressing EB_3 virus for use in the IFA technique:-

The cells were removed from the normal growth medium and grown for 4 days in minimal essential medium (M.E.M.) + 15% Foetal Calf Serum at 33°C. The medium was changed to arginine deficient M.E.M. (see Appendix) containing 15% foetal calf serum. After 3 days at 33°C, the cells were deposited by centrifugation at 500 g for 3 minutes before resuspending in fresh arginine deficient medium and incubating at 33°C. Epstein-Barr virus, which can be demonstrated by immunofluorescence, is apparent between day 6 and 7.

e) Human Embryo Lung Cells

These cells were used exclusively for the propagation of Cytomegalovirus (C.M.V.).

Human embryo lung cells were propagated by firstly stripping a 120 sq. cm. confluent monolayer of cells off the glass surface of a 24 oz. bottle in the following manner. The 30 ml of growth medium (see Appendix) was discarded and the monolayer was washed with 10 ml of sterile PBS pH 7.3 for 30 seconds. The PBS was then replaced with 10 ml of trypsin (0.025% w/v) and left at room temperature for approximately 1 minute until the monolayer produced an opaque appearance. The trypsin was discarded, 5 ml of Growth Medium was added and the solution was aspirated with a sterile 10 ml pipette to facilitate removal of the cells from the glass wall of the bottle. 2.5 ml aliquots of cell suspension were used to inoculate 2 new 24 oz. bottles and made up to 30 ml with Growth Medium. By incubating at 37°C a monolayer of cells developed in 3-4 days.

C.M.V. Propagation

Five, 4" x $\frac{1}{2}$" test tubes containing a confluent monolayer of human embryo lung cells were inoculated with CMV and incubated at 37°C until 50% of the monolayer showed signs of infection (plaque formation). This usually occurred within 3 days. The infected monolayers were then removed from the tubes by scraping with a sterile sealed pasteur pipette and transferred to a 24 oz. bottle containing a 120 sq. cm. confluent monolayer of human embryo lung cells and 30 ml of Growth Medium. The CMV inoculated 24 oz. bottle was then incubated at 37°C for 3 days by which time approximately 50% of the cells had become infected with CMV as shown by plaque formation. Microscope slides were then prepared from this material by the standard method (Section IV a).

III. Virus Stocks

In the course of the Forensic section of this project, 21 different viruses were propagated and harvested for use in the indirect fluorescent antibody technique.

The optimum inoculum of each virus for the production of suitable microscope slides for fluorescence (i.e. with the optimal number of cells containing virus) was determined by titrating 0.1 ml aliquots of 10 fold dilutions (10^{-1} to 10^{-4}) of virus in test tubes inoculated with the appropriate type of tissue cell monolayer. After incubation at 37°C, the cells were tested by the IFA technique with sera known to contain IgG antibodies to the type of virus under investigation. The dilution of virus yielding a 50% infection of tissue culture cells, as determined by the IFA test was chosen as the

inoculum for the production of a batch of virus in 40 sq. cm. bottles (approximately 50 slides could be prepared from one infected medical flat cell monolayer).

Different types of virus produce different patterns of infection when visualised by IFA. Some of the different patterns are shown in the colour plates at the end of this section. The cell requirements, culture conditions and types of fluorescence produced by each virus used in this study are listed in Table B.

IV. The Indirect Fluorescent Antibody Technique

a) Preparation of microscope slides

Under the conditions described in Table B approximately 50% of the cell population was infected with virus. In some cases, the extent of infection in the monolayer can be judged from the extent of the developing CPE's which may well be characteristic, e.g. enteroviruses cause cells to round up; Herpes simplex causes a ballooning appearance or the formation of giant multinucleate cells, while measles virus or respiratory syncytial virus tend to produce syncytia in media low in glutamine.

The cells are removed at the appropriate time as follows:-

The maintenance medium is decanted into hypochorite and replaced with 10 ml of 0.02% w/v versene solution and 1 ml of 0.25% w/v trypsin solution. The bottle is re-incubated at 37°C for approximately 10 minutes during which time the cells detach into the EDTA-enzyme solution.

The detached cells are centrifuged at 500 g for 3 minutes and resuspended in approximately 150μl of sterile PBS. Complete

resuspension of the pellet of cells is achieved by using a "whirlimixer".

The single cell suspension containing an ideal 50% infected cell population is then transferred as drops onto a clean microscope slide. The original suspension is repeatedly agitated during this procedure to prevent cell aggregation occurring. 8 replicate 5 mm diameter "spots" of cell suspension, in two columns of four, are applied to each microscope slide using a microdropper. The slides are left to dry at room temperature in a safety cabinet.

b) Fixation

Virus infected tissues are usually fixed in acetone at room temperature for 5 minutes. This follows the original report by Coons, & Kaplan (1950) that alcohol, formalin and Zenker's reagent were all unsuitable for use with virus antigens.

Acetone dehydrates the tissue culture cells and removes lipid from their cell membranes thereby allowing further processing and subsequent "staining" of the intracellular viral components.

The slides are then labelled (using a diamond marker) and stored at -70°C until required for use.

c) Indirect Fluorescent Antibody Procedure

The indirect procedure has been described already. In summary it involves two immunological reactions. In the first, viral antigens present in infected cells combine with specific, non-conjugated antibody. In the second, the cellular sites binding viral antibody are visualised by staining with FITC conjugated anti-species IgG.

The sera under investigation were diluted in sterile PBS from $\log_2$ 1 to $\log_2$ 7 using the wells of a microtitre plate for preparing the doubling dilutions. Previously prepared microscope slides with virus containing spots were removed from cold storage, allowed to reach room temperature, then washed by immersion in distilled water.

This procedure removed any traces of dirt which may have settled on their surfaces during storage. The slides were then dried in air and appropriate dilutions of serum were placed, as microdrops, to cover the spots. The last spot (positive control) received a drop of standard immune serum diluted 1:8 in PBS (see section IVd).

The slides were incubated at 37°C for 30 minutes in humidified plastic boxes before unreacted serum was removed by washing with PBS. The slides were washed twice in agitated PBS at room temperature for 5 minutes before rinsing briefly in distilled water.

Microdrops of a 1:50 dilution FITC conjugated goat anti-human IgG (Hyland Laboratories) in PBS were placed on the spots. The slides were immediately placed in humid boxes, incubated and washed as before. The slides were then ready for examination. Note: Naphthalein black powder was added to the diluted goat anti-human IgG conjugate to give a final concentration of 1 mg/ml. Naphthalein black is a mild protein binding stain which attaches to protein in inverse proportion to its molecular weight and therefore prevents autofluorescence.

d) Standard Immune Serum

This reagent was prepared by mixing 3 different serum samples chosen for their high content of specific antiviral IgG to many of the viruses employed in the Forensic Antibody Profile (Table B).

The resulting Standard Immune serum used at a 1:8 dilution contained sufficient amounts of IgG antibodies to all viral antigens detailed in Table B to allow monitoring of the quality and amount of the viral antigen on the microscope slides. It thereby provided a standard with which the fluorescence of the unknown test sample could be compared.

e) Interpretation of Results

Interpretation of the results of microscopy is always subjective. End points of titrations are recorded as the reciprocal of the dilution of serum showing maximal fluorescence as compared to the positive control.

With some preparations, e.g. polio virus and coxsackie B_4, the fluorescence diminishes sharply at the end point. In the case of Herpes simplex virus, Fc binding can make end points difficult to distinguish. With practice, however, the end point can be read with great accuracy.

Conclusions

The fluorescent antibody procedure was chosen for use in the forensic section of this project on the basis of its speed and simplicity. It is a rapid technique producing results in as little as 2 hours and can be used with eluted bloodstains in preference to other techniques such as the complement fixation test where the presence of haemoglobin might interfere with the end point reading.

These advantages over the other techniques detailed in the literature review (Section 1b) make it one of the techniques of choice for use in the forensic virus antibody profiling investigation.

Virus	Cell line	Inoculum	Temp./time Incubation	Type of fluorescence
Poliovirus 1, 2 & 3	Vero	1 ml Undiluted	37°C/18 hours	Cytoplasmic
Co.x.sackievirus B_1	Vero	1 ml 10^{-1}	37°C/18 hours	Cytoplasmic *
Echovirus 5, 9, 19 & 30	Monkey kidney	1 ml 10^{-1}	37°C/18 hours	Cytoplasmic
Mumps	Monkey kidney	1 ml 10^{-2}	37°C/3 days	Granular *
Measles	Vero	1 ml undiluted	37°C/2 days	Nuclear and cytoplasmic
Herpes Simplex	Vero	1 ml 10^{-3}	37°C/18 hours	Nuclear and cytoplasmic*
Adenovirus 2 & 5	Monkey kidney	1 ml undiluted	37°C/2-3 days	Crystaline inclusions in nucleus *
Respiratory syncytial virus	Monkey kidney	2ml undiluted	37°C/3 days	Syncytial
Parainfluenza 1 & 3	Monkey kidney	1 ml undiluted	37°C/1 day	Membrane
Influenza A(Scotland)	Monkey kidney	2 ml undiluted	37°C/$3\frac{1}{2}$ hr 18 hours	Nuclear Cytoplasmic
Influenza B	Monkey kidney	2 ml undiluted	37°C/18 hours	Cytoplasmic
Epstein-Barr virus	Lymphoblastoid	See text	See text	Nuclear and cytoplasmic*
Cytomegalovirus	Human embryo lung	2 ml undiluted	37°C/2-5 days	Inclusions in nucleus*
Rotavirus	Calf kidney	2 ml undiluted	37°C/2-3 days	Cytoplasmic*

TABLE B: Summary of viruses propagated for use in the Indirect Fluorescent Antibody Technique

* Colour plate available

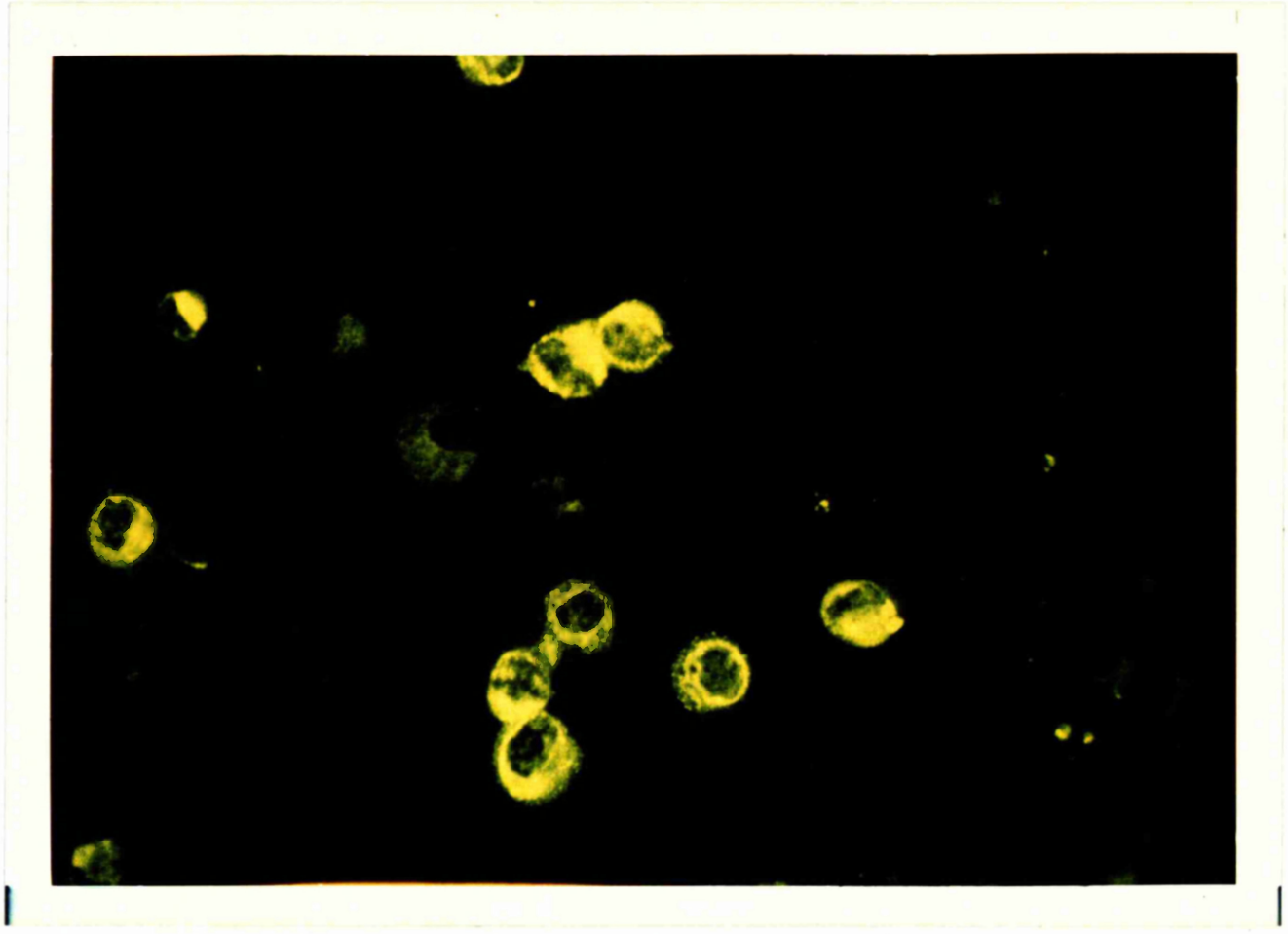


Plate 1 Vero cells infected with Coxsackievirus B_1

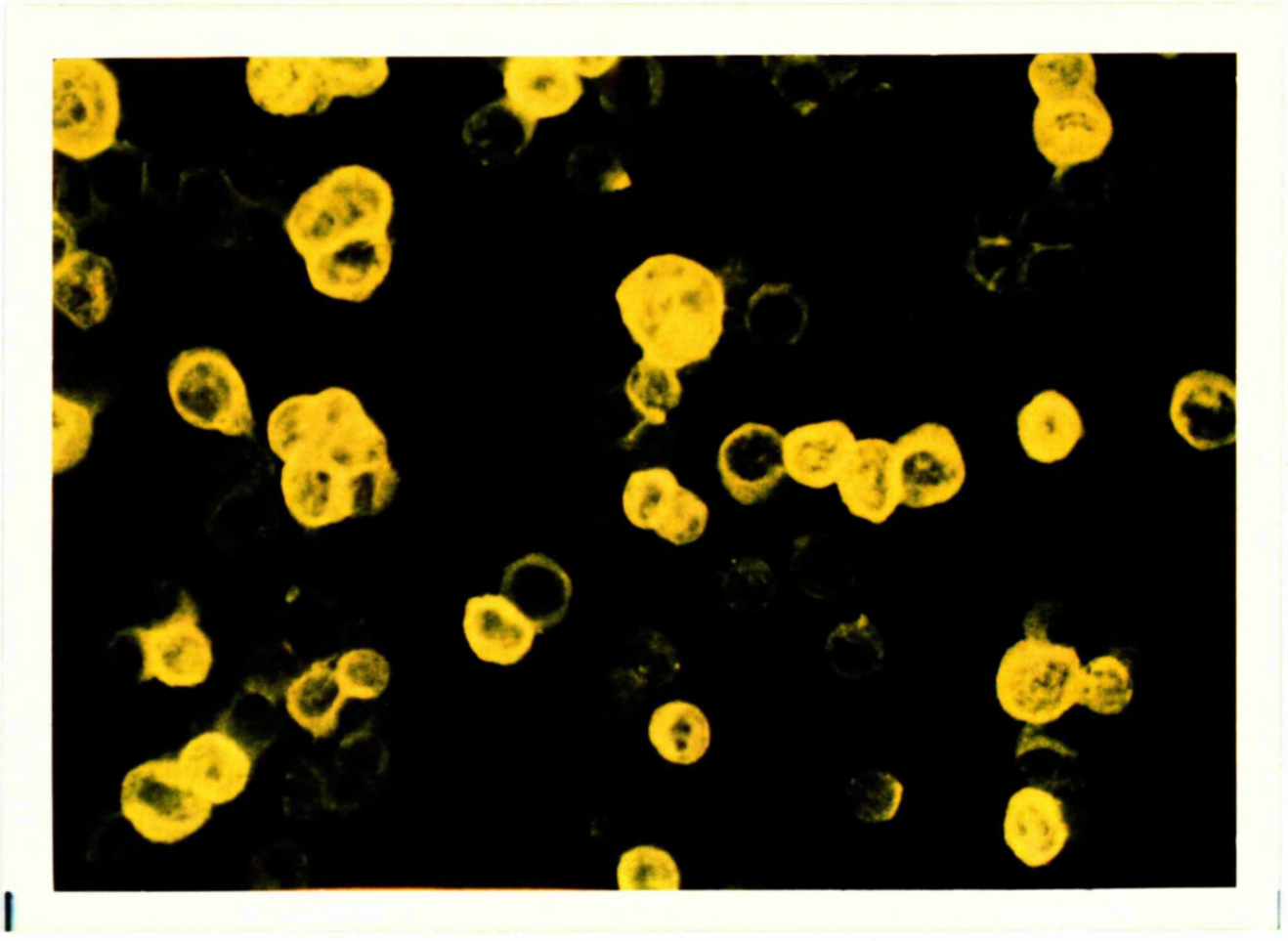


Plate 2 Vero cells infected with Herpes simplex virus type 1

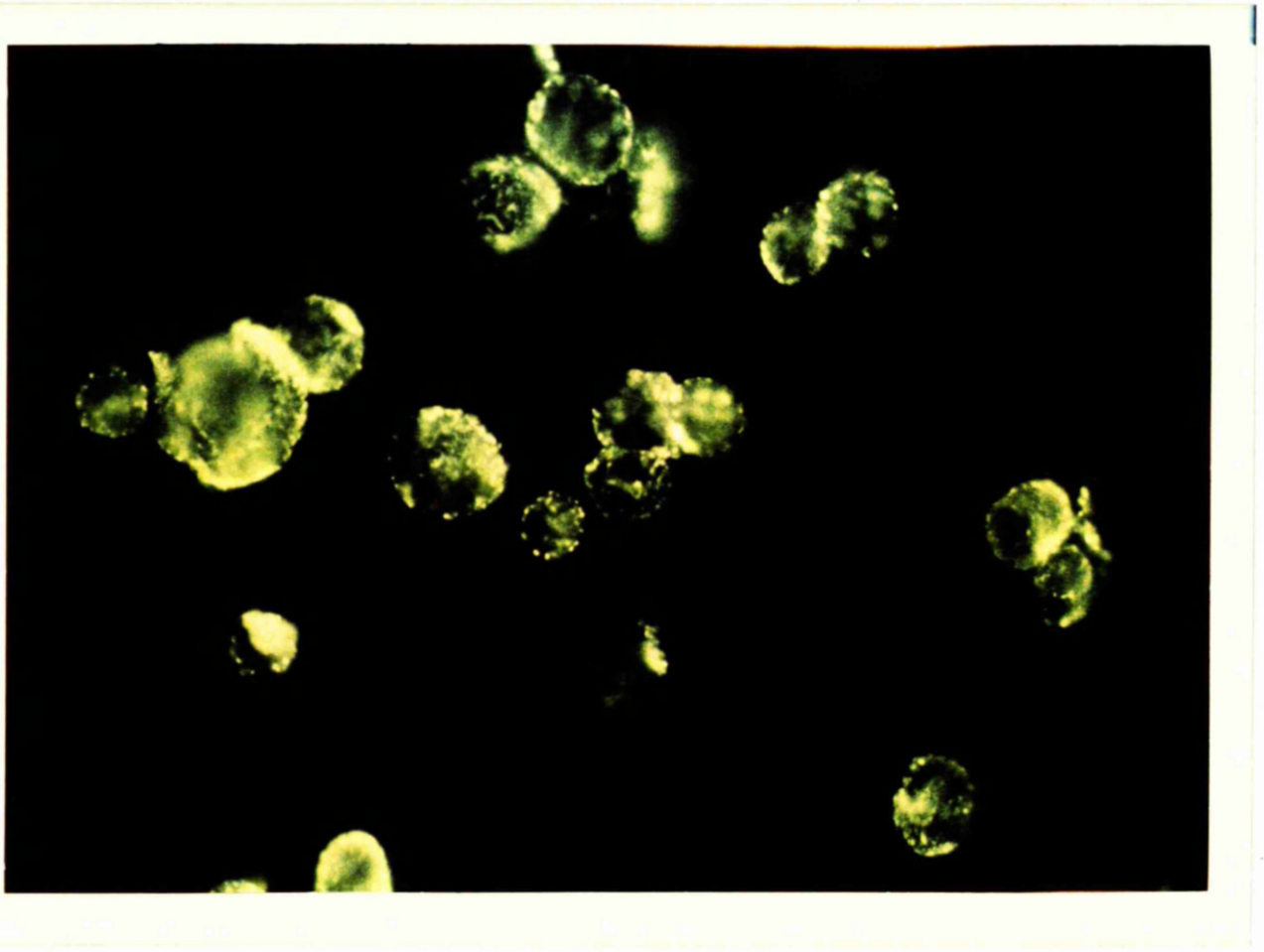
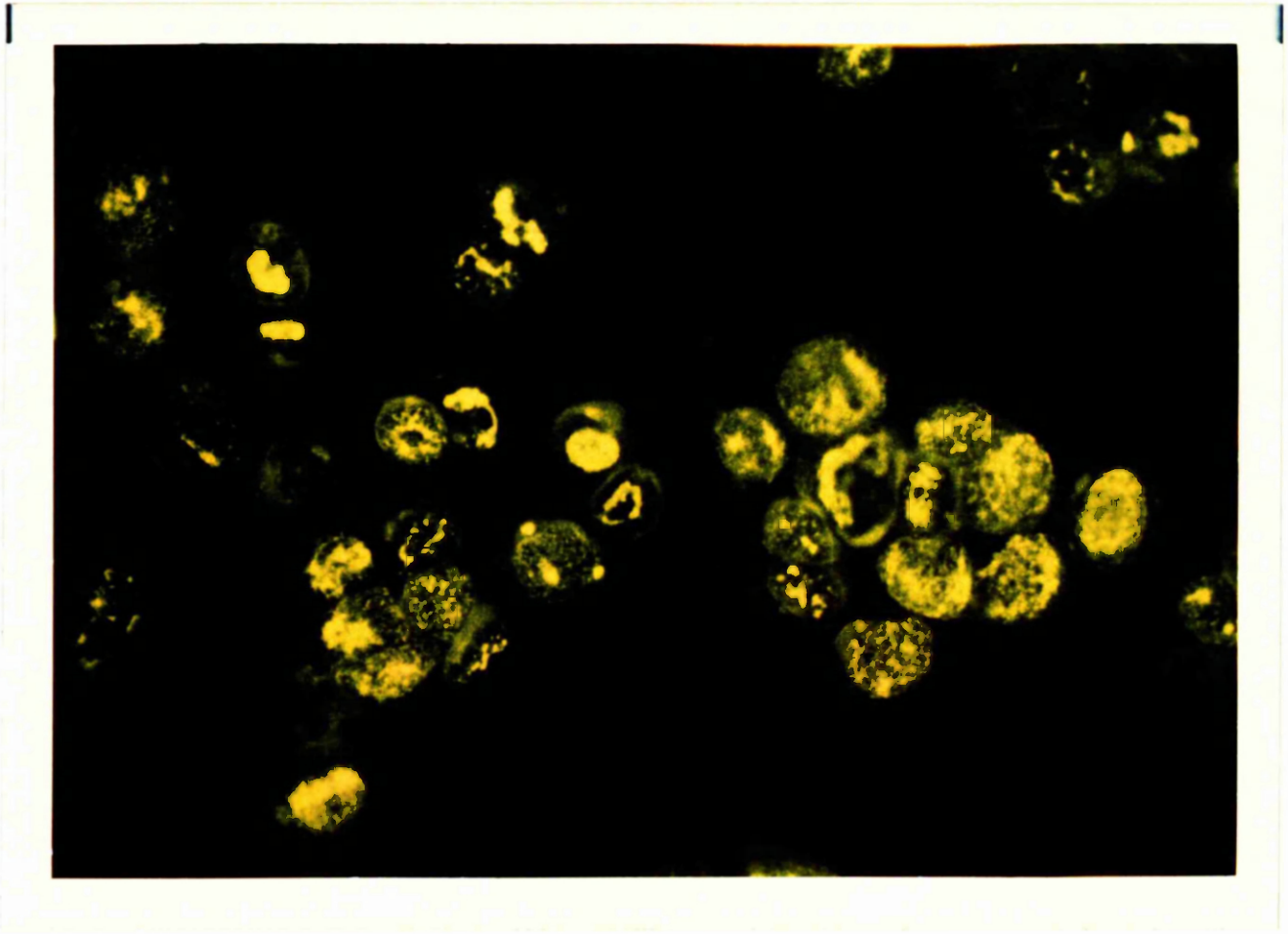


Plate 3 Monkey kidney cells infected with Mumps virus



Monkey kidney cells infected with Adenovirus type 5

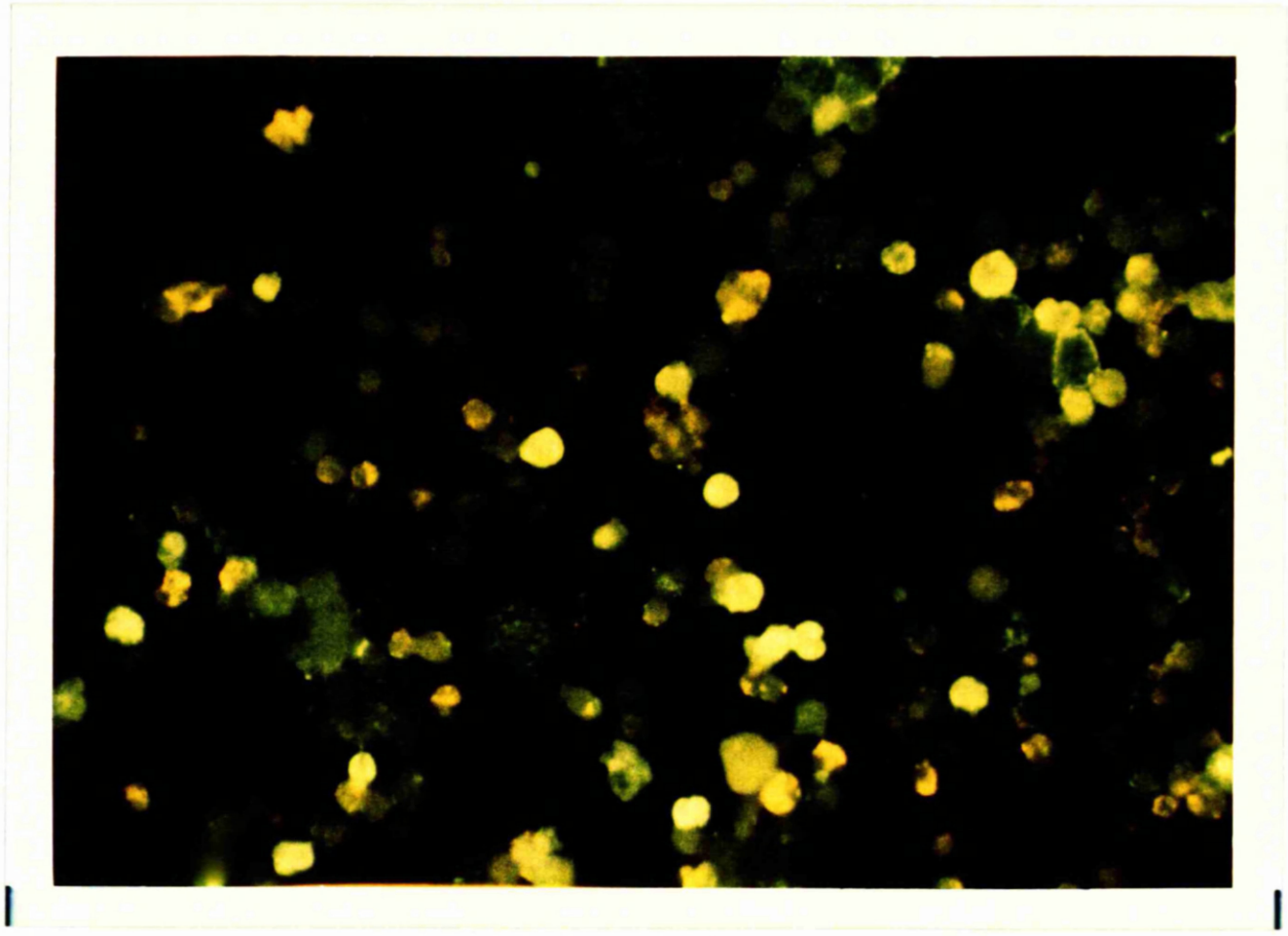


Plate 5 EB_3 cells infected with Epstein-Barr virus

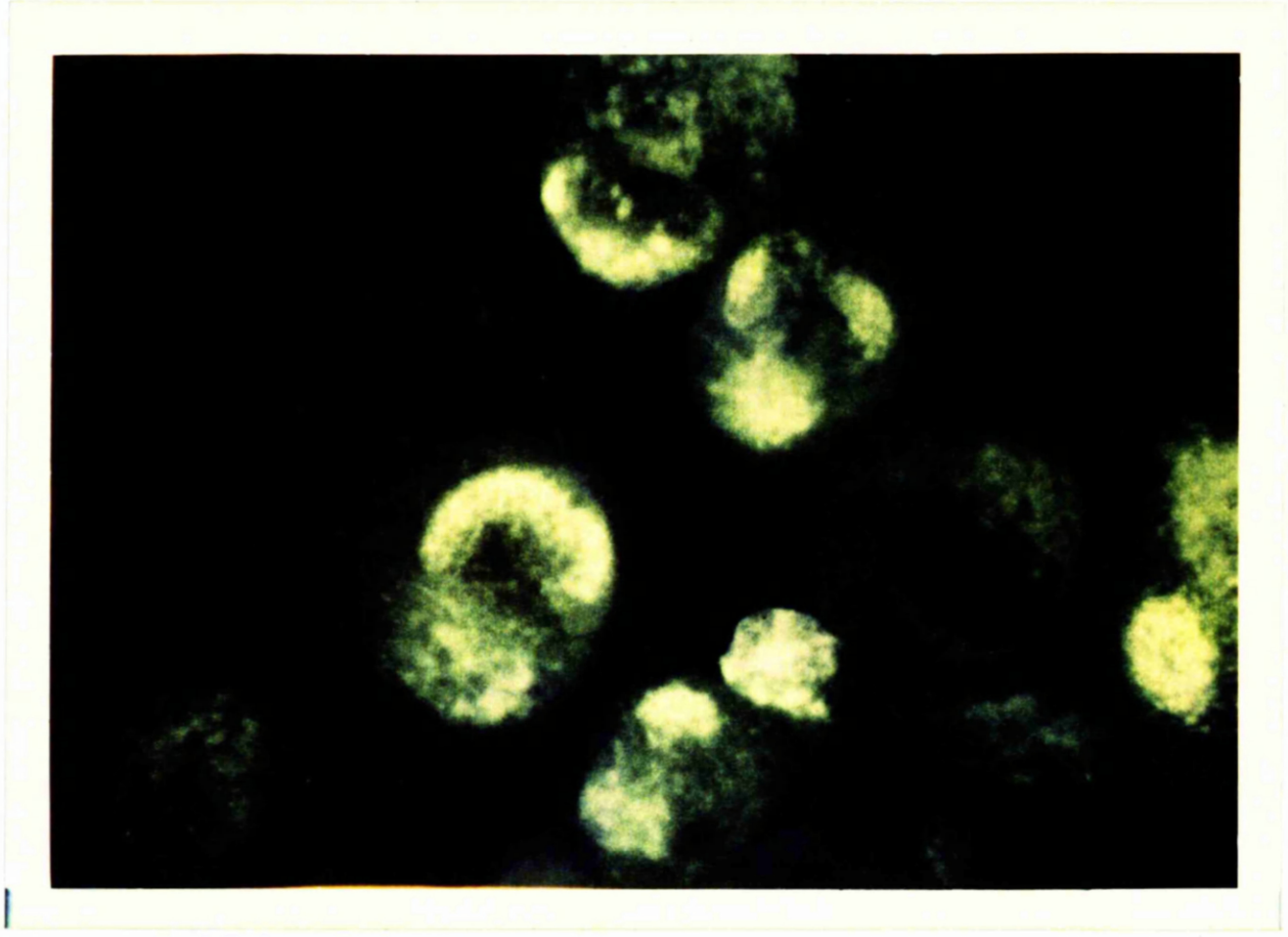


Plate 6 Human embryo lung cells infected with Cytomegalovirus

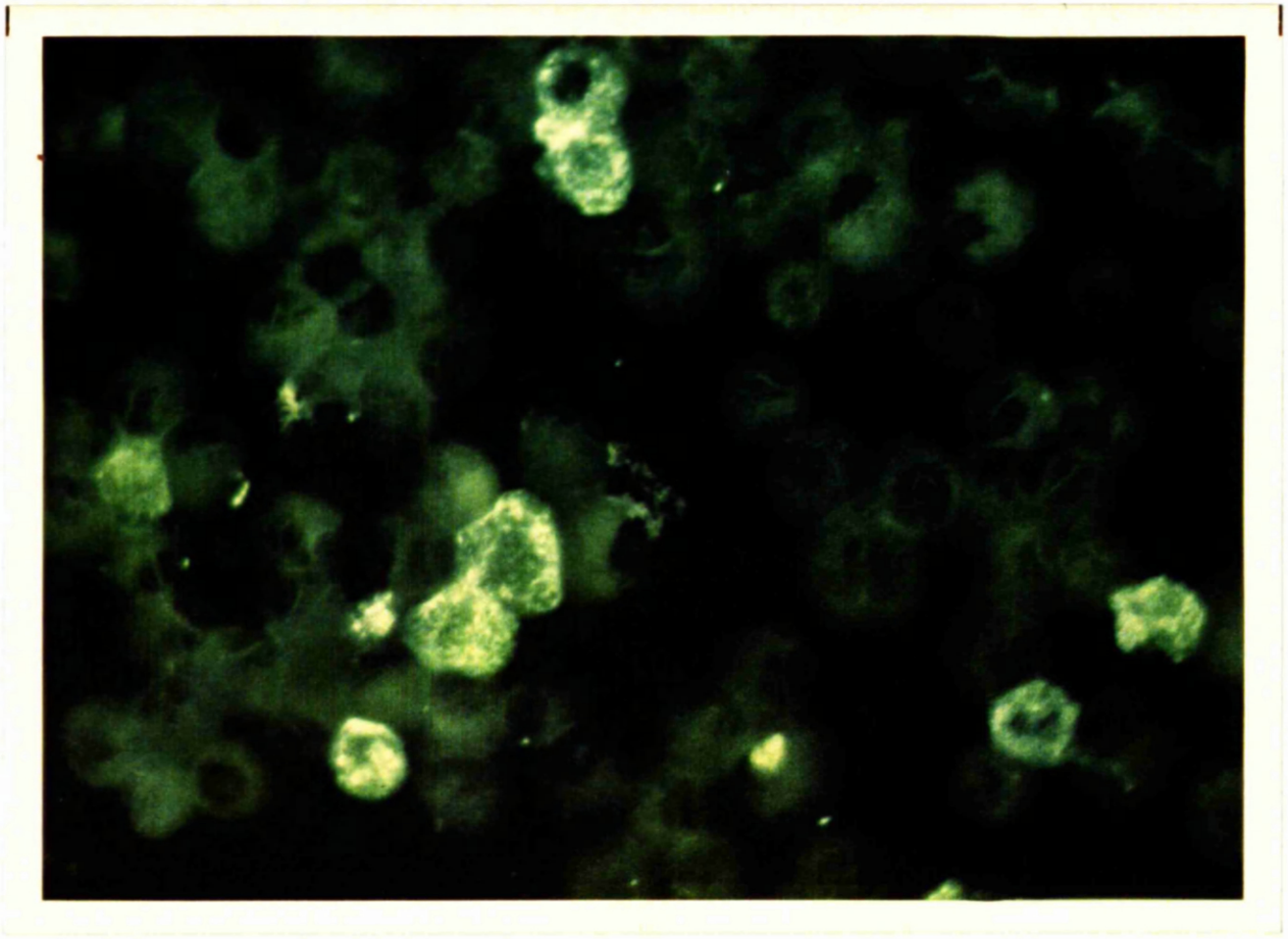


Plate 7 Calf kidney cells infected with Nebraska calf diarrhoea virus

b) Enzyme-linked immunosorbent assay (ELISA)

ELISA is a relatively recent addition to the field of serological testing and so it was important that the test be thoroughly investigated and evaluated before it could be adopted into the daily running of a routine virus diagnostic laboratory.

The advantages of the assay have already been outlined in the literature review but the main desirable feature was the ease with which the test could be performed (Engvall, et. al., 1972). After evaluation, however, it became evident that the ELISA as described by Engvall and Perlmann was deceptively simple - an observation confirmed by Bidwell, Bartlett & Voller (1977).

The ELISA procedure chosen for evaluation with regard to its application in virus serology was that described by Voller, Bidwell & Bartlett (1976a).

Of the three viruses to be investigated in the course of this project (Herpes simplex virus type I (HSV 1), Coxsackie B_1 virus (¢ B_1) and Rotavirus), HSV 1 was considered to be the best choice for evaluation of the ELISA technique for the following reasons.

a) Its ease of propagation in many different tissue culture cell lines.
b) The ready availability of human antisera containing different levels of anti-HSV 1 IgG (approximately 80% of the world's adult population have encountered herpes simplex virus type 1 either as a clinical or sub-clinical infection and therefore have developed IgG antibodies in their serum (Dulbecco & Ginsberg, 1973).

Note: Human antisera, suitable for use in the ELISA procedure were obtained from the serology unit of Belvidere Hospital. The amount of anti-HSV 1 IgG present in the sera was determined by the Indirect

fluorescent antibody technique (IFA). IgG titres of $\geq$ 128 by IFA were considered to be clinically significant.

I. Indirect ELISA procedure (Voller, et. al. 1976a).

1. Production of viral antigen

Voller, et. al. in their paper referred to above, advocated using virus extracts from tissue culture cells as stock virus antigen for use in ELISA. No details for the preparation of these extracts are given, however, but in a related paper concerning ELISA for toxoplasma antibody (Voller, et. al., 1976b) the protozoal antigen was prepared from Toxoplasma gondii organisms in the following manner.

Peritoneal exudates were obtained from mice infected with Toxoplasma gondii. The protozoa in the exudates were ruptured by freeze/thawing once, followed by sonication for 20 seconds at 4°C. The cell and other debris were removed by centrifugation at 10,000 g for 30 minutes and the supernatant fluid was used as the source of antigen for the assay.

A modification of this procedure was, therefore, adopted for the preparation of HSV 1 Ag for use in ELISA.

Experiment 1

An 8 oz. medical flat bottle containing a confluent monolayer of Vero tissue culture cells and 10 ml Vero cell maintenance medium was inoculated with 1 ml undiluted stock HSV 1. (The stock virus had been prepared by previous Vero cell propagation and stored in 1 ml aliquots at -196°C in liquid nitrogen).

After incubation at 37°C for 18 hours, visual observation of the cell monolayer, in the light microscope, revealed that approximately 80% of the Vero cells had become detached from the glass, implying that the majority of the Vero tissue culture cells had become infected and killed by the HSV 1.

The viral antigen was harvested by subjecting the culture to freezing and thawing three times. This procedure lysed any remaining intact cells (checked by microscopy) and liberated the HSV 1 into the maintenance medium. The tissue cell debris was deposited as a pellet by centrifugation at 500 g for 10 minutes and the supernatant liquid consisting of Vero cell maintenance medium and HSV 1 was used as the source of the HSV 1 virus antigen.

2. Sensitization of plates

The flat bottomed wells of polystyrene microtitre plates (Cooke Microtiter M129A; Dynatech Laboratories Ltd.) can be coated by passive absorption with solutions containing between 1 and 10 mg/ml of specific viral protein prepared in coating buffer pH 9.6 (see appendix for formula).

0.15 ml aliquots of a 1 : 100 dilution of viral antigen in coating buffer pH 9.6 were introduced into each well of a Dynatech microtiter plate (the last column was not inoculated and so acted as control) and incubated at 4°C for 18 hours to allow the viral antigen to absorb to (sensitize) the plastic surfaces of the wells.

