QUANTITATIVE STUDIES ON HAEMAGGLUTINATION:
THE MECHANISM AND KINETICS OF HAEMAGGLUTINATION
BY SEMLIK FOREST VIRUS

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Thesis presented for the Degree of Doctor of Philosophy of the University
of Edinburgh in the Faculty of Science

March 1969
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ACKNOWLEDGEMENTS

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SUMMARY
The aim of this study was to analyze quantitatively the mechanism and kinetics of the haemagglutination reaction between the arbovirus, Semliki Forest virus (SFV) and goose red blood cells (RBC).

A review of the literature is given of the mechanisms, kinetics and quantitative aspects of the reactions between viruses and red cells, and of some relevant properties of arboviruses.

Following a brief summary of the standard reagents and methods used, a detailed analysis of the conditions for the demonstration of haemagglutination by SFV is described. The haemagglutinin of SFV was characterized by gel filtration and by equilibrium density gradient centrifugation, and the influence on the reaction mixture of RBC species, pH, buffer ions, protein content and temperature was studied.

The quantitative studies were initiated by a critical investigation of the standard pattern method. The different pattern tests were considered, together with the influence of RBC concentration on pattern formation. This resulted in the definition of a constant, characteristic for the pattern test in WHO dimple plates. A more precise and quantitative presentation of the data obtained from haemagglutination and haemagglutination-inhibition tests is described.

Unsuccessful attempts with photometric procedures were made to confirm the 'dimers-only' hypothesis, using a wide variety of viral and non-viral haemagglutinating systems. However, under the present experimental conditions, a step-curve was only obtained with a non-agglutinating mixture of the different sized RBC from geese and sheep.

Automatic electronic cell counting by Coulter counter was studied to assess the possibility of determining the distribution of aggregates in a reaction mixture
of RBC and viral haemagglutinin. This method was unsuccessful since the instrumental conditions for the counting of aggregates were not optimal for the formation of aggregates.

These results led to the development of a new Sedimentation-Enumeration Method for the determination of haemagglutinin concentrations from the population distribution of aggregates formed in free suspension. The experimental and theoretical basis of this method is described. The method has been applied to the study of the influence of the concentrations of RBC and haemagglutinin, and of pH on the kinetics of the reaction.

The method is used to quantitate the haemagglutination reaction at low concentrations of haemagglutinin and shows that the reaction follows the Percentage Law. This leads to the enumeration of an Haemagglutination Index for an initial haemagglutinin preparation and of an Haemagglutination-Inhibition Index for an undiluted antiserum preparation.

The validity of this method is shown by analysis according to the statistical-mechanical theory of Goldberg for the antigen-antibody interaction.

An hypothesis is given to explain the complex mechanisms of the haemagglutination of RBC by arboviruses in terms of the competitive reactions of pH inactivation of haemagglutinin and its adsorption to RBC, and to explain the relationship between the numbers of bonds per RBC and the haemagglutinin concentration.

Finally, these results are discussed in relation to the data and hypotheses given by others in the published literature.
INTRODUCTION
In the laboratory study of arboviruses, three 'in vitro' reactions are principally used for the detection and identification of virus or specific antibody. These procedures utilize the fixation of complement, the detection and neutralization of virus infectivity and the haemagglutination of avian red blood cells. The 'all-or-none' observation of an activity due to the presence of virus is considered sufficient for many studies of these three different reactions. However, such data can only be related in statistical terms to the number or integrity of the virus particles responsible for the observed reaction.

Of these three widely applied and important reactions, the fixation of complement and the neutralization of infectivity have been most fully defined and interpreted in quantitative terms. Bradish and his colleagues have made detailed studies at the particle level on the nature of the complement fixation and neutralization reactions for the viruses of tobacco mosaic and tomato bushy stunt (Bradish and Crawford, 1960), vesicular stomatitis (Vaughan, 1966), and foot-and-mouth disease (Bradish and Farley, 1960; Bradish, Farley and Ferrier, 1962; Bradish, Jowett and Kirkham, 1964).

Haemagglutination by arboviruses has not been studied in similar terms and this study was undertaken in order to investigate the mechanism of the haemagglutination of goose red blood cells by Semliki Forest virus, a representative group of arbovirus, and to relate the results of the test to the concentration and specificity of virus and to the data of alternative test procedures.
I. HAEMAGGLUTINATION

a) The Interactions between Viruses and Red Blood Cells

Following the initial adsorption of virus particles to red cells (RBC), three types of reaction may occur.

(i) Haemolysis

Under specific conditions of temperature and ionic strength, the initial adsorption of virus may result in lysis of the red cell. This phenomenon has been demonstrated for several viruses, under the variety of conditions shown in Table I.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Red Cell Species</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Composition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arboviruses</td>
<td>1-day chick</td>
<td>6.0-6.2</td>
<td>37</td>
<td>Earle's saline</td>
<td>Karabatsos (1963, 1965)</td>
</tr>
<tr>
<td>Parainfluenza</td>
<td>rooster</td>
<td>5.8 and 10.0</td>
<td>37</td>
<td>PBS No divalent cations</td>
<td>Neurath (1965a,b)</td>
</tr>
<tr>
<td>Newcastle disease</td>
<td>fowl human</td>
<td>(7.2)</td>
<td>28</td>
<td>PBS No Ca ions</td>
<td>Burnet and Lind (1950)</td>
</tr>
<tr>
<td>Mumps</td>
<td>fowl</td>
<td>7.0-8.0</td>
<td>37</td>
<td>PBS</td>
<td>Morgan, Enders and Magley (1948)</td>
</tr>
<tr>
<td>Measles</td>
<td>monkey</td>
<td>7.2</td>
<td>37</td>
<td>PBS</td>
<td>Peries and Ohany (1960)</td>
</tr>
</tbody>
</table>

(N.B. In Tables 1 and 2, the values given in brackets are implied but not defined by the authors.)
(ii) Haemadsorption

Although the initial stage of virus adsorption to the red cell is properly called haemadsorption, this term is usually applied to the further interaction of the virus-RBC complex with the surfaces of either monolayers of infected cells (O'Connell, Barron, Milgrom and Witebsky, 1964) or single infected cells (Marcus, 1962). Since the first report of this phenomenon by Vogel and Shelokov (1957) for influenza virus growing in monkey kidney cell cultures, this form of haemadsorption has been demonstrated for many other viruses as illustrated in Table 2.

**TABLE 2. Conditions for Haemadsorption by Virus-infected Cells**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Tissue Culture System</th>
<th>Red Cell Species</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Composition</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arboviruses</td>
<td>chick embryo</td>
<td>goose</td>
<td>6.4</td>
<td>37</td>
<td>PBS + borate ions</td>
<td>Buckley (1959)</td>
</tr>
<tr>
<td></td>
<td>human embryo skin tissue</td>
<td>1-day chicks, sheep</td>
<td>7.2-7.8</td>
<td>23</td>
<td>PBS</td>
<td>Karpovitch (1961)</td>
</tr>
<tr>
<td>Influenza A</td>
<td>monkey kidney</td>
<td>human, guinea-pig</td>
<td>(7.2)</td>
<td>37</td>
<td>Hank’s buffer</td>
<td>Bukrinskaya (1960)</td>
</tr>
<tr>
<td>Para-influenza</td>
<td>monkey kidney, human embryo lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measles</td>
<td>monkey kidney</td>
<td>monkey</td>
<td>(7.2)</td>
<td>(37)</td>
<td>NaCl</td>
<td>Rosanoff (1961)</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>chick embryo liver</td>
<td>fowl</td>
<td>7.4</td>
<td>37</td>
<td>(PBS)</td>
<td>O'Connell et al. (1964)</td>
</tr>
<tr>
<td>Herpes</td>
<td>human embryo, HeLa</td>
<td>human, guinea-pig</td>
<td>(7.2)</td>
<td>(37)</td>
<td>(PBS)</td>
<td>Bocharov (1964)</td>
</tr>
<tr>
<td>Infectious bronchitis</td>
<td>chick embryo kidney</td>
<td>fowl</td>
<td>6.5-7.3</td>
<td>37</td>
<td>PBS</td>
<td>Biswal, Nazarian and Cunningham (1966)</td>
</tr>
</tbody>
</table>
(iii) **Haemagglutination**

The combination of the initially formed virus - RBC complexes to form larger aggregates is called haemagglutination. The discovery of this agglutination of red blood cells by viruses was made independently and simultaneously by Hirst (1941) and McClelland and Hare (1941). They observed that free chick red blood cells found in the allantoic fluids of chick embryos infected with influenza A (PR8 strain) and influenza B (Lee strain) viruses became agglutinated due to the adsorption of virus particles onto the cells, with the subsequent formation of 'bridges' between adjacent cells. They also noted that, after a reaction period dependent on time and temperature, the virus eluted from the surface of the red cells. This will be discussed later (Page 31).

It soon became apparent that this type of haemagglutination was not a characteristic of all viruses and that not all mammalian and avian red cells were agglutinable. Since this important observation, a large number of viruses from many different groups has been shown to agglutinate red blood cells from a wide variety of animal species, often under conditions unique for the virus. Table 3 shows the conditions for direct haemagglutination by a number of viruses and is arranged according to the type of viral nucleic acid and to the presence or absence of an outer envelope in the structure of the virus. Within each group, the data are arranged in order of particle size.
**TABLE 3. The Optimal Conditions for the Agglutination of Red Blood Cells by some Viruses**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Red Blood Cell Species</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VIRUSES CONTAINING DNA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyoma virus</td>
<td>guinea-pig</td>
<td>7.2</td>
<td>4</td>
<td>Hartley, Rowe, Chanock and Andrewes (1959)</td>
</tr>
<tr>
<td>Adenoviruses</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>Rhesus monkey</td>
<td>7.2</td>
<td>37</td>
<td>Rosen (1958, 1960a)</td>
</tr>
<tr>
<td>Group 2</td>
<td>Rhesus monkey and rat</td>
<td>7.2</td>
<td>37</td>
<td>Pereira and de Figueiredo (1962)</td>
</tr>
<tr>
<td>Group 3</td>
<td>rat</td>
<td>7.2</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Poxviruses</td>
<td>chicken</td>
<td>7.2</td>
<td>20,37</td>
<td>Burnet and Stone (1946)</td>
</tr>
<tr>
<td><strong>VIRUSES CONTAINING RNA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Echoviruses</td>
<td>human '0'</td>
<td>7.0-8.0</td>
<td>4</td>
<td>Podoplekin (1963)</td>
</tr>
<tr>
<td>Encephalomyocarditis virus</td>
<td>sheep, human '0', guinea-pig</td>
<td>8.0</td>
<td>4-25</td>
<td>Olitsky and Yager (1949)</td>
</tr>
<tr>
<td>Mouse encephalomyelitis</td>
<td>human '0'</td>
<td>7.2</td>
<td>0</td>
<td>Lahelle and Horsfall (1949)</td>
</tr>
<tr>
<td>Coxsackieviruses</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type: A7</td>
<td>fowl</td>
<td>7.2</td>
<td>20</td>
<td>Goldfield, Srilongse and Fox (1957)</td>
</tr>
<tr>
<td></td>
<td>human '0'</td>
<td>5.8-6.3</td>
<td>4</td>
<td>Rosen and Kern (1961)</td>
</tr>
<tr>
<td></td>
<td>human '0', guinea-pig</td>
<td>7.2</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>human '0', bovine</td>
<td>7.2</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>Reoviruses</td>
<td>human '0'</td>
<td>7.2</td>
<td>4,20,37</td>
<td>Rosen (1960b)</td>
</tr>
<tr>
<td></td>
<td>bovine</td>
<td>7.2</td>
<td>4</td>
<td>Eggers, Gonatos and Tamm (1962)</td>
</tr>
<tr>
<td>Virus</td>
<td>Red Blood Cell Species</td>
<td>pH</td>
<td>Temperature (°C)</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>------------------------</td>
<td>-----</td>
<td>------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Arboviruses</td>
<td>goose, 1-day chick</td>
<td>6.0-7.0</td>
<td>20,37</td>
<td>Casals and Brown (1954)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Porterfield (1957)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Clarke and Casals (1958)</td>
</tr>
<tr>
<td>Vesicular stomatitis virus</td>
<td>goose</td>
<td>6.2</td>
<td>4</td>
<td>Halonen (personal communication, 1967)</td>
</tr>
<tr>
<td>Rabies virus</td>
<td>goose</td>
<td>6.2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Pneumonia virus of mice</td>
<td>mouse, hamster</td>
<td>7.2</td>
<td>20</td>
<td>Mills and Dochez (1944)</td>
</tr>
<tr>
<td>Infectious bronchitis virus</td>
<td>chicken</td>
<td>6.5-7.3</td>
<td>4, 25, 37</td>
<td>Corbo and Cunningham (1959)</td>
</tr>
<tr>
<td>Myxoviruses</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Influenza: A</td>
<td>chicken, human 'O'</td>
<td>7.2</td>
<td>20</td>
<td>Andrews, Bang, Chanock and Zhdanov (1959)</td>
</tr>
<tr>
<td>Influenza: B</td>
<td>human 'O' and guinea-pig</td>
<td>7.2</td>
<td>20</td>
<td>Jensen, Minuse and Ackermann (1955)</td>
</tr>
<tr>
<td>Influenza: C</td>
<td>chicken, human 'O'</td>
<td>7.2</td>
<td>4</td>
<td>Chanock (1956)</td>
</tr>
<tr>
<td>Para-influenza: 1</td>
<td>chicken, human 'O', guinea-pig</td>
<td>7.2</td>
<td>4-30</td>
<td>Schmidt and Lennette (1961)</td>
</tr>
<tr>
<td></td>
<td>chicken</td>
<td>7.2</td>
<td>4-30</td>
<td>Johnson, Chanock, Cook and Huebner (1960)</td>
</tr>
<tr>
<td></td>
<td>human 'O', guinea-pig</td>
<td>7.2</td>
<td>4-30</td>
<td>Anderson (1947)</td>
</tr>
<tr>
<td></td>
<td>Rhesus monkey, guinea-pig</td>
<td>7.2</td>
<td>20, 37</td>
<td>Levens and Enders (1945)</td>
</tr>
<tr>
<td>Newcastle disease</td>
<td>chicken</td>
<td>7.2</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Mumps</td>
<td>chicken</td>
<td>7.2</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Measles virus</td>
<td>Rhesus monkey</td>
<td>7.2</td>
<td>37</td>
<td>Rosen (1961)</td>
</tr>
<tr>
<td>Rubella virus</td>
<td>2-day chicken</td>
<td>6.2</td>
<td>4</td>
<td>Halonen, Ryan and Stewart (1967)</td>
</tr>
</tbody>
</table>
One common application of the haemagglutination reaction is for the detection and assay of specific viral antibodies present in animal sera. If virus specific antibodies in an immune serum are allowed to combine with a known haemagglutinating virus, then the proportion of haemagglutinin (H) retaining the capacity to haemagglutinate added RBC may be estimated. The extent of inhibition of haemagglutination is a measure of the quantity of specific antibody in the serum (Fiset, 1964; Hoskins, 1967; Norrby, 1967; Smith, 1967; Tyrrell, 1967). This test has the specificity of the intact virus-antibody reaction and is widely used for the differentiation and identification of virus strains in clinical and epidemiological studies.

The initial adsorption stage and the subsequent elution of virus from red cells has been utilized for the purification and concentration of influenza viruses (Francis and Salk, 1942) and of adenoviruses (Simon, 1962).

Extensive reviews of haemagglutination by viruses have been made by Anderson (1959), Rosen (1964) and Sanna (1967).

(iv) Indirect Haemagglutination

Some of the viruses which have not yet been shown to adsorb directly to red cells and then to form aggregates may do so through an intermediate reaction. These methods can be grouped together as passive haemagglutination. The principle employed is that red blood cells may be modified to adsorb antigen more avidly by treatment with, for example, tannic acid (Boyden, 1951; Garabedian, 1965) or periodate (Kwapinski, 1965). Such antigen-coated RBC are agglutinated by antibodies which combine with
the adsorbed antigen. In this way, viruses can be detected and assayed using the quantitative techniques devised for direct haemagglutination. Other indicator, or carrier, particles which have also been used to adsorb antigens include latex (Aubert, Pavilanis and Starkey, 1962), bentonite (Bozicevich, Scott and Vincent, 1963), charcoal (Klein, Chaefsky and Muller, 1966) and collodion (Boand and Kempf, 1951).

Passive haemagglutination using tanned red cells has been demonstrated for animal viruses (adenoviruses, Fauconnier, 1966; poliovirus, McKenna, Zushek and Frankel, 1956; herpes simplex, Scott, Felton and Barney, 1957), for an insect virus (tipula iridescent virus, Cunningham, Tinsley and Walker, 1966) and for plant viruses (tobacco mosaic and barley stripe mosaic viruses, Saito and Iwata, 1964).

The sensitization of RBC with viral antigens for use in an indirect haemagglutination test has been described for arboviruses by Hale and Pillai (1960), Lim and Pang (1964), and Margolin and Kondrashova (1967), and for influenza virus by Gorbunova and Sokovykh (1967).

Extensive reviews of such passive and indirect methods of haemagglutination have been published by Stavitsky and Arquilla (1958), Shwartzman and Sinitsyn (1961), Stavitsky (1964), and Kwapinski (1965).

b) The Interaction between Red Blood Cells and other Agents

Although the haemagglutinating activity of viruses is a recent finding, the phenomenon has long been recognized for many other biological and non-biological agents.
(1) Haemagglutination by Bacteria

Neter et al. (1954) defined direct haemagglutination as "the agglutination of red blood cells resulting from the action of bacteria or bacterial products". Kraus and Ludwig (1902) and Flexner (1902) first observed the clumping of rabbit RBC in the presence of Staphylococci, Vibrio, Pseudomonas and Salmonella species. This agglutination was inhibited by specific antisera. Since then, direct haemagglutination has been shown for many species of Haemophilus, Clostridium and Corynebacterium (Kwapinski, 1965).

However, Collee (1962) has drawn a distinction between 'non-diffusible' haemagglutinins that are cell-bound or closely associated with the bacterial cell, and 'diffusible' haemagglutinins which enter the medium from the bacterial cell. The latter may be compared with the 'soluble' haemagglutinins of poxviruses (Cohen, 1963). A non-diffusible haemagglutinin for Escherichia coli was first demonstrated by Guyot (1908) and the development of this field has since been reviewed by Gillies (1959). This haemagglutinating activity is due to non-flagellar, filamentous appendages or fimbriae (Duguid, Smith, Dempster and Edwards, 1955).

Indirect haemagglutination by bacteria (Neter et al., 1954) is "the sensitization of RBC by exposure to extracts of bacteria which either adhere to the RBC surface, or alter it enzymically, followed by aggregation of these RBC by specific antisera". Such agglutination has been shown for Pasteurella, Aerobacter and Neisseria species (Kwapinski, 1965). The alternative method in which red cells are sensitized with specific antisera
has been used to detect the organisms of *Pasteurella tularensis*, *Pasteurella pestis*, *Brucella abortus* and *Bacillus anthracis* (Bayar, Konikova and Krylov, 1966).

Comprehensive reviews of microbial haemagglutination have been given by Neter (1956), Collee (1962), and Kwapinski (1965).

(ii) Haemagglutination by other Biological Agents

The agent of psittacosis, not now classified as a virus (Andrewes and Pereira, 1967), has been shown to haemagglutinate mouse RBC (Gogolak and Ross, 1955; Benedict and O'Brien, 1958; and Barron, Zakay-Rones, and Bernkoff, 1965).

Many mycoplasmas show both haemagglutination (John, Stahl and Fulginiti, 1966), and haemadsorption (Manchee and Taylor-Robinson, 1968), although there is no direct correlation between the two phenomena for the different mycoplasmas.

Some rickettsial agents have been shown to produce an "erythrocyte-sensitizing-substance" which can alter the surface of human RBC so that they can become agglutinated by specific immune sera (Chang, Murray and Snyder, 1954).

Agglutination of red blood cells can also be brought about by lectins or plant agglutinins (Boyd, 1963), and nucleic acids (Ishiyama, 1963; Leahu, 1966).

Also, many adult human sera agglutinate human red cells regardless of ABO specificity. This is called the Thomsen-Friedenreich phenomenon or panagglutination (Friedenreich, 1923; Neter, 1956), and is due to the alteration of the RBC surface by bacterial enzymes.
(iii) **Haemagglutination by Non-Biological Agents**

Although adsorption and agglutination are essentially simple physico-chemical reactions, there is a need for caution in the interpretation of the observed phenomena since similar effects may be produced in many different ways. It has been shown, for instance, that RBC can become agglutinated in the presence of tannic acid, trypsin, protamine sulphate, fatty acids, plant and animal tissue extracts, inorganic acids and bases, and salts of heavy metals. Such diversity of mechanisms must be considered in any application of haemagglutination to a virus system.

c) **Other Factors which Influence the Haemagglutinating Activity of Viruses**

The diversity of factors which influence haemagglutination by viruses is illustrated by the following examples.

1. **Virus Strain**

   It has been shown that of the several Echo virus type 6 strains not all can agglutinate human RBC (Goldfield, Srihongse and Fox, 1957).

2. **Passage Level of Virus**

   Burnet and Bull (1943) demonstrated that the affinity of influenza viruses for different species of RBC changed after about the fourth or fifth passage of these viruses in chick embryos.
(iii) Host of Origin of Virus

In studies with arboviruses, it has been found that the amount of haemagglutinating activity, both before and after extraction to remove inhibitors, is consistently higher for haemagglutinins derived from infected mouse brains than those obtained from chick embryo cell cultures which contain up to $10^{11}$ p.f.u. per ml. (Page 18).

(iv) Method of Virus Production and the Presence of Inhibitors

Inhibitors of haemagglutination derived from host material and components of the culture medium are frequently encountered in untreated myxovirus and arbovirus preparations. For arboviruses, these inhibitors may be conveniently removed by treatment with acetone and ether (Casals and Brown, 1953) or with fluorocarbon (Porterfield and Howe, 1960).

(v) Species of Red Blood Cell

Not all mammalian and avian RBC are agglutinable by arbovirus haemagglutinins (Banerjee, 1965). Goose, rooster and 1-day chick RBC are commonly used since these appear to be most widely useful in the test.

There may also be differences between individuals of the species. Thus, the poxviruses only agglutinate the RBC from certain chickens (Nagler, 1942).

(vi) Age of the RBC Donor

The RBC from 1- or 2-day old chicks are more suitable than those from adult chickens for the detection of haemagglutination by arboviruses (Chanock and Sabin, 1953) and rubella virus (Halonen et al., 1967).
(vii) **Influence of Medium Composition**

Haemagglutination by encephalomyocarditis virus (EMC) is inhibited by Na\(^+\), PO\(_4\)\(^{+++}\) and Ca\(^++\) and Mg\(^++\) ions at a concentration greater than about 0.04M, (Gard and Heller, 1951; Horvath and Jungeblut, 1952).

(viii) **Influence of Medium pH**

Haemagglutination by arboviruses is particularly dependent on the pH of the medium and normally occurs only over a limited pH range (6.0-7.0) and in certain buffer systems (Clarke and Casals, 1958). (Table 3).

(ix) **Influence of Temperature**

The optimum temperatures for haemagglutination by some viruses are given in Table 3 and extend over the range 0°C-37°C.
II. THE ARBOVIRUSES AND SOME OF THEIR PROPERTIES

a) Introduction

The name "arbovirus", now in general use, is a shortened form of arthropod-borne virus (Andrewes, 1962). A recent paper by Smith (1968a), discussed the difficulties encountered by a W.H.O. Scientific Study Group (1967) in defining the arbovirus group. The then accepted definition was that: "Arboviruses are viruses which are maintained in nature principally, or to an important extent, through biological transmission between susceptible vertebrate hosts by hematophagous arthropods; they multiply and produce viraemia in the vertebrates by the bites of arthropods after a period of extrinsic incubation". However, this is a very general and biologically-based definition which inevitably includes many agents which differ in fundamental morphological and physico-chemical properties. Consequently the arbovirus group is very large and heterogeneous.

The recent publication of the "Catalogue of Arthropod-borne Viruses of the World" (Taylor, 1967) is recognition of the importance of this complex group of viruses. The Catalogue contains epidemiological, serological and physico-chemical details of 204 viruses (as at February, 1967), although about 20 more recent isolations have also been classified as arboviruses.

The epidemiology and control of arbovirus diseases, together with the details of their serological grouping, have been reviewed by Smith (1966b) and Casals (1966), and will not be discussed here.
b) **Source and Production of Arboviruses**

Both experimental animals and cultured cells have been widely used in the study of arboviruses.

(i) **Experimental Animals**

One of the most common sources of arbovirus antigens is the suckling or adult mouse. Antigen suspensions are usually prepared from blood, liver or brain tissues by dilution or dispersion in borate-saline buffer at pH 9.0. (Clarke and Casals, 1958). The brains of suckling mice have been used in this study as the major source of viral haemagglutinin (Page 47).

(ii) **Cell Culture Systems**

A cytopathogenic effect (CPE) has been demonstrated with many cell culture systems infected by arboviruses. Buckley (1964) showed that 89 arboviruses caused CPE in HeLa cells and, more recently, (Karabatsos and Buckley, 1967) obtained CPE in the baby hamster kidney cell line, BHK-21 (Maepherson and Stoker, 1962), with 124 of 129 viruses tested. Other systems which have been studied for the growth of arboviruses are human diploid cells (tick-borne encephalitis viruses, Motajova and Libikova, 1965; and Rapoport, Stepanova and Andzhaparidze, 1965), stable lines of monkey and pig kidney cells (dengue viruses, Stim and Henderson, 1966), primary chick embryo cells (yellow fever virus, Fulton, 1965) and several cultures of tick tissues (4 group A and 11 group B viruses, Rehacek, 1965). Semenov, Karaseva and Rapoport (1966) made the observation that tick-borne encephalitis virus, cultured in diploid cells up to the 34th passage, failed to agglutinate goose red cells. However, haemagglutinating activity was restored after the infection of mice with the passaged preparations.
The production of Semliki Forest virus (SFV) has been described by Telling, Radlett and Mowat (1967) using BHK-21 cells in suspension in 4 litre culture vessels. At cell densities of up to $2 \times 10^6$ per ml. and with yields of about 3000 p.f.u. (plaque forming units) per cell, the infectivity of the culture supernatant fluid at 24 hours was about $10^{10}$ p.f.u. per ml. Zwartouw and Algar (1968), with primary chick embryo cells in suspension cultures under closely controlled conditions, obtained $10^3$ p.f.u. per cell or $10^{11}$ p.f.u. per ml. of culture supernate. This technique has been extended by Sargeant (personal communication, 1968) to cultures of 2-3 litre volumes with similar high infectivity.

An alternative method using primary chick embryo or human diploid (WI-38) cells in rolling multilayer cultures has also been used to produce similar high yields of vesicular stomatitis virus (Bradish and Kirkham, 1966) and of SFV and Langat viruses (Bradish, Thackeray and Allner, personal communication, 1968).

(iii) The Assay of Virus Infectivity

The quantity of infective virus produced by the above methods was assayed by the formation of plaques of lysed cells in monolayer (Dulbecco, 1952; Dulbecco and Vogt, 1954) or suspension cultures (Cooper, 1955; Bradish, Zwartouw, Allner and Algar, 1968). Such infectivity data cannot be related directly to the total number of virus particles present. The relationship between infectivity, number of virus particles and haemagglutinating activity is discussed later (Page 200).
(iv) The Replication of Arboviruses in Cell Cultures

In the few, well-documented cases, it appears that arboviruses mature either at the cell-wall or at the membranes of cytoplasmic vacuoles.

With Venezuelan equine encephalitis virus grown in KB cells, electron microscopical evidence suggested that the virus particles matured on the membranes surrounding such vacuoles (Musgay and Weibel, 1962). Similar observations have been made with Japanese B encephalitis virus in porcine kidney stable cells (Ota, 1965), Powassan virus in monkey kidney cells (Abdelwahab, Almeida, Doane and McLean, 1964), and with Semliki Forest virus in primary chick embryo cells (Acheson and Tamm, 1967) and in Hep-2 cells (Erlanson, Babcock, Southam, Bailey and Shipkey, 1967).

However, Chain, Doane and McLean (1966) studied Chikungunya virus in primary chick embryo fibroblasts and found that cytoplasmic vacuoles were absent. It was suggested that virus particles are formed adjacent to the endoplasmic reticulum and then released from the cell surface by budding.

Musgay (1964) has fully reviewed the multiplication and growth cycles of arboviruses in both vertebrate and arthropod cell and host systems.
e) Some Properties of Arboviruses

(i) Methods for the Detection of Antigens

The three most commonly used serological tests are the neutralization test, the haemagglutination-inhibition test and the complement-fixation test. Smith (1967) has discussed the standardization of these tests for epidemiological use. Casals (1961, 1963) and Porterfield (1961) have reviewed the procedures for the identification of arboviruses by cross-neutralization, cross-protection and other tests.


Lennette and Schmidt (1964) have described in detail the laboratory basis of these procedures.

(ii) The Structure-Function Relationship

(a) The Separation of Distinct Components from Suspensions of Arboviruses

(1) Arboviruses of Group A

Semliki Forest Virus (SFV)

The calcium phosphate chromatography of virus suspensions prepared from infected mouse brains (Smith and Holt, 1961) revealed several distinct physico-chemical fractions. Two haemagglutinating fractions were demonstrated of which only one showed infectivity and complement-fixing activity.

In studies on SFV extracted from infected mouse brains by treatment with
protamine sulphate and ultracentrifugation, Cheng (1961a, b) observed infectivity, haemagglutinating activity and complement-fixing activity in association with characteristic particles of 50 μm diameter. However, Faulkner and McGee-Russell (1968), using a similar preparation of SFV, showed by density gradient centrifugation in caesium chloride (CsCl) the presence of two distinct haemagglutinating components with densities of 1.240 and 1.205 gm per ml. of which only the more dense was infective. Similar studies in this laboratory with SFV extracted by fluorocarbon (trifluorotrichloroethane) and fractionated by gel filtration or centrifugation in CsCl have shown that the haemagglutinating activity is associated with a component corresponding in size and density (1.24 gm per ml.) with the infective particle (Page 59). Other non-infective, haemagglutinating components were not revealed.

Sindbis Virus

Mussgay and Rott (1964) obtained two haemagglutinating components of density 1.240 and 1.191 gm per ml. by the ultra-centrifugal analysis of supernatant fluids from cultures of Sindbis virus infected chick embryo cells. The more dense component was also associated with infectivity. Confirmatory results were obtained with calcium phosphate chromatography by Sarma, Fadda, Mandras and Feralta (1967).

Recent studies by Faulkner and Dobos (1968) using equilibrium density centrifugation in CsCl of Sindbis virus infected chick embryo culture fluids yielded three haemagglutinating components with characteristic buoyant densities of 1.240, 1.212 and 1.191 gm per ml. Infectivity was associated with the component of highest density. The least dense component (1.191 gm per ml.) contained non-infective, coreless particles and pleomorphic membranous structures derived from them.
On disruption of Sindbis virus by treatment with Tween-80 and ether (Mussgay and Horzinek, 1966), two complement-fixing components were produced with buoyant densities of about 1.27 and 1.16 g/ml. Two more dense components of densities 1.35 and 1.31 g/ml, associated with core material, were released when the virus was treated with cobra venom, an enzyme complex.

(2) Mosquito-Borne Viruses of Arbovirus Group B

Sabin and Buescher (1950) first suggested that the infective particle of Japanese B encephalitis virus was associated with haemagglutinating activity. More recently, it was reported by Kitaoka and Nishimura (1965) that sucrose-density gradient centrifugation of partially purified \( \beta \)-labelled Japanese B encephalitis virus grown in suckling mouse brains gave three haemagglutinating and complement-fixing components, two of which were infective.

Dengue virus (type 2), grown in cultures of KB cells and treated with fluorocarbon followed by equilibrium density centrifugation in CsCl, was shown to contain two haemagglutinating components of densities 1.24 and 1.19 g/ml. (Stevens and Schlesinger, 1965). The fraction containing the component of density 1.24 g/ml showed infectivity.

Smith and Holt (1961) showed by calcium phosphate chromatography that the mosquito-borne viruses of, amongst others, yellow fever, dengue and West Nile could be fractionated into two haemagglutinating and complement-fixing fractions, one of which was infective.

Similar results were obtained in studies of Langat virus as a representative of the tick-borne viruses of group B.

(3) Vesicular Stomatitis Virus

In studies of vesicular stomatitis virus from chick
embryo chorio-allantoic fluids by analytical and equilibrium density centrifugation in CsCl, Bradish and his colleagues identified two infective components of densities 1.217 and 1.191 gm per ml. and at least two complement-fixing components of densities 1.191 and 1.30-1.40 (Bradish, Brooksby and Dillon, 1956; Warrington, 1965). In contrast to this, vesicular stomatitis virus derived from BHK-21/13 cultures contained one infective component with a density of 1.225 gm per ml. and two complement-fixing components of densities 1.221 and 1.30-1.40 gm per ml.

(b) Distinctions due to the Conditions of Extraction of Arbovirus Suspensions

It is necessary to draw a distinction between native, or untreated, virus suspensions and those which receive a more or less disrupting extraction treatment prior to study. Any real correlation between physicochemical structure and biological activity is clearly dependent on the state of the virus suspension to be characterized.

Untreated virus suspensions may be studied directly by differential centrifugation (Cheng, 1961a), but it is more usual, particularly in studies of haemagglutinating activity, to treat such initial suspensions with lipid solvents in order to remove non-specific inhibitors of haemagglutination. Fluorocarbon extraction was used for this purpose by Porterfield and Rowe (1960) for four group A and 8 group B arboviruses, and by Takahashi (1965) for Japanese B encephalitis virus. This method destroys nearly all the infectivity of the preparation (reduced from $10^9$ to $10^2$ p.f.u. per ml.) without significant modification of the size and density of the virus particles (Page 59). Treatment with the detergent Tween-80 followed by extraction with diethyl ether (Musgay and Rott,
1964) is much more severe and the virus particles are broken down to non-infective, haemagglutinating components. Both of these methods are used in this study for the production of active haemagglutinating components from initial suspensions of Semliki Forest virus.

(c) The Relationship between Haemagglutinating Activity and the Concentration of Virus Components

The fundamental relationship between biological activity and the number of virus particles or derived active components, and particularly that between haemagglutinating activity and virus concentration, has been investigated by a number of authors. The discussion of such data will be deferred to a later section (Page 228) since this must depend upon a prior consideration of the mechanism and kinetics of haemagglutination which is the subject of the present experimental study.
d) Some Physical and Chemical Properties

(1) Size and Morphology

The available information on the size and morphology of arboviruses is meagre. Although attempts have been made to estimate the size of the infective particles of many arboviruses (Casals, 1968; Tauraso and Shelokov, 1967), it is difficult to draw sound conclusions since the methods of electron microscopy, ultra-filtration and ultracentrifugation are each subject to their own assumptions and limitations.

There are several divergent reports on the size of Semliki Forest virus (group A), as estimated by electron microscopy. Cheng (1961a) and Osterrieth and Calberg-Baeq (1966) favour a particle diameter of about 50 μm, whereas Faulkner and McGee-Russell (1968), also using a purified mouse brain preparation, and Simpson and Hauser (1968 a, b) with a primary chick embryo fibroblast preparation, consider the complete virion to have a diameter of about 70 μm. Muskay and Weibel (1963) showed that the diameter of the particle of Venezuelan equine encephalitis (group A), purified from chick embryos by centrifugation and treatment with protamine sulphate, varied from 40-75 μm depending on the methods of preparation and observation. Studies on the group C viruses Oriboca and Restan, and Manzanilla of the Simbu group, indicated physical particles of about 50 μm in diameter (Bastardo, Bergold and Munz, 1966).
Most arboviruses are quasi-spherical, possibly with cubical symmetry and are probably covered by an outer lipid envelope, like Semliki Forest virus, West Nile virus, western equine and Japanese B encephalitis viruses. The internal structure of Semliki Forest virus shows an electron-dense nucleoid with a diameter of about 50 μm (Faulkner and McGee-Russell, 1960; Simpson and Hauser, 1968a,b). The outer envelope is covered with a layer of fine projections, about 10-20 Angstroms long, which are assumed to be responsible for the haemagglutinating activity of the virus (Osterrieth and Calberg-Baeq, 1966).

Among the arboviruses, the vesicular stomatitis group is atypical in size and morphology. Preparations of vesicular stomatitis (New Jersey and Indiana strains) show bullet (172 x 78 μm) and cap (66 x 60 μm) shaped particles with a surface covering of fine projections of 10 μm in length associated with the lipid coat (Bradish and Kirkham, 1966; Bergold and Muns, 1967).

It is likely that most of the virus preparations examined contained a distribution of morphologically heterogeneous virus particles and this, together with the influence of different methods of preparation and observation, probably accounts for most of the inconsistencies in reports of the size and morphology of virus particles.
(ii) Chemical Composition

The data shown in Table 4 illustrate the available information concerning the protein, ribonucleic acid (RNA) and lipid composition of arboviruses. It is important to note that the methods used for the purification and analysis of the viruses were so different that strict comparisons are not possible.

Of some 204 arboviruses (Taylor, 1967), 22 have been analysed and shown to contain single-stranded RNA. The presence of lipid has been confirmed for 154 viruses by their sensitivity to the action of lipid solvents such as sodium deoxycholate, diethyl ether and chloroform (Theiler, 1957; Sunuga, Taylor and Henderson, 1960).

