A STUDY OF HAEMAGGLUTINATION WITH SPECIAL REFERENCE TO RUBELLA

by

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ABSTRACT

The work reported shows that:

Erythrocytes from chick embryos of 14-16 days of development may be used in rubella HA and HI tests.

Purified rubella-virus HA antigen contains a high stability (HS) and low stability (LS) fraction.

Rubella haemagglutination is reversed by the action of antibody. This is used in the HAR test which has been developed.

The absolute concentration of HA binding units may be calculated from the proportion of the erythrocytes remaining as monomers after agglutination is complete.

One HA binding unit is identical to one infective unit when complete influenza virus is used as the test reagent.

The relationship between the various dimensions of a virus suspension is best explained on a 'partial identity' basis.

SUMMARY

The rubella-virus haemagglutination-inhibition test is probably the most widely-used method for estimating antibody in patients' sera.

In general terms, the test has advantages which make it a good adjunct to the complement-fixation test and it is less cumbersome, and produces results more rapidly than the virus-neutralization test. There are, however, inherent disadvantages to the test as applied to rubella-virus. For a laboratory which lacks animal maintenance facilities, there is the problem of a constant supply of fresh erythrocytes of the appropriate species. Rubella-virus antigen has peculiar stability characteristics which, although masked by the standard method of antigen titration, need to be understood. The standard test requires the removal of non-specific inhibitors from the serum under test and the temperature-dependence of the test presents technical difficulties and areas of possible error in its performance.

In order to be maximally useful, the test must be as sensitive as possible whilst retaining high test stability. Ideally, it should estimate the absolute value of the binding-unit concentration, but this is not possible by an end-point evaluation test, and the aggregate-evaluation method devised by Cameron & Bradish (1972) although apparently possessing this potential ultimately fails to achieve it.

Finally, when considering the absolute values of the various dimensions of a virus population, the question arises as to the relationships between these. This question has not been considered formally nor empirically investigated with sufficient rigor to arrive at a valid conclusion.

Problems in the supply of fresh chick erythrocytes have been overcome by the use of chick embryo erythrocytes from eggs of 14 days of development and older.

Purified rubella-virus HA antigen appears to contain a high stability (HS) fraction and a low stability (LS) fraction. The LS fraction decays totally in approximately 35 minutes at 4°C, and although it constitutes 90% of the initial antigenic activity, the rubella-virus HI test is so constructed that it is the HS fraction which is the effective test antigen. Rubella-virus HA antigen, when adsorbed on erythrocytes, is totally stable for two hours, and shows a 20% decay after 5 1/2 hours, at 4°C. At room temperature there is a 56% decay after two hours. The adsorbed HA antigen does not elute, but the HA can be specifically reversed by serum antibody. This is the HA reversal (HAR) test and gives an 8-fold higher antibody titre than the conventional HI test. Goat anti-human IgG can be used to neutralize human IgG, but the commercial goat serum tested was effective against only 1/23rd of its own concentration of human serum. There is a need for hyperimmune anti-human IgG serum effective on at least a 1:1 basis.

In addition to containing agglutinin, normal human serum contains lysin which is active against chick erythrocytes at room temperature. Kaolin treatment and heating at 56°C for 30 minutes are both effective in removing the lytic activity. Human serum non-specific inhibitors are not effective in reversing the agglutination in the HAR test, but goat serum contains a non-specific reversing factor. The reversing factor is inactivated by kaolin treatment, but is re-activated by the presence of non-kaolin-treated human serum.

In order to maximize the sensitivity of the HI test, a method of HA antigen assay was developed using influenza-virus, PR8 strain. This method probably gives an absolute HA particle evaluation. The technique is simple in that it consists of assessing the proportion of the initial erythrocytes which remain as monomers after agglutination has proceeded to completion. The formula for conversion to binding unit concentration was derived from a theoretical model, the Random Distribution and Inter-Action Model. The conditions for the test and its sensitivity were evaluated, also the precision of the results obtained. The ratio of binding-units, as evaluated by this method, to tube HA test units was shown to be 1.06×10^6. The ratio of binding-units to egg-infective units was found to be compatible with a 1:1 relationship existing between them.

Various propositions as to the nature of the relationships existing between physical particle, infective unit, and HA unit as dimensions of a virus population were examined. By argument, the most acceptable proposition appears to be that of 'partial identity' where a population consists of physical particles, some having all of the virus characteristics and some lacking in one or more of the characteristics other than physical identity. Propositions involving 'equivalence' can only be eliminated by EM studies to determine how many physical particles are associated with a dimer. Native influenza-virus and that eluted from erythrocytes were found to have approximately the same egg-infective capacity. This and the von Magnus effect support the 'partial identity' proposition, but do not themselves eliminate the 'equivalence' propositions.

This work is seen as the basis for a very sensitive, and probably absolute, quantification method for specific antibody estimation.

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INTRODUCTION

A long-standing commitment to diagnostic virology must, necessarily, lead to an interest in serology since this area contains a potential, as yet largely unrealized in practice, for the identification of infecting viral agents as soon as the patient produces circulating antibody. In addition, it is the area which contains the most reliable tools for general epidemiological survey purposes and for the retrospective study of outbreaks of viral disease where stored sera are available for testing.

Serological tests for use in the diagnosis of viral disease must be both efficient and effective. They should give results that are reproducible within the limits of the test and over long periods of time; they should be sufficiently understood to make it possible to standardize all aspects of both the technique and the reagents; they should be capable of giving an answer within 48 hours under service conditions, and they should not require complex and expensive equipment for their application.

The complement-fixation test, with a history dating back to Bordet's immune cytolysis work between 1895 and 1912, has stood the test of time and has been used by so many workers in so many different fields during the past 60 years that it meets all of the criteria outlined above. This test is a sound foundation for any serological laboratory.

No single test, however, can be expected to supply all the required information in all cases of viral infection of patients.

The complement-fixation test has the advantage of broad-spectrum reaction, as with the adenoviruses and the influenza viruses, but this very aspect, so useful in the diagnosis of disease in the individual patient, detracts seriously from the usefulness of the test for epidemiological investigations. The test also entails disadvantages in the areas of cost and availability of antigens, and the preparation of antigens free from anti-complementary activity is frequently a problem. These disadvantages are, I think, the common experience of all who use the test on a regular basis for multiple serum-antibody titre determinations.

Lastly, there are currently no reliable and effective antigens available to make the complement-fixation test a useful tool for the assay of enterovirus serum-antibody.

Two tests have been developed to a sufficient extent to be useful routine diagnostic tools in serology, and the results of which complement those obtained by the use of the complement-fixation test. These are the virus-neutralization test and the haemagglutination-inhibition test.

The advantage of the virus-neutralization test is that it is potentially applicable to all viruses. It is based upon the principle of specifically neutralizing the capacity of a virus to infect sensitive host cells by means of antibody present in test sera. Inherent in this principle, however, are the two main disadvantages of the test. The first is the host cells; some agents, for example, many of the Coxsackie A viruses, will frequently infect a very limited range of host cells. Where the cells can

be used only in the environment of the whole animal, there are entailed the problems of animal supply and husbandry for the laboratory. The second is infectivity; the presence of a virus in a host is usually inferred by the generation of pathology, identifiable as specific by definable characteristics. It is in the nature of pathogenesis, whether in the whole animal or in a cell culture, that it develops over time and rarely is the period shorter than three or four days.

Perhaps the most useful application of the neutralization test is to enterovirus antibody estimation since there is no good substitute in this area. The impression gained from the results of routine enterovirus antibody estimations in the laboratory is that this group is, perhaps, the most commonly encountered cause of virus disease in the human population. Despite efforts to develop the one-step neutralization technique of Furesz and his associates (1969, 1972), and apply it to the enterovirus group, the problems of serum dilution and standardization of virus dose render it still a cumbersome technique, even by the micro-titration system.

The haemagglutination-inhibition (HI) test is a more attractive one as an adjunct to the complement-fixation test. It has the advantage of speed in producing results, of high specificity within virus groups, for example, with influenza and adenoviruses, and it has been demonstrated to be useful for work with a wide variety of viruses since the first report of the haemagglutination effect with influenza virus by Hirst & Pickels (1942).

The introduction of the Rubella HI test by Stewart et al. (1969) revealed a desirable tool for the estimation of rubella antibody in the sera of pregnant, or potentially pregnant, women. It is now a standard procedure in all laboratories having responsibilities to obstetric and paediatric areas of medicine. However, the responsibility resting on a laboratory which is called upon to supply definitive information on the immune status of newly pregnant women who have recently been in contact with possible rubella cases is great. The need here is for a test which is sufficiently sensitive and precise to give a true representation of the immune status of a patient who may have been infected many years prior to a current incident.

The work described here had two practical objectives:

1. To increase the sensitivity of the rubella-virus HI test, yet retain the stability necessary to give reproducible results in test-retest situations, and
2. To increase the precision of the antigen titration procedure in order to lay the foundation for a test characterized by both high precision and high sensitivity.

The first objective was pursued by addressing the rubella-virus HI test itself; a third objective was established in order to lay the foundation for the second, namely,

3. To construct and validate a theoretical model of the haemagglutination process in order to elucidate the dynamics involved.

The model was a statistical one based on the principle of random distribution and the validation was carried out using influenza-virus, PR8 strain, as the test agent.

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REVIEW OF LITERATURE

The haemagglutination-inhibition (HI) test is probably the most widely used test today for the assay of rubella-virus circulating antibody, yet it was only seven years ago that Stewart and his co-workers (Stewart et al. 1967) reported the successful demonstration of rubella-virus haemagglutination (HA), and the development of the HI test for antibody assay. Work published by Lennette et al. (1967), and by Sever et al. (1967), showed that the sensitivity of the HI test is comparable with that of the complement-fixation and the neutralization tests.

Rubella-virus haemagglutinin is a part of the virus particle (Schmidt et al. 1968; Furukawa et al. 1967); infectivity, complement-fixing antigen and haemagglutinin separate as virtually a single peak upon fractionation by both Sephadex G200 chromatography and sucrose density-gradient centrifugation.