The plate was then washed with PBS-Tween pH 7.4 (see appendix).

3. Plate washing

The washing procedure consisted of emptying the plate, refilling all the wells with PBS-Tween from a plastic wash bottle and gently agitating the plate by hand for 3 minutes at room temperature. This procedure was repeated 3 times and after the final wash the plate was shaken dry. The next step in the assay followed immediately.

4. Serum assay

Different human antisera containing known amounts of anti-HSV 1 IgG (as determined by the IFA test) were prepared in PBS-Tween to give doubling dilutions ranging from 1/2 through 1/256.

0.1 ml aliquots of each dilution were then added to appropriate sensitized wells in the plate and incubated at room temperature for 2 hours.

The sera were then discarded and the plate was washed as before.

5. Enzyme conjugate

Voller, et. al. (1976a) employed one of two different enzyme labelled anti-human IgG conjugates, i.e. alkaline phosphatase or horse-radish peroxidase conjugates.

The use of alkaline phosphatase conjugated goat anti-human IgG (Miles-Yeda Ltd.) was investigated in this experiment. The conjugate was diluted to a working strength of 1 : 500 in PBS-Tween pH 7.4 as suggested by Voller (1976a). 0.1 ml aliquots of the working strength conjugate were added to each well of the microtiter plate, including control wells, and incubated at room temperature for 3 hours after which the washing procedure was repeated.

6. Substrate

At least two different chemical compounds can be used in conjunction with alkaline phosphatase conjugate (see Section III). However, in the absence of an ELISA plate reader, and to avoid tedious manipulations with a conventional spectrophotometer a substrate was chosen which would produce results which were easily determined by visual observation.

For this purpose, 1 mg/ml thymolphthalein monophosphoric acid in 10% diethanolamine buffer pH 9.8 was suggested by Dr. D. Werrett (Personal Communication) as being the most suitable substrate.

0.1 ml of substrate was added to each well of the microtiter plate and incubated at room temperature overnight. The enzyme-substrate reaction was then stopped and the substrate colour developed by the addition of 50μl of 3 M NaOH to each well of the plate.

7. Results

No discernible pattern of results emerged from this experiment. Some wells produced a coloured reaction product but this was not found to be related to the dilution of antiserum employed in the test. The control wells, treated with alkaline phosphatase conjugate, PBS-Tween wash fluid and thymolphthalein monophosphoric acid substrate, however, did not produce any false positive colour reactions.

The reason for the occasional positive result obtained in the plate was, therefore, rather obscure.

Experiment 2

To investigate the use of the other enzyme conjugate suggested by Voller, i.e. horse radish peroxidase conjugate, the assay above was repeated precisely as before.

The same dilutions and inocula of HSV 1 Ag and human antisera were employed as before. A 1 : 500 dilution of horse radish peroxidase conjugated goat anti-human IgG conjugate (Nordic Immunological Laboratories) was prepared in PBS-Tween pH 7.4 and used in 0.1 ml aliquots. The substrate used was 5-aminosalicylic acid prepared as follows.

8 mg of 5-aminosalicylic acid was dissolved in 10 ml of warm distilled water. This solution was cooled and stored at $4^{o}C$. Immediately before use, a 10 ml aliquot of the solution was warmed to room temperature and a 1 mol/litre solution of Na OH was added to give pH 6.0. 1 ml of 0.05% hydrogen peroxide and 10 ml of 5-aminosalicylic acid solution were mixed and used immediately as 0.1 ml aliquots for inoculation into all the wells of the plate.

After incubation for 1 hour at room temperature in a darkened room, the reaction was stopped by the addition of 50μl of 3M Na OH to each well.

Result

More wells showed coloured reaction products (positive results), but again, no logical pattern of positive reactions, related to antiserum dilutions, was evident. Also, the control wells which were treated with horse radish peroxidase conjugate, PBS-Tween wash fluid and substrate, produced variable coloured reaction

products indicating false positive results. This conjugate at a dilution of 1 : 500 in PBS- Tween pH 7.4, therefore appeared to absorb to the plastic surfaces of the wells very readily and was not being removed by the standard washing procedure.

Conclusions

Since each step in these two assays (Experiments 1 and 2) had been performed in accordance with the protocol suggested by Voller, et. al. (1976a), (except for the preparation of the viral antigens) it was concluded that the failure of the assays was due to inadequate preparation of HSV 1 antigen. It was necessary, therefore, to investigate alternative sources of viral antigen which might be more suitable for use in ELISA.

Note: Because of the problem of non-specific binding to the microtiter plate plastic which was observed with the horse radish peroxidase conjugate, only alkaline phosphatase conjugate was employed in subsequent ELISA investigations.

In 1977, Leinikki and Passila, investigating RSV serology, reported the successful application of a complement fixing RSV antigen to the ELISA technique.

Accordingly, two complement fixing antigens (HSV 1 and ℄ B_1) were purchased from Immunitalia Laboratories Ltd.

The standard ELISA procedure described by Voller, et. al. (1976a) was repeated using Dynatech microtiter plates sensitized with a 1 : 100 dilution of either HSV 1 CF antigen or ℄ B_1 CF antigen prepared in coating buffer pH 9.6.

The human antisera with various antibody levels employed in these assays were again obtained from the hospital's serology unit and had been titrated previously by the IFA test.

Note: The initial experiment with the HSV 1 CF antigen coated plate revealed that with several sera, end point titres of $> 1:128$ were obtained by the ELISA test. The test was therefore, repeated using the same antisera in a wider dilution range, from 1 : 10 through 1 : 1280.

Results: Tables 1 and 2

The end point titres of the sera were assessed visually by determining the last dilution of serum to yield an obvious blue colouration of the substrate on the addition of the enzyme reaction terminator, 3 M NaOH.

Initial observation of the developed enzyme/substrate results in the plate, suggested that the assays had been successful in demonstrating end point titres to HSV 1 and γB_1 IgG serum antibodies.

Examination of the ELISA and corresponding IFA titres for each serum, however, revealed no correlation between results. Statistical analysis of the data by use of the correlation coefficient (r) suggested by Gilman and Docherty (1977) was then performed with the following results:-

Table 1 HSV 1 data

$r = 0.097$ corresponding to $P < 0.95$

No significant correlation was detected.

Table 2 γB_1 data

$r = 0.12$ corresponding to $P < 0.7$

No significant correlation detected.

Note: The correlation coefficient

The coefficient which relates x and y when these are measured in standard measure is called the correlation coefficient because it measures the association or correlation between the variables. It is represented by the symbol r when it is calculated from a finite sample.

$$r = \frac{\Sigma (x - \bar{x})(y - \bar{y})}{\sqrt{\Sigma (x - \bar{x})^2 \cdot \Sigma (y - \bar{y})^2}}$$

If there is a reason to believe that there is some association between two variables, the correlation coefficient is a good measure of that association, however, it is important not to assume that a high value for r must mean that the variables are directly related. Three situations can result in a high value for r, i.e.

1) the variables are related

2) each variable may be directly related to a common third variable

3) the correlation is due merely to chance.

Since it is possible by chance to obtain a high value for r when in fact there is no real correlation between the two variables, it is necessary to test the probability of obtaining an observed value of r in these conditions. The statistic t can be calculated from the correlation coefficient by means of the equation

$$t = r \sqrt{\frac{n - 2}{1 - r^2}}$$ and the probability of exceeding a particular value of t by chance in sampling can be determined by entering the table of t under n - 2 degrees of freedom.

Examination of the results in Table 1 reveals that several different human sera with a titre of 128 by the IFA test gave results ranging from 10 (serum No. 11/2524) to $\geq$ 1280(12/0393) by ELISA when the commercial CF antigen was used to sensitize the plates.

Similarly in Table 2, sera with a titre of 16 by IFA gave results ranging from 2 (serum No. 12/2538) to $\geq$ 128 (11/2953) with the ELISA test.

Further experiments were designed to investigate these discrepancies.

Well coating

(a) Half the total number of wells in a fresh Dynatech microtiter plate (uncoated) were inoculated with 0.1 ml aliquots of doubling dilutions of human antisera prepared from 1/2 through 1/256 in PBS - Tween pH 7.4. After incubation at room temperature for 2 hours the sera were discarded and the plate was given 3 x 5 minute washes with PBS - Tween as previously described.

0.1 ml aliquots of working strength alkaline phosphatase conjugate prepared in PBS -Tween pH 7.4 were added to all wells, incubated for 3 hours at room temperature, discarded and the plate washed as before. 0.1 ml aliquots of thymolphthalein monophosphoric acid substrate were then incubated in all the wells of the plate for 18 hours at room temperature. 50μl of 3M NaOH terminator were added to every well to stop and develop any enzyme reaction.

Result: No blue colour developed in any of the wells indicating that neither the serum IgG nor the conjugate were binding non-specifically to the wells of the plate.

(b) Since horse radish peroxidase conjugate at a dilution of 1 : 500 in PBS - Tween pH 7.4 had been found to absorb readily to uncoated microtiter plate wells in previous experiments, this reagent was used to investigate the ability of commercial CF antigen diluted in coating buffer to sensitize the wells of microtiter plates.

If sensitization of microtiter plate wells is achieved using commercial CF antigen prepared in coating buffer, the inside surface of the plastic wells should be coated with constituents of the CF antigen solution and no uncoated plastic should be available to absorb the peroxidase conjugate. On the other hand, if coating of the wells is incomplete using the CF antigen, then peroxidase conjugate could absorb to the remaining uncoated areas in the wells.

Procedure: 2 rows of wells of another fresh Dynatech microtiter plate were inoculated with 0.15 ml aliquots of either HSV 1 or ¢B$_1$ CF antigen prepared as a 1 : 100 dilution in coating buffer pH 9.6. After incubation at 4°C for 18 hours, the reagents were discarded and the plate was washed as usual with PBS - Tween.

0.1 ml aliquots of 1 : 500 dilution horse radish peroxidase conjugated goat anti-human IgG prepared in PBS - Tween were added to all the wells in the plate and incubated at room temperature for 3 hours.

The conjugate was discarded and the plate was washed as before.

0.1 ml aliquots of 5-aminosalicylic acid substrate was added and incubated at room temperature for 1 hour in a darkened room before the addition of 50μl of 3M NaOH to each well.

Result: Only the wells which had not been treated with CF antigen produced a "positive" substrate reaction.

Conclusion: The wells of Dynatech microtiter plates are adequately coated by some components of the commercial CF antigen solutions during the sensitization procedure.

(c) The enzyme alkaline phosphatase has been shown to exist in small quantities in normal serum.

To investigate whether the level of endogenous serum alkaline phosphatase might produce a detectable colour change in the thymolphthalein monophosphoric acid substrate, dilutions of serum No. 12/0393 from 1 : 2 to 1 : 256 were prepared in PBS - Tween and incubated as 0.1 ml aliquots with 0.1 ml aliquots of thymolphthalein monophosphoric acid substrate pH 9.8 for 18 hours at room temperature in a fresh microtiter plate.

Result: After addition of 50µl aliquots of 3 M NaOH, no colour developed in the substrate indicating that even at concentrations far in excess of those likely to be encountered during a conventional ELISA test, the amount of endogenous alkaline phosphatase present in normal serum was not sufficient to produce a demonstrable colour change in the substrate.

These findings indicated that the complete assay system, the coating Ag preparation, antiserum, conjugate and substrate had to be present before positive reactions were produced. The poor correlation of the ELISA titres with the IFA assay results must therefore be caused by the CF antigen preparation causing non-specific reactions.

Commercially prepared CF antigens were therefore not suitable for use in the ELISA technique, probably by virtue of impurities and so a procedure for preparing higher quality antigens for use in ELISA had to be investigated.

It was decided to concentrate on HSV 1 which can be grown easily and rapidly in several types of tissue culture cell monolayers.

Purification of the HSV 1 antigen was attempted using the following techniques:-

1) differential centrifugation

2) density gradient centrifugation.

Experiment 3 Differential centrifugation

Theory

The separation of soluble tissue cell fluid and maintenance medium constituents from particulate matter such as cell membranes, cell nuclei, mitochondria, ribosomes and infecting micro-organisms such as HSV 1, can be achieved by differential centrifugation.

The various components of tissue cells have different mass to volume ratios and therefore possess different densities. Heavier material will sediment under low gravitational forces (e.g. 500 g produced by low centrifuge speeds) while lighter particles will require higher gravitational forces (e.g. 100,000 g).

Consequently, efficient fractionation of the various cell components can be achieved by starting with a low gravitational force and performing a series of separate and successive centrifugations towards the higher forces.

Procedure

(a) Propagation of HSV 1

An 8 oz Medical flat bottle containing approximately a 40 sq cm confluent monolayer of Vero tissue culture cells and 10 ml of Vero maintenance medium, was inoculated with 2 ml of undiluted Herpes simplex type 1 virus. (This inoculum consisted of Vero tissue culture cells previously infected with HSV 1 and subsequently lysed by freezing and thawing 3 times, suspended in Vero cell maintenance medium. This stock virus preparation had been stored as 1 ml aliquots in liquid nitrogen until required for use).

After incubation at 37°C for 18 hours, approximately 80% of the Vero cells showed 4 + CPE. Many had detached. The remaining cells were scraped from the side of the bottle using a glass rod until all the Vero cells were observed by light microscopy to be floating in the maintenance medium.

(b) Purification of HSV 1

The virus/cell suspension was frozen and thawed 3 times to lyse the remaining intact Vero cells and to free aggregated HSV 1. The virus/cell suspension was then transferred to two 5 ml capacity centrifuge tubes and centrifuged at 500 g for 5 minutes to deposit Vero cell components.

The virus containing supernatant fluid was transferred to two fresh 5 ml capacity centrifuge tubes and subjected to ultracentrifugation at 100,000 g for 1 hour to sediment the remaining suspended material.

The supernatant fluid, now containing only the soluble components of the tissue culture cells and cell maintenance medium, was discarded and the pellets resuspended in 100µl of ELISA coating buffer pH 9.6. Sonication in a Dawes sonicator unit type 6441A was required to resuspend the aggregated material in the coating buffer.

Another 8 oz Medical flat bottle containing a 40 sq cm confluent uninfected monolayer of Vero cells and 10 ml of Vero maintenance medium was subjected to precisely the same differential centrifugation procedure as detailed above, for use as a control preparation in the ELISA technique.

Both antigen preparations were stored at -20°C until required for use in the ELISA procedure.

Note: On removal from cold storage, the virus was de-aggregated in a Dawes sonication unit type 6441A for 3 minutes.

(c) ELISA procedure

5 of the human antisera, previously employed in Experiment 2 were used to investigate the performance in the ELISA of the HSV 1 antigen purified by differential centrifugation.

A fresh Dynatech microtiter plate was coated with HSV 1 antigen and the control Vero cell preparations as follows:-

column 1 was not used (to facilitate blanking of the unreacted substrate at the end of the assay in the newly acquired ELISA plate reader) (See Section VII).

The wells in column 2 through 6 were sensitized with 0.15 ml aliquots of an arbitrary 1 : 100 dilution of Vero tissue culture partially purified HSV 1 antigen prepared in coating buffer pH 9.6.

The wells in columns 7 through 11 were sensitized with 0.15 ml aliquots of a 1 : 100 dilution of the Vero cell control preparation, again in coating buffer pH 9.6.

After incubation at 4°C for 18 hours, the coating fluids were discarded and the plate was washed with PBS-Tween as detailed in Experiment 1.

The 5 human antisera from Experiment 2, chosen to provide a range of HSV 1 IgG titres (IFA) were diluted in PBS-Tween pH 7.4 from 64 to 2048 and inoculated as 0.1 ml aliquots into 2 columns of wells; one column coated with the HSV 1 antigen and the other coated with the control preparation from uninfected Vero cells.

After incubation at room temperature for 2 hours, the sera were discarded, the plate was washed as before and the remainder of the assay performed as detailed in experiment 1.

Results (see Table 3 and Figures 1, 2, 3, 4 and 5).

Note: Results were now determined spectrophotometrically at 619 nm by a Multiskan ELISA reader. End points of the serum serial dilutions were taken as the last dilution to yield a >0.1 absorbance unit difference between the readings obtained with the HSV 1 antigen and corresponding uninfected control Vero antigen coated wells.

The specially prepared HSV 1 antigen yielded anti-HSV 1 IgG titres from the human sera under investigation in approximately the same pattern as those obtained in the IFA test -

Serum No. 11/2177 gave a relatively high antibody level (128) by IFA and a high titre with ELISA (1024) whereas serum No. 11/1986

producing an IFA titre of < 16 also yielded a low titre (< 64) by ELISA.

Conclusions

Partial purification of the HSV 1 antigen by differential centrifugation produced a more satisfactory correlation between ELISA and IFA than commercial complement fixing antigen.

The levels of antibody detected, however, were only marginally higher than those achieved by IFA testing, whereas ELISA has generally been quoted as being of greater sensitivity (Engvall, et. al. 1976).

To investigate the reason for this discrepancy it was decided to further purify the HSV 1 antigen by density gradient centrifugation (Gilman, et. al. 1977).

Experiment 4 HSV 1 Purification by 30% Sucrose

The purification procedure described by Gilman and Docherty (1977) was adopted for the preparation of highly purified HSV 1.

Theory: In equilibrium density gradient centrifugation, a centrifuge tube filled with a dense solution of either caesium chloride or sucrose has the material to be purified layered on top. During centrifugation a linear density gradient will be self-generating (low density at the top of the tube to high density at the bottom). The various components of the virus sample will sediment to a density value equal to their own densities. The purified components can then be recovered by either puncturing the bottom of the tube and collecting the contents as drips or after freezing by slicing the frozen tube and contents in small sections.

To avoid the need for further purification of the viral antigen from the sucrose, a density of sucrose can be chosen which will be lower than the density of the virions. In this case the virions will pass through the entire gradient and collect as a pellet. Any lighter density material will be retained in the sucrose.

(a) Propagation of HSV 1

The HSV 1 was propagated in a fresh 8 oz medical flat bottle containing a confluent monolayer of Vero cells as detailed in Experiment 3, section (a).

(b) Purification of HSV 1

The Vero cell suspension was subjected to 100,000 g for 1 hour to sediment the Vero cell and HSV 1 virions liberated from the cells during development of CPE. The supernatant Vero cell maintenance medium was discarded, the pellets resuspended in 2 ml phosphate buffered saline, sonicated at 20 Kc/sec for 20 seconds at 4°C to disintegrate any intact Vero cells and deaggregate the virions.

The resulting suspension containing lysed cell fragments and free viral antigens was layered in 0.8 ml aliquots onto each of 3 x 4.0 ml, 30% sucrose (W/V) preparations and centrifuged in a "swing out" rotor for 3 hours at 100,000 g.

The resulting purified HSV 1-containing pellets were resuspended in 100 μl of coating buffer pH 9.6 and sonicated to deaggregate viral antigens in the coating buffer.

Uninfected Vero tissue culture cells were treated in an identical manner for the preparation of control Vero antigenic material.

Both antigen preparations were stored at -20°C.

Note: These antigenic preparations were sonicated for 3 minutes as described in Experiment 3, section (B), before being used to sensitize microtitre plate wells.

The purified HSV 1 antigen was then used in a "checkerboard" titration according to the procedure described by Voller, Bidwell, Huldt & Engvall (1974) to ascertain the optimal dilution of antigen for use in the ELISA technique.

Checkerboard titration

High and low titred HSV 1 antisera, as determined by IFA, were used in the checkerboard titration (i.e. Serum No. 12/1578) found to be 512 by IFA and Serum No. 12/0679 found to be 2 by IFA). A series of arbitrary antigen dilutions (1 : 100, 1 : 250, 1 : 500, 1 : 750 and 1 : 1000) in coating buffer pH 9.6 were used in 0.15 ml aliquots to sensitize the top three wells of columns 2 through 6 and 7 through 11 in a fresh Dynatech microtiter plate. After overnight incubation at 4°C, the coating solutions were discarded and the plate was washed with PBS-Tween as described in Experiment 1.

The high and low titred sera were diluted 1 : 1000 in PBS-Tween. The high titred serum was inoculated in 0.1 ml aliquots to the top three wells of columns 2 through 6 and the low titred serum was inoculated in 0.1 ml aliquots to the top three wells of columns 7 through 11.

The plate was incubated at 37°C for 2 hours (Ambient temperature incubation was discontinued in accordance with the findings of Gilman, et. al. (1977) See Section V.)

The sera were discarded after incubation and the plate was washed with PBS-Tween as before.

0.1 ml aliquots of a 1 : 500 dilution of alkaline phosphatase conjugated goat anti-human IgG prepared in PBS-Tween were inoculated into the top three wells of columns 2 through 11 and incubated at 37°C for 2 hours.

The conjugate was then discarded and the plate was washed with PBS-Tween.

0.1 ml aliquots of 1 mg/ml thymolphthalein monophosphoric acid in 10% diethanolamine buffer pH 9.8, were added to every well in the plate and left overnight at room temperature. The reaction was stopped and the colour developed by the addition of 0.05 ml 3 M NaOH to each well.

Results See Table 4, Figure 11

The highest dilution of antigen to produce a strong reading i.e. approximately 0.5 absorbance units in the Multiskan plate reader at 619 nm with the high titred serum and a low reading i.e. approximately 0.05 absorbance units with the low titred serum was used in all subsequent tests i.e. 1 : 750.

Conclusions

The dilution of antigen chosen for use in subsequent ELISA tests for antibodies to HSV 1 was 1 : 750. Since the uninfected control antigen had undergone the same purification procedure, it was also used at a dilution of 1 : 750.

Experiment 5

The HSV 1 antigen and control uninfected Vero cell preparation purified by 30% sucrose were diluted 1 : 750 in ELISA coating buffer pH 9.6 and used in 0.15 ml aliquots to sensitize the wells of a fresh microtiter plate. Even numbered column wells (but not column 12) were coated with HSV 1 Ag and odd numbered column wells (but not column 1) were coated with the control Vero cell preparation. After overnight incubation at 4°C, the coating solutions were discarded and the plate was washed as usual with PBS-Tween.

The same sera as used in Experiment 3 were employed as a range of dilutions in PBS-Tween from 500 to 64,000 applied in 0.1 ml aliquots to both HSV 1 antigen and control antigen coated wells and incubated at 37°C for 2 hours.

After disposal of unbound antisera and washing of the plate with PBS-Tween, 0.1 ml aliquots of a 1 : 500 dilution of alkaline phosphatase conjugate were added to each well of columns 2 through 11 and incubated at 37°C for 2 hours. The plate was rewashed and 0.1 ml aliquots of thymolphthalein monophosphoric acid in 10% diethanolamine buffer pH 9.8 were added to each well of the plate and incubated overnight at room temperature. The reaction was stopped and developed by the addition of 0.05 ml, 3 M NaOH to each well.

Results See Table 5, Figures 6 to 10.

The end points of the serial serum dilutions in the ELISA tests were again taken as the last dilution to yield a > 0.1 absorbance unit difference between the readings obtained with the HSV 1 antigen and corresponding uninfected control Vero antigen coated wells.

Significantly higher titres of serum antibodies to HSV 1 were detected using the 30% sucrose purified viral preparation - results analogous to the range of HSV 1 antibody titres detected by Gilman, et. al. (1977), were obtained.

The ELISA and IFA titres of the 5 sera were also found to be in close agreement with each other. High titres by IFA produced high titres by ELISA. Similarly serum No. 11/1986 produced low results in both procedures.

Conclusion

Purification of the HSV 1 antigen by 30% sucrose had resulted in a much more sensitive assay procedure. It was concluded, therefore, that removal of contaminating proteins, etc. from the viral preparation was essential for the production of a suitable ELISA antigen.

General Considerations

II Choice of solid phase support

For a successful ELISA by the indirect method, a suitable solid carrier surface must be found to which the specific viral antigen will bind. Many proteins bind in a non-specific manner to plastic surfaces. This caused considerable difficulty in the early days of bio-engineering, where plastics were being investigated for use in making artifical heart valves, etc. Serum proteins tended to bind to the surfaces of these plastic components which were in contact with recipient's blood. This phenomenon caused a protein build up and produced an irregular surface on the plastic which initiated the blood clotting mechanism. Such plastics are now treated with quaternary

ammonium groups and heparin to prevent blood clotting and protein binding to the surface of the plastic (Leininger, Cooper, Falb & Grade, 1966).

The observation that proteins could bind to plastics was first used constructively by Catt and Tregear (1967), in a radioimmunoassay of human placental lactogen and human growth hormone. Since then, plastic tubes, plastic beads and plastic microtiter plates have all been investigated for use in both radioimmunoassay and ELISA.

Plastic beads require less reagents than tubes but the surfaces of the beads are prone to damage, especially during the washing procedure where addition of the wash fluid can cause bumping of the beads against the sides of their containers. Scratch damaged beads interfere with antigen coating and potentially introduce erroneous results in antibody titrations. The third type of solid phase support is the microtiter plate which was chosen for use in this project for the following reasons:-

a) ease of handling (each plate contains 96 separate tiny wells)

b) ease of reading results by using an automated plate reader

c) requirement for minute volumes of reagents, allowing considerable economy.

Not every type of microtiter plate is suitable for use in ELISA, however. Some plates are coated with a minute film of machine oil. This usually originates from the template used in the moulding process during manufacture (W.H. Stimson - Personal Communication). This film of oil coats the surface of the plastic and effectively prevents the antigen from binding to the plastic surface. For this reason, rigorous quality control measures are applied by the manufacturer to each batch of

plates designated for use in micro-ELISA.

A specific type of plastic (called Immulon) has also been chosen by Dynatech Laboratories Ltd. as being the best polystyrene derivative for use in the ELISA technique. Such plates were used in this project. Plates catalogued as Cooke Microtiter M129A were obtained from Dynatech Laboratories.

One disadvantage of using coated plastic, however, is that the antigen is only physically absorbed by electrostatic attraction and not covalently bound to the molecules comprising the solid phase surface. Since absorbed material is in equilibrium with soluble material during the assay reactions, the immune adsorbent potentially leaks into the assay solutions during incubations and washings. This potential leakage has been shown to result in a slight lowering of precision of the assay (Engvall, Jonsson & Perlmann, 1971) but such a disadvantage is compensated by the easy and rapid washing procedures which can subsequently be employed (Engvall & Carlsson, 1976).

III Conjugates and substrates

Although many investigators prepare their own enzyme-labelled conjugates for use in ELISA, this can be a difficult, time consuming procedure which demands a considerable amount of expertise and so before contemplating such an undertaking, two commercially available conjugates were purchased and examined for their suitability in ELISA. These conjugates were found to be adequate for use in this research project and preparation of an enzyme-labelled conjugate was not necessary.

Experience gained from initial ELISA experiments employing two types of anti-human IgG enzyme-linked conjugates and various corresponding substrates can be summarized as follows:-

A. Horse-radish peroxidase conjugated goat anti-human IgG. (Nordic Immunological Laboratories).

Peroxidase decomposes hydrogen peroxide (H2O2). The oxygen liberated in this process can then oxidise a hydrogen donor (DH_2) to a coloured compound (D).

i.e. $H_2O_2 + DH_2 \xrightarrow{\text{Peroxidase}} 2\,H_2O + D$ (Bernt & Bergmeyer, 1965).

Addition of a strong acid, e.g. 2 N $H_2\,SO_4$, after a specified period of time, inhibits further enzyme activity. The following substrates were examined for their suitability as hydrogen donors.

(1) 5-aminosalicylic acid + hydrogen peroxide (BDH Chemicals Ltd.). A positive reaction is indicated by a colour change varying from yellow through brown which can be detected visually and measured spectrophotometrically at 449 nm. The substrate is affected by light, however, and is very easily oxidised. Reaction conditions have to be strictly controlled. A further disadvantage lies in

the fact that its colour development is difficult to stop. Bullock & Walls (1977) tested a variety of terminators, i.e. 0.5 N, 1.0 N and 2.0 N Na OH, 10% formalin, 2% Na N_3, 3% H_2O_2 and absolute alcohol, but none of them markedly decreased the colour change. They concluded that this colour development was due to spontaneous alteration of the substrate and not due to continued enzyme activity.

(2) 3,3' diaminobenzidine tetrachloride + hydrogen peroxide (BDH Chemicals Ltd.).

Originally devised for use in histology for peroxidase staining (Avrameas & Ternynck, 1971).

Produces a light to dark brown colour change and a dark brown precipitate. Not suitable for use in ELISA as it is extremely susceptible to oxidation.

(3) o-phenylenediamine hydrochloride + hydrogen peroxide.

By far the best substrate tested for use with peroxidase conjugates because it produces a clearly observable pale yellow to dark orange colour change in a positive reaction. The colour can be read visually or spectrophotometrically at 492 nm.

All manipulations of o-phenylenediamine should be performed in subdued light, including the final reaction incubation period.

This substrate was found to be admirably suited for use in ELISA. It is supplied in tablet form by Organon Laboratories in their "Hepanostika" micro-ELISA kit for Hepatitis B surface antigen detection but the substrate is not available separately from Organon.

O-phenylenediamine is available from BDH Chemicals Ltd., but is difficult to dissolve and, like all the substrates mentioned above, is carcinogenic.

(4) 2,2'-Azino-di-(3-ethyl benzthioline sulphonic acid).

This is a new non-carcinogenic substrate for use with peroxidase conjugates which has recently (1978) been introduced by Sigma Chemical Company Ltd.

It produces a green colour in the presence of H_2O_2 to indicate a positive reaction. It is also suitable either for visual determination or can be measured spectrophotometrically at 650 nm.

B. Alkaline phosphatase conjugated goat anti-human IgG.

(Miles-Yeda Ltd.)

Alkaline phosphatase catalyses the hydrolytic cleavage of phosphoric acid esters (Linhardt & Walter, 1965).

e.g. $R - O - PO_3H_2 + H_2O \xrightarrow{\text{Phosphatase}} H_3PO_4 + R - OH$

substrate Phosphate Residue

Suitable substrates for use in ELISA are those whose phosphate free residues are coloured at alkaline pH.

After a predetermined incubation period, therefore, the assay mixture is rendered strongly alkaline with 3 M Na OH. The colour is formed and the enzyme is completely inhibited by the alkali.

(1) Thymolphthalein monophosphoric acid (Disodium salt) (Sigma Chemical Company Ltd.).

This substrate is initially golden-yellow in colour but when the enzyme reaction is finally stopped with strong alkali, the unreacted substrate turns colourless and positive results are indicated by the development of a dark blue colour.

The clear to dark blue range of colour development makes it an excellent substrate for use when results are to be determined by visual observation alone.

For quantitative results, spectrophotometric analysis should be performed at 619 nm.

Its only disadvantage is its requirement of a long-conjugate/substrate incubation time (18 hours).

(Note: The magnesium salt of thymolphthalein monophosphoric acid is difficult to dissolve and is therefore not as suitable as the disodium salt).

(2) p-Nitrophenyl phosphate (Disodium salt).

(Sigma Chemical Company Ltd.)

This is the most widely employed substrate for use with alkaline phosphatase conjugates. The optimum reaction time is very short (20-60 minutes) and positive reactions give a lemon coloured product from the original clear substrate.

Although results are perhaps not as easily read visually as those of thymolphthalein monophosphoric acid (a situation confirmed by Bullock, et. al. 1977) it is reliably measured spectrophotometrically at 405 nm.

Conclusion

Both enzyme-labelled conjugates (i.e. Horse radish peroxidase and alkaline phosphatase) are equally suited for use in ELISA for IgG determination.

(It should be noted, however, that although peroxidase labelled conjugates to many animal and human immunoglobulins are readily available commercially, the range of commercially produced

alkaline phosphatase labelled conjugates is relatively limited).

O-phenylenediamine is the substrate of choice for use with peroxidase conjugates, as it lends itself to both visual and spectroscopic determination, but its carcinogenic nature must be recognised as a disadvantage.

Thymolphthalein monophosphoric acid is the ideal substrate for alkaline phosphatase labelled conjugates when lack of a spectrophotometer or plate reader necessitates visual read out. A disadvantage lies in the fact that the assay requires an overnight incubation period.

The most suitable combination of conjugate and substrate for use in ELISA was found to be alkaline phosphatase conjugated goat anti-human IgG and p-Nitrophenyl phosphate substrate. This allows a short assay procedure (approximately 5 hours) and is easily determined colorimetrically.

IV Washing procedures

With a heterogeneous enzyme assay where the enzyme label is determined in the solid phase (such as the indirect ELISA technique) the procedure for washing the solid phase support must be given considerable attention. Incomplete removal of unbound components of the reaction system will seriously affect the accuracy and precision of the assay (Schuurs, et. al., 1977). Following the original procedure of Engvall, et. al. (1971) most investigators now wash the support 3 times with phosphate buffered saline containing a wetting agent such as 0.05% polysorbate 20 (Tween 20) or PBS-Tween. This solution is also used

for dilution of test sera and conjugate in the course of an ELISA reaction to minimise non-specific binding of proteins to the solid phase surface.

The greatest care must be taken during all washing procedures to ensure that the reactants on the solid phase carrier are not scraped off by dispensers which might come into contact with the surface of the solid phase support. Equipment is now available commercially which can both introduce and aspirate the wash solution either manually or automatically.

A small manually operated microtitre plate washer was evaluated for use in ELISA (Dynatech AM 50 Microwash) but it was found that much of the wash fluid was spilled over the plate during the washes, resulting in contamination of neighbouring wells.

With horse radish peroxidase conjugates, in particular, such cross contamination from one well to another during the washing procedure could easily introduce false positive results to negative wells in a microtitre plate.

More elaborate (and expensive) automatic plate washers are now available from Dynatech Laboratories Ltd. and Organon Teknicka Ltd. which have the advantage of containing the aspirated fluid in a waste container, the contents of which can later be disposed of safely, but these machines are still at the development stage. The following procedure was therefore adopted for use in the project.

After the appropriate incubation periods, the contents of the wells were shaken out of the plate into a powerful oxidising agent solution (Chloros). This procedure was performed in a safety cabinet

fitted with an extractor fan to avoid releasing aerosols of infective material into the atmosphere of the laboratory. The wells were then inoculated with 0.3 ml of PBS-Tween from an 8-channel pipette (Titertek Multichannel Pipette, Flow Laboratories).

After incubation of the wash fluid at room temperature for 5 minutes, the contents of the wells were again shaken out and the procedure above was repeated twice. After the third, 5 minute incubation period, the wells were shaken dry prior to inoculation with the next reactant in the test.

No false positive or negative results have yet been encountered which could be attributed to this washing protocol.

V Optimal - Temperature of antigen-antibody reactions

From the introduction of the ELISA technique, 3 temperatures have been employed for serum and conjugate incubations. Voller, et. al. (1976) suggested room temperature ($16^{o}C$) incubation (serum for 2 hours and conjugate for 3 hours) but problems in the reproducibility of results were encountered which could be attributed to the wide degree of fluctuation inherent in the choice of ambient temperature.

Engvall, et. al. (1972) favoured $25^{o}C$ as incubation temperature but 4 - 5 hours of incubation were required for the optimal binding of both antiserum and conjugate antibodies. When washing and substrate reaction times were taken into account it meant that in practice, their assay procedure took 24 hours to complete.

Gilman, et. al. (1977) studied the problem in detail and have shown that, for Herpes Simplex types 1 and 2, the specific activity of

ELISA is affected by the temperature of both the serum and conjugate incubations (see Figure 14).

Replicate assays of a high dilution of a strongly "positive" antiserum to HSV were incubated at various temperatures during both the serum and conjugate incubation periods. After a 30 minute reaction with substrate at room temperature, the virus-specific activities were determined for each temperature. The specific activity was found to increase with increasing temperature to a maximum at 37°C. Low levels of activity at low incubation temperatures were attributed to incomplete binding of available antibody. The decrease in activity at higher temperatures were considered to be due to a combination of factors which include temperature denaturation effects, possible dissociation of the enzyme-antiglobulin complex and probable release of viral antigens from the solid phase support at these elevated temperatures.

Since these observations were thought to be related to the antigen-antibody reaction, they can also probably be applied to ELISA techniques in general.

A temperature of 37°C (2 hours) was adopted for routine incubation of both serum and conjugate in all the ELISA experiments performed in the course of this project.

VI Determination of Results

Where a "yes/no" result only is required, visual assessment of ELISA tests may be adequate. Semi-quantitative visual readings

can be achieved by testing a series of dilutions of the test sample and establishing the end point as the dilution which produces no discernable substrate colour. The reliability of this procedure depends, of course, on the substrate used (see Section III) and on the visual acuity of the operator.

Where more precise readings are required, it is necessary to measure degradation of the substrate in a spectrophotometer. However, there is no generally accepted way of expressing ELISA results and a few possible methods are listed below.

a) A range of absorbance values given by the sera of a known normal uninfected population is determined. The mean absorbance reading is accepted as the negative value. Further unknown sera are then classified as positive if above and negative if equal to or below this value (Voller, et. al., 1976).

b) The ratio of the optical density (O.D.) of the test sample to a group of known reference negatives is determined (Wolters, et. al. 1976).

c) The actual concentration of the substance being determined is obtained by referring to a standard curve prepared by plotting O.D. values of a series of reference samples containing different amounts of the substance (Stimson, et. al. 1974).

d) All unknown sera can be titrated by serial dilution. An arbitrary absorbance value is then chosen and the dilution of serum yielding such an absorbance value is the "titre".

For the purposes of this project, a slight modification of this last procedure was adopted - mainly following the method described by Gilman, et. al. (1977), as follows:-

Serial dilutions of unknown antisera were prepared in PBS-Tween. The titre was recorded as the reciprocal of the highest dilution resulting in an absorbance of ≥ 0.2 absorbance units above that achieved by the same dilution of serum when processed in an identical manner but using uninfected tissue culture cells.

VII ELISA plate reader Evaluation

Two different procedures for the quantitative reading of ELISA results have been developed:

(a) The first procedure involved aspirating the substrate, at the end of the reaction, from the solid phase support and passing it through a conventional spectrophotometer calibrated to the appropriate wavelength. The advantage of this system lies in the fact that readings are made in the spectrophotometer's own optically perfect cuvettes. It is, however, a lengthy procedure and errors may be introduced from deterioration of the substrate with time, from inaccurate pipetting and from a variety of other reasons.

Automatic aspiration type ELISA readers are available commercially (Dynatech Ltd.) and these greatly increase the speed with which readings can be performed (approximately 15 minutes for a 96 well microtiter plate) but the use of the same feed lines and cuvettes within the spectrophotometer, for all the ELISA samples, means that there is a danger of cross contamination between samples.

(b) The other automated method for determining ELISA absorbance values in microtiter plates uses a specially designed spectrophotometer with a vertical light path. Readings can therefore be determined with the substrate remaining in the wells of the reaction plate. The equipment evaluated was a titertek Multiskan plate reader developed in Finland for Flow Laboratories Ltd., Scotland. It was designed for use with ELISA in microtiter plates and consists of an 8-channel photometer which simultaneously measures the absorbances of the contents of eight wells (1 column) of a 96 well (12 column)microtiter plate

The readings are printed onto paper tape in the form of absorbance units to 3 decimal places. All the Multiskan results quoted in this thesis are therefore in the form of absorbance units.

This system has 3 main advantages over the aspiration type system.

1) It reads a 96 well microtitre plate in 60 seconds.
2) Errors are unlikely to be introduced by substrate deterioration.
3) As the contents of the wells are not aspirated through feed lines, there is no danger of cross-contamination between samples (e.g. fluid remaining in the tube of a spectrophotometer).

Objections have been raised, however, to the fact that the readings are determined through the base of the plastic plate, which is not optically perfect.

It is claimed, however, that the instrument design overcomes the potential optical inferiority of the plastic.

Before this equipment was accepted for routine use in ELISA, its accuracy and reliability was investigated in the following series of experiments.

A. Performance of the Multiskan reader

The Multiskan reader was "blanked" and run without a plate in position. The readings produced were therefore a measure of the inherent variations in the machine itself.

Results : Table 8

The values obtained ranged from 0.002 to 9.999. When this procedure was repeated, the results shown in Table 9 were obtained. These ranged from 0.002 to 9.998. (Absorbance readings which are "negative" relative to the blank 0.000 are recorded in descending numerical value from 9.999).

Comparison of the two sets of figures revealed that the discrepancies above and below what was believed to be the true value of 0.000 occurred randomly. The inherent error of the Multiskan reader itself was therefore $\pm$ 0.002.