There is some evidence that the haemagglutinating activity is associated with the projections on the lipo-protein envelope of the virus particle. The enzymes pronase and caseinase C remove these projections from the surface of Semliki Forest virus with the subsequent loss of haemagglutinating activity (Osterrieth and Calberg-Bacq, 1966; Calberg-Bacq and Osterrieth, 1966). Mussgay and Rott (1964) treated Sindbis virus with Tween-80 and diethyl ether and observed the formation of star-shaped particles composed of rolled-up fragments of envelope. These particles showed haemagglutinating activity.
<table>
<thead>
<tr>
<th>Group</th>
<th>Virus</th>
<th>Mass Composition (%)</th>
<th>Reference</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Phospho-Cholesterol</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Sindbis</td>
<td>47.2/8.8/33/11</td>
<td>Pfefferkorn and Hunter (1963)</td>
</tr>
<tr>
<td></td>
<td>Eastern equine encephalitis</td>
<td>49.1/4.4/35/13</td>
<td>Taylor et al. (1943)</td>
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<tr>
<td>B</td>
<td>Dengue</td>
<td>7/7</td>
<td>Stollar et al. (1966)</td>
</tr>
<tr>
<td></td>
<td>Murray Valley encephalitis</td>
<td>82/7.9/0.8/1.0</td>
<td>Ada et al. (1962)</td>
</tr>
<tr>
<td></td>
<td>Japanese B encephalitis</td>
<td>30-35/7/55-60</td>
<td>Nosima et al. (1964)</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular stomatitis</td>
<td>20-30/3/60-70</td>
<td>Prevec and Whitmore (1963)</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Base Ratios (%)</th>
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<tbody>
<tr>
<td>Adenine (A)</td>
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<tr>
<td>Guanine (G)</td>
</tr>
<tr>
<td>Cytosine (C)</td>
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<tr>
<td>Uracil (U)</td>
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<tr>
<td>A</td>
</tr>
<tr>
<td>Sindbis</td>
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<tr>
<td>B</td>
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<tr>
<td>Murray Valley encephalitis</td>
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III. THE MECHANISMS AND KINETICS OF HAEMAGGLUTINATION BY VIRUSES

a) Haemagglutination by Myxoviruses

The myxo-group of viruses has been intensively studied since the demonstration by Hirst (1943) that many of the reactions between influenza virus and RBC also occur with host tissue cells. This subject has been reviewed by Burnet (1952).

(i) The Attachment of Virus to Red Cells

Under suitable ionic conditions, myxoviruses attach to and elute from RBC over a wide range of temperatures above 4°C. The initial attachment has been shown to be of an electrostatic nature by the dependence on the presence of electrolytes and on the pH of the medium (Flick, Sanford and Mudd, 1949; Puck, Garen and Cline, 1951; Valentine and Allison, 1959). The presence of cations reduces the electrostatic repulsion between the negatively charged RBC and virus particles and enhances the forces of attraction responsible for haemagglutination. Ackermann (1964) has shown that in a sucrose solution, RBC are agglutinated in the absence of virus due to the reduction of the negative charge on the RBC. The addition of negatively charged virus particles to this reaction mixture modifies the charge distribution and causes the separation of the RBC aggregates.

Other non-specific forces may contribute towards the binding of RBC and virus. Cohen (1963) has suggested that since the rate of attachment is mainly independent of temperature, the energy of activation of such forces must be low. Therefore, hydrogen-bonding may be an additional mechanism of the binding of virus particles to RBC.
The rate of attachment of virus to RBC is dependent on the concentrations of both and on the frequency of their collision, as defined by Brownian movement. If the RBC concentration is sufficiently high to be effectively in excess with respect to the concentration of virus, then the reaction approximates to first order kinetics (Cohen, 1963). With vaccinia or fowl plague viruses reacting with fowl RBC, Allison and Valentine (1960a, b) found that the reaction tended to conform to first order kinetics from about one hour after the initiation of the reaction. They concluded that only one third of the collisions led to the irreversible binding of virus particles. During the later stages of the reaction, the rates of adsorption decreased due to the agglutination of the RBC and the consequent reduction in their available surface area.

A more complete review of these possible mechanisms of attachment has been given in a thesis by Biswal (1965).

More detailed studies of the physico-chemical nature of haemagglutination by non-viral agglutinins, such as blood group antibodies, have been made by Filitti-Wurmsen, Jacquot-Armand, Aubel-Lesure and Wurmsen (1954) and Pollack and Hager (1964).

(ii) The Adsorption of Virus to Red Cells

The initial adsorption of influenza virus is rapid (Buzzell and Hanig, 1958) and RBC can become almost completely covered with virus particles within a few minutes, clearly visible in the electron microscope (Dawson and Elford, 1949). Donald and Isaacs (1954) suggested, on the basis of rather limited data, that, after adsorption of influenza virus by RBC, a negligible proportion of virus particles remained free in the medium. Magill (1951) showed that repeated adsorptions of a range \(10^6\) of virus dilutions with RBC for 30
minutes at 0°C showed that 0.3%–3% of the virus particles remained unadsorbed and were detectable by their infectivity. However, according to Hirst and Pickels (1942) there were never more than 8% of the RBC in the form of aggregates even after treatment with nominally high virus concentrations. This may be interpreted either as the presence of non-agglutinable RBC in the suspension or as the proportion of RBC completely saturated with haemagglutinin which can then be agglutinated only by the addition of further unsaturated RBC, as in the iso-haemagglutination system (McKerns and Denstedt, 1950).

(iii) The Elution of Virus from Red Cells

Above 4°C, adsorbed myxoviruses elute spontaneously from RBC with the destruction of the RBC receptors. The mechanism of this enzymatic reaction has been defined mainly through the work of McCrea (1953) and Gottschalk (1960). The viral enzyme, neuraminidase, destroys the RBC receptor by splitting the carboxyl group of the N-acetylneuraminic acid which is attached to an adjacent sugar residue. After elution, this receptor is no longer able to adsorb a virus particle but the eluted virus can repeatedly adsorb onto new RBC receptor sites. The rate of elution depends on temperature, enzymatic activity and on the amount of virus adsorbed to each RBC. (Sagik, Puck and Levine, 1954; Sagik and Levine, 1957). This eluting behaviour is different for each member of the myxovirus group. Burnet, McCrea and Stone (1946) have shown that these viruses could be arranged in a receptor gradient according to their capacity for agglutinating RBC which have been previously treated with other members of the group. For example, the influenza viruses destroy receptors more actively than Newcastle disease virus which, itself, is higher in gradient position than the weakly active mumps virus.
The damage of receptor sites and adsorption-elution equilibrium rates may also be responsible for the incomplete adsorption of virus particles to red cells described above.

(iv) The Structure of Haemagglutinating Components

There have been many studies of the nature of the haemagglutinating components of myxoviruses. Eckert (1967) reported that influenza virus envelope protein, the haemagglutinin, is adversely sensitive to high temperatures (23°C), concentrated urea, sulphhydryl-reducing reagents and trypic digestion at high salt concentrations. His conclusion was that the haemagglutinin becomes stabilized when associated with the lipid micelle at the virus surface. The detailed peptide structure of three strains of influenza virus has been correlated with some of the biological activities of the virus, including haemagglutination (Laver, 1964). The chemical structure of the haemagglutinin appears to be strain specific. In addition, Almeida, Himmelweit and Isaacs (1966) studied the "rosette" and "bottle-brush" forms of the intracellular haemagglutinating component of fowl plague virus. These occurred in the normal state, whereas they can only be obtained for other myxoviruses by treatment with solvents such as ether.

More complete discussions on the mechanisms involved in the interaction between RBC receptors, analogues and inhibitors have been given by Buzzell and Hanig (1958), Cohen (1963), Philipson (1963) and Sauna et al. (1967).

(b) Haemagglutination by Arboviruses

There are very few reports concerning the mechanisms of haemagglutination by arboviruses.
(i) The Attachment and Adsorption of Haemagglutinin to Red Cells

Using the Belyanchikov strain of the tick-borne encephalitis virus complex and rooster RBC, Salminen (1960) observed that 98% of the haemagglutinin prepared by sucrose-acetone extraction adsorbed to RBC only in the pH range of 6.2-6.3 and at 0°C. He considered that adsorption occurs at pH values between the iso-electric points of the haemagglutinin and of the receptor on the RBC surface, and therefore the reaction is an electrostatic salt linkage between ionised amino- and carboxyl groups.

Also using group B viruses such as Japanese B encephalitis virus, West Nile and yellow fever viruses, Hale and Pillai (1960) studied the properties of haemagglutination with 1 day-old chick RBC. Haemagglutinin only adsorbed to RBC over the pH range 6.5-7.0, although there was total inactivation of the haemagglutinin at these low pH values in the absence of RBC (Page 181).

(ii) The Elution of Haemagglutinin from the Red Cell

Salminen (1960) observed the elution of tick-borne encephalitis virus haemagglutinin from the red cell above pH 6.8 and found this process to be independent of temperature. After elution, the RBC continued to adsorb fresh haemagglutinin and it was concluded from this that no specific receptor sites were involved.

On the other hand, Hale and Pillai (1960) were unable to show elution of some group B virus haemagglutinins from the stable complex with RBC. However, the inability to recover free, unadsorbed haemagglutinin might be due to its rapid inactivation at pH 6.5-7.0.

(iii) The Physico-Chemical Nature of the Haemagglutinating Components

The physico-chemical data for the components of Semliki Forest and Sindbis viruses showing haemagglutinating activity have been
described on Page 25.

c) **Haemagglutination by Other Viruses**

Very limited information is available concerning the mechanisms of haemagglutination by viruses other than those of the myxo- and arbo- groups described above.

(i) **Viruses which show Elution from Red Cells**

At temperatures above that at which adsorption occurs, several viruses elute spontaneously from RBC with the concomitant destruction of the receptor sites. Encephalomyocarditis (EMC) virus has been shown to agglutinate sheep RBC under specified conditions at 4°C in the absence of potassium ions (Olitsky and Yager, 1949) and then to elute at higher (23°C) temperatures (Hallauer, 1951). There is some evidence from experiments using receptor-destroying enzyme (RDE) that the RBC receptor for EMC virus is similar, if not identical to, that for the myxoviruses (Verlinde, De Baan, Kret and Waller-Fetter, 1951; Kodza and Jungeblut, 1958).

Isabelle and Horsfall (1949) showed that the GD VII strain of mouse encephalomyelitis virus agglutinated human group 'O' RBC at 0°C and that the virus was released from the cells if the temperature was raised. Similar observations have been made for polyoma virus (Sachs, Fogel and Winocour, 1959), for reoviruses (Lemer, Cherry and Finland, 1963), for several Echo and Coxsackie viruses (Philipson and Bengtsson, 1962; Heberling and Cheever, 1965), and for bovine ECHO viruses (Moscovici and Maisel, 1958). In all of these cases, the RBC receptors were different from those for the myxoviruses and the elution reaction was independent of any enzymatic mechanisms.
(ii) Viruses which do not Elute from Red Cells

Although haemagglutination occurs with adenoviruses (Rosen, 1958, 1960a; Pereira and De Figueiredo, 1962), measles virus (Norrby, 1964, 1968; Norrby, Magnusson, Falksveden and Gronberg, 1964), and poxviruses (Nagler, 1942), elution of the virus from the complex with RBC has not been reported.

d) General Conclusion

The absence of a lipid-containing envelope in the structure of a virus, as shown in Table 3 (Page 7), does not necessarily imply the absence of the capacity to agglutinate red cells.

From the above data, it is clear that the mechanism of haemagglutination by arboviruses cannot be assumed to be the same as that for other groups. It cannot even be assumed that agents classified as arboviruses will necessarily show the same mechanism of haemagglutination, particularly in view of their complex behaviour in haemagglutination with respect to pH and other environmental factors. This problem is discussed later (Page 234).
IV. THE QUANTITATION OF HAEMAGGLUTINATION BY VIRUSES

It has long been clear that considerable practical importance attaches to the quantitative relationship between the amount of virus present in an experimental sample and the extent of agglutination produced when this is mixed under specified conditions with an appropriate suspension of red blood cells.

The principle of methods for the assay of viruses by haemagglutination is the attachment of otherwise undetectable virus particles to the red blood cell (RBC) as a visible carrier. The consequent agglutination of RBC due to adsorbed virus may then be detected visually or with the aid of optical instrumentation. If the adsorption reaction of virus as haemagglutinin (H) to RBC may be suitably controlled, then the quantitation of virus as haemagglutinin, at the particle level, should be possible by appropriate interpretation of the extent of agglutination.

The formation of aggregates may be represented as shown in Figure 1. The initial attachment of haemagglutinin to individual RBC is the first or the adsorption stage of the reaction. In the second stage of the reaction, individual virus-coated RBC agglutinate to form a suspension of dimers and larger aggregates. Finally, in the third stage of the reaction, the aggregates settle to the bottom of the container and, under the influence of charge effects and the shape of the container wall, form large, surface-bound aggregates which form a characteristic pattern dependent on the number and size of aggregates present.
**FIRST STAGE**
The initial attachment of haemagglutinin to red cells in free suspension.

**SECOND STAGE**
The intermediate reaction in free suspension with increase of sedimentation rate as dimers and small aggregates are formed.

**THIRD STAGE**
The terminal reaction at the tube or cup surface to form large surface-bound aggregates which settle in a characteristic pattern.
a) **The Pattern Method**

The Pattern Method is the technique most commonly used in the laboratory for assaying the haemagglutinating activity of a virus preparation. This procedure for haemagglutination was first described by Salk (1944) using test-tubes, and was recommended as a standard procedure by the World Health Organisation (1953) in the modification using Perspex or plastic agglutination or dimple plates. A micro-technique was devised by Takatay (1955) and later modified and developed by Sever (1962) and Takatay (1967); this micro-procedure is very economical of test reagents and well-suited for survey studies on large numbers of samples. To determine the haemagglutinating activity of a virus suspension by this pattern method (Appendix III), dilutions of the virus are mixed in an appropriate medium with equal volumes of red blood cells in either tubes or dimple plates. After a suitable reaction period, the end-point dilution is shown as that at which there is a standard but arbitrary level of partial agglutination. This state of partial agglutination is indicated by the adhesion of some aggregates to the curved surface of the tube or dimple in the presence of some free RBC which roll down to form a central ring or button. The resulting measure of haemagglutinating activity is simply observed, and is adequate for many purposes, but, through the complexity of the reactions involved, is not readily related to the initial concentration of haemagglutinin or of virus particles. This is the subject of the investigations to be described.
b) **Photometric Procedures**

Hirst, using chick red blood cells and influenza virus, made the first attempt to quantitate the haemagglutination reaction. The initial method (Hirst, 1942a) was to add RBC to different dilutions of the virus preparations and allow the reaction mixtures to stand at room temperature for 75 min. The percentage of RBC not agglutinated was then measured by visual comparison with standard, unagglutinated RBC suspensions. The endpoint was taken as the dilution which agglutinated 33%-50% of the available RBC. This technique was later improved by using a simple photoelectric densitometer to estimate the extent of agglutination, (Hirst, 1942b).

This method has been further developed for the estimation of antibodies, again using influenza virus, by Hirst and Pickels (1942), Miller and Stanley (1944), and Belyavin, Westwood, Please and Smith (1951).

Hirst and Pickels (1942) improved the procedure by measuring the optical densities of the middle layer of virus-RBC reaction mixtures. The 50% agglutination end-point of a particular virus suspension was then determined from a standard optical density - virus dilution curve. When used for measuring antibody activities in sera, the method was "quick, more objective, accurate and reproducible". An important finding was that even with very high concentrations of virus, total agglutination was not observed. This was later confirmed by Miller and Stanley (1944) who showed that over a wide range of RBC concentrations, a constant amount of virus caused the agglutination and settling out of a constant percentage of RBC per unit time. Belyavin *et al.* (1951) applied statistical techniques to Hirst and
Pickels' method in order to reduce the error in virus and serum antibody activities derived from such haemagglutination procedures.

In 1953, Levine, Puck and Sagik, undertook experiments to obtain a direct relationship between the infectivity and the haemagglutinating activity of the same virus suspension. Using the photometric technique with influenza virus, they claimed that an initial and dominating dimer stage (Figure 1) of the haemagglutination reaction could be detected and measured. The presence of mainly RBC dimers in a reaction mixture was shown by the change of optical density resulting from the introduction of virus. The relationship obtained was of 1 haemagglutinating particle to 0.41 egg-infective units and to 1.33 physical particles identified by electron microscopy. Horsfall (1954), using the photometric technique, established a 1:1 ratio of haemagglutinating particles to infective influenza virus particles. In 1953, Horsfall and Tamm devised a "fractional dilution procedure" in tubes in which dilution steps of 0.1 logarithmic unit were used. The precision and reproducibility of the method compared favourably with the results using the photometric method. These and other published data and conclusions will be more fully discussed later (Page 227).
A major development in the photometric assay of the activities of viral haemagglutinin and of homologous and heterologous antibodies occurred in the work by Drescher and his colleagues using strains of influenza virus (Drescher, 1957; Drescher, Hennessy and Davenport, 1962; Drescher, Davenport and Hennessy, 1962). The basis of their method was that the haemagglutinating activities of virus suspensions were expressed in terms of the highest virus dilution causing maximal agglutination of a RBC suspension. This procedure was adopted because it was found empirically that the variability in reactivity of RBC with virus decreased as the concentration of agglutinated RBC approached a maximum level. In practice, this state of maximal agglutination was determined by extrapolation of experimental data obtained for reaction mixtures with constant RBC concentrations and a range of virus concentrations. This method is discussed on Page 88. The technique was used to analyse the antigenic composition of ether-treated influenza A virus haemagglutinin suspensions (Davenport, Hennessy, Drescher and Webster, 1964; Drescher, 1967), and for enhancing the sensitivity of antibody assay using ether-treated virus (Drescher, 1966). No further progress or modifications to this have been made (Dr. F.M. Davenport, personal communication, June, 1967).

The photometric method has been used to count red blood cells (Godal, 1966) and to determine the haemagglutinating activity of proteins such as soyin and soybean extracts (Liener, 1955).
c) **Automated Procedures**

(1) **The Auto-Analyser**

Modifications designed to automate the above methods have been made in the following way. A reaction mixture containing RBC aggregates was passed through an apparatus in which the agglutinated RBC settle out to leave free, un-agglutinated RBC in free suspension. The free and now separate RBC were lysed with 0.1N sodium hydroxide and the concentration of the released haemoglobin was measured with a photometer and related to the fraction of cells lysed. This is the principle of the Auto-Analyser (Technicon Instruments Inc.) automated technique which has been described as an accurate and reproducible method applicable to several serological tests. Ferrari (1964), Grunmeier, Gray and Ferrari (1965), Morris, Jenkins and Horswood (1965) and Hebeka, Brandon and Molteni (1967) have used the Auto-Analyser successfully for the assay of haemagglutination by influenza viruses. Morris *et al.* (1965) met with some difficulties in the operation of the Auto-Analyser and suggested that the 4-16 times higher sensitivity of the Klett-Summerson photometric method was due to the assay of a different substance. Cohen (1966) compared the assay of antibody activity to influenza viruses by the Auto-Analyser and by photometric procedures. With the former, he reported inaccuracy, lack of reproducibility and slow sampling rate.
Ginzburg and Traub (1959), Finter (1964, 1967) and Goedemans and Peters (1968) have shown that it is possible to make use of the haemadsorption activity of several viruses in the quantitation of interferon activity and neutralising antibodies. The principle is similar to that of the Auto-Analyzer technique in that the RBC that are adsorbed to infected tissue cultures are lysed and the optical density of the resulting solution is a measure of the RBC adsorbed, and hence of the virus concentration.

The Auto-Analyzer has been applied to the routine haematological procedures of blood grouping and typing (McNeil, Helmick and Ferrari, 1963; Sturgeon, Cedergren and McQuiston, 1963). Dybkjaer (1966) has studied non-viral blood antibody systems and has adapted the technique of lysing free RBC and estimating the haemoglobin concentration photometrically. The free RBC are separated from the aggregates of an equilibrated reaction mixtures by centrifugation on a 40% (w/v) dextrose solution (Renton and Hancock, 1964). The free RBC remain in the upper part of the dextrose solution and are removed for haemoglobin estimation.

It is evident that considerable difficulties have been encountered in efforts to establish an acceptable and economical technique for virological and serological work.
Another commercial apparatus, the Fragiligraph (Kohn and Danon, 1965, 1967), has been used for the rapid estimation of the haemagglutinins of influenza virus, Newcastle disease virus, Semliki Forest virus, adenovirus type 3 and encephalomyocarditis virus. Haemagglutination inhibition by specific antibody has also been studied with a variety of RBC species. The Fragiligraph was designed to measure the osmotic fragility of RBC (Danon, 1963) and measures the optical density of a reaction mixture of virus with RBC at high concentration. With increasing agglutination and the settling out of aggregates, the optical density decreases to a plateau value corresponding to the proportion of unagglutinated RBC. The published quantitative data are incomplete.

**d) Electronic Counting Methods**

(i) **The Coulter Counter**

All of the above procedures yield an integrated measure of the average reaction and provide data which cannot readily be interpreted at the particle level in terms of the kinetics of the reaction between viral haemagglutinin and red blood cell. To provide the data required for the interpretation of haemagglutination reactions at the particle level, it is necessary to count individual RBC and aggregates. Such techniques using electronic cell counters (Coulter Electronics Inc.) have been extensively developed for the quantitation and study of blood group substances (Brecher, Schneiderman and Williams, 1956; Mattern, Brackett and Olson, 1957; Peacock, Williams and Mengoli, 1960; Goodman, 1962; Mengoli, Fruitt and Carpenter, 1963; Bowdler and Swisher, 1964; Gibbs et al., 1963,
1965a, b; Solomon et al., 1965). However, there is apparently no report of an electronic counting device being used to quantitate the haemagglutinating activity of a virus preparation. The electronic counting procedure was, therefore, one of the methods investigated in this study. (Page 110).

(ii) The Celloscope

A similar alternative, which has not been studied, is the Celloscope (Ohlin, 1958) in which the RBC are passed through a capillary tube and are counted, as before, in terms of local conductivity changes. The Celloscope may be unsuitable for the study of agglutinated suspensions in view of the adhesion of aggregates to the greater area of the capillary wall. Also, shear forces within the capillary channel may lead to breakdown of the aggregates. These problems are discussed later (Page 124). The Celloscope could be used for the quantitation of free, single cells in such reaction mixtures if the aggregates were first removed by centrifugation (Ronton and Hancock, 1964).

e) The Sedimentation-Enumeration Method

Since the results obtained from those investigations using the photometric and electronic counting methods have not clarified the mechanisms of the interaction between viral haemagglutinin and red blood cell, an alternative procedure was sought for counting cells and aggregates. A possibility was the direct observation with a light microscope of an undisturbed, settling reaction mixture. The Sedimentation-Enumeration Method which has now been developed for this purpose has been used as a quantitative tool in the study of the mechanism and kinetics of the haemagglutination reaction between the arbovirus, Semliki Forest virus and goose red blood cells. (Page 125).
MATERIALS AND METHODS
a) Viruses

(i) Vaccinia Virus

In the preliminary experiments with photometric procedures vaccinia virus was used. The virus was grown according to the method of Zwartouw, Westwood and Appleyard (1962). Virus grown on the skin of rabbits was purified and concentrated by sucrose-density gradient centrifugation. The haemagglutinin separated from the infective particles as an upper band in the 30% sucrose layer. This haemagglutinin was stored at 4°C and showed a haemagglutinating activity of up to about 3,600 HAU per ml., depending on the susceptibility of the RBC from individual chickens (Nagler, 1942).

(ii) Influenza Virus, Type A (PR8 strain)

A seed virus suspension was obtained from the Virus Reference Laboratory, Colindale, and grown in the chorio-allantoic cavity of chick embryos by a method, modified after Lief and Henle (1956). Aliquots of 0.1 ml. of a 10^-5 dilution of the seed suspension were inoculated into fertile eggs by the allantoic route. The eggs were incubated at 37°C for 48 hr. and the surviving embryos chilled at 4°C for 2 hr. The allantoic fluids were then removed and individually tested for the presence of haemagglutinating activity. Allantoic fluids containing RBC were incubated at 37°C for 30 min. to elute virus before centrifugation at 2,000 rev. per min. for 10 min. to deposit RBC. The pooled supernatant fluids were stored at -30°C as the stock haemagglutinin. This usually had a haemagglutinating activity of about 3,000 HAU per ml.
(iii) **Semliki Forest Virus (SFV)**

This group A arbovirus, isolated from mosquitoes from the Semliki Forest in western Uganda (Smithburn and Haddow, 1944), was obtained from the American Type Culture Collection (VR 67 : original strain) as a suspension of mouse brains at the twelfth passage. The stock virus was a suspension of mouse brains at the fifteenth passage.

(iv) **Lengat Virus**

The TP-21 strain was isolated from an ixodid tick in Malaya (Smith, 1956) and is included in the tick-borne encephalitis complex of group B arboviruses. The haemagglutinin used in this study was derived from a mouse brain suspension by sucrose-acetone extraction and was obtained from Dr. C.E.G. Smith.

b) **The Preparation of Haemagglutinins from Semliki Forest Virus**

For the three passages of virus in this laboratory, suckling mice were inoculated by the intracerebral (IC) route with aliquots of 0.02 ml. of a 10% (w/v) suspension of the virus seed in borate buffer (EB/BSA, Page 50). After about 36 hours when the mice were sick and moribund, they were killed by chloroform. Their brains were removed by aseptic techniques and weighed after freezing by solid CO₂. The brains were homogenized in borate buffer at pH 9.0, containing 0.75% bovine serum albumin, to give a 20% (w/v) suspension which was stored at -80°C.

Some later experiments used SFV grown in suspension cultures of primary chick embryo cells (Zwartouw and Algar, 1968).

Initially the sucrose-acetone extraction procedure of Clarke and Casals (1958) for the preparation of haemagglutinins for arboviruses was used. This required large volumes of solvents and in later work the following more rapid
and economical procedures were adopted. Lipid haemagglutination inhibitors were removed by homogenization of the virus suspension with fluorocarbon (trifluorotrichloroethane, Porterfield and Rowe, 1960) or by treatment with the detergent Tween-80 (polyoxy-ethylene sorbitan mono-oleate) followed by extraction with diethyl ether (Musgay, Fadda and Feralta, 1967; Saturno, 1967). These methods are given in detail in Appendix II.

The following code is adopted for the different preparations of arbovirus haemagglutinins which have been used in this study:

<table>
<thead>
<tr>
<th>Stock Virus</th>
<th>Host and Tissue of Origin</th>
<th>Method of Extraction</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Senliki Forest (SFV)</td>
<td>mouse-brain suspension (M)</td>
<td>None</td>
<td>SFV(M)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fluorocarbon (F)</td>
<td>SFV(M)/F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sucrose/acetone (SA)</td>
<td>SFV(M)/SA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tween-80/ether (TE)</td>
<td>SFV(M)/TE</td>
</tr>
<tr>
<td></td>
<td>supernatant fluid of chick embryo cell culture (C)</td>
<td>None</td>
<td>SFV(C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fluorocarbon (F)</td>
<td>SFV(C)/F</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sucrose/acetone (SA)</td>
<td>SFV(C)/SA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tween-80/ether (TE)</td>
<td>SFV(C)/TE</td>
</tr>
<tr>
<td>Langat (TP-21)</td>
<td>mouse brain suspension (M)</td>
<td>sucrose/acetone (SA)</td>
<td>TP-21(M)/SA, etc.</td>
</tr>
</tbody>
</table>

c) Red Blood Cells (RBC)

All RBC were bled into modified Asevers' Solution (Appendix I).

Geese: Blood was taken weekly, from about six birds, pooled and stored at 4°C.

Fowls: Blood was taken individually when required and then tested with vaccinia haemagglutinin. The sensitive individual chickens were kept for further use.
Sheep: Blood was taken weekly and pooled. RBC more than 5 days old were not used in this study.

d) Preparation of Goose and Fowl RBC for Use in Haemagglutination Tests

RBC were washed twice by deposition and resuspension in physiological saline before final deposition by centrifugation at 2000 rev. per min. for 5 min. The packed RBC were then suspended to a nominal concentration in the appropriate buffer solution prior to the determination of RBC concentration by counting in a Neubauer haemocytometer.

It should be noted that in this study all concentrations and dilutions are given as the final values in the preparation or reaction mixture.

e) The Preparation and Treatment of Antiserum

Specific anti-SFV sera for use in haemagglutination-inhibition (HAI) tests were prepared in rabbits. An initial intravenous inoculation of $10^6$ p.f.u. per ml. of stock SFV(M) was followed 4-6 weeks later by a similar inoculation. This programme of inoculation was continued until at the second or third course the antiserum showed a Serum Neutralization Index (SNI) of $10^4$-$10^5$. Before being used with SFV in the HAI test, the inactivated ($56^\circ$C for 30 min.) antiserum was treated at $0^\circ$C with packed goose RBC to adsorb natural rabbit agglutinins for goose RBC. The adsorbed serum was then treated with acetone or acid-washed kaolin in HB/BSA at pH 9.0 to remove the non-specific inhibitors of haemagglutination (Clarke and Casals, 1958).

In some later experiments, a RBC-antibody agglutinating system was used in comparison with the usual RBC-virus system. Rabbit anti-goose RBC antiserum was prepared by the intravenous inoculation of $10^3$ goose RBC, with further inoculations at intervals of one month.
Horse anti-sheep RBC antiserum was obtained from Burroughs Wellcome Ltd., Kent.

f) Buffer Solutions  (For details see Appendix I ).

All chemicals and reagents were of Analytical grade (British Drug Houses, Ltd., Dorset), unless otherwise stated.

All solutions were made in glass distilled water and sterilized by Seitz filtration or by autoclaving at a pressure of 10 lb. per in.² for 15 min. The Beckman, model G, pH meter with glass and calomel electrodes was used for the determination of the pH values of all solutions.

The buffers used were:

(i) Borate Buffer/Bovine Serum Albumin (BB/BSA): Borate buffer at pH 9.0 containing bovine serum albumin at a concentration of 0.2% (w/v) is the diluent normally used for the haemagglutinins of arboviruses.

(ii) Phosphate Buffered Saline (PBS): This PBS (see Appendix I ) is used to prepare suspensions of packed RBC for use in the haemagglutination test.

(iii) In the detailed studies on the conditions for haemagglutination by arboviruses, the following buffer solutions were also used:

Melville's citrate buffer, Sorensen's phosphate buffer, Tris buffer and Veronal buffer.

(iv) Calcium/magnesium saline and physiological saline were used as diluents for both the RBC and the haemagglutinins of vaccinia and influenza viruses.

g) Preparation of RBC Membranes

In early work, membranes of fowl RBC were required and these were prepared conveniently by lysis of RBC with saponin, after the method of Carlisle
and Elrod (1949). Washed, packed fowl RBC (1 ml.) were added to 100 ml. of 0.125% saponin. The mixture was shaken by hand for 5 min. to ensure complete lysis followed by centrifugation at 2000 rev. per min. and washing twice with physiological saline. To allow the identification of membranes when used in the pattern test, they were stained by the addition of 2 ml. of 0.5% aqueous methyl green to the saponin solution. This did not affect the ability to form the usual range of patterns in the presence of haemagglutinin.

h) Protocol for Haemagglutination Tests by the Pattern Method.

This is based on the general methods described by WHO (1953) and Sever (1962) and, for arboviruses in particular, by Clarke and Casals (1958).

The following procedures were adopted using the WHO designed Perspex pattern or dimple plates.

(i) For Influenza and Vaccinia Viruses

The haemagglutinin was diluted in two-fold steps in physiological or calcium/magnesium saline. Aliquots of 0.25 ml. of these dilutions were distributed into a row of cups in a dimple plate. To each cup were added 0.25 ml. of saline and 0.50 ml. of a suspension of RBC at a concentration sufficient to give $5.5 \times 10^6 - 6.5 \times 10^6$ RBC per ml. of the reaction mixture.

Thus, the four-volume test mixture as used was:

1 volume (0.25 ml.) dilution of haemagglutinin
1 volume (0.25 ml.) buffer solution
2 volumes (0.50 ml.) RBC suspension

(ii) For Arboviruses

A four-volume test mixture similar to that given above was used with adjustment of the final pH of the reaction mixture to the optimal value.
required for haemagglutination by arboviruses. This was done by mixing equal volumes of haemagglutinin in 
BB/BSA and RBC in PBS of appropriate pH to yield reaction mixtures of predetermined pH in the range 6.0-7.0.

The test then became:

1 volume (0.25 ml.) dilution of haemagglutinin in BB/BSA, pH 9.0
1 volume (0.25 ml.) BB/BSA, pH 9.0
2 volumes (0.50 ml.) RBC in PBS as required.

The dimple plate was incubated at room temperature (23°C) for about 1 hour before the pattern was read.

In the haemagglutination-inhibition test, the volume of buffer (BB/BSA) was replaced by 1 volume of inhibitor or antiserum diluted in BB/BSA. This test was carried out in two steps. The initial reaction at 23°C for 30 min. between the antiserum or inhibitor and a standardized '4' quantity of haemagglutinin was followed by the reaction of residual or available haemagglutinin with the then added RBC suspension.

The method of reading the pattern and the calculation of the haemagglutinating activity as haemagglutinating units per ml. (HAU per ml.) of reaction mixture, are given in Appendix III.

i) Assay of the Infectivity of Arbovirus Suspensions

Plaque assays were carried out on chick embryo cells suspended in agar (Bradish, Zwartouw, Allner and Algar, 1968). To each of a series of tenfold dilutions of virus (0.5 ml.) were added 4.5 ml. of chick embryo cells (2 x 10^7 per ml.) suspended in Parker's 199 medium (Glaxo Ltd., Middlesex) containing 10% calf serum. After holding for 15 min. at 37°C, 5 ml. of 1.2% agar in 199 medium at 42°C without sodium bicarbonate were added and the whole reaction mixture rapidly poured into a disposable Petri dish of 9 cm. in diameter. After incubation at 34°C for 2 days in an atmosphere of 5% CO₂: 95% air, the
plates or dishes were stained with 0.005% neutral red in saline. The areas of virus replication then appeared as circular areas or plaques of lysed cells which were clearly distinguished from the background of intact, stained cells. The concentration of infective units, or plaque forming units (p.f.u.), was then obtained by counting.

j) The Fractionation of SFV by Gel Filtration

(i) Gel Filtration on Columns of G-200

Sephadex G-200 (Pharmacia, Uppsala, Sweden) was prepared and set up at room temperature (23°C) in glass columns of length 25 cm. and of diameter 1.5 cm, with a bed volume of 20 ml. and a void volume of 3 ml. A volume (1 ml.) of haemagglutinin or virus was carefully layered onto the column and was eluted with PBS buffer at a flow rate of 0.75 ml. per min. The eluate was collected in 5 ml. fractions and each was tested for infectivity and haemagglutinating activity.

(ii) Gel Filtration on Columns of Sepharose-4B

Columns of length 25 cm. and diameter 3 cm. were set up containing Sepharose-4B (Pharmacia, Uppsala, Sweden) with a bed volume of 75 ml. and a void volume of 10 ml. As for filtration with columns of G-200, a known volume of haemagglutinin (usually 1 ml.) was layered onto the column and eluted with PBS at room temperature (23°C) at a flow rate of 2.0 ml. per min. The eluate was collected in 2 ml. fractions and tested for haemagglutinating activity.

d) Fractionation of SFV by Equilibrium Density Centrifugation in Caesium Chloride

Equal volumes of SFV haemagglutinin and 60% (w/v) caesium chloride
(CsCl) in BB pH 9.0 were mixed and centrifuged to equilibrium in the SW-39 rotor of a Beckman Spinco centrifuge (Model L) at 37,000 rev. per min. for 24 hr. The rotor was allowed to come to rest without the use of the brake. The bottom of the tube was pierced and fractions of 0.5 ml. volume were collected and tested for haemagglutinating activity. Densities were estimated from the refractive indices measured with an Abbe refractometer and related to density by data derived from published tables (The Chemical Rubber Co., U.S.A.).
RESULTS
I. HAEMAGGLUTINATION BY ARBOVIRUSES

a) Introduction

To show specific haemagglutination by arboviruses, it is necessary for the haemagglutinin preparation to be free from non-specific inhibitors, for the RBC to be derived from a suitable animal species and for the pH of the overall reaction mixture to be at the optimal pH value. Similarly, precise conditions are also required for haemagglutination by rubella virus and by the bullet-shaped or vesicular stomatitis-like viruses (Halenen et al., 1967; 1968).

The present study of the optimal conditions for haemagglutination by arboviruses was undertaken in order to enhance the sensitivity of the procedure and to provide an acceptable and defined basis for the later quantitation of the mechanisms and kinetics of the reaction.

b) Characterization of the Haemagglutinins used in this Study

In many cases, arbovirus suspensions are derived from the brains of mice and the products consequently contain large amounts of host-derived lipid inhibitors of haemagglutination. To demonstrate haemagglutinating activity, these inhibitors (Salminen, 1962) must be removed by extraction with lipid solvents such as acetone, ether, fluorocarbon or a combination of Tween-80 and ether. In early experiments, the sucrose-acetone and acetone-ether methods of Clarke and Casals (1958) were used successfully to produce haemagglutinins. In later studies the simpler and more convenient methods using fluorocarbon (Gessler, Bender and Parkinson, 1953) and Tween-80/ether (Musagay et al., 1967) were adopted since they gave preparations with haemagglutinating activities of at least 2,500-3,000 HAU per ml. which were entirely satisfactory for further studies. These methods are described in Appendix III.
Table 5 shows the influence of different extraction procedures on the infectivity and haemagglutinating activity of preparations of arboviruses derived from mouse-brains and chick embryo cells. It is clear that extraction with sucrose-acetone or Tween-80/ether results in the complete inactivation of infectivity. Treatment with fluorocarbon is clearly less severe since it results in less enhancement of haemagglutinating activity and less complete inactivation of infectivity. Three or more extractions with fluorocarbon of a mouse-brain suspension of SFV failed to show a significant increase in haemagglutinating activity.

<table>
<thead>
<tr>
<th>Number of Extractions</th>
<th>None</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemagglutinating Expt. A</td>
<td>200</td>
<td>800</td>
<td>1,800</td>
<td>2,500</td>
<td>3,300</td>
<td>3,600</td>
</tr>
<tr>
<td>Activity (HAU per ml.) Expt. B</td>
<td>70</td>
<td>700</td>
<td>640</td>
<td>900</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Since the object of this study was the investigation of the quantitation of haemagglutination by arboviruses, it was necessary to characterize the haemagglutinins (SFV(M)/F and SFV(C)/TE) with which the work was to be carried out. The techniques of gel filtration (Page 53) and equilibrium density gradient centrifugation (Page 53) were adopted for this purpose.