The HA reaction is pH dependent. Both HA and HI titres are two to eight-fold higher at pH 6.5 than at pH 6.8 (Sever et al. 1967). HA activity is maximal between pH 6.0 and pH 6.5, and this is independent of the type of buffer used (Lieberhaber, 1970). This means that the optimum pH for the test is the least advantageous for virus stability since this optimum is pH 9.0 (Holmes & Warburton, 1967).

The roles of calcium ions, gelatin, and albumin in achieving a stable test situation were defined by Lieberhaber (1970); "A 0.14M NaCl solution buffered with N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid (HEPES) at pH 6.05 to 6.15, containing 0.001M $CaCl_2$, 1.0% bovine albumin

and 0.00025% gelatin was shown to provide conditions for a maximally sensitive, stable and clear test. Under these conditions, the outcome of the haemagglutination test is solely a function of the concentration of haemagglutinin in the test specimen". This appears to be the basis for the commercially available diluents for the rubella-virus HI test.

It is not necessary to use the complete virus particle as HA antigen. Tween-80-ether treated material is effective (Lieberhaber, 1970), and this material can be purified to yield an antigen which is a pure polysaccharide (Dr. J. Polley, personal communication). The results of the work carried out by Lieberhaber imply that the Tween-80-ether extracted antigen is relatively stable. When the material was diluted at 25°C to give eight HA units and an aliquot immediately cooled in an ice-bath, it was found to be stable for at least 12 hours at 4°C, and at least an hour at room temperature.

Serum normally contains non-specific inhibitors which block the HA reaction, and therefore, mask the HI reaction. Kaolin treatment was recommended by Stewart et al. (1967) for their removal, but this method was rejected by Lieberhaber (1970) on the grounds of non-specificity of action, variation in results between laboratories, and the elimination of low titre antibodies. Lieberhaber compared two procedures involving specific reactants with β-lipoproteins, the class to which non-specific inhibitors belong. Heparin-manganese chloride was rejected as it requires a lower ionic strength than exists in serum

for complete reaction; and complexed chylomicrons (lipid-amino acid complexes) form a clear pellicle at the surface on centrifugation, and tend to be re-incorporated in the supernatant fluid when this is removed. Lieberhaber considered the method of choice to be dextran-calcium chloride. Low density serum β-lipoproteins form irreversible complexes with dextran sulphate and these are precipitated in the presence of calcium ions. The reaction is maximal in the range pH 5.5 to pH 7.5 and at physiological ionic strength. Careful experimentation showed that the non-globulin inhibitors were reduced to undetectable levels by this method without reducing the immune inhibitors or the quantitative levels of IgM, IgA and IgG.

The range of species whose erythrocytes are agglutinated by rubella-virus haemagglutinin has been extended to cover a useful range. Day-old chick and adult goose are both suitable (Stewart et al. 1967; Lieberhaber, 1970). Stewart et al. reported that sheep erythrocytes are less efficiently agglutinated than those of the other two species; more recently it has been reported that trypsinized human group 'O' erythrocytes can be substituted for the avian cells (Quirin et al. 1972) and this has been confirmed by Iwakata and his colleagues (Iwakata et al. 1973).

Rubella-virus has been included in the Togavirus group (Melnick, 1971). This possibility was suggested by Holmes & Warburton (1967) who listed and compared the characteristics of rubella-virus and those of the arboviruses. They pointed out the striking similarity in the areas of virion diameter (40-60 mμ), core diameter (25-30 mμ), budding at the cell

surface, possession of RNA, sensitivity to ether and 1:1000 sodium deoxycholate, agglutination of erythrocytes of the same species, pH optimum of 6.2 to 6.6 for HA, stability of infectivity at pH 9.0, and sera contain non-specific HA inhibitors which are removed by Kaolin in both cases.

The arbovirus literature then is a source of possible solutions to problems associated with rubella-virus.

Channock & Sabin (1954) found positive evidence in the case of WEE, and circumstantial evidence in the case of Japanese B virus and St. Louis, for greatly enhanced stability of virus bound to erythrocytes as against the low stability of free virus under the same conditions. Bound WEE virus was not eluted when the cell-virus complex was transferred to conditions of pH under which HA does not occur.

Ping-Yao Cheng (1967), working with Semliki Forest virus, reported that one virus particle will form one dimer, and is identical with one physical particle and one plaque-forming unit. These findings are similar to those of Furukawa et al. (1967) and Schmidt et al. (1968) with rubella-virus, namely, that the physical particle was indistinguishably associated with infectivity, complement-fixing and HA activity. It must, of course, be realized that association does not imply identity, so that the two sets of findings do not directly support each other.

Also, Cameron & Bradish (1972) found that, in a mixture of agglutinin and erythrocytes, a wide range of aggregate sizes could be directly observed; this casts serious doubt upon the basic assumption made by Cheng, and by Levine (1953) whose work he was following, that the population of aggregates in a mixture of agglutinin and erythrocytes consists entirely of monomers and dimers. It is probable that the findings of Furukawa et al. (1967) and Schmidt et al. (1968) are the only ones which are currently valid.

Cameron & Bradish (1972) reported the results and theoretical interpretation of studies carried out on Semliki Forest virus. They used a 'bond enumeration' method in which the population of aggregates was assayed by counting, and determining the size of, aggregates falling in a spectrophotometer cuvette past a reference line after an initial equilibration time of 15 minutes at room temperature. From this was calculated the actual concentration of aggregates and the average number of bonds per erythrocyte by the use of two pre-determined constants and a formula which is derived in the paper. They reported that, at bond/cell ratios between 0.22 and 0.50, there is a linear relationship between the bond/cell ratio and the $\log_2$ value of the overall dilution of the haemagglutinin, and that extrapolation of this linear portion of the curve to the control baseline gives a precise and reproducible estimation of the limiting dilution at which agglutinating activity is first detectable. By using the pattern method they found that the optimum pH for agglutination was 6.4, and this was confirmed by sedimentation

enumeration, that the minimum pH for haemagglutinin stability was 7.6, and that adsorption of agglutinin to erythrocytes in a mixture which was held stationary at room temperature obeyed a 'percentage law' in which a constant percentage of the initial agglutinin was adsorbed, regardless of the initial concentration, when the concentration of erythrocytes was held constant.

Cameron & Bradish characterized five virus suspensions along three different dimensions, physical particle, infective units, and haemagglutinating units and compared the results obtained. The concentrations of physical units varied from 1.0×10^{10} to 2.0×10^{13} per ml. in the suspensions tested. The estimates of physical particles per infective unit varied from 9.4 to 32.0, the higher values tending to be associated with the higher physical particle concentrations. The estimates of physical particles per RBC-RBC bond varied from 2.6 to 83, the higher the initial physical particle count the higher the obtained estimate of the ratio with one exception, at the 1.9×10^{13} level. The ratio of infective units to haemagglutinating units was not given, but when calculated from the data presented, they were found to vary from 0.86 to 8.33, with no clear evidence of any relationship between ratios and physical particle concentration. In addition, they found that monomers were observed at all haemagglutinin concentrations. They found that 16% of the total erythrocytes counted at a dilution of 1:8 were monomers compared with 39% at a dilution of 1:2,560; a dilution giving 75% agglutination in a pattern test.

Cameron & Bradish's interpretation of the above results is as follows:

1. The presence of monomers at high agglutinin concentrations is due to saturation with agglutinin, which inhibits the formation of aggregates by these cells.
2. The extent of bond-formation is a function of initial bond concentration and the rate of inactivation at the reaction temperature.
3. As the concentration of physical particles increases, there is a falling efficiency of agglutination as an increasing number of virus particles are wastefully adsorbed and sterically hindered in reactions at higher concentrations of agglutinin.
4. Three to ten particles are required initially for the formation of a bond. This is not an estimate of the number of particles per bond, but is the sum of the number of particles per bond, the number wastefully adsorbed to unfavourable sites on the erythrocytes, the number sterically hindered from bond formation, the number adsorbed to the walls of the vessel, and the number lost due to inactivation during the reaction time.

Levine et al. (1953) compared erythrocyte-virus mixtures and erythrocyte suspensions free of virus densitometrically to determine the proportions of cells remaining as monomers. It was assumed that the cells not accounted for as monomers had settled out as dimers, and that each dimer represented a single agglutinin particle. This system was assumed to give an absolute evaluation of the agglutinin concentration

in the suspension. Levine and his co-workers were using influenza-virus and one HA particle, as assayed by their method, was calculated to be equivalent to 0.41 egg-infecting particles and 1.33 physical particles. As stated above, the observation by Cameron & Bradish (1972) of aggregates at least as high as 10 would appear to invalidate the fundamental hypothesis on which this work was based, the 'dimers only' hypothesis, so that the only value which may be seriously considered is that of 3.24 physical particles being equivalent to one egg-infecting unit.

MATERIALS AND METHODS

Alsever's solution: Prepared according to the procedure recommended by Kalter (1963).

Phosphate Buffered Saline (PBS): This was prepared according to the formula given by Kalter (1963), but omitting the calcium and magnesium chloride.

Dextrose-Gelatin-Veronal Buffer (DGV) and Hepes Buffer (HSAG:Hepes-Saline-Albumin-Gelatin): Purchased from Flow Laboratories, Inc., Rockville, Maryland, U.S.A.

Kaolin: A 25% suspension of washed kaolin in HSAG was supplied by Flow Laboratories, Inc.

Bovine Albumin: Obtained as a crystalline powder from General Biochemicals; it was prepared as a 10% solution in PBS. This was stored at 4°C after filtration through a 0.4μ membrane. Freezing rendered the solution less effective as a stabilizing agent for erythrocytes in a tube haemagglutination test.

Microtitre plates for HA and HI tests: Disposable 96-well plates were supplied by ICN Canada Ltd., Montreal, P.Q.

Pipettes and test-tubes: Disposable glass pipettes and test-tubes were used at all times. This avoided any uncertainty as to the cleanliness of the equipment.

Centrifuge tubes: These were Kimble disposable 15 ml. conical plastic tubes.

Room temperature varied between 70°F and 82°F.

Eggs: Fertile eggs were purchased newly laid and stored at 4°C until laid down for incubation. For development, the eggs were placed in an incubator at 101.5°F, ±.5°F with the relative humidity maintained at >90% and constant fresh-air ventilation. Hatched chicks were from the same supplier as the eggs.