B. Performance with Dynatech microtiter plates (empty)

An empty Dynatech microtiter plate was placed in the machine and "blanked" by reading O.D. from the first empty column. The absorbance values from the remainder of the columns were then measured.

Results : Table 10

In this experiment the largest random variation observed, attributable to the plate construction and not to dirt, was ± 0.011 in column 7 row 7. The large absorbance reading in column 4 row 6 (0.035) was found to be due to an opaque area of plastic in the base of this well.

When these results are compared with those from Experiment A above, it is obvious that the use of the Dynatech plates increased the inherent Multiskan error of ± 0.002 to ± 0.011.

Conclusion

Reading results to 2 decimal places must be regarded as having an error of at least ± 0.01 absorbance units when Dynatech microtiter plates are used. The microtiter plates must also be carefully examined before use to ensure that no well bases are seriously damaged.

C. Performance of Multiskan with plate and substrate

When any liquid is added to the plate wells, another potential variable is introduced to the spectrophotometric system because when a solution of substrate is placed in the path of a beam of light (of appropriate wavelength) the intensity of the light after passing through the solution (I) will depend upon the intensity of the light after passing through an equivalent column of solvent (Io), the thickness of the column of solution (l) and the concentration of the substrate in mg/ml (c) according to the Beer-Lambert Law.

$$\log_{10} \frac{Io}{I} = k\ c\ l$$ (Kabat and Mayer, 1964)

where k is a constant called the specific extinction.

Since the pathlength (l) affects the absorbance reading, the amount of liquid present in the well of a microtiter plate is a critical factor in the production of reliable results. The accuracy of pipetting of reagents will, therefore, be of prime importance to ensure that the volume of liquid present in all the wells prior to photometric evaluation is the same.

This basic assumption was proved by the following experiment.

A 0.005% solution of naphthalein black dye was prepared in distilled water. The first column of wells in a microtiter plate was left empty so as not to introduce a pathlength error into the blank wells (other than the slight discrepancies arising from the variations in thickness of the plastic base). 100μl of naphthalein black solution from a calibrated Finnpipette (Jencons Scientific Instruments Ltd.) were introduced into each well of column 2. 150μl were added to the wells of column 3 and in column 4, 50μl to 100μl of naphthalein black solution were added in 10μl increments from row 1 to row 6 respectively.

The O.D. of the plates were then read three times in the Multiskan plate reader at 619 nm.

Results : Table 11

Discrepancies of up to 0.03 absorbance units were recorded on reading the 100μl aliquots of naphthalein black solution (column 2) at 619 nm. This discrepancy increased to 0.05 absorbance units when the larger volumes of 150μl (column 3)

were read in the Multiskan reader.

Replicate readings (I, II and III) of the 100μl and 150μl results produced only small variations, the maximum being 0.01 absorbance units.

From the results in column 4, the 10μl differences in volumes produced a mean increase of 0.105 absorbance units.

Conclusions

The amount of liquid present in the microtiter plate wells is a critical factor in the overall accuracy of the absorbance reading system.

Accurate pipetting of reagents is critical to minimise discrepancies. In view of this, the accuracy of two different types of pipette was assessed for possible use in the ELISA system.

D. A Comparison of the performance of the two types of adjustable pipette

Column 1 of a fresh microtiter plate was left empty to allow for "blanking" of the Multiskan unit. Columns 2, 3 and 4 were inoculated with 150μl of a fresh % solution of naphthalein black dispensed by a Finnpipette. Columns 5, 6 and 7 were inoculated with 150μl of the same solution dispensed by a Pipetman pipette manufactured by Gilson in France.

The plate was then read as before at 619 nm in the Multiskan plate reader.

Results Table 12

With the Finnpipette a mean discrepancy of 0.042 absorbance units was produced. The Pipetman instrument produced a mean discrepancy of 0.043 absorbance units. Both types of

adjustable pipette are therefore equally suitable for use in the ELISA test.

Conclusions

The Multiskan ELISA plate reader is a very reliable instrument, being accurate to within $\pm$ 0.002 absorbance units. However, due to inherent manufacturing discrepancies in Dynatech plates and in the adjustable dispensers, the accuracy of the instrument is reduced.

From the results of Experiment D it was concluded that a reasonable safety margin for an ELISA system would allow for variation by $\pm$ 0.05 absorbance units. Since such a discrepancy might theoretically overlap the 0.1 absorbance unit difference between positive and control wells previously used in recording ELISA titres, the absorbance unit value of the serum end point titre was increased to 0.2 absorbance units in all subsequent experiments detailed in the main discussion section of this thesis.

VIII Summary of the ELISA test adopted for all subsequent experiments

1. The even numbered column wells (except column 12) of a Dynatech microtiter plate (M 29 AR) were "sensitized" by inoculating them with 150μl of an "optimal" checkerboard predetermined dilution of antigen in coating buffer pH 9.6. A similar dilution of uninfected tissue culture control antigen was used in 150μl aliquots to coat the odd numbered column wells in the same plate. Column 1, however, was left empty to facilitate "blanking" of the substrate at the end of the assay. The only reagents to be added to the wells of column 1 were the substrate and its

terminator. The plate was incubated at 4°C for 18 hours.

2. The sensitizing solutions were then carefully discarded into Chloros in a safety hood and all the wells were given 3 x 5 minute washes with 300µl of PBS-Tween p H 7.4.

   The plate was then shaken dry.

3. Suitable dilutions of unknown human sera prepared in PBS-Tween p H 7.4 were inoculated in 100µl aliquots to appropriate wells of the microtiter plate and incubated for 2 hours at 37°C.

   Note: The use of 150µl of coating antigen and subsequent use of 100µl of reagents is a safeguard to prevent non-specific binding of the antiserum and conjugate to uncoated plastic in the wells.

4. The antiserum dilutions were discarded into Chloros and the plate was washed as before.

5. 100µl aliquots of a 1 : 500 dilution of alkaline phosphatase conjugated Goat anti-human IgG prepared in PBS-Tween were introduced into the wells of the plate and incubated at 37°C for 2 hours.

6. The conjugate was discarded to Chloros and the plate was washed as before.

7. 100µl of a 1 mg/ml solution of p-nitrophenylphosphate in 10% diethanolamine buffer p H 9.8 was added to all the wells in the plate. The ensuing enzyme reaction was carefully timed and stopped by adding 50µl of 3 M Na OH after 30 minutes at room temperature.

   Note: The Na OH was added to the wells in the same sequence as the inoculation of substrate to allow replicate timing of the enzyme reaction in each well.

8. The plates were then read in the Multiskan plate reader using column 1 to "blank" the instrument.

9. The end point titres were recorded as the highest dilution of antiserum to yield a $\geqslant 0.2$ absorbance unit difference over the corresponding antiserum dilution in the control antigen well.

c) Blood stain elution

Introduction

Possible methods for elution of bloodstains from absorbent materials (filter paper discs and various types of fabric) was relevant to both sections of my work. For forensic purposes, blood on clothing must be solubilized before its origin can be determined. In the clinical situation, filter paper discs are frequently used for transport and storage of blood or serum specimens (Kalter, 1963; Wolff & Hudson, 1974).

The efficient recovery of immunoglobulins from bloodstains is, therefore, of great importance if reliable results are to be obtained in subsequent serological tests.

The performance of various solvents in extracting bloodstains for analysis by the indirect fluorescent antibody technique was investigated.

1. Estimation of bloodstain eluate concentration

The accurate measurement of the degree of dilution in the eluate derived from a bloodstain is important and therefore two procedures for the determination of blood concentration were evaluated.

A. The haemoglobin concentration of the blood stain eluate was measured by the cyanmethaemoglobin method of Drabkin and Austin (1932).

Procedure: A 1:300 dilution of whole blood or stain eluate was prepared in Aculute diluent (Ortho Diagnostics Ltd.). Aculute contains potassium cyanide which converts oxyhaemoglobin to cyanmethaemoglobin.

The pipette used was rinsed carefully with the diluent. (Note: Since the Aculute diluent pellet is originally dissolved in distilled water, whole erythrocytes lyse spontaneously). The solution of blood and diluent is mixed and left at room temperature for 10 minutes to permit the coloured compound, cyanmethaemoglobin to form. The colour of this solution is read at 540 nm (using Aculute diluent as the blank) to give an optical density reading which can be converted to the concentration of haemoglobin present (in g/100 ml) by reference to a standard curve. (Diagram 15).

Where the haemoglobin concentration of the original whole blood could be determined, the dilution factor of the resulting bloodstain eluate was obtained by calculating the haemoglobin percentage recovered from the stain with regard to the initial haemoglobin concentration of the sample.

Where the bloodstain eluate was of unknown origin, its dilution factor was estimated on the basis that normal adult blood has a mean haemoglobin concentration of 15.0 g/100 ml (Men 16.0 g/100 ml; women 14.0 g/100 ml) (Documenta Geigy, 1962).

This procedure was, obviously, only suitable for whole blood as stain eluate evaluation. In the clinical situation, involving serum samples on filter paper discs, the total protein concentration was investigated as a method of determining the serum eluate dilution factor.

B. Lowry Protein Estimation (Lowry, Rosebrough, Farr & Randall, 1951).

A 1:16 dilution of serum or serum eluate and a 1:32 dilution of whole blood or bloodstain eluate in distilled water was used as a sample for the automatic analysis of total protein concentration in a Technicon protein analyser (Lowry, 1970). This equipment automatically added the correct quantities of 2% $Na_2 CO_3$ in O.IN Na OH, 0.5% Cu $SO_4 . 5H_2O$ in 1% sodium tartrate and Folin-Ciocalteau reagent diluted 1:1 with distilled water, to the reaction mixture via a series of small catheters before finally reading the absorbance of the resulting solution at 750 nm.

The results were converted to total protein concentration (in g/100 ml) by reference to a standard curve (Diagram 16) Calculation of a dilution factor for samples of unknown origin were based on the fact that sera from normal, healthy adults had a mean total protein concentration of 6.55 g/100 ml, plasma had a mean total protein concentration of 6.95 g/100 ml and whole blood had a mean total protein concentration of 21.8 g/100 ml (Spector, 1956).

2. Correlation of protein and haemoglobin determination procedures in bloodstain recovery

A sample of whole blood was obtained from the Haematology Department and analysed with regard to its haemoglobin and protein concentration, as follows:-

16, 0.1 ml bloodstains were prepared on cotton cloth. 8 stains were stored at 4°C and 8 at room temperature (R.T.). After 1 day and 7 days, 4 stains from each temperature were recovered with PBS (see Section 4). The haemoglobin and protein concentrations of the eluted stains were measured (see Section 1). immediately after recovery and used to determine the percentage recovery of bloodstain from the cloth.

Results: Table 13

Mean bloodstain recovery rates of 41.2% and 47.7% were obtained by protein estimation and haemoglobin estimation procedures, respectively.

These figures indicated that the stains were being eluted at between $\frac{1}{3}$ and $\frac{1}{2}$ the concentrations of the original whole blood. Eluate antibody titres would, therefore, be expected to be decreased by 1 $\log_2$ dilution.

3. Elution procedures

Various extraction fluids have been used by different investigators in the course of their experiments. These fluids were therefore examined for their suitability in virus antibody profiling.

Randomly selected blood samples were obtained from the Haematology Departments of two local hospitals. These whole blood specimens were then examined by the indirect fluorescent antibody technique (IFA) for the presence of IgG to selected viral antigens. The "antibody profiles" of the specimens were therefore determined in terms of the antigens used. 0.1 ml bloodstains of specimens

showing antibody profiles containing high titres of IgG to one or more viruses were prepared on white cotton cloth and left at room temperature for 2 days.

The efficiency of the following elution fluids, for recovering antiviral IgG from the stains, was then examined:-

Weak solvents: a) 1 M phosphate buffered saline (PBS) pH 7.3

b) 25% kaolin in PBS

Strong solvents: a) 5% ammonia

b) 2-8 M urea in PBS

Enzyme solutions: a) 1 mg/ml subtilopeptidase in PBS

b) 0.025 - 0.1% trypsin in PBS.

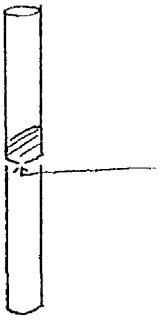
Any resulting antiviral IgG in the eluted stains was titrated by the IFA test and the dilution of the eluted blood was estimated by the cyanmethaemoglobin procedure.

To allow a comparison to be made of the various elution fluids, the PBS extraction procedure was regarded as being the standard procedure for use in the elutions of bloodstains.

4. Standard bloodstain elution procedure

The 0.1 ml dry bloodstains were cut out of the cloth. As much cloth as possible which had not been soaked with the blood was trimmed off. The bloodstained material was then placed in a small plastic test tube to which 0.15 ml of IM PBS pH 7.3 was added and left to soak for 2 hours at $4^{o}C$.

A small hole was then pierced in the base of the test tube and a second similar tube was taped underneath it (See diagram



This double extraction device devised by Baxter and Rees (1974) was then centrifuged at 600 g for 10 minutes to transfer the eluate from the upper to the lower tube. The resulting bloodstain extract was then ready for analysis by the IFA and cyanmethaemoglobin techniques.

Note: The eluate can also be removed from the cloth by placing the bloodstained material and 0.15 ml PBS in a disposable 5 ml syringe, using the plunger to squeeze the bloodstained material, This, however, requires considerable physical effort to achieve the maximum removal of the eluate.

5. Solvents

a) IM phosphate buffered saline, pH 7.3 is the normal solvent for bloodstains on various materials. It was first used in the context of antibody profiling with the fluorescent antibody test by King in 1974 who used 0.1 ml bloodstains eluted from white cotton cloth with 0.15 ml aliquots of PBS.

Antibody molecules are therefore eluted into a medium of suitable pH and osmotic pressure for use in the fluorescent antibody and enzyme-linked immunosorbent assay techniques.

b) Brody, McAllister, Haseley and Lee (1964) used a suspension of 25% kaolin in PBS to elute bloodstains from filter paper discs. After soaking the bloodstained disc overnight at 4°C, the tube was placed at room temperature for 20 minutes and shaken every 5 minutes. The disc was then squeezed against the side of the tube with a wooden applicator and discarded. The kaolin was removed by centrifugation for 20 minutes at 2,500 rpm and the supernatant used as the source of eluted bloodstain.

This procedure was very effective when applied in Brody, et.al.'s adenovirus complement fixation and measles haemagglutination-inhibition tests. When used in conjunction with the IFA test for antibody profiling, it was found to reduce the non-specific fluorescing property of the eluate and had the added advantage of providing some mechanical abrasion which helped to remove the bloodstain from the cloth. It was therefore a useful procedure for use with stains which had been stored for considerable lengths of time at room temperature. (Such stains were not efficiently recovered by PBS extraction).

c) Kind and Lang (1976) obtained good results in conventional bloodstain grouping techniques (e.g. ABO, rhesus systems) by extracting their bloodstains for 30 minutes with 5% v/v ammonia in deionised water.

When applied to the IFA technique, however, the resulting eluate was found to be unreadable.

d) MacConkey (Personal Communication) suggested using 8 M urea in PBS to remove blood from stains. Solutions of 2, 4 and 8 M urea in PBS at 4°C were used with bloodstains on cloth, but although an increase in haemoglobin recovery was detected, the eluates were unsuitable for IFA.

e) Two enzymic methods of digesting dried bloods were considered and examined.

The first involved the use of a protease called subtilopeptidase A (Sigma Chemicals Ltd.) as follows-

0.1 ml bloodstains dried on cloth were treated with 0.15 ml of a 1 mg/ml solution of subtilopeptidase A at 37°C following the usual procedure. The haemoglobin recovery from 2 day old stains and from stains 6-12 months old was marginally increased, but no IgG type antibody could be detected by IFA.

f) More promising results were obtained with weak solutions of trypsin in PBS at 37°C. Trypsin specifically hydrolyses peptides, amides and esters, but has only weak action on serum globulins and haemoglobin. Solutions of 0.025%, 0.05%, 0.075% and 0.1% trypsin in PBS were used to elute 2 day old bloodstains from cotton cloth following the standard procedure already described. The resulting haemoglobin concentrations of the bloodstain eluates were found to be greater than those from "normal" PBS elution. 0.025% and 0.05% trypsin solutions gave correspondingly higher antibody titres and a "cleaner" eluate.

for fluorescent microscopy. With the higher concentrations of trypsin (0.075% and 0.1%), antibody titres were found to decrease because of peptide cleavage.

Standard elution with 0.05% trypsin, therefore, was effective in increasing the IgG recovery and yielded an eluate which was suitable for the IFA technique. This procedure was subsequently employed in some of the later forensic work.

6. Elution volume

0.1 ml bloodstains were eluted with various volumes of PBS in the range 0.1 ml to 5.0 ml following the standard elution procedure. The resulting eluates were concentrated to 0.1 ml again using a polyacrylamide gel (lyphogel, Gelman Instrument Company). 1 gram of gel removed 5 ml of water and low molecular weight substances, peptides etc. but excluded proteins and other large molecules.

The larger extraction volumes gave a better recovery of haemoglobin from the stain but no significant improvement was found in the IgG titres.

7. Conditions of soaking for bloodstains

0.1 ml bloodstains were left to soak in 0.15 ml PBS for various times and temperatures. From experiments which involved soaking the stain for between 15 minutes and 24 hours, it was concluded that most elution occurred in the first 30 minutes and little benefit was gained by extending the time period beyond this. It was technically convenient, however, to soak the stains for 1 to 2 hours.

It was also found that the extraction procedure was not greatly influenced by temperature in the range 4°C to 37°C. Higher temperatures than 37°C are not recommended because protein may denature and become insoluble (Schleyer, 1962).

It was also important to wet the stain completely with the solvent. Prolonged agitation on a whirlimixer was required to wet stains left on cloth for more than 6 months.

Conclusions

For the recovery of bloodstains from cotton cloth, it was found that treatment 0.15 ml PBS or 25% kaolin in PBS at 4°C or 0.15 ml 0.05% trypsin in PBS at 37°C for 2 hours were suitable procedures for yielding eluates for use in the IFA technique.

It was also found that haemoglobin and IgG were extracted fairly easily from fresh stains using the three elution procedures outlined above whereas old stains proved to be more difficult in that IgG was always recovered less successfully than haemoglobin.

d) Comparative Serology

Introduction

Three viruses, namely: Herpes simplex virus (HSV 1), Coxsackie B_1 virus (¢ B_1) and Rotavirus, were chosen to investigate the comparative sensitivity and relevance of various serological techniques in detecting antiviral antibodies in human serum samples.

Experiment 1 Comparative Serology with Herpes simplex virus (Table 14)

45 randomly chosen adult sera were obtained from the Serology Department at Belvidere Hospital. Each serum was titrated for the presence of Herpes simplex IgG by the following techniques:-

a) Indirect fluorescent antibody test.

See Chapter 2, section a

b) Enzyme-linked immunosorbent assay.

See Chapter 2, section b

c) Neutralisation test.

See Appendix.

Experiment 2 Comparative Serology with ¢B_1 virus (Table 15)

30 sera of known IgG titre to ¢B_1 pre-determined by the indirect fluorescent antibody test (Chapter 2, section a) were obtained from the Serology Department of the hospital. The reciprocal anti-¢B_1 IgG titres determined by IFA ranged from <8 to 128. Each serum was then tested for anti-¢B_1 IgG by

a) Neutralisation test

See Appendix

b) Enzyme-linked immunosorbent assay

$\underline{\text{¢B}_1 \text{ ELISA procedure}}$

1. Propagation of ¢B_1 virus

3 x 8 oz 'medical flat' bottles, each containing a 40 sq. cm. confluent monolayer of Vero tissue culture cells and 10 ml Vero maintenance medium were inoculated with 2 ml aliquots of a 10^{-1} dilution of stock ¢B_1 virus. The cultures were incubated for 2 days at 37°C.

2. Purification of ¢B_1 virus

The ¢B_1 infected Vero cell suspension was subjected to 100,000 g for 1 hour to sediment the cells. The supernatant Vero cell maintenance medium was discarded, the pellets resuspended in 2 ml phosphate buffered saline and sonicated at 20 Kc/sec for 20 seconds at 4°C to disintegrate the Vero cells and disaggregate virions.

The resulting suspension containing cell fragments and free ¢B_1 virus was layered in 0.8 ml aliquots onto each of 3 x 4.0 ml, 30% (w/v) sucrose preparations and centrifuged in a "swing out" rotor for 3 hours at 100,000 g. The supernatant sucrose was discarded and the resulting purified ¢B_1, containing pellets were resuspended in 100 µl of coating buffer pH 9.6 and sonicated to disaggregate viral antigens in the coating buffer before being used in a checkerboard titration to determine the optimal dilution for use in ELISA.

3. Checkerboard titration

High and low titred CB_1 antisera, as determined by IFA, were used in the checkerboard titration (Serum No. 2/0145:64 by IFA; Serum No. 2/0619:< 8 by IFA).

A series of arbitrary antigen dilutions (1:100, 1:250, 1:500, 1:750 and 1:1000) in coating buffer pH 9.6 (see Appendix) were used in 0.15 ml aliquots to sensitize the top three wells of columns 2 through 6 and 7 through 11 in a fresh Dynatech microtitre plate. After overnight incubation at 4°C, the coating solutions were discarded and the plate was washed with PBS-Tween (see Appendix).

The high and low titred sera were diluted 1:500 in PBS-Tween. The high titred serum was inoculated in 0.1 ml aliquots to the top three wells of columns 2 through 6 and the low titred serum was inoculated in 0.1 ml aliquots to the top three wells of columns 7 through 11. The plate was then incubated at 37°C for 2 hours.

The sera were discarded after incubation and the plate was washed with PBS-Tween as before.

0.1 ml aliquots of a 1:500 dilution of alkaline phosphatase conjugated goat anti-human IgG prepared in PBS-Tween were inoculated into the top three wells of columns 2 through 11 and incubated at 37°C for 2 hours.

The conjugate was then discarded and the plate was washed with PBS-Tween.

0.1 ml aliquots of 1 mg/ml p-nitrophenylphosphate in 10% diethanolamine buffer pH 9.8 were added to every well in the plate and left overnight at room temperature. The reaction was

stopped by the addition of 0.05 ml 3M NaOH to each well.

Results. See Table 6 Figure 12

The highest dilution of antigen to produce a strong reading, i.e. approximately 0.5 absorbance units in the Multiskan plate reader at 405 nm with the high titred serum and a low reading, i.e. approximately 0.1 absorbance units with the low titred serum was used in all subsequent tests.

Conclusion

The dilution fulfilling these requirements was 1:750. Uninfected Vero tissue culture control antigen purified in an identical manner by centrifugation through 30% sucrose was also used at a dilution of 1:750.

The 30 sera were then investigated for anti-$\mathcal{C}B_1$ IgG by ELISA using dilutions in the range 1:500 through 1:64000 following the procedure described in Chapter 2, section b.

Experiment 3 Comparative Serology with Rotavirus (Table 16)

25 sera of known antibody titre to Rotavirus as determined by the revised complement fixation technique (Zissis, et. al. 1974) performed by Dr. G. Zissis in Brussels, were received from Belgium dried in 0.1 ml aliquots on filter paper discs.

The stains were eluted with 0.15 ml PBS by the standard procedure described in Chapter 2, section c. Analysis of the serum eluates for protein concentration (Chapter 2, section c) revealed that approximately 50% of the serum was being recovered from the filter paper.

The serum eluates were then investigated for anti-Rotavirus IgG by a) the indirect fluorescent antibody test

(Chapter 2, section a)

b) ELISA.

ELISA procedure

1) Propagation of Rotavirus

3 x 8 oz medical flat bottles, each containing a 40 sq. cm. confluent monolayer of primary calf kidney cells and 10 ml of maintenance medium were inoculated with 1 ml aliquots of stock Nebraska calf diarrhoea virus. The cultures were incubated at 37°C for 4 days by which time extensive CPE was observed in the monolayers.

2) Purification of Rotavirus

The Rotavirus infected primary calf kidney cell suspension was subjected to 100,000 g for 1 hour to sediment the calf kidney cells. The supernatant maintenance medium was discarded, the pellets resuspended in 2 ml phosphate buffered saline and sonicated at 20 Kc/sec for 20 seconds at 4°C to disintegrate any intact calf kidney cells and disaggregate the virions.

The resulting suspension containing lysed cell fragments and free viral antigens was layered in 0.8 ml aliquots onto each of 3 x 4.0 ml, 30% (w/v) sucrose preparations and centrifuged in a "swing out" rotor for 3 hours at 100,000 g.

The resulting purified Rotavirus-containing pellets were resuspended in 100 µl of coating buffer pH 9.6 and sonicated to disaggregate the viral antigens in the coating buffer before being used in a checkerboard titration to determine the optimal dilution for use in ELISA.

3) Checkerboard titration

High and low titred Rotavirus antisera, as determined by IFA were used in the checkerboard titration (Serum No. 4832:128, by IFA, and Serum No. 1187:<8 by IFA).

A series of arbitrary antigen dilutions (1:100, 1:250, 1:500, 1:750 and 1:1000) in coating buffer pH 9.6 were used in 0.15 ml aliquots to sensitize the top three wells of columns 2 through 6 and 7 through 11 in a fresh Dynatech microtitre plate. The remainder of the checkerboard titration was performed in an identical manner to that employed for the $\mathcal{C}B_1$ virus titration.

Results: See Table 7 Figure 13

The highest dilution of antigen to produce a strong reading, i.e. approximately 0.4 absorbance units in the Miltiskan plate reader at 405 nm with the high titred serum and a low reading, i.e. approximately 0.1 absorbance units with the low titred serum was used in all subsequent tests.

Conclusion

The dilution of antigen chosen for use in ELISA tests for estimating anti-Rotavirus IgG was 1:100. Uninfected primary calf kidney tissue culture control antigen purified by centrifugation through 30% sucrose was also used at a dilution of 1:100.

The 25 sera were then investigated for Rotavirus IgG by ELISA using dilutions in the range 1:32 through 1:4096. (Table 16)

e) Virus Antibody Profiling (VAP)

Introduction

At the beginning of this part of the investigation, 21 different viruses were propagated in appropriate tissue culture cells to prepare microscope slides for the indirect fluorescent antibody technique. Examination of random sera obtained from the Virus Laboratory at Belvidere Hospital, revealed that some of the chosen viruses would be easier to use than others. For example, the two parainfluenza viruses (PF1 and PF3), produced only "membrane" fluorescence under the conditions of incubation, time, etc. chosen. This appearance was difficult to quantitate reproducibly. Initial experiments, therefore, involved only 19 different viruses.

Experiment 1 Replicate Antibody Profiles (Tables17 through 33)

Before any detailed examination of virus antibody profiling could be undertaken, reproducibility had to be evaluated. It was decided to investigate this by repeatedly blind titrating different aliquots of several specimens to a total of 10 times.

Procedure: Fresh plasma from 5 different randomly selected donors was obtained from the Haematology Department of the hospital. Each specimen was distributed into 0.1 ml amounts and stored in small sterile glass vials at -20^{o}C until required for profiling. Usually 3 or 4 large profiles could be processed in a working day.

5 different whole oxalated blood samples were also obtained from the Haematology Department and similarly used to investigate the reproducibility of the indirect fluorescent antibody test on bloodstain eluates.

Procedure: 10 replicate 0.1 ml bloodstains were prepared on freshly laundered, white cotton cloth from each sample of whole blood. The stains were dried and stored for 4 days at room temperature before being extracted with 0.15 ml PBS in the standard elution procedure (Chapter II, Section c). The recovered bloodstain eluate (approximately 0.1 ml) was stored at -20°C until required for use in the virus antibody profile.

Experiment 2 Virus antibody profile survey (Tables 34 through 47)

Having established that the indirect fluorescent antibody technique could be relied upon repeatedly to detect the same levels of virus antibodies in identical serum or bloodstain eluate specimens, a series of randomly selected whole oxalated bloods from several different donors was obtained to determine whether the virus antibody profile could discriminate between individual bloods.

Procedure: 100 different randomly selected oxalated whole blood samples were frozen to -20°C. The resulting lysed blood specimens were then profiled against 19 viruses by the indirect fluorescent antibody technique.

Experiment 3 In vivo Stability of VAP (Tables 48 and 49)

For the successful matching of a bloodstain eluate with a blood specimen from a suspect, the virus antibody profile must be shown to be stable in vivo.

Procedure: 7 individuals were found from the hospital records to have had plasma samples examined on at least 2 occasions over various time intervals. All the plasma specimens had been stored at -20°C. Virus antibody profiles were performed on each pair of specimens to confirm the stability of the IgG antibodies in vivo, and to demonstrate that

current infection with one virus did not significantly alter the antibody status to different viruses.

Experiment 4 VAP stability in vitro (effect of temperature) (Tables 50a through 57)

In forensic work, bloodstains may lie undiscovered for a considerable length of time and may be exposed to extremes of temperature, humidity, etc. To examine the effects of potential adverse temperature storage, 8 different randomly chosen whole oxalated blood specimens (Haematology Department) were inoculated onto white cotton cloth in 0.1 ml aliquots and left for various lengths of time at $4^{o}C$ and room temperature before being eluted with 0.15 ml PBS. Virus antibody profiles were then performed on the eluates and the results compared with the original profile obtained on Day 1 from a sample of the same blood which had been lysed by freezing to $-20^{o}C$.

Experiment 5 VAP stability in vitro (effect of humidity) (Table 58)

Experiments were also conducted to investigate the stability of the IgG antibodies under simulated humid conditions.

Procedure: 0.1 ml bloodstains were prepared on white cotton cloth from a whole oxalated blood sample and placed in several small plastic boxes containing tissue paper each moistened with various quantities of water; 0.1 ml, 0.5 ml, 1 ml or 5 ml. The boxes were left for 7 days at room temperature during which time the water evaporating from the tissue created a humid atmosphere in the boxes. The different amounts of water on the tissues created differing degrees of humidity for different times, as may be judged by the following observation:-

0.1 ml water evaporated to dryness in approximately 1 day
0.5 ml water evaporated to dryness in approximately 4 days
1.0ml water maintained a damp atmosphere for 7 days
5.0 ml water maintained a very humid atmosphere for 7 days.

The stains were eluted with 0.15 ml PBS after 7 days and stored at -20°C until profiled (within 2 days). Some degree of fungal contamination was evident on the stains which had been exposed to the 1 ml and 5 ml atmospheres at room temperature, and as this is a very common experience in forensic laboratories where specimens are stored in plastic bags in humid conditions, the presence of fungus growth was carefully noted.

Experiment 6 VAP Blind Trials (Tables 59 through 67)

To investigate the usefulness of Virus Antibody Profiling in a forensic situation, 6 blind trials were conducted to investigate the capability of this technique to discriminate between bloodstains.

Procedure: Various whole oxalated blood samples were obtained from the Victoria Infirmary, Glasgow, Haematology Department. Part was inoculated onto clean white cotton cloth as 0.1 ml stains. After drying at room temperature, this material was presented to the investigator as coded stains (A, B, C, etc.) and the remaining original liquid blood samples were numbered 1, 2, 3, etc. The whole bloods were lysed by freezing to -20°C and stored at this temperature until required for use.

The stains in Blind Trials 1 through 5 were left at room temperature for 3 days before being extracted in 0.15 ml PBS by the standard elution procedure. In Blind Trial 6, the stains were removed, after storage for 3 days at room temperature, with 0.15 ml, 0.05% trypsin as detailed in Chapter 2, Section C, 5*f*). The eluates from all 6 trials were stored at -20°C until required for use.

Haemoglobin (Hb) concentrations were noted to provide an indication of the dilution of eluate recovered from the cloth. Blood group information was also made available for trials 2 through 6.

Experiment 7 VAP by ELISA (Table 68)

Immunofluorescence depends upon subjective visual assessment of the fluorescing image by microscopy. Since the IFA test does not readily lend itself to simple instrumental quantitation, the ELISA technique was evaluated as a possible test for producing an objective quantitative result.

An initial experiment was conducted to determine how ELISA would perform with lysed blood samples. Since it is probable that many proteins other than specific antibodies will adhere to sites on the solid phase surface not occupied by the coating antigen, the possibility exists that false positive or negative results will be obtained. In order to decrease such possible background staining, Ruitenberg, Steerenberg, Brosi and Buys (1976) incorporated 4% bovine serum albumin (BSA) into their 1:500 dilution of conjugate and found that background reactions were minimised. Gilman, et.al. (1977) included the addition of 4% BSA in their PBS-Tween diluent for serum and conjugate preparations.

Consequently an experiment was performed to investigate the ability of ELISA to detect anti-HSV 1 IgG antibody in 2 lysed bloods and 2 corresponding eluted blood stains of known HSV 1 antibody titre (as determined by IFA) and also observe the effect that 4% BSA had on the assay when incorporated into the PBS-Tween diluent solution.

Procedure: Two Dynatech microtitre plates were sensitized as follows.

The even numbered column wells (2, 4, 6 and 8) in each plate were inoculated with 0.15 ml of a 1:750 dilution of HSV 1 antigen

(purified by 30% sucrose). The odd numbered column wells (3, 5, 7 and 9) in each plate were inoculated with 0.15 ml of a 1:750 dilution of uninfected control Vero cells also subjected to the 30% sucrose purification procedure. After incubation at 4°C for 18 hours these solutions were discarded and the plates were given 3 x 5' washes with PBS-Tween.

Serial doubling dilutions of the four specimens under test were prepared in both PBS-Tween and PBS-Tween + 4% BSA from 1:500 through 1:64000 and inoculated as 0.1 ml aliquots into the appropriate wells of the plate. Each serial dilution was therefore incubated in wells sensitized by both HSV 1 antigen and a control uninfected Vero cell preparation.

After incubation of the plates at 37°C for 2 hours, the specimens were discarded and the plates were given 3 x 5' washes in PBS-Tween.

0.1 ml aliquots of a 1:500 dilution of alkaline phosphatase conjugated goat anti-human IgG prepared in PBS-Tween were inoculated into each well of the plates, except those in column 1, and the plate was incubated at 37°C for 2 hours.

The conjugate was then discarded and after carefully washing the plates as before, 0.1 ml aliquots of 1 mg/ml p-nitrophenyl-phosphate substrate in 10% diethanolamine buffer pH 9.8 were added to each well of the plate. After incubation at room temperature for 30 minutes, the reactions were stopped with 50μl aliquots of 3 M Na OH.

Experiment 8 VAP Blind Trial by ELISA (Table 69)

Duplicate 0.1 ml stains from Blind Trial No. 6 which had previously been performed by the IFA technique were used to investigate the suitability of ELISA for discriminating between bloodstains.

The use of 0.15 ml of 0.05% trypsin solution in PBS for the elution of one set of 10 bloodstains compared with 0.15 ml of PBS alone was also investigated in this experiment.

Procedure: 2 sets of 10 x 0.1 ml coded bloodstains on cloth were eluted by the standard elution procedure. One set of stains was eluted with 0.15 ml PBS and the other set with 0.15 ml of 0.05% trypsin in PBS. Both sets of stain eluates were used along with the original coded lysed bloods in a conventional ELISA test for detecting anti-HSV1 IgG antibodies and anti-$\not{C}B_1$ IgG antibodies.

Since low results had been obtained from the lysed blood samples used in the previous ELISA experiment, dilutions of lysed blood and stain eluate were prepared in PBS-Tween + 4% BSA from 1:125 through 1:16000.

ELISA Protocol

9 Dynatech microtitre plates were sensitized with HSV 1 antigen, $\not{C}B_1$ antigen and control uninfected Vero cell antigen (all purified by 30% sucrose). See diagram H.

Column 1 of every plate was left empty to facilitate "blanking" each plate in the Multiskan ELISA reader at the end of the experiment. 0.15 ml aliquots of a 1:750 dilution of HSV 1 antigen in coating buffer pH 9.6 were inoculated into each well of column 2 of the first plate and also into the wells of every third column from plate 1 to plate 9. Similarly 0.15 ml aliquots of a 1:750 dilution of $\not{C}B_1$ antigen in coating

buffer were inoculated into every well of column 3 of plate 1 and into every third column of wells in the plates. Finally, 0.15 ml aliquots of a 1:750 dilution of the control uninfected Vero cell preparation were inoculated into all the wells of column 4 and every third column of wells from plate 1 to 9.

After incubation at 4°C for 18 hours the sensitizing solutions were discarded and the plates were given 3 x 5' washes with PBS-Tween.

Dilutions of lysed blood, PBS extracted stains and trypsin extracted stains were prepared in PBS-Tween + 4% BSA from 1:125 through 1:16000 and inoculated in 0.1 ml aliquots to the appropriate wells as shown in diagram H.

The plates were then incubated at 37°C for 2 hours before the test solutions were discarded and the plates washed with PBS-Tween as before.

0.1 ml aliquots of a 1:500 dilution of alkaline phosphatase conjugated goat anti-human IgG prepared in PBS-Tween were inoculated into every well in each plate, except column 1, and incubated at 37°C for 2 hours. The conjugate solutions were then discarded and the plate was rewashed with PBS-Tween. 0.1 ml aliquots of 1 mg/ml p-nitrophenylphosphate substrate in 10% diethanolamine buffer pH 9.8 were added to each well of plates 1 to 9. After incubation at room temperature for 30 minutes, the reactions were stopped with 50μl aliquots of 3 M Na OH.

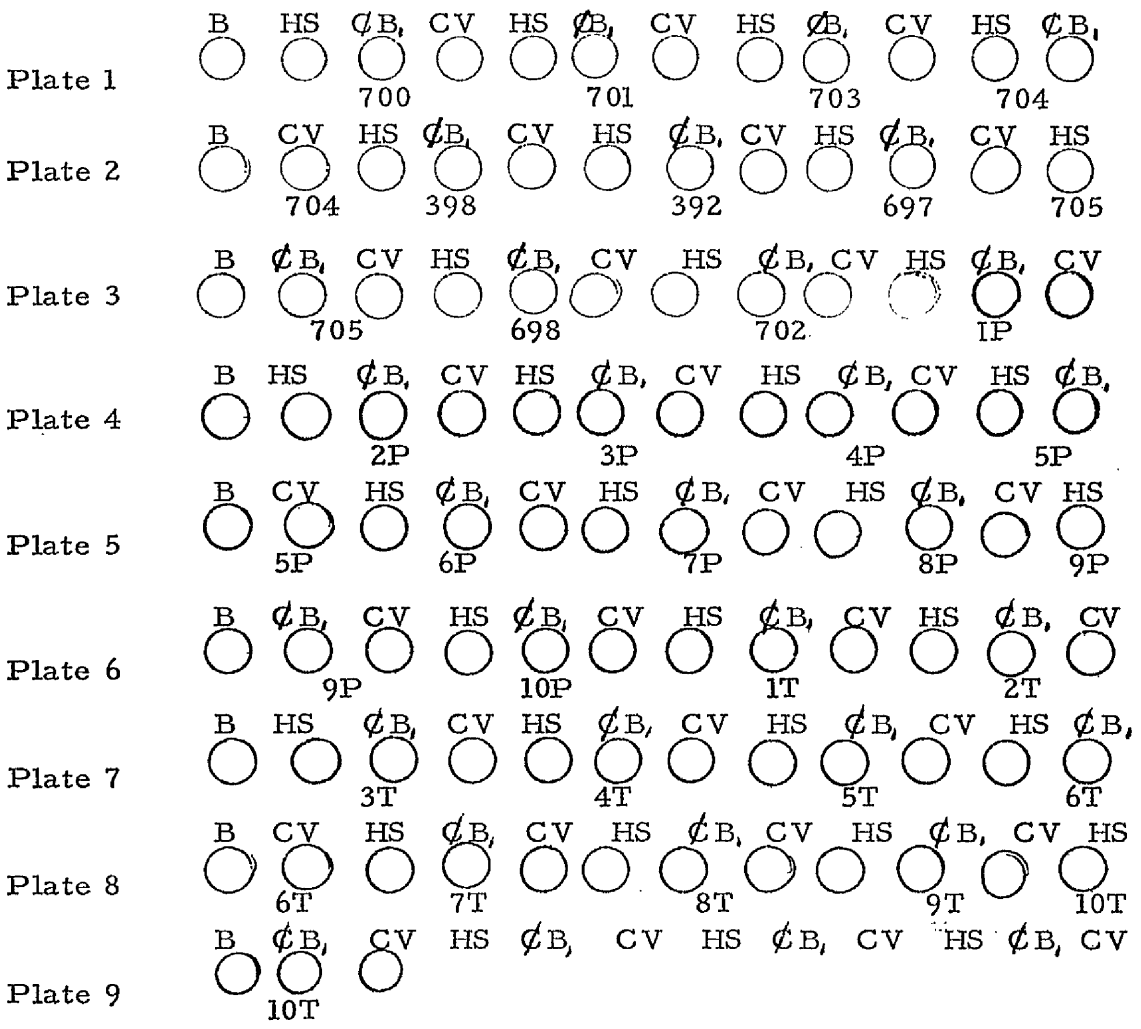


Diagram H Sequence of antigens in plates involved in Experiment 8.