(i) Gel Filtration on Columns of Sephadex G-200 and Sepharose-4B

Sephadex G-200 selects particles within the molecular weight range $5 \times 10^4 \text{g} \cdot \text{mol}^{-1}$-$1 \times 10^6 \text{g} \cdot \text{mol}^{-1}$ by a molecular sieving action. Several experiments, typified by the data of Figure 2, have shown that both infective Semliki Forest virus (SFV(M)) and non-infective, haemagglutinating components obtained from SFV by extraction with fluorocarbon of infected mouse-brains (SFV(M)/F) are eluted in the same volume fraction immediately
### TABLE 5. The Infectivity and Haemagglutinating Activity of Mouse-brain and Chick Embryo Cultures of Sanliki Forest Virus (SFV) Treated with Different Solvents.

<table>
<thead>
<tr>
<th>Host and Tissue of Origin</th>
<th>Method of Extraction and (Code)</th>
<th>Infectivity (log p.f.u. per ml.)</th>
<th>Haemagglutinating Activity (HAU per ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse-brain suspension (M)</td>
<td>None (SFV(M))</td>
<td>9.01 8.20</td>
<td>100 640</td>
</tr>
<tr>
<td></td>
<td>fluorocarbon, F. (SFV(M)/F)</td>
<td>2.78 1.73</td>
<td>3,300 5,000</td>
</tr>
<tr>
<td></td>
<td>sucrose/acetone, SA. (SFV(M)/SA)</td>
<td>&lt;1.0 &lt;1.0</td>
<td>8,700 -</td>
</tr>
<tr>
<td></td>
<td>Tween-80/ether, TE. (SFV(M)/TE)</td>
<td>&lt;1.0 &lt;1.0</td>
<td>- 32,000</td>
</tr>
<tr>
<td>supernatant fluid of chick embryo cell culture (C)</td>
<td>None (SFV(C))</td>
<td>8.95 9.40</td>
<td>320 100</td>
</tr>
<tr>
<td></td>
<td>fluorocarbon, F. (SFV(C)/F)</td>
<td>&lt;1.0 -</td>
<td>640 -</td>
</tr>
<tr>
<td></td>
<td>sucrose/acetone, SA. (SFV(C)/SA)</td>
<td>&lt;1.0 &lt;1.0</td>
<td>- 820</td>
</tr>
<tr>
<td></td>
<td>Tween-80/ether, TE. (SFV(C)/TE)</td>
<td>&lt;1.0 &lt;1.0</td>
<td>33,000 2,000</td>
</tr>
</tbody>
</table>
FIGURE 2. Elution of Infectivity and Haemagglutinating Activity from a Sephadex G-200 Column before and after Extraction with Fluorocarbon.

INFECTIVITY AS \( \log_{10} \) p.f.u. PER ML.

HAEMAGGLUTINATING ACTIVITY AS \( \log_{10} \) H.A.U. PER ML.

[Graph showing elution profile with points labeled SFV(M) and SFV(M)/F]

SFV(M) - mouse-brain suspension of Semliki Forest virus.

SFV(M)/F - Semliki Forest virus extracted from mouse-brain suspension by treatment with fluorocarbon.
following the void volume.

A similar pattern of elution was observed using the agarose Sepharose-4B which selects particles within the molecular weight range, \(3 \times 10^5-3 \times 10^6\). Figure 3 shows the typical peaks of haemagglutinating activity in the volume fractions immediately following the void volume for SFV(M)/F and for the supernatant fluids of chick embryo cells infected with SFV and extracted with Tween-80/ether (SFV(C)/TE). The subsidiary peak obtained with SFV(C)/TE suggested that a second haemagglutinating component was also present but refractionation showed that this component was not distinct from that at the first peak of activity.

It was concluded that the predominating haemagglutinating components of SFV(M)/F and SFV(C)/TE as used in this study were of a similar molecular size to that of the infective virus particles. These data will be considered further in later sections on quantitation.

(ii) Equilibrium Density Gradient Centrifugation in Caesium Chloride

Figure 4 illustrates typical data for the centrifugation to equilibrium of SFV(M)/F in a caesium chloride (CsCl) gradient. A sharp peak of haemagglutinating activity was obtained at the fraction with a density of about 1.24 g/mL, a value also obtained by Faulkner and McGee-Russell (1969) for the fraction corresponding with whole, infective virus particles or virions (Lwoff and Tournier, 1966).

From these data it was concluded that the preparations used in this study, which were SFV(M)/F and to a lesser extent SFV(C)/TE, were composed of particles of a similar size and density (1.24 g/mL) to that of the intact, infective virion. The modified infectivity and haemagglutinating activity of these haemagglutinins were discussed earlier (Page 56).
FIGURE 3. Elution of Haemagglutinating Activity from a Sepharose-4B Column after Extraction of Semliki Forest Virus with Fluorocarbon and Tween-80/Diethyl Ether.

HAEMAGGLUTINATING ACTIVITY AS % OF MAXIMUM

$\text{SFV(M)/F} - \text{Semliki Forest virus extracted from mouse-brain suspension by treatment with fluorocarbon.}$

$\text{SFV(C)/TE} - \text{Semliki Forest virus extracted from chick embryo cell suspension by treatment with Tween-80 and ether.}$
FIGURE 4. The Fractionation of Senliki Forest Virus Haemagglutinin (SFV/H/P) by Equilibrium Density Gradient Centrifugation in Caesium Chloride.

![Graph showing haemagglutinating activity and buoyant density against tube volume sampled.](image)
c) The Influence of Red Blood Cell Species

Following the observation that RBC from 1-day old chicks were optimally agglutinated by Japanese B encephalitis virus (Sabin and Buescher, 1950), it was suggested that RBC from other avian species might be even more suitable (pigeon, Macdonald, 1952; goose, Porterfield, 1957; rooster, Salminen, 1959). A survey by Banerjee (1965) compared the relative agglutinating capacities of RBC derived from 15 mammalian and avian species. He concluded that, in comparison with the widely used goose RBC, horse RBC (5.5µ in diameter) were about twice as sensitive, and the RBC of langur monkeys, rabbits (7.5µ), hamsters (5.6µ) and guinea-pigs (7.4µ) were up to twice as sensitive.

However, in this study with arboviruses, RBC from a group of geese (Page 48) have been found to be satisfactory for all haemagglutination reactions. Occasionally fowl RBC have been used with a resultant 4-fold decrease in detectable haemagglutinating activity. This reduction is not easily accounted for by the difference in surface area between the RBC of geese (12.2 x 7.2µ) and fowls (11.2 x 5.6µ). No attempt has been made in this study to characterize further the relative virus-adsorbing capacities of different RBC species.

One of the main advantages in the use of goose RBC in arbovirus haemagglutination is related to their size and morphology. Being nucleated and large, they settle quickly to form a clear pattern within 1 hour of setting up the reaction mixtures in dimple plates. In comparative experiments with sheep and rabbit RBC, no satisfactory control patterns showing negative haemagglutination were obtained.
d) **The Ranges and Optimal Values of pH Necessary for the Detection of Haemagglutination by Semliki Forest and Langat Viruses**

The pH ranges for maximal haemagglutinating activity in the standard pattern test (Page 51) were determined with sucrose-acetone extracted preparations of mouse brains infected with SFV or Langat virus (SFV(M)/SA or TP-21(M)/SA) as shown in Figure 5. It is clear that the extracted haemagglutinin of Langat virus shows a much broader range of pH acceptance than that of SFV. This is in agreement with the generalisation that viruses of group B agglutinate RBC over a wider range of pH than do those of group A (Andrewes and Pereira, 1967).

It should also be noted that under given test conditions, the pH value for optimal haemagglutinating activity is characteristic for each virus. For the haemagglutinin of SFV the optimum activity occurs at pH 6.35 ± 0.05 and for the haemagglutinin of Langat virus at pH 6.55 ± 0.05.

e) **The Influence of Buffer Ions**

  (i) **Borate ions**

In experiments designed to show the influence of ion concentration on the haemagglutination of Semliki Forest virus extracted by fluorocarbon from infected mouse-brains, the haemagglutinin (SFV(M)/F) was diluted at pH 9.0 in borate buffer solutions at different ion concentrations (0.003M, 0.006M, 0.012M, 0.018M, and 0.025M) and then tested at pH 6.3 in the standard pattern method. It was found that the haemagglutinating activity was unchanged (175 ± 10% HAU per ml.) over this range of ion concentrations.

The present indication that the concentration of borate ions does not influence the haemagglutinating activity of the haemagglutinin of SFV may be contrasted with the report by Rangonen and Vikberg (1964) that borate ions (0.004M-0.037M) inhibit the haemagglutinating activity of plant agglutinins.
FIGURE 5. The Influence of pH on the Demonstration of Haemagglutinating Activity by Semliki Forest and Langat Viruses

HAEMAGGLUTINATING ACTIVITY AS % OF MAXIMUM.

TP-21(M)/SA (MAX. ACTIVITY = 20,100 HAU PER ML)

SFV(M)/SA (MAX. ACTIVITY = 5000 HAU PER ML)

pH OF REACTION MIXTURE

SFV(M)/SA - Semliki Forest virus extracted from mouse-brain suspension by treatment with sucrose and acetone.

TP-21(M)/SA - Langat virus extracted from mouse-brain suspension by treatment with sucrose and acetone.
In further experiments using four other buffer systems in comparison with the standard borate buffer-BSA-FBS system, it was shown that borate ions were not essential for haemagglutination. The four buffer solutions tested were McIlvaine's citrate, Sorensen's phosphate and the Tris and Veronal systems (Appendix I). The haemagglutinin (SFV(M)/F) was diluted in each of these buffers at pH 8.0 or above and the subsequent reaction mixtures with RBC in PBS were adjusted to give a range of final pH values from 6.0 to 7.0. Typical data in Figure 6 show that McIlvaine's citrate buffer solution gave a haemagglutinating activity 10% greater than that with the standard borate system; an advantage shown consistently in three experiments. The other buffer systems gave peak activities greatly below that for the borate system control. This suggested that an appropriate citrate/phosphate system might prove to be more advantageous in haemagglutination studies than the borate/phosphate system.

(ii) Citrate ions

When the standard haemagglutinin was diluted in a range of dilutions of citrate ions in PBS at pH 6.0 and then mixed with RBC in PBS to give a reaction mixture at pH 6.3, the haemagglutinating activity was unchanged until the pattern became indistinct at low citrate ion concentrations (< 0.006 mM)

<table>
<thead>
<tr>
<th>Reaction Medium</th>
<th>Borate-BSA-PBS (Standard Test)</th>
<th>Citrate-PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall Molarity of Citrate Ions (mM) in reaction mixture</td>
<td>None</td>
<td>0.006</td>
</tr>
<tr>
<td>Haemagglutinating Activity (HAU per ml.) Expt. A</td>
<td>1,000</td>
<td>800</td>
</tr>
<tr>
<td>Activity</td>
<td>Expt. B</td>
<td>1,200</td>
</tr>
</tbody>
</table>
FIGURE 6. The Influence of pH on the Haemagglutinating Activity of Semliki Forest Virus Suspended in Five Different Buffer Solutions.

HAEMAGGLUTINATING ACTIVITY AS % OF BORATE BUFFER CONTROL (I)

BUFFER SOLUTION
I BORATE
II McILVAINE'S CITRATE
III SORENSEN'S PHOSPHATE
IV TRIS
V VERONAL

pH OF REACTION MIXTURE
However, in view of the greater convenience in the preparation of the standard borate-BSA-PBS system, the citrate-PBS system showed no practical advantage.

(iii) Phosphate ions

In experiments similar to those above the standard medium was subjected to changes in the concentration of phosphate. The haemagglutinin was diluted in Sorensen's phosphate buffer solution at pH 8.0 and allowed to react with goose RBC suspended in further solutions of phosphate ions to give terminal ion concentrations of 0.22M (standard), 0.03M and 0.01M. The reaction mixture was at pH 6.3 throughout. The haemagglutinating activity was unchanged at 1,600 ± 10% HAU per ml. under these conditions and indicated that the concentration of phosphate was not critical.

(iv) Chloride ions

The influence of sodium chloride concentration on the haemagglutinating activity of SFV(M) in the standard borate/PBS system at pH 6.3 was also studied. Over the range of concentrations used (0.14M, 0.20M, 0.26M, 0.32M and 0.56M) the haemagglutinating activity was constant at 2,900 ± 600 HAU per ml. up to 0.32M sodium chloride. At 0.56M, the RBC were damaged and formed unsatisfactory control patterns in the absence of haemagglutinin.

(f) The Influence of Protein Content

Protein in the form of BSA is normally included in the diluent for the haemagglutinin in order to ensure stability of activity in stored suspensions. When the concentration of BSA in the standard reaction medium was adjusted, the data shown overleaf were obtained. Protein is a necessary component of the reaction mixture and must be present to at least 0.001 gm. per ml., if clear dimple plate patterns are to be formed.
### (g) The Influence of Reaction Sequence

The extent of haemagglutination shown by a suspension of haemagglutinin derived from SFV is dependent on the pH value of the reaction mixture. In later experiments, it is shown that the level of haemagglutinating activity actually observed is a result of the competition between one reaction, in which haemagglutinin is being inactivated in a medium of adverse pH, and a second and simultaneous reaction in which available haemagglutinin is adsorbed by RBC in the preliminary stage of agglutination. The nature of these reactions and their influence on the kinetics and the outcome of the haemagglutination test are discussed later (Page 189).

### (h) The Influence of Temperature

The influence of temperature on the haemagglutinating activity of standard haemagglutinin (SFV(M)/P) was tested at pH 6.3 in the pattern test procedures used in the preceding sections. It was found that the activity was the same in tests performed at 4°C, 23°C and 37°C.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>4</th>
<th>23</th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemagglutinating Expt. A</td>
<td>700</td>
<td>850</td>
<td>700</td>
</tr>
<tr>
<td>Activity (HAU per ml.) Expt. B</td>
<td>2,500</td>
<td>2,500</td>
<td>2,500</td>
</tr>
</tbody>
</table>
For this reason, all further experiments were made at room temperature (23°C). Other temperature factors which are relevant to the sedimentation-enumeration method will be discussed later (Page 142).

(i) Conclusion

As a result of these confirmatory investigations on the reaction conditions in the pattern test, the borate-BSA (0.002 gm. per ml.)-PBS system at a final pH of 6.3 was adopted as standard for the quantitative studies on the agglutination of goose red cells by the haemagglutinin of Senliki Forest virus (Page 125).
II QUANTITATIVE HAEMAGGLUTINATION

a) Introduction

Since the Pattern Method is commonly used in the estimation of the haemagglutinating activity of a virus preparation, the method was studied in detail in order to evaluate the factors which influence the formation of a typical pattern. As a result of this study, a more precise presentation and interpretation of the data became possible.

However, the pattern method utilizes only the overall pattern arising from a complex reaction which is necessarily influenced by additional wall factors. Consequently, any estimate of haemagglutinating activity is a comparative statement. For this reason the alternative Photometric Method for the quantitation of the reaction in suspension between haemagglutinin and red blood cell was studied with a number of viral and non-viral agglutinating systems. These two methods use only the change in population characteristics of whole reaction mixtures and therefore do not take into account the distribution and size of aggregates.

Following the lack of success with the above procedures, an alternative approach was adopted. It was considered that a more meaningful method of quantitation would be the analysis of the populations of aggregates formed in a red cell suspension due to the presence of haemagglutinin. The use of an electronic cell counting device, described for other agglutinating systems (Page 44), was thus considered for a virus-red cell agglutinating system. However, the Coulter Counter proved unsatisfactory for this purpose, and an alternative method of population analysis was sought. This gave rise to the Sedimentation-Enumeration Method.

A scheme summarizing the various experimental procedures for the quantitation of haemagglutination by viruses is shown in Figure 7.
FIGURE 7. Scheme for the Quantitation of Haemagglutination by Viruses

Initial Infective Virus Suspension, V

Extraction methods to remove inhibitors of haemagglutination (p. 47)

Derived haemagglutinin, H

Haemagglutination reaction conditions (p. 51)
(pH, medium, temperature, time)

Characteristic distribution of aggregates of various sizes from single cells to n-fold aggregates of n red cells

Method of observation of agglutination

<table>
<thead>
<tr>
<th>Method</th>
<th>Following separation of free, single red cells</th>
<th>By the change in the characteristics of whole suspensions</th>
<th>By analysis of population distributions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual</td>
<td>-</td>
<td>Pattern (p. 72)</td>
<td>-</td>
</tr>
<tr>
<td>Instrumental</td>
<td>-</td>
<td>Photometric (p. 87)</td>
<td>Sedimentation-Enumeration (p. 125)</td>
</tr>
<tr>
<td>Automatic</td>
<td>Auto-Analyzer (p. 42)</td>
<td>-</td>
<td>Coulter counter (p. 110)</td>
</tr>
</tbody>
</table>
b) **A Study of the Pattern Method in the Estimation of the Haemagglutinating Activity of Viruses**

The Pattern Method, as previously defined (Page 38), is the most usual method used for the detection and estimation of the haemagglutinating activity of viruses. Reaction mixtures containing haemagglutinating components and the appropriate red blood cells in a suitable buffer system show the formation of characteristic patterns on the curved surfaces of the dimple cups. Typical dimple cup patterns are shown in Figure 8, (Page 75).

The formation of such patterns has been investigated by Chesbro and Hedrick (1937). A layer of red cells (RBC) becomes attached to the curved surface of the dimple cup, probably by an electrostatic mechanism. Experiments in this study show that in the absence of haemagglutination free, single RBC roll over this surface layer to form a compact central button or pellet. In the presence of haemagglutinin, the small aggregates, which are formed in free suspension, or the residual single RBC with attached haemagglutinin then settle onto the surface layer where they become attached to form the shield pattern characteristic of maximum haemagglutination for a selected red cell concentration (Figure 8, Page 75).

At lower, intermediate concentrations of haemagglutinin, a pattern showing partial haemagglutination is formed in which some RBC are unagglutinated and roll down the surface layer while the few formed aggregates adhere to this layer. This results in the formation of a ring whose diameter increases with the concentration of the haemagglutinin. The pattern of the transition from no haemagglutination to maximum haemagglutination is scored on a '0 to 4' scale of values as illustrated below and in Appendix III (Page 244). From the pattern scores, the dilution of haemagglutinin giving a central '2' score may be calculated and this leads to a
convenient numerical statement of the activity or concentration of haemagglutinin in the experimental sample (Page 81).

(i) A Consideration of Different Pattern Tests

Preliminary experiments were mounted to obtain the most reliable and consistent procedure for the estimation of haemagglutinating activity of viruses for use as a comparison with other methods.

The original method of Salk (1944), using small (10 x 75 mm) round-bottomed glass test-tubes was discarded as unreliable since, unless the bottoms of the tubes were uniformly hemispherical, irregular patterns were formed and these did not allow a confident interpretation to be made.

Takata (1967) and Sever et al. (1964) have advocated the use of the microtitre test in which a microloop system is used to prepare dilutions of small volumes (0.025 ml.) in the cups of disposable, Incite haemagglutination micro-plates. These plates are available with either U- or V-shaped cups. This microtitre system is widely used in serological and epidemiological studies because of economies in time and in quantities of reagents. However, significant differences were found in many comparisons between the macromethod using the WHO dimple plate (Page 38) and the micromethod with plates of either U- or V-shaped cups. In these comparisons, the SFV(M)/F haemagglutinin and goose RBC were used in the appropriate buffer system (Page 52). In the micromethod, the end-point of detectable haemagglutinating activity was variable over a four-fold range in tests on twenty samples from a series of dilutions of haemagglutinin. This inconsistency was probably largely due to the variation in the volume taken up by the microloop during the distribution of samples. The air-bubble inside the loop was often difficult to dislodge despite vigorous agitation in the diluent and careful prior cleaning by sodium hypochlorite and flaming.
The end-points were more easily observed with the V-shaped cups but the test was about two-fold more sensitive in the U-shaped cups. In addition, it was not possible to grade the end-point on a '0 to 4' scale as described below for the macromethod (Page 81).

For these reasons, the dimple plate pattern test was adopted as standard for comparison with the haemagglutinating activities obtained by the alternative and more quantitative photometric and sedimentation-enumeration methods.

It may be noted that an observation by Sever (1962), and later developed by Hardy (unpublished information, 1965), showed that when a dimple plate containing a typical haemagglutination pattern was tilted and maintained at an angle of $45^\circ$ for about 60 seconds, the cup containing the first '0' score pellet could be shown to contain some agglutinated RBC. The free, unagglutinated RBC were displaced down the side of the cup leaving the aggregates adhered to the centre of the original button. In this way, it was possible to increase the end-point dilution by a factor of about two, depending on the volume of the reaction mixture and the geometry of the cup. This test procedure was not adopted in the present study in view of the already complex mechanisms related to the reactants.

(ii) The Influence of Red Cell Concentration on Pattern Formation

The effect of red cell concentration on the formation of the dimple plate pattern was investigated since this was likely to be a key parameter in the definition of the sensitivity of the haemagglutination test.

A range of two-fold dilutions of Semliki Forest virus haemagglutinin (SFV[H]/F) and dilutions of goose RBC in approximately 1.2-fold steps were reacted together in the standard borate-ESB-PBS buffer system (Page 52). The series of patterns obtained with red cell concentrations (R) from $2.0 \times 10^6$ to $44.5 \times 10^6$ RBC per ml.
FIGURE 8. The Pattern Method: Typical Dimple Plate Patterns for a Range of Red Cell Concentrations (R).

\[ R \times 10^6 \] = red cell concentration.

\[ D \] = haemagglutinin dilution denominator.

(See also Salk (1944) and Waterson (1968)).
are shown in Figure 8. The end-point dilutions (D) for haemagglutination were calculated from the score-grades as shown in Appendix III, and are presented on linear and logarithmic scales against red cell concentration in Figure 9. It is clear that the end-point dilution is inversely proportional to the concentration of red cells in the reaction mixture, or

\[ D \propto \frac{1}{[R]} \quad (1) \]

If \([H]\) is the initial concentration of particles of haemagglutinin, then the concentration at the end-point dilution is \([H]_D\) so that equation (1) may be written in the form

\[ [H]_D = K_v \cdot [R] \quad (2) \]

where \(K_v\) is characteristic of the experimental method for a reaction mixture of volume, \(v\) ml. and is, in fact, the constant number of particles of haemagglutinin per red cell at the end-point dilution for the detection of haemagglutinin. This is evaluated later (Page 223). This relationship shows that the lower the concentration of red cells the greater the sensitivity of detection of haemagglutinin. However, the sensitivity of the pattern test is limited by the concentration below which a complete central button is not formed in the control cups without haemagglutinin.

Rosen (1964) has emphasised that it is the total number of settled red cells in a dimple cup which is important and not the volume of the reaction mixture in the cup, although no data were presented in support of this assertion. This generalization has been investigated by the calculation of \([H]_D\) for volumes (v) of standard four-volume reaction mixtures over the range 1 to 5 ml., with overall red cell concentrations of \(6 \times 10^6\) and \(12 \times 10^6\) cells per ml. of reaction mixture.

HAEMAGGLUTINATING ACTIVITY AS LOG H.A.U. PER ML. (LOG D)

HAEMAGGLUTINATING ACTIVITY AS H.A.U. PER ML. (D)
FIGURE 10. The Pattern Method: The Relationship between the Reaction Constant, $K_v$, and the Volume of the Reaction Mixture, $v$, for the Pattern Test in WHO Dimple Plates.

\[ \frac{[H]_0}{K_v \times 10^{-9}} = D[R] \]

Linear (ideal) relationship

Actual data

Optimum condition

$[R]$ 6 x 10^6 per ml.

12 x 10^6 per ml.
An almost linear relationship was obtained when the values of \( D \cdot \frac{[R]}{K_v} \) in Table 6 were presented against the reciprocal of the total volume, \( \frac{1}{v} \) as shown in Figure 10. This is the limit to which quantitative interpretation of pattern test data may be taken without the introduction of more than one empirical constant.

For the ideal case of linearity, we may write,

\[
K_v = k \cdot v
\]

where \( k \) is a reaction constant which characterizes the pattern test and is independent of the volume of the reaction mixture for this ideal case. The constants \( K_v \) and \( k \) are evaluated in terms of \( \frac{[H]}{K_v} \) and \( \frac{[H]}{k} \) in Table 6. Further evaluation requires an independent and absolute estimate of \( [H]_o \), the initial concentration of particles of haemagglutinin.

Thus combining equations \((2)\) and \((3)\), we obtain

\[
[H]_o = k \cdot v \cdot [R] \cdot D
\]

which shows that the concentration of particles of haemagglutinin, \( [H]_o \), may be expressed in terms of the observed quantities \( v \), \( [R] \) and \( D \), together with a single method constant which, as stated above, may itself be evaluated if an absolute estimate of \( [H]_o \) is available. The evaluation of \( k \) is given on Page 223.

The simplifying assumptions of equation \((4)\) represent an ideal case from which the practical test at \( v = 1 \text{ ml.} \) departs by \(-16\%\) in \( [H]_o \) value due to non-linearity in Figure 10.

Observations in this study show that for the most reproducible results in the WHO Perspex dimple plate at room temperature, a minimum concentration of approximately \( 6 \times 10^6 \) cells per dimple cup containing 1 ml. of reaction mixture is necessary to obtain a clearly defined central button, without haemagglutinin. Under test conditions near optimal, an identical concentration of particles of haemagglutinin
### TABLE 6. The Reaction Constants $K_v$ and $k$ for Haemagglutination Tests in WHO Dimple Plates

<table>
<thead>
<tr>
<th>Total Volume of Reaction Mixture, $v$ ml.</th>
<th>Overall Red Cell Concentration in Reaction Mixture, $[R]$</th>
<th>Mean Value for $\frac{[H]_0}{k}$ ($x 10^9$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$6 \times 10^6$ cells per ml.</td>
<td>$12 \times 10^6$ cells per ml.</td>
</tr>
<tr>
<td>* 1.0</td>
<td>$4.98 \times 10^9$</td>
<td>$5.00 \times 10^9$</td>
</tr>
<tr>
<td>1.5</td>
<td>$3.88 \times 10^9$</td>
<td>-</td>
</tr>
<tr>
<td>3.0</td>
<td>$2.12 \times 10^9$</td>
<td>$2.10 \times 10^9$</td>
</tr>
<tr>
<td>4.0</td>
<td>$1.65 \times 10^9$</td>
<td>$1.63 \times 10^9$</td>
</tr>
<tr>
<td>5.0</td>
<td>$1.37 \times 10^9$</td>
<td>$1.34 \times 10^9$</td>
</tr>
</tbody>
</table>

* Reaction conditions for the optimal four-volume pattern test (Page 51).
is indicated over a range of values of red cell concentration and test volume according to equation (4).

(iii) The Interpretation and Presentation of Data from Haemagglutination and Haemagglutination-Inhibition Pattern Tests

(a) Justification for a reconsideration of the pattern test

There is a necessity for a consideration of the presentation of pattern data in order to eliminate the need for the display of complete blocks of experimental data which the reader is not readily able to interpret in terms of quantitative end-points or haemagglutinating activities since this requires the appraisal of several sets of three or four score-grade entries.

It is clear that the information given by the several score-grades between '0' and '4' is not fully expressed unless the dilution end-point for the '2' score is established by a numerical best-fit procedure.

The incorporation of experimental dilution factors in the correct manner then allows the haemagglutinating activity to be presented in terms of standard units rather than in terms of reciprocal dilution end-points.

A unified system of presentation also provides a basis for the estimation of errors and a quantitative correlation with data obtained by other methods. The maximum amount of information in the available experimental data may thus be extracted and simply expressed.

(b) The protocol for the standard pattern test

The protocol adopted in this study as a standard technique for both arboviruses and other haemagglutinating viruses has already been described (Page 51). The criteria for the selection of volume and pH values have
been defined. The transition of pattern from no haemagglutination at score-grade '0' to maximum haemagglutination at score-grade '4' is shown in Figure 67 (Page 244).

(c) The interpolation method for score-grades in haemagglutination tests

Consider a standard reaction mixture in which the haemagglutinin (H) to be assayed is present at an overall dilution D with respect to the initial suspension of haemagglutinin. If the dilution D, is that at which the defined '2' pattern is observed, then the reaction mixture contains, by definition, one '2' score-grade haemagglutinating unit (HAU) per ml. and the initial haemagglutinin sample contained,

\[ [H]_o = D \cdot \text{HAU per ml.} \quad (5) \]

or,

\[ \log [H]_o = \log D \]

\[ = \log (E, \text{ overall dilution to '4' score}) + \log (F, \text{ further dilution between '4' score and the best-fit '2' score}) \quad (6) \]

However, the score pattern is rarely of the '4, 2, 0' form and cannot, in general, be interpreted by inspection. The more usual extended sequence of score-grades requires a standardized interpolation and this is based on the sigmoid dose-response curve relating score-grades to dilution, as shown in Figure 11. The '2' score represents the centre-point of the range of score-grades and occurs at a dilution of haemagglutinin analogous to the 50% end-point dilution in an assay of infectivity. Thus, the accumulation principle of Reed and Muench may be applied to the estimation of the best-fit dilution giving a '2' score.

The last term, \( \log F \), of equation (6) has been determined by accumulation
FIGURE 11. The Pattern Method: Typical Dimple Plate Data Showing the Sigmoid Dose-Response Relationship.
using the empirical equation,

\[ \log F = 0.15 \left( 1 + \frac{s}{2} \right) \]  (7)

where \( s \) is the sum of the score-grades for all cups above that showing '4' and the constant 0.15 is the log dilution change for an increment of '1' in score-grade (Figure 11). Numerical values for \( \log F \) and \( s \), according to equation (7), are presented in Table 7 together with the required logarithmic value of the dilution \( E \) showing the highest '4' score. The final dilution giving the best-fit '2' score is then given on the logarithmic scale by,

\[ \log D = \log E + \log F \]  (8)

A worked example for the calculation of haemagglutinating activity by this method is shown in Appendix III (Page 244).

(d) The interpolation method for haemagglutination-inhibition tests

Consider an initial haemagglutinin sample of activity \([H]_o\) HAU per ml. which is present at an overall dilution \( D \) in a reaction mixture with specific antiserum at overall dilution, \( d \).

If, after equilibration and mixing with an equal volume of red cell suspension, as in the standard test, the reaction mixture shows the '2' score pattern in the haemagglutination test, then each unit volume contains, by definition, one haemagglutinating unit (HAU). However, since the reaction mixture has been diluted two-fold with the RBC suspension, then of the \([H]_D\) units originally present, the number \([H]_D - 2\) have been inhibited or blocked by the antiserum at the overall dilution \( d \). Hence the initial antiserum sample represents an inhibitory activity of,

\[ [I] = d \left( [H]_D - 2 \right) \text{ HAU per ml.} \]  (9)
TABLE 7. Interpolation Data for the Calculation of Dilution (D) giving Score-Grade '2' in the Standard Pattern Test

\[ \log D = \log E + \log F \]

<table>
<thead>
<tr>
<th>Overall Dilution Denominator to '4' Score</th>
<th>Log Overall Dilution Denominator to '4' Score = Log E</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.301</td>
</tr>
<tr>
<td>4</td>
<td>0.602</td>
</tr>
<tr>
<td>8</td>
<td>0.903</td>
</tr>
<tr>
<td>16</td>
<td>1.21</td>
</tr>
<tr>
<td>32</td>
<td>1.51</td>
</tr>
<tr>
<td>64</td>
<td>1.81</td>
</tr>
<tr>
<td>128</td>
<td>2.11</td>
</tr>
<tr>
<td>256</td>
<td>2.41</td>
</tr>
<tr>
<td>512</td>
<td>2.71</td>
</tr>
<tr>
<td>1024</td>
<td>3.01</td>
</tr>
<tr>
<td>2048</td>
<td>3.31</td>
</tr>
<tr>
<td>4096</td>
<td>3.61</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total Score above '4'</th>
</tr>
</thead>
<tbody>
<tr>
<td>( s )</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>( \frac{1}{2} )</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>( 1\frac{1}{2} )</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>( 2\frac{1}{2} )</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>( 3\frac{1}{2} )</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>( 4\frac{1}{2} )</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>( 5\frac{1}{2} )</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>( 6\frac{1}{2} )</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>( 7\frac{1}{2} )</td>
</tr>
<tr>
<td>8</td>
</tr>
</tbody>
</table>
but, since the control mixture without antiserum shows a dilution end-point for
the '2' score of \( D_0 = [H]_o \), when \( [B'] = 0 \), we may write,

\[
[B'] = 2 \bar{d} \left( \frac{D_0}{D} - 1 \right) \text{ inhibitory units (IU) per ml.} \tag{10}
\]

This represents the antibody activity in terms of inhibition of haemagglutination.
From equation (10), the inhibitory activity may be represented on the
logarithmic scale by the expression,

\[
\log [B'] = 0.30 + \log \bar{d} + \log \left( \frac{D_0}{D} - 1 \right) \tag{11}
\]

which is the form most convenient in the reduction of dimple plate data.
A worked example is given in Appendix III (Page 245).

This presentation of haemagglutination-inhibition data uses the interpolation
procedures already defined for the treatment of haemagglutination pattern scores
and offers the same advantages in terms of quantitation and extraction of information.

(e) **Estimation of operational errors in the haemagglutination test on WHO Perspex dimple plates**

Throughout this study replicate experiments were
regularly set up to estimate the reproducibility of the estimates of haemagglutinating
activity. These data showed that when ten replicate series of dilutions from a
common stock haemagglutinin suspension were set up in the standard dimple plate test
(Page 51), there was negligible variation in the series. Of ten replicates, only
two differed from the remaining eight by a '1' score and indicated that the range of
score-grades does not exceed \( \pm \frac{1}{2} \) in this series. Thus, the percentage error in
the estimation of haemagglutinating activity in a single test is not greater than
\( \pm 10\% \) or \( \pm 0.04 \) on a logarithmic scale.
o) Quantitation by Photometric Procedures

The pattern method is widely used for the estimation of haemagglutinating activity for a large number of viruses. Although this test procedure is rapid, convenient and may be economical in the use of reagents, it has been shown (Page 74) that it cannot be used directly for the estimation of the absolute concentration of haemagglutinin since the indicated end-point is not independent of the concentration of red cells. It was also shown that the end-point was the result of a complex reaction involving the container wall at the third stage of the agglutination sequence outlined in Figure 1 (Page 37).

It was therefore considered more appropriate to devise a method for the quantitation of the free suspension reaction at the second stage of the agglutination sequence.

The photometric method of Levine et al. (1953), based on earlier work (Page 39), endeavoured to determine the absolute numbers of influenza virus particles in a reaction mixture containing settling aggregates of red cells by photometric observations of optical density. The principle of this procedure is that aggregates of different sizes show characteristic sedimentation velocities which may be used to determine the extent of agglutination. The higher the concentration of haemagglutinin, the larger and more numerous the aggregates subsequently formed, and thus over a period of time, more red cells in the form of aggregates settle out of the free suspension. Optical density measurements of such a reaction mixture thus provide a measure of the number of red cells settling out as aggregates which, under limiting conditions, may be proportional to the concentration of haemagglutinin.

According to Levine et al. (1953), presumably under conditions of lowest haemagglutinin concentration, the population is composed entirely of a mixture of
free, single red cells and of dimers in which two red cells are bound by haemagglutinin. In this 'Dimers-Only' hypothesis, which defines the maximum sensitivity for the detection of haemagglutination, the dimers are assumed to settle more rapidly than single cells and thus, as shown in Figure 12, the optical density at a given level of observation gives a 'step-curve' when plotted against the time of settling. From this, these authors suggested, as will be discussed later, that the number of haemagglutinating particles may be estimated by comparison with the optical density curve obtained for a reaction mixture without haemagglutinin. It was also reported that, with high concentrations of haemagglutinin and red cells, an 'avalanche' effect of very large, rapidly settling aggregates was observed. However, in the present study with arboviruses, a distribution showing single cells and dimers in the absence of larger aggregates has not been observed (Page 139).

Most studies by this photometric procedure have been confined to the myxovirus system, but it seemed likely that the method could be applied to other haemagglutinating systems. Although the published data are incomplete and allow no consideration of the 'dimers-only' hypothesis, the method has been applied by Cheng (1961b) to Semliki Forest virus and by Nozima et al. (1964) to Japanese B encephalitis virus. However, when the method was applied to haemagglutination by antibody molecules, Somers, Brown and Makinodan (1966) found it to be unsatisfactory for the determination of the absolute number of haemagglutinating antibody molecules in antisera prepared against both sheep and chicken red blood cells. The reason for this failure was that at low concentrations of antibody, the frequency of dimer formation was not a linear function of antibody concentration.

The alternative photometric method of Drescher and his colleagues (see Page 41),
FIGURE 12. The Photometric Method: The Step-Curve Associated with the 'Dimers-Only' Hypothesis

OPTICAL DENSITY (O.D.)

\( M = \) monomers
\( D = \) dimers
\( d = \) distance from meniscus to observation slit.

\( \text{TIME OF SETTLING (MIN.)} \)

\( \text{1) monomers only} \)
\( \text{2) monomers and dimers} \)
\( \text{3) dimers only} \)
which has been applied only to myxoviruses, has not been used in this study. By this method, haemagglutinating activities are expressed in terms of the highest haemagglutinin dilution which causes maximal agglutination of a red cell suspension. This is based on the observation that the variation in the quantity of haemagglutinin required to agglutinate different batches of red cells decreased as the state of maximal agglutination was approached. In this method, reaction mixtures are observed photometrically after remaining undisturbed for two hours at room temperature. The resulting optical density values are measures of the slowly settling, unagglutinated red cells and small aggregates remaining in the area of observation after the larger and more rapidly settling aggregates have sedimented. In this respect, the measurement of the concentration of these remaining single cells is equivalent to the measurement of the haemoglobin content of lysed, single red cells, as in the Autoanalyzer technique (Page 42). Thus, any estimation of haemagglutinating activity obtained by either of these two methods is unrelated to the size or distribution of settled aggregates.

(4) Attempts to Confirm the 'Dimers-Only' Hypothesis

(a) Experimental Conditions

Viral and Non-Viral Haemagglutinating Systems

In the present study, attempts were made with influenza virus type A (FR8 strain) to obtain a satisfactory protocol by the photometric method of Levine et al. (1953). Several other experiments were carried out with the soluble haemagglutinin of vaccinia virus in order to provide a further readily available distinct haemagglutinin.

The readily available non-viral haemagglutinating system of horse anti-sheep RBC serum and sheep RBC was studied in order to test the 'dimers-only' hypothesis.
without reference to viral haemagglutinin. Protamine (salmine) sulphate and copper sulphate were also used to agglutinate fowl red cells.