Chick erythrocytes: These were obtained fresh on the morning of the day of use. For details, see text.

Standardization of chick erythrocytes: Erythrocytes which had been thrice-washed using DGV at room temperature were made up to an approximate 1.0% suspension in DGV. A 1:15 dilution of the suspension was evaluated photometrically and the transmittance converted to cell concentration by reference to a standard curve. The standard chick erythrocyte dose for a microtitre well was one drop (0.025 ml.) of a 0.3% suspension, (4.0×10^5 cells per well), in DGV.

Human erythrocytes: Fresh human blood was obtained in a heparinized capillary, 0.15 ml. by finger-prick on the day required, washed three times in 15 ml. quantities of PBS and made up to the required concentration. This was standardized by counting 18 samples of 0.1 mm^3.

Human embryo lung cells: Derived from a human foetus of approximately 12 weeks of development, they were stored frozen at -80°C at the 9th pass level. For use, an ampoule of cells was washed once in 15 ml. of Eagles MEM medium containing 10% of fetal calf serum and seeded into 250 ml. bottles, 3×10^6 living cells per bottle. After four to six days

of incubation at 37°C, the bottles were trypsinized and the cells seeded into Microtest II plates, 96 wells per plate, 2×10^4 cells per well in 0.2 ml. The plates were sealed with adhesive covers and incubated at 37°C. When useable cell sheets had developed (two days), the cultures were washed twice with a serum-free medium, inoculated with 0.025 ml. of virus suspension and 0.2 ml. of serum-free medium was added to each well. The cultures were incubated at 36°C until haemadsorption tests were carried out on the wells.

Rubella haemagglutinating antigen: This was kindly supplied by Dr. J. Polley, Bureau of Virus Diseases, Ottawa. It was a purified preparation freeze-dried in 0.5 ml. quantities in glass ampoules and sealed in vacuo and stored at 4°C.

Rubella HA antigen titration: All equipment, materials, and operations were at 4°C. Serial two-fold dilutions of re-constituted antigen were prepared in 0.5 ml. quantities in tubes using HSAG as the diluent. Each dilution was dropped out in a plate using standard droppers, 0.025 ml. per well, to give six replicates of the dilution series. To each well was added 1 drop of HSAG and the plate was allowed to stand for two hours (see text); one drop of 0.3% chick erythrocyte suspension was added to each well and the contents mixed by shaking the plate. The titrations were read after standing for four to 18 hours at 4°C. The end-point was taken as that well showing "50%" agglutination.

Rubella HI test: All sera were treated with kaolin and chick erythrocytes according to the instructions given by Flow Laboratories (1973). A known positive and a known negative serum were treated with each group of sera to be tested. All materials, equipment and operations were at 4°C.

Two-fold serum dilutions were prepared, in micro-titre plates, from 1:10 to 1:320 in duplicate. To one set of dilutions was added rubella-virus HA antigen in HSAG, six units of antigen, one drop per well, and to the other, one drop of HSAG. A back titration of the antigen and a cell control were included in each test. After standing for two hours, one drop of 0.3% chick erythrocytes was added to each well and the contents mixed by shaking the plate. The results were read after standing for 4-18 hours. There should be no agglutination in the serum-HSAG wells, the cell controls should show a clean 'button', and the back-titration should show >4, but less than eight units of antigen to be present in the test. The positive wells should show smooth, even plaques of agglutinated cells. The serum titre is taken to be that dilution showing less than '50%' agglutination.

Goat anti-human IgG serum: Supplied by Hyland Co. Inc., Lot # 8201R002A1, antibody potency 3.36 mg. per ml.

Complement: Supplied by Microbiological Associates, Inc., Bethesda, Md., as a 2.0 ml. volume of fresh guinea pig serum, free of antibody, freeze-dried in a rubber-capped vial and stored at 4°C.

Influenza-virus infected allantoic fluid: Eggs at the 10th or 11th day of development were inoculated intra-allantoically with 0.1 ml. of a 10^{-6} dilution of seed virus, corresponding to 10-50 egg infecting units of virus. The eggs were incubated at 37°C for 40-49 hours and 1.0 ml. of allantoic fluid harvested from a warm egg, avoiding contamination with blood, for use in the test situation.

Statistical computations: Standard error of the mean and t-Test computations were carried out using a programmed SCM Cogito 1016 PR desk computer. For other statistics, the formulae given by Hardyck and Petrinovich were used.

Other procedures are given in the appropriate places in the text.

EXPERIMENTAL

The experimental work is described in four parts:

1. Empirical observations on some of the properties of rubella-virus haemagglutinin with work designed to develop an HI test capable of giving the most sensitive possible, yet reproducible, estimate of the immune status of a patient with respect to this virus.

2. The construction of a random distribution and interaction model of the HA process to serve as a frame of reference by which to interpret the results of aggregate evaluation.

3. Experimental work carried out with the object of simultaneously validating the theoretical model and developing a test by which an absolute quantification of haemagglutinin may be made.

4. An evaluation of the relationship between haemagglutinin binding-units, calculated by monomer-count, and infective units, tube HA units, and haemagglutinin binding-units calculated by direct evaluation of the aggregate population in a reaction mixture.

EXPERIMENTAL - PART 1A

Rubella-virus Haemagglutination and Haemagglutination-inhibition

Erythrocytes

The ideal condition of erythrocytes to be used in a serological test is that they should be fresh at the time of use and should have been washed free of host material using the fluid in which the cells are to be suspended for use in the test. If this condition is met, then many of the variables which can confound the test results, such as variation in the age of the erythrocytes, storage in less-than-ideal suspending fluids, and effects of temperature during storage or transportation will be eliminated.

The techniques for carrying out the rubella-virus HI test in current use call for using erythrocytes from day-old chicks, from geese, or from pigeons. All of the species of erythrocytes mentioned entail problems of supply. Few laboratories maintain constantly available specimens of any of the avian species required, and human group 'O' blood must usually be obtained as out-dated blood from the local blood-bank since group 'O' staff-members seem reluctant to be bled on a regular basis.

Most virus laboratories carry fertile eggs at varying stages of development and the rational answer would appear to be to allow unused eggs to hatch to provide day-old chicks to be heart-punctured. This solution, however, appears to involve marked emotional reactions on the part of many staff-members, whereas causing haemorrhage in fertile eggs invokes

no such response. Fertile eggs, therefore, were investigated as a source of suitable erythrocytes for use in rubella-virus HA and HI tests.

Fertile eggs, of ages from 13 days of incubation through to the point of hatching, were tested as sources of chick-embryo erythrocytes. The shells over the air sacs of eggs fresh from the incubator were chipped away and the chorioallantoic membrane torn to cause bleeding into the allantoic fluid. If the allantoic vein was easily available, this was torn too. The egg contents were stirred gently at intervals in order to mix the blood and allantoic fluid, and so avoid formation of a blood clot. When embryo activity had fallen to a negligible level, the allantoic fluid was harvested and the erythrocytes sedimented by centrifugation. They were washed three times in DGV and standardized for use in the test. With eggs of 16 days and older, debris derived from the allantoic fluid is usually present. This deposits as a discrete layer underlying the packed erythrocytes and may be either removed by pipette, or left undisturbed if the erythrocytes are resuspended very gently and removed for the first washing.

Hatched chicks, of ages varying from newly hatched through the seventh day post-hatching, were bled by heart-puncture and the blood mixed with twice its volume of Alsever's solution. The erythrocytes were separated by centrifugation, washed three times in DGV, and standardized for the test.

All of the above erythrocytes were tested in parallel for sensitivity for demonstrating rubella-virus HA antigen in a two-fold dilution test.

The results showed that the ratio of the lowest antigen titre obtained to the highest one was <2. Since a ratio of >2 is normally required in order that the result may be considered significant, the variation among the titres obtained was considered to be within the limits of the error of the test and not attributable to differences in sensitivity among the erythrocytes tested. A similar result was obtained when the same erythrocytes were tested in a full rubella-virus HI test using negative (<1:10), low titre (1:10), moderate titre (1:40), and high titre (1:160) sera, one at each level. The ratio of the lowest titre to the highest titre at each level was <2.

One 17 day-old egg yields approximately 0.35 ml. of packed erythrocytes. This is sufficient to absorb seven sera or to carry out 250 HI tests when using the microtitre system.

In conclusion, erythrocytes from embryos in shell may be used with confidence for the rubella HA and HI tests. Chick erythrocytes can be obtained fresh on the day required by the test, and the ideal conditions met, by making use of out-dated eggs that have been laid down against the eventuality of work being received in the laboratory which requires egg-inoculation procedures.

Rubella HI antigen stability

Problems have frequently been met in the area of antigen standardization using the highly purified rubella-virus HA antigen. These have usually occurred upon starting a new batch of freeze-dried material.

It appeared possible that the antigen may be unstable under the condition of being held at 4°C for two hours for the test. When the antigen standardization procedure was changed, and each new batch assayed under the conditions of the test, test-to-test variation was reduced to a level compatable with experimental error. The level aimed for was always six units of antigen and the antigen control titration normally showed between four and eight units.

An alternative possibility, that the agglutinin elutes off the cells during, or following, the settling process in the plates was investigated as follows:

1. An HA titration was carried out making four replicates in separate plates. Two plates were allowed to settle at 4°C and two plates allowed to settle at room temperature. Settling time was two hours.

   There was no detectable difference in the HA titres derived from the tests at either temperature.

2. The cells in the plates prepared above were resuspended in the wells by shaking the plates. Re-settling for two hours was allowed at the same temperatures as previously.

   There was no change in the antigen titre at either temperature.

Two conclusions were drawn from the above data:

1. Antigen adsorbed on to erythrocytes is stable both at 4°C and at room temperature. This result is compatible with the results obtained by Channock & Sabin (1954) who reported the haemagglutinin of several Togaviruses to be stable when adsorbed on to erythrocytes. Work arising from this observation is reported later under 'HA reversal'.
2. There may be an unstable component in the antigen which leads to anomalies when the results of assay of fresh antigen are compared with those of assay of the same antigen stored for a period of hours at 4°C.