B = Blank well

HS = Well coated with HSV1 antigen

$\not{C}B_1$ = Well coated with $\not{C}B_1$ antigen

CV = Well coated with control uninfected Vero cell antigen

700, 701, etc. = Coded lysed blood specimens

1P, 2P, etc. = Coded PSB extracted stains

1T, 2T, etc. = Coded trypsin extracted stains

CHAPTER III

EXPERIMENTAL DATA (2)

RESULTS

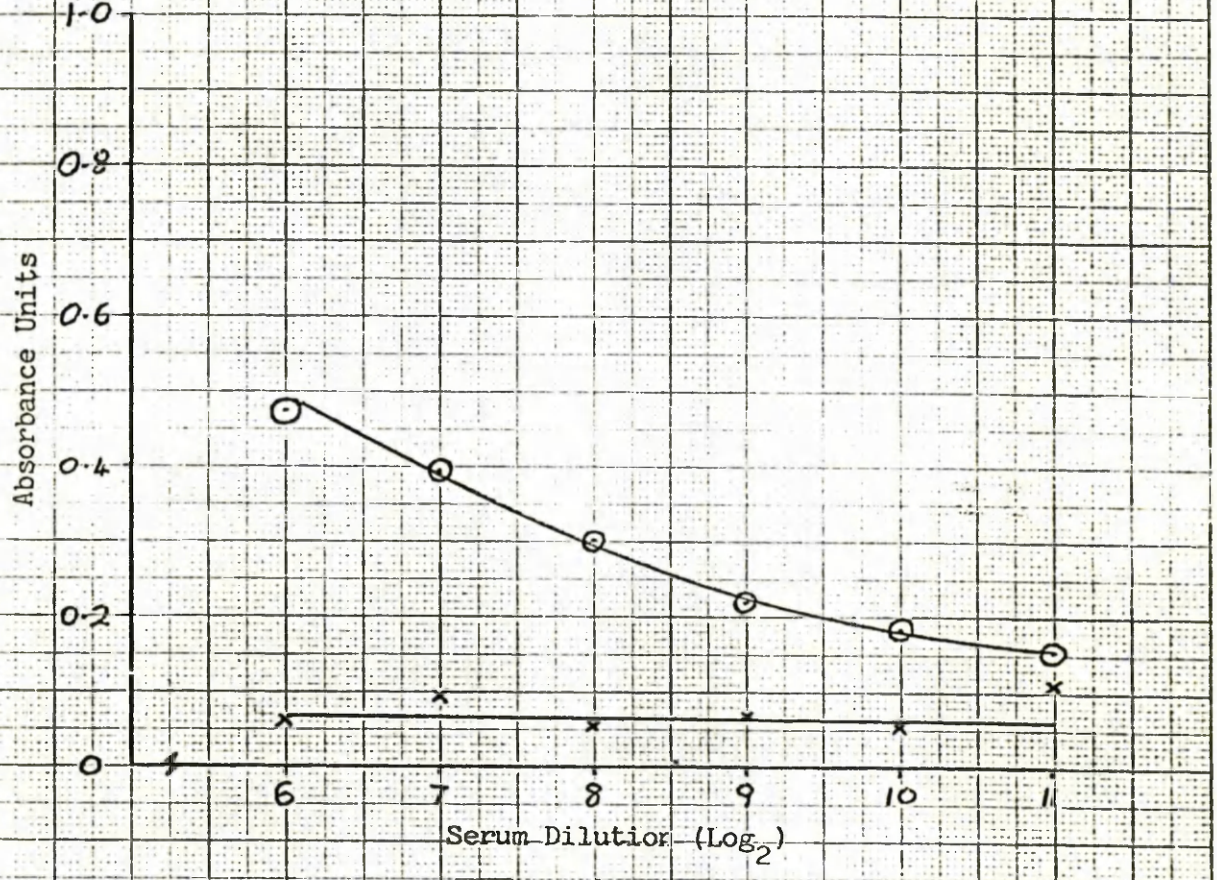


Figure 1 ELISA absorbance readings of Serum No. 11/2177 in HSV1 (Differential Centrifugation) Ag coated wells (⊙) and uninfected Vero cell control wells (x) showing the end point of the serum titration to be Log_2 10. (1:1024)

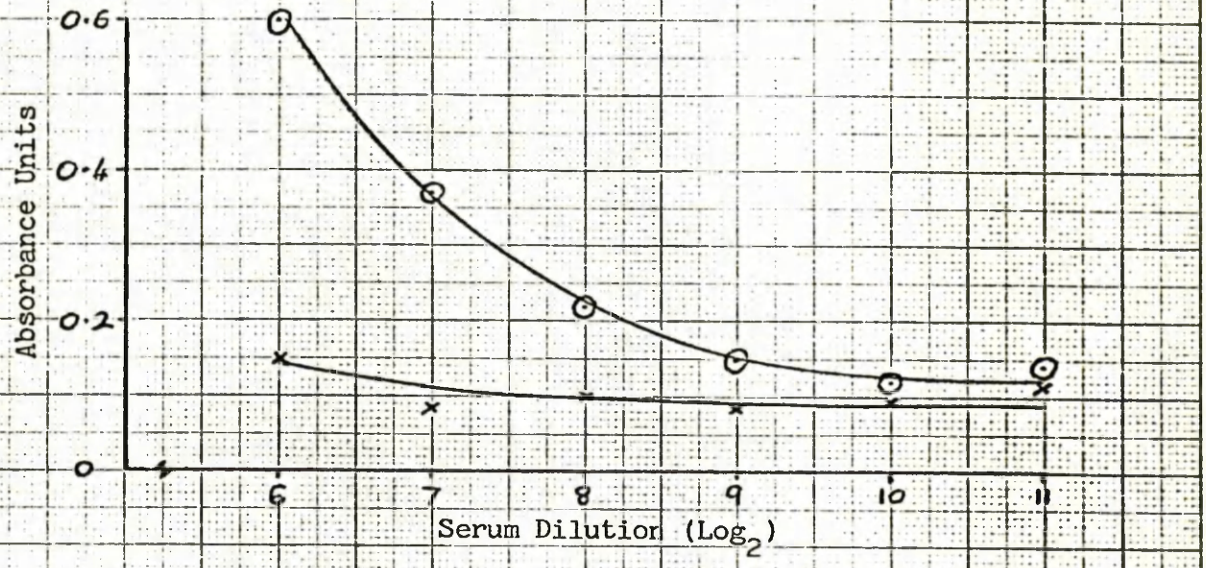


Figure 2 ELISA absorbance readings of Serum No. 11/2823 in HSV1 (Differential Centrifugation) Ag coated wells (⊙) and uninfected Vero cell control wells (×) showing the end point of the serum titration to be Log_2 8. (1:256)

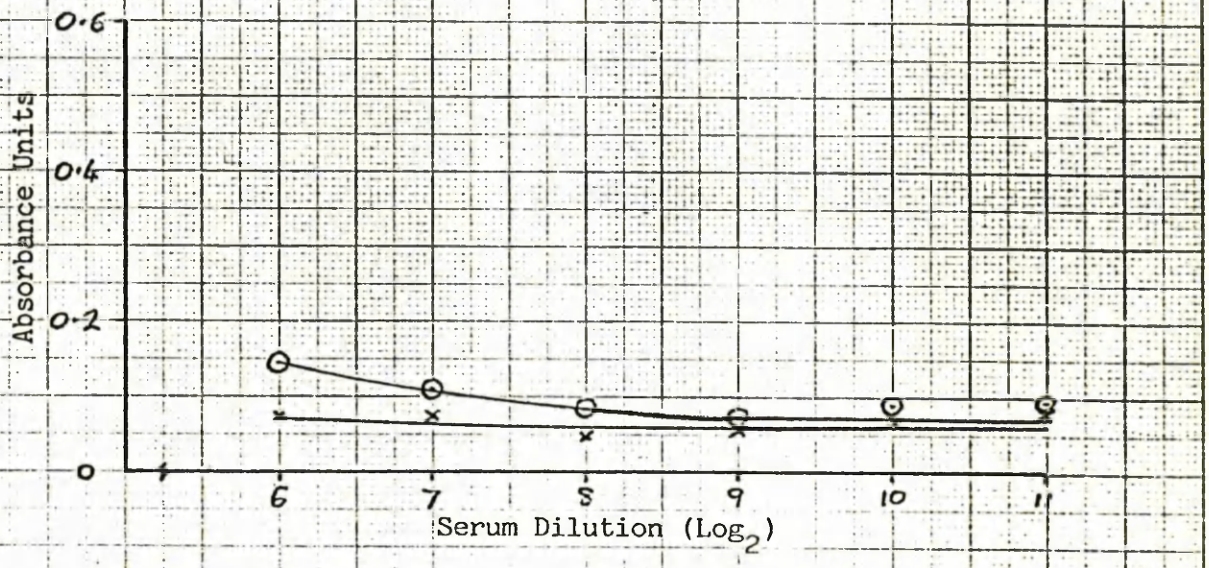


Figure 3 ELISA absorbance readings of Serum No. 11/1986 in HSV1 (Differential Centrifugation) Ag coated wells (o) and uninfected Vero cell control wells (×) showing the end point of the serum titration to be $<Log_2$ 6. ($<$1:64)

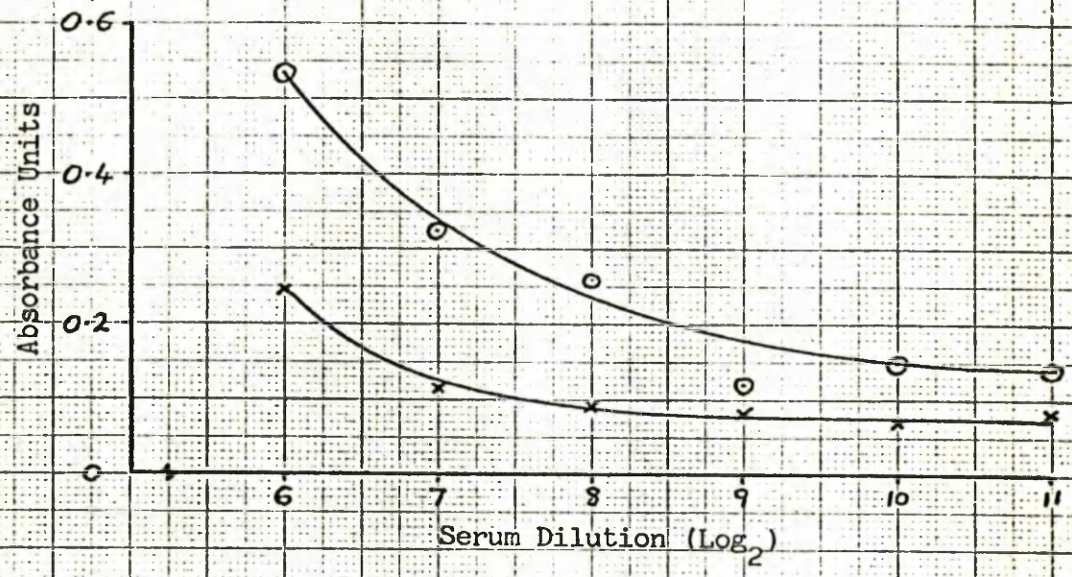


Figure 4 ELISA absorbance readings of Serum No. 11/2439 in HSV1 (Differential Centrifugation) Ag coated wells (o) and uninfected Vero cell control wells (x) showing the end point of the serum titration to be Log_2 8. (1:256)

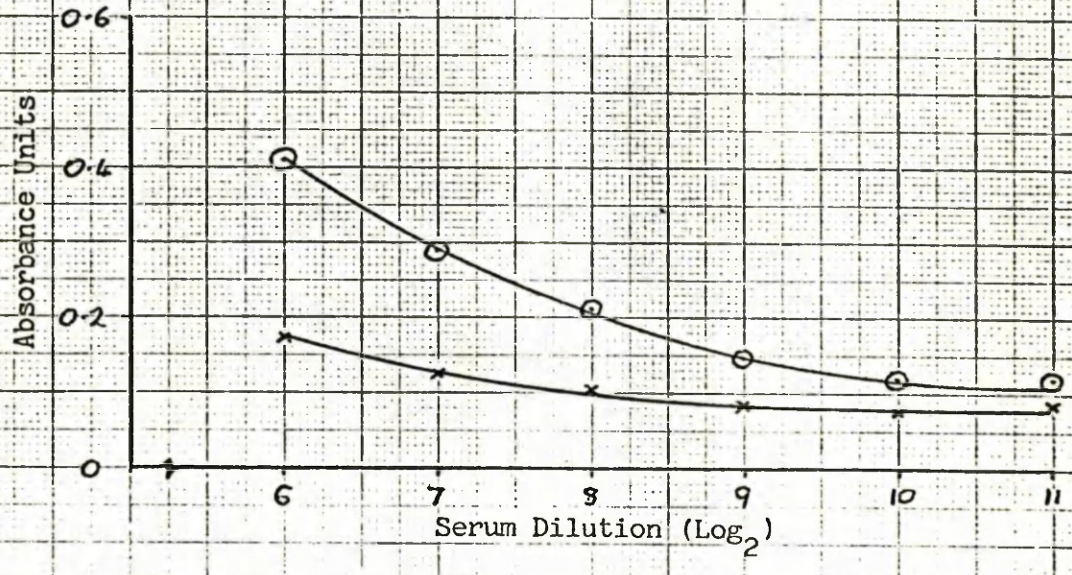


Figure 5 ELISA absorbance readings of Serum No. 11/1650 in HSV1 (Differential Centrifugation) Ag coated wells (o) and uninfected Vero cell control wells (x) showing the end point of the serum titration to be Log_2 8. (1:256)

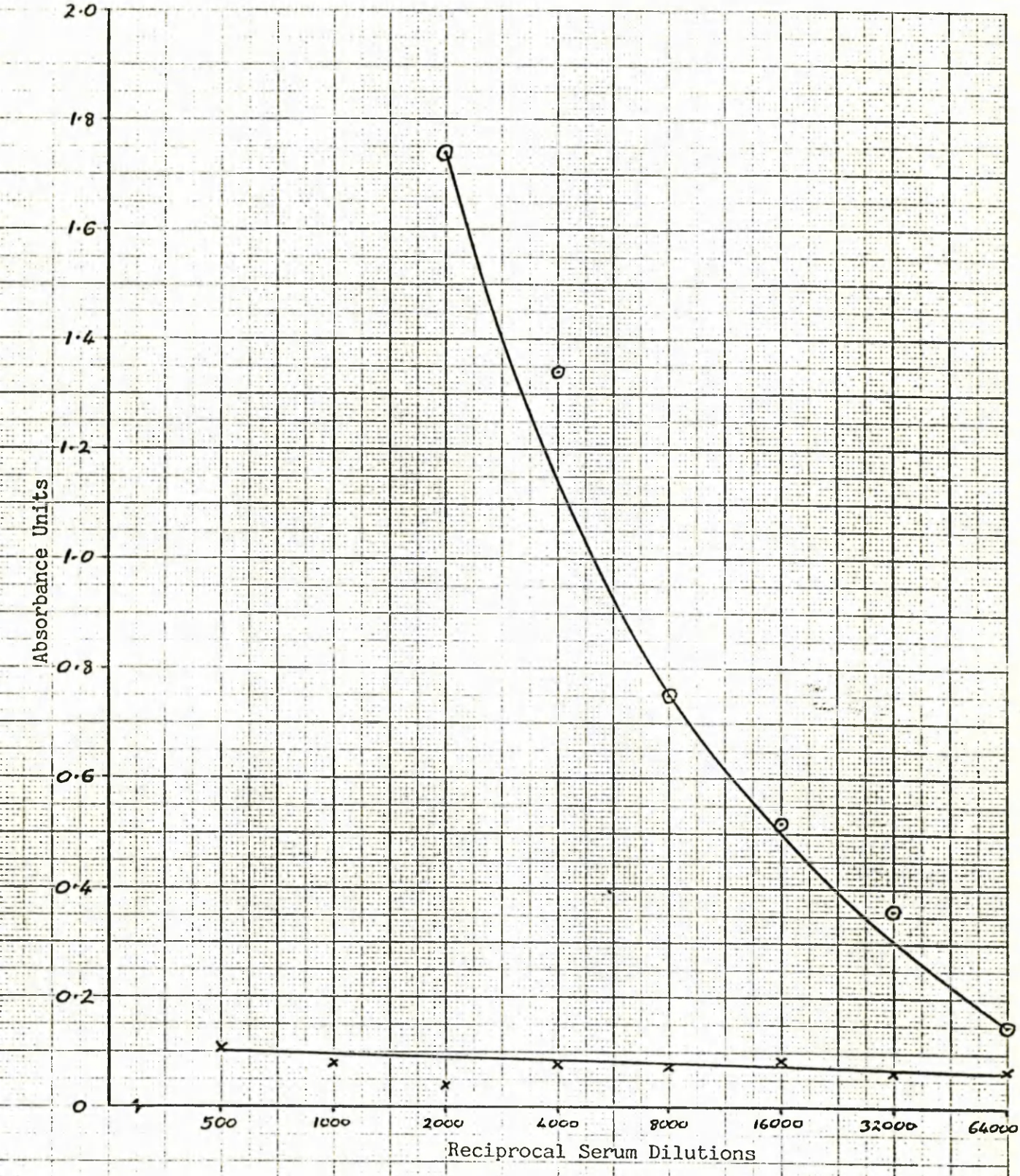


Figure 6 ELISA absorbance readings of Serum No. 11/2177 in HSV1 (30% Sucrose) Ag coated wells (⊙) and uninfected Vero cell control wells (×) showing the end point of the serum titration to be 1:32000.

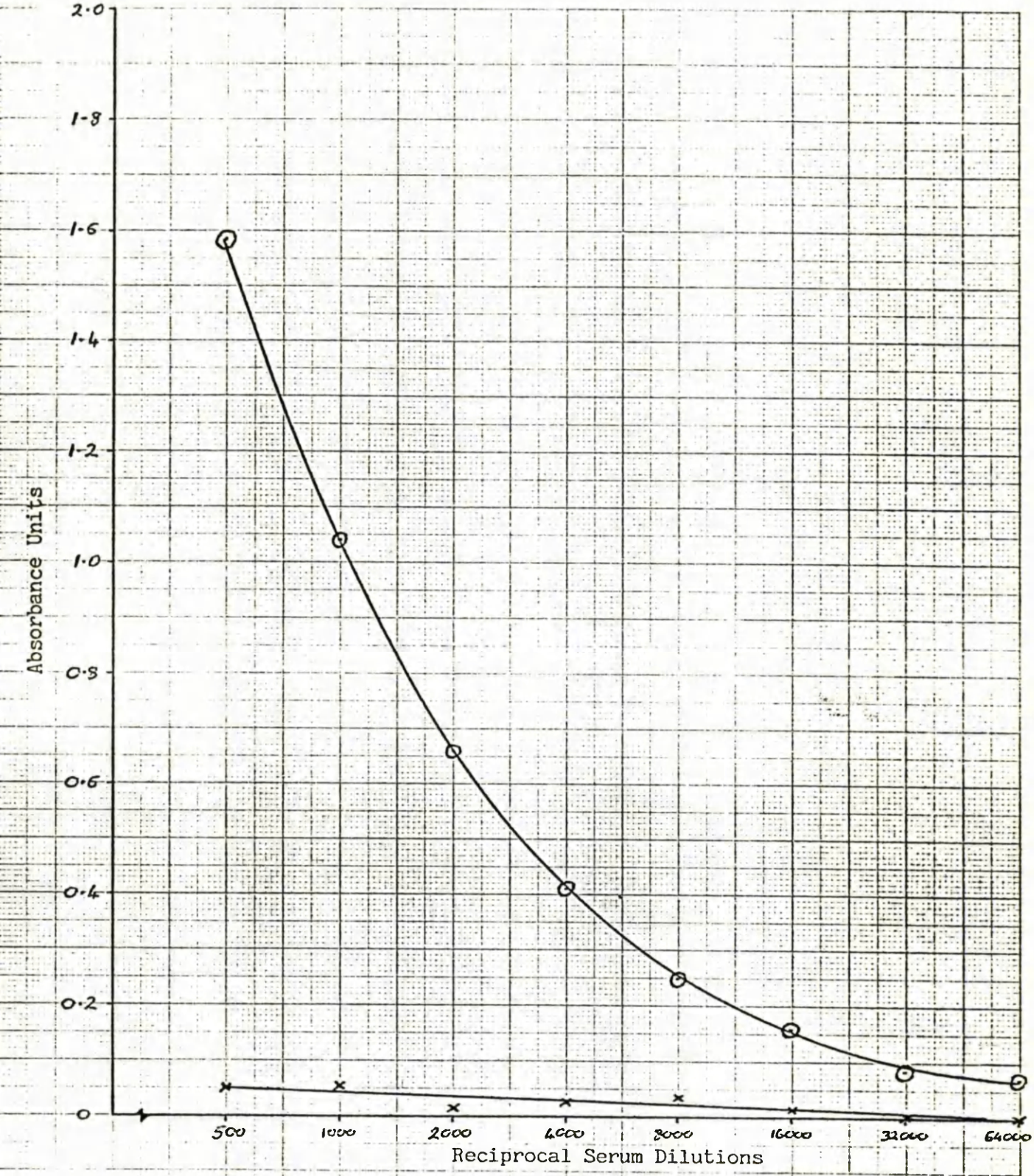


Figure 7 ELISA absorbance readings of Serum No. 11/2823 in HSV1 (30% Sucrose) Ag coated wells (o) and uninfected Vero cell control wells (x) showing the end point of the serum titration to be 1:16000.

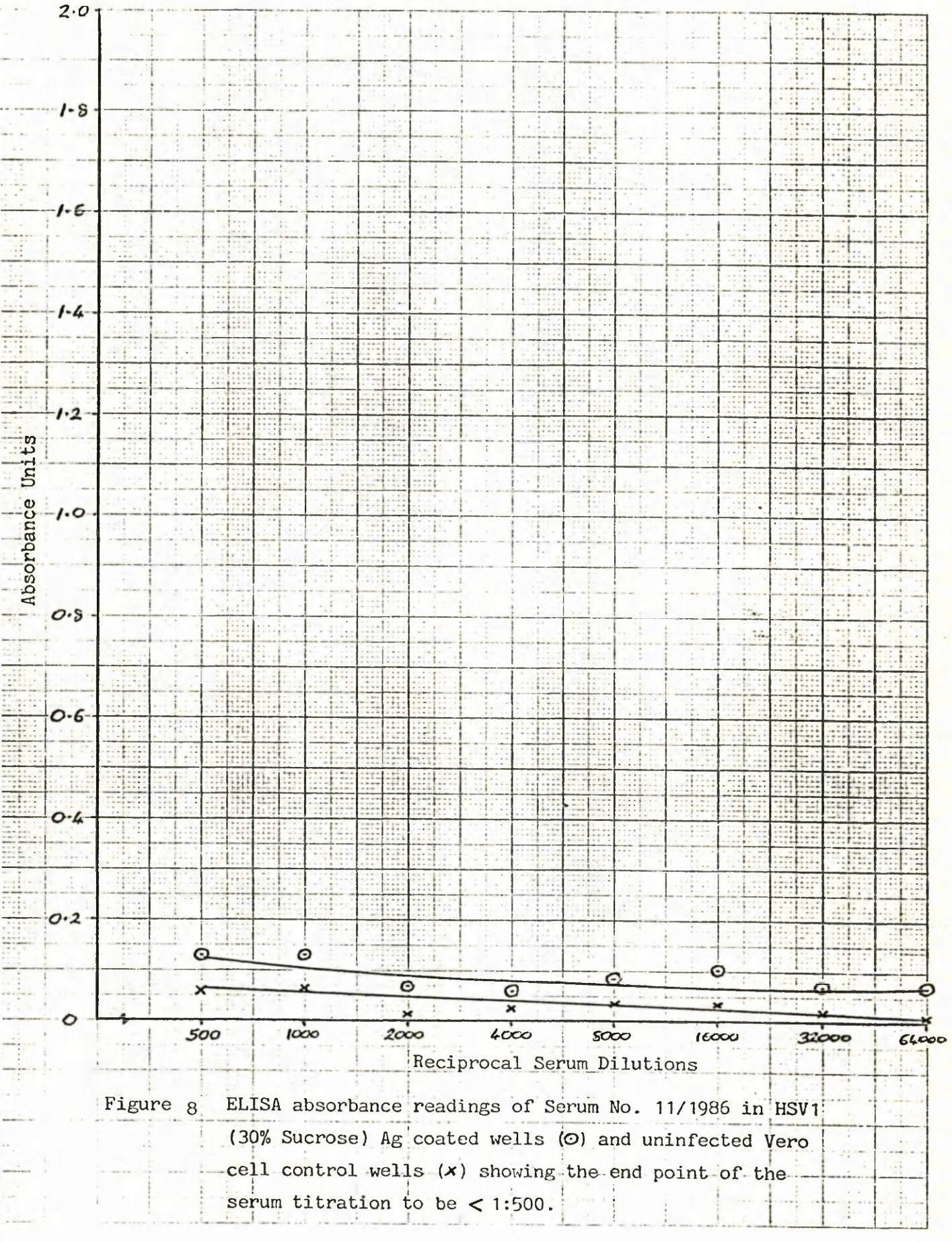


Figure 8 ELISA absorbance readings of Serum No. 11/1986 in HSV1 (30% Sucrose) Ag coated wells (⊙) and uninfected Vero cell control wells (×) showing the end point of the serum titration to be < 1:500.

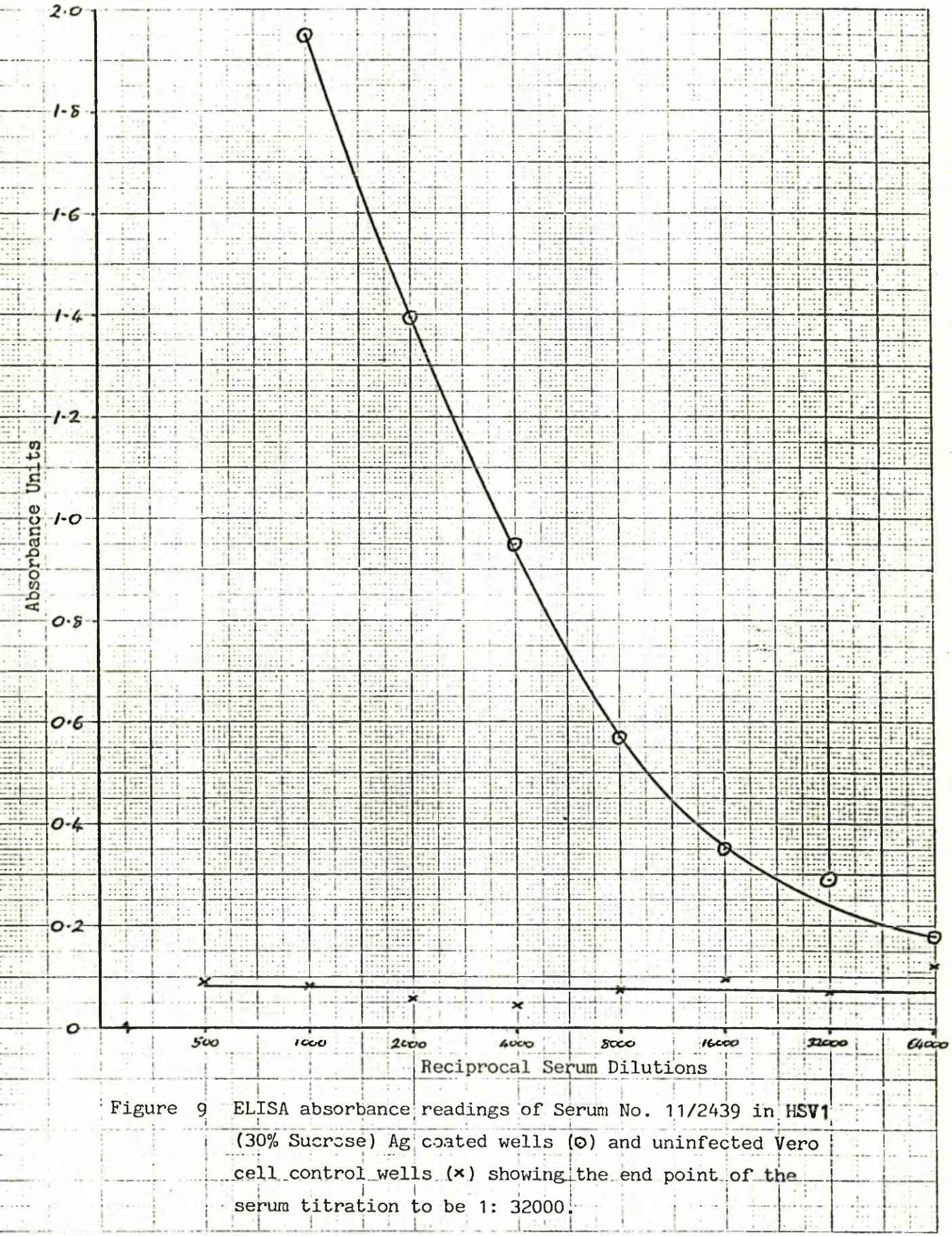


Figure 9 ELISA absorbance readings of Serum No. 11/2439 in HSV1 (30% Sucrose) Ag coated wells (⊙) and uninfected Vero cell control wells (×) showing the end point of the serum titration to be 1: 32000.

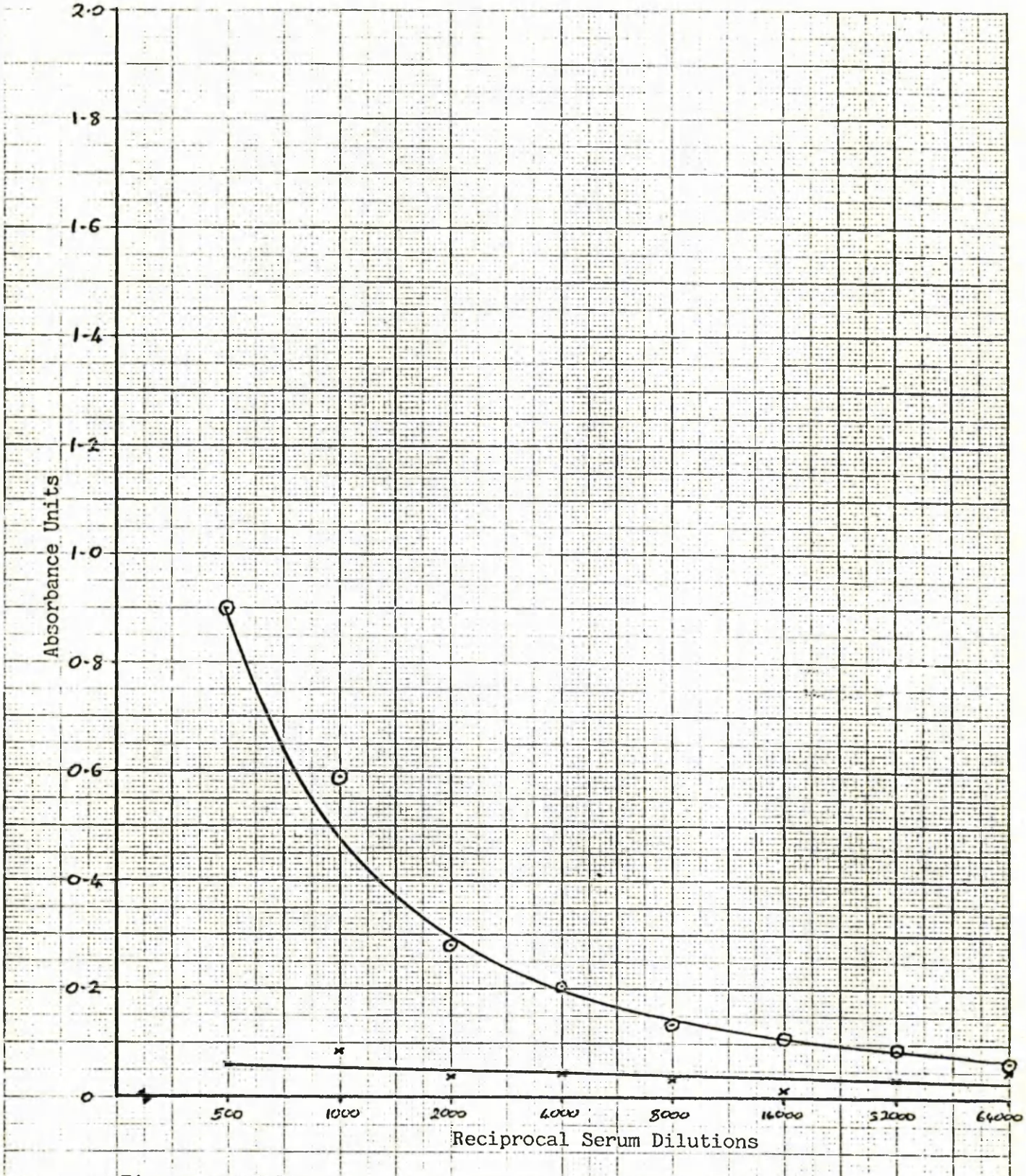


Figure 10 ELISA absorbance readings of Serum No. 11/1650 in HSV1 (30% Sucrose) Ag coated wells (⊙) and uninfected Vero cell control wells (×) showing the end point of the serum titration to be 1:8000.

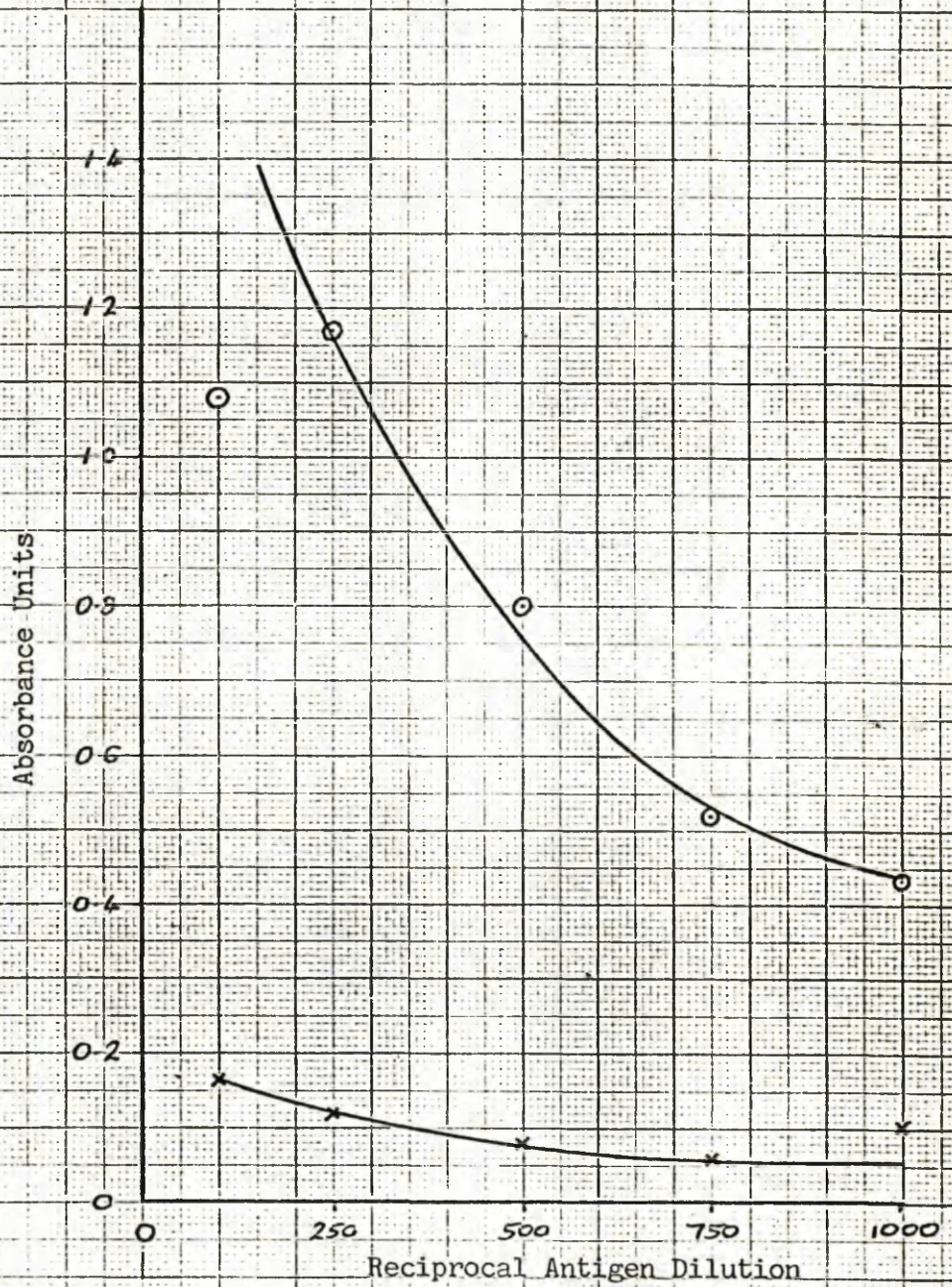


Figure 11 ELISA absorbance readings for a checkerboard titration of HSV1 Ag purified by 30% Sucrose
Serum No 12/1578 (⊙) Serum No 12/0679 (×)
Optimum Coating Dilution = 1:750

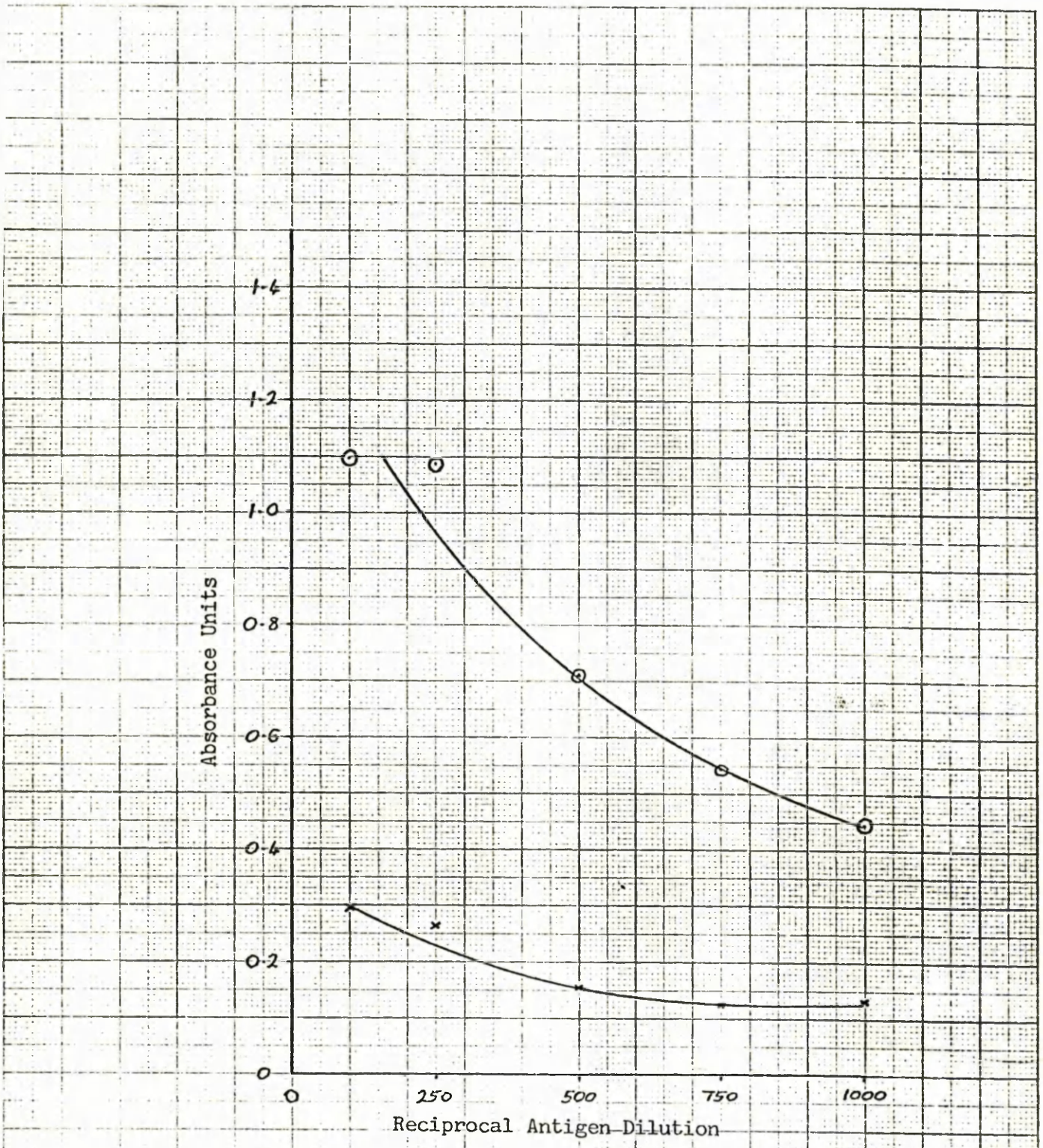


Figure 12 ELISA absorbance readings for a checkerboard titration of $\emptyset B_1$ Ag purified by 30% Sucrose
Serum No 2/1264 (⊙) Serum No 2/0619 (×)
Optimum Coating Dilution = 1:750