In all experiments, the red cells and the haemagglutinating agents were suspended in either physiological saline or calcium/magnesium saline at pH 7.2 (Appendix I), and the reaction took place at room temperature (23°C).

**Photometric Apparatus**

In the first experiments, the Hilger Biochem-Absorptiometer (H.810) was used with small test-tubes (10 x 75mm) containing the reaction mixture.

The Unicam SP500 Spectrophotometer was generally used in subsequent experiments in order to increase the sensitivity of the optical density measurements under more closely defined experimental conditions.

In most cases, the overall red cell concentration of the reaction mixture was adjusted to give an optical density value which would cover the most sensitive region of the scale near 0.3 optical density units. This corresponded to a concentration of about 5 x 10^7 cells per ml. for fowl red cells.

**(b) Results**

Undisturbed reaction mixtures containing fowl red blood cells and the haemagglutinins of influenza or vaccinia virus at different concentrations were observed continuously. Typical results are shown in Figure 13. The data for several concentrations of haemagglutinin are compared with the corresponding data for free fowl red cells in the absence of haemagglutinin. The 1/2560 dilution of influenza virus and the 1/1280 dilution of vaccinia haemagglutinin gave minimal agglutination ('1' score, Page 244) in the pattern test. It is clear that at this red cell concentration of about 2 x 10^7 cells per ml. of reaction mixture there is very little difference between the control without haemagglutinin
The Photometric Method: Optical Density Data for the
Agglutination of Fowl Red Cells by the Haemagglutinins of Vaccinia and Influenza Viruses
and those reaction mixtures containing haemagglutinin and showing a satisfactory '1' score in the pattern test. These results were independent of the volume of the reaction mixture (1 to 4 ml.) and of the reaction and observation time (up to 2 hr.).

Thus, with these minimal concentrations of haemagglutinin, no step-curves (Figure 12, Page 89) due to the formation of dimers were obtained, despite the observation of positive scores in the pattern test. There was thus no confirmation either of the observations of Levine et al. (1953) or of the 'dimer-only' hypothesis.

(ii) Attempts to Increase the Sensitivity of Detection of Haemagglutination

Further studies were designed to increase the ratio of aggregates to free red cells, in order to increase the difference in optical density between reaction mixtures containing haemagglutinin and aggregates and those without haemagglutinin. This was attempted in several ways.

(a) Sensitization of Red Cells with Tannic Acid

Red blood cells were treated by the method of Boyden (1951) and Stavitsky (1954), (see Page 9), with 1/20,000 tannic acid in attempts to make them more susceptible to the adsorption of antigen. Preliminary attempts with fowl red cells and influenza virus showed a marginal two-fold increase of haemagglutinating activity. Recent detailed study by Smith and Courtney (1966) showed that for antigens of viral origin, the successful application of sensitization techniques required the prior treatment of red cells with virus-specific gamma globulins under defined conditions. This is an indirect technique and was not pursued further.
(b) The Influence of Agitation on the Formation of Red Cell Aggregates

From the previous results (Figure 13), it was concluded that no RBC aggregates of a significant size were formed in free suspension even with high concentrations of haemagglutinin. This might be due to the low rate of collision between red cells and the consequent slow rate of agglutination when RBC have combined with haemagglutinin. Methods of agitation were therefore devised in order to increase the rate of collision without damaging the red cells or reducing the extent of subsequent agglutination. This might be particularly important at low red cell concentrations. The three methods of rolling, shaking and tumbling were tested. Gentle, regular inversion of the reaction mixtures in tubes fixed to a drum rotating at about 9 rev. per min. produced a tumbling motion which appeared to enhance haemagglutination without subsequent dissociation of the fragile aggregates. The effect on the sedimentation patterns for influenza virus for different periods of tumbling is shown in Figure 14. There is a significant displacement of the graph for the reaction mixture containing haemagglutinin from that for a similarly agitated mixture without haemagglutinin. This was independent of the time of tumbling. This increased extent of agglutination only occurred with high concentrations of haemagglutinin. In further studies on the influence of the concentrations of red cells and haemagglutinin (below), all reaction mixtures were tumbled for a standard time of 30 min. This method of agitation was more satisfactory than rolling or shaking.

It was possible that agitation for long periods might damage the red cells and cause the cells to lyse and produce stromata which could influence the observed extent of agglutination. Stromata prepared by lysis of red cells with saponin
FIGURE 14. The Photometric Method: The Effect of Tumbling on the Agglutination of Fowl Red Cells by Influenza Virus Haemagglutinin
were mixed with different proportions of whole red cells and subsequently used in a standard pattern test with dilutions of vaccinia haemagglutinin. Although in all cases the characteristic dimple pattern score was unchanged (320 HAU per ml.), with 75% stomata present in a mixture, the pattern was only complete after 2 hr. Therefore, the presence of some stomata due to tumbling is unlikely to influence the overall effect of an agglutinated reaction mixture when observed by the photometric method.

(c) The Influence of Red Cell Concentration on the Formation of Aggregates in Free Suspension

An increase in the red cell concentration results in an increased rate of collision between red cells with a consequently greater extent of agglutination in the presence of haemagglutinin. This may be particularly significant at low concentrations of haemagglutinin at which the highest sensitivity of detection is required to reveal the few aggregates formed in the presence of excess red cells.

A range of red cell concentrations was combined (8 x 10⁷ to 1 x 10⁷ per ml.) with a constant dilution (1/20) of influenza virus haemagglutinin, and these reaction mixtures were tumbled for 30 mins at 9 rev. per min. The typical values of photometric observations expressed as a percentage of the initial optical density are shown in Figure 15. Under this condition of constant high haemagglutinin concentration, it was concluded that red cell concentration does not affect the extent of agglutination in free suspension.

(d) The Influence of Haemagglutinin Concentration on the Formation of Aggregates in Free Suspension

The results of a typical experiment designed to demonstrate
The Photometric Method: The Influence of Red Cell Concentration on the Agglutination of Fowl Red Cells by Influenza Virus Haemagglutinin

Optical Density (O.D.) as percentage of initial O.D.

<table>
<thead>
<tr>
<th>[RBC] per ml.</th>
<th>8 x 10^7</th>
<th>4 x 10^7</th>
<th>1 x 10^7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of Settling (min.)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
the dependence of the extent of agglutination on the haemagglutinin concentration are presented in Figure 16. Reaction mixtures containing a constant red cell concentration \((10^7 \text{ cells per ml. of reaction mixture})\) and varying dilutions of influenza virus haemagglutinin \((1/10 \text{ to } 1/10,000)\) were tumbled and then observed photometrically in the usual way. The graphs show a significant displacement for high concentrations of haemagglutinin which decreases with decreasing concentrations of haemagglutinin. It is again evident that in no case is there a step-curve corresponding to a population consisting entirely of single red cells and dimers. In addition, the avalanche effect was only obtained with high concentrations of both red cells and haemagglutinin.

(iii) A Study of Non-Viral Haemagglutinating Agents

In view of the failure to confirm the 'dimer-only' hypothesis for viral haemagglutination it seemed possible that a non-viral haemagglutinating system might nevertheless allow the photometric demonstration of a step-curve in optical density.

(a) Sheep RBC and Horse Anti-Sheep RBC Antiserum

Dilutions of antiserum were prepared in saline and reacted with sheep RBC \((2 \times 10^6 \text{ per ml. of reaction mixture})\). When the reaction mixtures were then observed photometrically in the usual way, the resulting sedimentation curves showed no significant difference between samples with or without antiserum. Since it was possible that agitation or tumbling might increase the extent of agglutination, reaction mixtures were set up with constant red cells \((2 \times 10^6 \text{ per ml.})\) and a range of antiserum dilutions \((1/40 \text{ to } 1/2560)\) and tumbled for periods of 30, 60 and 120 mins. For this system a time of 60 min. was optimal. A typical distribution of sedimentation curves is shown in Figure 17. Again, no
FIGURE 16. The Photometric Method: The Influence of the Concentration of Influenza Virus Hemagglutinin on the Agglutination of Fowl Red Cells.
FIGURE 17 • The Photometric Method: The Influence of the Concentration of Anti-Sheep Red Cell Serum on the Agglutination of Sheep Red Blood Cells.

The graph shows the optical density over time of settling for different Ab dilutions. The data points are as follows:

- **no Ab**
- 1/2560
- 1/320
- 1/160
- 1/80
- 1/40

The graph plots the optical density on the y-axis and the time of settling on the x-axis, ranging from 0 to 120 minutes.
step-curve was demonstrated for any dilution of antiserum and there was evidence of an 'avalanche' effect only at high concentrations of antiserum (1/40 dilution). With low concentrations, there was no significant difference between reaction mixtures with and without antiserum although all the dilutions of antiserum gave a '4' reaction in the standard pattern test in dimple plates.

The data in Figure 17 suggested that the curves might be characteristic of a haemagglutinating system which is in a state of reversible equilibrium. This was tested by the several experiments illustrated in Figure 18. The antiserum dilution (1/160) giving the linear settling curve in Figure 17, was used in the control reaction mixture after tumbling for 60 min. To this reacted mixture was added a further volume of red cell suspension, or a further volume of antiserum dilution (1/260) or a volume of saline. These three mixtures were then tumbled for a further 60 min. and observed photometrically in the usual way. The displacements of the control curve following the addition of antiserum or red cells demonstrate that the reaction between sheep red cells and the corresponding antiserum is in a state of reversible equilibrium which changes to a new condition of either more or less agglutination when further reactants are added.

Thus, this haemagglutinating system did not show a population consisting of predominantly free cells and dimers even at minimal concentrations of antiserum.

(b) Copper Sulphate and Protamine (Salmine) Sulphate and Fowl Red Cells

Preliminary attempts were made to prepare aggregates of fowl red cells of defined size using solutions of copper sulphate and protamine sulphate. However, even with concentrations of < 0.00025% and varying concentrations of fowl RBC in saline, it was impossible to obtain a population of single cells and dimers
The Photometric Method: The Agglutination of Sheep Red Cells by Anti-Sheep Red Cell Serum and the Reversal of this Reaction by the Addition of Further Reactants.

Optical Density (O.D.) as Percentage of Initial O.D.

controls with and without Ab (serum)

- (RBC + Ab) 120 min.
- (RBC + Saline) 120 min.
- ((RBC + Ab) 60 min. + Ab) 60 min.
- ((RBC + Ab) 60 min. + RBC) 60 min.
- (RBC + Ab) 60 min. + Saline 60 min.
or small aggregates. Microscopic examination showed a wide range of aggregate size and consequently such suspensions in sedimentation experiments did not give rise to step-curves open to quantitative interpretation according to Levine et al. (1953).

(iv) The Formation of a Step-Curve by Photometric Observation of a Non-Haemagglutinating System

As a final test of the feasibility of obtaining a satisfactory demonstration of the presence of two distinct entities differing in size in free suspension, the haemagglutination phenomenon was abandoned.

The distinct red blood cells of sheep and goose were mixed together and observed photometrically. These red cells were selected because their shorter dimensions differed by a factor of about two and in this way a reaction mixture consisting solely of slowly (sheep RBC of diameter ~4μ) and rapidly (goose RBC of diameter ~9μ) sedimenting species was simulated. These two species of cells do not interact and constitute an irreversible system equivalent to the 'ideal' system of free cells and dimers only.

The RBC were each washed and suspended in saline to give the same optical density reading. The sheep RBC suspension contained $1.5 \times 10^6$ cells per ml. and the goose RBC suspension contained $7.5 \times 10^5$ cells per ml. Sedimentation curves were obtained for each of the two RBC suspensions and then for a 50 : 50 mixture. The resultant optical density sedimentation curves are shown in Figure 19. It is evident that a 'step-curve' is now obtained which shows clearly the distinct sedimentation rates of the two species of red cell.
(v) An Analysis of Data on the Basis of the Distinct Sedimentation Velocities of Aggregates

The characteristic sedimentation velocities of different cells may be related to their diameters. Data for cells of different species are presented in Figure 20 and show a linear relationship between sedimentation velocity \((s)\) and diameter squared \((d^2)\).

The sedimentation velocities for aggregates of defined size are also known from the data presented in Figure 32 (Page 133). These settling curves for defined aggregates may then be superimposed on the data for a typical reaction mixture of goose red cells with minimal ('2' score in the pattern test) concentration of influenza virus haemagglutinin, as shown in Figure 21.

According to Levine et al. 1953, such a reaction mixture contains predominantly single red cells with dimers of two red cells. It is evident, however, that the step-curve predicted by this 'dimer-only' hypothesis (Figure 12, Page 89) is not consistent with the present data. On the contrary, the observed settling curve indicates that the reaction mixture is composed of aggregates, the majority of which are greater than dimers. In fact, the reaction mixture giving a '2' score in the pattern test shows the following distribution of aggregates in terms of the contribution to optical density. It may be noted that about 60% of the optical density is due to aggregates greater than dimers.

<table>
<thead>
<tr>
<th>Aggregate Size, n.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Optical Density</td>
<td>25</td>
<td>16</td>
<td>24</td>
<td>15</td>
<td>7</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

The derivation of these data from Figure 22 may be illustrated with reference to the points A and B. The optical density for the settling curve for the reaction
FIGURE 20. The Photometric Method: The Sedimentation Velocities for Single Cells of Different Species

(ERK = embryonic rabbit kidney cells)
The Photometric Method: Comparison of Settling Curves for Single Cells, Defined Aggregates and a Reaction Mixture with Haemagglutinin

(ERK = embryo rabbit kidney cells)
FIGURE 22. The Photometric Method: Reduction of Optical Density Data to a Distribution of Defined Aggregates

OPTICAL DENSITY (O.D.) AS % OF INITIAL O.D.

TIME OF SETTLING (MIN.)

OD contribution by trimers

by dimers

n = 10 9 8 7 6 5 4 3 2 1
mixture above point A, the intersect of the curve with the corresponding curve for aggregates of three red cells (trimers), is given by aggregates of three cells or more. Similarly, the optical density for the curve at point B is given by aggregates of four cells or more. Therefore the distance AB is an indication of the optical density due to trimers.

(vi) Conclusions

It is clear that a step-curve, relating optical density to settling time, has not been obtained in the present photometric study of a dynamic viral haemagglutinating system. The results show that, at any instant, the reaction mixture of haemagglutinin and red blood cells consists not of singles and dimers only but of a population of aggregates of varying sizes. Therefore, the optical density values for these reaction mixtures, although characteristic, are only mean data for a population of aggregates, and consequently the quantitation of the haemagglutination reaction by this method is limited by lack of information on aggregate distribution.

In the methods of both Levine et al. (1953) and Drescher (Page 40) there is no consideration of possible spontaneous agglutination nor of any red cells with adsorbed haemagglutinin which fail to form aggregates. These factors must be considered in any quantitative estimate of haemagglutinating activity.
d) **Automatic Electronic Cell Counting with the Coulter Counter**

It has been mentioned previously (Page 45) that the automatic electronic cell counting technique offered by the Coulter counter and commonly used in the study of many agglutinating systems has not been applied to haemagglutination by viruses. It was considered that this technique offered the possibility of not only counting individual aggregates but also of grouping on a basis of size, in contrast to the mean population data obtained by photometric procedures (Page 109).

(i) **The Coulter counter, Model A**

In the Coulter counter (Coulter Electronics Inc., Illinois, U.S.A.), an electrical current passes between two platinum electrodes arranged on either side of a very small aperture (50-200μ diameter) mounted at the end of a glass tube. When the suspension of cells is drawn through this aperture, the passage of each cell produces a transitory increase in electrical resistance. This change of resistance appears as a voltage pulse which is proportional to the volume of the cell and is registered on a counter. By adjustment of the voltage pulse threshold, only cells or aggregates above a certain size are registered on the counter. In this way, the size distribution of cells and aggregates may be obtained from successive counts at various settings of the voltage threshold control.

Details of the principles, operation and calibration of the Coulter counter have been given elsewhere, (Brecher, Schneiderman and Williams, 1956; Mattern, Brackett and Olson, 1957; Peacock, Williams and Mengoli, 1960).
For the present study, the sampling and aperture tube were simplified to allow smaller volumes (0.5 ml.) of cell suspension to be sampled and counted. The whole apparatus was enclosed by a wire mesh screen to reduce external electrical interference.

(ii) Experimental Conditions

The use of an aperture of approximately ten times the diameter of the particles to be counted is recommended by the manufacturer to avoid blockage of the aperture. Thus, an aperture of 100μ in diameter was chosen to count fowl cells of approximate dimensions, 12μ x 7μ in diameter.

To reduce the possibility of disruption of aggregates due to shear forces at the aperture, it was necessary to reduce to a minimum the rate of flow of the reaction mixture through the aperture. Such an acceptable flow rate of about 0.1 ml. per sec. was obtained at a negative pressure of 250 mm. of mercury.

(a) Calibration of the Coulter counter with the 100μ aperture

It was necessary to determine the optimal values for the voltage threshold and the aperture current before the instrument could be used for the particle system under investigation.

The relationship between the voltage threshold and the number of cells counted for a range of aperture currents was obtained using a suspension of fowl cells (RBC) at a concentration of about 3 x 10⁴ cells per ml. This relationship (Figure 23) can be represented by the expression:

\[
\text{Voltage threshold} = k \cdot (\text{Aperture current})^2 \quad (12)
\]

In Figure 24, the square of the aperture current is plotted against the voltage threshold required for half the maximum count (50% threshold).
FIGURE 23. The Coulter Counter: The Relationship between the Voltage Threshold and the Count of Red Cells for a Range of Aperture Currents.

RED CELL COUNT ($\times 10^{-4}$)  
(IN 0.5 ML SAMPLE)

APERTURE CURRENTS  
1-7

VOLTAGE THRESHOLD  
(ARGUMENT SCALE UNITS)

\((\text{Aperture Current})^2\)

Voltage threshold for 50% maximum count (arbitrary scale units)
The optimal conditions (Figure 23, curve 4) were those giving a broad plateau for the cell count with a rejection of particle sizes below a size limit set by the voltage threshold. For the fowl red cell system, a voltage threshold setting of 20 and an aperture current setting of 4 were adopted as optimal. All of the instrumental settings are quoted in arbitrary scale units.

(b) The haemagglutinating system under study

In tests with the Coulter counter, influenza virus, type A, PR 8 strain (Page 46) was allowed to react with fowl RBC at room temperature in calcium/magnesium saline (Appendix I) at pH 7.2. It was found necessary to incorporate bovine serum albumin at a concentration of 0.1% in the medium to reduce the loss of cells by adsorption to the walls of the reaction vessel.

The diluting fluid for the sample was phosphate buffered saline at pH 7.2 (Dulbecco and Vogt, 1954; Appendix I). This was filtered through sintered glass before use to remove particles which might otherwise contribute to a background count.

From the experiments on photometric procedures (Page 94), it was clear that reaction mixtures should be agitated to obtain the maximal formation of aggregates. In the present experiments, the reaction mixtures were tumbled for periods of up to 60 min. For this purpose, the reaction tubes were fixed to a drum rotating at about 9 rev. per min. and arranged so that the tubes were inverted completely at each revolution. On completion of the reaction, an appropriate sample was taken with a calibrated Pasteur pipette into 20 ml. of diluting fluid.
This gave a concentration of about $10^5$ cells per ml suitable for the Coulter counter.

Thus, when haemagglutination occurs, the number of aggregates and free cells to be counted will be less than the initial count of free cells obtained for the control sample without haemagglutinin. All counts were corrected for the reduction due to the coincidence of two cells or aggregates passing through the aperture at the same time.

(iii) Results

(a) The influence of red cell concentration

Reaction mixtures containing one of three high concentrations of red cells and one of a range of dilutions of haemagglutinin were mixed by tumbling for 15 min and then sampled and diluted for counting. The relationship between the actual count and the log of the overall dilution of haemagglutinin is shown in Figure 25.

A more revealing relationship is obtained when these data are considered in a way similar to that adopted for the Sedimentation-Enumeration method, (Page 128).
FIGURE 25. The Coulter Counter: The Influence of Red Cell Concentration on the Count of Red Cells in the Presence of Haemagglutinin.

RED CELL COUNT ($\times 10^4$) (IN 0.5 ML. SAMPLE) vs INITIAL CELL COUNT (PER ML.)

- control without haemagglutinin
- control
- control

$\log$ (overall dilution of haemagglutinin) = $\log$ D
Thus, if \( A_n \) is the number of aggregates of \( n \) red cells counted in the presence of haemagglutinin, then the total count of aggregates is given by,

\[
A_o = A_1 + A_2 + A_3 + \ldots + A_n
\]

Similarly, the minimum number of bonds between red cells formed due to the presence of haemagglutinin is given by,

\[
B = A_2 + 2A_3 + \ldots + (n-1)A_n
\]

and the initial concentration of free cells by,

\[
C_0 = A_1 + 2A_2 + 3A_3 + \ldots + nA_n
\]

Then,

\[
A_o + B = C_0
\]

or,

the number of bonds = the number of cells - the number of aggregates.

Since \( C_0 \) is the initial concentration of red cells, \( R \), as used in the sedimentation-enumeration method (Page 128), we may then write for the number of bonds per red cell,

\[
\frac{B}{R} = 1 - \left( \frac{A}{R} \right)_0
\]

The numbers of bonds per red cell, \( \frac{B}{R} \), can thus be related, as shown in Figure 26, to the \( \log \) of the overall dilution of haemagglutinin, \( \log D \).

The data for the experiments at different red cell concentrations lie on a single curve and indicate that \( \frac{B}{R} \) is independent of the RBC concentration but dependent on the haemagglutinin concentration. This suggests that the haemagglutinin concentration is in excess to the RBC concentration and that the Percentage Law (Page 165) operates with respect to the latter, and,
FIGURE 26. The Coulter Counter: The Relationship between Red Cell Concentration and the Subsequent Extent of Agglutination.

![Graph showing the relationship between red cell concentration and the extent of agglutination.](image)

**Extent of Agglutination as Bonds per Red Cell (B/R)**

**Log(Overall Dilution of Haemagglutinin) = Log D**

**Initial Red Cell Count (per ml.).**

- ○ $1.00 \times 10^8$
- △ $0.50 \times 10^8$
- □ $0.25 \times 10^8$
therefore, that the intercept on the abscissa (Figure 26) is a numerical constant (Haemagglutination Index) which characterizes the concentration of haemagglutinin in the reaction mixture.

It should be noted that under these conditions, the value of \( \frac{B}{R} \) will never be more than unity since this is the asymptote for maximum agglutination in which all available haemagglutinin and red cells appear as aggregates.

A similar relationship was derived from observations of the optical densities due to haemoglobin released when free or unagglutinated cells in these reaction mixtures were lysed with distilled water (Figure 27). The supernate containing predominantly free cells was sampled after allowing the reaction mixture to stand for a period of 60 min. at room temperature. Since the haemoglobin (Hb) concentration is proportional to the number of cells, the data can be expressed in terms of the fraction of cells agglutinated, \( \frac{C_A}{C_o} \), but cannot be further reduced to a statement of the concentration of bonds, B, associated with these aggregates.

Thus,

\[
\frac{C_A}{C_o} = 1 - \frac{[Hb]_H}{[Hb]_o}
\]  \hspace{1cm} (18)

where \([Hb]_o\) and \([Hb]_H\) are the optical densities of the lysed control and reaction mixtures, respectively. The derivation of \( \frac{C_A}{C_o} \) is given in Appendix IV.

This expression can only be reduced to a form involving the number of bonds by the provision of a theoretical statement of aggregate distribution or by the acceptance of an equivalent assumption.
FIGURE 27. The Coulter Counter: The Fraction of Red Cells in Aggregates as a Function of Haemagglutinin Concentration following Lysis of Free Red Cells in the Supernate.
The characteristic lines of Figures 26 and 27 are plotted on common axes in Figure 28 and show a Haemagglutination Index of \( \log D_0 = 2.27 \) for both different methods. A similar Index can be obtained using the sedimentation-enumeration method to which the percentage law also applies. (Page 230).

(b) The effect of the time of mixing on the haemagglutination reaction

Attempts were made with the Coulter counter to observe the extent of agglutination of a reaction mixture containing haemagglutinin and red cells in relation to the time of mixing by tumbling. A standard reaction mixture containing a high concentration of haemagglutinin (\( \log D = 1.8 \)) and about \( 7 \times 10^6 \) RBC per ml. was tumbled and samples were then removed at intervals of up to three hours, for dilution and counting (Page 114). The data expressed as bonds per red cell, \( \frac{D}{R} \), are shown in Figure 29. The implication of these data is that a reaction time of 30 min. is sufficient to achieve maximum agglutination for the selected concentration of haemagglutinin. This result is consistent with that shown in Figure 14 (Page 95).

(iv) Discussion

Although these results suggested that the Coulter counter may be used successfully in the study of haemagglutination, the method has certain difficulties.
FIGURE 28. The Coulter Counter: Comparison of the Haemagglutination Index Indicated by Two Methods for the Reaction between Haemagglutinin and Red Cells.

The lines are those from the experimental data of Figures 26 and 27.

EXTENT OF AGGLUTINATION
AS BONDS PER RED CELL \( \frac{B}{R} \)

TIME OF MIXING IN MINUTES, t.
Agglutinated reaction mixtures with high concentrations of haemagglutinin and red cells (Figure 25) cannot be counted directly because the aperture is very easily blocked. It was therefore essential, as recommended by the manufacturer, for the aperture to be monitored continuously by observation with a microscope. Alternatively, the reaction mixture may be diluted to a cell concentration \(10^5\) cells per ml. more suitable for uninterrupted counting. Such a dilution step is unsatisfactory since it changes the pattern of reactant concentrations and may lead to the disruption of fragile aggregates and thereby distort the size distribution characteristic of the reaction mixture.

It was concluded that the ideal conditions for the use of the Coulter counter were not well matched to those required for the optimal formation of aggregates between influenza virus and fowl red cells. Since it is necessary to manipulate an agglutinated reaction mixture before counting with this technique, an alternative method was desired which offered as little disturbance as possible. The use of the Coulter counter was discontinued for these reasons.

Also, with the instrument available at that time (Coulter model A), it was not possible to obtain the size distributions of aggregates which are the necessary data for the correlation of the observed haemagglutinating activity with the concentration of haemagglutinating particles, (Page 191, equation 34). It would be interesting to assess the potential in haemagglutination studies of the more advanced Coulter instruments which are provided with upper and lower threshold settings and other refinements which contribute to the determination of size distributions.
e) The Direct Quantitation of Haemagglutinin by the Sedimentation-Enumeration Method

Although it was observed that small aggregates could be sampled from a suspension of red blood cells and viral haemagglutinin before they settled to the bottom of a tube, the quantitative analysis of this stage of the reaction by the photometric (Page 87) and cell counting procedures (Page 110) was blocked by a number of technical and interpretation difficulties (Page 124).

An alternative direct approach was therefore considered in which reaction mixtures of red cells and haemagglutinin were observed, by light microscopy, for the formation of aggregates in free suspension. These settling aggregates and free cells were then identified and counted, under standardized experimental conditions, to give population distributions which were shown to be characteristic of the reaction mixtures. It was also demonstrated that such distributions could be interpreted in terms of a quantitative estimate of viral haemagglutinin.

Following the quantitation of the reaction, this Sedimentation-Enumeration Method was also used to study the kinetics of the reaction and the influence of key parameters such as pH, medium and red cell concentration. The influence of specific antibody in the inhibition of haemagglutination was also studied.

Most of these detailed studies were restricted to the Semliki Forest virus - goose red blood cell system.
(i) The Development of the Sedimentation-Enumeration Method

(a) The observation of red cell aggregates in free suspension

The first indication of the formation of aggregates in free suspension was obtained when a standard reaction mixture was gently sampled by Pasteur pipette at a position 3-4 mm. above the bottom of the reaction tube. Samples taken at intervals of up to 60 min. were examined directly on a slide with the low power objective of a light microscope. When formed aggregates were thus disturbed as little as possible, they were first detected as clumps of up to 3 RBC in size in the 45-60 min. samples. Such preliminary experiments demonstrated an increase in aggregate size with increasing haemagglutinin concentration but did not indicate any simple, direct relationship between aggregate size and RBC concentration. The volume of the reaction mixture did not influence the final pattern observed at the bottom of the tube.

In further exploratory experiments, the haemagglutinin-RBC reaction mixtures were placed in glass spectrophotometer cuvettes with path-lengths of 1, 2 and 5 mm. These were then observed with the detached optical tube of a light microscope fixed in a horizontal plane. The eyepiece was fitted with a graduated graticule so that RBC and aggregates sedimenting under the influence of gravity could be counted as they passed a horizontal reference line. With this simple arrangement (Figure 30), it was possible to observe continuously the number, size and velocity of settling aggregates and to explore quantitatively the mechanism of haemagglutination by representative viruses.
FIGURE 30. The Experimental Apparatus for the Sedimentation-Enumeration Method.

- M - microscope
- C - cuvette containing reaction mixture
- SL - spirit level
- S - screen
- W - water-bath
- F - ground glass filter
- L - lamp
- B - base
(b) **The preliminary theoretical approach**

Consider 1 ml. of a reaction mixture at equilibrium containing \( R \) red blood cells and \( [H]_0 \) particles of haemagglutinin, where \( [H]_0 \) is the initial haemagglutinin concentration and \( D \) is the overall dilution denominator in the reacting mixture with cells.

Then, if haemagglutination occurs, each unit volume contains, \( A_1 \) single cells, \( A_2 \) dimers, and \( A_n \) \( n \)-fold aggregates. The available \( R \) red cells are then distributed so that,

\[
R = A_1 + 2A_2 + 3A_3 + \ldots + nA_n \quad (19)
\]

Each \( n \)-fold aggregate has a minimum of \( (n-1) \) bonds due to the presence of haemagglutinin, so that the minimum concentration of bonds per ml. is defined by,

\[
B = A_2 + 2A_3 + \ldots + (n-1)A_n \quad (20)
\]

or,

\[
B = R - (A_1 + A_2 + A_3 + \ldots + A_n) \quad (21)
\]

Hence, the distribution of red cells and aggregates defines the average number of RBC-RBC bonds per red blood cell \( \left( \frac{B}{R} \right) \), as a population parameter.

Thus, from expressions (19) and (21),

\[
\frac{B}{R} = 1 - \frac{\sum A_n}{\sum nA_n} \quad (22)
\]

where \( A_n \) is the true concentration of aggregates, each containing \( n \) red cells.

However, \( A_n \) is not observed directly but in terms of the number, \( C_n \), of \( n \)-fold aggregates sedimenting past the reference base-line of length 1 in the observation time \( t \). The relationship between \( C_n \) and \( A_n \) may be determined.
in terms of the sedimentation velocity, $s_n$, and depth of focus, $f_n$, for $n$-fold aggregates as shown in Figure 31. The values of $s_n$ and $f_n$ were determined in separate experiments as described below (Page 131).

We may then write,

$$A_n = \frac{C_n}{s_n f_n l.t.} \quad (23)$$

Then, substituting this expression for $A_n$ (equation(23)) into equation (22), we obtain,

$$B = 1 - \frac{1}{l.t.} \left( \frac{c_1}{s_1 f_1} + \frac{c_2}{s_2 f_1} + \cdots + \frac{c_n}{s_n f_1} \right)$$

$$= 1 - \frac{1}{l.t.} \left( \frac{c_1}{s_1 f_1} + \frac{2 c_2}{s_2 f_2} + \cdots + \frac{n c_n}{s_n f_n} \right)$$

$$= 1 - \frac{1}{l.t.} \left( \frac{c_1}{s_1 f_1} + \frac{2 (s_1 f_1)}{s_2 f_2} \cdot c_2 + \cdots + n (s_1 f_1) \cdot c_n \right) \quad (24)$$

Now writing,

$$F_1 = \frac{s_1 f_1}{s_1 f_1}, \quad F_2 = \frac{s_1 f_1}{s_2 f_2} \quad \text{and} \quad F_n = \frac{s_1 f_1}{s_n f_n} \quad (25)$$

to express the calculation constants, $F_n$, in terms of the ratios of the sedimentation velocities and depths of focus for single red cells to those for $n$-fold aggregates, we obtain,

$$B = 1 - \frac{\sum C_n F_n}{\sum n C_n F_n} \quad (26)$$
FIGURE 31. The Sedimentation-Enumeration Method: Diagram of the Volume Occupied by n-fold Aggregates Subsequently Counted in Time \( t \) in Passing the Observation Slit of Length \( l \).

\[ s_n = \text{sedimentation velocity} \]
\[ f_n = \text{depth of focus} \]
(c) The evaluation of the ratio constants, \( F_a \)

In accordance with the requirements of the theoretical considerations outlined above, the sedimentation velocities and the depths of focus for single RBC and aggregates were observed during gravitational settling.

A standard reaction mixture of SFV(M)/F, (Page 52) and goose RBC at pH 6.3 was used for these calibration experiments. The rates of settling were measured in the apparatus described on Page 141 for a large number (Table 8) of each aggregate size over a range of distances, and at different observation levels and depths of focus. The range of velocities in \( \mu \) per sec. for each aggregate size, together with the percentage frequency of each class, is shown in Table 8. The data for the mean sedimentation velocities, and their appropriate weightings, were used to obtain the line of best-fit for the relationship between sedimentation velocity and aggregate size (Figure 32).

Similarly, Table 9 shows the scatter of the values of the depths of focus for aggregates of different sizes, with the percentage frequencies, under the conditions mentioned above. Similarly, a line of best-fit for the mean depth of focus (\( \mu \)) and the aggregate size (\( n \)) was obtained, as shown in Figure 33.
**TABLE 8**. The Sedimentation-Enumeration Method: The Distribution of Sedimentation Velocities for Defined Aggregates of Goose Red Blood Cells

<table>
<thead>
<tr>
<th>Sed. Velocity (μm per sec.)</th>
<th>Number (n) of RBC in Aggregate</th>
<th>Number (and %) of n-fold Aggregates of Given Sedimentation Velocity</th>
<th>Mean Velocity (sₙ)</th>
<th>sₙ/s₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-16</td>
<td>1</td>
<td>-</td>
<td>28.6</td>
<td>1.00</td>
</tr>
<tr>
<td>16-23</td>
<td>2</td>
<td>6(24)</td>
<td>31.2</td>
<td>1.09</td>
</tr>
<tr>
<td>23-29</td>
<td>3</td>
<td>3(12)</td>
<td>33.8</td>
<td>1.18</td>
</tr>
<tr>
<td>29-36</td>
<td>4</td>
<td>11(44)</td>
<td>36.4</td>
<td>1.27</td>
</tr>
<tr>
<td>36-42</td>
<td>5</td>
<td>4(16)</td>
<td>39.6</td>
<td>1.39</td>
</tr>
<tr>
<td>42-49</td>
<td>6</td>
<td>1(4)</td>
<td>42.3</td>
<td>1.48</td>
</tr>
<tr>
<td>49-55</td>
<td>7</td>
<td>-</td>
<td>45.5</td>
<td>1.59</td>
</tr>
<tr>
<td>55-62</td>
<td>8</td>
<td>-</td>
<td>48.1</td>
<td>1.68</td>
</tr>
<tr>
<td>62-68</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mean Velocity (sₙ)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sₙ/s₁</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 32. The Sedimentation-Enumeration Method: The Sedimentation Velocities for Defined Aggregates of Goose Red Blood Cells

SEDIMENTATION VELOCITY (µ per sec) OF n-FOLD AGGREGATES

NUMBER OF RED CELLS IN AGGREGATE (n)

<table>
<thead>
<tr>
<th>Depth of Focus (μ)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (n) of RBC in Aggregate</td>
<td>7(70)</td>
<td>3(27)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Number (and %) of n-fold Aggregates of Given Depth of Focus</td>
<td>3(30)</td>
<td>5(45)</td>
<td>5(50)</td>
<td>3(23)</td>
<td>1(8.5)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mean Depth of Focus ($f_n$)</td>
<td>21.0</td>
<td>25.5</td>
<td>26.5</td>
<td>29.5</td>
<td>32.5</td>
<td>35.0</td>
<td>38.0</td>
<td>-</td>
<td>44.0</td>
<td></td>
</tr>
<tr>
<td>$f_n / f_1$</td>
<td>1.00</td>
<td>1.16</td>
<td>1.30</td>
<td>1.47</td>
<td>1.64</td>
<td>1.80</td>
<td>1.95</td>
<td>2.11</td>
<td>-</td>
<td>2.44</td>
</tr>
</tbody>
</table>
FIGURE 33. The Sedimentation-Enumeration Method: The Depths of Focus for Defined Aggregates of Goose Red Blood Cells

DEPTH OF FOCUS (in μ)
OF n-FOLD AGGREGATES

NUMBER OF RED CELLS IN AGGREGATE (n)
The normalised sedimentation velocities, \( s_n \), may then be expressed from the above best-fit data (Figure 32) as,

\[
   s_n = s_1 \left( 1 + \frac{(n-1)}{k} \right)
\]

and, similarly, for the depths of focus, \( f_n \) (Figure 33) as,

\[
   f_n = f_1 \left( 1 + \frac{(n-1)}{K} \right)
\]

Hence, the constants,

\[
   k = \frac{s_1(n-1)}{s_n-s_1} = 10.05 \quad (29)
\]

and

\[
   K = \frac{f_1(n-1)}{f_n-f_1} = 6.28 \quad (30)
\]

characterize the lines of best-fit in Figures 32 and 33.