Support for the antigen-instability hypothesis was obtained in an antigen-comparison experiment.

Samples of five batches of freeze-dried antigen were received from Dr. J. Polley for cross-checking with his own assay results. All materials, equipment, and operations were at 4°C. An ampoule of each batch of antigen was re-constituted with distilled water, after which it was diluted in HSAG. The dilution series used was a 1:10 to 1:640 doubling series in 0.5 ml. quantities. Six replicates of each dilution were made by transferring one drop (.025 ml.) to each of six wells in a microtitre plate. To each well was added one drop of 0.3% chick erythrocytes (1.5×10^7 cells/ml.) in DGV. The plates were allowed to settle at 4°C. The dilution tubes were held for four hours at 4°C, after which time six replicates were dropped out as previously, erythrocytes added, and all plates were read after standing overnight at 4°C.

The results, shown in Table 1, indicate that only a two-fold variation in titre among the batches was obtained on initial titration. The replicate results within each batch were entirely consistent so it is possible that the two-fold variation is a reflection of true differences among the materials titrated. The end-points, set at the single + level, varied from 1:80 to 1:160.

After the dilutions had been held for four hours at 4°C, there was a significant fall in the titre of each material. As in the first set of titrations, there was total internal consistency in each group, but the differences between the batches were more pronounced, probably reflecting variable stability characteristics among them.

The test-re-test results for each batch showed a fall in antigen titre varying from ∿4-fold to ∿10-fold.

A detailed investigation of the stability of purified rubella-virus HA antigen was carried out. All materials, equipment, and procedures were at 4°C.

In the first experiment, an aliquot of antigen which had been re-constituted, distributed in aliquots, and stored at -80°C was thawed and immediately diluted in HSAG to give dilutions from 1:15 x 1.5, to 1:576.65. The dilutions were dropped out into a plate at varying intervals of time and one drop of .3% chick cells in DGV was immediately added to each. The times were: 1-5 minutes after thawing, 15 minutes after thawing, and every 15 minutes thereafter up to 120 minutes after

TABLE 1

LEGEND

Table 1: Stability of purified rubella-virus HA antigen

The results of duplicate titrations of five different batches of purified rubella-virus HA antigen immediately after being re-constituted and after being held for four hours at 4°C after being re-constituted.

TABLE 1

Batch	No. of replicates	Titre immediately after reconstitution	Titre after being held for four hours at 4°C	%Δ
3-72	6	1:160	1:40	-75
10-72(a)	6	1:160	1:15	-91
11-72	6	1:80	1:20	-75
12-72	6	1:160	1:20	-88
10-72(b)	6	1:80	1:13	-84

thawing. The dilution showing a single + reaction was taken as the end point, and the reciprocal of this was used as the activity level. At the 75 minutes level, the single + reaction was between two of the dilutions used so the mean of the reciprocals of those two dilutions was used.

Table 2 shows the activity level of the antigen and the proportion of the activity at 1.5 minutes remaining after each time interval. Figure 1 shows the activity levels on the ordinate and time after thawing on the abcissa. It appears that there was a rapid decay of activity during the first 90 minutes of the experiment. Since zero time in this experiment is the time after the original re-constituting, distributing and thawing, a second experiment was carried out to test the dynamics of the degradation of newly constituted material.

For the second experiment, two ampoules, totalling 1.0 ml. of rubella-virus HA antigen, were re-constituted with distilled water and pooled. This material was immediately distributed, one drop (.025 ml.), into each of a series of prepared volumes of HSAG and one drop into each of a series of prepared volumes of .15% chick erythrocytes in 50:50 HSAG:DGV. The resulting dilutions in the HSAG were 1:22.5 x 1.5, to 1:865. The dilution in the tubes containing erythrocytes were equivalent to the dilutions in HSAG in the above series, with an equal volume of .3% chick erythrocytes in DGV added. An aliquot of each was transferred to room temperature. The HSAG dilutions were dropped out after various time intervals and one drop of .3% chick erythrocytes in DGV added.

TABLE 2

LEGEND

Table 2: Stability experiment #1

The activity level of frozen re-constituted purified rubella-virus HA antigen held at 4°C for varying times after being thawed.

TABLE 2

Minutes after being thawed	Activity level	Activity level as a proportion of the 1.5 minutes activity level
1.5	126	1.00
15	114	0.90
30	76	0.60
45	51	0.40
60	34	0.27
75	28	0.22
90	23	0.18
105	23	0.18
120	15	0.12

FIGURE 1

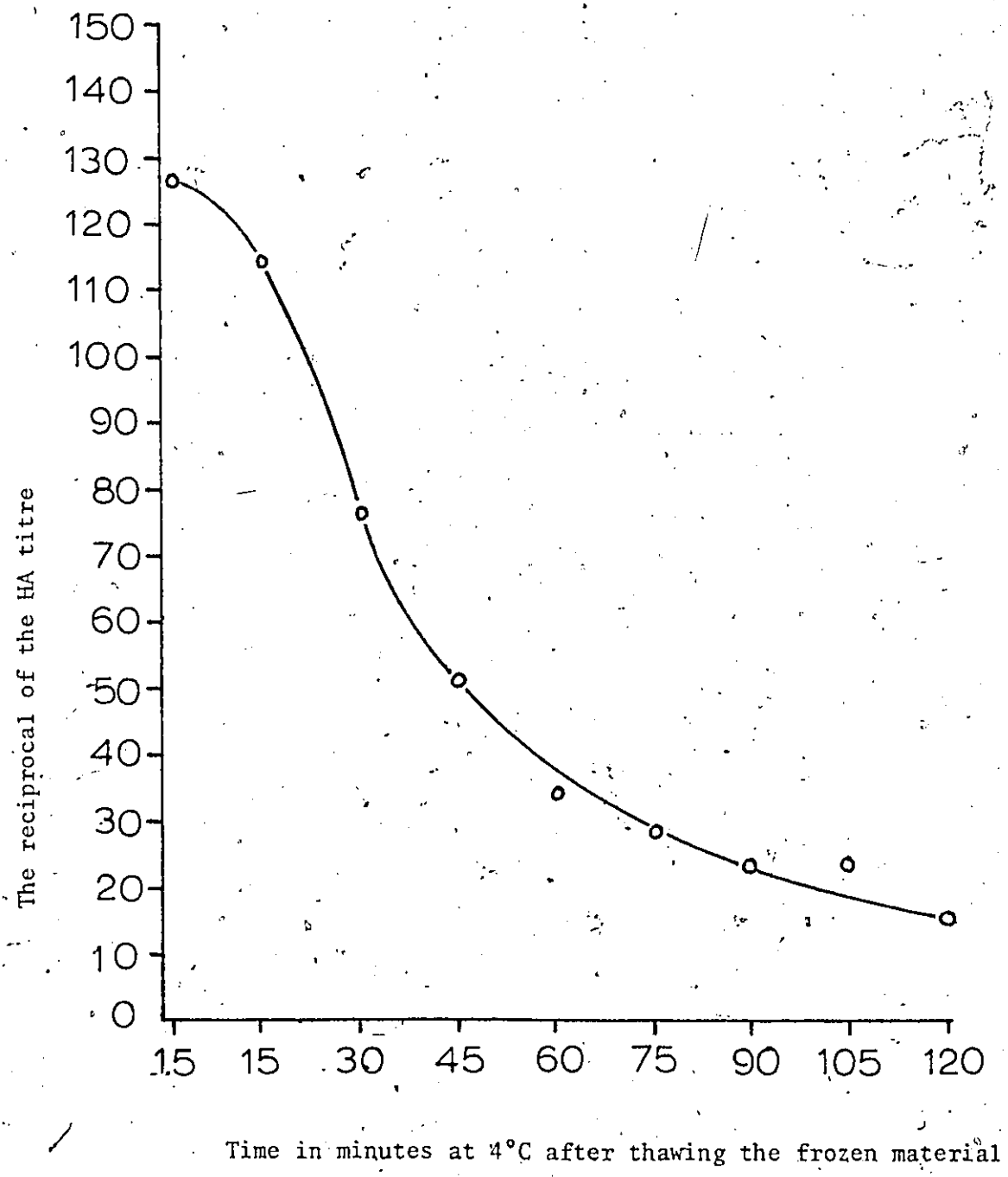
LEGEND

Figure 1: Rubella-virus HA stability. The activity level of purified HA antigen after varying holding times at 4°C. The material had been re-constituted and frozen at -70°C. The point in time immediately after thawing constitutes zero minutes in this experiment.

Ordinate: The reciprocal of the HA titre.

Abcissa : Time in minutes at 4°C after thawing the frozen material.

FIGURE 1


150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
The reciprocal of the HA titre
1.5
15
30
45
60
75
90
105
120
Time in minutes at 4°C after thawing the frozen material

The sensitized chick erythrocytes were dropped out, two drops (.05 ml.), per well, after varying time intervals at the two holding temperatures. In addition, at one hour and 24 hours, the sensitized erythrocytes stored at 4°C were dropped out into plates containing dilutions of a kaolin treated positive rubella serum with a titre in the standard test of 1:40. Two drops of sensitized cell suspension were added to each serum well. All plates were read after settling overnight. Activity levels were recorded as in the previous experiment.

A number of things are apparent from the results of this experiment (Table 3 & Figure 2):

1. The instability of rubella haemagglutinin at 4°C is confirmed.
2. The degree of decay is far greater when the starting value is nearer the true value than was apparent when the antigen had been re-constituted, frozen and thawed, and presumably already partially degraded.
3. Adsorption on to erythrocytes is very rapid (note the high activity level indicated in Table 4), and in this form the antigen is very stable at 4°C and relatively stable at room temperature. This confirms the finding reported above. These results are shown in Table 4 and illustrated in Figure 2.
4. The degraded antigen is not totally decayed, but in some way rendered 'incomplete', as indicated by the long 'tails' of partial HA after all time periods (see Table 5). This 'incomplete' antigen is degraded to approximately 0.25 of its total initial activity after two hours at 4°C.

TABLE 3

LEGEND

Table 3: Stability experiment #2.

The activity level of freshly re-constituted purified rubella-virus HA antigen held at 4°C for varying times after being re-constituted.