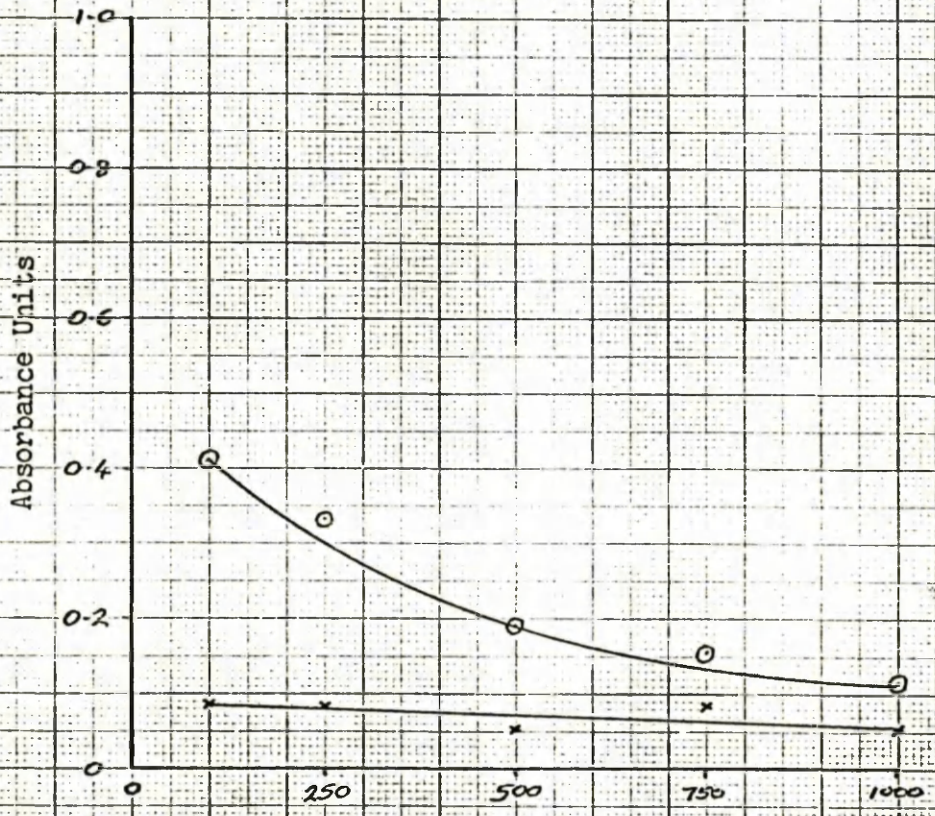


Figure 13 ELISA absorbance readings for a checkerboard titration of Rotavirus Ag purifird by 30% Sucrose
Serum No 4832 (o) Serum No 1187 (x)
Optimum Coating Dilution = 1:100

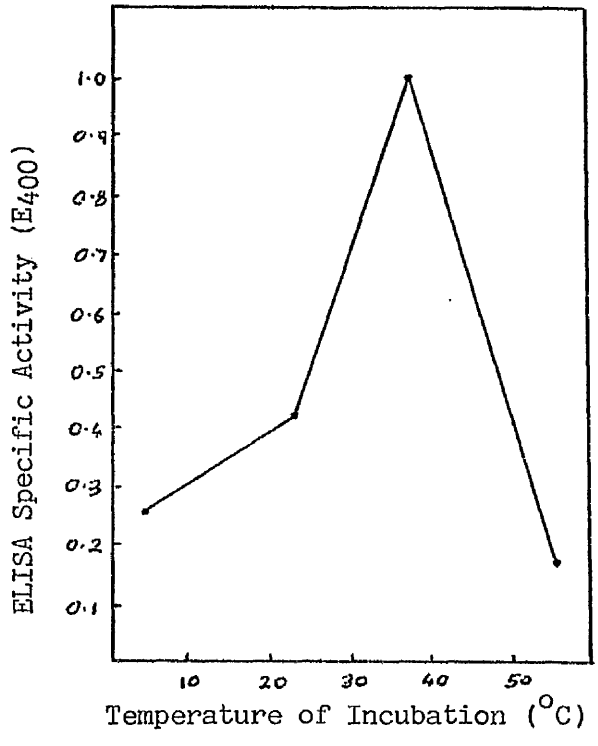


Figure 14 The effect of the temperature of serum and conjugate incubations on the specific activity in enzyme-linked immunosorbent assay (ELISA) for antigens of Herpes simplex virus type 1. (Gilman and Docherty, 1977.) Showing the optimum temperature of incubation resulting in maximum ELISA specific activity to be 37°C.

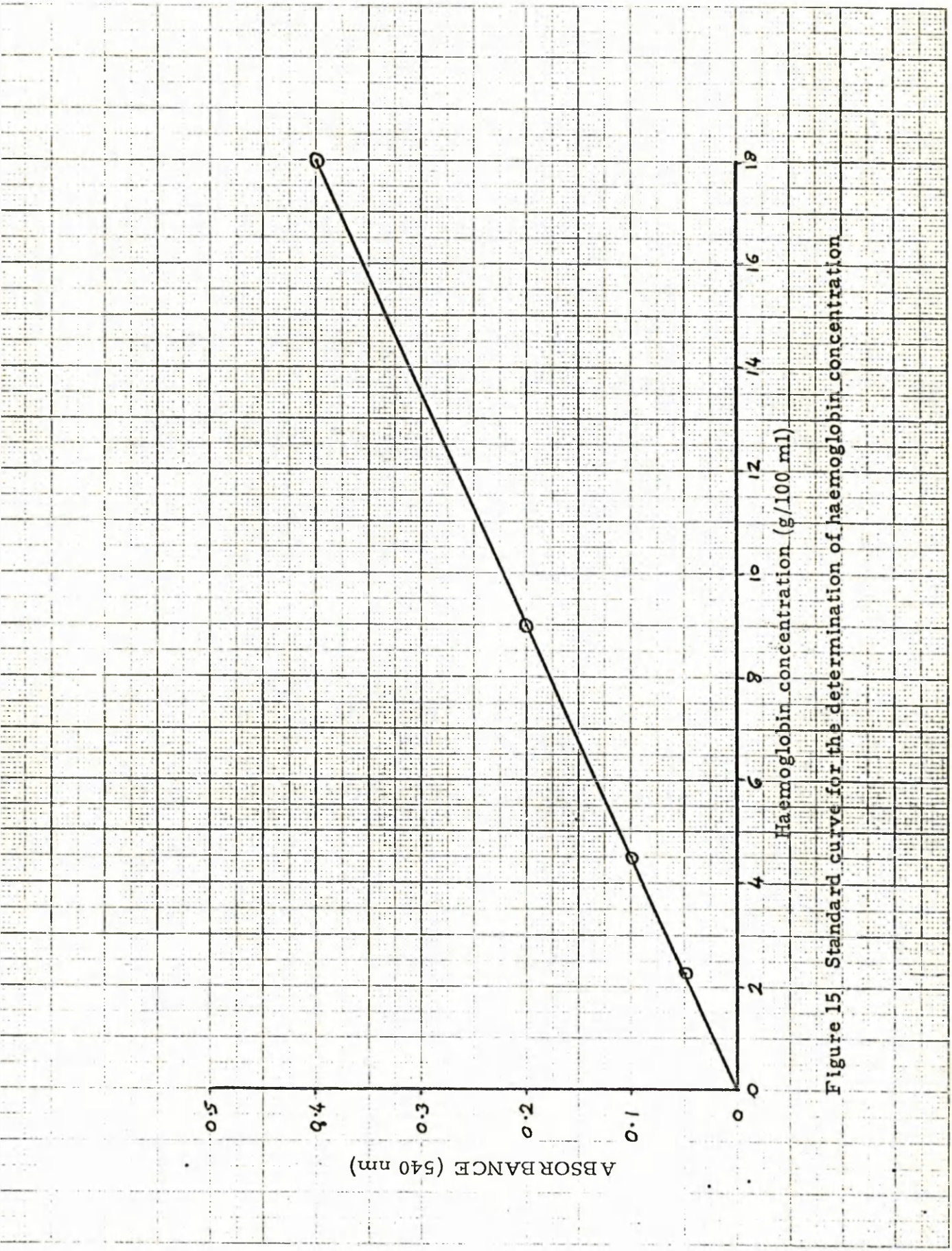


Figure 15 Standard curve for the determination of haemoglobin concentration

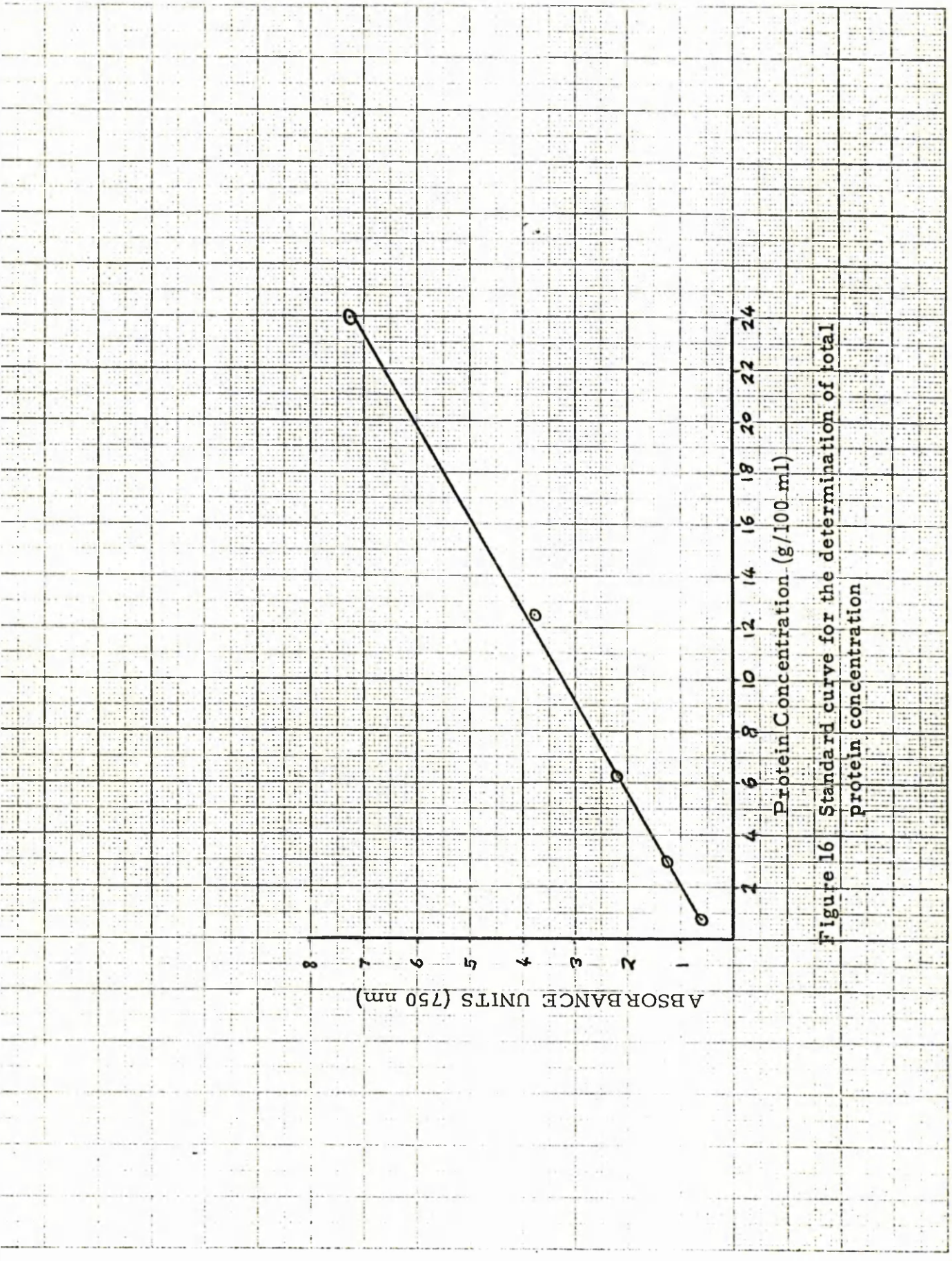


Figure 16 Standard curve for the determination of total protein concentration

SERUM No.	Reciprocal Titre I F A	ELISA
9/1806	16	40
9/2247	16	160
9/2399	32	< 10
9/0127	128	160
11/2177	128	40
11/2524	128	10
11/2823	64	320
11/1986	< 2	640
11/2693	32	⩾ 1280
11/2842	64	< 10
11/1650	32	80
11/2439	512	40
12/0393	128	⩾ 1280
12/0991	32	10
12/0988	128	40
12/1469	128	⩾ 1280

TABLE 1 Comparison of I F A and ELISA titres using Immunitalia HSV 1 CF antigen to senstize the ELISA plates.

SERUM No.	Reciprocal Titre I F A	ELISA
12/0471	16	16
12/0895	16	16
12/0311	<16	16
12/2741	32	128
12/0678	16	128
12/0646	128	⩾ 128
12/0593	64	⩾128
12/2538	16	2
11/2437	256	2
11/1531	16	32
11/2953	16	⩾ 128
11/3085	16	⩾ 128
11/3104	16	4
11/1681	32	⩾ 128
11/1988	64	2
11/3084	16	8
11/2178	32	2
9/1460	32	< 10
9/1216	16	< 10
9/2142	64	20
8/2908	16	80

TABLE 2 Comparison of I F A and ELISA titres using Immunitalia ℄ B_1 CF antigen to sensitize the ELISA plates.

Serum No	Reciprocal Titres		
	IFA	ELISA (CF Ag)	ELISA (DC)
11/2177	128	< 40	1024
11/2823	64	320	256
11/1986	< 16	640	< 64
11/2439	512	< 40	256
11/1650	32	80	256

Table 3 Comparison of IFA and ELISA titres using Immunitalia HSV1 Complement Fixing antigen (CF Ag) or HSV1 Ag purified by Differential Centrifugation (DC) to sensitize the ELISA plates.

Antigen Dilution	Serum No 12/0679	Serum No 12/1578
1:100	0.169	1.083
1:250	0.119	1.172
1:500	0.085	0.799
1:750	0.060	0.518
1:1000	0.108	0.435

Table 4 ELISA results (in absorbance units) for a checkerboard titration to find the optimal coating dilution of HSV1 Ag purified by 30% Sucrose.

Note: Each result in the table above is the mean value of 3 replicate assays.

Serum No	IFA	ELISA
11/2177	128	32000
11/2823	64	16000
11/1986	< 16	< 500
11/2439	512	32000
11/1650	32	8000

Table 5 Correlation of IFA and ELISA titres using HSV1 Ag purified by 30% Sucrose to sensitize the ELISA plates.

Antigen Dilution	Serum No 2/0619	Serum No 2/1264
1:100	0.299	1.099
1:250	0.266	1.089
1:500	0.152	0.710
1:750	0.124	0.544
1:1000	0.131	0.448

Table 6 ELISA results (in absorbance units) for a checkerboard titration to find the optimum coating dilution of ØB_1Ag purified by 30% Sucrose.

Note: Each result in the table above is the mean value of 3 replicate assays.

Antigen Dilution	Serum No 1187	Serum No 4832
1:100	0.088	0.411
1:250	0.085	0.331
1:500	0.054	0.191
1:750	0.088	0.151
1:1000	0.054	0.118

Table 7 ELISA results (in absorbance units) for a checkerboard titration to find the optimum coating dilution of Rotavirus Ag purified by 30% Sucrose.

Note: Each result in the table above is the mean value of 3 replicate assays.

COLUMN No. / ROW No.	1	2	3	4	5	6	7	8	9	10	11	12
1	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.000	0.000	0.000
2	0.000	0.000	0.000	0.000	0.000	0.000	9.999	0.000	9.999	9.999	9.999	9.999
3	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.000	0.000	0.001	0.000
4	0.000	0.001	0.001	0.001	0.002	0.001	0.001	0.000	0.001	0.001	0.000	0.001
5	0.000	0.001	0.001	0.001	0.001	0.001	0.001	0.000	0.000	9.999	0.000	0.000
6	0.001	0.001	0.001	0.002	0.002	0.002	0.001	0.001	0.001	0.000	0.001	0.001
7	9.999	9.999	9.999	0.000	0.000	0.000	9.999	9.999	9.999	9.999	9.999	9.999
8	0.000	0.000	0.000	0.001	0.001	0.001	0.001	0.000	0.000	0.000	0.000	0.000

TABLE 8 Absorbance values from Multiskan Reader containing no micrititre plate.

ROW No. \ COLUMN No.	1	2	3	4	5	6	7	8	9	10	11	12
1	0.000	9.999	9.999	9.999	0.000	0.000	0.000	9.999	0.000	9.999	0.000	0.000
2	9.999	9.998	9.999	9.999	9.999	9.999	9.999	9.998	9.998	9.998	9.999	9.999
3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	9.999	9.999	0.000	0.000
4	0.002	0.000	0.001	0.000	0.001	0.001	0.001	0.001	0.001	0.000	0.001	0.001
5	0.000	0.000	0.000	0.000	0.000	0.001	0.000	0.000	0.000	0.000	0.000	0.001
6	9.999	9.998	9.999	9.999	9.999	0.000	9.999	9.999	9.998	9.998	9.999	0.000
7	9.999	9.999	9.999	9.999	9.999	9.999	9.999	9.999	9.999	9.999	9.999	0.000
8	0.000	0.000	9.999	9.999	0.000	0.000	0.000	9.999	9.999	9.999	0.000	0.000

TABLE 9 Repeat experiment of absorbance values from Multiskan Reader containing no plate.

ROW No. \ COLUMN No.	1	2	3	4	5	6	7	8	9	10	11	12
1	0.002	0.003	0.001	0.000	0.005	0.004	0.000	9.999	0.006	0.006	0.000	0.003
2	0.001	9.999	0.002	9.998	9.998	0.000	0.000	9.997	9.998	9.998	9.998	9.997
3	0.001	0.000	0.002	0.000	0.001	0.000	0.002	9.999	0.000	0.000	9.999	9.999
4	0.002	0.002	0.003	0.004	0.002	0.003	0.003	0.002	0.004	0.003	0.002	0.002
5	0.002	0.005	0.005	0.004	0.001	0.001	0.004	0.001	0.003	0.003	0.003	0.002
6	0.001	0.001	0.005	0.035	0.000	0.002	0.006	9.999	0.000	9.999	0.000	0.000
7	0.000	9.991	9.991	9.995	9.989	9.990	9.989	9.990	9.990	9.989	9.990	9.992
8	0.000	0.001	0.005	0.003	0.003	0.005	0.004	0.004	0.002	0.002	0.003	0.004

TABLE 10 Multiskan Reader absorbance values for an empty Dynatech Microtitre plate.

	I				II				III			
COLUMN No. / ROW No.	1	2	3	4	5	6	7	8	9	10	11	12
1	0.000	1.153	1.705	0.605	0.000	1.151	1.701	0.606	0.001	1.149	1.702	0.601
2	0.000	1.158	1.717	0.696	9.999	1.154	1.716	0.697	9.999	1.148	1.712	0.695
3	9.999	1.169	1.667	0.851	0.000	1.163	1.671	0.851	0.001	1.165	1.666	0.853
4	0.001	1.159	1.709	0.960	0.000	1.153	1.712	0.958	0.001	1.149	1.711	0.959
5	0.001	1.140	1.712	1.059	0.000	1.137	1.713	1.063	0.001	1.137	1.714	1.060
6	0.000	1.141	1.700	1.134	9.999	1.142	1.698	1.135	0.000	1.142	1.700	1.135
7	9.998	1.149	1.691	9.999	9.998	1.144	1.695	0.000	9.999	1.148	1.694	9.999
8	0.000	1.139	1.673	0.000	0.000	1.736	1.677	0.000	0.000	1.136	1.667	9.999

TABLE 11 Replicate Optical Density readings (I, II & III) of various quantities of 0.005% naphthalene black solution.

COLUMN No. / ROW No.	1	2	3	4	5	6	7
1	0.001	1.690	1.677	1.679	1.742	1.739	1.727
2	0.000	1.669	1.680	1.671	1.720	1.735	1.725
3	0.000	1.661	1.666	1.660	1.716	1.710	1.718
4	0.002	1.686	1.690	1.693	1.731	1.724	1.733
5	0.001	1.683	1.674	1.675	1.736	1.729	1.722
6	0.001	1.682	1.692	1.700	1.747	1.739	1.744
7	0.000	1.677	1 702	1.691	1.745	1.734	1.753
8	0.001	1.667	1.690	1.685	1.730	1.730	1.727

TABLE 12 Optical Density readings obtained from 150 ul aliquots of 0.005% naphthalene black solution inoculated into the plate wells by :-

a) Finnpipette (2, 3 & 4)

b) Pipetteman (5, 6 & 7)

Specimen	Protein conc. (g/100 ml)	% of protein recovered	Haemoglobin conc. (g/100 ml)	% of haemoglobin recovered
Original whole blood	16.0	-	12.1	-
Day 1 4°C eluate	5.8	36	6.5	53.7
Day 1 RT eluate	5.75	36	5.8	47.9
Day 7 4°C eluate	7.85	49.1	5.9	48.8
Day 7 RT eluate	6.95	43.5	4.9	40.5

Table 13 Bloodstain eluate recovery experiment showing the correlation between percentage protein and percentage haemoglobin recovered from bloodstains as a function of time and temperature

Serum No	IFA	ELISA	NEUT*
11/1986	< 2	< 500	< 10
12/0679	< 2	< 500	< 10
11/2693	< 2	< 500	< 10
1/2680	< 2	< 500	< 10
2/0838	< 2	< 500	< 10
2/2049	< 2	< 500	< 10
2/2047	< 2	< 500	< 10
1/2678	< 2	< 500	< 10
1/2686	< 2	< 500	< 10
2/0720	16	500	< 10
11/0667	< 2	1000	< 10
1/2467	8	1000	< 10
1/2897	8	2000	10
1/2333	16	2000	10
1/2280	8	2000	10
11/2547	32	4000	100
11/1650	32	4000	10
1/2281	16	4000	10
1/2674	16	4000	10
1/2809	16	4000	10
11/2524	64	8000	50
12/1578	512	8000	10
11/2658	32	8000	50
1/2683	32	8000	10
11/1136	16	8000	< 10
2/2050	32	8000	10
2/0873	32	8000	10
2/1570	8	8000	50
2/1600	64	8000	< 10
1/2884	512	8000	100
1/1424	256	8000	100
12/0393	32	16000	10
11/2823	64	16000	10
11/2663	16	16000	50
11/2842	32	16000	100
11/0246	32	16000	100
2/0549	64	16000	500
2/1571	32	16000	100
11/2439	512	32000	100
11/2177	128	32000	100
11/0250	128	32000	100
2/0193	256	32000	100
11/1469	256	≥ 64000	100
2/0614	1024	≥ 64000	5000
1/2317	1024	≥ 64000	10000

Table 14 Correlation of IFA, ELISA and Neutralisation HSV1 IgG titres of 45 different sera.

* = Neutralisation test.

Serum No	IFA	ELISA	NEUT*
2/0619	< 8	< 500	< 10
2/0786	32	< 500	< 10
1/2812	8	< 500	< 10
1/2811	8	< 500	< 10
11/2437	128	< 500	< 10
12/1646	128	500	10
10/3104	8	500	< 10
10/3085	64	500	< 10
10/3084	16	500	< 10
11/2953	32	500	< 10
2/1046	32	1000	< 10
2/0853	32	1000	< 10
11/1988	64	1000	< 10
11/1531	16	1000	< 10
11/2178	32	1000	50
1/2082	16	1000	< 10
1/2800	8	1000	50
2/0102	32	2000	10
2/1149	32	2000	< 10
2/0843	8	2000	< 10
11/1681	32	2000	< 10
12/0846	64	2000	< 10
12/1702	32	2000	< 10
2/1947	8	4000	10
2/2052	8	4000	< 10
12/0471	16	4000	< 10
12/0593	128	4000	> 100
11/2741	32	4000	50
12/0594	128	4000	10
2/0145	64	8000	< 10

Table 15 Comparison of IFA, ELISA and Neutralisation Ø B_1 IgG titres of 30 different sera.

* = Neutralisation test.

Serum No	IFA	ELISA	CF
1187	< 8	< 32	0
3225	64	< 32	32
3540	32	< 32	16
3733	< 8	< 32	16
4751	8	< 32	32
4794	8	< 32	2
5201	< 8	< 32	4
4179	16	< 32	4
2460	8	< 32	32
0494	8	32	16
3193	8	32	32
0904	32	64	32
0894	8	64	32
4923	8	64	8
2920	8	64	4
5528	16	64	32
1770	64	128	16
1408	64	128	64
5470	64	256	32
3912	64	256	32
2898	< 8	256	16
4119	32	512	0
4832	128	512	32
2586	16	512	64
1147	8	512	8

Table 16 Comparison of IFA, ELISA and CF Rotavirus IgG titres of 25 different sera.

VIRUS	A	B	C	D	E	F	G	H	I	J
Polio 1	3	N	N	N	3	N	N	N	N	N
Polio 2	N	N	N	3	3	N	N	N	N	3
Polio 3	3	N	N	3	3	1	N	N	3	3
Ø B_1	N	N	3	N	N	2	N	N	3	N
ECHO 5	N	N	N	N	N	3	N	N	4	N
ECHO 19	3	N	N	3	3	N	N	N	N	N
Measles	3	4	-	4	3	2	3	3	2	N
Herpes S	7	6	4	5	6	8	4	4	-	3
Adeno 2	N	N	N	3	3	2	N	N	N	N
Adeno 5	N	N	N	N	N	N	N	N	N	N
R S V	5	5	5	4	5	N	4	3	4	4
Flu A 3½hr	N	-	-	3	3	-	N	3	N	N
Flu A 18hr	3	3	4	3	5	1	3	3	N	3
Flu B	6	6	-	5	6	3	5	5	N	6
E B V	6	4	5	5	5	5	4	3	6	5
C M V	7	4	4	4	5	4	4	4	4	3

TABLE 17 Replicate Antibody Profiles (A - J) of plasma from a 66 year old Glasgow female.

Note: In Tables 17 through 65,

N = No specific antiviral antibody detected

- = No result available

nt = Antibody detected in undiluted plasma sample

1, 2, 3 etc. = Antibody titre recorded as Log_2

VIRUS	A	B	C	D	E	F	G	H	I	J
Polio 1	4	N	3	5	4	3	3	5	4	4
Polio 2	4	4	4	4	5	3	3	3	3	3
Polio 3	5	4	5	6	6	5	6	5	5	5
Ø B_1	N	N	N	4	4	4	4	4	3	3
ECHO 5	4	4	5	5	5	4	5	5	6	4
ECHO 19	N	N	N	5	5	N	N	3	4	2
Measles	4	4	N	4	3	5	5	4	3	N
Herpes S	-	4	4	6	6	7	7	6	7	5
Adeno 2	N	3	N	3	3	N	N	N	N	N
Adeno 5	N	3	4	3	4	3	3	2	2	2
R S V	6	6	5	6	6	6	7	6	6	7
Flu A 3½hr	N	6	3	5	5	4	4	3	4	5
Flu A 18hr	3	5	4	4	4	4	5	-	-	2
Flu B	5	5	5	5	5	5	5	5	5	5
E B V	5	6	6	6	6	6	6	6	6	6
C M V	4	5	4	6	6	N	N	5	4	6

TABLE 18 Replicate Antibody Profiles of plasma from a 64 year old Glasgow male.

VIRUS	A	B	C	D	E	F	G	H	I	J
Polio 1	2	2	2	3	-	-	N	N	3	2
Polio 2	3	2	1	3	2	3	3	1	3	2
Polio 3	5	5	3	5	3	4	5	N	4	4
Ø B_1	4	4	4	N	4	3	3	3	4	4
ECHO 5	4	4	4	4	3	4	4	4	3	4
ECHO 19	-	-	N	4	-	-	N	3	3	4
Measles	5	4	-	4	-	4	N	N	N	5
Herpes S	7	6	8	-	-	-	7	-	-	4
Adeno 2	N	N	N	N	N	N	N	2	1	-
Adeno 5	-	-	N	-	-	-	N	N	N	N
R S V	4	5	3	N	4	5	-	5	5	4
Flu A 3½hr	N	N	N	N	N	N	3	N	N	2
Flu A 18hr	N	N	N	N	N	N	3	3	2	-
Flu B	2	2	4	3	2	2	5	4	-	-
E B V	6	5	5	5	6	6	6	5	6	6
C M V	N	5	6	3	6	5	N	5	6	5

TABLE 19 Replicate Antibody Profiles of plasma from a 65 year old Glasgow male.

VIRUS	A	B	C	D	E	F	G	H	I	J
Polio 1	N	1	2	N	N	N	N	N	N	N
Polio 2	N	N	1	-	4	2	4	3	3	1
Polio 3	2	3	5	4	3	1	2	2	N	1
Ø B_1	N	3	3	4	3	3	N	3	2	N
ECHO 5	3	4	5	3	4	4	4	4	3	3
ECHO 19	3	N	N	2	2	2	2	N	3	2
Measles	N	4	4	3	3	3	N	4	3	1
Herpes S	4	5	6	5	4	N	4	7	5	7
Adeno 2	-	N	4	2	2	2	2	-	4	2
Adeno 5	N	3	5	2	3	3	3	2	4	3
R S V	5	6	6	6	5	7	7	5	6	5
Flu A $3\frac{1}{2}$hr	4	N	N	N	N	4	5	-	-	-
Flu A 18hr	-	N	3	N	3	5	4	-	-	-
Flu B	-	6	-	5	4	4	4	-	-	-
E B V	5	6	8	7	7	7	8	6	7	-
C M V	5	7	6	7	5	6	5	7	6	7

TABLE 20 Replicate Antibody Profiles of plasma from a 77 year old Glasgow male.

VIRUS	A	B	C	D	E	F	G	H	I	J
Polio 1	4	N	3	5	4	3	3	5	4	4
Polio 2	4	4	4	4	5	3	3	3	3	3
Polio 3	5	4	5	6	6	5	6	5	5	5
Ø B_1	N	N	N	4	4	4	4	4	3	3
ECHO 5	4	4	5	5	5	4	5	5	6	4
ECHO 19	N	N	N	-	-	N	N	3	4	N
Measles	4	4	-	4	3	5	5	4	3	-
Herpes S	-	4	4	6	6	7	7	6	7	6
Adeno 2	N	3	N	3	3	N	N	N	N	N
Adeno 5	N	3	4	3	4	3	3	2	2	2
R S V	6	6	5	6	6	6	7	6	6	7
Flu A 3½hr	N	6	3	5	5	4	4	3	4	5
Flu A 18hr	3	5	4	4	4	4	5	-	-	2
Flu B	5	5	5	5	5	5	5	5	5	5
E B V	5	6	6	6	6	6	6	6	6	6
C M V	4	5	4	6	6	-	-	5	4	6

TABLE 21 Replicate Antibody Profiles of plasma from a 66 year old Glasgow male.

VIRUS	A	B	C	D	E	F	G	H	I	J
Polio 1	4	3	3	2	2	3	N	N	N	N
Polio 2	5	3	2	2	2	2	3	4	3	4
Polio 3	6	3	3	3	3	3	3	N	N	4
Ø B_1	3	N	N	N	2	2	N	N	3	3
ECHO 5	6	2	3	4	2	2	4	6	5	5
ECHO 19	4	2	N	2	1	1	N	N	5	4
Measles	4	N	N	N	3	3	4	N	N	N
Herpes S	-	N	7	6	6	6	N	N	8	8
Adeno 2	-	N	N	1	1	2	N	2	3	2
Adeno 5	N	N	N	N	N	N	N	N	5	2
R S V	5	3	3	3	5	4	4	5	4	3
Flu A 3½hr	2	N	3	1	N	1	N	3	N	3
Flu A 18hr	3	2	N	1	2	2	3	4	4	4
Flu B	6	4	4	4	5	5	3	4	3	-
E B V	5	5	5	5	5	5	5	5	6	7
C M V	N	N	N	N	N	N	N	N	N	N
Hb (%)	45	38	45	44	43	45	36	36	42	44

TABLE 22 Replicate Antibody Profiles of bloodstain eluates. Donor: 20 year old Glasgow male.

VIRUS	A	B	C	D	E	F	G	H	I	J
Polio 1	N	1	-	-	-	-	-	-	N	N
Polio 2	1	1	4	3	4	2	3	2	N	N
Polio 3	1	2	4	3	5	3	5	4	4	4
Ø B_1	3	2	5	3	4	3	2	4	4	4
ECHO 5	2	3	5	4	5	5	5	5	4	4
ECHO 19	N	N	3	3	5	4	6	4	2	2
Measles	N	N	5	N	3	N	3	N	1	3
Herpes S	3	3	6	5	8	4	8	5	4	5
Adeno 2	-	-	N	2	N	1	2	N	N	N
Adeno 5	-	-	N	N	3	2	2	N	N	N
R S V	3	4	5	4	5	4	4	5	4	4
Flu A 3½hr	3	3	3	4	5	N	4	2	5	4
Flu A 18hr	2	3	3	2	4	2	3	2	2	3
Flu B	2	2	6	4	5	5	5	4	4	4
E B V	5	5	6	6	8	5	7	6	5	5
C M V	N	N	N	3	2	N	2	N	2	2
Hb (%)	53	46	52	51	49	49	51	49	51	49

TABLE 23 Replicate Antibody Profiles of bloodstain eluates. Donor: 56 year old Glasgow male.

VIRUS	A	B	C	D	E	F	G	H	I	J
Polio 1	N	N	N	N	N	N	N	N	N	N
Polio 2	N	N	N	N	N	N	N	N	N	N
Polio 3	N	N	N	N	N	N	N	N	N	N
Ø B_1	N	N	N	N	N	N	N	1	1	1
ECHO 5	2	2	N	N	2	N	N	2	2	N
ECHO 19	2	N	N	N	N	N	N	N	N	N
Measles	3	2	5	N	4	2	4	3	3	3
Herpes S	4	3	7	6	4	4	5	6	5	3
Adeno 2	N	N	3	N	N	N	N	N	N	N
Adeno 5	N	N	N	1	-	-	5	N	N	N
R S V	4	4	3	N	3	N	3	3	2	4
Flu A 3½hr	N	N	N	N	-	2	2	3	2	2
Flu A 18hr	N	2	2	2	2	N	N	2	3	1
Flu B	5	5	4	4	4	5	4	4	3	4
E B V	6	5	7	7	7	6	6	5	5	6
C M V	2	3	4	N	3	5	5	4	4	2
Hb (%)	53	64	57	53	57	50	53	53	52	53

TABLE 24 Replicate Antibody Profiles of bloodstain eluates. Donor: 52 year old Glasgow female.

VIRUS	A	B	C	D	E	F	G	H	I	J
Polio 1	N	N	N	N	N	N	N	N	N	N
Polio 2	1	1	N	N	N	N	N	N	N	N
Polio 3	N	N	N	N	N	N	N	N	N	N
Ø B_1	N	N	N	N	N	N	N	N	2	2
ECHO 5	2	1	3	3	1	N	N	N	3	3
ECHO 19	N	N	N	N	N	N	N	N	1	1
Measles	2	2	2	3	2	1	N	N	N	N
Herpes S	6	7	7	6	7	7	8	8	6	6
Adeno 2	N	1	1	N	1	1	N	N	2	3
Adeno 5	1	1	1	1	2	1	1	2	1	2
R S V	4	3	3	3	4	3	3	3	2	2
Flu A 3½hr	1	2	4	4	4	4	-	-	-	-
Flu A 18hr	2	3	4	4	2	2	2	2	2	3
Flu B	5	5	5	6	6	5	-	-	6	6
E B V	4	4	4	5	5	5	-	-	6	6
C M V	N	3	3	3	5	5	5	5	5	5
Hb (%)	46	46	46	56	55	51	49	51	56	56

TABLE 25 Replicate Antibody Profiles of bloodstain eluates. Donor: 61 year old Glasgow male.

VIRUS	A	B	C	D	E	F	G	H	I	J
Polio 1	N	N	N	N	N	N	N	N	N	N
Polio 2	2	2	2	3	3	2	4	4	N	1
Polio 3	2	2	N	4	N	N	2	2	N	2
Ø B_1	N	N	4	5	2	2	4	3	1	2
ECHO 5	4	4	5	6	4	4	-	-	1	-
ECHO 19	N	N	5	5	2	2	6	5	2	2
Measles	5	6	5	-	4	4	5	5	2	5
Herpes S	7	7	8	8	6	7	8	8	5	6
Adeno 2	1	1	4	4	2	2	4	3	2	1
Adeno 5	N	N	3	4	2	2	3	4	2	N
R S V	4	4	5	5	3	3	5	4	2	2
Flu A 3½hr	N	N	-	-	1	1	3	3	4	3
Flu A 18hr	2	2	3	3	1	1	3	N	1	1
Flu B	6	6	6	7	6	5	5	5	4	4
E B V	5	5	7	6	6	6	8	7	5	7
C M V	N	N	N	N	2	N	N	N	N	N
Hb (%)	73	72	74	73	70	68	73	72	71	72

TABLE 26 Replicate Antibody Profiles of bloodstain eluates. Donor: 48 year old Glasgow male.

VIRUS	A	B	C	D	E	F	G	H	I	J
Polio 1	3	4	3	5	4	3	4	4	4	5
Polio 2	3	5	3	5	4	3	3	5	4	5
Polio 3	8	6	5	5	6	7	6	6	6	6
$\varnothing$ B_1	5	5	-	-	5	5	5	5	4	5
ECHO 5	4	3	-	-	-	-	-	-	6	4
ECHO 19	4	3	4	4	5	4	4	5	-	-
Measles	-	-	-	-	-	-	-	-	-	-
Herpes S	5	4	5	6	8	8	7	6	7	6
Adeno 2	N	N	N	N	N	N	N	N	N	N
Adeno 5	-	-	-	-	-	-	-	-	-	-
R S V	5	5	4	4	5	4	4	5	4	5
Flu A 3½hr	-	-	-	-	-	-	-	-	-	-
Flu A 18hr	-	-	-	-	-	-	-	-	-	-
Flu B	5	4	4	-	-	-	-	4	4	6
E B V	4	4	4	4	4	4	4	5	5	4
C M V	4	4	4	4	4	4	4	4	4	5

TABLE 27 Replicate Antibody Profiles of plasma from a 41 year old Glasgow female.