Then, substituting \( s_1 \) and \( f_1 \) from equations (27) and (28) into equation (25), we obtain,

\[
   F_n = \frac{s_1 f_1}{s_n f_n} = \left( \frac{1}{\left(1 + \frac{(n-1)}{k} \right)} \right) \left( \frac{1}{\left(1 + \frac{(n-1)}{K} \right)} \right)
\]

Table 10 shows the ratio constants, \( F_n \), thus obtained for different values of \( n \), which are required for substitution into equation (26),

\[
   \frac{R}{R} = 1 - \sum \frac{C_n F_n}{n \cdot C_n F_n}
\]

These values of \( F_n \) are characteristic for the optical arrangement and for the goose red cells used in this study.
### TABLE 10. The Sedimentation-Enumeration Method: Evaluation of the Constant, $F_n$, for Defined Aggregates of n Goose Red Blood Cells

<table>
<thead>
<tr>
<th>Aggregate Size, $n$</th>
<th>Mean Sedimentation Velocity, $a_n$ ($\mu$ per sec.)</th>
<th>$\frac{s_1}{s_n}$</th>
<th>Mean Depth of Focus, $f_n$ (m)</th>
<th>$\frac{f_1}{f_n}$</th>
<th>$F_n = \frac{s_1 f_1}{s_n f_n}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28.6</td>
<td>1.00</td>
<td>18.0</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>31.2</td>
<td>0.91</td>
<td>21.0</td>
<td>0.85</td>
<td>0.77</td>
</tr>
<tr>
<td>3</td>
<td>33.8</td>
<td>0.84</td>
<td>23.5</td>
<td>0.76</td>
<td>0.63</td>
</tr>
<tr>
<td>4</td>
<td>36.4</td>
<td>0.78</td>
<td>26.5</td>
<td>0.69</td>
<td>0.52</td>
</tr>
<tr>
<td>5</td>
<td>39.6</td>
<td>0.72</td>
<td>29.5</td>
<td>0.61</td>
<td>0.43</td>
</tr>
<tr>
<td>6</td>
<td>42.3</td>
<td>0.67</td>
<td>32.5</td>
<td>0.55</td>
<td>0.36</td>
</tr>
<tr>
<td>7</td>
<td>45.5</td>
<td>0.62</td>
<td>35.0</td>
<td>0.51</td>
<td>0.31</td>
</tr>
<tr>
<td>8</td>
<td>48.1</td>
<td>0.59</td>
<td>38.0</td>
<td>0.47</td>
<td>0.27</td>
</tr>
<tr>
<td>9</td>
<td>50.7</td>
<td>0.56</td>
<td>41.5</td>
<td>0.43</td>
<td>0.24</td>
</tr>
<tr>
<td>10</td>
<td>53.3</td>
<td>0.53</td>
<td>44.0</td>
<td>0.40</td>
<td>0.21</td>
</tr>
</tbody>
</table>
(d) **Presentation of the experimental data**

Preliminary work indicated that the values of \( H \) may be conveniently represented as the ordinate against the overall \( R \) dilution denominator \( (\log D) \) of viral haemagglutinin \( (H) \) in the reaction mixture. This gives a reproducible relationship which shows three distinct regions and which are illustrated in Figure 34 by the typical experimental data from Table II:

A = a plateau region of high haemagglutinin concentration,

B = a linear region at critical intermediate haemagglutinin concentrations,

and C = a base-line region defining the spontaneous non-viral agglutination of RBC at very low haemagglutinin concentrations.

The further interpretation of this relationship is presented later (Page 234).

It has been noted that in control reaction mixtures without haemagglutinin some small aggregates are always present. This is referred to as spontaneous agglutination and contributes a small but significant number of bonds \( (B_0 \text{ per ml.}) \) which are unrelated to the presence of haemagglutinin. The number of haemagglutinin specific bonds per ml. thus becomes \( (B-B_0) \) and the mean number of virus specific bonds per RBC:

\[
\frac{B}{R} = \left( \frac{R}{R} \right)_o.
\]

These corrections for spontaneous agglutination are applied to all experimental data obtained by the sedimentation enumeration method (Figure 34).

For a given reaction mixture, the values of \( \frac{R}{R} \) approach \( \left( \frac{R}{R} \right)_o \) as the haemagglutinin concentration is reduced. The value \( \left( \frac{R}{R} \right)_o \) is given by the controls without haemagglutinin (Table II).
### Table II: Typical Population Distributions of Aggregates of Goose Red Blood Cells Formed in Free Suspension by Semliki Forest Virus.

<table>
<thead>
<tr>
<th>Aggregate Size (n)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Haemagglutinin Dilution Denominator, D</strong></td>
<td>( \log D )</td>
<td>Number of Aggregates of a Given Size</td>
<td>Bonds per Red Cell, ( \frac{B}{R} )</td>
<td>Pattern Test</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>0.9</td>
<td>68</td>
<td>40</td>
<td>23</td>
<td>21</td>
<td>5</td>
<td>8</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>80</td>
<td>1.9</td>
<td>57</td>
<td>40</td>
<td>16</td>
<td>16</td>
<td>13</td>
<td>12</td>
<td>2</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>160</td>
<td>2.2</td>
<td>75</td>
<td>40</td>
<td>19</td>
<td>14</td>
<td>4</td>
<td>8</td>
<td>3</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>320</td>
<td>2.5</td>
<td>98</td>
<td>51</td>
<td>17</td>
<td>19</td>
<td>3</td>
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<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>2560</td>
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<td>144</td>
<td>41</td>
<td>18</td>
<td>11</td>
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<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>10240</td>
<td>4.0</td>
<td>156</td>
<td>32</td>
<td>14</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20480</td>
<td>4.3</td>
<td>153</td>
<td>33</td>
<td>9</td>
<td>2</td>
<td>1</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>40960</td>
<td>4.6</td>
<td>138</td>
<td>22</td>
<td>10</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Control without Haemagglutinin</strong></td>
<td>140</td>
<td>24</td>
<td>11</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*NB: Pattern Test: pattern for a sample of haemagglutinin initially containing 3,100 HAU per ml.*
FIGURE 34. The Sedimentation-Enumeration Method: Relationship between the Extent of Agglutination as Bonds per Red Cell, $\frac{B}{R}$, and the Logarithm of the Overall Dilution of Haemagglutinin ($\log D$).
The intersection of the line B with the line \( \frac{B}{R} = \left( \frac{R}{B} \right)_o \) gives the logarithm of the limiting dilution of haemagglutinin (log \( D_L \)) at which haemagglutination is first detectable. The relationship between the limiting dilution, \( D_L \), and the actual, initial concentration of virus \([V]_o\) or haemagglutinin \([H]_o\) will be discussed later (Page 223).

A Meteor Mercury computer has been programmed to evaluate \( \frac{B}{R} \) and related quantities (Appendix V).

Table II shows the values of \( \frac{B}{R} \) which are characteristic of the distribution of aggregates for a typical reaction mixture with Semliki Forest virus (SFV) haemagglutinin (SFV(H)/SA) and goose red cells \((10^7\) cells per ml.) at pH 6.3. The corresponding patterns obtained in the dimple plate test are included for comparison.

(e) **The Construction of the Apparatus and the Conditions for the Observation of Sedimenting Aggregates**

Experiments showed that the optimal conditions for the observation and counting of settling aggregates were obtained using spectrophotometer cuvettes of 2 mm. path-length with internal dimensions of 43 x 10 x 2 mm. After use and before the cleaning process, the cuvettes were steeped in \( \frac{H}{10} \) hydrochloric acid for 1-2 hours for the disinfection of haemagglutinin. Since it was essential for these Spectrosil cuvettes (Thermal Syndicate Ltd., Northumberland) to be absolutely clean, they were then boiled in Decon 75 detergent (Medical Pharmaceutical Developments Ltd., Sussex) and, after thorough washing with distilled water, were dried in a stream of filtered air. This procedure avoided irregular results due to bubble and cell adhesion effects.
The cuvettes (c) were mounted in a brass/Perspex holder as shown in Figure 35. The holder consists of a Perspex yoke with a brass plate on each side. Two cuvettes could be held simultaneously so that one sample was counted while the other was held for thermal and mechanical stabilization. The rear plate was fitted with spring-loaded screws to hold the cuvettes with gentle, even pressure in a vertical plane. This cuvette-holder was mounted on a fixed pillar, fitted with a two-way spirit-level (SL) in a horizontal plane. Thus, the glass cuvettes could be accurately aligned by screw adjustment to a vertical position. This was essential in order to prevent settling RBC from sticking to the otherwise sloping walls of the cuvette and allowing the formation of stationary aggregates.

The reaction mixture was observed with the optical unit and focussing adjustments of a light microscope (M) which had been adapted to allow three-dimensional movement (Figure 36). Thus the reaction mixture and cuvettes were stationary and the optical system was moved into position for observation of either sample. This arrangement was necessary for the stabilization of mechanical swirling caused by pipetting of the sample and to avoid further physical disturbance.

The light source was a simple lamp (L) mounted on the optical axis of the microscope and fitted with a ground glass filter (F) to give uniform, flat illumination. Since the sedimentation of red cells and aggregates was very susceptible to disturbance by thermal convection from external sources of heat, such as that due to the observer (o) and the light source, a Perspex water-bath (w) of dimensions, 10 x 8 x 8 cm. containing 1% copper sulphate solution was placed, together with a black screen (S), between the cuvette and the light source. All surfaces of the water-bath and the cuvette-holder were blackened.
FIGURE 35. The Sedimentation-Enumeration Method: Design of the Holder for the Cuvettes Containing the Reaction Mixture.
FIGURE 36. Apparatus Used in the Sedimentation-Enumeration Method.

0 - observer  
M - microscope  
C - cuvette containing reaction mixture  
SL - spirit level  
S - screen  
W - water-bath  
F - ground glass filter  
L - lamp  
B - base
The whole apparatus (Figure 36) was mounted on a single base (B) fitted with three levelling screws to be used in conjunction with the spirit-level to maintain the cuvettes in a vertical plane.

(f) The counting of the RBC aggregates

It was convenient to count cells and aggregates as their leading edges crossed the horizontal reference line of a graduated eyepiece graticule during brief periods of observation. This line was of effective length 1000μ. To ensure an efficient sampling of the population, counts were made for 4 sec. in each minute during a total settling or reaction time of 15 min. The data (Table 12) show that the total number of RBC counted, regardless of their distribution as aggregates, remains constant if counting is completed under defined conditions.

After a preliminary period of 15-20 minutes for stabilization of disturbances due to mechanical swirling and thermal convection in the 0.5 ml. sample volume, observations were made at the slit positioned at a level 15 mm. below the meniscus and 10 mm. above the base. This allowed counts to be made for a reaction time of 15 minutes before the upper boundary of settling cells (B) entered the observation volume (0). The observation volume was sufficiently above the bottom of the cuvette to avoid deceleration of the aggregates. Counts were made in the centre of the cuvette, equidistant from the two sides, to reduce frictional effects. For similar reasons, observations were made at a convenient distance of about 300μ from the near face (F₁) of the cuvette. Ideally this distance should be 1000μ, equidistant between the faces F₁ and F₂, but this made it more difficult to identify and count aggregates. It was confirmed that aggregates sedimented uniformly within this observation volume (0) (Figure 37).
TABLE 12. The Independence of Number of Red Blood Cells, their Distribution as Aggregates and Total Reaction Time for Different Concentrations of Haemagglutinin

<table>
<thead>
<tr>
<th>Aggregate Size (n)</th>
<th>Computed Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of Red Cells (R) x (l.t.s.^1.)</td>
</tr>
<tr>
<td></td>
<td>Bonds per Red Cell (R) (Page 250)</td>
</tr>
<tr>
<td></td>
<td>(Page 129)</td>
</tr>
<tr>
<td>1</td>
<td>63</td>
</tr>
<tr>
<td>2</td>
<td>64</td>
</tr>
<tr>
<td>3</td>
<td>88</td>
</tr>
<tr>
<td>4</td>
<td>76 - 72</td>
</tr>
<tr>
<td>5</td>
<td>69</td>
</tr>
<tr>
<td>6</td>
<td>77</td>
</tr>
<tr>
<td>7</td>
<td>73</td>
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<td>8</td>
<td>82</td>
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<tr>
<td>9</td>
<td>66 - 72</td>
</tr>
<tr>
<td>10</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>80 - 70</td>
</tr>
<tr>
<td></td>
<td>69</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total Reaction Time, (min)</th>
<th>Haemagglutinin Dilution Denominator, D</th>
<th>Log D</th>
<th>Number of Aggregates of a Given Size Counted in 20 seconds</th>
<th>Number of Red Cells (R) x (l.t.s.^1.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
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<td>1.8</td>
<td>19 8 4 5 2 2 1 0 0 1</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>256</td>
<td>2.4</td>
<td>25 9 6 2 0 3 0 1 0 0</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>2048</td>
<td>3.3</td>
<td>44 17 6 1 1 1 0 0 0 0</td>
<td>88</td>
</tr>
<tr>
<td></td>
<td>4096</td>
<td>3.6</td>
<td>44 10 5 1 1 1 0 0 0 0</td>
<td>76 - 72</td>
</tr>
<tr>
<td>Control without Haemagglutinin</td>
<td>-</td>
<td></td>
<td>48 8 1 1 1 1 0 0 0 0</td>
<td>69</td>
</tr>
<tr>
<td>10</td>
<td>64</td>
<td>1.8</td>
<td>13 9 8 6 2 6 1 1 0 0</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>256</td>
<td>2.4</td>
<td>27 10 5 6 2 0 0 2 0 0</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>2048</td>
<td>3.3</td>
<td>37 15 7 1 1 1 0 0 0 0</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>4096</td>
<td>3.6</td>
<td>39 12 3 1 0 0 0 0 0 0</td>
<td>66 - 72</td>
</tr>
<tr>
<td>Control without Haemagglutinin</td>
<td>-</td>
<td></td>
<td>43 7 4 1 0 0 0 0 0 0</td>
<td>64</td>
</tr>
<tr>
<td>15</td>
<td>64</td>
<td>1.8</td>
<td>13 6 4 3 2 1 3 0 0 0</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>256</td>
<td>2.4</td>
<td>27 10 7 2 1 0 1 0 0 0</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>2048</td>
<td>3.3</td>
<td>42 12 5 3 0 1 0 0 0 0</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>4096</td>
<td>3.6</td>
<td>46 14 4 0 2 0 0 0 0 0</td>
<td>80 - 70</td>
</tr>
<tr>
<td>Control without Haemagglutinin</td>
<td>-</td>
<td></td>
<td>47 7 5 1 0 0 0 0 0 0</td>
<td>69</td>
</tr>
</tbody>
</table>
FIGURE 37. The Sedimentation-Enumeration Method: Definition of the Observation Volume for a Reaction Mixture Containing Settling Aggregates.

N.B. NOT TO SCALE

F₁ = near face
F₂ = far face
B = cell boundary layer
O = observation volume
M = microscope
The sedimenting aggregates were only counted when they were within the defined observation volume (0) and when, after equilibration, they were moving steadily downwards. The observed field was, of course, subject to optical inversion.

In all the preliminary experiments using RBC concentrations (about 6 x 10^6 per ml.) close to that used in the pattern test, it was noticed that in most cases none of the aggregates contained more than ten RBC and thus were easily identified and counted by this sedimentation-enumeration method. This is in contrast to the massive aggregates which were observed microscopically after gently sampling from the surface of a dimple plate cup containing a '4' pattern. It is likely, as will be discussed later, (Page 226) that the proximity of the surface induces the formation of very large aggregates due to mechanisms which do not occur in free suspension.

(c) The influence of temperature

All experiments with the sedimentation-enumeration method were made at room temperature (23°C). Preliminary tests at 4°C showed that there was a tendency for the buffer solutions to release bubbles of air which adhered to the faces of the cuvette and interfered with the sedimentation of the cells and aggregates. For this reason, all media and buffer solutions were equilibrated at room temperature before use. It was also found that at the higher temperature of 37°C, the evaporation of the sample induced the formation of strong and persistent convection currents which prohibited the application of the sedimentation-enumeration method at this temperature.
The Experimental Validity of the Sedimentation-Enumeration Method

With any new procedure it is necessary to confirm that the experimental results are both internally consistent and bear the correct relationship to data obtained by alternative and independent methods.

A. Consistency

Reproducibility of the $\frac{B}{R}$ v. log D Relationship

The replicate data shown in Figure 36 demonstrate that the experimental conditions (see Page 145) allow a consistent observation of the relationship between the number of RBC-RBC bonds per red cell ($\frac{B}{R}$) and the log of the overall dilution of haemagglutinin (log D). Each experiment was performed in the standard borate-BSA-PBS buffer system at pH 6.3 with the same dilutions of haemagglutinin (SFV(M)/F) but with fresh preparations of goose red cells. The day to day reproducibility and consistency of results for the same haemagglutinating system is shown by the close correspondence of the slopes for the linear region B (Figure 34, Page 140).

Distribution of Red Cells as Aggregates

A further demonstration of the consistency of this method of counting settling aggregates is obtained through the observation that the function

$$\sum_n c_n s_n F_n$$ (Appendix VI)

computed from the overall number of red cells counted, after correction (Page 126) for the different sedimentation velocities of aggregates, is proportional to the time of counting for a given red cell concentration, regardless of the concentration of haemagglutinin or of the distribution of red cells as aggregates. Such data are given in Table 12 (Page 146) and in Figure 39. It is shown in Appendix VI that these data lead directly to a valid estimate of the absolute concentration of red cells.
FIGURE 3B: The Sedimentation-Enumeration Method: The Reproducibility in Three Separate Experiments of the Standard $B$ v. log $D$ Relationship for Savannah Forest Virus (SFV(M)/F) and Goose Red Cells.
The Sedimentation-Enumeration Method: The Proportionality of the Function \( \sum n \cdot C_n \cdot F_n \) to the Time of Counting, to

\[ \frac{\sum n \cdot C_n \cdot F_n}{\sum n \cdot C_n \cdot F_n} = \text{Red Cell Concentration} \times (1.4 \pm 0.1) \]

FIGURE 39. 

![Graph showing the sedimentation-Enumeration method relationship between time of counting and red cell concentration](image-url)
Estimates of Bonds Per Red Cell Ratio, $\frac{B}{R}$

If the sedimentation-enumeration method is consistent, then it is also necessary that, for a given reaction mixture, the value for the bonds per red cell ratio, $\frac{B}{R}$, should remain constant regardless of the observed reaction time. This is confirmed by the typical data shown in Table 12 (Page 146) and in Figure 40.

Although counting for 20 sec. in 5 min. produced consistent results and suggested that the haemagglutination reaction had reached equilibrium, it was decided to standardize on counting for a total time of 60 sec. in 15 min. in order to maintain the statistical significance of the counts on a sampling basis to within $\pm 10\%$.

B. Independent Controls

Since the red cell concentration, $[R]$, is a necessary and critical parameter for the haemagglutination reaction, and the bonds per red cell ratio, $\frac{B}{R}$, is an expression of its outcome, it was desirable to find independent check estimates for $[R]$. Alternative absolute methods for the check estimation of haemagglutinin concentration do not exist so that the consistency of the present sedimentation-enumeration method can only be checked by internal consistency with respect to the estimate of haemagglutinin concentration and of its variation with dilution and its independence of red cell concentration.

Concentration of Red Blood Cells, $[R]$

In order to test the validity of the observational procedures used in the sedimentation-enumeration method, a comparison was made between the red cell concentration estimated directly from haemocytometer counts and the alternative estimate obtained from counts on red cells in aggregates in the experimental
FIGURE 40. The Sedimentation-Enumeration Method: The Independence of the Bonds Per Red Cell Ratio \( \frac{B}{R} \) of the Reaction or Settling Time for Different Dilutions of Haemagglutinin (SFV[D]/F).

![Graph showing the extent of agglutination as bonds per red cell (B/R) for different log D values and reaction/settling times.](image-url)
settle cuvette. The data presented in Appendix VI, (Page 250), show that the red cell concentration estimated by sedimentation-enumeration, \( [R_{SB}] \), is 75\% ± 4\% of the concentration estimated by haemocytometer counts, \( [R_H] \). This somewhat low estimate by sedimentation-enumeration of the red cell concentration reflects uncertainty in the estimation of short counting times which does not influence the time independent ratio, \( \frac{E}{H} \).
(ii) Investigation of the Kinetics of the Haemagglutination Reaction

In the study of any reaction, it is necessary to observe some consequence of the reaction and to characterize this in terms of the nature and concentration of the components of the reacting system.

The observable effect in the present study is the agglutination of red blood cells (RBC) and this may be investigated in quantitative terms by the sedimentation-enumeration method devised for that purpose. The concentration of haemagglutinin is a definitive parameter in this method and detailed observations may be made at many concentrations over the experimental range. The influence of RBC concentration on the reaction is described below (Page 155).

In contrast, the pattern test, as performed in dimple plates (Page 51), allows only an observation of haemagglutinating activity at that single concentration of haemagglutinin which identifies the nominal end-point (Page 76). This limits severely the value of the pattern test for the detailed study of the haemagglutination reaction. The quantitative relationship between the sedimentation-enumeration and pattern methods will be discussed later (Page 223).

The influence of pH and of the nature of the reactants on the haemagglutination reaction are then examined followed by consideration of the reaction between haemagglutinin and virus-specific antibody.

(a) The influence of red cell concentration

Preliminary experiments with the sedimentation-enumeration method showed that it was not possible to count the cells or aggregates in reaction mixtures containing high concentrations (> 2.5 x 10^7 RBC per ml.) of red blood cells (RBC). This was because individual cells and aggregates could not be identified and counted when the field of view contained more than about 4 x 10^5 (per cm^2) particles of any kind. Investigation of the effect of RBC concentration
was therefore limited to the range of concentration up to about $1.5 \times 10^7$ RBC per ml.

Haemagglutinin derived by fluorocarbon extraction of suspensions of mouse-brains infected with Semliki Forest virus (SFTV(M)/F) was serially diluted in PBS/BSA at pH 9.0 and allowed to react with goose RBC suspended in PBS to give a final mixture at pH 6.3. The agglutination of red cells in the reaction mixture was observed under the standard conditions previously defined (Page 51) and the results of several observations at different RBC concentrations are summarized in Figure 41. These data show that there is a central linear region in the characteristic which indicates that, if RBC are in excess, the number of bonds per red cell is independent of the RBC concentration. Also, with increasing haemagglutinin concentration, the extent of agglutination rises to a maximum and then maintains a distinct plateau at a bonds per RBC ($\frac{B}{R}$) value which is determined by the concentration of available RBC. The common base-line is defined by the control reaction mixtures containing no haemagglutinin (See Figure 34, Page 140) and this expresses the number of bonds per red cell due to the spontaneous agglutination of RBC.

The optimal RBC concentration for most subsequent experiments was selected as $10^7$ RBC per ml. of reaction mixture. This concentration allowed the practised observer to identify and count all aggregates in the field of view and permitted the demonstration of a conveniently wide extension of the linear portion of the relationship between $\frac{B}{R}$ and log D.

(b) The influence of the haemagglutinating system

In view of the wide range of types of haemagglutinating systems mentioned in the Introduction (Page 3), it was necessary to select a
The Sedimentation-Enumeration Method: The Influence of Increasing Red Cell Concentration on the Formation of Aggregates in Free Suspension.

**Figure 41**: Extent of agglutination as bonds per red cell ($B/R$) as a function of log(overall dilution of haemagglutinin) = log D. Control without haemagglutinin.
limited number for study. For this purpose it was considered essential to compare the kinetics of a viral haemagglutinating system with those of a non-viral system. Since the experimental constants determined for the sedimentation-enumeration method (Page 125) are characteristic for goose red cells, the agglutination of the same goose RBC by the specific rabbit antiserum prepared against these RBC was selected as an appropriate system for study. This system is described in detail later (Page 178).

The diversity of the haemagglutinating activity of viruses may be considered under three classes:

A) Similar haemagglutinating effects may be produced by quite different viruses.

B) A given virus preparation may contain several distinct haemagglutinating components.

C) The components of a virus preparation may be variously modified by different methods of extraction, as in the case of arboviruses.

These distinctions must be considered in any study of haemagglutination by viruses.

A) Haemagglutination by different viruses.

In this study, three viral haemagglutinating systems were considered for use in the sedimentation-enumeration method. Two arboviruses were studied, (Semliki Forest (SFV) of group A and Langat (TP-21) of group B), together with influenza virus (type A, PR8 strain) representing the early classical system. The preparation of haemagglutinins for these viruses was described on Page 47.

Sucrose-acetone extracted haemagglutinins of the arboviruses (SFV(M)/SA and TP-21 (M)/SA) were used in the standard borate-BSA-FBS buffer system, with goose RBC,
at the optimum pH values of 6.3 and 6.5 for SFV and Langat virus, respectively. Physiological saline at pH 7.2 and goose RBC were used for the haemagglutination of influenza virus.

Using a concentration of about $10^7$ RBC per ml. of reaction mixture, the data from many experiments with these three viruses are summarized in Figure 42. This shows a common slope for the critical linear region (Figure 34, Page 140) which indicates the same relationship for all three viruses between the extent of agglutination, $\frac{B}{R}$, in free suspension and the log of the overall dilution of haemagglutinin (log D). However, it cannot be assumed that with a common RBC concentration, a given extent of agglutination defines the same numbers of physical particles for these different virus systems. Donald and Isaacs (1954) showed that for influenza virus, filamentous particles had a 7-8 fold greater agglutinating activity than the more compact quasi-spherical particles.

B) **Multiple haemagglutinins in one virus preparation.**

It has been reported by Smith and Holt (1961) that arboviruses have two haemagglutinating components in native preparations and Faulkner and Dobos (1963) have shown, by equilibrium density gradient centrifugation in caesium chloride, that preparations of Sindbis virus (group A) contain three distinct haemagglutinins. The data obtained in this study for Semliki Forest virus (SFV(M)/F), in analysis by equilibrium density gradient centrifugation or gel filtration (Page 56), have shown only one haemagglutinating component. This component can be identified with the initial infective particle.

C) **The influence of different extraction procedures on the haemagglutinin of Semliki Forest virus.**

Satisfactory haemagglutinins for arboviruses are only obtained from either mouse-brain or tissue culture supernatant fluid after the removal of lipid
FIGURE 42. The Sedimentation-Enumeration Method: The Extent of Agglutination Obtained for the Haemagglutinins of Semliki Forest, Langat and Influenza Viruses.
and other inhibitors of haemagglutination (Clarke and Casals, 1958; and Page 55).

Various solvent extraction techniques using sucrose/acetone, Tween-80/ether and fluorocarbon have been employed and a comparison of these three methods using SFV(M) and the sedimentation-enumeration method is shown in Figure 43. The standard reaction relationship (Page 140) was observed in each case, with the fluorocarbon method giving a reduced yield of haemagglutinating activity when compared with the activity recovered following extraction by the other two methods.

To interpret this result in terms of virus particles, it must be recognised that these methods may produce distinct haemagglutinins. Tween-80/ether treatment usually causes the disruption of whole virus particles to smaller haemagglutinating fragments, probably lipo-protein components of the capsid, which retain complement-fixing and antibody-combining activities (G. Appleyard, personal communication, 1968). The different types of haemagglutinating particles are likely to differ in terms of their capacity or avidity to form a stable bond between two red blood cells. Thus, the same value for the extent of agglutination ($\frac{R}{R}$) may be given by distinct virus components due to different concentrations of haemagglutinating activity or distinct bond-forming capacities. However, the Tween-80/ether treatment used in this study does not disrupt the virus particles, as shown by gel filtration experiments (Page 56). Therefore whole virus particles give the haemagglutinating activities shown in Figure 43.

(o) The influence of pH on the haemagglutination reaction.

The earlier studies (Page 63) on the conditions for haemagglutination by arboviruses have demonstrated the criticality of the pH of the reaction mixture. In these and published studies, the pattern test was invariably used and consequently only the terminal agglutination at the dimple
FIGURE 43. The Sedimentation-Enumeration Method: The Extent of Agglutination Shown by the Haemagglutinins Derived from Semliki Forest Virus by Different Extraction Procedures.
plate surface was observed (Figure 8, Page 75). It was possible therefore that pH might be critical for the final reaction at the wall surface but not for the initial attachment or for the later formation of small aggregates in free suspension.

Preliminary experiments with extracted Semliki Forest virus (SFV(II)/F) in the sedimentation-enumeration method showed that in the presence of this haemagglutinin, known to be active at pH 6.3, no new RBC-RBC bonds were formed in reaction mixtures at pH 7.6 and 9.0 (Figure 44). In studying the relationships between haemagglutinating activity, infectivity and physical particles for a given virus preparation, it would be ideal if the same preparation, buffer system and pH could be used for all of these assays. However, the result of these experiments confirms other observations that, for arboviruses, a high pH (9.0) is necessary to preserve the infectivity and the haemagglutinating activity of virus suspensions, a neutral pH (7.4-7.6) is required for virus-cell interaction and a low pH (6.3) is essential for the demonstration of haemagglutination.

More detailed experiments were set up to define and compare the optimal pH ranges for the indication of haemagglutinating activity in the pattern test and by the sedimentation-enumeration method. The haemagglutinating activity of a sample of SFV(II)/F was determined in the standard borate-BSA-PBS buffer system from pH 6.1 to 6.7 by both methods. To enable a valid comparison to be made, it was necessary to adjust the data since different concentrations of red cells are used in the tests and the haemagglutinating activity and the extent of agglutination (D/R) are normally expressed in different terms. With the aid of the summary graphs shown in Figure 9, (Page 77), the end-point dilutions obtained in the pattern test were adjusted to correspond to the red cell concentration (10^7 RBC per ml. of reaction mixture) used in the sedimentation-enumeration method. The data from both methods were then expressed as the logarithm of the limiting dilution D_L at (D/R). (Figure 45,
FIGURE 44. The Sedimentation-Enumeration Method: The Influence of pH on the Extent of Agglutination Shown by the Haemagglutinin of Semliki Forest Virus

pH 6.3: optimal for haemagglutination
pH 7.6: optimal for the assay of infectivity
pH 9.0: optimal for the preservation of haemagglutinin
Page 166) at which virus-specific haemagglutinating activity was first detectable.

With the data normalised in this way (Figure 45), it is clear that both the optimum and range of pH for the formation of aggregates in free suspension are almost identical in the pattern test and by the sedimentation-enumeration method.

(a) The inhibition of haemagglutination by antibody.

A further very important reaction to be considered is the inhibition of haemagglutinating activity by specific antibody. This reaction is widely used as a test in laboratory studies of arboviruses (see Page 3) and its observation by the sedimentation-enumeration method offered a further approach to the study of the kinetics of haemagglutination.

The standard laboratory method for estimating the quantity of virus-specific antibody in an antiserum by the inhibition of the haemagglutinating activity of a virus suspension has been described on Page 52. Following the interaction between antibody and haemagglutinin under standard conditions of time and temperature, any remaining available haemagglutinin was detected by the addition of susceptible RBC and the observation of the extent of subsequent agglutination in the pattern test. The sedimentation-enumeration method lends itself to the study of this reaction in more quantitative terms than the pattern method.

Before such experimental data are discussed, it is appropriate to consider the theoretical treatment applied to the virus-antibody reaction in other test procedures. Under given experimental conditions, with an excess of antiserum, the fraction of infective virus remaining after neutralization of infectivity by antibody is constant regardless of the initial level of virus infectivity. This is the Percentage Law of Andrewes and Elford (1933) which has been studied in detail by Bradish, Farley and Ferrier (1962) for the neutralization of the virus of foot-and-mouth disease by

\[ \log (\text{overall dilution of haemagglutinin at the end-point}) = \log D_L \]
specific antibody. Thus, if $[V]_0$ is the initial virus infectivity and $[V]_{Ab}$ is the virus infectivity remaining after reaction with antibody, then the log depression of infectivity ($\log \frac{[V]_0}{[V]_{Ab}}$) may be related to the log of the overall dilution of antibody ($\log D_{Ab}$), by the expression,

$$\log S = \frac{1}{n} \left( \log \frac{[V]_0}{[V]_{Ab}} \right) + \log D_{Ab} \tag{32}$$

This equation defines the straight line obtained when the log depression of infectivity is plotted against the log dilution of antiserum. Thus, $n$ is the slope constant and $\log S$, the intersection of the $\log D_{Ab}$ axis, is the log of the antibody activity (Serum Neutralization Index, S.N.I.) for the undiluted antiserum.

As mentioned previously (Page 441), the characteristic linear region $B$ of the relationship between the extent of agglutination ($\frac{R}{R}_o$) and the log of the overall dilution of haemagglutinin ($\log D$) may be represented by the limiting dilution of haemagglutinin ($D_L$) at which virus-specific haemagglutinating activity is first detectable. In this case, $\log D_L$ is the intercept on the abscissa corresponding to the extent of spontaneous agglutination, $\left( \frac{R}{R}_o \right)_o$, and $D_L$ is proportional to the initial concentration of haemagglutinin $[H]_o$.

It follows from this that the initial haemagglutinin, $[H]_o$, may be related to the antibody concentration and to the concentration of haemagglutinin remaining after interaction with antibody, $[H]_{Ab}$, in a similar way to that considered for the neutralization of infectivity as described above.

Experiments were set up with dilutions of antiserum reacting with extracted Semliki Forest virus (SFV(M)/F) in borate-BSA buffer, pH 9.0 at room temperature ($23^\circ\text{C}$) for 30 minutes. The antiserum used was hyperimmune, rabbit anti-SFV serum which was treated before use with kaolin, to remove non-specific inhibitors of haemagglutination, and with goose RBC to remove natural agglutinins for goose RBC (Page 49). Following the interaction between haemagglutinin and antibody, goose
RBC suspended in PBS were added to give a final reaction pH of 6.3 and an overall RBC concentration of about $10^7$ RBC per ml. of reaction mixture. Each reaction mixture was then sampled and observed by the sedimentation-enumeration method under standard conditions (Page 145) to give a value for the extent of agglutination $\left( \frac{B}{A} \right)$ for the residual haemagglutinating activity. Data for one of three such experiments are presented in Figure 46, and show a series of parallel lines for the linear region B for all of the antiserum dilutions used. It is clear that the greater the concentration of antibody, the lesser the quantity of detectable haemagglutinin remaining in suspension for the agglutination of the added red cells.

The consideration of the data from the above experiment in terms of log depression of haemagglutinating activity $\left( \log \frac{B}{A} \right)$ is presented in Table 13. Data from a similar experiment with the same antiserum preparation are given in Table 14. The values shown in brackets relate to slight inhibition at high dilutions of antibody and are not considered in the mean values given in the last column of the tables. The data thus give an approximately constant value for $\log S$ (equation (32)) and this has been designated the Haemagglutination Inhibition Index (H.I.I.) by analogy with the Serum Neutralization Index (S.N.I.) applicable to the neutralization expression for the undiluted serum. These data may also be expressed graphically as shown in Figure 47 where the intersection of the characteristic line with the ordinate axis $\left( \log D_{\text{Ab}} \right)$ defines the haemagglutination inhibition index. These data confirm that the percentage law offers a valid expression of the haemagglutination-inhibition reaction and that, under present experimental conditions, antiserum may be characterized by a Haemagglutination Inhibition Index, or H.I.I. (In this case, from Figure 47, $H.I.I. = 2.90$ log units).
### TABLE 13. The Characterization of Haemagglutination Inhibition \( \text{H.I.I.} \)

<table>
<thead>
<tr>
<th>Dilution from stock Haemagglutinin before Reaction with Antibody</th>
<th>( \log D_{\text{Ab}} ) (Antibody)</th>
<th>Bonds per Red Cell</th>
<th>( \log ) of Final Haemagglutinin Concentration ( (\log [H_b]) )</th>
<th>( \log ) Depression of Haemagglutinin Concentration ( (\log [H_a]_b) )</th>
<th>Haemagglutination Inhibition Index ( (\frac{\log[H_a]}{[H_b]} + \log D_{\text{Ab}}) )</th>
<th>Mean Value of H.I.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \frac{1}{32} )</td>
<td>1.9</td>
<td>0.270</td>
<td>0.55</td>
<td>1.35</td>
<td>3.25</td>
<td>3.04</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>0.300</td>
<td>1.28</td>
<td>0.62</td>
<td>2.82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.425</td>
<td>1.57</td>
<td>0.33</td>
<td>2.83</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.8</td>
<td>0.400</td>
<td>1.42</td>
<td>0.48</td>
<td>3.28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.1</td>
<td>0.435</td>
<td>1.64</td>
<td>(0.26)</td>
<td>(3.36)</td>
<td></td>
</tr>
<tr>
<td>NIL</td>
<td></td>
<td>0.502</td>
<td>1.9 = ( \log [H_b] )</td>
<td>0.00</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>( \frac{1}{128} )</td>
<td>1.9</td>
<td>0.258</td>
<td>0.52</td>
<td>0.90</td>
<td>2.80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>0.298</td>
<td>0.80</td>
<td>0.62</td>
<td>2.82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.392</td>
<td>1.42</td>
<td>(0.00)</td>
<td>(-)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.1</td>
<td>0.368</td>
<td>1.22</td>
<td>(0.20)</td>
<td>(3.30)</td>
<td></td>
</tr>
<tr>
<td>NIL</td>
<td></td>
<td>0.392</td>
<td>1.42 = ( \log [H_b] )</td>
<td>0.00</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>( \frac{1}{512} )</td>
<td>1.9</td>
<td>0.180</td>
<td>0.00</td>
<td>0.92</td>
<td>2.82</td>
<td>2.90</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>0.235</td>
<td>0.35</td>
<td>0.57</td>
<td>2.77</td>
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<tr>
<td></td>
<td>2.5</td>
<td>0.260</td>
<td>0.52</td>
<td>0.40</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>2.8</td>
<td>0.269</td>
<td>0.60</td>
<td>0.32</td>
<td>3.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.1</td>
<td>0.297</td>
<td>0.76</td>
<td>(0.16)</td>
<td>(3.26)</td>
<td></td>
</tr>
<tr>
<td>NIL</td>
<td></td>
<td>0.317</td>
<td>0.92 = ( \log [H_b] )</td>
<td>0.00</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>( \frac{1}{1024} )</td>
<td>1.9</td>
<td>0.196</td>
<td>0.14</td>
<td>0.62</td>
<td>2.52</td>
<td>2.74</td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>0.195</td>
<td>0.14</td>
<td>0.62</td>
<td>2.82</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.233</td>
<td>0.38</td>
<td>0.38</td>
<td>2.88</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.8</td>
<td>0.269</td>
<td>0.61</td>
<td>(0.15)</td>
<td>(2.95)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.1</td>
<td>0.252</td>
<td>0.46</td>
<td>(0.30)</td>
<td>(3.40)</td>
<td></td>
</tr>
<tr>
<td>NIL</td>
<td></td>
<td>0.297</td>
<td>0.76 = ( \log [H_b] )</td>
<td>0.00</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
### Table 14: The Characterization of Haemagglutination Inhibition: The H.I.I.