TABLE 3

Minutes after being re-constituted	Activity level	Activity as a proportion of activity at time zero	Log_{10} activity level
0	577	1.00	2.76193
2	480	0.83	2.68124
6	171	0.30	2.23300
11	114	0.20	2.05690
15	76	0.13	1.88081
25	34	0.06	1.53184
35	34	0.06	1.53184
48	28	0.05	1.44716
60	28	0.05	1.44716
80	23	0.04	1.36173
100	23	0.04	1.36173
120	23	0.04	1.36173

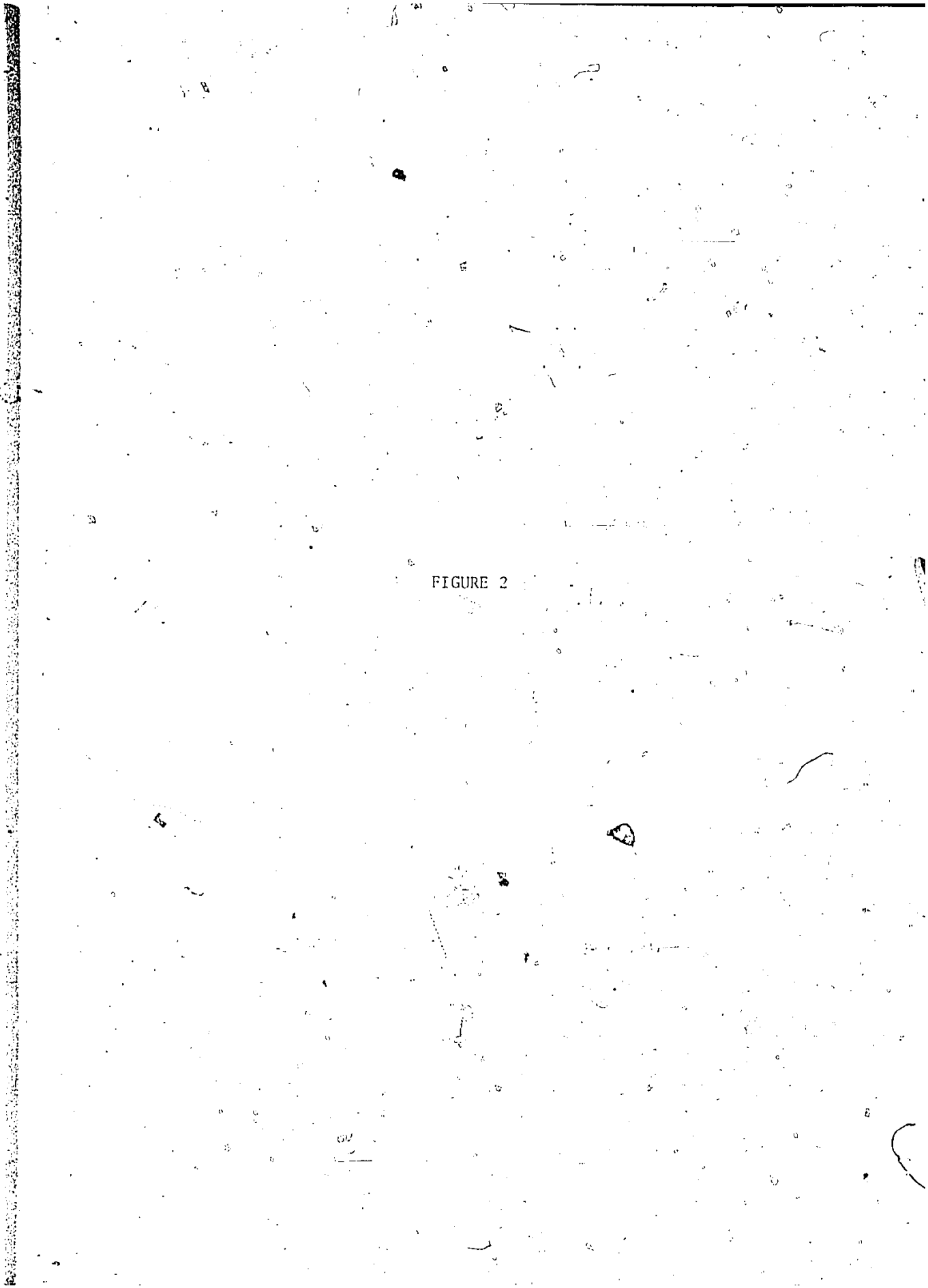


FIGURE 2

LEGEND

Figure 2: Rubella-virus HA stability. The activity level of free purified HA antigen after varying holding times at 4°C compared with the activity level of antigen adsorbed to erythrocytes after varying holding times at 4°C and room temperature. The point in time at which the antigen was re-constituted is zero-hours.

o ——— o = free antigen at 4°C

● ——— ● = adsorbed antigen at room temperature

+ ——— + = adsorbed antigen at 4°C

Ordinate: The reciprocal of the HA titre.

Abcissa : Time in hours after re-constituting the antigen.

FIGURE 2

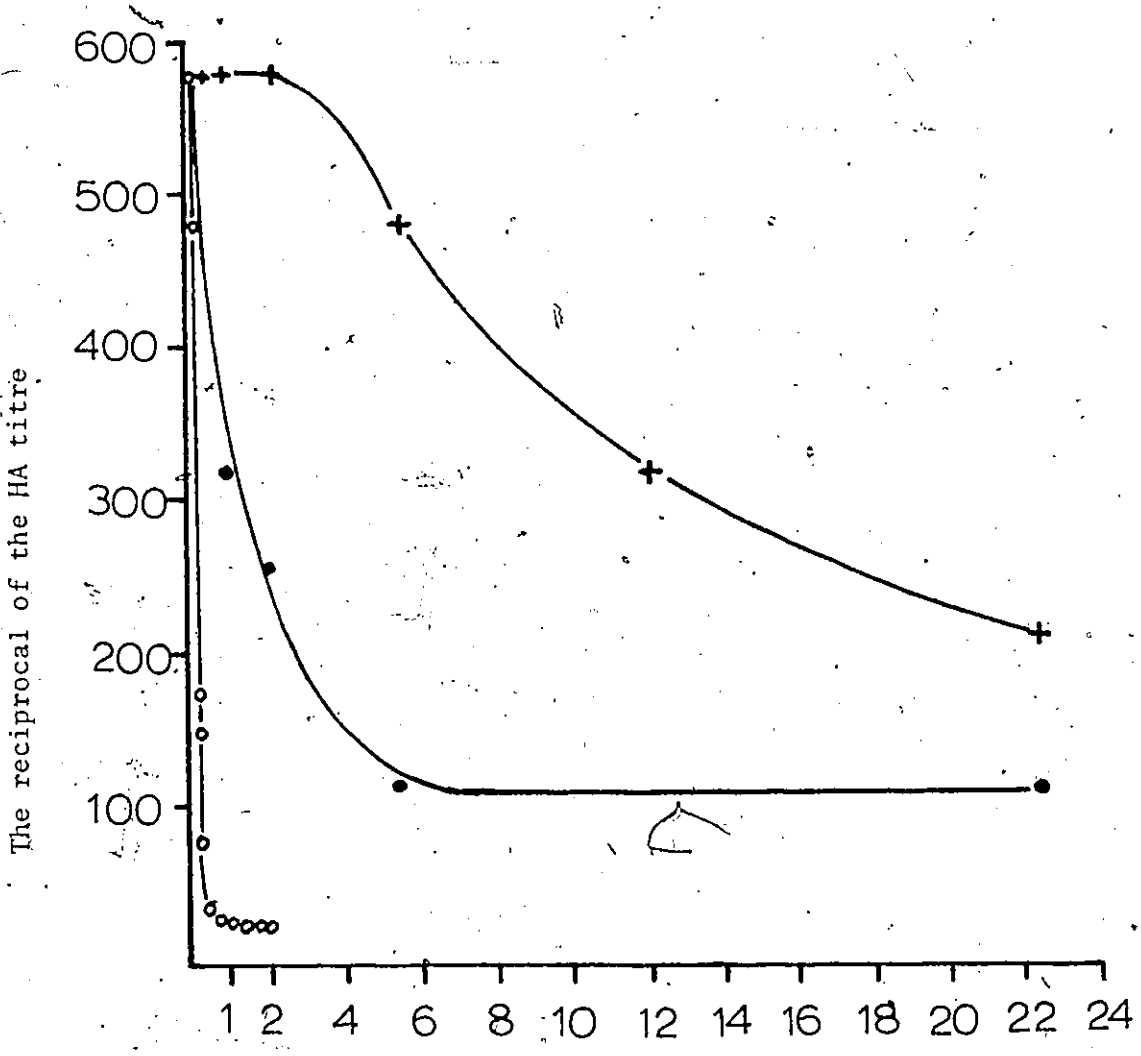

600
500
400
300
200
100
The reciprocal of the HA titre
1 2 4 6 8 10 12 14 16 18 20 22 24
Time in hours after re-constituting the antigen

TABLE 4

LEGEND

Table 4: Stability of freshly re-constituted purified rubella-virus HA antigen. The antigen was adsorbed on washed chick-embryo erythrocytes and stability was indicated by the ability of the antigen-erythrocyte mixtures to form positive patterns in wells at varying times after adsorption. The mixtures were made at 4°C and held at 4°C and 25°C.

TABLE 4

Hours after re-constituting the antigen and making the antigen-RBC mixtures	Activity level			
	4°C		25°C	
	Aggl. units	Units as a proportion of time zero activity	Aggl. units	Units as a proportion of time zero activity
0	577	1.00	578	1.00
1/6th	577	1.00	NT	NT
1	577	1.00	320	0.56
2	577	1.00	256	0.44
5 1/2	480	0.83	214	0.37
12	320	0.56	NT	NT
22 1/2	214	0.37	114	0.20

TABLE 5

LEGEND

Table 5: 'Incomplete' antigen remaining after decay of freshly re-constituted antigen at 4°C.