VIRUS	A	B	C	D	E	F	G	H	I	J
Polio 1	3	N	N	N	3	N	N	N	-	N
Polio 2	N	N	N	3	3	N	N	N	N	3
Polio 3	3	N	N	3	3	N	N	N	3	3
Ø B_1	N	N	3	N	N	N	N	N	3	N
ECHO 5	N	N	N	-	-	3	N	N	4	N
ECHO 19	3	3	N	3	3	-	N	N	N	N
Measles	3	4	-	4	3	2	N	N	N	N
Herpes S	7	6	4	5	6	8	4	4	-	3
Adeno 2	N	N	-	3	3	N	N	N	N	N
Adeno 5	-	-	-	-	-	-	-	-	-	-
R S V	5	5	5	4	5	N	4	3	4	4
Flu A 3½hr	N	-	-	N	N	-	N	3	N	N
Flu A 18hr	3	3	4	5	1	3	3	N	3	3
Flu B	6	6	-	5	6	3	5	5	N	6
E B V	6	4	5	5	5	5	4	3	6	5
C M V	7	4	4	4	5	4	4	4	4	3

TABLE 28 Replicate Antibody Profiles of plasma from a 68 year old Glasgow female.

VIRUS	A	B	C	D	E	F	G	H	I	J
Polio 1	1	N	1	N	1	1	N	nt	N	1
Ø B_1	N	N	1	1	nt	N	1	1	nt	N
ECHO 19	1	nt	1	nt	nt	nt	2	2	nt	nt
Herpes S	3	1	3	2	2	2	3	3	3	3
Adeno 5	N	N	N	N	N	N	N	N	N	nt
E B V	2	2	3	3	3	2	2	3	4	2
C M V	N	N	N	N	N	N	N	N	N	N
Hb (%)	23	23	25	24	27	25	23	25	25	21

TABLE 29 Replicate Antibody Profiles of bloodstain eluates. Donor: 16 year old Glasgow male.

VIRUS	A	B	C	D	E	F	G	H	I	J
Polio 1	nt	N	N	N	nt	N	N	nt	N	N
Ø B_1	N	N	N	N	N	N	N	nt	N	N
ECHO 19	nt	1	N	nt	1	N	nt	1	nt	N
Herpes S	4	3	4	3	4	3	3	3	3	3
Adeno 5	N	N	N	N	N	N	N	N	N	N
E B V	3	2	2	1	2	3	2	1	1	2
C M V	N	N	N	N	N	N	N	N	N	N
Hb (%)	20	19	14	14	14	17	16	14	13	10

TABLE 30 Replicate Antibody Profiles of bloodstain eluates. Donor: 34 year old Glasgow female.

VIRUS	A	B	C	D	E	F	G	H	I	J
Polio 1	1	nt	1	1	nt	1	2	1	2	1
Ø B_1	2	2	3	2	3	2	3	3	2	3
ECHO 19	3	3	2	1	1	1	3	3	3	3
Herpes S	2	2	2	2	2	3	3	3	4	3
Adeno 5	1	nt	nt	nt	nt	2	1	1	1	N
E B V	nt	1	1	1	1	2	2	1	1	3
C M V	N	N	N	N	N	N	N	N	N	N
Hb (%)	24	25	29	33	24	24	24	25	36	35

TABLE 31 Replicate Antibody Profiles of bloodstain eluates. Donor: 7 year old Glasgow female.

VIRUS	A	B	C	D	E	F	G	H	I	J
Polio 1	2	1	1	2	2	2	2	1	1	1
Ø B_1	N	N	1	nt	1	1	1	1	N	N
ECHO 19	2	1	1	1	1	2	1	1	2	1
Herpes S	3	2	2	2	3	3	2	4	2	2
Adeno 5	N	N	N	N	N	N	N	N	N	N
E B V	2	2	2	2	3	3	2	2	2	1
C M V	5	5	4	4	4	4	4	4	4	4
Hb (%)	27	17	32	30	29	38	29	28	29	22

TABLE 32 Replicate Antibody Profiles of bloodstain eluates. Donor: 27 year old Glasgow female.

VIRUS	A	B	C	D	E	F	G	H	I	J
Polio 1	2	2	2	2	1	2	1	1	1	1
Ø B_1	1	1	N	N	nt	nt	N	nt	nt	nt
ECHO 19	N	N	N	N	N	N	N	N	N	N
Herpes S	N	N	N	N	N	N	N	N	N	N
Adeno 5	N	N	N	N	N	N	N	N	N	N
E B V	N	N	N	N	N	N	N	N	N	N
C M V	N	N	N	N	N	N	N	N	N	N
Hb (%)	28	28	28	24	28	32	33	37	27	28

TABLE 33 Replicate Antibody Profiles of bloodstain eluates. Donor: $1\frac{1}{2}$ year old baby girl.

PROFILE No.	SEX	AGE	OCCUPATION	LOCATION
1.	M	6	-	Glasgow
2.	-	64	-	Glasgow
3.	M	24	Salesman	Glasgow
4.	-	-	-	-
5.	-	-	-	-
6.	F	52	Housewife	Glasgow
7.	F	22	Virologist	Bellshill
8.	-	27	-	Glasgow
9.	M	61	Retired	Glasgow
10.	F	18	Salesgirl	Glasgow
11.	-	20	-	Glasgow
12.	F	23	-	Glasgow
13.	F	-	-	Glasgow
14.	F	31	-	Glasgow
15.	M	42	Porter	Glasgow
16.	F	70	-	Glasgow
17.	M	73	Teacher	Glasgow
18.	M	-	-	Glasgow
19.	-	29	-	Glasgow
20.	F	-	-	Glasgow
21.	M	23	Virologist	Glasgow
22.	F	35	Virologist	Dunblane
23.	M	-	Engineer	Glasgow
24.	M	71	Retired	Glasgow
25.	F	77	Retired	Glasgow

TABLE 34 Antibody Profile Survey. - Additional Information.

PROFILE No.	SEX	AGE	OCCUPATION	LOCATION
26.	-	24	-	Glasgow
27.	-	24	-	Glasgow
28.	-	-	-	-
29.	F	36	Nurse	Glasgow
30.	M	-	-	Glasgow
31.	M	51	Unemployed	Glasgow
32.	-	23	-	Glasgow
33.	F	54	Housewife	Uddingston
34.	F	-	-	Glasgow
35.	M	31	-	Glasgow
36.	F	62	-	Glasgow
37.	F	-	-	Glasgow
38.	M	-	-	Glasgow
39.	F	64	-	Kilmarnock
40.	M	-	-	Glasgow
41.	M	52	Clerk	E. Kilbride
42.	-	-	-	-
43.	-	-	-	-
44.	-	-	-	-
45.	-	-	-	-
46.	-	-	-	-
47.	-	-	-	-
48.	M	72	Driver	Glasgow
49.	M	56	-	E. Kilbride
50.	F	65	Housewife	Hamilton

TABLE 35 Antibody Profile Survey. - Additional Information.

(Contd.)

PROFILE No.	SEX	AGE	OCCUPATION	LOCATION
51.	M	51	Clerk	Glasgow
52.	F	28	-	Glasgow
53.	F	58	-	Glasgow
54.	F	76	Retired	Glasgow
55.	-	-	-	-
56.	-	-	-	-
57.	M	54	Labourer	Glasgow
58.	F	29	-	Glasgow
59.	-	-	-	-
60.	-	-	-	-
61.	F	53	-	Glasgow
62.	F	80	Retired	Paisley
63.	M	-	-	Glasgow
64.	M	68	-	Glasgow
65.	F	22	-	Glasgow
66.	F	41	-	Glasgow
67.	M	65	-	Glasgow
68.	M	57	-	Glasgow
69.	M	55	-	Glasgow
70.	M	6	-	Glasgow
71.	F	26	-	Glasgow
72.	M	56	-	Glasgow
73.	M	69	-	Glasgow
74.	M	73	-	Glasgow
75.	M	74	-	Glasgow

TABLE 36 Antibody Profile Survey. - Additional Information

(Contd.)

PROFILE No.	SEX	AGE	OCCUPATION	LOCATION
76.	F	-	-	Glasgow
77.	M	9	-	Glasgow
78.	M	28	Clerk	Glasgow
79.	F	44	-	Glasgow
80.	M	63	-	Argyll
81.	F	77	-	Glasgow
82.	M	1	-	Glasgow
83.	F	41	-	Glasgow
84.	F	31	-	Glasgow
85.	F	58	-	Glasgow
86.	-	52	-	Glasgow
87.	M	17	-	Glasgow
88.	F	70	-	Glasgow
89.	M	78	-	Glasgow
90.	F	23	-	Glasgow
91.	M	73	-	Glasgow
92.	-	20	-	Glasgow
93.	F	-	-	Glasgow
94.	M	12	-	Glasgow
95.	M	20	-	Glasgow
96.	F	-	-	Glasgow
97.	F	58	-	Glasgow
98.	F	56	-	Glasgow
99.	M	53	-	Glasgow
100.	F	71	-	Glasgow

TABLE 37 Antibody Profile Survey. - Additional Information

(Contd.)

VIRUS	1	2	3	4	5	6	7	8	9	10
Polio 1	4	1	3	4	N	3	6	3	2	4
Polio 2	4	1	2	5	1	N	4	3	2	3
Polio 3	5	2	3	5	N	N	5	3	2	4
Ø B_1	4	N	N	N	N	1	1	N	2	5
ECHO 5	6	-	4	5	3	3	5	-	2	5
ECHO 9	4	3	3	5	N	1	4	3	N	4
ECHO 19	5	3	3	4	1	N	3	3	1	3
ECHO 30	3	-	3	4	1	N	3	3	N	2
Mumps	-	-	N	3	N	1	-	-	N	2
Measles	N	3	4	4	1	N	N	3	3	3
Herpes S	N	5	N	5	4	5	N	5	5	5
Adeno 2	N	2	4	3	1	N	N	N	2	3
Adeno 5	N	1	4	N	N	N	N	1	N	2
R S V	7	-	N	6	1	3	3	-	2	N
Flu A 3½hr	N	8	N	4	N	N	N	3	N	N
Flu A 18hr	5	6	N	5	N	N	1	3	1	N
Flu B	N	6	N	3	N	N	3	3	3	3
E B V	N	-	-	6	-	-	4	-	-	-
C M V	N	-	-	-	-	-	4	-	-	-

TABLE 38 Antibody Profile Survey.

VIRUS	11	12	13	14	15	16	17	18	19	20
Polio 1	5	5	4	1	5	N	3	1	5	3
Polio 2	3	4	3	1	4	-	1	N	4	3
Polio 3	3	4	2	N	5	N	3	3	4	5
Ø B_1	2	4	N	N	N	N	N	1	N	1
ECHO 5	-	6	-	3	4	N	N	N	-	4
ECHO 9	3	5	3	N	5	N	N	1	3	3
ECHO 19	3	3	3	N	4	2	N	N	3	2
ECHO 30	-	-	-	N	4	N	N	1	-	2
Mumps	-	N	N	N	-	2	N	N	-	N
Measles	N	4	N	5	N	N	N	1	N	-
Herpes S	5	5	2	5	7	N	1	3	4	N
Adeno 2	N	1	N	N	3	N	N	N	3	-
Adeno 5	2	1	N	N	N	N	1	2	3	-
R S V	4	6	5	2	6	3	2	1	7	3
Flu A 3½hr	2	2	N	N	4	4	2	N	2	2
Flu A 18hr	4	5	1	2	3	4	4	1	1	3
Flu B	-	4	N	5	4	3	N	1	3	3
E B V	-	-	-	-	5	3	-	-	-	N
C M V	-	-	-	-	N	N	-	-	-	2

TABLE 39 Antibody Profile Survey. (Contd.)

VIRUS	21	22	23	24	25	26	27	28	29	30
Polio 1	3	2	5	N	2	4	3	3	N	1
Polio 2	3	1	3	N	N	2	N	1	N	4
Polio 3	4	3	4	N	3	2	4	3	N	4
Ø B_1	N	N	N	N	3	N	N	N	N	5
ECHO 5	2	1	5	4	2	2	5	1	N	4
ECHO 9	N	3	4	N	2	4	5	2	N	4
ECHO 19	3	2	4	N	3	1	2	N	N	4
ECHO 30	3	2	2	1	2	2	4	1	N	3
Mumps	2	1	N	N	N	N	2	N	N	N
Measles	3	N	1	2	N	N	N	1	N	5
Herpes S	N	N	6	N	6	2	N	1	N	N
Adeno 2	N	1	2	3	2	N	3	1	N	4
Adeno 5	N	2	3	N	N	N	5	2	N	3
R S V	3	3	2	3	4	3	4	1	N	6
Flu A 3½hr	N	1	2	N	N	N	N	N	N	2
Flu A 18hr	1	2	3	N	N	3	1	2	N	4
Flu B	N	2	3	N	N	N	1	N	N	5
E B V	-	-	-	-	-	-	-	-	-	-
C M V	-	-	-	-	-	-	-	-	-	-

TABLE 40 Antibody Profile Survey. (Contd.)

VIRUS	31	32	33	34	35	36	37	38	39	40
Polio 1	N	3	2	N	2	2	3	5	8	2
Polio 2	N	1	2	N	1	N	1	3	3	N
Polio 3	-	1	N	2	3	N	3	3	3	1
℄ B_1	N	N	N	N	3	N	4	6	3	N
ECHO 5	2	-	N	2	2	3	4	6	3	N
ECHO 9	1	3	3	N	3	N	2	5	3	1
ECHO 19	1	3	2	-	3	2	3	5	4	N
ECHO 30	1	-	N	-	2	3	3	2	2	N
Mumps	N	N	N	-	N	N	1	N	4	N
Measles	-	N	2	N	5	3	3	4	7	N
Herpes S	6	3	N	-	4	5	4	6	6	N
Adeno 2	1	N	2	N	2	4	3	N	5	N
Adeno 5	-	3	N	N	2	N	2	4	3	N
R S V	1	5	2	4	5	3	3	4	5	1
Flu A 3½hr	4	-	N	5	N	N	N	5	6	1
Flu A 18hr	4	N	2	-	4	N	1	N	2	N
Flu B	1	1	N	1	3	3	N	N	3	N
E B V	6	-	-	5	-	-	5	7	2	4
C M V	N	-	-	N	-	-	-	-	-	-

TABLE 41 Antibody Profile Survey. (Contd.)

VIRUS	41	42	43	44	45	46	47	48	49	50
Polio 1	3	6	4	5	4	4	5	4	2	N
Polio 2	2	4	1	5	2	3	2	N	3	3
Polio 3	6	5	3	6	3	2	3	3	2	3
℄ B_1	3	6	4	5	5	4	3	N	N	N
ECHO 5	6	4	7	5	5	3	3	6	5	4
ECHO 9	4	5	2	3	5	3	3	6	3	3
ECHO 19	6	5	4	4	4	2	4	3	3	1
ECHO 30	4	4	4	N	5	4	N	4	4	1
Mumps	N	N	1	1	N	N	N	N	2	4
Measles	N	3	4	3	2	4	5	4	1	6
Herpes S	2	4	5	4	5	5	6	7	4	6
Adeno 2	N	N	N	N	N	1	2	2	3	2
Adeno 5	2	2	N	N	1	N	4	1	2	N
R S V	1	2	3	3	N	3	2	5	5	5
Flu A 3½hr	3	5	2	3	3	2	2	N	N	3
Flu A 18hr	N	5	N	N	1	2	N	2	N	3
Flu B	2	2	1	N	N	2	N	4	5	2
E B V	-	-	-	-	-	-	-	-	-	-
C M V	-	-	-	-	-	-	-	-	-	-

TABLE 42 Antibody Profile Survey. (Contd.)

VIRUS	51	52	53	54	55	56	57	58	59	60
Polio 1	4	3	5	3	3	3	5	4	5	2
Polio 2	4	1	4	6	3	5	2	4	5	2
Polio 3	4	1	3	4	6	3	5	3	5	5
Ø B_1	N	3	5	N	N	4	N	4	1	N
ECHO 5	4	3	4	5	5	5	4	4	3	N
ECHO 9	3	3	3	5	4	3	3	6	1	3
ECHO 19	3	N	3	4	5	4	1	4	4	2
ECHO 30	3	2	N	3	5	4	3	N	3	3
Mumps	N	N	N	1	N	N	2	N	4	N
Measles	4	4	4	5	5	4	2	4	2	-
Herpes S	6	4	5	4	8	6	6	4	5	7
Adeno 2	3	1	3	3	3	2	N	N	N	-
Adeno 5	3	N	1	N	N	3	N	4	3	-
R S V	3	2	2	4	6	4	3	5	6	-
Flu A 3½hr	2	2	N	N	5	N	4	4	N	6
Flu A 18hr	4	4	2	2	5	2	4	1	3	3
Flu B	2	3	N	5	4	N	7	-	N	3
E B V	-	-	-	4	6	-	-	5	4	5
C M V	-	-	-	-	-	-	-	-	N	N

TABLE 43 Antibody Profile Survey.)Contd.)

VIRUS	61	62	63	64	65	66	67	68	69	70
Polio 1	N	N	6	3	5	2	3	2	4	3
Polio 2	2	-	4	4	N	3	4	4	2	4
Polio 3	-	2	5	2	N	2	3	3	3	4
Ø B_1	-	1	4	1	4	1	3	5	2	4
ECHO 5	3	4	4	N	3	4	1	2	N	N
ECHO 9	N	2	4	4	5	N	2	2	N	2
ECHO 19	N	3	4	2	5	2	2	3	3	4
ECHO 30	N	N	-	N	-	2	N	N	N	N
Mumps	-	2	2	-	-	-	-	-	N	-
Measles	N	3	N	4	7	5	8	5	3	5
Herpes S	4	5	6	5	7	5	7	8	N	5
Adeno 2	N	1	N	N	N	N	3	-	N	N
Adeno 5	N	N	N	N	4	1	N	2	2	N
R S V	N	4	4	5	5	5	-	-	-	5
Flu A 3½hr	N	3	1	-	6	2	7	4	N	N
Flu A 18hr	-	3	N	3	4	1	3	4	N	3
Flu B	N	2	N	-	N	N	-	-	1	-
E B V	6	5	3	3	5	4	5	5	3	N
C M V	N	5	N	N	N	4	6	N	N	-

TABLE 44 Antibody Profile Survey. (Contd.)

VIRUS	71	72	73	74	75	76	77	78	79	80
Polio 1	3	5	5	N	2	2	4	3	2	5
Polio 2	2	4	5	N	3	3	4	4	4	5
Polio 3	2	N	5	N	3	N	4	2	3	5
℄ B_1	2	N	6	N	2	4	4	3	4	5
ECHO 5	1	N	2	N	N	N	N	2	N	N
ECHO 9	3	-	4	N	3	1	2	1	4	6
ECHO 19	4	4	4	3	4	N	2	4	4	3
ECHO 30	N	-	N	N	N	N	N	N	N	N
Mumps	-	-	N	-	-	N	-	N	N	N
Measles	2	4	2	4	5	4	4	-	-	-
Herpes S	5	5	-	4	5	N	3	N	5	N
Adeno 2	N	3	2	N	N	N	N	N	N	3
Adeno 5	N	-	3	N	N	N	-	3	N	N
R S V	3	4	7	-	3	2	-	-	4	4
Flu A 3½hr	1	5	-	4	7	1	2	3	2	7
Flu A 18hr	3	N	4	2	4	4	4	4	3	3
Flu B	-	N	-	-	1	-	-	1	-	-
E B V	3	4	6	5	6	4	5	4	3	6
C M V	-	N	4	-	2	2	N	N	3	N

TABLE 45 Antibody Profile Survey. (Contd.)

VIRUS	81	82	83	84	85	86	87	88	89	90
Polio 1	N	4	2	3	-	4	N	2	2	5
Polio 2	-	4	2	4	1	5	2	3	2	4
Polio 3	2	4	3	4	4	4	2	N	-	N
℄ B_1	1	N	N	N	3	6	N	4	2	5
ECHO 5	N	N	N	N	4	6	1	N	N	N
ECHO 9	N	4	N	1	N	5	2	2	N	2
ECHO 19	3	N	4	1	2	5	3	2	2	2
ECHO 30	N	N	N	N	3	N	N	N	N	N
Mumps	2	-	N	-	-	5	1	-	-	N
Measles	5	N	2	5	3	2	4	4	4	4
Herpes S	7	N	5	5	6	7	6	2	6	-
Adeno 2	N	N	N	N	2	3	N	N	N	N
Adeno 5	4	N	2	N	3	2	4	N	2	N
R S V	N	1	-	7	4	-	-	4	-	3
Flu A 3½hr	3	N	2	N	3	5	1	-	3	N
Flu A 18hr	5	N	2	N	2	4	3	5	3	N
Flu B	2	-	3	3	3	-	N	-	4	-
E B V	4	N	3	3	5	6	4	4	7	4
C M V	N	1	N	3	4	N	N	N	2	N

TABLE 46 Antibody Profile Survey. (Contd.)

VIRUS	91	92	93	94	95	96	97	98	99	100
Polio 1	N	N	3	2	4	–	4	5	3	2
Polio 2	N	1	2	–	6	N	N	4	2	N
Polio 3	3	2	N	N	N	3	N	N	N	1
Ø B_1	N	3	4	5	5	5	5	6	4	N
ECHO 5	N	4	2	N	N	4	4	N	N	2
ECHO 9	–	4	2	2	6	6	1	6	4	–
ECHO 19	4	3	3	4	1	4	2	5	4	N
ECHO 30	–	N	N	N	N	–	N	N	–	–
Mumps	–	–	–	N	N	N	N	1	–	–
Measles	3	4	3	5	7	–	–	–	–	N
Herpes S	6	6	6	N	N	N	4	5	5	4
Adeno 2	N	N	2	N	N	–	N	N	–	N
Adeno 5	3	3	N	N	N	4	N	N	3	N
R S V	7	–	2	4	2	1	N	7	3	3
Flu A 3½hr	5	N	N	2	1	3	3	2	–	2
Flu A 18hr	4	1	N	2	N	2	2	5	4	N
Flu B	5	–	–	–	–	–	–	–	4	2
E B V	4	5	4	2	4	5	5	4	4	4
C M V	N	N	N	N	N	5	N	6	3	N

TABLE 47 Antibody Profile Survey. (Contd.)

VIRUS	Time Interval (Months) A (1)	B (2)	C (1)	D (1)
Polio 1	3 (3)	5 (4)	3 (4)	3 (2)
Polio 2	3 (2)	4 (3)	4 (2)	4 (3)
Polio 3	5 (5)	4 (3)	4 (4)	4 (5)
∅ B_1	1 (N)	4 (4)	4 (3)	N (N)
ECHO 5	4 (2)	6 (5)	N (N)	N (N)
ECHO 9	3 (3)	5 (4)	2 (3)	1 (N)
ECHO 19	2 (3)	3 (4)	4 (5)	1 (N)
ECHO 30	2 (3)	- (-)	N (N)	N (N)
Mumps	N (N)	N (N)	- (-)	- (-)
Measles	- (-)	3 (3)	5 (6)	5 (3)
Herpes S	N (N)	5 (5)	5 (5)	5 (6)
Adeno 2	- (-)	1 (1)	N (N)	N (N)
Adeno 5	- (-)	1 (1)	N (2)	N (N)
R S V	3 (1)	6 (6)	5 (5)	7 (7)
Flu A 3½hr	2 (2)	2 (3)	N (N)	N (N)
Flu A 18hr	3 (2)	5 (5)	3 (3)	N (N)
Flu B	3 (2)	4 (4)	- (-)	3 (3)
E B V	N (N)	- (-)	N (N)	3 (3)
C M V	2 (1)	- (-)	- (-)	3 (4)

TABLE 48 Antibody profiles of plasma specimens A-D.

The antibody profiles obtained from the same blood donors A - D after the specified number of months (detailed at the top of the table) are recorded in parentheses

VIRUS	Time Interval (Months) E (½)	F (3)	G (6)
Polio 1	5 (5)	N (N)	1 (N)
Polio 2	N (N)	N (N)	4 (4)
Polio 3	N (N)	2 (2)	4 (4)
℄ B_1	4 (4)	N (N)	5 (5)
ECHO 5	3 (3)	2 (2)	4 (7)
ECHO 9	N (5)	N (N)	4 (5)
ECHO 19	6 (6)	- (-)	4 (6)
ECHO 30	- (-)	2 (3)	3 (4)
Mumps	- (-)	- (-)	N (N)
Measles	5 (7)	N (N)	5 (5)
Herpes S	4 (7)	- (-)	N (N)
Adeno 2	N (N)	N (N)	4 (4)
Adeno 5	5 (4)	N (N)	3 (4)
R S V	5 (5)	4 (5)	6 (6)
Flu A 3½hr	5 (6)	5 (5)	2 (N)
Flu A 18hr	3 (4)	- (-)	4 (4)
Flu B	N (N)	1 (1)	5 (4)
E B V	4 (5)	5 (5)	- (-)
C M V	N (N)	N (N)	- (-)

TABLE 49 Antibody profiles of plasma specimens (E - G).

The antibody profiles obtained from the same blood donors E - G after the specified number of months (detailed at the top of the table) are recorded in parentheses

VIRUS	ORIGINAL BLOOD	4^{o} C Storage (Days) 2	28	56
Polio 1	5	N	2	4
Polio 2	2	N	N	N
Polio 3	3	2	1	3
Ø B_1	N	N	1	N
ECHO 5	4	4	2	3
ECHO 9	3	3	N	3
ECHO 19	1	N	N	3
ECHO 30	3	2	2	-
Mumps	2	1	N	-
Measles	2	2	N	-
Herpes S	6	5	5	4
Adeno 2	N	-	N	-
Adeno 5	N	N	N	-
R S V	3	3	5	6
Flu A 3½hr	4	2	2	5
Flu A 18hr	4	4	3	3
Flu B	7	5	N	5
E B V	-	-	-	-
C M V	-	-	-	-
Hb (%)	-	58	64	72

TABLE 50a Antibody Profiles of bloodstain eluates.

arter storage at 4^{o} C.

Donor: 54 year old Glasgow male.

VIRUS	ORIGINAL BLOOD	Room Temp. Storage (Days) 28	57
Polio 1	5	2	N
Polio 2	2	N	N
Polio 3	3	1	N
℄ B_1	N	N	N
ECHO 5	4	1	N
ECHO 9	3	N	N
ECHO 19	1	N	N
ECHO 30	3	N	N
Mumps	2	N	-
Measles	2	N	-
Herpes S	6	4	2
Adeno 2	N	N	-
Adeno 5	N	N	-
R S V	3	6	4
Flu A 3½hr	4	N	2
Flu A 18hr	4	3	2
Flu B	7	1	2
E B V	-	-	-
C M V	-	-	-
Hb (%)	-	47	50

TABLE 50b Antibody Profiles of bloodstain eluates after storage at room temperature.

Donor: 54 year old Glasgow male.

VIRUS	ORIGINAL BLOOD	4^{o} C Storage (Days) 2	29	57
Polio 1	3	3	N	4
Polio 2	1	1	2	3
Polio 3	1	1	3	4
ø B_1	3	N	N	N
ECHO 5	3	3	4	3
ECHO 9	3	2	2	3
ECHO 19	N	N	4	2
ECHO 30	2	N	4	-
Mumps	N	N	1	-
Measles	4	3	N	-
Herpes S	4	5	5	5
Adeno 2	1	-	3	-
Adeno 5	N	N	N	-
R S V	2	3	4	4
Flu A 3½hr	2	1	2	2
Flu A 18hr	4	2	4	3
Flu B	3	2	4	N
E B V	-	-	-	-
C M V	-	-	-	-
Hb (%)	-	65	58	66

TABLE 51a Antibody Profiles of bloodstain eluates after storage at 4^{o} C.

Donor: 28 year old Glasgow female.

VIRUS	ORIGINAL BLOOD	Room Temp. Storage (Days) 4	29	57
Polio 1	3	3	3	N
Polio 2	1	2	3	N
Polio 3	1	2	3	N
$\emptyset$ B_1	3	N	2	N
ECHO 5	3	4	3	2
ECHO 9	3	3	3	N
ECHO 19	N	N	2	2
ECHO 30	2	2	3	2
Mumps	N	N	1	-
Measles	4	3	N	-
Herpes S	4	4	4	4
Adeno 2	1	2	3	-
Adeno 5	N	N	N	-
R S V	2	3	4	3
Flu A $3\frac{1}{2}$hr	2	1	N	N
Flu A 18hr	4	4	4	1
Flu B	3	4	2	N
E B V	-	-	-	-
C M V	-	-	-	-
Hb (%)	-	56	63	29

TABLE 51b Antibody Profiles of bloodstain eluates after storage at Room Temperature.

Donor: 28 year old Glasgow female.

VIRUS	ORIGINAL BLOOD	4^{o} C Storage (Days) 2	29
Polio 1	4	3	2
Polio 2	4	2	4
Polio 3	4	3	4
Ø B_1	N	N	4
ECHO 5	4	2	5
ECHO 9	3	3	4
ECHO 19	3	2	2
ECHO 30	3	3	2
Mumps	N	N	3
Measles	4	5	N
Herpes S	6	5	5
Adeno 2	3	3	3
Adeno 5	3	1	3
R S V	3	5	5
Flu A 3½hr	2	N	4
Flu A 18hr	4	4	4
Flu B	2	2	N
E B V	-	-	-
C M V	-	-	-
Hb (%)	-	57	61

TABLE 52a Antibody Profiles of bloodstain eluates after storage at 4^{o} C.

Donor: 51 year old Glasgow male.

VIRUS	ORIGINAL BLOOD	Room Temp. Storage (Days) 2	29
Polio 1	4	1	2
Polio 2	4	N	2
Polio 3	4	3	2
Ø B_1	N	N	N
ECHO 5	4	N	2
ECHO 9	3	3	2
ECHO 19	3	N	N
ECHO 30	3	1	3
Mumps	N	N	N
Measles	4	4	N
Herpes S	6	4	6
Adeno 2	3	2	2
Adeno 5	3	N	2
R S V	3	4	4
Flu A 3½hr	2	N	4
Flu A 18hr	4	4	4
Flu B	2	1	N
E B V	-	-	-
C M V	-	-	-
Hb (%)	-	73	46

TABLE 52b Antibody Profiles of bloodstain eluates after storage at Room Temperature.

Donor: 51 year old Glasgow male.

VIRUS	ORIGINAL BLOOD	4^{o} C Storage (Days) 1	47	67
Polio 1	N	N	3	3
Polio 2	3	5	2	3
Polio 3	3	4	3	-
Ø B_1	N	2	N	N
ECHO 5	4	4	2	4
ECHO 9	3	3	N	3
ECHO 19	1	2	3	1
ECHO 30	1	4	2	N
Mumps	4	3	2	3
Measles	6	5	-	-
Herpes S	6	7	3	6
Adeno 2	2	1	-	N
Adeno 5	N	N	-	-
R S V	5	6	5	5
Flu A 3½hr	3	3	3	2
Flu A 18hr	3	3	N	-
Flu B	2	2	2	2
E B V	5	5	5	6
C M V	N	N	N	N
Hb (%)	-	56	47	42

TABLE 53a Antibody Profiles of bloodstain eluates after storage at 4^{o} C.

Donor: 65 year old Glasgow female.

VIRUS	ORIGINAL BLOOD	Room Temp. Storage (Days) 47	66
Polio 1	N	2	N
Polio 2	3	N	1
Polio 3	3	3	1
Ø B_1	N	N	N
ECHO 5	4	1	2
ECHO 9	3	N	1
ECHO 19	1	2	2
ECHO 30	1	1	N
Mumps	4	2	N
Measles	6	-	-
Herpes S	6	5	3
Adeno 2	2	-	N
Adeno 5	N	-	-
R S V	5	4	4
Flu A 3½hr	3	1	1
Flu A 18hr	3	N	-
Flu B	2	3	2
E B V	5	5	4
C M V	N	N	N
Hb (%)	-	35	18

TABLE 53b Antibody Profiles of bloodstain eluates after storage at Room Temperature.

Donor: 65 year old Glasgow female.

VIRUS	ORIGINAL BLOOD	Storage (Days) 4^{o} C (30)	R.T. (30)	R.T.(120
Polio 1	3	N	N	N
Polio 2	6	4	2	N
Polio 3	4	2	1	N
℄ B_1	N	N	N	N
ECHO 5	5	4	3	N
ECHO 9	5	N	N	N
ECHO 19	4	3	2	N
ECHO 30	3	2	N	N
Mumps	1	N	1	N
Measles	-	-	-	-
Herpes S	4	6	5	N
Adeno 2	3	N	N	N
Adeno 5	N	N	N	N
R S V	4	2	1	N
Flu A 3½hr	N	N	N	N
Flu A 18hr	2	N	N	N
Flu B	5	-	N	N
E B V	4	6	5	1
C M V	5	5	3	N
Hb (%)	-	52	22	9

TABLE 54 Antibody Profiles of bloodstain eluates after long term stain storage.

Donor: 76 year old Glasgow female.

VIRUS	ORIGINAL BLOOD	Storage (Days) 4^{o} C (28)	R.T. (28)
Polio 1	2	2	N
Polio 2	3	3	2
Polio 3	2	2	N
Ø B_1	N	N	N
ECHO 5	5	3	N
ECHO 9	3	4	N
ECHO 19	3	3	1
ECHO 30	4	2	2
Mumps	2	2	N
Measles	1	N	1
Herpes S	4	6	5
Adeno 2	3	4	4
Adeno 5	2	3	N
R S V	5	5	5
Flu A 3½hr	N	2	N
Flu A 18hr	N	N	N
Flu B	5	4	3
E B V	4	4	4
C M V	-	-	-
Hb (%)	-	75	61

TABLE 55 Antibody Profiles of bloodstain eluates after long term stain storage.

Donor: 56 year old Glasgow male.

VIRUS	ORIGINAL BLOOD	Storage (Days) 4^{o} C (30)	R.T. (30)
Polio 1	4	2	N
Polio 2	N	2	3
Polio 3	3	2	3
Ø B_1	N	N	N
ECHO 5	6	5	3
ECHO 9	5	N	2
ECHO 19	3	3	N
ECHO 30	4	4	N
Mumps	N	4	2
Measles	4	N	N
Herpes S	7	7	5
Adeno 2	2	2	N
Adeno 5	1	3	N
R S V	5	7	6
Flu A 3½hr	N	5	2
Flu A 18hr	2	3	2
Flu B	4	4	N
E B V	5	5	4
C M V	5	4	5
Hb (%)	-	41	44

TABLE 56 Antibody Profiles of bloodstain eluates after long term storage as stain.

Donor: 72 year old Glasgow male.

VIRUS	ORIGINAL BLOOD	Storage (Days) 4^{o} C (48)	R.T. (48)
Polio 1	4	4	N
Polio 2	5	5	2
Polio 3	5	5	2
℄ B_1	3	N	N
ECHO 5	5	4	1
ECHO 9	5	4	2
ECHO 19	4	4	2
ECHO 30	5	4	1
Mumps	3	3	1
Measles	4	-	-
Herpes S	5	7	3
Adeno 2	3	-	-
Adeno 5	N	N	N
R S V	6	5	5
Flu A 3½hr	4	4	3
Flu A 18hr	5	4	2
Flu B	3	2	3
E B V	6	6	6
C M V	6	6	1
Hb (%)	-	69	42

TABLE 57 Antibody Profiles of bloodstain eluates after long term stain storage.

Donor: 36 year old female, Glasgow.

VIRUS	ORIGINAL BLOOD	Chamber Water Content (ml.) 0.1	0.5	1.0	5.0
Polio 1	N	N	N	N	N
Ø B_1	3	2	3	3	N
ECHO 19	4	-	-	-	-
Herpes S	4	3	4	2	N
Adeno 5	N	N	N	N	N
R S V	2	2	4	1	N
E B V	4	4	4	4	2
C M V	N	N	N	N	N
Hb (%)	-	46	49	46	17

TABLE 58 Antibody Profiles of bloodstain eluates from identical stains exposed to varying degrees of humidity for 7 days at room temperature.

TABLE 59 Antibody Profiles - Blind Trial No. 1.

VIRUS	Original Blood Specimens									
	1	2	3	4	5	6	7	8	9	10
Polio 1	2	N	3	4	3	3	3	2	N	5
Ø B_1	N	N	3	3	3	1	4	5	N	6
ECHO 19	1	N	N	3	3	1	3	4	N	6
Herpes S	N	5	6	N	5	6	5	6	5	5
Adeno 5	2	N	3	N	3	2	3	3	N	2
E B V	6	6	5	N	6	2	4	6	5	N
C M V	4	6	N	5	4	4	1	5	5	5
Hb (g/100ml)	13.9	9.4	16.5	13.5	14.6	14.7	16.5	13.9	12.8	19.1

VIRUS	Bloodstain Eluates									
	A	B	C	D	E	F	G	H	I	J
Polio 1	3	3	4	2	3	3	1	4	5	2
Ø B_1	N	N	4	N	4	3	N	5	2	2
ECHO 19	3	4	4	N	N	3	N	4	4	4
Herpes S	5	5	5	7	2	4	5	6	7	7
Adeno 5	3	2	3	N	N	N	N	3	4	2
E B V	2	5	N	3	3	3	3	6	2	5
C M V	4	4	4	7	5	N	3	4	N	4
Hb (g/100ml)	7.7	9.7	8.1	7.1	8.8	7.6	10.3	10.6	12.4	9.2

TABLE 60 Antibody Profiles - Blind Trial No. 2.

VIRUS	Original Blood Specimens 1	2	3	4	5	6	7	8	9	10
Polio 1	4	4	1	4	4	4	4	3	4	4
Ø B_1	3	N	3	4	3	4	4	3	N	3
ECHO 19	4	4	4	4	4	4	4	3	3	4
Herpes S	4	4	N	N	4	4	4	4	3	3
Adeno 5	3	3	3	3	4	4	3	3	3	5
R S V	N	N	3	4	3	4	4	4	N	3
E B V	4	3	N	4	N	4	N	4	N	4
C M V	3	4	4	N	4	4	N	N	3	4
Blood Group	O+	O+	A−	O+	B−	B−	A−	A−	A−	O+

VIRUS	Bloodstain Eluates A	B	C	D	E	F	G	H	I	J
Polio 1	2	3	2	2	N	N	N	2	2	N
Ø B_1	N	3	N	3	N	2	3	2	2	2
ECHO 19	2	2	N	4	N	N	2	3	N	3
Herpes S	3	2	3	2	N	3	3	N	3	1
Adeno 5	2	3	N	N	N	N	3	N	3	3
R S V	3	3	3	3	3	2	2	2	N	N
E B V	3	3	N	N	2	3	3	N	N	3
C M V	2	2	3	N	3	N	2	3	3	2
Blood Group	O+	B−	O+	A−	A−	A−	O+	A−	B−	O+

TABLE 61 Antibody Profiles - Blind Trial No. 3.

VIRUS	Original Blood Specimens 1	2	3	4	5	6	7	8	9	10
Polio 1	3	3	4	5	4	4	3	4	3	3
Ø B_1	N	N	4	4	3	3	N	3	N	3
ECHO 19	N	3	4	5	3	N	3	3	3	3
Herpes S	4	4	1	4	4	4	4	4	3	3
Adeno 5	N	3	N	3	3	4	N	3	3	3
R S V	3	4	4	4	4	3	3	4	3	3
E B V	3	4	5	5	5	3	4	3	N	4
C M V	N	3	4	4	4	3	4	N	N	3
Hb (g/100ml)	14.4	13.0	15.3	13.9	15.3	18.7	12.4	10.5	11.9	17.5
Blood Group	O+	A−	AB+	B−	O−	A+	O+	A+	A+	A+

VIRUS	Bloodstain Eluate A	B	C	D	E	F	G	H	I	J
Polio 1	1	2	N	N	N	2	N	N	N	2
Ø B_1	2	1	N	N	N	N	2	N	2	2
ECHO 19	N	3	N	N	N	2	2	N	3	3
Herpes S	2	2	3	3	3	2	2	2	3	3
Adeno 5	2	2	N	N	3	2	N	N	3	2
R S V	3	2	2	N	2	3	N	2	N	N
E B V	3	3	2	2	2	2	3	N	3	4
C M V	2	2	N	2	2	N	3	N	3	2
Hb (g/100ml)	11.9	9.0	7.7	6.0	9.0	9.6	9.4	11.2	7.6	9.0
Blood Group	A+	A+	O+	O+	A−	A+	AB+	A+	B−	O−

TABLE 62 Antibody Profiles - Blind Trial No. 4.