<table>
<thead>
<tr>
<th>Dilution from stock Haemagglutinin before Reaction with Antibody</th>
<th>Log $D_{Ab}$ (Antibody)</th>
<th>Bonds per Red Cell (R/R)</th>
<th>Log of Final Haemagglutinin Concentration (Log $H_{no}$)</th>
<th>Log Depression of Haemagglutinin Concentration (Log $H_{no}$)</th>
<th>Haemagglutination Inhibition Index (H.I.I.) $= \log H_{no} + \log D_{Ab}$</th>
<th>Mean Value of H.I.I.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/32</td>
<td>1.6</td>
<td>0.280</td>
<td>0.80</td>
<td>1.40</td>
<td>3.00</td>
<td>2.92</td>
</tr>
<tr>
<td></td>
<td>1.9</td>
<td>0.320</td>
<td>1.25</td>
<td>0.95</td>
<td>2.85</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.2</td>
<td>0.380</td>
<td>1.70</td>
<td>0.50</td>
<td>2.70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.8</td>
<td>0.415</td>
<td>1.85</td>
<td>0.35</td>
<td>3.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3.1</td>
<td>0.480</td>
<td>2.00</td>
<td>(0.20)</td>
<td>(3.30)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NIL</td>
<td>0.550</td>
<td>2.20 = Log $H_{no}$</td>
<td>0.00</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
**FIGURE 47.** The Sedimentation-Enumeration Method: The Determination of the Haemagglutination Inhibition Index for a Rabbit Antiserum Prepared Against Semliki Forest Virus.

- data from Table 13
- data from Table 14

\[
\log(\text{overall dilution of antibody}) = \log D_{Ab}
\]

---

**Legend:**

- HAEMAGGLUTINATION INHIBITION INDEX, (H.I.I) = 2.90

- **Log Depression of Haemagglutinating Activity:** \(\log \frac{H_o}{H_{Ab}}\)
It may be concluded, in accordance with the percentage law, that in the presence of excess antibody, a constant proportion of haemagglutinin combines with specific antibody regardless of the initial concentration of haemagglutinin. Thus it appears that the reaction between viral haemagglutinin and virus-specific antibody may be described in the same terms as those previously confirmed for the neutralization reaction (Page 165). Since all virus particles are rapidly coated by antibody molecules (Bradish and Crawford, 1960), it follows that the subsequent agglutination of red blood cells is by these haemagglutinin-antibody complexes and that this agglutination is not inhibited by the excess of unattached antibody. The results from exploratory experiments to confirm this hypothesis suggest that the presence of anti-antibody (goat anti-rabbit serum) reduces the capacity of these haemagglutinin-antibody complexes to agglutinate goose red blood cells. This is observed as an enhancement of the inhibition of haemagglutination. More detailed studies of the enumeration of haemagglutinating particles and of antibody molecules require electron microscopical techniques or analytical ultracentrifugation.
(iii) The Quantitation of Haemagglutination at Low Concentrations of Haemagglutinin

Since it was shown that the extent of agglutination between red blood cells and viral haemagglutinins, as expressed by the ratio \( \frac{B}{R} \), was independent of red cell concentration (Page 155), the data obtained by the sedimentation-enumeration method were considered in relation to the estimation in absolute terms of the particle concentration of haemagglutinin. The data of Figure 34 (Page 140) show that the extent of agglutination, \( \frac{B}{R} \), diminishes along the linear slope \( B \) as the concentration of haemagglutinin decreases. The data also show that, in the linear region \( B \) the number of RBC-RBC bonds is not directly proportional to the concentration of haemagglutinin. Thus an absolute estimate of the number of particles of haemagglutinin is not available from data in this region.

In the region immediately above the limiting dilution \( (D_L) \), at which viral haemagglutination is first detectable, the number \( (n) \) of particles of haemagglutinin per bond between two red cells is at a minimum and may be taken to be unity or a small integer. The consistency of experimental data in this region of limiting haemagglutinin dilution was tested by using closer dilution intervals than in previous experiments. The standard conditions (Page 69) of pH 6.3 and red cell concentration \( (\sim 10^7 \text{ cells per ml.}) \) for the haemagglutinin of Semliki Forest virus (SFV(M)/F) were used. Typical data from one of three experiments are shown in Figure 48. These data may be presented in the alternative form in which the extent of agglutination, \( \frac{B}{R} \), as the ordinate is shown against the haemagglutinin concentration expressed as the reciprocal of the overall dilution, \( \frac{1}{D} \). The data then confirm that at low haemagglutinin concentrations the extent of agglutination, \( \frac{B}{R} \), is proportional to the particle
FIGURE 48. The Sedimentation-Enumeration Method: The Analysis of the $\frac{B}{R}$ vs. $\log D$. Relationship at Low Concentrations of Haemagglutinin.

**Extent of Agglutination as Bonds per Red Cell**

$B/R$

**Log (Overall Dilution of Haemagglutinin) vs. Log D**

Control without haemagglutinin

**Extent of Agglutination as Bonds per Red Cell**

$B/R$

Initial region of proportionality

Control without haemagglutinin

Reciprocal of overall dilution of haemagglutinin, $\frac{1}{D}$
concentration of haemagglutinin. To ensure that these data are consistent, the precision of the observations of \( \frac{H}{R} \) in this initial region was enhanced by repeated observations on different samples of the same haemagglutinin dilution for longer periods of time and for correspondingly larger numbers of aggregates. A dilution of haemagglutinin was selected from each end of this region and replicate (7) observations were made of reaction mixtures for each dilution. These data are shown in Figure 49 as a straight line derived from the arithmetic means of the replicate observations. The initial slopes in Figures 48 and 49 are the same (105).

Thus over this range of dilutions near the limiting dilution \( D_L \), the minimum number \( (h_m) \) of particles of haemagglutinin per EBC-RBC bond is constant and is given by,

\[
h_m = \frac{[H]_0}{D} \cdot \left( \frac{1}{b \cdot [R]} \right)
\]

or,

\[
b = \frac{1}{D} \cdot \left( \frac{[H]_0}{[R] \cdot h_m} \right)
\]

where \([H]_0\) is the initial particle concentration of haemagglutinin, \( D \) is the dilution denominator for haemagglutinin, \([R]\) is the red cell concentration and \( b \) is the average number of haemagglutinin-specific, EBC-RBC bonds per red cell,

\[
\left( \frac{H}{R} - \left( \frac{H}{R} \right)_0 \right).
\]

From equation (34), if \( b \) is presented on the ordinate axis against \( \frac{1}{D} \) on the abscissa axis then the slope of the initial linear region in Figure 48 is equal to

\[
\left( \frac{[H]_0}{[R] \cdot h_m} \right).
\]
The Sedimentation-Emigration Method: The Consistency of the Analysis of the $\frac{B}{R}$ v. log D Relationship in the Initial Region of Proportionality at Low Haemagglutinin Concentrations.

**EXTENT OF AGGLUTINATION AS BONDS PER RED CELL** \( \left( \frac{B}{R} \right) \)

![Graph showing the relationship between agglutination extent and log D.]

- Control without haemagglutinin.

**EXTENT OF AGGLUTINATION AS BONDS PER RED CELL** \( \left( \frac{B}{R} \right) \)

![Graph showing the relationship between agglutination extent and reciprocal of overall dilution of haemagglutinin.]

- Control without haemagglutinin.
This offers a direct estimate of the number of particles of haemagglutinin, \( \text{H}^\circ \), in the initial sample.

The validity of equation (3.4) was tested in terms of the variation of slope for this initial region of limiting haemagglutinin dilution as a function of red cell concentration for a given preparation of haemagglutinin. The data from four experiments given in Figure 50 show that, contrary to equation (3.4), the slope remained constant and was therefore independent of the red cell concentration over the range \( 2 \times 10^6 \) to \( 10 \times 10^6 \) cells per ml. The control level, \( \left( \frac{B}{R} \right)_0 \), altered slightly depending on the initial haemagglutinating activity in each experiment.

Since these data are inconsistent with equation (3.4) in relation to the dependence on red cell concentration, it was desirable to confirm that this was not peculiar to the Senliki Forest virus-goose red cell system. For this reason, a non-viral haemagglutinating system was selected to provide a comparison with the viral system. Since in the sedimentation-enumeration method only single or aggregated goose red cells are observed, then the alternative agglutinating system of goose RBC and specific rabbit anti-goose RBC serum was used. The agglutination of goose red cells by antibody was tested in a variety of buffer systems. The standard borate-BSA-PBS buffer system at pH 6.3 was used in addition to PBS buffer at pH 7.2, in the presence or absence of BSA (See Appendix I ). Reaction mixtures were analyzed for haemagglutination by the sedimentation-enumeration method as before and the data for several experiments are summarized in Figure 51. A relationship between \( \frac{B}{R} \) and \( \log D \) was obtained similar to that for viral haemagglutinin and showed the same three characteristic regions (A, B and C of Page 138). It was also noted that the goose RBC-anti-goose RBC sera agglutinating system was not influenced by the pH of the reaction mixture or by the presence or absence of BSA at this standard concentration of 0.002 gm. per ml.
FIGURE 50.  The Sedimentation-Enumeration Method. The Influence of Red Cell Concentration on the Slope of the Initial Linear Region for the Haemagglutination of Goose Red Cells by the Haemagglutinin of Senlíki Forest Virus (SFVII) x.

<table>
<thead>
<tr>
<th>[RBC] Per ML.</th>
<th>Extent of Agglutination (B/R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2 x 10^6</td>
<td>0.4</td>
</tr>
<tr>
<td>4.0 x 10^6</td>
<td>0.3</td>
</tr>
<tr>
<td>4.5 x 10^6</td>
<td>0.2</td>
</tr>
<tr>
<td>10.9 x 10^6</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Diagram: Extent of Agglutination as Bonds per Red Cell (B/R) vs. Reciprocal of Overall Dilution of Haemagglutinin, 1/D.
FIGURE 51: The Sedimentation-Enumeration Method: The \( \frac{R}{R} \) v. log \( D \) Relationship for the Agglutination of Goose Red Cells by Anti-Goose Red Cell Serum.

**Extent of Agglutination**

As Bonds per Red Cell \( \left( \frac{B}{R} \right) \)

\[ \log \left( \text{Overall Dilution of Antibody} \right) = \log D_{\text{Ab}} \]

- **A** - Plateau region of high antibody concentration.
- **B** - Linear region at intermediate antibody concentrations.
- **C** - Base-line region defining spontaneous haemagglutination at very low antibody concentrations.

- \( \bullet \) Borate-PBS at pH 6.3
- \( \circ \) Borate-BSA-PBS at pH 6.3
- \( \triangle \) PBS at pH 7.2
- \( \triangle \) PBS-BSA at pH 7.2
To test equation (34) for this non-viral agglutinating system, analyses were made of the influence of red cell concentration on the initial slope. The data for RBC concentrations ranging from $2.7 \times 10^6$ to $9.3 \times 10^6$ cells per ml. are shown in Figure 52 and confirm that the initial slope is independent of red cell concentration for the haemagglutinin of SFV but that for agglutination by anti-goose RBC antibody, the slope increases with RBC concentration in such a way that the number of antibody molecules per RBC-RBC bond is lower at higher red cell concentrations. Thus these experiments demonstrate that haemagglutination by viral haemagglutinin differs in mechanism from haemagglutination by antibody since responses to pH and to red cell concentration are distinct in each case. From these limited data, it appears that haemagglutination by antibody may fail to conform to equation (34) but in a different way from that shown by viral haemagglutination.

(a) The influence of pH on the haemagglutination reaction

It was clear from these results that experimental parameters were involved in the haemagglutination reaction other than those incorporated in the considerations leading to equation (34). Since the pH of the reaction mixtures is particularly critical for the demonstration of haemagglutination by arboviruses, it was possible that this also had a controlling influence on the course of the reaction between haemagglutinin and red cells at the optimum pH. The following experiments were set up to test the extent to which the rate of inactivation of haemagglutinin by pH and the rate of adsorption of haemagglutinin by red cells may be critically competitive reactions.

1. The rate of inactivation of haemagglutinin by pH

An experiment was set up in which equal volumes of dilutions of SFV haemagglutinin (SFV(M)/F), in borate-BSA buffer at pH 9.0, and PBS were
The Sedimentation-Enumeration Method: The Influence of Varying Red Cell Concentration on the Slope of the Initial Linear Region for the Haemagglutination of Goose Red Cells by Segalidi Forest Virus (SFV(H/F)) and by Anti-Goose Red Cell Serum.

**Figure 52.**

**Extent of Agglutination**

As Bonds per Red Cell ($B/R$)

<table>
<thead>
<tr>
<th>[RBC] per ML</th>
<th>SFV</th>
<th>Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.7 x 10^6</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>6.1 x 10^6</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>9.3 x 10^6</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

Control without haemagglutinin or antibody

**Extent of Agglutination**

As Bonds per Red Cell ($B/R$)

<table>
<thead>
<tr>
<th>[RBC] per ML</th>
<th>SFV</th>
<th>Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.7 x 10^6</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>6.1 x 10^6</td>
<td>▲</td>
<td>▲</td>
</tr>
<tr>
<td>9.3 x 10^6</td>
<td>□</td>
<td>□</td>
</tr>
</tbody>
</table>

Control without haemagglutinin or antibody

**Log (Overall Dilution of Haemagglutinin or Antibody)**

$\log(D) = \log(D_{Ab})$

**Reciprocal of Overall Dilution of Haemagglutinin or Antibody,**

$\frac{1}{D}$ or $\frac{1}{D_{Ab}}$
mixed to give reaction mixtures with final pH values of 6.0, 6.3, 6.4, 6.6 and 7.6. These were left undisturbed at room temperature until sampled at defined times. The samples were appropriately diluted in borate-BSA buffer, pH 9.0, to raise the pH to about pH 6.0 and thus prevent further pH inactivation. Dilutions in two-fold steps were then made in borate-BSA buffer at pH 9.0 and the residual haemagglutinating activity was estimated in a standard four-volume pattern test (Page 51), with goose RBC suspended in PBS to give an optimum test pH of 6.3.

In Figure 53 the percentage haemagglutinating activity of the initial uniniactivated control is shown against the reaction time. The reaction constant k is evaluated later (Page 194). It is clear that the haemagglutinating activity was reduced in reaction mixtures of lower pH. There was complete and rapid inactivation at pH 6.0, but at the optimum of pH 6.3 only about 50% of the haemagglutinin was inactivated after a reaction time of 15 to 30 min. This was consistently observed in other experiments. It was also shown that 10 to 20% of haemagglutinating activity was still detectable even after 72 hr. at pH 6.3.

2. The rate of adsorption of haemagglutinin to goose red cells

Further experiments were set up to study the effect on the estimation of haemagglutinating activity of the rate of adsorption of haemagglutinin to red cells in the standard reaction at pH 6.3. It must be recognized that in such experiments, for the adsorption of haemagglutinin to be observed as haemagglutination, the reaction mixture must be at or near the optimal pH 6.3 for Semliki Forest virus (SFV). It was shown previously (Page 183) that inactivation occurred rapidly at this pH and consequently such inactivation also took place during the time in which the haemagglutinin was being adsorbed to RBC.

In these experiments, a constant dilution (1/10) of SFV haemagglutinin
FIGURE 53. The Sedimentation-Enumeration Method: The Inactivation by pH of Semliki Forest Virus Haemagglutinin (SFV(H)/F) over a Range of pH Values.

RESIDUAL HAEMAGGLUTINATING ACTIVITY AS % OF INITIAL ACTIVITY

Reactivity vs. Reaction Time (min)
SFV(M) was reacted at pH 6.3 with goose RBC suspended in PBS at concentrations over a range from \(2.5 \times 10^6\) to \(3.8 \times 10^7\) cells per ml. The reaction mixtures were sampled at defined times, the RBC and aggregates centrifuged down and the cell-free supernate tested, as previously described (Page 51), for residual haemagglutinating activity. This residual activity was expressed as a percentage of the initial haemagglutinating activity and is presented against the adsorption or reaction time in Figure 54. The data show that the greater the concentration of red cells the greater the rate of haemagglutinin adsorption. The reaction constant \(K_f\) for this reaction is calculated later (Page 194).

An additional experiment was carried out in which a range of different dilutions of haemagglutinin (1/10 to 1/160) were each reacted with a constant concentration of goose RBC (\(\sim 10^7\) cells per ml.) in the standard borate-BSA-PBS buffer system at pH 6.3. As before, samples were removed from the reaction mixtures after defined intervals of time and the agglutinated cells removed by centrifugation. The cell-free supernate was then adjusted to pH 8.0 with borate-BSA buffer, pH 9.0, to eliminate pH inactivation and the residual haemagglutinating activity estimated in the standard four-volume pattern test at pH 6.3. Again, expressing this residual activity as the percentage haemagglutinating activity remaining after adsorption (Figure 55), the data correspond closely with those in Figure 54 for a red cell concentration of \(\sim 10^7\) cells per ml. and further confirm that, under these experimental conditions, the proportion of haemagglutinin adsorbed is dependent on the overall red cell concentration.

When the data for the pH inactivation of haemagglutinin and for its adsorption to red cells at pH 6.3 are presented together (Figure 56), it appears that, at this red cell concentration of about \(10^7\) cells per ml., adsorption occurs more rapidly
The Sedimentation-Enumeration Method: The Adsorption of Haemagglutinin (SFV1/H) over a Range of Goose Red Cell Concentrations.
FIGURE 55. The Sedimentation-Enumeration Method: The Adsorption of Haemagglutinin (SPV(1)/F) by Goose Red Cells from a Series of Different Concentrations of Haemagglutinin.

RESIDUAL HAEMAGGLUTINATING ACTIVITY AS % OF INITIAL ACTIVITY

[RBC] = 10^7 CELLS PER ML

HAEMAGGLUTININ DILUTION
- 1/10
- 1/20
- 1/40
- 1/80
- 1/160

REACTION TIME (MIN)
The Sedimentation-Enumeration Method: A comparison of the inactivation of haemagglutinin (SF13/2) by pH and the adsorption of haemagglutinin to goose red cells (10⁷ cells per ml) at pH 6.3.

**FIGURE 56.**

RESIDUAL HAEMAGGLUTINATING ACTIVITY AS % OF INITIAL ACTIVITY

A - after exposure to pH 6.3 for indicated time before reaction with RBC in the standard pattern test at pH 6.3.

B - after adsorption to RBC at pH 6.3 for indicated time and removal of cell-free supernate for further reaction with RBC in the standard pattern test at pH 6.3.
than inactivation by pH at reaction times up to about 15 min. After this time, the slopes of the two graphs are very similar.

It is concluded that the adsorption of haemagglutinin to red cells and the inactivation of haemagglutinin at low values of pH are competitive reactions, and that their dynamic balance controls the overall haemagglutination reaction.

3. The influence of reaction sequence in the pattern test

This was mentioned previously (Page 68) and is considered here because it involves the two competitive reactions of inactivation of haemagglutinin and of adsorption of haemagglutinin by red cells. Since these two reactions occur simultaneously, the sequence of the addition of reactants and buffers in the operation of the pattern test is important if haemagglutinating activity is to be detected with maximal sensitivity.

In the experiment shown in Table 15, haemagglutinin derived from Semliki Forest virus (SFV(M)/F) and goose RBC were diluted in either borate-BSA buffer at pH 9.0, or PBS at pH ~ 6.1 and then immediately mixed, together with the appropriate compensating buffer to give a final reaction mixture at pH 6.3. The data in Table 15 show clearly that the maximal haemagglutinating activity is obtained only when the haemagglutinin enters the test after dilution in borate-BSA buffer at pH 9.0. Thus, even for the shortest period at low pH, many potentially haemagglutinating particles may be inactivated by pH before RBC-RBC bonds can be formed.
TABLE 15. A Summary of the Influence of Reaction Sequence on the Haemagglutination of Goose Red Cells by the Haemagglutinin of Semliki Forest Virus (SFV(H)/F) in the Borate-BSA-PBS Buffer System at pH 6.3

<table>
<thead>
<tr>
<th>Reactants in Order of Rapid Mixing</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Resultant Haemagglutinating Activity (HAU per ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemagglutinin in Borate Buffer at pH 9.0 (BB)</td>
<td>Red Cells in PBS</td>
<td>BB</td>
<td>PBS</td>
<td>910</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PBS</td>
<td>Red Cells in PBS</td>
<td>BB</td>
<td>910</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Red Cells in BB</td>
<td>PBS</td>
<td>PBS</td>
<td>910</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Red Cells in BB</td>
<td>BB</td>
<td>PBS</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>Haemagglutinin in Phosphate Buffered Saline at pH ~6.1 (PBS)</td>
<td>BB</td>
<td>Red Cells in BB</td>
<td>PBS</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Red Cells in PBS</td>
<td>BB</td>
<td>BB</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BB</td>
<td>BB</td>
<td>Red Cells in PBS</td>
<td>&lt;10</td>
<td></td>
</tr>
</tbody>
</table>
(b) The quantitation of haemagglutination in terms of the competitive reactions between pH inactivation and RBC adsorption of haemagglutinin.

It was shown above that the inactivation of haemagglutinin by pH competes with its adsorption to red cells. Thus if \([H]_0\) is the initial particle concentration of haemagglutinin and a fraction \(\alpha\) of these are inactivated by pH before successful union with a red cell can occur, then only the fraction \((1-\alpha)\) of particles are available to form RBC-RBC bonds.

Thus the equations,

\[
\frac{\text{Particles of haemagglutinin per ml.}}{\text{RBC-RBC bond}} = \frac{\text{Particles of haemagglutinin per ml.}}{\text{Haemagglutinin-specific bonds}} \cdot \text{(RBC per ml.)}
\]

or,

\[
h_m = \frac{[H]_0}{\frac{B}{R} - \frac{B}{R}_0} \cdot \frac{[R]}{D}
\]

which were discussed on Page 176 must be modified to,

\[
[H]_0 = h_m D \cdot [R] \cdot \frac{\frac{B}{R} - \frac{B}{R}_0}{(1-\alpha)}
\]

which express haemagglutination in terms of those particles which escape pH inactivation and are successfully adsorbed to red cells to contribute to an RBC-RBC bond.

If the data concerning the influence of the pH of the reaction mixture on haemagglutination in the pattern test, (Figure 5, Page 64) and the percentage haemagglutinating activity inactivated after treatment at individual pH values for 15 min. and detected in the standard pattern test at pH 6.3 (Figure 56, Page 88) are presented together, then Figure 57 is obtained. The optimum of pH for the detection of haemagglutinating activity is at that pH at which \((1-\alpha)\) is maximal (nearest to unity) and the increasing inactivation of haemagglutinin with falling...

![Graph showing observed haemagglutinating activity as % of maximum activity vs. pH of reaction mixture.]

- **A** - after treatment at individual pH values for 15 min. followed by standard pattern test at pH 6.3.
- **B** - by standard pattern test.
pH is compensated by an increase in the efficiency of adsorption and bond formation.

1. The estimation of \( \alpha \)

Consider a suspension containing \([H]\)_o particles of haemagglutinin per ml and \([R]\) red cells per ml. Then the rate of combination of particles of haemagglutinin with red cells and their conversion to a state in which they are no longer susceptible to pH inactivation is given by,

\[
- \frac{d[H]}{dt} = K_1[H][R]
\]

or

\[
[H]_t = [H]_o e^{-K_1[R]t}
\]

where \( K_1 \) is an adsorption rate constant independent of red cell concentration defined by the environmental conditions of pH, medium and temperature.

Similarly the rate of pH inactivation is given by,

\[
- \frac{d[H]}{dt} = k_1[H]
\]

or

\[
[H]_t = [H]_o e^{-k_1t}.
\]

where \( k_1 \) is an inactivation rate constant independent of red cell concentration and defined again by the environmental conditions and in particular by the pH of the reaction mixture. The overall rate of loss of free and potentially active particles of haemagglutinin, by the combination of adsorption and inactivation, is,

\[
- \frac{d[H]}{dt} = K_1[H][R] + k_1[H]
\]

or

\[
[H]_t = [H]_o e^{-(K_1[R] + k_1)t}.
\]

Thus, the total number of particles of haemagglutinin which adsorb to red cells before pH inactivation and thereby escape inactivation by pH is given by,
\[
[H]_{\text{ads}} = \frac{K_1[R][H]_o}{K_1[R] + k_1} \left(1 - e^{-(K_1[R] + k_1)t}\right) \quad (39)
\]

at the terminal state of the equilibrated reaction when \( t = \infty \) and this becomes,

\[
[H]_{\text{ads}} = \frac{K_1[R][H]_o}{K_1[R] + k_1} \quad (40)
\]

Therefore, by the definition, of \( \alpha \) as the fraction of particles lost by pH inactivation before adsorption,

\[
(1 - \alpha)[H]_o = [H]_{\text{ads}} = \frac{K_1[R][H]_o}{K_1[R] + k_1} \quad (41)
\]

or,

\[
\alpha = \frac{k_1}{K_1[R] + k_1} \quad (42)
\]

Thus, equation \((35)\) becomes,

\[
[H]_o = \frac{k_1}{k_1}[R] \left(\frac{R}{[H]_o} - \frac{[R]}{[H]_o} + k_1\right) \quad (43)
\]

where \( k_1 \) is the rate constant for pH inactivation of haemagglutinin at pH 6.3 and \( K_1 \) is the rate constant for the adsorption of haemagglutinin to red cells under these same environmental conditions.

2. The evaluation of the reaction constants \( k_1 \) and \( K_1 \)

By equation \((37)\), \( \log_{10} \frac{[H]}{[H]_o} t = k_1 t \) for pH inactivation alone and by equation \((36)\), \( \log_{10} \frac{[H]}{[H]_o} t = \frac{K_1[R] + k_1}{2.303}t \)

for the total reaction in the presence of red cells. Here \([H]_t\) is the concentration of still active but unadsorbed particles of haemagglutinin after reaction time \( t \).

The data in Figure 56 (Page 188) suggest that at 15 min, the adsorption of
haemagglutinins to RBC is complete and that the remaining haemagglutinin is then
inactivated by pH to give a decay curve parallel to that for the pH inactivation
control series. Thus the corresponding data from Figure 53 (Page 184) and
Figure 54 (Page 186) for the pH inactivation and RBC adsorption reactions at pH
6.3 for reaction times up to 15 min. may be expressed as the log depressions of
haemagglutinating activity, \( \log_{10} \left[ \frac{[R]}{[R]_0} \right] \), and are given in Table 16. If
these values are presented against the reaction time, \( t \), in sec. as in Figure 58 ,
the corresponding slopes obtained enable the evaluation of the reaction constants
\( k_1 \) and \( K_1 \) for the haemagglutination reaction between goose RBC and SFV haemagglutinin
at the optimum pH 6.3.

Thus, \( k_1 = 0.000197 \times 2.303 = 4.54 \times 10^{-4} \) reciprocal sec.
and \( k_1 [R] + k_1 = 0.000423 \times 2.303 = 9.76 \times 10^{-4} \) reciprocal sec.
but for these data, \([R] = 1.09 \times 10^7 \) cells per ml., therefore,
\[
K_1 = \frac{1.09 \times 10^7}{2.76 \times 10^{-4} - 4.54 \times 10^{-4}} \text{ ml. per cell per sec.}
= 4.79 \times 10^{-11} \text{ ml. per cell per sec.;}
\]
and,
\[
\frac{k_1}{K_1} = \frac{4.54 \times 10^{-4}}{4.79 \times 10^{-11}} = 9.47 \times 10^6 \text{ cells per ml. as required for equation (43)}.
\]

An alternative evaluation of these reaction constants may be derived from the
data for \( \log_{10} \left[ \frac{[R]}{[R]_0} \right] \) in Table 16 for a range of red cell concentrations, \([R] \) from
0 (the control curve for pH inactivation) to \( 3.8 \times 10^7 \) cells per ml. for a constant
reaction time, \( t = 900 \) sec.). Since the fraction of adsorbed haemagglutinin
(equation (42)) is proportional to \( K_1 [R] + k_1 \), it follows that in Figure 59 ,
the intercept on the ordinate is equal to the reaction constant \( k_1 \) and that the
remaining haemagglutinin is proportional to \( K_1 \) at any particular value of \([R] \).
The Log Depressions of Haemagglutinating Activity, $\log_{10} \left[ \frac{[H]}{[H]_0} \right]$, for the pH Inactivation of SFV Haemagglutinin and its Adsorption to Goose Red Cells at pH 6.3

<table>
<thead>
<tr>
<th>Red Cell Concentration $[R]$, (cells per ml.)</th>
<th>$0$</th>
<th>$2.5 \times 10^6$</th>
<th>$4.2 \times 10^6$</th>
<th>$1.09 \times 10^7$</th>
<th>$2.14 \times 10^7$</th>
<th>$3.8 \times 10^7$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction</td>
<td>$0$</td>
<td>$150$</td>
<td>$300$</td>
<td>$600$</td>
<td>$900$</td>
<td></td>
</tr>
<tr>
<td>Time, $t$, (sec)</td>
<td>$0.00$</td>
<td>$-0.08$</td>
<td>$-0.20$</td>
<td>$-0.26$</td>
<td>$-0.17$</td>
<td></td>
</tr>
<tr>
<td>Log Depression of Haemagglutinating Activity, $\log_{10} \left[ \frac{[H]}{[H]_0} \right]$</td>
<td>$-0.14$</td>
<td>$0.27$</td>
<td>$0.40$</td>
<td>$0.60$</td>
<td>$1.05$</td>
<td></td>
</tr>
</tbody>
</table>
FIGURE 58. The Sedimentation- Enumeration Method: The Evaluation of the Reaction Constants $k_1$ for the pH Inactivation of SFV Haemagglutinin and $k_1$ for the Adsorption of SFV Haemagglutinin to Goose RBC at pH 6.3.

LOG DEPRESSION OF HAEMAGGLUTINATING ACTIVITY AS $\log_{10} \left( \frac{[H]_t}{[H]_0} \right)$

$[RBC] = 1.09 \times 10^7$ CELLS PER ML.

- Adsorption of haemagglutinin to red cells, $\frac{k_1 [R]}{2.303}$
- pH inactivation of haemagglutinin, $\frac{k_1}{2.303}$
FIGURE 59. The Sedimentation-Enumeration Method: An Alternative Evaluation of the Reaction Constants $k_1$ for the pH Inactivation of SFV Haemagglutinin and $k_1$ for the Adsorption of SFV Haemagglutinin to Goose RBC at pH 6.3 after a Reaction Time of 15 min.

\[
\text{LOG DEPRESSION OF HAEMAGGLUTINATING ACTIVITY AS } \log_{10} \frac{[H]_0}{[H]_\infty}.
\]

\[
\text{REACTION TIME = 15 min. (900 sec.)}
\]

\[
slope = \frac{k_1 \cdot 900}{2.303}
\]

\[
\text{intercept} = \frac{k_1 \cdot 900}{2.303}
\]

\[
\text{OVERALL RED CELL CONCENTRATION } [R] \text{ (CELLS PER ML)}
\]
Thus, \( k_1 = \frac{0.1335 \times 2.303}{900} = 3.43 \times 10^{-4} \) reciprocal sec.

and \( K_1 = \frac{0.0236 \times 10^{-6} \times 2.303}{900} = 6.06 \times 10^{-11} \) ml. per cell per sec.

and therefore the ratio \( \frac{k_1}{K_1} \) is given by,

\[
\frac{k_1}{K_1} = \frac{3.43 \times 10^{-4}}{6.06 \times 10^{-11}} = 5.65 \times 10^6 \text{ cells per ml.}
\]

These values for \( k_1, K_1 \) and \( \frac{k_1}{K_1} \) are summarised below:

<table>
<thead>
<tr>
<th>Reaction Constant (units)</th>
<th>Constant ([R]) Variable (t)</th>
<th>Constant ([R]) Variable (t)</th>
<th>Mean Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_1 ) (reciprocal sec.)</td>
<td>( 4.54 \times 10^{-4} )</td>
<td>( 3.43 \times 10^{-4} )</td>
<td>( 4.0 \times 10^{-4} )</td>
</tr>
<tr>
<td>( K_1 ) (ml. per cell per sec.)</td>
<td>( 4.79 \times 10^{-11} )</td>
<td>( 6.06 \times 10^{-11} )</td>
<td>( 5.4 \times 10^{-11} )</td>
</tr>
<tr>
<td>( \frac{k_1}{K_1} ) (cells per ml.)</td>
<td>( 9.47 \times 10^6 )</td>
<td>( 5.65 \times 10^6 )</td>
<td>( 7.6 \times 10^6 )</td>
</tr>
</tbody>
</table>

The precision of the test is reflected in an uncertainty of about \( \pm 12\% \) in the estimates of the values of the constant \( k_1 \) or \( K_1 \) derived from data (Table 16) at constant time (900 sec.) or at constant red cell concentration (\( 10^7 \) per ml.).
An estimate for $\alpha$, the fraction of particles of haemagglutinin inactivated by pH may be now obtained from equation (42),

$$\alpha = \frac{k_1}{K_1[R] + k_1} = \frac{4.0 \times 10^{-4}}{(5.4 \times 10^{-11} \times 1.09 \times 10^7 + 4.0 \times 10^{-4})} = 0.40.$$

3. The quantitation of particles of haemagglutinin by equation (43)

By the substitution of the mean value for the constant ratio $\frac{k_1}{K_1}$ ($= 7.6 \times 10^6$) from Page 199 into equation (43) (Page 194), it is now possible to obtain an estimate for the initial concentration of haemagglutinin, $[H]_0$.

Assuming $[H]_0$ to be constant for a particular preparation of haemagglutinin then, if $h_m$ is taken to be a constant small integer in the region of low haemagglutinin concentrations, the ratio of these two unknowns is a constant. Therefore equation (43) for a reaction mixture at pH 6.3 becomes,

$$\frac{[H]_0}{h_m} = \text{b} \cdot \left(\frac{[R]}{K_1} + \frac{k_1}{K_1}\right)$$

where $b = \left(\frac{3}{R} - \frac{[H]}{R} \cdot \frac{[H]_0}{h_m}\right)$. Thus, when the values of b from Figures 48, 49, and 52 (Table 17) are presented against the corresponding values for $\frac{1}{D \cdot \left(\frac{[R]}{K_1} + \frac{k_1}{K_1}\right)}$, the resultant best-fit slopes should be proportional to the ratio $\frac{[H]_0}{h_m}$ (Figure 60).

Estimates for $[H]_0$ and $h_m$ may be obtained in the following way. The initial infectivity (I) of this preparation of Semiliki Forest virus was $1.02 \times 10^9$ p.f.u. per ml. before extraction with fluorocarbon. Many observations in this laboratory (D.H.J. Titmuss, personal communication) have shown that the number (N) of characteristic physical particles is about ten times greater than the number of infective units. Since there is quantitative recovery of haemagglutinating activity after
Table 17. The Estimation of the Number of Particles of Haemagglutinin per RBC-RBC Bond, \( h_m \), in the Region of Low Haemagglutinin Concentrations

<table>
<thead>
<tr>
<th>Red Cell Concentration ([R])</th>
<th>([R] + \frac{k_1}{K_1})</th>
<th>Dilution Denominator of Haemagglutinin, ( b = \frac{b}{[R]} - \left(\frac{b}{[R]}\right)_0)</th>
<th>Extent of Agglutination</th>
<th>( \frac{1}{b \left(\frac{[R]}{[R]} + \frac{k_1}{K_1}\right)} )</th>
<th>Slope, ** ( \frac{[H]_0}{h_m} )</th>
<th>Particles of Haemagglutinin per RBC-RBC Bond, ( h_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>(10.9 \times 10^6)</td>
<td>(18.5 \times 10^6)</td>
<td>5120</td>
<td>0.01</td>
<td>(9.47 \times 10^{10})</td>
<td>(0.106 \times 10^{10})</td>
<td>(5(5.26))</td>
</tr>
<tr>
<td>5120</td>
<td>0.00</td>
<td>9.47 \times 10^{10}</td>
<td>0.106 \times 10^{10}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5120</td>
<td>0.00</td>
<td>9.47 \times 10^{10}</td>
<td>0.106 \times 10^{10}</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>5120</td>
<td>0.00</td>
<td>9.47 \times 10^{10}</td>
<td>0.106 \times 10^{10}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5120</td>
<td>0.03</td>
<td>9.47 \times 10^{10}</td>
<td>0.106 \times 10^{10}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5120</td>
<td>0.04</td>
<td>9.47 \times 10^{10}</td>
<td>0.106 \times 10^{10}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>0.13</td>
<td>1.48 \times 10^{10}</td>
<td>0.676 \times 10^{10}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>0.14</td>
<td>1.48 \times 10^{10}</td>
<td>0.676 \times 10^{10}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>0.14</td>
<td>1.48 \times 10^{10}</td>
<td>0.676 \times 10^{10}</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>0.11</td>
<td>1.48 \times 10^{10}</td>
<td>0.676 \times 10^{10}</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Ratio of reaction constant \( k \) for pH inactivation and \( K_1 \) for the adsorption of haemagglutinin to red cells = \(7.6 \times 10^6\) cells per ml. (Page 199)

**Estimated initial number of particles of haemagglutinin, \([H]_0 = 1.02 \times 10^{10}\) per ml.
<table>
<thead>
<tr>
<th>[R]</th>
<th>[R] + ( \frac{k_1}{K_1} )</th>
<th>D</th>
<th>( b = \frac{R}{R} - \left( \frac{R}{R} \right)_0 )</th>
<th>( \frac{1}{D([R] + \frac{k_1}{K_1})} )</th>
<th>**[H]_0 ( h_m )</th>
<th>Particles of Haemagglutinin per RBC-RBC Bond, ( h_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0 x 10^6</td>
<td>17.6 x 10^6</td>
<td>5000</td>
<td>0.0054</td>
<td>8.80 x 10^10</td>
<td>0.114 x 10^10</td>
<td>7(7.18)</td>
</tr>
<tr>
<td>10.0 x 10^6</td>
<td>17.6 x 10^6</td>
<td>4000</td>
<td>0.00</td>
<td>7.04 x 10^10</td>
<td>0.142 x 10^10</td>
<td></td>
</tr>
<tr>
<td>10.0 x 10^6</td>
<td>17.6 x 10^6</td>
<td>3160</td>
<td>0.03</td>
<td>5.55 x 10^10</td>
<td>0.180 x 10^10</td>
<td></td>
</tr>
<tr>
<td>10.0 x 10^6</td>
<td>17.6 x 10^6</td>
<td>2560</td>
<td>0.05</td>
<td>4.50 x 10^10</td>
<td>0.220 x 10^10</td>
<td></td>
</tr>
<tr>
<td>10.0 x 10^6</td>
<td>17.6 x 10^6</td>
<td>2000</td>
<td>0.03</td>
<td>3.52 x 10^10</td>
<td>0.280 x 10^10</td>
<td></td>
</tr>
<tr>
<td>10.0 x 10^6</td>
<td>17.6 x 10^6</td>
<td>1600</td>
<td>0.08</td>
<td>2.82 x 10^10</td>
<td>0.355 x 10^10</td>
<td>1.42 x 10^3</td>
</tr>
<tr>
<td>10.0 x 10^6</td>
<td>17.6 x 10^6</td>
<td>1400</td>
<td>0.08</td>
<td>2.46 x 10^10</td>
<td>0.406 x 10^10</td>
<td>7-8 (7.59)</td>
</tr>
<tr>
<td>10.0 x 10^6</td>
<td>17.6 x 10^6</td>
<td>1280</td>
<td>0.07</td>
<td>2.25 x 10^10</td>
<td>0.440 x 10^10</td>
<td></td>
</tr>
<tr>
<td>10.0 x 10^6</td>
<td>17.6 x 10^6</td>
<td>1100</td>
<td>0.04</td>
<td>1.93 x 10^10</td>
<td>0.520 x 10^10</td>
<td></td>
</tr>
<tr>
<td>10.0 x 10^6</td>
<td>17.6 x 10^6</td>
<td>1000</td>
<td>0.10</td>
<td>1.76 x 10^10</td>
<td>0.568 x 10^10</td>
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</tr>
<tr>
<td>10.0 x 10^6</td>
<td>17.6 x 10^6</td>
<td>640</td>
<td>0.11</td>
<td>1.13 x 10^10</td>
<td>0.835 x 10^10</td>
<td></td>
</tr>
<tr>
<td>9.3 x 10^6</td>
<td>16.9 x 10^6</td>
<td>2560</td>
<td>0.030</td>
<td>4.33 x 10^10</td>
<td>0.231 x 10^10</td>
<td>1.34 x 10^3</td>
</tr>
<tr>
<td>9.3 x 10^6</td>
<td>16.9 x 10^6</td>
<td>640</td>
<td>0.125</td>
<td>1.08 x 10^10</td>
<td>0.326 x 10^10</td>
<td>7-8 (7.59)</td>
</tr>
<tr>
<td>6.1 x 10^6</td>
<td>13.7 x 10^6</td>
<td>2560</td>
<td>0.010</td>
<td>3.51 x 10^10</td>
<td>0.285 x 10^10</td>
<td>1.13 x 10^3</td>
</tr>
<tr>
<td>6.1 x 10^6</td>
<td>13.7 x 10^6</td>
<td>640</td>
<td>0.135</td>
<td>0.88 x 10^10</td>
<td>1.14 x 10^10</td>
<td>9(8.96)</td>
</tr>
<tr>
<td>2.7 x 10^6</td>
<td>10.3 x 10^6</td>
<td>2560</td>
<td>0.025</td>
<td>2.64 x 10^10</td>
<td>0.38 x 10^10</td>
<td>7.54 x 10^3</td>
</tr>
<tr>
<td>2.7 x 10^6</td>
<td>10.3 x 10^6</td>
<td>640</td>
<td>0.115</td>
<td>0.66 x 10^10</td>
<td>1.51 x 10^10</td>
<td>13(13.52)</td>
</tr>
</tbody>
</table>
FIGURE 60. The Sedimentation-Enumeration Method: The Estimation of the Number of Particles of Haemagglutinin per RBC-RBC Bond. $h_m$, in the Region of Low Haemagglutinin Concentrations.