++ = Complete agglutination

\+ = 50% of " (approximately)

$\pm$ = 25% " " "

tr = 10% " " "

tr = =<10% " " "

TABLE 5

Time in minutes after being re-constituted	Reciprocal of the antigen dilution									
	22.5	33.8	50.6	75.9	113.9	170.9	256.3	384.4	576.7	865.0
0	++							++	+	tr
2	++	++	++	++	++	++	++	+-	tr	tr
6	++	++	++	++	++	+	+-	+-	tr	tr
11	++	+-	+-	+-	+	+-	+-	tr	tr	-
15	+-	+-	+-	+	+-	+-	tr	tr	tr	-
25	+-	+	+	+	+-	+-	tr	tr	-	-
35	+-	+	+	+-	+-	tr	tr	tr	-	-
48	+-	+-	+-	+-	+-	tr	tr	tr	-	-
60	+-	+-	+-	+-	+-	tr	tr	-	-	-
80	+	+-	tr	tr	tr	tr	tr	-	-	-
100	+	+-	tr	tr	tr	tr	tr	-	-	-
120	+	+-	tr	tr	tr	tr	tr	-	-	-

'Incomplete' antigen

5. The agglutination of the erythrocytes can be reversed using specific antiserum. This result confirms previous experience with influenza-virus, (unpublished data), that haemadsorption can be specifically reversed with immune globulin. It can be seen from Table 6 that the test gives an antibody titre at least as high as that obtained by using the HI test. Furthermore, although the optimum antigen concentration is 1.5 to 3 units, gross excess of antigen does not appear to invalidate an interpretation of the antibody titre to within a two-fold dilution of that obtained using the optimum antigen level. This method of antibody estimation will be examined in more detail later.

Figure 3 shows the $\log_{10}$ values of the activity levels of free antigen, during degradation with time, at 4°C. It appears that the degradation was exponential from 48 to 120 minutes; however, there was a very rapid degradation taking place during the first 48 minutes.

Analysis of the stability data. It was hypothesized that this data could be satisfied by postulating the existence of two types of antigen.

1) A high stability (HS) antigen represented by the exponential curve from 48 to 120 minutes.
2) A low stability (LS) antigen which has entirely degraded after 48 minutes at 4°C.

If this hypothesis is supported, then the 0-48 minutes section of the curve comprises the combined degradation curves of both the LS and HS antigens.

TABLE 6

LEGEND

Table 6: Immune reversal of agglutination

Rubella-virus HA/chick embryo erythrocyte system was added to kaolin-RBC treated rubella-immune human serum. All processes and reactions were at 4°C.

Degrees of scoring as in Table 5.

TABLE 6

Reciprocal of antigen dilutions	Antigen units	Reciprocal of serum dilutions 10	20	40	80	160	320	640	1280	2560	No. serum
865	0.4	-	-	-	-	-	-	-	tr	tr	tr
577	0.7	-	-	-	-	-	-	tr	tr	tr	tr
384	1.0	-	-	-	-	-	+	+-	+-	+-	+-
256	1.5	-	-	-	-	+	++	++	++	++	++
171	2.3	-	-	-	tr	+-	++	++	++	++	++
114	3.4	-	-	-	tr	+-	++	++	++	++	++
76	5.1	tr	tr	tr	+-	++	++	++	++	++	++
51	7.6	+	+	+	++	++	++	++	++	++	++
34	11.4	+	+	+	++	++	++	++	++	++	++
23	17.1	+	+	+	++	++	++	++	++	++	++

FIGURE 3

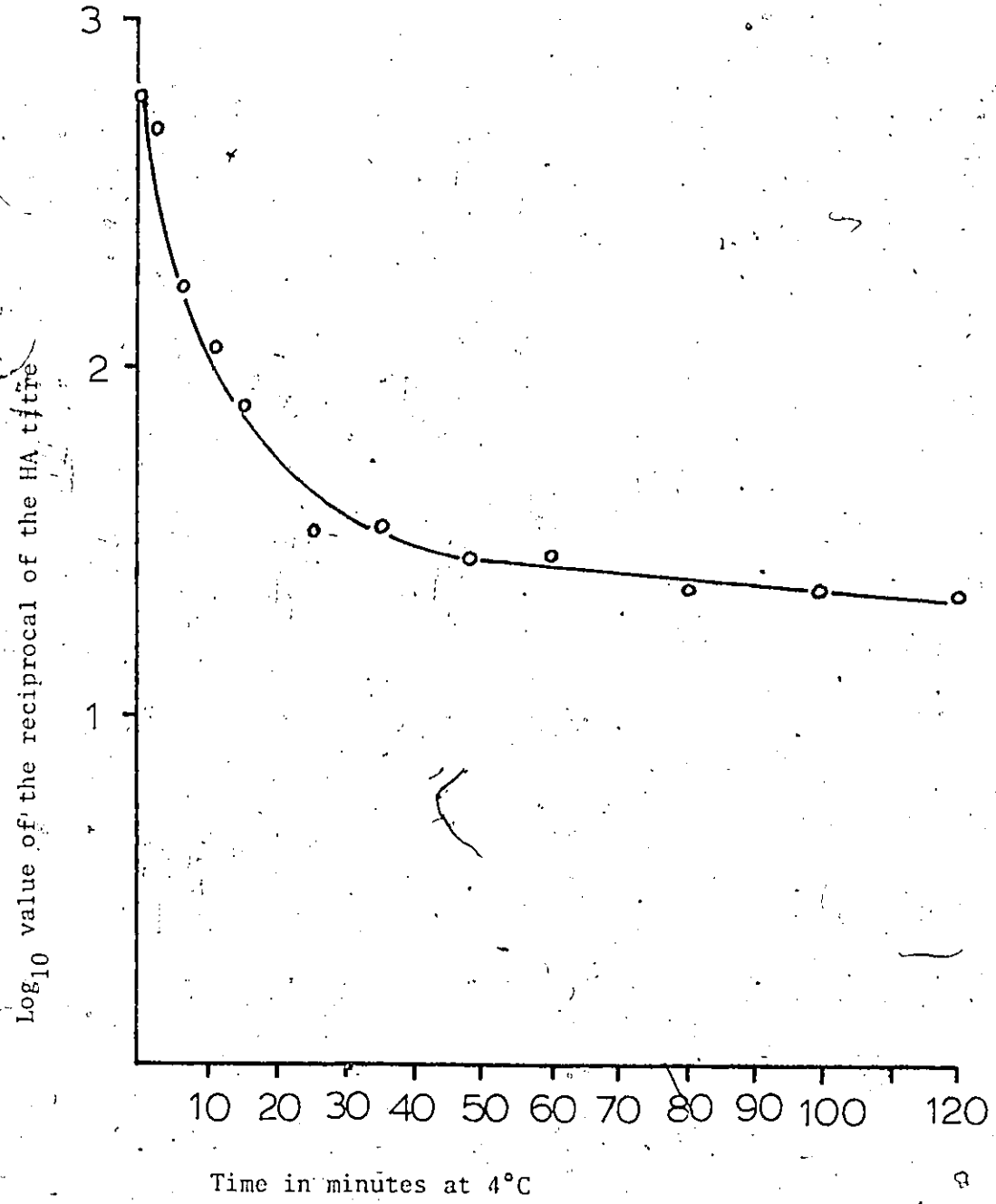
LEGEND

Figure 3: Rubella-virus HA stability. The $\log_{10}$ values of the activity level of free purified antigen after varying holding times at 4°C.

Ordinate: $\log_{10}$ value of the reciprocal of the HA titre.

Abcissa : Time in minutes at 4°C.

FIGURE 3


3
2
1
Log_{10} value of the reciprocal of the HA titre
10 20 30 40 50 60 70 80 90 100 120
Time in minutes at 4°C

The situation was analyzed mathematically as follows:
The best-fit line was calculated for the 48-120 minute $\log_{10}$ values.
Y' values were calculated for the times 0-48 minutes.
The antilogs of the calculated Y' values (representing the HS activity components) were subtracted from the raw score values for the 0-48 minutes period. The remainders represent the LS components of the raw score data. The $\log_{10}$ values of the LS component were plotted with the best-fit line along with those for the HS component. The result of this analysis is shown in Figure 4.

Since the data can be analyzed into two components, each degrading exponentially, the hypothesis would appear to be supported.

Experience suggests that the proportions of the total activity represented by the LS and HS components is variable. This can lead to problems in carrying out the conventional test which have been overcome, as stated previously, by titrating the antigens under the conditions of the test, when the LS component degrades beyond detection and only the HS component is used in the test. It would be better to stabilize the antigen, and use it in complete form, than accommodate the test to a mixture of stable and degraded antigen. This is particularly so where the degraded antigen is likely to constitute the major portion of the antigenic material used in the test and may possibly interfere in the test reaction. Adsorbing the antigen on erythrocytes appears to offer a stabilizing system applicable to rubella-virus HA antigen. The feasibility of using adsorbed antigen for rubella antibody assay was, therefore, investigated as a factor in the haemagglutination-reversal test reported in the next section.

FIGURE 4

LEGEND

Figure 4: Rubella-virus HA stability. Plot of the two exponential curves which, when summated, satisfy the empirical data for the decay of purified antigen at 4°C.

+ — + — o — o = Best fit line calculated for the point 48 minutes to 120 minutes (a = 1.50851 b = 0.00138) and extended to the Y intercept. This is termed the High Stability (HS) component.