VIRUS	Original Blood Specimens 1	2	3	4	5	6	7	8	9	10	11	12
Polio 1	N	4	4	3	6	4	3	N	N	N	N	5
Ø B_1	N	N	4	4	3	3	3	N	N	N	N	4
ECHO 19	N	N	5	4	6	5	4	N	N	N	N	N
Herpes S	N	5	5	5	3	5	4	N	6	7	6	6
Adeno 5	N	N	4	3	N	4	N	N	N	N	N	N
R S V	6	6	5	4	6	4	4	5	6	4	4	5
E B V	7	4	6	N	4	4	4	5	5	N	N	N
C M V	7	4	N	5	N	4	4	3	5	7	3	3
Polio 2							N	3	N			
ECHO 5							3	3	5			
Mumps							N	N	5	N	N	
Measles							N	7	3	3	4	
Flu A $3\frac{1}{2}$hr							3	-	-	1	3	
Flu B							3	N	2	3	4	
Rota							N	7	7	4	4	
Hb (g/100ml)	13.0	9.0	8.1	13.0	12.6	9.9	16.1	12.6	7.9	11.4	15.7	14.8
Blood Group	O+	O+	A+	O-	B+	AB-	A+	A+	A+	O+	O+	B+

TABLE 63 Antibody Profiles - Blind Trial No. 4. (Contd.)

VIRUS	Bloodstain Eluates A	B	C	D	E	F	G	H	I	J	K	L
Polio 1	2	3	N	5	N	2	N	4	N	N	N	2
Ø B_1	1	1	N	3	N	2	N	4	N	N	N	2
ECHO 19	1	N	N	2	N	3	N	3	N	N	N	N
Herpes S	4	4	1	4	2	4	4	4	5	N	3	4
Adeno 5	2	N	N	2	N	1	N	N	N	N	N	N
R S V	3	4	2	4	4	2	N	4	4	4	2	3
E B V	3	4	N	4	2	N	N	2	N	4	2	2
C M V	3	4	N	N	1	2	2	N	4	4	2	N
Polio 2			N		N						N	
ECHO 5			N		N						1	
Mumps			N		N		N		N		N	
Measles			N		N		N		1		3	
Flu A 3½hr			N		3		N		1		1	
Flu B			N		N		1		2		N	
Rota			N		3		N		1		3	
Hb (g/100ml)	6.3	7.2	3.6	9.9	2.7	9.0	9.0	7.2	9.0	6.3	6.3	9.5
Blood Group	AB-	O+	A+	A+	A+	O-	O+	B+	O+	O+	A+	B+

TABLE 64 Antibody Profiles - Blind Trial No 5.

VIRUS	Original Blood Specimens 1	2	3	4	5	6	7	8	9	10
Polio 1	4	5	N	N	4	4	4	5	4	6
Ø B_1	4	3	N	N	4	3	3	3	4	3
ECHO 19	5	4	3	4	3	N	N	N	4	N
Herpes S	5	6	6	6	5	6	6	6	7	6
Adeno 5	N	3	3	N	N	N	1	4	4	4
R S V	5	N	3	4	3	6	6	N	5	N
E B V	N	N	5	N	4	5	6	N	4	N
C M V	N	N	N	5	4	4	3	6	4	4
Hb (g/100ml)	16.8	15.2	7.4	13.9	12.8	13.5	18.0	10.8	15.2	19.0
Blood Group	A+	A+	O+	A+	A+	O-	O-	O+	A+	O+

VIRUS	Bloodstain Eluate A	B	C	D	E	F	G	H	I	J
Polio 1	3	N	2	1	3	1	N	N	1	2
Ø B_1	5	N	2	2	3	2	N	N	N	N
ECHO 19	3	1	1	1	2	N	2	N	N	N
Herpes S	-	-	-	-	5	5	5	5	1	5
Adeno 5	1	N	N	2	1	N	2	N	N	N
R S V	N	N	N	N	N	4	4	2	N	4
E B V	N	N	2	4	N	4	3	3	N	N
C M V	N	5	N	5	N	1	N	1	5	1
Hb (g/100ml)	6.7	7.2	7.7	9.0	6.7	10.1	5.9	17.0	11.7	17.5
Blood Group	A+	A+	A+	A+	A+	O-	O+	O-	O+	O+

TABLE 65 Antibody Profiles - Blind Trial No. 6.

VIRUS	Original Blood Specimens 1	2	3	4	5	6	7	8	9	10
Polio 1	7	N	N	N	4	N	N	3	N	4
Ø B_1	N	N	3	N	6	N	N	4	N	3
ECHO 19	7	N	N	3	5	N	N	N	N	4
Herpes S	7	4	7	7	4	6	3	6	5	7
Adeno 5	3	N	N	N	3	4	N	4	N	3
R S V	3	3	N	3	N	N	4	3	4	4
E B V	6	N	6	3	4	N	3	3	4	5
C M V	-	-	-	-	-	-	-	-	-	-
Hb (g/100ml)	10.8	9.7	17.0	14.6	9.7	11.9	19.3	19.3	15.7	16.8
Blood Group	O+	B+	O+	A+	O-	B-	B+	A+	O+	O+

VIRUS	Bloodstain Eluate A	B	C	D	E	F	G	H	I	J
Polio 1	N	4	N	6	N	3	N	N	N	N
Ø B_1	N	5	N	2	N	2	N	N	2	2
ECHO 19	3	3	N	6	2	2	N	N	N	3
Herpes S	6	6	2	6	5	5	2	2	3	2
Adeno 5	N	3	N	N	N	N	N	N	3	N
R S V	-	-	-	-	-	-	-	-	-	-
E B V	3	2	N	4	N	3	3	2	2	2
C M V	-	-	-	-	-	-	-	-	-	-
Hb (g/100ml)	6.7	10.3	5.2	8.1	12.6	10.8	8.5	9.0	9.0	9.7
Blood Group	A+	O-	B+	O+	B-	O+	O+	B+	A+	O+

Blind Trial No 1

LYSED BLOOD	VAP ANSWER	CORRECT ANSWER
1	F	F
2	A	H
3	J	J
4	B	B
5	D	D
6	C	G
7	I	I
8	H	A
9	G	C
10	E	E

Blind Trial No 2

LYSED BLOOD	VAP ANSWER	CORRECT ANSWER
1	A	A
2	C	C
3	E	E
4	J	G
5	I	I
6	B	B
7	D	D
8	F	F
9	H	H
10	G	J

Blind Trial No 3

LYSED BLOOD	VAP ANSWER	CORRECT ANSWER
1	C	C
2	E	E
3	G	G
4	I	I
5	J	J
6	B	A
7	D	D
8	F	F
9	H	H
10	A	B

Blind Trial No 4

LYSED BLOOD	VAP ANSWER	CORRECT ANSWER
1	J	J
2	B	B
3	D	D
4	F	F
5	H	H
6	A	A
7	K	C
8	C	K
9	E	E
10	I	I
11	G	G
12	L	L

TABLE 66 Blind Trial Results.

Blind Trial No 5

LYSED BLOOD	VAP ANSWER	CORRECT ANSWER
1	A	A
2	C	E
3	G	G
4	B	B
5	D	C
6	F	F
7	H	H
8	I	I
9	E	D
10	J	J

Blind Trial No 6

LYSED BLOOD	VAP ANSWER	CORRECT ANSWER
1	D	D
2	C	C
3	G	J
4	A	A
5	B	B
6	E	E
7	H	H
8	I	I
9	J	G
10	F	F

TABLE 67 Blind Trial Results.

SPECIMEN	DILUENT	IFA	ELISA
Lysed Blood 697	PBS - Tween	8	500
Lysed Blood 697	PBS-Tween + 4% BSA	8	500
PBS Eluate 697	PBS - Tween	4	500
PBS Eluate 697	PBS-Tween + 4% BSA	4	500
Lysed Blood 705	PBS - Tween	64	? *
Lysed Blood 705	PBS-Tween + 4% BSA	64	2000
PBS Eluate 705	PBS - Tween	32	? *
PBS Eluate 705	PBS-Tween + 4% BSA	32	2000

Table 68 Comparison of HSV 1 antibody titres obtained from lysed bloods & PBS eluted stains by the IFA and ELISA techniques

* = End point of titration unreadable

Table 69 Blind Trial involving ELISA & IFA.

SAMPLE	HSV I IFA	HSV I ELISA	ϕB_1 IFA	ϕB_1 ELISA
700	128	1000	N	125
701	16	< 125	N	< 125
703	128	1000	8	125
704	128	1000	N	125
389	16	250	64	250
393	64	1000	N	125
697	8	125	N	500
705	64	2000	16	125
698	32	1000	N	125
702	128	250	8	125
700P	64	1000	4	250
701P	4	125	4	125
703P	4	500	16	500
704P	64	250	N	125
389P	64	250	64	1000
393P	32	500	N	125
697P	4	125	4	500
705P	8	2000	16	500
698P	4	500	4	250
702P	32	250	16	250
700T	32	4000	4	250
701T	4	250	N	125
703T	4	2000	4	125
704T	32	500	N	500
389T	32	500	32	1000
393T	32	2000	N	125
697T	4	125	N	125
705T	8	1000	4	125
698T	4	2000	N	125
702T	32	500	4	125

CHAPTER IV

DISCUSSION

a) Comparative Serology

Experiment 1 Comparative Serology with Herpes Simplex Virus type 1 (HSV 1)

HSV1 is known to produce a wide variety of diseases in humans. It is most frequently acquired in childhood (from 2-4 years) and is usually retained in the sensory cells of the trigeminal nerve ganglion for life (Timbury, 1974). The fact that 80% of adults have relatively high titres of neutralising and complement fixing antibodies (Davies, et. al. 1973) indicates that HSV 1 is a very prevalent human pathogenic virus. When the initial infection (primary infection) recedes virus persists producing a latent infection despite the presence of a high antibody titre. The form in which the virus exists during the occult stage remains unknown but the balance is readily upset by many different factors such as heat, cold, sunlight and emotional disturbances, provoking the second form of herpes simplex, recurrent disease. Although primary herpetic infections are frequently mild with localized lesions, recurrent infections may result in serious damage and even death of the host.

Gilman, et. al. (1977) investigated an ELISA technique and a complement fixation test for determining antibodies to HSV 1 in 30 human sera. Statistical evaluation of this data by correlation coefficient and Students t-test revealed a significant relationship between CF and ELISA ($r = 0.54$; $P<0.001$). In two instances, however, conflicting results were found. In one, a high CF result (256) produced only a minimal ELISA titre (4096) and in the other, a high ELISA titre (32,768) produced only a minimal CF titre (32). Gilman, et. al. suggested that these differencies reflected the type

of immunoglobulin detected by the individual procedures. CF detects IgM and three of the four subclasses of IgG indiscriminantly whereas ELISA (because of the nature of the anti-IgG-enzyme conjugate used in the assay) is primarily directed at IgG (all subclasses) although some IgM may be detected because of the presence of antibodies to light chain determinants (Engvall and Perlmann, 1972).

The above experiment of Gilman, et. al. involved correlating a classical reaction (CF) with a globulin-antiglobulin based technique (ELISA). The present study involved determining the HSV1 antibody content of 45 human sera by means of two globulin-antiglobulin based techniques (IFA and ELISA) to investigate whether discrepancies could still arise. The neutralising power of a serum reflects the degree of protection against a virus. In HSV 1 infection, neutralising antibodies prevent the virus disseminating to other cells in the host although it does reach contiguous cells. The relevance of the titres obtained with the ELISA and IFA assays was therefore investigated by correlating them with the results obtained by neutralisation tests performed on the same sera.

The results are detailed in Table 14.

Examination of the results revealed several anomalies. Sera 1/2884 and 1/1424 repeatedly recorded mid range ELISA values (8000) but very high IFA titres (512 and 256 respectively). The IFA results, therefore, paralleled the high neutralisation titres (both = 100) more closely than the ELISA results. Sera 12/0393 and 11/2823 each recorded ELISA antibody titres of 16000 but very

low neutralisation titres (10) which again paralleled the IFA titres of 32 and 64 respectively. Antibodies detected by ELISA are therefore not a reliable judge of neutralising antibody present in serum.

Anomalous results were also recorded with the IFA technique, however. Serum 2/1570 repeatedly produced a low IFA result (8) but a significant neutralisation titre (50). Serum 2/1600 produced a mid range IFA titre of 64 but no detectable neutralising antibodies. Serum 12/1578 produced a high IFA titre (512) but a very low neutralising titre (10). The possibility of Fc binding accounting for this latter IFA result was discounted since the same serum produced only a mid range ELISA titre (8000).

Analysis of the results by correlation coefficient (r) and Students t-test revealed the following relationships:-

	r
Ab titres obtained with IFA cf Ab titres obtained with ELISA	0.76
Ab titres obtained with IFA cf Ab titres obtained with neutralisation	0.79
Ab titres obtained with ELISA cf Ab titres obtained with neutralisation	0.47

Antibody titres obtained with the IFA and ELISA techniques showed a highly significant correlation ($r = 0.76$; $P < 0.001$). The value of 0.76 for r is substantially higher than the 0.54 value obtained by Gilman, et. al. (1977) in their similar study involving the comparison of ELISA and CF techniques for detecting HSV1 antibodies in human sera. This is to be expected since the IFA and ELISA reactions are based on the same fundamental theory, that of detecting IgG by an anti-IgG antibody labelled with either

a fluorescent marker or an enzyme. It was concluded that ELISA was as capable of detecting HSV1 IgG as IFA but approximately 250 times more sensitive. When the IFA results were compared with the neutralisation test results, a highly significant correlation was obtained ($r = 0.79$; $P < 0.001$) but the ELISA results gave a lower correlation coefficient ($r = 0.47$) by the same comparison.

Martin, Palmer and Kissling (1972) investigated the use of 3 fractions of HSV 1 as CF antigen. The 3 fractions separated by density gradient centrifugation were a) capsid Ag, b) envelope Ag and c) non-virion associated soluble components. Serological studies showed that convalescent phase sera contained higher levels of CF antibodies to envelope antigens than capsid antigens and sera from human who had experienced natural HSV 1 infections contained very low titres of CF antibody (< 8) to soluble antigens.

During the preparation of HSV1 antigen for use in the ELISA experiments, sonication and centrifugation through 30% sucrose was involved which probably resulted in the production of non-enveloped capsid antigen devoid of any soluble components for sensitization of the ELISA plates. This explains the discrepancies between the ELISA and neutralisation results.

The ELISA technique measured antibodies directed against the capsid antigen alone but neutralising antibodies are directed against the virion envelope. This fact coupled with the low correlation coefficient obtained with ELISA and neutralisation

suggests that the ELISA technique is capable of missing the relevant protective antibodies produced in HSV1 infection. IFA, however, can also produce "spurious" results but on the basis of a high correlation coefficient with the neutralisation test results, it appears to detect the more relevant protective antibodies.

Experiment 2 Comparative Serology with Coxsackie B_1 virus ($\not{C}B_1$)

Coxsackie viruses were first identified by Dalldorf and Sickles (1948). Coxsackie viruses belong to two groups on the basis of the lesions which they produce in suckling mice. Group A viruses produce a diffuse myositis with acute inflammation and necrosis of fibres of skeletal muscles; Group B viruses cause focal areas of degeneration in the brain, focal necrosis in skeletal muscle and inflammatory changes in the dorsal foot pads, pancreas and occasionally the myocardium (Davis, et. al. 1973).

In humans the coxsackieviruses can produce a remarkable variety of illnesses and the same virus may be responsible for quite different types of disease. Coxsackie B viruses can produce sore throats, "aseptic" meningitis, epidemic pleurodynia or pericarditis. Group B viruses may also produce myocarditis of new-born babies (Davis, et. al. 1973).

Diagnosis of coxsackievirus infections depends upon isolation of the causative agent from faeces, throat secretions or cerebrospinal fluid, however, since many enteroviruses can exist as commensals in the intestinal tract of man, identification of the pathogenic nature of a newly isolated virus demands the demonstration of a serological response by neutralization, immunofluorescence, complement fixation or haemagglutination-inhibition titrations as appropriate.

The immune response in humans to infection with a group B coxsackievirus consists of the development of IgM type specific antibodies (Schmidt and Lennette, 1962) as well as IgG antibodies. These IgG antibodies tend to have cross reactivity with other coxsackieviruses of the B group.

This experiment investigated the IgG antibody titres obtained with the IFA and ELISA techniques and examined the relevance of these antibodies by measuring their degree of correlation with the titres of type specific neutralising antibodies present in the same serum specimens.

The results in Table 15 were analysed by the correlation coefficient and Student's t-test and yielded the following information:

Ab titres obtained with IFA cf Ab titres obtained with ELISA $r = 0.17$

Ab titres obtained with IFA cf neutralisation Ab titres $r = 0.29$

Ab titres obtained with ELISA cf neutralisation Ab titres $r = 0.24$

Neither the IFA results ($r = 0.29$; $P < 0.1$) nor the ELISA results ($r = 0.24$; $P < 0.2$) showed any statistically significant correlation with the neutralisation results. Comparison between the IFA and ELISA results produced less correlation ($r = 0.17$; $P < 0.3$). Furthermore, the IFA and ELISA tests frequently detected antibodies in the absence of any corresponding neutralising antibodies e.g. Serum Nos. 2/1149; 11/1681; 12/0846; 12/1702 and 2/0145.

Schmidt, Dennis, Frommhagen and Lennette (1963) showed that the diagnostic value of the CF test in Coxsackievirus infections was limited by a high proportion of heterotypic reactions. Studies which have been conducted on the development, specificity and persistence of HAI antibodies in enterovirus infections have shown that homotypic HAI antibody responses parallel neutralising antibody responses (Schmidt, Dennis, Lennette, Ho, Shinomoto, 1965). In echovirus infections, however, heterotypic HAI antibody and antibody titre rises have been demonstrated in the absence of corresponding neutralising antibody (Bussell, Karzon, Barron and Hall 1962). Heterotypic CF reactivity has been elucidated by

Schmidt, et. al. (1963) by the finding that most of the coxsackievirus CF antigen which reacts with antibodies in human sera is comprised of noninfectious, empty virus capsids which have broader serologic reactivity than the intact virion in CF and precipitation tests.

The results of my experiment (above) indicate that the IFA and ELISA tests are detecting heterotypic antibodies directed against empty capsid antigen whereas the neutralisation test detects homotypic antibodies directed against intact virions.

Schmidt, et. al. (1965) showed that in coxsackievirus or echovirus infections, antibodies to the empty capsid antigen are generally present in the acute phase serum, apparently due to past infections with viruses sharing the group reactive antigen. Type specific antibodies to the virion antigen develop relatively early in the course of infection and disappear within a few weeks while antibodies to the empty capsid antigen persist. In the case of coxsackievirus infections, this type of antibody response has been elucidated by the demonstration that the antibodies reacting with the virion antigen are IgM in nature, while those reacting with empty capsid component are IgG (Schmidt, Lennette and Dennis, 1968).

Thus, the IFA and ELISA techniques appear to detect a large variety of the heterotypic IgG antibodies inherent to the coxsackieviruses. They, therefore, supply little relevant information to the serologist to enable him to confirm or refute a diagnosis, although IFA is marginally superior to ELISA (see page 259)

Experiment 3 Comparative Serology with Rotavirus

The study of Rotaviruses and their importance in disease is relatively new to clinical research. It had been recognised for many years that the neonates of many species of animals frequently suffered from a disease of unknown origin (certainly not bacterial) characterised by fever and diarrhoea of varying severity.

In 1969, Mebus, Underdahl, Rhodes and Twiehaus reported the experimental production of diarrhoea in "colostrum deprived" calves by inoculation of faecal material from field cases. Bacteria cultured from the faeces of calves with diarrhoea did not cause disease when administered orally to healthy calves whereas bacteria-free faecal filtrates appeared to cause diarrhoea. Numerous virus-like particles, approximately 65 nm in diameter were observed in extracts of faeces from such diarrhoeic animals. Further characterisation of this virus showed that it was similar to the reovirus group in morphology but was serologically unrelated to reovirus types 1 and 3 (Fernelius, Ritchie, Classick, Norman and Mebus, 1972).

In 1973, Bishop, Davidson, Holmes and Ruck, also using an electron microscope observed the presence of orbi-virus-like particles in biopsy material from children with acute non-bacterial gastroenteritis. Virus particles, morphologically indistinguishable from the calf reo-like virus have since been detected in the faeces of children with gastroenteritis throughout the world. Similar orbi- or reo-like viruses have been detected in the faeces of a number of animal species and it has been suggested by Davidson, Bishop, Townley, Holmes and Ruck (1975) that these viruses should

be included as a new genus within the family Reoviridae. As yet no official name for this group has been adopted. The names "duovirus" (Davidson, et. al. 1975) and "Rotavirus" (Flewett, Bryden, Davies, Woode, Bridger and Derrick, 1974) have been proposed: Rotavirus has proved the most popular.

The most common age for rotavirus infection in children is between 6 months and 1 year (Madely, Cosgrove, Bell and Fallon, 1977) but infection of adults, though less severe symptomatically, has also been reported (von Bonsdorff, Hovi, Makela, Hovi and Tevalroto-Aarnio, 1976). Rotavirus infections are often encountered where humans live in close proximity to one another, e.g. children's homes, hospitals and army barracks.

Rotaviruses are stable in faeces (Woode and Bridger, 1975) and are relatively resistent to commonly used disinfectants such as chloros and lysol (Snodgrass and Herring, 1977). In humans there is a seasonal incidence of rotavirus gastroenteritis with a greater incidence of infection during the winter months (Davidson, et. al. 1975).

The diagnosis of rotavirus infections is usually based on the detection of virus or viral antigen in faecal samples. A number of techniques have been employed:- electron microscopy (Bishop, Davidson, Holmes and Ruck, 1974), immunofluorescent staining (Foster, Peterson and Spendlove, 1975), complement fixation (Kapikian, Cline, Mebus, Wyatt, Kalica, James, Vankirk, Chanock and Kim, 1975). Radioimmunoassay (Middleton, Holdaway, Petrie, Szymanski and Tam, 1977), immunoelectrophoresis (Grauballe, Genner, Meyling and Hornsleth, 1977) and ELISA (Yolken, Kim, Clem, Wyatt, Kalica, Chanock and Kapikian, 1977).

Von Bondsdorff, Hovi, Makela and Morttinen (1978) question the use of these techniques alone in Rotavirus disease diagnosis without a serological technique for comparison. In their examination of diarrhoea faecal samples for Rotavirus particles by electron microscopy alone, 25% of examined cases were found to be Rotavirus positive. This figure increased to 40% when a combination of electron microscopy and examination of the antibody status by complement fixation test using Nebraska calf diarrhoea virus (NCDV) antigen was employed for each patient. Von Bonsdorff, et. al. (1978) concluded that the relatively poor efficacy of electron microscopy compared to serology was due to the fact that in many cases the first stool sample was obtained only after the worst of the diarrhoea was over. Rotavirus infection has a short incubation period, before symptoms appear, ranging from 15 hours to 3 or 4 days (Middleton, Szymanski, Abbott, Bortolussi and Hamilton, 1974) therefore since diarrhoea is present for only 24 to 48 hours (McNulty, 1978) it is easy to miss the peak excretion of virus in the faeces, if the virus is the main irritant causing diarrhoea.

Examination of methods for the demonstration of antibody to Rotavirus is, therefore, of great importance and for this reason three serological techniques were investigated.

Human Rotavirus can only be cultured in human kidney cells and so the IFA and ELISA tests were conducted with Nebraska Calf Diarrhoea Virus (NCDV) which can be cultured in primary calf kidney cells. This virus is closely related antigenically to human rotavirus but it was considered pointless to evaluate the

detection of relevant antibodies by neutralisation tests with a calf Rotavirus instead of a human Rotavirus.

The complement fixation test, however, developed by Zissis involved testing the human sera for CF antibodies using a human faecal rotavirus as antigen in a faecal extract and so the question of detecting relevant antibodies was confined to investigating which of the two serological tests, IFA or ELISA correlated best with the CF results.

The results in Table 16 were analysed by the correlation coefficient (r) and Student's t-test and yielded the following information:-

	r
Ab titres obtained with IFA cf Ab titres obtained with ELISA	0.41
Ab titres obtained with ELISA cf Ab titres obtained with CF	0.2
Ab titres obtained with IFA cf Ab titres obtained with CF	0.38

A statistically significant correlation ($r = 0.41$; $P < 0.025$) was obtained between the IFA and ELISA results. This is to be expected since both tests involved the same strain of Rotavirus. The ELISA test was approximately 3 times more sensitive than the IFA technique.

When the IFA and ELISA results were compared with the CF results, however, only the IFA results produced a statistically significant correlation with the CF antibody titres ($r = 0.38$; $P < 0.05$). By contrast the ELISA results showed no significant correlation with the CF results ($r = 0.2$; $P < 0.3$).

The following explanation is offered to account for these findings.

The antibody titres obtained by IFA were obtained by examining calf kidney cells infected with NCDV for 2-3 days, for

intracytoplasmic fluorescent staining. These cells contain many incomplete viral particles. In the ELISA procedure, however, the NCDV was obtained from calf kidney cells after 4 days by which time the tissue culture cells were observed to have detached from the glass surface indicating that mature complete virus was being released into the maintenance medium. The NCDV was then purified by centrifugation through 30% sucrose. In this procedure, therefore, any soluble and unassembled viral antigens would be separated and only complete virus or viral capsid would be obtained to sensitize the microtitre plate wells.

Intact Rotaviruses have been shown to have a double layered capsid structure (Holmes, Ruck, Bishop and Davidson, 1975). Woode, Bridger, Jones, Flewett, Bryden, Davies and White (1976) showed that single-shelled rotavirus particles from different animal species were agglutinated by all the convalescent sera tested, regardless of the speciesof origin and antibodies could be detected adhering to the inner capsid layer by immune electron microscopy. Double-shelled human rotavirus particles, however, reacted only with antiserum to the human virus. These results indicated that the rotavirus group antigen is located on the inner capsid layer of the virus.

Inaba, Sato, Takahashi, Kuogi, Satoda, Omori and Matumato (1977) discovered that a haemagglutinin is present in preparations of calf rotavirus. Separation of double and single shelled virus particles on caesium chloride density gradients demonstrated that double-shelled particles agglutinated erythrocytes but single-shelled particles did not. This finding suggested that

the species-specific antigen resided on the outer capsid layer of the virus.

The results from the ELISA experiment detailed above appear to confirm these findings since the 30% sucrose purification procedure would ensure that the Rotavirus antigen involved inthe ELISA experiment is double shelled NCDV capsid. These capsids are sufficiently different antigenically from the human antigen involved in the CF test to produce many conflicting results:-

Serum No.	ELISA titre	CF titre
3225	< 32	32
3540	< 32	16
3733	< 32	16
4751	< 32	32
2460	< 32	32
4119	512	0
1147	512	8

The IFA test, however, because antibodies to the unassembled virus antigens including the group antigen are detected, is therefore a more reliable technique for detecting the relevant human antibodies to human rotavirus.

b) Virus Antibody Profiling

At the outset of the forensic section of this project, a serological test had to be chosen which would meet the following requirements of the proposed virus antibody profiling procedure.

1) Capable of utilizing very small volumes of eluted bloodstain
2) Specific enough to yield reliable and reproducible results with antibodies which may have been exposed to adverse "storage" conditions in the form of a bloodstain
3) Capable of measuring antibody levels against a wide spectrum of viruses in the bloodstain
4) Capable of handling large numbers of such specimens quickly and efficiently.

Many of the serological tests discussed in Section a of the literature review were obviously incapable of satisfying some or all of these requirements.

The complement fixation test, for example, depending upon the development of haemolysis to indicate the existence of antiviral antibodies in a serum or plasma specimen, is therefore unsuitable for use with lysed blood specimens in the form of bloodstain eluates. Complement fixation is also very prone to anticomplementary reactions which are commonly associated with microbially contaminated bloodstain eluates.

The haemagglutination-inhibition test is more sensitive but would similarly not be capable of registering positive or negative results in the presence of lysed blood. This technique also has problems associated with the large number of potential non-specific

inhibitors which can produce false positive results.

Neutralisation tests can fulfil most of the proposed requirements since they are sensitive, specific and not influenced by bacterial products or haemoglobin but have the disadvantage of being labour intensive and time consuming.

The three techniques which, it was considered, could fulfil the aforementioned requirements were, Radioimmunoassay (RIA), Enzyme-linked immunosorbent assay (ELISA) and the Indirect fluorescent antibody technique (IFA).

RIA, unfortunately, could not be evaluated for use in virus antibody profiling since it required specialised equipment which was not available to this laboratory. ELISA is a relatively new technique which still requires development and so its possible part in the present context was investigated for only two viruses.

IFA, however, was in routine use within this laboratory and was considered to have the potential to meet all the requirements detailed above.

The major part of this discussion, therefore, deals with the IFA test and its application to forensic virus antibody profiling.

Before any bloodstain discrimination trials could be attempted, however, a thorough evaluation of the IFA test as applied to virus antibody profiling was performed.

Experiment 1 Replicate Antibody Profiles

Tables 17 through 21 contain the results of 10 replicate virus antibody profiles on each of 5 different plasma samples. Tables 22 through 26 show the replicate virus antibody profiles from 5 different bloodstain eluates.

These results show a fairly poor reproducibility. For example in Table 17 the antibody titres obtained by IFA from replicate samples of the same plasma examined within 2-3 days show Herpes simplex values ranging from Log_2 3 to Log_2 8. In Table 18, the antibody titres obtained for Poliovirus type 1, . Echo 19, Measles, Flu A $3\frac{1}{2}$ hr and CMV range from zero to $Log_2 6$.

Similar variations in antibody titres to many viruses are also evident in Tables 19, 20 and 21.

The same observations of poor reproducibility can be detected in the replicate virus antibody profiles resulting from bloodstain eluates recorded in Tables 22 through 26.

To account for these variations, three explanations were considered:-

1) Lack of skill of the microscopist in recording the fluorescent antibody end point results. This is notoriously difficult and subjective errors may occur - particularly with operator fatigue. There is no doubt that this is the most important problem in using IFA for antibody level determination. It is a problem which can only be overcome by the introduction of metered reading of specimens, e.g. by reflection spectrometry or by integrating circuitry.

2) Genuine differences exist in the amount of antibody present in each sample from the same specimen.
This cannot occur with replicate plasma specimens from the same plasma sample which have been stored and handled in identical conditions.

Differences might arise, however, between antibody levels in plasma and bloodstain eluates because the amounts of IgG type antibody present in the eluate would depend upon the efficiency of the elution procedure. However, if one assumes that haemoglobin and IgG are eluted from cotton cloth with the same efficiency (as suggested by Werrett- Personal Communication) a low percentage recovery of haemoglobin should similarly give the lowest values in the virus antibody titre ranges detected. This does not always occur.

In Table 22 it can be seen that two eluates, G & H, have been recovered with the same efficiency (36% of the haemoglobin has been recovered in both specimens). Variations still occur, however, between the amount of antibody detected to certain viruses in these two eluates. With measles virus, for example, an antibody titre of $Log_2 4$ was detected in specimen G whereas no anti-measles IgG was detected in specimen H. Paradoxically, specimen C with a greater haemoglobin recovery value (45%) shows no anti-measles IgG whereas specimens A & F (also with 45% haemoglobin recovered) produced anti-measles IgG titres of $Log_2 4$ and $Log_2 3$ respectively.

In Table 23, the same type of anomaly is evident with Herpes simplex virus. Specimen A with a haemoglobin recovery of 53%, gave a titre of $Log_2 3$ for anti-Herpes simplex IgG, while specimen E, with slightly less of a haemoglobin recovery (49%) recorded a titre of $Log_2 8$ for anti-Herpes simplex IgG.

Numerous similar anomalous results can be found in the 3 other bloodstain reproducibility experiments recorded in Tables 24 through 26.

Poor reproducibility was therefore evident in these initial experiments but this could not be attributed to differences in the amount of IgG recovered from a bloodstain.

3) Varying quality of the virus antigen preparations used to make the slides used for the indirect fluorescent antibody technique. It is most unfortunate that due to the large numbers of slides used in the course of these experiments, more than one batch of antigen carrying slides was often involved in performing a replicate antibody profiling experiment.

Although each batch of antigen slides was carefully quality controlled by performing an IFA test, with an antiserum with known titre, on several randomly selected slides, differences in quality were found with different slide batches as follows:

a) Despite meticulous care, variations inevitably occurred in the amount of infected cell material placed on each spot during slide preparation.

b) Long term cold storage ($-70^{o}C$) had a deleterious effect on pre-spotted slides. This appeared to be due to damage caused to the infected cells by the accumulation of ice crystals on the spots and from slides frozen together by a thin film of ice.

It was therefore decided to include a standard reference serum spot on each slide to monitor the quality of the antigen spots.

Tables 27 and 28 record the results of two further plasma replication VAP experiments incorporating the standard dilution of reference serum on one spot on each slide examined and also utilising a single batch of virus antigen slides for each experiment performed. These tables show better correlation of replicate antibody titres to most of the viruses investigated. Occasional discrepancies are evident within the undiluted (N) to Log_2 3 range of antibody titres (see Table 28, Polio 1, Polio 2, Polio 3, ¢B_1, Echo 5, Echo 19, Adeno 2, Flu A $3\frac{1}{2}$). These discrepancies are caused by the extreme sensitivity of the IFA technique where faint non-specific fluorescence often appears with titres in the range Log_2 1 to Log_2 3.

Tables 29 through 33 record the results of 5 replicate bloodstain eluate VAP experiments performed in a similar manner with the reference serum sample.

In these experiments the virus antibody profile was considerably shortened following discussion with Dr. D. Werrett (Central Research Establishment, Aldermaston) after statistical analysis of the results from Experiment 2 (Tables 29-33). Analysis of the frequency of occurrence of IgG type antibodies to significant levels (i.e. $\geq Log_2 3$) against the 19 viruses in the profile revealed that the best viruses for discriminating between blood samples were Polio 1, ¢B_1, Echo 19, Herpes simplex, Adeno 5, Respiratory Syncytial virus, Epstein Barr and cytomegalovirus.

The dramatic improvement in reproducibility (to within $\pm$ 1 Log_2 dilution in the majority of the antigens investigated) is clearly evident from Tables 29 through 33.

A reference serum was therefore included in all subsequent experiments.

Experiment 2 Virus Antibody Profile Survey

Having established that the indirect fluorescent antibody technique could be made sufficiently accurate and reproducible, the potential of virus antibody profiling for characterization of a blood sample was investigated.

Plasma samples from one hundred randomly selected adult individuals (Tables 34 through 37) were profiled and the results are recorded in Tables 38 through 47.

This experiment was performed for two reasons:-

1. To find out whether each plasma sample was unique in terms of VAP
2. To provide information for statistical analysis (performed by Dr. D. Werrett, Central Research Establishment, Aldermaston).

1. No VAP's were found which were exactly alike. In a population consisting of m objects (virus antibodies) with n possible values for each object (antibody titre), the probability of obtaining 2 sets of identical values is $1:m^n$. With up to 80 different viruses and 8 antibody titres (N through $Log_2 7$), 80^8 different antibody profiles are possible. Even with the shorter profile consisting of 8 viruses, 8^8 different profiles can, theoretically, be obtained.

   In practice, despite these theoretical considerations, profiles may well show relatively minor differences. This can be observed from profiles 37 and 38 (Table 41) where the plasma

donors were siblings. Their immunological experience has obviously been very similar with only Flu A $3\frac{1}{2}$ hr antigen and possibly Adeno 2 antigen providing any evidence of individuality within the short profile used in this experiment.

2. As a result of computer analysis to determine the frequency of occurrence of antibody titres to the 19 viruses in the 100 antibody profiles, 8 viruses were selected from the original 19 which showed the greatest potential for discrimination between individual blood samples.

   In general, the computer analysis revealed the expected cross reactivity between the Polioviruses, Echoviruses, and Adenoviruses employed in the full profile. Other viruses such as mumps, measles, influenza A and B seldom produced antibody titres outwith the basal range (N to $Log_2 4$) and so provided less potential discrimination between individuals. These viruses, however, could not be totally rejected. They would provide further characteristics which might enable a discrimination to be achieved between blood samples when the 8 viruses in the primary profile failed to discriminate.

Experiment 3 *In vivo* stability of VAP

With the conventional methods of blood typing and identification, the time interval between receipt of the stain and arrival of blood for comparative purposes is not particularly critical. The normally used tests rely upon genetically controlled polymorphisms which occur in blood (blood group, rhesus factor and red cell iso-enzymes) and do not normally change with time. These factors could possibly be changed slightly only by blood transfusion.

If, for example, the victim of an assault requires a blood transfusion, he will be grouped in respect of ABO and rhesus type only. Therefore a person with the red cell enzyme phosphoglucomutase 1 (PGM 1) may receive blood of type PGM 2 or PGM 2-1. Such an event would alter his blood type for 4-6 weeks (the life-span of the transfused red blood cells). Similarly, transfused plasma containing immunoglobulin to viruses encountered by the donor would be metabolised within 3 months. The dilution effect would, however, make it extremely unlikely that its presence could ever be detected.

The next experiment was designed to investigate the stability of the virus antibody profile in vivo.

Since IgG is not a static material but is constantly being produced by B-lymphocytes, the concentration, type and specificity of IgG must be expected to change with time, e.g.

1. On encountering new virus Ag, B-lymphocytes involved with humoral immunity will secrete new IgG molecules specifically directed against the new invading antigen.
2. A previously high titre of IgG to one particular virus, possibly indicative that the blood sample has been taken during the convalescent stage of an illness may slowly fall. (The level of IgG in serum reflects the balance between rates of synthesis and degradation).

Tables 48 and 49 record the results of virus antibody profiles from pairs of plasma samples taken at intervals of up to 6 months apart from 7 different donors. In most cases a maximum of only 2 $\log_2$ dilutions was found between the antibody titres in the profiles of each pair of specimens.

In specimen E, however, anti-Echo 9 IgG which had not been present in the first specimen was detected at a titre of Log_2 5 in the second specimen taken 2 weeks later. This suggested that the donor was currently or recently infected with Echo 9 virus or a close antigenically related agent.

Inspection of the results also reveals that on 5 occasions no antibody was found in the second samples to certain viruses which had given positive titres in the first samples. This must be regarded as experimental error since in each case the first recorded level was low enough to fall into the zone where interpretation is difficult.

It was concluded that apart from the obvious example of recent infection with a virus which was to be included in the profile, virus antibody profiles change very little between samples taken at intervals of up to 6 months apart.

Experiment 4 VAP temperature stability in vitro

Bloodstains are usually left in a random manner and may not be found until many days or weeks have passed. They may also be buried on clothing. In this experiment I investigated the effect of storage at 4°C and ambient temperature on stain recovery and the resulting virus antibody profile. The results are recorded in Tables 50a through 57.

After storage at 4°C for up to 67 days it is evident that haemoglobin is recovered adequately (Table 53a). Storage of the same blood as a stain at room temperature for the same time (Table 53b) yielded a much lower quantity of haemoglobin.

Room temperature storage generally yielded lower haemoglobin recovery than 4°C storage (Tables 50a through 57). Room temperature storage of bloodstains therefore presents greater difficulties of elution for the forensic scientist than low temperature storage.

Table 53b shows a low haemoglobin recovery (18%) from an eluate recovered from a stain exposed to ambient temperature for 66 days. Many of the antibody titres from this eluate were also low (mumps IgG titre had decreased from $Log_2 4$ to N after 66 days). Antibodies to Herpes simplex RSV and EBV, however, appeared to be more stable after room temperature storage in the bloodstain eluate.

Similar figures can be seen in Table 54 where relatively high IgG titres were recorded for Echo 5, Herpes simplex, EBV and CMV from an eluate yielding a haemoglobin recovery of only 22%.

In Table 50a, IgG to Polio 1 was not detected in a stain eluted after 2 days at 4°C whereas it was present at a titre of $Log_2 5$ in the original blood sample. Antibody to Flu B was similarly not detected in the same eluate recovered after 28 days at 4°C.

Table 52a shows the apparent 'de novo' appearance of anti-Coxsackie B, antibody and anti-mumps IgG in an eluate recovered after 29 days at 4°C. This can only be the microscopist's error and is simply faulty reading of the slides.

The results of this experiment can be summarized as follows:-

a) From examination of the percentage haemoglobin recovery of stains stored at 4°C and room temperature it was apparent that stains can be eluted more easily and efficiently after low temperature storage.

b) Long term room temperature storage of bloodstains (120 days), Table 54, results in very little haemoglobin and almost no antiviral IgG being eluted from the sample when the standard elution procedure is employed.

c) Herpes simplex, EBV, CMV and CB_1 antibodies appear to be more easily recovered than others from stains stored at room temperature and more easily detected by the indirect fluorescent antibody test.

d) Serious inconsistencies can occur when comparing the profiles of eluted bloodstains with their original lysed blood profiles.