EXTENT OF AGGLUTINATION AS BONDS PER RED CELL, $b = \left( \frac{B}{R} - \frac{B}{R_0} \right)$

- [RBC] per ml.
  - $2.7 \times 10^6$: 13
  - $6.1 \times 10^6$: 9
  - $9.3 \times 10^6$: 7-8
  - $10.0 \times 10^6$: 7
  - $11.0 \times 10^6$: 5

$\frac{1}{D(\left[R]\right) + 7.6 \times 10^6}$
extraction and also little significant disruption of physical particles, then it is likely that the number of initial physical particles is closely equivalent to the concentration of particles of haemagglutinin. The integrity of physical particles in the haemagglutinin preparation was confirmed by gel filtration and by equilibrium density gradient centrifugation (Page 55). The initial infectivity of $1.02 \times 10^9$ p.f.u. per ml. may then be regarded as equivalent to $1.02 \times 10^{10}$ physical or haemagglutinin particles per ml. However, for the haemagglutination reaction in the region of low haemagglutinin concentrations and at the optimum red cell concentration ($\sim 10^7$ cells per ml.) we have found that,

$$\frac{[H]_0}{n} = 1.42 \times 10^9 \text{ bonds (Table 17)},$$

and putting $[H]_0 = N = 1.02 \times 10^{10}$ particles per ml. we obtain,

$$h_m = 1.02 \times 10^{10} \frac{1.42 \times 10^9}{1.02 \times 10^{10}} = 7.18 \text{ particles per bond}.$$

Thus, in this region of limiting haemagglutinin concentration, it is probable that about 7 particles of haemagglutinin are initially present for every RBC-RBC bond subsequently formed.

The variation in the estimation of $h_m$ according to the values of the ratios, $n$,

$n = \text{concentration of physical particles per ml. in initial sample.}$

$\text{concentration of infective units per ml. in initial sample.}$

and

$r = \text{concentration of particles of haemagglutinin per ml. in extracted sample.}$

$\text{concentration of physical particles per ml. in initial sample.}$

are shown below where,

$h_m = \text{concentration of particles of haemagglutinin per ml. in initial sample.}$

$\text{concentration of RBC-RBC bonds formed in test reaction.}$
The value \( h_m \) near 7 remains as the likely central estimate since the values of \( n \) and \( r \) marked by an asterisk are not consistent with the experimental estimates of \( n \sim 10 \), and \( r \sim 0.5 \) to 1.0.

Thus, using these data, we may propose a scheme (Figure 61) for the distribution of particles of haemagglutinin at each stage of the haemagglutination reaction from the initial infective virus preparation to the formation of haemagglutinin-specific RBC-RBC bonds under experimental conditions of low haemagglutinin concentration and optimum red cell concentration. The sedimentation-enumeration method estimates the concentration of bonds rather than of potentially haemagglutinating particles and therefore does not include the numbers of particles unavailable for the formation of bonds due to aggregation or to adsorption to the walls of the reaction vessel. Also, it is possible that not all of the available particles will be directly involved in the formation of an RBC-RBC bond since some may be wastefully adsorbed to the red cell surface and others sterically hindered. Thus the value \( h_m \approx 7 \) is an overall estimate of the number of particles of haemagglutinin required to form one bond but not of the number directly involved in each RBC-RBC bond.
**Figure 61.** A Scheme for the Distribution of Particles of Haemagglutinin (SFV(M)/F) in the Haemagglutination Reaction

**Initial Infective Virus Preparation (SFV(M))**

Number of physical particles, \( N \approx 1.02 \times 10^{10} \) per ml.

Number of infective particles, \( I = 1.02 \times 10^9 \) per ml.

\[
N = I \times n.
\]

Extraction of Inhibitors with Fluorocarbon

\[\rightarrow\] Haemagglutinin (SFV(M)/F).

Particles of Haemagglutinin recovered after extraction

\[
r = \left[ \frac{H}{N} \right] = 1, \text{ for quantitative recovery.}
\]

Initial number of particles of haemagglutinin for the haemagglutination test, \( [H]_0 = I \times n \times r = 1.02 \times 10^{10} \) per ml.

**Haemagglutination Test**

\( \alpha \), fraction of particles inactivated by pH = 0.4

\[
\alpha = [H]_0 \times \alpha = 0.4 \times 1.02 \times 10^{10} = 4.08 \times 10^9 \text{ per ml.}
\]

\( 1 - \alpha \), Number of particles available for the formation of RBC-RBC bonds

\[
[H]_0 \times (1 - \alpha) = 1.02 \times 10^{10} \times 0.6 = 6.12 \times 10^9 \text{ per ml.}
\]

One of the most fundamental observations of the sedimentation-enuculation method is that a population distribution of aggregates is formed in haemagglutination reaction mixtures containing red cells and low concentrations of viral haemagglutinin. This differs from the implications of work published by Levine, et al. (1953) and Cheng (1961a) and others (Page 39) who interpret their data in terms of the 'dimers-only' hypothesis (Page 88). In the present studies, wide distributions of aggregates greater than the dimer have been consistently observed and an attempt has been made to analyse such data fully in order to obtain directly a value for the extent of reaction which does not require the manifestly erroneous assumption of 'dimers-only'. It is not unreasonable to consider the red cell-virus interaction as an aggregation which may be treated according to the statistical-mechanical theory of Goldberg (1952, 1953) which has already been frequently and successfully applied to aggregation processes in antigen-antibody or virus-antibody interactions.

The Goldberg theory will be considered in the simplified form presented by Bradish and Crawford (1960). If the aggregation/agglutination processes in the antigen-antibody and RBC-haemagglutinin systems are analogous and if RBC are considered to be in excess with haemagglutinin as the limiting reactant, then the fractional concentrations of red cells which appear free or as dimers, trimers, tetraters and n-mers, etc. may be defined as $F_1, F_2, F_3, F_4$ and $F_n$, so that,

$$1 = F_1 + F_2 + F_3 + F_4 + \ldots + F_n$$

(45)

and,

$$\frac{1}{R^m} \left[ \frac{H}{R} \right] = \frac{B}{R} = 1 - F_1 - \frac{F_2}{2} - \frac{F_3}{3} - \frac{F_4}{4} - \ldots - F_n$$

(46)
For a given red cell concentration, this ratio is proportional to the concentration of haemagglutinin in the reaction mixture if the number of particles of haemagglutinin per bond \( h_m \) is constant.

The valency of the red cell for combination with haemagglutinin, \( f \), may be estimated (Bradish and Crawford, 1960, equations (9) and (10)) from the observed distributions of aggregates by the use of the equations,

\[
\frac{F_2^2}{F_1^2 F_3^2} = \frac{2f}{3(f-1)} \quad \text{or,} \quad f = \frac{3F_2^2}{3F_2^2 - 2F_1^2 F_3^2} \quad (47)
\]

\[
\frac{F_2 F_3}{F_1 F_4} = \frac{9}{4} \cdot \frac{f}{4f-5} \quad \text{or,} \quad f = \frac{20F_2 F_3}{16F_2 F_3 - 9F_1 F_4} \quad (48)
\]

These definitions of the valency of the excess reactant, in this case the red cell, are a direct consequence of Goldberg's statistical-mechanical theory of aggregation.

Since RBC are in excess, we may also write, the extent of reaction,

\[
P = \frac{2}{f} \cdot \frac{B}{R} = \frac{2}{f} \cdot \frac{1}{h_m} \cdot \frac{[H]}{[R]} \quad (49)
\]

The key parameters for each agglutination mixture, \( \frac{B}{R} \), \( f \) and \( p \) may thus be estimated from the observed agglutination distribution data. If \( f \) is estimated for different aggregate distributions over a range of values of \( \frac{B}{R} \), a value between 2 and 4 is found (Figure 62).

If \( f \), the effective valency of the red cell, is taken to be 2 then,

\[
P = \frac{B}{R} = \frac{1}{h_m} \cdot \frac{[H]}{[R]} \quad (50)
\]

and the extent of reaction, \( p \), is equal to the extent of agglutination, \( \frac{B}{R} \).
FIGURE 62. The Sedimentation-Enumeration Method: The Estimation of the Effective Valency, \( f \), of the Red Cell in the Haemagglutination Reaction

\[
\begin{align*}
\text{EFFECTIVE VALENCY OF RED CELL, } f.

f &= \frac{3F_2^2}{3F_2^2 - 2F_1F_3} \\
\text{or } f &= \frac{20F_2F_3}{16F_2F_3 - 9F_1F_4}
\end{align*}
\]
The Goldberg distribution then reduces to,

\[ F_n = n \cdot p^{n-1} \cdot (1-p)^2 \]  

(51)

to express the distribution resulting from the interaction between divalent red cells in excess and divalent particles of haemagglutinin as the limiting reactant.

The fractional concentration of n-fold aggregates of red cells then becomes,

\[ A_n = \frac{F_n}{n} \cdot R = p^{n-1} \cdot (1-p)^2 \cdot R \]  

(52)

or,

\[ \log A_n = (n-1) \log p + \log A_1 \]  

(53)

since the concentration of single red cells is given by,

\[ A_1 = (1-p)^2 \cdot R \]  

(54)

If values for \( \log A_n \) are presented against values for \( (n-1) \), then the resultant plot should be linear with a slope equal to \( \log p \), or \( \log \frac{R}{R_0} \), and the intercept on the ordinate at \( \log A_1 \), (Figure 63). The scatter in the points of the lines at \( (n-1) \) values above 6 is due to the smaller number of aggregates counted and the uncertainty in the identification of \( n \) for larger aggregates. These data nevertheless justify the application of the Goldberg analysis to the red cell-viral haemagglutinin system under the present experimental conditions.

Thus, the distribution of aggregates following haemagglutination provides two distinct estimates of the extent of agglutination \( p = \frac{R}{R_0} \):

\[ (i) \quad \frac{R}{R_0} = 1 - F_1 - \frac{F_2}{2} - \frac{F_3}{3} - \ldots \]  

(46)

and derived forms which have been used as the basis of the sedimentation-enumeration method and do not require knowledge of the form of the distribution \( F_1', F_2', F_3', F_n' \).
The Sedimentation- Enumeration Method: Confirmation of \( \frac{B}{R} \) Values by Analysis of the Goldberg Distribution

Equation (53): \( \log A_n = (n - 1) \log \frac{B}{R} + \log A_1 \)

Mean Values of \( \frac{B}{R} \) for Groups of Data:

- \( a = 0.19 \)
- \( b = 0.26 \)
- \( c = 0.32 \)
- \( d = 0.41 \)
- \( e = 0.49 \)

as calculated by equation (46) and presented in Table 18.
(ii) \[ \log \frac{B}{R} = \log A_n - \log A_1 \quad \text{or} \quad \log \frac{nF_n}{R} - \log F_1 \] (53)

which yields an estimate for \( \frac{B}{R} \) for each term \( A_n \) or \( F_n \) in the distribution for which the number of aggregates is significant. The distribution of spontaneously formed aggregates of RBC in the control mixture without haemagglutinin indicates the zero or base-line correction \( \left( \frac{B}{R} \right) \) which must be subtracted from the corresponding values of \( \frac{B}{R} \) estimated in the presence of haemagglutinin.

Table 18 shows the comparison of data calculated by these two methods and serves to provide a further confirmation of the validity of the proposed distribution.

**TABLE 18. The Sedimentation-Enumeration Method: A Comparison of Estimates of the Extent of Agglutination, \( \frac{B}{R} \), Derived from the Distribution of Aggregates**

<table>
<thead>
<tr>
<th>Groups of Data</th>
<th>Equation (46)</th>
<th>Equation (53)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \frac{B}{R} = 1 - F_1 - \frac{F_2}{2} - \frac{F_3}{3} - \cdots - \frac{F_{n}}{n} )</td>
<td>( \log A_n = (n-1) \log \frac{B}{R} + \log A_1 ) (used to calculate ( \frac{B}{R} ) from distribution data of Figure 63)</td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>0.19</td>
<td>0.17</td>
</tr>
<tr>
<td>b</td>
<td>0.26</td>
<td>0.32</td>
</tr>
<tr>
<td>c</td>
<td>0.32</td>
<td>0.38</td>
</tr>
<tr>
<td>d</td>
<td>0.41</td>
<td>0.49</td>
</tr>
<tr>
<td>e</td>
<td>0.49</td>
<td>0.59</td>
</tr>
</tbody>
</table>
(v) **An Analysis of the Distribution of Particles of Haemagglutinin following Multiple Adsorption to Red Blood Cells**

The previous discussion and analysis of the distribution of aggregates in terms of the formation of bonds as the primary reaction (Page 207) was not related to the concentration of particles of haemagglutinin except by the assumption that under conditions of limiting dilution every particle of haemagglutinin gave rise to a single bond. It is therefore necessary to consider the more general relationship between the initial concentration of particles of haemagglutinin, \([H]_0\), their subsequent distribution among red cells and the consequent formation of HBC-RBC bonds.

Consider unit volume (1 ml.) of a reaction mixture containing \(H\) potentially haemagglutinating particles and the much greater concentration of red blood cells, \(R\). If, at equilibrium, all particles of haemagglutinin are randomly adsorbed onto the surfaces of red cells, then the number of red cells \(R_n\) carrying \(n = 0, 1, 2\) etc., particles of haemagglutinin will be defined by the binomial distribution as,

\[
\begin{align*}
R_0 &= R \cdot \left(1 - \frac{1}{R}\right)^H \\
R_1 &= H \cdot \left(1 - \frac{1}{R}\right)^{H-1} \\
R_2 &= \frac{H(H-1)}{2!} \cdot \left(1 - \frac{1}{R}\right)^{H-2} \\
& \vdots \\
R_n &= \frac{H(H-1) \cdots (H-n+1)}{n!} \cdot \left(1 - \frac{1}{R}\right)^{H-n}
\end{align*}
\]

or

\[
R_n = \frac{H(H-1) \cdots (H-n+1)}{n! \cdot (H-n)!} \cdot \left(1 - \frac{1}{R}\right)^{H-n}
\]  \( (55) \)

The number of red cells carrying one or more particles of haemagglutinin will be given by,

\[
R_{1+} = R - R_0 = R \cdot \left(1 - \left(1 - \frac{1}{R}\right)^H\right)
\]  \( (56) \)
If the presence of one or more particles of haemagglutinin on a red cell ensures that that red cell will form one agglutinating RBC-RBC bond, then the number of bonds formed will be given by,

\[ B = vR^* = R \left( 1 - \left(1 - \frac{1}{R}\right)^H \right) \]

or,

\[ B = 1 - \left(1 - \frac{1}{R}\right)^H \]  (57)

Therefore,

\[ 1 - \frac{B}{R} = \left(1 - \frac{1}{R}\right)^H \]  (58)

or, \( \log \left(1 - \frac{B}{R}\right) = H \log \left(1 - \frac{1}{R}\right) \)  (59)

\[ = -0.434 \frac{H}{R} \text{ if } R \gg 1 \]

\[ = -0.434 \frac{[H]}{D \cdot [R]} \]  (60)

where \( D \) is the overall dilution denominator for the initial sample of haemagglutinin containing \([H]_0\) particles per ml.

Thus, \( [H]_0 = -\frac{D \cdot [R]}{0.434} \log \left(1 - \frac{B}{R}\right) \)  (61)

which offers a direct evaluation of \([H]_0\) in terms of the quantities observed in the sedimentation-enumeration method, subject only to the assumptions that particles of haemagglutinin are randomly distributed after total adsorption to red cells and that the presence of one or more particles of haemagglutinin on the red cell surface ensures the formation of a single agglutinating RBC-RBC bond.

This treatment provides a theoretical relationship between the concentration of particles of haemagglutinin and the number of bonds formed between red cells and, as such, is complementary to the previous treatment of the formation and distribution of aggregates in which bond formation was taken as a completed primary reaction.
(a) The evaluation of $[H]_0$ by equation (61)

With haemagglutination by arboviruses, the influence of pH inactivation must be considered (Page 181). The fraction $\alpha$ of particles of haemagglutinin inactivated by pH before adsorption by red cells varies with red cell concentration and is given by equation (42) (Page 194),

$$\alpha = \frac{k_1}{K_1 + k_1}$$

where $k_1$ is an inactivation rate constant and $K_1$ is an adsorption rate constant.

Then, replacing $[H]_0$ by $(1-\alpha)[H]_0$, equation (61) becomes,

$$-\log (1-b) = \left(0.434(1-\alpha)[H]_0\right) \cdot \frac{1}{D}$$

$$= \left(0.434 \frac{[H]_0}{[R] + \frac{k_1}{K_1}}\right) \cdot \frac{1}{D}$$

(62)

where $K_1/k_1 = 7.6 \times 10^6$ cells per ml. (Page 199).

The best-fit values for the product $-D \log (1-b)$ are given in Table 19, for the experimental data from Table 17 (Page 201) used in Figure 60 (Page 203). The corresponding values of $[H]_0$ as estimated by equation (62) are also given. These values are low in comparison with the estimate of $[H]_0 = 1.02 \times 10^{10}$ particles per ml. by electron microscopy (Figure 61, Page 206). This difference, however, may be anticipated since the two estimates can only be identical if every physical particle recognized by electron microscopy is potentially haemagglutinating and is adsorbed by the red cell at sites which are effective and without steric hindrance.
The Estimation of the Initial Concentration of Particles of Haemagglutinin, $[H]_0$, by Equation (62).

<table>
<thead>
<tr>
<th>Red Cell Concentration $[R]$ (cells per ml.)</th>
<th>$[R] + k^*$</th>
<th>$-D \cdot \log (1-b)$</th>
<th>$[H]_0 = -D \cdot \log (1-b) \cdot \frac{[R] + k}{K_k}$</th>
<th>$0.434$ (particles per ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10.9 \times 10^6$</td>
<td>$18.5 \times 10^6$</td>
<td>$48.74$</td>
<td>$2.14 \times 10^9$</td>
<td></td>
</tr>
<tr>
<td>$10.0 \times 10^6$</td>
<td>$17.6 \times 10^6$</td>
<td>$36.67$</td>
<td>$1.54 \times 10^9$</td>
<td></td>
</tr>
<tr>
<td>$9.3 \times 10^6$</td>
<td>$16.9 \times 10^6$</td>
<td>$36.98$</td>
<td>$1.49 \times 10^9$</td>
<td></td>
</tr>
<tr>
<td>$6.1 \times 10^6$</td>
<td>$13.7 \times 10^6$</td>
<td>$38.67$</td>
<td>$1.27 \times 10^9$</td>
<td></td>
</tr>
<tr>
<td>$2.7 \times 10^6$</td>
<td>$10.3 \times 10^6$</td>
<td>$33.69$</td>
<td>$0.82 \times 10^9$</td>
<td></td>
</tr>
</tbody>
</table>

* Ratio of reaction constants $k^*$, for pH inactivation, and $K_k$, for the adsorption of haemagglutinin to red cells, $= 7.6 \times 10^6$ cells per ml. (Page 199).
However, the values of $[H]_o$ in Table 19 were obtained for reaction mixtures in the region of limiting dilution of haemagglutinin in which $b$ is small and usually below 0.1. When larger values of $b$ are encountered for reaction mixtures containing a range of higher concentrations of haemagglutinin with a constant red cell concentration ($10.2 \times 10^6$ cells per ml.), the data may be presented as in Figure 64. Here $-\log (1-b)$ is shown as a function of $\frac{1}{D}$ to give, according to equation (62), an initial linear region of slope proportional to $[H]_o$. Proportionality ceases when $\frac{1}{D}$ exceeds about $2 \times 10^{-3}$ ($D = 500$) corresponding with the haemagglutinin particle to red cell ratio of $\frac{1}{2}$. The range of values for $[H]_o$ obtained from these data is given in Table 20.

The results of Tables 19 and 20 are consistent in that the extent of agglutination is dependent on the ratio of the numbers of particles of haemagglutinin and red cells, $\frac{H}{R}$. The present treatment is acceptable for calculations of $[H]_o$ only if $b \left(= \frac{H}{R}\right)$ is below 0.35 and red cells are not saturated by haemagglutinin.

The value of $[H]_o$ of $2 \times 10^9$ to $4 \times 10^9$ particles per ml. at a red cell concentration of $10^7$ cells per ml. is shown in Tables 19 and 20 for data within the acceptable non-saturation region. This estimate of initial haemagglutinin concentration $[H]_o$ is about a third of the value indicated by electron microscopic counting of characteristic particles.

(b) **The relationship between the extent of agglutination ($b$) and the dilution of haemagglutinin ($D$).**

Using equation (62), it is now possible to compare the theoretical anticipation with the experimental observation for the relationship between the extent of agglutination ($b = \frac{H}{R}$) and the overall dilution denominator of haemagglutinin ($D$). The anticipated theoretical curve is derived directly and only
FIGURE 64. The Sedimentation-Enumeration Method: The Estimation of $[H]_0$
from Equation (62).

$$-\log (1-b) = \left( \frac{0.434 \cdot [H]_0}{[M] + \frac{k_1}{K_1}} \right) \cdot \frac{1}{D} \quad (62)$$

**NB.** The straight lines indicate the calculated slopes for $[H]_0$:

- **I** = $0.1 \times 10^{10}$ particles per ml.
- **II** = $0.5 \times 10^{10}$ particles per ml.
The Estimation of $[H]_0$ by Equation (62) for a Reaction Mixture at Constant Red Cell Concentration $[R]$ ($10.2 \times 10^6$ cells per ml.)

$$[H]_0 = -D \cdot \log (1-b) \cdot \left( [R] + \frac{k_1}{K_1} \right)$$

(62)

where the ratio $\frac{k_1}{K_1} = 7.6 \times 10^6$ cells per ml. (Page 199)

<table>
<thead>
<tr>
<th>Overall Dilution Denominator of Haemagglutinin, $D$</th>
<th>Reciprocal of $D$, $\frac{1}{D}$</th>
<th>Extent of Agglutination $b = \frac{B}{R} - \left( \frac{B}{R} \right)_0$</th>
<th>$- \log (1-b)$</th>
<th>$-D \cdot \log (1-b)$</th>
<th>$[H]_0$ by Equation (62). (particles per ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>0.25000</td>
<td>0.384</td>
<td>0.211</td>
<td>0.644</td>
<td>$3.46 \times 10^7$</td>
</tr>
<tr>
<td>8</td>
<td>0.12500</td>
<td>0.359</td>
<td>0.195</td>
<td>1.560</td>
<td>$6.40 \times 10^7$</td>
</tr>
<tr>
<td>32</td>
<td>0.03120</td>
<td>0.345</td>
<td>0.184</td>
<td>5.89</td>
<td>$2.42 \times 10^8$</td>
</tr>
<tr>
<td>64</td>
<td>0.01560</td>
<td>0.365</td>
<td>0.198</td>
<td>12.7</td>
<td>$5.20 \times 10^8$</td>
</tr>
<tr>
<td>256</td>
<td>0.00390</td>
<td>0.215</td>
<td>0.106</td>
<td>27.2</td>
<td>$1.12 \times 10^9$</td>
</tr>
<tr>
<td>640</td>
<td>0.00160</td>
<td>0.190</td>
<td>0.091</td>
<td>32.0</td>
<td>$1.31 \times 10^9$</td>
</tr>
<tr>
<td>2048</td>
<td>0.00049</td>
<td>0.124</td>
<td>0.058</td>
<td>119.0</td>
<td>$4.88 \times 10^9$</td>
</tr>
<tr>
<td>3160</td>
<td>0.00032</td>
<td>0.090</td>
<td>0.041</td>
<td>129.5</td>
<td>$5.30 \times 10^9$</td>
</tr>
<tr>
<td>4096</td>
<td>0.00024</td>
<td>0.063</td>
<td>0.028</td>
<td>115.0</td>
<td>$4.72 \times 10^9$</td>
</tr>
</tbody>
</table>
from the initial concentrations of haemagglutinin and red cells and the rate constants for the pH inactivation of haemagglutinin and for the adsorption of haemagglutinin to red cells. Thus when the typical values for red cell concentration \([R] = 10.2 \times 10^6\) cells per ml., rate-constant ratio \(\frac{k_1}{k_3} = 7.6 \times 10^6\) cells per ml., and haemagglutinin concentration \([H]_0 = 0.1 \times 10^{10}\) to \(1.0 \times 10^{10}\) particles per ml., are substituted into equation (62), the calculated curves may be compared with those for an actual experiment, as shown in Figure 65. The differences between the theoretical and experimental data at higher concentrations of haemagglutinin \((\frac{H}{R} > 0.25\) and \(b > 0.25)\) is due to the increasing saturation of the red cell surface and the consequent wasteful adsorption of particles of haemagglutinin which cannot be expressed by agglutination due to increasing steric interference. The experimental curve eventually develops a saturation plateau (Figure 34, Page 140) whereas the theoretical curve continues to rise smoothly. Each experimental point lies on a theoretical curve determined by the concentration of particles of haemagglutinin available after pH inactivation. This residual haemagglutinin concentration must then be increased by the already discussed (Page 194) factor \(\left(\frac{R}{[R]_0} + \frac{k_1}{k_3}\right)\) to compensate for pH inactivation prior to haemagglutination (Table 20). Under conditions for which steric hindrance is negligible \((b < 0.25)\) the experimental and theoretical curves do not diverge significantly and the present treatment therefore offers an acceptable and quantitative interpretation of the haemagglutination reaction in terms of an absolute estimate of haemagglutinin concentration.

(c) The application and simplification of equation (62)

The equation (62) which characterizes the haemagglutination reaction may be simplified to a convenient practical form for the interpretation of the reaction in free suspension as observed by the sedimentation-enumeration method.
The Sedimentation-Enumeration Method: A Comparison between the Theoretical and Observed Relationships between the Extent of Haemagglutinin-Specific Agglutination (h) and the Overall Dilution Denominator of Haemagglutinin (D).

\[ [R] = 10.2 \times 10^6 \text{ cells per ml.} \]

Theoretical Curves for \([H]_0\):

- **I** - \(0.1 \times 10^{10}\) particles per ml.
- **II** - \(0.25 \times 10^{10}\) particles per ml.
- **III** - \(1.0 \times 10^{10}\) particles per ml.
Since, under the present standard reaction conditions and with a red cell concentration of about $10^7$ cells per ml., the range of values for $b$ is about 0.0 to 0.4, then the slope of the central linear region of the relationship between the extent of agglutination and the dilution of haemagglutinin (Figure 65) may be characterized by the mean mid-point dilution denominator ($D_M$) for $b = \frac{0.4}{2} = 0.2$. Then substituting $b_M = 0.2$ and the $D_M$ to be observed into equation (62) we obtain,

$$
[H]_o = \frac{-D_M \cdot ([R] + \frac{k_1}{K_1}) \cdot \log (1-b_M)}{0.434}
$$

(63)

$$
= 0.625 \cdot D_M \cdot ([R] + 7.6 \times 10^6)
$$

(64)

Then for the typical reaction mixture of Figure 65 containing $[R] = 10.2 \times 10^6$ cells per ml,

$$
[H]_o = (11.16 \times 10^7) \cdot D_M \text{ particles per ml.}
$$

(65)

but $D_M = 400$ to give the mean central extent of agglutination of $b_M = 0.2$, so that,

$$
[H]_o = 4.46 \times 10^9 \text{ particles per ml.}
$$

A similar simple calculation may be applied for the estimation of absolute haemagglutinin concentrations from other sets of agglutination data.
The Evaluation of the Method Constants for the Pattern and Sedimentation-Enumeration Methods

The Pattern Method is characterized by the general equation

\[ \frac{[H]}{D} \circ = k \cdot v \cdot [R] \]  (4)

where \( \frac{[H]}{D} \circ \) is the concentration of particles of haemagglutinin at the end-point dilution, \([R]\) is the concentration of red cells in the dimple cup, and \(k \cdot v = k_v\) (equation 3) is characteristic of the experimental method for a reaction mixture of volume, \(v\) ml.

The method constant \(k\) may now be estimated in terms of the absolute determination of \([H]_\circ\) by the sedimentation-enumeration method and by electron microscopy, (Page 200). The value of \(D \cdot [R]\) is obtained as the characteristic slope in the presentation of \(D\) against \([R]\) as in Figure 9 (Page 77).

Thus, for a single preparation of haemagglutinin of Sealiki Forest virus (SFV(M)/F), \([H]_\circ = 1.02 \times 10^{10}\) particles per ml. and the slope \(D \cdot [R] = 1.22 \times 10^{10}\) cells per ml. Therefore,

\[ k \cdot v = \frac{[H]_\circ}{D \cdot [R]} = \frac{1.02 \times 10^{10}}{1.22 \times 10^{10}} = 0.85 \text{ particles per red cell} \]

and \(k = 0.85\) particles per red cell, since \(v = 1\) ml. in the present experiments with the pattern test. It thus appears that at the end-point dilution for the detection of haemagglutinin, there is approximately one particle of haemagglutinin per red cell in the pattern test.

By the Sedimentation- Enumeration Method,

\[ [H]_\circ \propto D_L \]

where \(D_L\) is the limiting end-point dilution indicated by extrapolation to zero.
extent of agglutination for viral haemagglutinin-specific haemagglutination (Figure 34). This end-point dilution is independent of the overall red cell concentration in the reaction mixture.

Thus, $[H]_0 = K_{SE} \cdot D_L$ (66)

where $K_{SE}$ is a characteristic constant for the sedimentation-enumeration method.

For a single preparation of haemagglutinin of Semliki Forest virus (SFV(M)/F) the mean value of $D_L$ obtained from five experiments is $2750 \pm 6\%$. Therefore, with the value of $[H]_0$ from Figure 61 (Page 206), the method constant $K_{SE}$ becomes,

$$K_{SE} = \frac{[H]_0}{D_L} = \frac{1.02 \times 10^{10}}{2750} = 3.7 \times 10^6 \text{ per ml.}$$
DISCUSSION
This thesis is concerned with the definition and quantitation of the sequence of reactions by which goose red cells are agglutinated by Semliki Forest virus (SFV), a representative Group A arbovirus. The components and reaction conditions were defined before any attempt was made to obtain quantitative results or interpret them in terms of component activities and concentrations.

(1) Characterization of Components and Reaction Conditions (Page 55)

Although several authors (Page 21) have indicated that preparations of Group A arboviruses may contain two or three haemagglutinating components, the results obtained here in studies by gel-filtration and equilibrium density gradient centrifugation show that preparations of fluorocarbon-extracted SFV (SFV(M)/F) contain only one haemagglutinating component, of size and density comparable to those of the infective particle.

It has been confirmed that for the demonstration of haemagglutination by arboviruses it is necessary to treat the virus preparation with lipid solvents to remove inhibitors of haemagglutination. Such treatments enhance the haemagglutinating activity but also destroy infectivity. However, many purified preparations of viruses of high infectivity \( (\sim 10^{10} \text{ p.f.u. per ml.}) \) fail to show haemagglutinating activity even after extraction (D.H.J. Titmuss, personal communication); thus infectivity and haemagglutinating activity are unlikely to be potential activities of the same particle. To establish absolute relationships between such biological activities and the concentration of characteristic physical particles, it is necessary to show that the count of defined particles is regularly and consistently proportional to the appropriate expression of the specific biological activity.

The most important condition which dominates the expression of haemagglutinating activity is the pH of the reaction mixture containing haemagglutinin and red cells.
Although the haemagglutinating activity of arboviruses is significantly inactivated in free solution at values of pH below about 7.0, a condition of pH 6.0 to 7.0 has been established by most investigators as necessary for the attachment of arboviral haemagglutinin to red cells. As will be discussed later, this is the consequence of the competition between the inactivation of haemagglutinin by pH and the adsorption of haemagglutinin to red cells. Thus many initially active particles fail to contribute to the formation of aggregates.

(ii) The Quantitation of the Haemagglutination Reaction

The sedimentation-enumeration method was developed in order to investigate the distributions of single cells and aggregates formed under different reaction conditions. The expression of haemagglutinating activity was thus observed in terms of the number of haemagglutinin-specific red cell-red cell bonds formed in free suspension. Unlike the pattern method, this observation of the reaction in free suspension is not controlled by the shape and charge of the container surface.

The theoretical basis of the sedimentation-enumeration method has been established by application of the statistical-mechanical treatment developed by Goldberg (1952, 1953) for the antigen-antibody reaction (Bradish and Crawford, 1960). It has been concluded that, under the present experimental conditions, both red cells and particles of haemagglutinin may be regarded as divalent (Page 210). It may be noted that a similar series of expressions were used by Davidson, Macosko and Collins (1967) for the analysis of the size of latex particles by flow ultramicroscopy.

Analysis by sedimentation-enumeration of control reaction mixtures without haemagglutinin have regularly shown that the extent of spontaneous agglutination is significant in comparison with that produced by haemagglutinin-specific bonds.
Although this spontaneous agglutination has been noted by Smith and Courtney (1965) as responsible for a background effect, the phenomenon has not been emphasized in other studies of haemagglutination. It is difficult to provide an exact theoretical treatment of the influence of spontaneously formed aggregates but an effective practical correction may be made by regarding these as due to an initial concentration of spontaneous haemagglutinin.

It has been shown that a high proportion (about 25%) of red cells remained unagglutinated even at high concentrations of haemagglutinin. This may be explained in terms of the saturation of some cells by adsorbed haemagglutinin. These 'saturated' cells cannot combine to form aggregates and thus constitute the 'free-cell' phenomenon of McKerns and Denstedt (1950); this effect is most probable under reaction conditions of high relative concentrations of haemagglutinin.

The several photometric procedures for the observation of haemagglutination provide information on aggregate formation in terms of the change of optical density as aggregates settle and, as such, these methods can only yield information which is averaged over the distribution of aggregates. The results of such methods cannot therefore give a quantitative estimate of haemagglutinin concentration unless either the form of the aggregate distribution is known or one or more limiting assumptions is accepted.

One such limiting assumption is the 'dimers-only' hypothesis of Levine et al. (1953) (Page 88). The present study has shown conclusively that at low concentrations of haemagglutinin, a distribution of single cells and aggregates is formed which fails to confirm this 'dimers-only' hypothesis. It has been found that the haemagglutinin concentration is such that as soon as any dimers are formed, then a smaller but significant number of trimers, tetramers, etc., are also formed. It
has also been found (Page 103) that the simple step-curve anticipated as a consequence of the 'dimers-only' hypothesis cannot be observed for haemagglutination reaction mixtures but only for synthetic and non-agglutinating mixtures of sheep and goose red cells of distinct sedimentation rate.

In addition to assumptions concerning the distribution of aggregates, several other assumptions are usually made concerning the nature or efficiency of the cell-haemagglutinin reaction. These assumptions, which are frequently applied but rarely stated, may be summarized as follows:

(a) No virus particles or active haemagglutinating components are lost by pH inactivation, by adsorption to container wall or by aggregation before adsorption to red cells.

(b) All virus particles or active haemagglutinating components are rapidly adsorbed to red cells.

(c) No red cell adsorbs more than one virus particle or active haemagglutinating component.

(d) Every adsorbed particle of virus or haemagglutinin then 'bridges' to a second red cell to form a dimer.