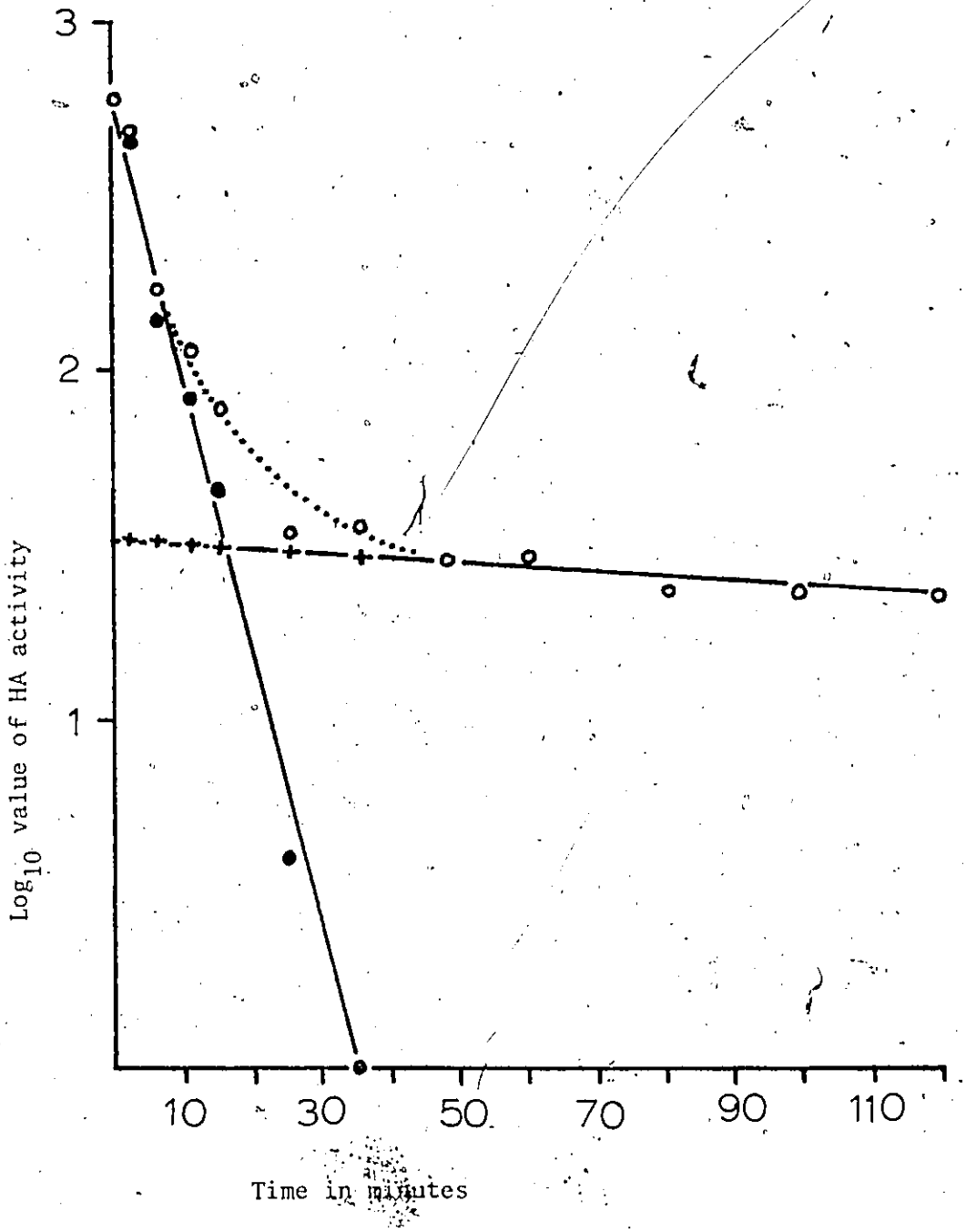
o —— o = The best fit line for the difference between the points corresponding to the HS component and the raw scores (a = 2.75469, b = 0.08042). This is termed the Low Stability (LS) component

o —— o = The raw score datum points.

Ordinate: Log_{10} value of HA activity

Abcissa : Time in minutes

FIGURE 4


3
2
1
Log10 value of HA activity
10
30
50
70
90
110
Time in minutes

EXPERIMENTAL - PART 1B

Some Aspects of the use of a Haemagglutination Reversal (HAR) Test

It was shown in Part 1A (Table 6), that rubella-virus haemagglutination is reversed by antibody and that the optimum sensitizing dose-range of antigen is 1.5 to 3.4 units.

Experiments were carried out to determine:

1. The conditions under which the natural chick-cell lysins, found to be present in human serum, function and the method of their removal.
2. The effect of non-specific inhibitors on the test.
3. The effect of temperature on the test.
4. The possibility of using goat anti-human IgG in the test to expose reacting IgM antibody.

Chick erythrocyte lysins in human sera

It appears that human sera contain lysins active against chick erythrocytes.

An experiment was carried out to compare the effects of different treatments and their interactions on human lysins for normal chick erythrocytes, the effect of time on the lytic process, and whether the lytic action is complement dependent.

Human serum was from a donor (JP) whose serum was known to be negative in the routine HI test at 1:10 dilution, but who had a high level of chick erythrocyte lysins. Complement was an untreated 1:20 dilution of re-constituted lyophilized guinea pig serum (approximately three units of complement).

Chick erythrocyte adsorption was carried out by adding to the serum 1/20th volume of 50% chick embryo erythrocytes, allowing to react at 4°C for one hour, then removing the erythrocytes by centrifugation. Inactivation was by placing 0.5 ml. of serum in a 12 x 75 mm tube in a waterbath at 56°C for 30 minutes. For kaolin treatment, 1 ml. of 25% suspension of kaolin in HSAG was added to 0.2 ml. of serum and 0.8 ml. of HSAG. The mixture was left at room temperature for 30 minutes, with intermittent shaking, then centrifuged in the cold to remove the kaolin. All sera were adjusted to give a dilution of 1:10 to be the first tube in a doubling dilution series from 1:10 to 1:160. Serum dilutions were made in a microtitre plate. To each well were added two drops of 0.2% normal chick embryo erythrocytes in DGV at room temperature.

The results shown in Table 7 indicate that the normal lysins are slow acting at room temperature, the titre rising from 1:20 at the one hour reading to 1:160 after standing overnight.

Treatment with erythrocytes removed all of the agglutinins, but was less effective in removing the lysins. Inactivation at 56°C destroyed all of the lytic activity of the serum; kaolin was also

TABLE 7

LEGEND

Table 7: Removal of chick-erythrocyte lysins and agglutinins from human serum by various treatments.

Rubella-virus HA antigen was not present in this test. Guinea-pig complement was added to show C' independence of the lytic process. All processes and equipment were at room temperature.

L = Lysis, A = Agglutination.

TABLE 7

Serum treatment	Reciprocal of serum dilutions														
	10	20	40	80	160	10	20	40	80	160	10	20	40	80	160
No treatment	L	L	A	A	A	L	L	L	LA	A	L	L	L	A	A
Erythrocyte absorbed	-	-	-	-	-	Trace L	-	-	-	-	L	L	L	-	-
56°C x 30 minutes	No reaction					No reaction					No reaction				
Kaolin															
Inactivated + GP C'															
C' only															
No serum															
	1 hour					2 hours					20 hours				
Time from setting up to reading the test															

effective for this purpose. Addition of guinea pig complement did not reactivate the heat inactivated lysins so it is concluded that the effectiveness of heating is not due to complement inactivation and that the lysins do not function in a complement mediated reaction.

Lysis of erythrocytes has not been observed at 4°C.

Non-specific inhibitors and temperature in HA reversal

In order to determine whether the non-specific inhibitors in human serum, for the HI test, are active in the HAR test, a rubella positive and a negative serum were each treated by two methods.

1. Kaolin and erythrocytes.
2. Erythrocytes and 56°C for 30 minutes.

The resultant four sera were tested at dilutions from 1:10 to 1:1280 against chick erythrocytes sensitized with 2.3 units of rubella-virus HA antigen. The tests were in duplicate, one set being at 4°C and the other at room temperature. It was found that there was no reversal with the negative serum with either method of serum treatment, regardless of the temperature at which the test was carried out (see Table 8). These test results also confirm the equal effectiveness of kaolin and inactivation at 56°C in eliminating the lysins present in the serum.

The HAR test appeared to be more sensitive in assaying antibody level than the conventional test. Sensitivity was most marked at room temperature, where the antibody titre was 1:640, as against 1:80 by the conventional HI test method.

LEGEND

Table 8: Rubella-virus HA-reversal test.

Determination of the effect of two serum treatments on the sensitivity of the test, carried out at two temperatures using a positive and a negative serum selected from sera tested by the conventional rubella-virus HI test.

4°C = all equipment and procedures at 4°C

RT = all equipment and procedures at room temperature

Antigen dose = 2.3 sensitizing units

Degrees of scoring as in Table 5.

TABLE 8

Serum	Serum treatment	Temperature	Reciprocal of serum silutions							
			10	20	40	80	160	320	640	1280
+	Kaolin & cells	4°C	-	-	-	-	-	+	++	++
-			++	++	++	++	++	++	++	++
+		RT	-	-	-	-	-	-	+ -	++
-			++	++	++	++	++	++	++	++
+	Cells & 56°C	4°C	-	-	-	-	-	-	+	++
-			++	++	++	++	++	++	++	++
+		RT	-	-	-	-	-	-	+ -	++
-			++	++	++	++	++	++	++	++
+	Standard HI test	4°C	-	-	-	+	++	++	++	++
-			++	++	++	++	++	++	++	++

The use of anti-human IgG serum

It was considered that, with the increased sensitivity of the antibody assay test, it may be possible to neutralize the IgG antibody fraction in a serum and directly assay the IgM antibody titre. The advantage of such a system for rubella-virus antibody data interpretation by medically qualified personnel is self-evident.

Goat serum and the HAR test. Goat anti-human IgG serum (Hyland Co.), was assessed for performance in a possible test. The serum was inactivated at 56°C for 30 minutes and absorbed with chick erythrocytes. It was tested against rubella sensitized cells in a reversal test at room temperature using 1.5 fold dilutions.

The serum was found to possess HAR activity to a titre of >1:30. Since this activity was removed by kaolin treatment, it was assumed that the reversal was non-specific (Table 9).

The non-specific factor system in the goat is apparently different from that in humans, in that it is capable of reversing agglutination, whereas the human non-specific factor is capable only of inhibition.

Human IgG-neutralization. Dilutions of human rubella-immune serum, heated at 56°C for 30 minutes and absorbed with RBC's, were mixed with dilutions of goat anti-human IgG serum which had been treated with kaolin, absorbed with RBC's and heated at 56°C for 30 minutes. The mixtures were allowed to react at 37°C for one hour, then chick erythrocytes sensitized with 2.1 units of agglutinin were added at room temperature and allowed to settle.

TABLE 9

LEGEND

Table 9: Rubella-virus HAR test.