Experiment 5 VAP Stability in vitro (Effect of Humidity)

Bloodstained material may encounter various degrees of humidity either at its place of disposal or in the forensic laboratory itself. It is a common occurrence for rain soaked bloodstained material to be transported and stored in sealed polythene bags. Humid conditions are therefore frequently produced by accident and these provide ideal conditions for bacterial and fungal growth.

Table 58 records the results of an experiment in which bloodstained material was left exposed to various degrees of humidity for 7 days at room temperature before being profiled for comparison with a profile from the original lysed blood.

After 7 days slight fungal contamination was evident on the bloods stored in the chamber containing 1 ml of water whereas the stain in the chamber containing 5 ml of water was heavily contaminated with fungal colonies.

Despite slight fungal growth, the results in Table 58 show a good correlation of results from the bloodstains stored in 0.1 ml, 0.5 ml and 1.0 ml chambers for 7 days. The results also reveal the overall decrease in antibody titres and haemoglobin recovery obtained with the eluate from the stain exposed to the most humid conditions (5.0 ml H_2O).

It was concluded that storage under slightly humid conditions made no appreciable difference to the antibody profile but severe fungal contamination of the bloodstain resulted in an eluate depleted in antibody and haemoglobin, because these and other blood proteins were acting as substrates for the fungal growth.

Experiment 6 VAP Blind Trials

Having investigated Virus Antibody Profiling in some depth, a few potential areas of difficulty were observed which could lead to false results being recorded in the antibody profiles from bloodstain eluates. Being aware of these potential difficulties, a series of blind trials was organised to determine how well the VAP technique could discriminate between different bloodstains and aid in matching the correct eluates with their original blood samples (Tables 59 through 67).

Blood group information (ABO and Rhesus) was made available for Trials 2 through 6.

The pairing of profiles from eluted bloodstains with lysed blood samples by their Virus Antibody Profiles was performed visually following four basic rules.

a) A lysed blood sample which showed no antibody to a particular virus antigen should not be expected to show antibodies to the same antigen in the form of an eluted bloodstain.

Stains which showed high titres of antibodies to various antigens could confidently be rejected as possible pairs to lysed blood samples showing no detectable antibodies to these antigens. e.g. In blind trial No. 3, stains C and D have many similarities. From blood group data, the only 2 possible matches (O +) are bloods 1 and 7.

The correct matching was achieved by means of CMV antigen where antibodies were only found in blood No. 7

($\log_2 4$). Stain C showed no antibodies whereas stain D produced antibody to a titre of $\text{Log}_2 2$. It was therefore concluded that blood 1 and stain C (No CMV antibodies) and blood 7 and stain D should be the correct pairs.

The haemoglobin concentrations of the stains also aided in substantiating this choice of pairing since blood 1 (14.4 g/100 ml) and stain C (7.7 g/100 ml) showed higher haemoglobin concentrations than blood 7 (12.4 g/100 ml) and stain D (6.0 g/100 ml).

It must be noted, however, that in some cases this procedure of eliminating blood/stain matches was complicated by the apparent de novo appearance of antibodies in some stains - occurring merely as a result of operator error. Several examples of this phenomenon can, unfortunately, be found in Blind Trial No. 1 and can only be explained as being due to poor technique on the part of the operator on his first Blind Trial Experiment.

b) Virus antigens showing high antibody titres ($\geqslant \log_2 5$) in both lysed blood and eluted stains were taken as indicative of pairing. e.g. In Blind Trial No. 6, stain D shows high antibody titre ($\log_2 6$) to Polio 1, Echo 19 and Herpes Simplex antigens. The only lysed blood to show similar high antibody titres to all 3 antigens is blood 1 ($\log_2 7$ to Polio 1, Echo 19 and Herpes simplex). Blood group data was also indicative of a match (both being 0+).

c) Patterns of closely related titres in both lysed bloods and eluted stains were taken as indicative of pairing.

e.g. In Blind Trial No. 2 the blood group data showed that bloods 5 and 6 must be paired with stains B and I. Blood 6 presented a remarkable profile in that every antigen had an antibody titre of $\log_2 4$. Stain B showed a similar profile in that every antigen had antibodies to a titre of $\log_2 2$ or $\log_2 3$. The absence of Epstein-Barr Virus antibodies in blood 5 and stain I was also indicative of correct pairing.

d) Where the results obtained using the primary profile antigens were incapable of differentiating between bloods or stains, further antigens were easily introduced into the system to provide additional virus antibody profile information (see Blind Trial No. 4).

Tables 66 and 67 show the matches of the 6 Blind Trials as suggested by virus antibody profiling. The correct matches are also listed.

In Blind Trial No. 1 where no blood group information was available 6 of the 10 blood stain eluates could be matched correctly with their original bloods.

In Blind Trials 2 and 3, with the inclusion of the blood group information, an 80% matching of blood to stains was achieved.

Blind Trial No. 4 required the use of additional virus antigens to discriminate between 5 of the 12 bloodstains. An 83% success rate was thereby achieved.

Blind Trial No. 5 proved very difficult to analyse. The abundance of A+ blood groups did not help to eliminate potential pairs and the investigation had to rely very heavily on virus antibody profiling. A 70% success rate was achieved.

Blind Trial No. 6 again recorded an 80% success rate. In this trial, the bloodstains were eluted with 0.05% trypsin. These eluates were easier to quantitate in the IFA test but still produced errors which resulted in two stains being mismatched.

Overall, the correct stains and bloods were matched on 75.8% of occasions in the 6 Blind Trials.

Experiment 7 VAP by ELISA

The previous experiment had shown that the indirect fluorescent antibody technique was capable of successfully matching eluted bloodstains with their original lysed blood samples on up to 80% of occasions. IFA achieved this with antibody titres in the 1:2 through 1:128 dilution range. ELISA is a much more sensitive technique able to detect some specific antiviral antibodies within the dilution range 1:500 through 1:64,000 (Chapter IV, section a). ELISA's potential contribution to virus antibody profiling in forensic applications was therefore investigated.

This experiment was designed to observe the effect which lysed blood might have on ELISA results. See Table 68.

Lysed whole bloods and eluted stains prepared from 1:500 through 1:64000 in PBS-Tween produced no discernable end point in their titrations in ELISA plates sensitized with HSV 1. The inclusion

of 4% BSA in the PBS-Tween diluent, however, rectified this situation and end points were easily determined as the last dilution to produce a ≥ 0.2 absorbance unit difference over the corresponding specimen at the same dilution in the Vero control antigen well. The ELISA test was also found to be 30-40 times more sensitive than IFA when used to examine lysed blood or eluted bloodstain samples.

Experiment 8 VAP Blind Trial by ELISA

ELISA has been shown to be a very sensitive technique for the determination of antiviral antibodies in serum (Chapter IV, section a). The previous experiment demonstrated its ability to detect antibodies to HSV 1 in lysed blood samples and eluted bloodstains. The results of this experiment (Table 69) indicate that it is also suitable for use in forensic virus antibody profiling.

Analysis of the antibody titres obtained with HSV1 antigen by the correlation coefficient (r) produced the following results.

IFA titres of lysed bloods cf IFA titres of PBS eluted stains $r = 0.37$

IFA titres of lysed bloods cf IFA titres of trypsin eluted stains $r = 0.44$

ELISA titres of lysed bloods cf ELISA titres of PBS eluted stains $r = 0.88$

ELISA titres of lysed bloods cf ELISA titres of trypsin eluted stains $r = 0.45$

The correlation of lysed blood titres with eluted stains titres determined by IFA is increased from $r = 0.37$ to $r = 0.44$ when trypsin is used to elute the bloodstain. Neither correlation, however, is statistically significant ($P < 0.2$ and $P < 0.1$ respectively). This substantiates the findings of Experiment 7 Blind Trial No. 6, that

although the trypsin eluted stains were better suited to the IFA technique than those eluted with PBS alone, false positive results could still be mistakenly observed in the stains.

The correlation of lysed blood HSV1 antibody titres with PBS eluted stain titres is greatly improved when the ELISA technique is employed ($r = 0.88$, $P < 0.001$). The use of trypsin to elute the stains, however, decreases the correlation of the results in ELISA ($r = 0.45$, $P < 0.1$). This is probably due to the trypsin in the stain eluate degrading the antigen coating on the surface of the ELISA plate wells.

This experiment shows that ELISA is a better technique than IFA in determining the HSV1 antibody content of lysed blood and PBS eluted stains for use in forensic virus antibody profiling.

The same statistical analysis was performed on the results obtained from the γB_1 antibody determinations

IFA titres of lysed blood cf IFA titres of PBS eluted stains

$r = 0.99$

IFA titres of lysed blood cf IFA titres of trypsin eluted stains

$r = 0.97$

ELISA titres of lysed blood cf ELISA titres of PBS eluted stains

$r = 0.49$

ELISA titres of lysed blood cf ELISA titres of trypsin eluted stains

$r = 0.31$

The correlation of lysed blood γB_1 antibody titres with eluted stain titres determined by IFA is highly significant (PBS eluted stains $r = 0.99$, $P < 0.001$: trypsin eluted stains $r = 0.97$, $P < 0.001$).

"Almost perfect" correlation was achieved by the IFA technique using φB_1 virus. This improvement over the corresponding HSV1 values detailed above probably reflects the fact that antibodies to φB_1 virus are less frequently found in blood specimens than HSV1 antibody and, therefore, their occurrence is more significant statistically.

The ELISA correlation results again show that stains extracted with 0.05% trypsin ($r = 0.31$) are less suited to use in ELISA than stains extracted with PBS ($r = 0.49$).

The results also show that the IFA technique produces a better correlation of results for φB_1 virus than ELISA.

CHAPTER V

CONCLUSIONS

CONCLUSIONS

From the experiments performed in the course of this project, the sensitivity of the ELISA technique has been shown to be greater than that of the IFA test but its increased sensitivity is dependent upon the purity of the viral antigen preparation. With HSV 1 antigen purified through 30% sucrose, ELISA antibody titres were approximately 250 times higher than the corresponding antibody titres obtained from human sera with the IFA technique. Purification of Rotavirus and coxsackievirus B_1 by the same procedure produced increases in ELISA antibody titres over IFA antibody titres of 10 and 100 times respectively. The ELISA procedure was therefore concluded to be a highly sensitive technique for the determination of antiviral antibody in serum.

Comparison of the HSV 1, & B_1 and Rotavirus antibody titres in human sera detected by IFA and ELISA tests revealed startling discrepancies. Experiments showed that for HSV1 and Coxsackie B_1 virus, the IFA antibody titres, although being lower than ELISA results, nevertheless correlated more closely with the neutralisation results and were therefore more relevant for determining the presence of protective antibody in the sera.

see p 260

Due to the particular problems posed by the fastidious growth requirements of human Rotavirus, calf rotavirus has generally been employed as antigen in the IFA and ELISA tests for the determination of human Rotavirus antibody. The IFA antibody titre results again correlated better than the ELISA results with CF antibody titres obtained in a CF test using a human Rotavirus

faecal isolate as antigen. The IFA results were, therefore, more relevant diagnostically than the ELISA results.

These anomalies between ELISA and IFA results were ascribed to the different types of antigen involved in the respective serological techniques.

The ELISA technique holds great promise for the future in providing a very sensitive, quantitative serological assay. However, a great deal of research will have to be concentrated on identifying which viral antigens will provide the most relevant antibody titre results in virus disease diagnosis or immunity screening, etc.

Virus antibody profiling by the IFA technique, when applied to human blood samples and eluted bloodstains produced results which enabled the matching of stains with original blood samples with a 60% success rate. The inclusion of additional serological information such as blood and rhesus groups aided in the matching of the samples so that a maximum success rate of 83% was achieved.

Where the primary profile of 8 viruses failed to yield sufficient information to enable a matching pair to be identified, further viruses could be introduced into the profile to increase the discriminative power of the technique. This procedure was limited only by the amount of blood sample and stain eluate available.

A limited trial of the ELISA technique showed that it also was suitable for use in virus antibody profiling and that it potentially gave an increase in sensitivity of 30-40 times over the IFA test.

APPENDIX

APPENDIX

Tissue culture media

A. Monkey kidney growth medium

75 ml distilled H_2O

10 ml Eagles MEM + Earles salts

10 ml FBS

1 ml Na HCO_3

1 ml PSNG *

B. Monkey kidney maintenance medium

80 ml distilled H_2O

10 ml Eagles MEM + Earles salts

1 ml FBS

1 ml Na HCO_3

1 ml PSNG

C. Vero growth medium

75 ml distilled H_2O

10 ml 199 + Earles salts

5 ml FBS

2 ml Hepes

2 ml Na HCO_3

1 ml PSNG

D. Vero maintenance medium

80 ml distilled H_2O

10 ml 199 + Earles salts

1 ml FBS

2 ml Hepes

2 ml Na HCO_3

1 ml PSNG

E. Calf kidney growth medium

65 ml distilled H_2O

10 ml 10x Hanks BSS

10 ml FBS

10 ml lactalbumin hydrolsate

1 ml glucose

1 ml Na HCO_3

1 ml PSNG

F. Calf kidney maintenance medium

75 ml distilled H_2O

10 ml 10x Hanks BSS

10ml FBS

10 ml lactalbumin hydrolysate

1 ml glucose

1 ml Na HCO_3

1 ml PSNG

* PSNG = Antibiotic solution + glutamine (100 units/ml penicillin,
10 ug/ml streptomycin, 25 units/ml nystatin
300 ug/ml glutamine)

G. EB3 cell growth medium

100 ml Eagles MEM

10 ml foetal calf serum

2 ml 4.4% Na HcO_3

1 ml PSNG

(MEM with or without arginine)

H. Human embryo lung growth medium

375 ml distilled H_2O

50 ml Hanks BSS

50 ml foetal bovine serum

10 ml Hepes

5 ml non-essential amino acids

2.5 ml Na HCO_3

1 ml PSNG

I. Human embryo lung maintenance medium

100 ml MEM containing 0.85 g/l $NaHCO_3$

2 ml foetal bovine serum

2 ml Hepes

1 ml non-essential amino acids

1 ml PSNG

1 ml Kanamycin and Gentamycin

APPENDIX

ELISA Solutions

Coating buffer (p H 9.6)

$Na_2 Co_3$	1.59 g
$Na H CO_3$	2.93 g
$Na N_3$	0.2 g
Distilled water	1000 ml

PBS - Tween (p H 7.4)

Na Cl	8.0 g
$K H_2 PO_4$	0.2 g
$Na_2HPO_4 12 H_2O$	2.9 g
K Cl	0.2 g
Tween 20	0.5 ml
$Na N_3$	0.2 g
Distilled water	1000 ml

10% Diethanolamine buffer

Diethanolamine	97 ml
Distilled H_2O	800 ml
$Na N_3$	0.2 g

Add a 1 M solution of H Cl and adjust to give p H 9.8

Make total volume 1000 ml with distilled water.

Neutralisation Test

1. Appropriate dilutions (1:10, 1:50, etc.) of the antiserum under investigation were prepared in sterile P.B.S.

2. 0.1 ml of each antiserum dilution was mixed with a 0.1 ml aliquot of virus solution and incubated at 37°C for one hour.

3. Duplicate 0.1 ml aliquots of each serum/virus mixture was used to inoculate a 4" x $\frac{1}{2}$" test tube containing a confluent monolayer of tissue culture cells and 1 ml of maintenance medium.

4. The tubes were incubated at 37°C for 18 hours.

5. The result of the neutralisation test was determined by noting the highest dilution of serum to produce only 50% infection in the tissue culture cell monolayer.

REFERENCES

Armstrong, M.A., Fraser, K.B. and Shirodaria, P.V. (1979)
Journal of General Virology, 44, 541-545

Avrameas, S. (1969)
Immunochemistry, 6, 43-52

Avrameus, S. and Ternynck, T. (1971)
Immunochemistry, 8, 1175-1179

Axen, R., Porath, J. and Ernback, S. (1967)
Nature, 214, 1302-1304

Barbour, H.M. (1976)
Journal of Immunological Methods, 11, 15-23

Bartlett, A., Dormandy, K.M., Hawkey, C.M., Stableforth, P. and Voller, A. (1976)
British Medical Journal, 1, 994-996

Bastiani, R. (1978)
Proceedings of 2nd R.S. First Symposium on Immunoassays, Karger, Basel

Baxter, S.J. and Rees, B. (1974)
Medicine Science and the Law, 14, 231

Belanger, L., Hamel, D., Dufour, D. and Pouliot, M. (1976)
Clinical Chemistry, 22, 198-204

Bernt, E. and Bergmeyer, H.U. (1965)
Inorganic Peroxidase, in "Methods of Enzymatic Analysis", H.U. Bergmeyer Ed. Academic Press.

Bidwell, D.E., Bartlett, A. and Voller, A. (1977)
Journal of Infectious Disease, 136, Supplement, S274-S278

Bishop, R.F., Davidson, G.P., Holmes, I.H. and Ruck, B.J. (1973)
Lancet, ii, 1281-1283

Bishop, R.F., Davidson, G.P., Holmes, I.H. and Ruck, B.J. (1974)
Lancet, i, 149-151

Booth, J.C., Hannington, G., Azis, T.A.G. and Stern, H. (1979)
Journal of Clinical Pathology, 32, 122-127

Bordet, J. (1896)
Annals Institute Pasteur, 10, 193

Bosch, A.M.G., Van Hell, H., Brands, J.A.M., Van Weemen, B.K. and Schuurs, A.H.W.M. (1975)
Clinical Chemistry, 21, 1009

Bradish, C.J., Brooksby, J.B. and Tsubahara, H. (1960)
Journal of General Microbiology, 22, 392-415

Breindl, M. (1971)
Virology, 46, 962

Brody, J.A., McAlister, R., Haseley, R. and Lee, P. (1964)
Journal of Immunology, 92, 854-857

Brown, F. and Crick, J. (1958)
Virology, 5, 133

Bullock, S.L. and Walls, K.W. (1977)
Journal of Infectious Disease, 136, Supplement S279-S285

Burnet, F.M. and Faris, D.D. (1942)
Journal of Bacteriology, 44, 241-248

Burnet, F.M. and Lush, D. (1939)
Journal of Pathology and Bacteriology, 48, 275-286

Burroughs, J.N., Rowlands, D.J., Sangar, D.V., Talbot, P. and Brown, F. (1971)
Journal of General Virology, 13, 73

Bussell, R.H., Karzon, D.T., Barron, A.L. and Hall, F.T. (1962)
Journal of Immunology, 88, 47-54

Carlsson, H.E., Lindberg, A.A. and Hammarström, S. (1972)
Infection and Immunity, 6, 703-708

Casals, J. (1947)
Journal of Immunology, 56, 337-341

Casals, J. and Brown, L.V. (1954)
Journal of Experimental Medicine, 99, 429-449

Catt, K. and Tregear, G.W. (1967)
Science, 158, 1570-1571

Ceglowski, W.S. (1965)
Virology, 25, 328

Chadwick, C.S., McEntegart, M.G. and Nairn, R.C. (1958a)
Lancet, 1, 412-414

Chadwick, C.S., McEntegart, M.G. and Nairn, R.C. (1958b)
Immunology, 1, 315-327

Chanock, R.M. and Sabin, A.B. (1953)
Journal of Immunology, 70, 302-316

Choppin, P.W., Murphy, J.S. and Tamm, I. (1960)
Journal of Experimental Medicine, 112, 945

Chow, N. and Simpson, R.W. (1971)
Proceedings of the National Academy of Sciences, 68, 752

Chu, C.M. (1951)
Journal of General Microbiology, 5, 739-758

Clark, M.F. and Adams, A.N. (1977)
Journal of General Virology, 34, 475-483

Comoglio, S. & Celada, F. (1976)
Journal of Immunological Methods, 10, 161-170

Compans, R.W., Klenk, H., Caliguiri, L.A. and Choppin, P.W. (1970)
Virology, 42, 880

Coombs, R.R.A., Bedford, D. and Rouillard, L.M. (1956)
Lancet, 1, 461-463

Coombs, R.R.A. and Dodd, B.E. (1961)
Medicine Science and the Law, 1, 359-377

Coons, A.H., Creech, H.J. and Jones, R.N. (1941)
Proceedings of the Society for Experimental Biology and Medicine, 47, 200-202

Coons, A.H., Creech, H.J., Jones, R.N. and Berliner, E. (1942)
Journal of Immunology, 45, 159-170

Coons, A.H. and Kaplan, M.H. (1950)
Journal of Experimental Medicine, 91, 1-13

Cowan, K.M. (1968)
Journal of Immunology, 101, 1183

Cowan, K.M. and Graves, J.H. (1966)
Virology, 30, 528

Creech, H.J. and Jones, R.N. (1941)
Journal of the American Chemical Society, 63, 1670

Culliford, B.J. (1964)
Nature, 201, 1092-1093

Culliford, B.J. (1971)
National Institute of Law Enforcement and Criminal Justice
(PR 71-7), U.S. Department of Justice. Law Enforcement
Assistance Administration

Dalldorf, G. and Sickles, G.M. (1948)
Science, 108, 61

Davidson, G.P., Bishop, R.F., Townley, R.R.W., Holmes, I.H.
and Ruck, B.J. (1975)
Lancet, i, 242-246

Davis, B.D., Dulbecco, R., Eisen, H.N., Ginsberg, H.S. and Wood, W.B. (1973)
Microbiology, 2nd Ed. Harper and Row, Inc.

Documenta Geigy (1962)
Editor: Konrad Diem, Geigy Pharmaceutical Company Ltd.

Dodd, B.E. (1972)
Medicine, Science and the Law, 12, 195-199

Drabkin, D.L. and Austin, J.H. (1932)
Journal of Biological Chemistry, 98, 719-733

Dudgeon, J.A. (1950)
Journal of Clinical Pathology, 3, 239-247

Duesberg, P.H. (1968)
Proceedings of the National Academy of Sciences, 59, 930

Dulbecco, R. and Ginsberg, H.S. (1973)
"Herpesviruses" in Microbiology, 2nd Edition. Harper International.

Eagle, H. (1955)
Journal of Experimental Medicine, 102, 595

Engvall, E. and Carlsson, H.E. (1976)
First International Symposium on Immunoenzymatic Technique
INSERM Symposium No. 2. Editors Fieldman, et.al.
North-Holland Publishing Company, Amsterdam.

Engvall, E., Jonsson, K. and Perlmann, P. (1971)
Biochimica Biophysica Acta, 251, 427-434

Engvall, E. and Perlmann, P. (1971)
Immunochemistry, 8, 871-874

Engvall, E. and Perlmann, P. (1972)
Journal of Immunology, 109, 129-135

Espana, C. and Hammon, W.M. (1948)
Journal of Immunology, 59, 31-44

Fazekas de St. Groth, S., Webster, R.G. and Davenport, F.M. (1969)
Journal of Immunology, 103, 1099

Fernelius, A.L., Ritchie, A.E., Classick, L.G., Norman, J.O. and Mebus, C.A. (1972)
Archiv fur die gesamte Virusforschung, 37, 114-130

Flewett, T.H., Bryden, A.S., Davies, H., Woode, G.N., Bridger, J.C. and Derrick, J.M. (1974)
Lancet, ii, 61-63

Forsgren, M. (1968)
Acta Pathologica et Microbiologica Scandinavica, 74, 591

Foster, L.G., Peterson, M.W. and Spendlove, R.S. (1975)
Proceedings of the Society for Experimental Biology and Medicine, 150, 155-160

Fothergill, J.E. (1964)
Fluorochromes and their conjugation with proteins in "Fluorescent protein tracing". Ed. R.C. Nairn. E.A.S. Livingstone.

Francis, T. (1947)
Journal of Experimental Medicine, 85, 1-7

Frommhagen, L.H., Knight, C.A. and Freeman, N.K. (1959)
Virology, 8, 176

Gilman, S.C. and Docherty, J.J. (1977)
Journal of Infectious Disease, 136, Supplement S286-S293

Ginsberg, H.S. and Horsfall, F.L. Jnr. (1949)
Journal of Experimental Medicine, 90, 475-495

Goldstein, G., Slizys, I.S. and Chase, M.W. (1961)
Journal of Experimental Medicine, 114, 89-109

Goldwasser, R.A. and Shepard, C.C. (1958)
Journal of Immunology, 80, 122-131

Gottschalk, A. (1966)
"Glycoproteins: Their composition structure and function".
Elsevier, Amsterdam.

Grabar, P. and Williams, C.A. (1953)
Biochimica Biophysica Acta, 10, 193

Grauballe, P.C., Genner, J., Meyline, A. and Hornsleth, A. (1977)
Journal of General Virology, 35, 203-218

Gravell, M., Dorsett, P.H., Gutenson, O. and Ley, A.C. (1977)
Journal of Infectious Disease, 136, Supplement S300-S303

Griffiths, P.D., Buie, K.J. and Heath, R.B. (1978)
Journal of Clinical Pathology, 31, 827-831

Guimaraes, M.C.S., Camargo, M.E., Ferreira, A.W., Castilho, E.A. and Nakahara, O.S. (1978)
American Journal of Tropical Medicine and Hygiene, 27, 350-353

Halbert, S.P. and Anken, M. (1977)
Journal of Infectious Disease, 136, Supplement S318-S323

Harboe, A. (1963)
Acta Pathologica et Microbiologica Scandinavica, 57, 317

Hazard, M.E. (1949)
British Journal of Experimental Pathology, 30, 520-529

Henle, G., Harris, S. and Henle, W. (1948)
Journal of Experimental Medicine, 88, 133-147

Hilleman, M.R. and Werner, J.H. (1953)
Journal of Immunology, 71, 110-117

Hirst, G.K. (1941)
Science, 94, 22-23

Hirst, G.K., Rickard, E.R., Whitman, L. and Horsfall, F.L. (1942)
Journal of Experimental Medicine, 75, 495-511

Holmes, I.H., Ruck, B.J., Bishop, R.F. and Davidson, G.P. (1975)
Journal of Virology, 16, 937-943

Hoste, B., Brocteur, J., Donea, B. and Andre, A. (1977)
Forensic Science, 10, 229-234

Inaba, Y., Sato, K., Takahashi, E., Kurogi, H. and Satoda, K. (1977)
Microbiology and Immunology, 21(9), 531-534

Isaacs, A. and Bozzo, A. (1951)
British Journal of Experimental Pathology, 32, 325-335

Jerne, N.K. (1960)
Annual Review of Microbiology, 14, 341

Kabat, E.A. and Mayer, M.M. (1964)
Experimental Immunochemistry. Charles C. Thomas, U.S.A.

Kalter, S.S. (1963)
Journal of Laboratory Clinical Medicine, 62, 525-534

Kapikian, A.Z., Cline, W.L., Mebus, C.A., Wyatt, R.G., Kalica, A.R., James, H.D., Vankirk, D., Chanock, R.M. and Kim, H.W. (1975)
Lancet, i, 1056-1061

Katagiri, S., Hinuma, Y. and Ishida, N. (1967)
Virology, 32, 337

Kato, K., Hamaguchi, Y., Fukui, H. and Ishikawa, E. (1975)
FEBS Letters, 56, 370-372

Keller, Peitchel, Goldman and Goldman (1976)
Journal of Immunology, 116, 772-777

Kilbourne, E.D. (1951)
Journal of Immunology, 67, 321-329

Kind, S.S. and Lang, B.G. (1976)
Journal of the Forensic Science Society, 16(1), 47-54

Kind, S.S. and Overman, M. (1972)
Science against crime. Aldus Books Limited, London

King, L.A. (1974)
Journal of the Forensic Science Society, 14, 117-121

King, L.A., Werrett, D.J. and Whitehead, P.H. (1976)
Forensic Science, 7, 1-4

King, L.A. and Whitehead, P.H. (1975)
Forensic Science, 6, 197-203

Kingsbury, D.W. and Webster, R.G. (1969)
Journal of Virology, 4, 219

Kirkpatrick, A., Wepsic, H.T. and Nakamura, R.M. (1977)
Clinical Chemistry, 23, 50-59

Kriss, S.L. and Mandel, B. (1972)
Virology, 48, 666

Landsteiner, (1899)
Centr Bakteriol, 25, 546

Lauer, R.C. and Erlanger, B.F. (1974)
Immunochemistry, 11, 533-536

Laver, W.G. (1971)
Virology, 45, 275

Laver, W.G. and Valentine, R.C. (1969)
Virology, 38, 105

Le Bouvier, G.L. (1955)
Lancet, 2, 1013

Le Bouvier, G.L. (1959)
British Journal of Experimental Pathology, 40, 605

Leinikki, P.O. and Passila, S. (1977)
Journal of Infectious Disease, 136, Supplement S294-S299

Leininger, R.I., Cooper, C.W., Falb, R.D. and Grode, G.A. (1966)
Science, 152, 1625-1626

Lennette, E.H. and Schmidt, N.J. (1964)
American Public Health Association, Inc.

Lighter, A.G. (1953)
A.M.A. Archives Dermatology and Syphilology, 67, 362-368

Linhardt, K. and Walter, K. (1965)
Phosphatases (Phosphomonoesterases) in "Methods of Enzymatic Analysis", H.U. Bergmeyer, Ed. Academic Press.

Lowry, O.H. (1970)
Protein (Lowry Reaction) Technicon Autoanalyser Method, No. N-721/11

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951)
Journal of Biological Chemistry, 193, 265-275

McCrea, J.F. (1946)
Australian Journal of Experimental Biology and Medical Science, 24, 283-291

MacFarlane, D.E. (1972)
Ph.D. Thesis, Glasgow University

MacFarlane, D.E., McLure, A.R. and Sommerville, R.G. (1971)
Archiv für die gesamte Virusforschung, 34, 346-350

McNulty, M.S. (1978)
Journal of General Virology, 40, 1-18

Madeley, C.R., Cosgrove, B.P., Bell, E.J. and Fallon, R.J. (1977)
Journal of Hygiene, 78, 261-273

Maiolini, R. and Masseyeff, R. (1975)
Journal of Immunological Methods, 8, 223-234

Marrack, J. (1934)
Nature, 133, 292-293

Martin, M.L., Palmer, E.L. and Kissling, R.E. (1972)
Infection and Immunity, 5, 248-254

Mayer, M.M., Rapp, H.J., Roizman, B., Klein, S.W., Cowan, K.M., Lukens, D., Schwerdt, C.E., Schaffer, F.L. and Charney, J. (1957)
Journal of Immunology, 78, 435

Mebus, C.A., Underdahl, N.R., Rhodes, M.B. and Twiehaus, M.J. (1969)
University of Nebraska Agricultural Experiment Station Research Bulletin, No. 233

Middleton, P.J., Szymanski, M.T., Abbott, G.D., Bortolussi, R. and Hamilton, J.R. (1974)
Lancet, i, 1241-1244

Middleton, P.J., Holdaway, M.D., Petric, M., Szymanski, M.T. and Tam, J.S. (1977)
Infection and Immunity, 16, 439-444

Nairn, R.C. (1964)
"Fluorescent protein tracing". R.C. Nairn, Ed.
E & S Livingstone

Nakane, P.K., Sri Ram, J. and Pierce, G.B. (1966)
Journal of Histochemistry and Cytochemistry, 14, 789-791

Nickolls, L.C. and Pereira, M. (1962)
Medicine Science and the Law, 2, 172-179

Norrby, E. and Gollmar, Y. (1972)
Infection and Immunity, 6, 240-247

Ouchterlony, O. (1962)
Progress in Allergy, 6, 30

Paul, J. (1975)
Cell and Tissue Culture, 5th Edition. Churchill Livingstone

Pesce, A.J., Mendoza, N., Boreisha, I., Gaizutis, M.A. and Pollack, V.E. (1974)
Clinical Chemistry, 20, 353-359

Pinon, J.M. and Dropsy, G. (1977)
Journal of Immunological Methods, 16, 15-22

Planterose, D.N., Cartwright, B. and Brown, F. (1963)
Journal of Experimental Medicine, 117, 951

Polatnick, J. and Arlinghaus, R.B. (1967)
Virology, 31, 601-615

Polesky, H.F. and Kraus, H.D. (1977)
Transfusion, 17, 521-524

Rappaport, I., Siegel, A. and Haselkorn, R. (1965)
Virology, 25, 325

Riggs, J.L., Seiwald, R.J., Burckhalter, J.H., Downs, C.M. and Metcalf, T.G. (1958)
American Journal of Pathology, 34, 1081-1097

Roizman, B., Mayer, M.M. and Roane, P.R. Jnr. (1959)
Journal of Immunology, 82, 19

Rose, H.M. (1952)
Journal of Immunology, 68, 687-691

Rowlands, D.J., Sangar, D.V. and Brown, F. (1971)
Journal of General Virology, 13, 85

Rubenstein, K.E., Schneider, R.S. and Ullman, E.F. (1972)
Biochemical Biophysical Research Communications, 47, 846-851

Ruitenberg, E.J., Steerenberg, P.A., Brosi, B.J.M. and
Buys, J. (1976)
Journal of Immunological Methods, 10, 67-83

Ruitenberg, E.J. and Van Knapen, F. (1977)
Journal of Infectious Disease, 136, Supplement S267-S273

Sabin, A.B. and Buescher, E.L. (1950)
Proceedings of Society for Experimental Biology and Medicine,
74, 222-230

Salmi, A.A., Norrby, E. and Panelius, M. (1972)
Infection and Immunity, 6, 248-254

Schild, G.C. (1970)
Journal of General Virology, 9, 191

Schleyer, F. (1962)
Investigation of biological stains with regard to species of origin in
"Methods of Forensic Science", Volume 1, Ed. F. Lundquist.
Interscience Publishers.

Schluederberg, A., Horstmann, D.M., Andiman, W.A. and
Randolf, M.F. (1978)
Journal of Infectious Disease, 138, 877-883

Schmidt, N.J., Dennis, J., Frommhagen, L.H. and Lennette, E.H.
(1963)
Journal of Immunology, 90, 654-662

Schmidt, N.J., Dennis, J., Lennette, E.H., Ho, H.H. and
Shinomoto, T.T. (1965)
Journal of Immunology, 95, 54-69

Schmidt, N.J. and Lennette, E.H. (1962a)
Journal of Immunology, 89, 85-95

Schmidt, N.J., Lennette, E.H. and Dennis, J. (1968)
Journal of Immunology, 100, 99-106

Schubert, J.H., Stanford, S.M. and Tiffany, E.J. (1951)
Journal of Laboratory and Clinical Medicine, 37, 388-393

Schulman, J.L. (1969)
Bulletin of the World Health Organisation, 41, 641

Schulze, I.T. (1972)
Virology, 47, 181

Schuurs, A.H.W.M. and Van Weemen, B.K. (1977)
Clinica Chimica Acta, 81, 1-40

Sever, J.L. (1962)
Journal of Immunology, 88, 320-329

Shaffer, M.F. & Enders, J.F. (1939)
Journal of Immunology, 37, 383-411

Skehel, J.J. and Schild, G.C. (1971)
Virology, 44, 396

Smith, K.O. and Gehle, W.D. (1977)
Journal of Infectious Disease, 136, Supplement S329-S336

Smith, W. and Westwood, J.C.N. (1950)
British Journal of Experimental Pathology, 31, 725-738

Snodgrass, D.R. and Herring, J.A. (1977)
Veterinary Record, 101, 81

Sommerville, R.G. (1967)
Archiv fur die gesamte Virusforschung, 20, 452-458

Spector, W.S. (1956)
Editor of Handbook of Biological Data, W.B. Saunders Company

Spencer, N., Hopkinson, D.A. and Harris, H. (1964)
Nature, 201, 299-300

Stelos, P. (1967)
Isolation of Immunoglobulin in "Handbook of Experimental Immunology",
Ed. by D.M. Weir. Blackwell Scientific Publications

Stimson, W.H. and Sinclair, J.M. (1974)
FEBS Letters, 47, No. 1, 190-192

Talbot, P. and Brown, F. (1972)
Journal of General Virology, 15, 163

Taran, A. (1946)
Journal of Laboratory and Clinical Medicine, 31, 1037-1039

Timbury, M.C. (1974)
"Notes on Medical Virology". Churchill Livingstone

Tyrrel, D.A.J. and Horsfall, F.L. Jnr (1953)
Journal of Experimental Medicine, 97, 845-861

Udenfriend, S. (1962)
"Florescence assay in Biology and Medicine". Academic Press.

Van Weemen, B.K. and Schuurs, A.H.W.M. (1971)
FEBS Letters, 15, 232-236

Van Weemen, B.K. and Schuurs, A.H.W.M. (1972)
FEBS Letters, 24, 77-81

Van Weemen, B.K. and Schuurs, A.H.W.M. (1974)
FEBS Letters, 43, 215-218

Voller, A., Bartlett, A. and Bidwell, D.E. (1978)
Journal of Clinical Pathology, 31, 507-520

Voller, A. and Bidwell, D.E. (1975)
British Journal of Experimental Pathology, 56, 338-339

Voller, A. and Bidwell, D.E. (1976)
British Journal of Experimental Pathology, 57, 243-247

Voller, A., Bidwell, D.E. and Bartlett, A. (1976a)
Bulletin of the World Health Organisation, 53, 55-64

Voller, A., Bidwell, D.E., Bartlett, A., Fleck, D.G., Perkins, M. and Oladehin, B. (1976b)
Journal of Clinical Pathology, 29, 150-153

Voller, A., Bidwell, D.E., Huldt, G. and Engvall, E. (1974)
Bulletin of the World Health Organisation, 51, 209-211

Voller, A., Draper, C., Bidwell, D.E. and Bartlett, A. (1975)
Lancet, i, 426-428

Voller, A., Huldt, G., Thors, C. and Engvall, E. (1975)
British Medical Journal, 1, 659-661

Von Bonsdorff, C.H., Hovi, T., Makela, P., Hovi, L. and Tevalvoto-AArnio, M. (1976)
Lancet, ii, 423

Von Bonsdorff, C.H., Hovi, T., Makela, P. and Morttinen, A. (1978)
Journal of Medical Virology, 2, 21-28

Weber, G. (1952)
Biochemical Journal, 51, 155-167

Webster, R.G. (1970)
Virology, 40, 643

Webster, R.G., Laver, W.G. and Kilbourne, E.D. (1968)
Journal of General Virology, 3, 315

Weller, T.H. and Coons, A.H. (1954)
Proceedings of the Society of Experimental Biology and Medicine, 86, 789-794

Werrett, D.J. and King, L.A. (1976)
Clinical Allergy, 6, 75-77

Werrett, D.J., King, L.A. and Whitehead, P.H. (1976)
Journal of the Forensic Science Society, 16, 121-126

Wide, L. and Porath, J. (1966)
Biochimica et Biophysica Acta, 130, 257-259

Wisdom, G.B. (1976)
Clinical Chemistry, 22, 1243-1255

Wolff, K.L. and Hudson, B.W. (1974)
Applied Microbiology, 28, 323-325

Wolters, G., Kuijpers, L.P.C., Kacaki, J. and Schuurs, A.H.W.M. (1976)
Journal of Clinical Pathology, 29, 873-879

Woode, G.N. and Bridger, J.C. (1975)
Veterinary Record, 96, 85-88

Woode, G.N., Bridger, J.C., Jones, J.M., Flewett, T.H., Bryden, A.S., Davies, H.A. and White, G.B.B. (1976)
Infection and Immunity, 14, 804-810

Yolken, R.H., Kim, H.W., Clem, T., Wyatt, R.G., Kalica, A.R., Chanock, R.M. and Kapikian, A.Z. (1977)
Lancet, ii, 263-267

Yolken, R.H., Wyatt, R.G., Zissie, G., Brandt, C.D., Rodriguez, W.J., Kim, H.W., Parrott, R.H., Urrutia, J.J., Mata, L., Greenberg, H.B., Kapikian, A.Z. and Chanock, R.M. (1978)
New England Journal of Medicine, 299, No. 21, 1156-1161

Yorde, D.E., Sasse, E.A., Wang, T.Y., Hussa, R.O. and Garancis, J.C. (1976)
Clinical Chemistry, 22, 1372-1377

Zissis, G. and Clinet, G. (1974)
Lancet, Vol. 1, No. 7860, 20th April, 754-755