(e) No aggregates greater than the dimer are formed.

(f) No aggregates are formed in the absence of viral haemagglutinin.

These assumptions apply to the studies by Cheng (1961b), on Sealiki Forest virus, and by Nosima et al. (1964), on Japanese B encephalitis virus. The photometric method of Levine et al. (1953) was used to obtain estimates of 'erythrocyte-dimer forming units' and it was concluded in both cases that infectivity and haemagglutinating activity could be identified with the characteristic physical particle.
above assumptions were neither justified nor allowed for in interpretation.

With the recognition of the aggregate distribution applicable to viral haemagglutination, it is now possible to interpret more fully the results of quantitative methods for which limiting assumptions have previously been made. Thus the Technicon Autoanalyzer (Page 42) depends upon the validity of the principle that unagglutinated single red cells may be completely separated by decantation from formed aggregates. These single cells are then lysed and the increase of optical density due to the released haemoglobin serves as a measure of their concentration. Thus, if use is now made of the aggregate distribution established in this investigation, the extent of agglutination, \( p \), may be calculated from the concentration of single or unagglutinated cells by the expression,

\[
F_1 = (1-p)^2 \quad \text{or} \quad \frac{F}{R} = 1 - \sqrt{F_1}
\]  

(67)

The Autoanalyzer method clearly depends upon the efficiency of separation of single cells and upon the theoretical basis, now proposed, by which the concentration of free cells may be related to the concentration of haemagglutinin.

Similar considerations and equations apply to the counting of single red cells by the Coulter Counter technique (Page 110) although this is unsatisfactory for the study of viral haemagglutination systems for reasons related to its lack of aggregate size discrimination. The difficulties previously described may be overcome by a method of "hydrodynamic focusing" recently described by Spielman and Goren (1968). They showed that since the electrical pulse produced by a particle passing through the aperture depends in part on its path near and through the aperture, the resolution and size discrimination is improved if all particles approach the counting aperture in the same streamline flow and in a direction normal to the plane of the aperture.
Quantitative studies with the sedimentation-enumeration method have shown consistently that the characteristic linear region (Page 138) of the relationship between the extent of agglutination \( \frac{B}{R} \) and the logarithm of the overall dilution denominator of haemagglutinin \( \log D \) defines the limiting dilution \( D_L \) at which virus-specific haemagglutinating activity is first detectable; this may be called the Haemagglutination Index (H.I. = \( \log D_L \)) for the preparation of haemagglutinin and is independent of the red cell concentration. In contrast to this, the findings for the pattern method (Page 72) have shown that, under standard conditions and at a defined pattern end-point, the number of particles of haemagglutinin per red cell is constant. In the present experiments this number is about 1 (Page 223), so that, given such numerical calibration for a system of known red cell concentration, the pattern method provides a precise numerical, but empirical estimate of the concentration of particles of haemagglutinin.

However, there are several objections to the use of the pattern method for quantitative studies: the '50%' or '2-score' pattern is subjective and arbitrary and the method cannot easily be used to resolve the mechanisms or assumptions relating to the primary free solution reactions. These objections have not been considered in the many studies, (Schwerdt, 1959), on the haemagglutination of fowl red cells by suspensions of influenza virus. In all the reports using both the pattern method (Fazekas de St. Groth and Cairns, 1952; Werner and Schlesinger, 1954) and photometric procedures (Friedewald and Pickels, 1944; Levine et al., 1953) it was assumed or concluded that at the end-point of haemagglutinating activity every virus particle was associated with one bond between two red cells. This assumption was also adopted by Bateman, Davis and McCaffrey (1955) in their study of the agglutination of guinea-pig erythrocytes by influenza virus; and by Morris (1953) in the study of
the agglutination of human group '0' red cells by the virus of murine encephalomyelitis (3D VII strain). The present study has shown the necessity for the critical appraisal of assumptions which underlie the interpretation of results by these methods before any valid conclusions may be drawn concerning the relationship between observed activity and active particle concentration.

Isaacs (1957) and Tyrrell and Valentine (1957), as a result of studies by electron microscopical techniques to estimate the numbers of virus particles adsorbed to the membranes of lysed red cells (Donald and Isaacs, 1954; Isaacs and Donald, 1955), concluded that the method of Levine et al. (1953) underestimated the virus particle count by a factor of about ten. The assumption that every virus particle forms a dimer-bond between two red cells implies a 100% efficiency of attachment to red cells which was shown to be erroneous by Isaacs and Donald (1955). Fazekas de St. Groth and Stone (quoted by Fazekas de St. Groth, 1962) have suggested that the reason for this underestimation is that cells only form aggregates by binding edge to edge due to high negative charge effects and rarely by binding face to edge or face to face. Since 90% of the virus particles continue with receptors on the 'flat' faces of cells, these are then not used for edge to edge haemagglutination but can combine with specific antibody. Thus many potentially haemagglutinating particles must be considered as wastefully adsorbed or sterically hindered.

The several distinct methods for the quantitative observation of haemagglutination by viruses are summarized in Table 2.

This study has established the importance of the influence of the pH of the reaction mixture on the level of haemagglutinating activity subsequently observed. A significant proportion, about 40%, of the initial number of particles of Semliki Forest virus haemagglutinin is inactivated at pH 6.3, the optimal condition for the
### Table 21: Methods for the Observation of the Haemagglutination Reaction between Viruses and Red Cells

<table>
<thead>
<tr>
<th>Method</th>
<th>Reference</th>
<th>Observation</th>
<th>Limiting Assumptions required for Interpretation in terms of Conc. of Haemagglutinin (unless detailed theoretical treatment is given)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedimentation-Enumeration</td>
<td>Present study</td>
<td>Distribution of cells and aggregates observed directly by light microscopy.</td>
<td>None</td>
</tr>
<tr>
<td>Electron Microscopy</td>
<td>Donald and Isaacs (1954)</td>
<td>'Direct' counting of physical particles adsorbed to lysed red cells.</td>
<td>(f) No aggregates formed in absence of viral haemagglutinin.</td>
</tr>
<tr>
<td></td>
<td>Salk (1944)</td>
<td>Visual observation of pattern formed by settled reaction mixture in hemispherical cup.</td>
<td>(a) No particles of virus or haemagglutinin are lost by pH inactivation, by adsorption to container wall or by aggregation before adsorption to red cells.</td>
</tr>
<tr>
<td></td>
<td>W.H.O. (1953)</td>
<td></td>
<td>(b) All particles of virus or haemagglutinin are rapidly adsorbed to red cells.</td>
</tr>
<tr>
<td>Autoanalyzer</td>
<td>Ferrari (1964)</td>
<td>Fraction of separated single cells estimated by optical density of released haemoglobin.</td>
<td>(c) No red cell adsorbs more than one particle of virus on haemagglutinin.</td>
</tr>
<tr>
<td></td>
<td>Morris et al. (1965)</td>
<td></td>
<td>(d) Every adsorbed particle of virus or haemagglutinin then 'bridges' to a second red cell to form a dimer</td>
</tr>
<tr>
<td>Coulter Counter</td>
<td>Present study</td>
<td>Distribution of cells and aggregates estimated in terms of electrical pulses.</td>
<td>(e) No aggregates greater than the dimer are formed.</td>
</tr>
<tr>
<td>Photometric</td>
<td>Hirst (1942b)</td>
<td>Whole populations of cells and aggregates characterized by optical density while settling under gravity.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Levine et al. (1953)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kohn and Danon (1965)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Fragiligraph)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Drescher (1957)</td>
<td>Predominantly single cells characterized by optical density while settling under gravity.</td>
<td></td>
</tr>
</tbody>
</table>
demonstration of haemagglutination. These inactivated particles are therefore not available for the formation of red cell-red cell bonds and the expression of haemagglutinating activity. Thus, there is direct competition between pH inactivation and adsorption of haemagglutinin to red cells, and any quantitation of the overall reaction must take account of the two appropriate rate constants. This important feature of arboviral haemagglutination has not been emphasized previously.

It has also been shown (Page 191) that the optimal pH for the formation of haemagglutinin-specific RBC-RBC bonds is below pH 6.0 and that for the stability of haemagglutinating activity is above pH 7.0. Thus the optimal pH of 6.3 to 6.4 for the detection of haemagglutinating activity is a compromise between these two requirements.

Although the sedimentation-enumeration method counts RBC-RBC bonds and cannot directly identify the actual numbers of particles of haemagglutinin per bond, it has been shown that the overall number (h) of particles associated with each RBC-RBC bond varies with the ratio of haemagglutinin and red cell concentrations (Figure 60, Page 203). At the optimal concentration of about $10^7$ red cells per ml. and limiting concentrations of haemagglutinin, $h$ is about seven. However, the actual number of particles of haemagglutinin in each bond may be inferred from the likely distribution of particles of haemagglutinin across the available concentration of red cells (Page 213).
(iii) The Mechanism of Arboviral Haemagglutination

The physico-chemical detail of the mechanism by which viral haemagglutinin attaches to red blood cells has not been established although considerable elucidation has been achieved of the reaction kinetics which control the rates of adsorption and of aggregate formation.

The charge-basis for the initial attachment of virus particles to red cells proposed by Valentine and Allison (1959) has been criticised by Ogston (1963) on the grounds that a suitable mathematical model for the calculation of collision frequency between two moving particles is not yet available. For arboviruses, the reaction is further complicated since there is evidence that attachment is primarily due to ionic and van der Waals forces (Hale and Pillai, 1960). This may be the reason for the pH dependence of the haemagglutination reaction.

The results of this study in relation to the influence of pH, reactant concentrations, spontaneous agglutination and the number of particles of haemagglutinin per RBC-RBC bond are summarized in Figure 66.
An Interpretation of the $H \text{ vs. } \log D$ Relationship for the Haemagglutination Reaction between Semliki Forest Virus Haemagglutinin (SFV(H)/F) and Goose Red Cells at pH 6.30

**EXTENT OF AGGLUTINATION**

**AS BONDS PER RED CELL, $\frac{B}{R}$**

---

**STANDARD CURVE**

**CONTROL WITHOUT HAEMAGGLUTININ**

- **$h = \sim 30$**
- **$h = 8-10$**
- **$h = 4-5$**
- **$h = 1-2$**

---

**LOG (OVERALL DILUTION OF HAEMAGGLUTININ (H)) = LOG D.**

<table>
<thead>
<tr>
<th>HAEMAGGLUTININ (H)</th>
<th>IN EXCESS</th>
<th>NOT IN EXCESS</th>
<th>DECREASING TO SMALL INTEGER (1 to 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RED CELLS (R)</td>
<td>NOT IN EXCESS</td>
<td>CHANGING TO EXCESS</td>
<td>IN EXCESS</td>
</tr>
<tr>
<td>PARTICLES PER RED-RED BOND (h)</td>
<td>HIGH</td>
<td>DECREASING TO SMALL INTEGER (1 to 7)</td>
<td>NONE</td>
</tr>
<tr>
<td>BONDS PER RED CELL, $\frac{B}{R}$</td>
<td>DEPENDENT ON $[R]$</td>
<td>INDEPENDENT OF $[R]$, BUT DEFINED BY $\frac{B}{D}$</td>
<td>NONE</td>
</tr>
<tr>
<td>RATE OF pH INACTIVATION AT pH 6.3</td>
<td>HIGH</td>
<td>LOW</td>
<td></td>
</tr>
</tbody>
</table>
CONCLUSIONS
1. The haemagglutinin extracted by fluorocarbon from the brains of suckling mice following infection with Semliki Forest virus consists of particles of a size and density similar to those of the infective particle.

2. The critical pH requirements for the haemagglutination of red cells have been confirmed. The influence of buffer ions on haemagglutinating activity is marginal.

3. The pattern method is a simple practical technique which may be used to give a rapid estimate of haemagglutinating activity provided it is calibrated for the numbers of particles of haemagglutinin present at the defined pattern end-point.

4. A new presentation and interpretation is proposed for the results of pattern tests for haemagglutination and haemagglutination-inhibition.

5. The 'dimers-only' hypothesis frequently accepted for the interpretation of photometric procedures in haemagglutination studies is not valid for quantitative haemagglutination in viral systems.

6. Estimates of haemagglutinin concentration by any method must be based upon an acceptable analysis of the continuous distribution of aggregates of different sizes.

7. Theoretical and technical difficulties block the application of the Coulter counter to the quantitative analysis of the distributions of cells and aggregates in a virus-red cell reaction mixture.

8. A new sedimentation-enumeration method has been developed by which the haemagglutination of red cells by viruses may be observed quantitatively as a distribution of aggregates. This leads to the quantitative estimation of haemagglutinin concentration in terms of the number of red cell-red cell bonds formed. This method satisfies the criterion that the estimate of haemagglutinin concentration for an initial preparation of haemagglutinin is independent of the red cell concentration used in an assay test.
9. The theoretical basis of the sedimentation-enumeration method has been confirmed by analysis of results in terms of Goldberg's statistical-mechanical theory for antigen-antibody reaction. Under present conditions, both red cell and haemagglutinating particle act as divalent entities.

10. The haemagglutination reaction, with red cells as excess reactants, and the haemagglutination-inhibition reaction, with antibody as excess reactant, follow the Percentage Law.

11. The specific activity of an antiserum sample may be defined by an Haemagglutination-Inhibition Index (H.I.I.), analogous to the Serum Neutralization Index (S.N.I.) for the neutralization of virus infectivity by specific antibody.

12. The detectable haemagglutinating activity for a suspension of Semliki Forest virus is limited by the competitive rates of reaction for pH inactivation of haemagglutinin and for adsorption of haemagglutinin to red cells. In consequence of competitive inactivation by pH about seven initial particles of haemagglutinin must be available for every haemagglutinin-specific red cell-red cell bond subsequently formed.

13. The observed relationship between the extent of agglutination as bonds per red cell and the overall dilution denominator of haemagglutinin corresponds closely with that derived theoretically in terms of the random adsorption of particles of haemagglutinin by red blood cells during competitive pH inactivation. This formulation leads to the interpretation of the haemagglutination reaction in terms of an absolute estimate of haemagglutinin concentration.
14. The constant $k$ which characterizes the kinetics of the pattern method with a reaction mixture of volume 1 ml. indicates that, at the end-point dilution, each red cell is associated, on the average, with one particle of haemagglutinin. Thus the detection of haemagglutinin in WHO Perspex agglutination plates may be extended to the estimation of the concentration of particles of haemagglutinin.
APPENDICES
APPENDIX I

Buffer Solutions

a) **Alsowere Solution** (Bukantz, Rein and Kent, 1946).

- **Dextrose** 20.50 gm
- **Sodium citrate** 8.00 gm
- **Sodium chloride** 4.20 gm
- **Citric acid** 0.55 gm

made up to 1 litre with glass distilled water → pH 6.1

b) **Phosphate Buffered Saline, PBS** (Dulbecco and Vogt, 1954)

Three solutions (i), (ii) and (iii) were prepared.

(i) **Sodium chloride** 8.00 gm
**Potassium chloride** 0.20 gm
**Disodium hydrogen phosphate** 1.15 gm
**Potassium dihydrogen phosphate** 0.20 gm
**Glass distilled water** 800 ml.

(ii) **Calcium chloride** 0.10 gm
**Glass distilled water** 100 ml.

(iii) **Magnesium chloride (MgCl₂.6H₂O)** 0.10 gm
**Glass distilled water** 100 ml.

The three solutions were autoclaved separately, allowed to cool and mixed together to give a final pH of 7.2.

c) **Calcium/Magnesium Saline** (Fenner, 1958)

- **Sodium chloride** 8.00 gm
- **Boric acid** 1.20 gm
- **Sodium borate (Na₂B₄O₇.10H₂O)** 0.05 gm
- **Calcium chloride (CaCl₂.6H₂O)** 0.06 gm
- **Magnesium chloride (MgCl₂.6H₂O)** 0.17 gm

made up to 1 litre with glass distilled water → pH 7.2
d) **McIlvaine's Citrate Buffer Solution** (Hale, 1965)

\[ A = 0.1M \text{ citric acid} \]

\[ B = 0.2M \text{ disodium hydrogen phosphate} \]

Mix \( x \) ml. of \( A \) and \( y \) ml. of \( B \).

<table>
<thead>
<tr>
<th>pH</th>
<th>( A(x) )</th>
<th>( B(y) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>7.37</td>
<td>12.63</td>
</tr>
<tr>
<td>6.2</td>
<td>6.78</td>
<td>13.22</td>
</tr>
<tr>
<td>6.4</td>
<td>6.15</td>
<td>13.85</td>
</tr>
<tr>
<td>6.6</td>
<td>5.45</td>
<td>14.55</td>
</tr>
<tr>
<td>6.8</td>
<td>4.55</td>
<td>15.45</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH</th>
<th>( A(x) )</th>
<th>( B(y) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>3.53</td>
<td>16.47</td>
</tr>
<tr>
<td>7.2</td>
<td>2.61</td>
<td>17.39</td>
</tr>
<tr>
<td>7.4</td>
<td>1.83</td>
<td>18.17</td>
</tr>
<tr>
<td>7.6</td>
<td>1.27</td>
<td>18.73</td>
</tr>
<tr>
<td>7.8</td>
<td>0.85</td>
<td>19.15</td>
</tr>
</tbody>
</table>

e) **Sorensen's Phosphate Buffer Solution** (Hale, 1965)

\[ A = 0.066M \text{ disodium hydrogen phosphate} \]

\[ B = 0.066M \text{ potassium dihydrogen phosphate} \]

Mix \( x \) ml. of \( A \) and \( y \) ml. of \( B \).

<table>
<thead>
<tr>
<th>pH</th>
<th>( A(x) )</th>
<th>( B(y) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.91</td>
<td>1.0</td>
<td>9.0</td>
</tr>
<tr>
<td>6.24</td>
<td>2.0</td>
<td>8.0</td>
</tr>
<tr>
<td>6.47</td>
<td>3.0</td>
<td>7.0</td>
</tr>
<tr>
<td>6.64</td>
<td>4.0</td>
<td>6.0</td>
</tr>
<tr>
<td>6.81</td>
<td>5.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>pH</th>
<th>( A(x) )</th>
<th>( B(y) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.98</td>
<td>6.0</td>
<td>4.0</td>
</tr>
<tr>
<td>7.17</td>
<td>7.0</td>
<td>3.0</td>
</tr>
<tr>
<td>7.38</td>
<td>8.0</td>
<td>2.0</td>
</tr>
<tr>
<td>7.73</td>
<td>9.0</td>
<td>1.0</td>
</tr>
<tr>
<td>8.04</td>
<td>9.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>
f) **Tris Buffer Solution** (Hale, 1965)

0.2M tris (hydroxymethyl) aminomethane

A = 0.1M hydrochloric acid

To 25 ml. 0.2M Tris add x ml. A, and dilute to 100 ml.

<table>
<thead>
<tr>
<th>pH</th>
<th>A(x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.19</td>
<td>45.0</td>
</tr>
<tr>
<td>7.36</td>
<td>42.5</td>
</tr>
<tr>
<td>7.54</td>
<td>40.0</td>
</tr>
<tr>
<td>7.66</td>
<td>37.5</td>
</tr>
<tr>
<td>7.77</td>
<td>35.0</td>
</tr>
<tr>
<td>7.87</td>
<td>32.5</td>
</tr>
<tr>
<td>7.96</td>
<td>30.0</td>
</tr>
<tr>
<td>8.05</td>
<td>27.5</td>
</tr>
</tbody>
</table>

g) **Veronal Buffer Solution** (Hale, 1965)

A = 0.1M sodium diethylbarbiturate

B = 0.1M hydrochloric acid

Add x ml. of A to y ml. of B.

<table>
<thead>
<tr>
<th>pH</th>
<th>A(x)</th>
<th>B(y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.8</td>
<td>5.22</td>
<td>4.78</td>
</tr>
<tr>
<td>7.0</td>
<td>5.36</td>
<td>4.64</td>
</tr>
<tr>
<td>7.2</td>
<td>5.54</td>
<td>4.46</td>
</tr>
<tr>
<td>7.4</td>
<td>5.81</td>
<td>4.19</td>
</tr>
<tr>
<td>7.6</td>
<td>6.51</td>
<td>3.49</td>
</tr>
<tr>
<td>7.8</td>
<td>6.62</td>
<td>3.38</td>
</tr>
<tr>
<td>8.0</td>
<td>7.16</td>
<td>2.84</td>
</tr>
<tr>
<td>8.2</td>
<td>7.69</td>
<td>2.31</td>
</tr>
</tbody>
</table>
h) **Borate Buffer Solution (Bi), pH 9.0** (Clarke and Casals, 1953)

- 0.5M Boric acid 100 ml
- 1.5M Sodium chloride 80 ml
- 1.0M Sodium hydroxide 24 ml
- Glass distilled water 796 ml

pH adjusted to 9.0 with 1.0M sodium hydroxide.

i) **Bovine Serum Albumin (BSA)**

Fraction V of bovine serum obtained from Armour Pharmaceuticals Co., Illinois, U.S.A.

A stock solution was prepared as 10% BSA (w/v) in borate buffer, pH 9.0, and stored at -80°C before use.

j) **Phosphate Buffered Saline (PBS) for Haemagglutination Tests with Arboviruses** (Begum, 1963)

- A = 1.5M sodium chloride
- B = 0.5M disodium hydrogen phosphate
- C = 1.0M sodium dihydrogen phosphate
- D = glass distilled water

<table>
<thead>
<tr>
<th>pH</th>
<th>6.0</th>
<th>6.1</th>
<th>6.2</th>
<th>6.3</th>
<th>6.4</th>
<th>6.5</th>
<th>6.6</th>
<th>6.7</th>
<th>6.8</th>
<th>6.9</th>
<th>7.0</th>
<th>7.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>B</td>
<td>32</td>
<td>47</td>
<td>62</td>
<td>92</td>
<td>112</td>
<td>136</td>
<td>160</td>
<td>176</td>
<td>192</td>
<td>222</td>
<td>240</td>
<td>274</td>
</tr>
<tr>
<td>C</td>
<td>184</td>
<td>177</td>
<td>169</td>
<td>154</td>
<td>144</td>
<td>132</td>
<td>120</td>
<td>112</td>
<td>104</td>
<td>89</td>
<td>80</td>
<td>63</td>
</tr>
<tr>
<td>D</td>
<td>684</td>
<td>676</td>
<td>669</td>
<td>654</td>
<td>644</td>
<td>632</td>
<td>620</td>
<td>612</td>
<td>604</td>
<td>589</td>
<td>580</td>
<td>563</td>
</tr>
</tbody>
</table>
APPENDIX II

Preparation of Haemagglutinin from Semliki Forest Virus (SFV)

a) Extraction using Fluorocarbon

Dilute stock SFV(M) or SFV(C) \( \frac{1}{5} \) in BB/BSA, pH 9.0.


Homogenize the mixture at about 2,000 rev. per min. for 2 min. at room temperature (23°C).

Centrifuge the homogenate at 2,000 rev. per min. for 10 min. to separate the upper, aqueous layer containing the haemagglutinin from the lower, often solid deposit containing fluorocarbon, inhibitors and cellular debris.

Repeat the extraction of the supernate with an equal volume of fresh fluorocarbon.

Remove the clear supernatant fluid and store at -30°C as the stock haemagglutinin (H). This usually has an activity of at least 2,600 HAU per ml.

b) Extraction with Tween-80 and Ether

Dilute stock SFV(M) or SFV(C) \( \frac{1}{5} \) in BB/BSA, pH 9.0.

Add an equal volume of 2% Tween-80 (from Honeywill Atlas Ltd., Surrey) and allow the mixture to stand at 23°C (room temperature) for 15 min. with occasional shaking.

Add a further volume of diethyl ether to the mixture and shake for 10 min.

Centrifuge the reaction mixture at 2,000 rev. per min. for 10 min.

Remove the aqueous phase and store at 4°C as the stock haemagglutinin (H).
APPENDIX III

Calculation of Haemagglutinating Activity and Haemagglutination-Inhibitory Activity

a) Haemagglutinating Activity (HA)

The dimple plate patterns and the corresponding scores on the '0 to 4' scale for two samples of haemagglutinin, A and B, are shown below in Figure 67.

**FIGURE 67**

![Dimple plate patterns](image)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Score</th>
<th>Grades</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>½</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**Sample A**

Using equation (6) on Page 82,

\[
\log [H] = \log D = \log E + \log F
\]

where \(E\) is the overall dilution to '4' score

and \(F\) is the further dilution between '4' score and the best-fit '2' score.

Thus, \(E\) at final '4' score = 2048, or \(\log E = 3.31\) (Table 7, Page 85).

The total score, \(s\), above '4' at \(E = 2048\) is 1, and from Table 7, \(\log F = 0.23\) for \(s = 1\).

Then, substituting these two values into the above equation, we obtain,

\[
\log [H] = 3.31 + 0.23
\]

\[
= 3.54 \text{ HAU per ml.}
\]

or,

\[
[H] = 3,500 \text{ HAU per ml.}
\]
Sample B

A similar calculation for sample B gives a haemagglutinating activity of

\[ [H] = 2,600 \text{ HAU per ml.} \]

b) Haemagglutination-Inhibitory Activity (B)

The pattern and score-grades obtained for the stock haemagglutinin in a standard four-volume test were:

<table>
<thead>
<tr>
<th>( D_0 )</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>160</th>
<th>320</th>
<th>640</th>
<th>1280</th>
<th>2560</th>
<th>5120</th>
<th>CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score-Grades</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>1 ½</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

As previously described,

\[ \log D_0 = \log E + \log F \]
\[ = 2.51 + 0.41 \]
\[ = 2.92 \]

or,

\[ D_0 = 830 \text{ HAU per ml.} \]

The overall dilution, \( D \), of haemagglutinin used in the reaction mixture with antiserum was 320.

The pattern and score-grades for the haemagglutination-inhibition test using a constant dilution of haemagglutinin, \( D = 320 \), throughout in a four-volume test are given below:

<table>
<thead>
<tr>
<th>( d )</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>256</th>
<th>512</th>
<th>1024</th>
<th>CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score-Grades</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

The best-fitting overall dilution, \( d \), of the antiserum in the reaction mixture required to give a '2' score in the pattern test is calculated from the scores as,

\[ \log d = \log E - \log F \]

(68)
where \( E \) is the overall dilution to '4' score, and \( F \) is the dilution between '4' score and the best-fit '2' score

or, from the data of Table 7, (Page 85),

\[
\log d = 3.01 - 0.45 = 2.56.
\]

Then, substituting into equation (11) on Page 86, we obtain,

\[
\log [B] = 0.30 + \log d + \log \left(\frac{D_2}{D_1} - 1\right)
\]

\[
= 0.30 + 2.56 + \log \left(\frac{320}{320} - 1\right)
\]

\[
= 0.30 + 2.56 + \log 1.6
\]

\[
= 0.30 + 2.56 + 0.20
\]

\[
= 3.06
\]

Therefore, \([B] = 1200 \text{ IU per ml} \).
APPENDIX IV

The Derivation of $\frac{B}{R}$ from Coulter counter data, and $\frac{C_A}{C_o}$ from the

Haemoglobin (Hb) Content of free red cells

(1) Bonds per Red Cell, $\frac{B}{R}$

If $A_n$ is the number of aggregates of $n$ red cells (RBC) counted in the
presence of haemagglutinin (H), then the total number of aggregates counted
by the Coulter counter (for conditions, see Page 114) is given by,

$$A_o = A_1 + A_2 + A_3 + \ldots + A_n$$  \hspace{1cm} (13)

Similarly, the minimum number of bonds between red cells due to the presence
of haemagglutinin is given by,

$$B = A_2 + 2A_3 + \ldots + (n-1)A_n$$  \hspace{1cm} (14)

and the initial concentration of free cells by,

$$C_o = A_1 + 2A_2 + 3A_3 + \ldots + nA_n$$  \hspace{1cm} (15)

Then,

$$A_o + B = C_o$$  \hspace{1cm} (16)

Since $C_o$ is the initial concentration of red cells, $R$, (see
sedimentation-enumeration method, Page 128), we may then write for the
number of bonds per red cell,

$$\frac{B}{R} = 1 - \left(\frac{A_o}{C_o}\right)$$  \hspace{1cm} (17)
(ii) The Fraction of Red Cells as Aggregates, $\frac{C_A}{C_0}$

The concentration of haemoglobin (Hb) released by lysis of red cells and measured as optical density is proportional to the number of cells present, regardless of their distribution as aggregates.

Thus, on lysis of the free and unagglutinated cells in the cell distribution characterized by equation (13), the released haemoglobin (Hb), indicated by optical density, is proportional to $A_1$:

$$[\text{Hb}]_H = K A_1$$  \hfill (69)

Similarly, in the control reaction mixture without haemagglutinin,

$$[\text{Hb}]_0 = K C_0$$  \hfill (70)

but by equation (15), the concentration of cells in aggregates of any size ($C_A$) is given by,

$$C_A = C_0 - A_1$$  \hfill (71)

Therefore,

$$\frac{C_A}{C_0} = 1 - \left(\frac{A_1}{C_0}\right)$$  \hfill (72)

or, in terms of the observed release of haemoglobin, the fraction of cells in aggregates = $1 - \frac{[\text{Hb}]_H}{[\text{Hb}]_0}$  \hfill (18)
APPENDIX V

Program in NAC (Mercury Autocode) for the computation of $\frac{B}{R}$ and related quantities

1) READ J : Number of data sheets in group

Set Constants

READ D : Dilution

READ $R_1$

READ $A_k$ : Column totals, Sizes

Calc. $X = X + A_k P_k$ : $Y = Y + KA_k P_k$

Calc. $R_1/R_2$ Calc. $B$ Calc. $R_1/R_2$

PRINT $D_1 R_1 R_2 R_1/R_2 B B/R$

IF $J = 0$ THEN PRINT

Set $Q = 1$

GO TO 1

END
APPENDIX VI

The Consistency of the Red Cell Concentration as Determined by the Sedimentation-Enumeration Method, [*R*<sub>SE</sub>] and by Haemocytometer Count, [*R*<sub>H</sub>]

Let [*R*<sub>SE</sub>] and [*R*<sub>H</sub>] be the concentration of red cells per ml. calculated from the sedimentation-enumeration data and by haemocytometer counts, respectively.

From equations (23) and (26) on Page 128 for the sedimentation-enumeration method, the denominator in equation (26) is the red cell concentration [*R*<sub>SE</sub>].

Thus,

\[
[R_{SE}] = \frac{1}{l \cdot t \cdot s_1 \cdot f_1} \left( \sum^n_{n=1} c_n \cdot F_n \right)
\]

(73)

where *l* is the length of the observation slit (0.1 cm.), *t* is the total time of counting (60 sec.), *s_1* and *f_1* are the sedimentation velocity and depth of focus for a single goose red cell (from Table 10, Page 137, *s_1* is 0.0029 cm. per sec. and *f_1* is 0.0018 cm.). For the present experimental arrangement, the value of the method constant (1.*t*.*s_1*.*f_1*) is 3.1 \times 10^{-5} cm.\(^3\) and therefore,

\[
[R_{SE}] = 3.3 \times 10^4 \left( \sum^n_{n=1} c_n \cdot F_n \right)
\]

(74)

Values of [*R*<sub>SE</sub>] were calculated for ten experiments and then compared with the values of [*R*<sub>H</sub>] in the ratio [*R*<sub>SE</sub>]/*R*<sub>H</sub> as shown in Table 22. Since the significance of haemocytometer counts is about 16% [*R*<sub>H</sub>](Berkson, Magath and Hurn, 1940), the mean value for the ratio [*R*<sub>SE</sub>] of 0.75 and its standard error of ± 0.037 confirm, within the limit of error set by the definition of short counting times, the identity of the estimates of red cell concentration by sedimentation-enumeration and by direct haemocytometer counts. It should be noted that the time of counting, *t*, is required for the calculation of the ratio [*R*<sub>SE</sub>]/*R*<sub>H</sub> but not for the ratio [*R*<sub>H</sub>]/*R*<sub>H</sub>, which is independent of time of counting since *t* is eliminated in equation (24).
TABLE 22. Comparisons of the Determination of Red Cell Concentration by Sedimentation-Enumeration and by Haemocytometer Count

<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>Red Cell Concentration by Haemocytometer, ( [R_H] ) per ml.</th>
<th>Mean Computed Value, ( \sum n_i C_i F_i )</th>
<th>Mean Red Cell Concentration by Sedimentation-Enumeration, ( [R_{SE}] = \sum n_i C_i F_i \times 3.3 \times 10^4 ) per ml.</th>
<th>( \frac{[R_{SE}]}{[R_H]} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.7 x 10^6</td>
<td>217</td>
<td>8.6 x 10^6</td>
<td>0.67</td>
</tr>
<tr>
<td>2</td>
<td>13.5 x 10^6</td>
<td>272</td>
<td>10.9 x 10^6</td>
<td>0.67</td>
</tr>
<tr>
<td>3</td>
<td>8.3 x 10^6</td>
<td>181</td>
<td>7.2 x 10^6</td>
<td>0.72</td>
</tr>
<tr>
<td>4</td>
<td>9.0 x 10^6</td>
<td>222</td>
<td>8.9 x 10^6</td>
<td>0.82</td>
</tr>
<tr>
<td>5</td>
<td>8.3 x 10^6</td>
<td>200</td>
<td>8.1 x 10^6</td>
<td>0.80</td>
</tr>
<tr>
<td>6</td>
<td>9.2 x 10^6</td>
<td>222</td>
<td>8.9 x 10^6</td>
<td>0.80</td>
</tr>
<tr>
<td>7</td>
<td>6.4 x 10^6</td>
<td>155</td>
<td>6.2 x 10^6</td>
<td>0.80</td>
</tr>
<tr>
<td>8</td>
<td>2.6 x 10^6</td>
<td>79</td>
<td>3.1 x 10^6</td>
<td>1.01</td>
</tr>
<tr>
<td>9</td>
<td>7.5 x 10^6</td>
<td>137</td>
<td>5.5 x 10^6</td>
<td>0.60</td>
</tr>
<tr>
<td>10</td>
<td>8.6 x 10^6</td>
<td>170</td>
<td>6.7 x 10^6</td>
<td>0.65</td>
</tr>
</tbody>
</table>

\( 0.75 \pm 0.037 \)
Estimate of Red Cell Concentration $[R_{SE}]$ from the Relationship between $\sum n_i c_i F_i$ and Time of Counting, $t$ (Figure 39, Page 151)

The red cell concentration is given above as,

$$[R_{SE}] = \frac{\sum n_i c_i F_i}{1. t. s_i f_1}$$ (73)

Thus, when the function $\sum n_i c_i F_i$ is presented against the time of counting, $t$,

the slope of the resulting graph is given by,

$$\text{Slope} = \frac{\sum n_i c_i F_i}{t} = [R_{SE}] \cdot (1.s_i f_1)$$ (75)

or,

$$[R_{SE}] = \frac{\text{slope}}{1. s_i f_1}$$ (76)

$$= \frac{3.6}{5.1 \times 10^{-7}}$$

$$\therefore [R_{SE}] = 7.1 \times 10^6 \text{ cells per ml.}$$

When this value is related to the red cell concentration as determined by haemocytometer count, $[R_H]$, for the same experiment, the following ratio is obtained,

$$\frac{[R_{SE}]}{[R_H]} = \frac{7.1 \times 10^6}{9.2 \times 10^6} = 0.77$$

which agrees closely with the ratio given in Table 22.
APPENDIX VII

Nomenclature and Units

[R] - the concentration of red blood cells (number per ml.).

p.f.u. - plaque forming units assayed by titration of virus infectivity (Page 52).

[V] - the concentration of virus particles which may or may not be infective (number per ml. or p.f.u. per ml.)

[V] Ab - the activity (infectivity) detected after interaction with specific antibody (p.f.u. per ml.).

[H] o - the concentration of particles of haemagglutinin (number per ml.).

H.A.U. - haemagglutinating units assayed in the haemagglutination test by the Pattern method (Page 51).

[H] Ab - the activity (in haemagglutination) detected after interaction with specific antibody (H.A.U. per ml.).

[H] t - the activity (in haemagglutination) as H.A.U. per ml. detected after reaction time t (sec. or min.).

[H] Ads - the concentration of particles of haemagglutinin adsorbed to red cells (number per ml.).

D - the overall dilution, as dilution denominator, of the initial concentration of haemagglutinin.

D Ab - the overall dilution, as dilution denominator, of the initial concentration of antibody.

D L - the overall dilution, as dilution denominator, of the haemagglutinin concentration at which haemagglutinin-specific red cell-red cell bonds are first detectable.

n - the number of red cells (number per ml.).

A n - the number of aggregates of n red cells (number per ml.).
- the number of red cells in aggregates of n red cells (number per ml.).
- the sedimentation velocity for an n-fold aggregate (μ per sec.).
- the depth of focus for an n-fold aggregate (μ).
- the extent of agglutination as the number of bonds per red cell in the presence of viral haemagglutinin (bonds per cell).
- the extent of spontaneous agglutination in the absence of viral haemagglutinin (bonds per ml.).
- the extent of agglutination by haemagglutinin-specific red cell-red cell bonds (bonds per ml.).
- the pattern method constant for a reaction mixture of volume v ml., in W.H.O. agglutination plates, (particles per ml.).
- the rate constant for the inactivation of viral haemagglutinin by pH (reciprocal sec.).
- the rate constant for the adsorption of viral haemagglutinin to red cells (ml. per cell per sec.).
- the fraction of the initial concentration of particles of haemagglutinin inactivated by pH before successful adsorption to red cells.
- the number of particles of haemagglutinin per haemagglutinin-specific red cell-red cell bond at minimal concentrations of haemagglutinin.
- the effective valency of the red cell in combination with particles of haemagglutinin in free suspension.
- the method constant for the sedimentation-enumeration method (per ml.).
ACKNOWLEDGEMENTS
I should like to express my gratitude to Dr. C. E. Gordon Smith, Director of the Microbiological Research Establishment, for permission to present this part of the departmental programme in virology as a Ph.D. thesis.

I am indebted to Dr. C. J. Bradish for his continuous encouragement and guidance and to Dr. R. H. A. Swain for his helpful advice. In addition, I am grateful to Mr. D. H. J. Titmuss for the use of some Coulter counter data, to Mr. S. Peto for statistical advice and to Mr. B. J. Maidment for the preparation of programmes for the Mercury computer.

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