Effect of different treatments on the non-specific-reversing factors present in non-rubella-immune goat serum. The test was carried out at room temperature using 2.3 sensitizing units of antigen.

I = inactivated at 56°C for 30 minutes.

E = absorbed with an equal volume of packed chick-embryo erythrocytes.

K = absorbed with the kaolin from five times the serum volume of 25% kaolin.

Degrees of scoring as in Table 5.

TABLE 9

Serum treatment	Reciprocal of serum dilutions →									No serum
	1.5	2.3	3.4	4.1	6.1	9.1	13.7	20.6	30.9	
I, E	-	-	-	-	-	-	-	±	±	++
I, E, K	++	++	++	++	++	++	++	++	++	++

The human serum showed normal HAR to a dilution of 1:137 or greater. The goat serum showed no HAR at 1:1.5 dilution (Table 10). Comparison of the rows in the checker-board reveals that the goat serum is effective in neutralizing the reversal effect of the human serum only at 1:1.5, the highest goat serum concentration, and then only with human serum dilutions of 1:33.8 and higher. Human serum, therefore, requires 23 times its own concentration of this particular anti-IgG serum for complete IgG neutralization.

A further feature revealed by this experiment is the re-activation of non-specific reversal by the serum mixtures. This suggests that the goat reversal factor consists of two fractions; one is removed by kaolin treatment and the other is not. The kaolin-sensitive fraction is common to both human and goat serum, the non-kaolin sensitive fraction must be present in goat serum only, or human serum would show non-specific reversal under the conditions of the test. Neutralization of the human serum HAR by the goat anti-human IgG serum in this test infers that the HAR is by specific antibody.

The conclusions that may be drawn from these experiments are:

1) Human serum must have a rubella antibody titre of at least 1:90 before a significant reduction in titre can be demonstrated if non-diluted commercial anti-human IgG serum is used.

2) In order for this test to be useful, the anti-human IgG serum must completely neutralize the IgG in human serum which is no more dilute than 1/10th of its own concentration. Preferably it should be even more potent than this.

3) Regardless of whether the conventional HI or the HAR test is used to demonstrate IgG neutralization, both the human and the goat serum must be kaolin-treated. Table 11 shows an effective HAR inhibition test when this procedure is used.

TABLE 10

LEGEND

Table 10: Rubella-virus HAR test.

Checker-board of dilutions of goat anti-human IgG absorbed with kaolin and chick-embryo erythrocytes and inactivated at 56°C for 30 minutes against dilutions of rubella-virus immune human serum absorbed with chick-embryo erythrocytes and inactivated at 56°C for 30 minutes. The serum dilutions reacted at 37°C for one hour. The erythrocytes were sensitized with 2.1 units of antigen and the HAR test was carried out at room temperature.

Degrees of scoring as in Table 5.

TABLE 10

Reciprocal of dilutions of goat serum	Reciprocal of dilution of human serum								No human serum
	10	15.	22.5	33.8	50.6	75.9	113.9	170.9	
1.5	+	+	+	+±	++	+	+	+	++
2.3	±	±	±	±	+	+	+	+	++
3.4	tr	tr	tr	tr	tr	±	+	+	++
5.1	tr	tr	tr	tr	tr	tr	+	+	++
7.6	-	-	-	-	-	-	tr	tr	++
11.4	-	-	-	-	-	-	tr	tr	++
17.1	-	-	-	-	-	-	tr	tr	++
No goat serum	-	-	-	-	-	tr	±	+	++

TABLE 11

LEGEND

Table 11: IgG neutralization by goat anti-human IgG serum in the rubella-virus HAR test.

Both the human and goat sera were treated with kaolin and chick embryo erythrocytes for the test. The sera were allowed to react for one hour at 37°C. The chick-embryo erythrocytes were sensitized with 2.1 units of agglutinin. Reversal was at room temperature.

Degrees of scoring as in Table 5.

TABLE 11

	Reciprocal of dilutions of human serum						
	10	20	40	80	160	320	640
No goat serum	-	-	-	-	+	++	++
With 1.2 goat anti-human IgG serum	-	-	+	++	++	++	++

EXPERIMENTAL - PART 2

A Random Distribution and Interaction Model for Haemagglutination

Introduction

As a result of the experimental work reported in Part 1, it may be stated that:

1. The conventional rubella HI test using purified haemagglutinin possesses the inherent disadvantage that not more than a small part of the initially available haemagglutinin can be used due to the instability of the 19S fraction.
2. The HAR test, although showing higher sensitivity than the HI test, is still not sufficiently sensitive to make the routine use of commercial anti-human globulin sera economical and useful.

The conventional HI test could be made more sensitive by reducing the antigen dose, but general experience with serological tests suggests that hypersensitive tests lead to test instability, control difficulties, and erratic results in test-retest situations. All of the current antigen and antibody assay methods possess a further built-in difficulty. They are end-point tests. The reaction limit in any end-point test system is a function of the conditions of the test, which must be sufficiently sensitive to show a reaction with low-level reactors, but must not be so sensitive as to give test instability. Furthermore, end-point tests are very difficult to standardize between laboratories due to the problems of standardizing all the conditions of the test for more than one work situation.

A method of absolute assay of the test reagent in a serum antibody assay system would appear to offer a way to overcome the above mentioned problems. The work published by Cameron and Bradish (1972) appeared, at first sight, to demonstrate such an absolute method for the assay of haemagglutinin; however, closer examination revealed problems inherent in their system. Throughout the paper, the basic assumption was made that erythrocytes were in excess in the system, yet the only evidence for this was that there were non-agglutinated cells remaining in the system. Whilst this reasoning may be intuitively valid for the high agglutinin dilutions, it leads to the problem of accounting for the continued presence of single cells despite an increase in agglutinin concentration of some 1,200 times. Cameron and Bradish invoked the 'free-cell' phenomenon of McKerns and Denstedt (1950) and suggested that the free cells were saturated with agglutinin and, therefore, could not agglutinate. They did not, however, suggest at what concentration saturation occurs, nor did they account for the absence of a single-cell-free zone between the conditions of erythrocyte excess and agglutinin excess implied by the saturation hypothesis.

Since the hypotheses of erythrocytes-in-excess and erythrocyte-saturation are mutually exclusive, the two conditions must be shown to be distinct in the system. This was not done, nor does it appear possible to do it using the data given. The erythrocytes-in-excess hypothesis can only be validated by demonstrating the absolute relationship between erythrocytes and agglutinin concentration. Since the sedimentation enumeration technique gives an end-point estimation, not an absolute bond value at zero dilution,

the technique cannot be used for this purpose. The test is a refinement on the standard end-point methods rather than a method taking full advantage of actual bond enumeration.

The reasons for failure to take full advantage of the conception of a bond enumeration technique are revealed in the data derived. The results obtained are a compromise between 1) allowing agglutinin adsorption to proceed to completion, and thereby having a large proportion lost by inactivation, and 2) taking an early reading when maximum concentration is present and accepting the inherent error due to incomplete agglutinin adsorption. Furthermore, from the B/R values given in the example by Cameron and Bradish, it is evident that the absolute bond-estimate at zero dilution is a function of the dilution used for bond enumeration; gross absolute-value estimates vary from 2.304×10^{10}/ml., using 1:10240 dilution, to 4.304×10^{8}/ml. when using a 1:80 dilution. A technical problem inherent in the technique is that of counting, with any sort of precision, aggregates greater than five in the time it takes for them to cross the line. Attempts to use this technique lead to the conclusion that estimations at >8 magnitudes are unreliable, also it appears to be strange that there are no aggregate magnitudes >10 reported. Intuitively, it is difficult to accept ten as the upper limit of aggregate magnitude in an agglutination system with a very high agglutinin concentration.

Such speculation is, however, mere armchair theorizing and has no more theoretical or empirical basis than have the assumptions entertained by Cameron and Bradish.

The problem is a basic one. There is no theoretical or empirical model against which to measure speculative conclusions. The only recourse, therefore, is to produce a model; this has been done on a theoretical, random distribution basis, and is reported in the next section.

Bases of the construction of the model

Two hypotheses were entertained in the construction of this model:

1. If an agglutinin suspension and the appropriate erythrocytes are mixed, the agglutinin units will distribute randomly among the erythrocytes present.
2. In a reaction mixture, agglutination will occur randomly and all erythrocytes will be available for interaction until all of the binding units have been utilized.

Given that hypothesis 2. holds true, it is proposed that the final distribution of aggregate magnitudes in the system will be a characteristic of the binding unit/cell ratio in the initial reaction mixture.

For the purpose of the model, a binding-unit is defined as a divalent entity capable of agglutinating two erythrocytes.

In the construction of the model, certain assumptions were made:

1. An aggregate of erythrocytes contains n-1 binding units where n is the number of erythrocytes.
2. Distribution and aggregation had proceeded to completion without any loss of agglutinin due to inactivation.

3. Each erythrocyte had sufficient agglutinin reaction sites to satisfy the random distribution demanded by the model, with one in addition to enable the cell to take part in the agglutination reaction.

4. An aggregate, once formed, was stable.

Construction procedure

Distribution. The numerals 1 to 1000 were listed; these were the erythrocytes. Varying numbers of binding units were distributed on the erythrocytes; the erythrocyte to which each binding unit was assigned was determined by taking a 3-digit number from a table of random digits (Rand Corporation, 1955).

Figure 5 illustrates the distribution of binding units on the erythrocytes at varying binding unit/cell (BU/C) ratios. The lines in this figure show the Poissonian distribution of events between $\bar{X} = 0$ and $\bar{X} = 1$. It can be seen that some of the erythrocytes appear to have adsorbed multiple binding units even at very low BU/C ratios and that the distribution tends towards higher adsorbed multiplicities rather than rapidly increasing numbers of erythrocytes with a single adsorbed binding unit and a corresponding rapid reduction in the number of erythrocytes free of them.

Agglutination. The binding unit distribution data for BU/C ratios .004, .016, .040, .077, .169, .244, .509, .709 and .925 were used to determine the aggregate distribution after agglutination has proceeded to completion.

LEGEND

Figure 5: The distribution of binding-units on erythrocytes at varying binding-unit/cell multiplicities.

Ordinate : The number of cells.

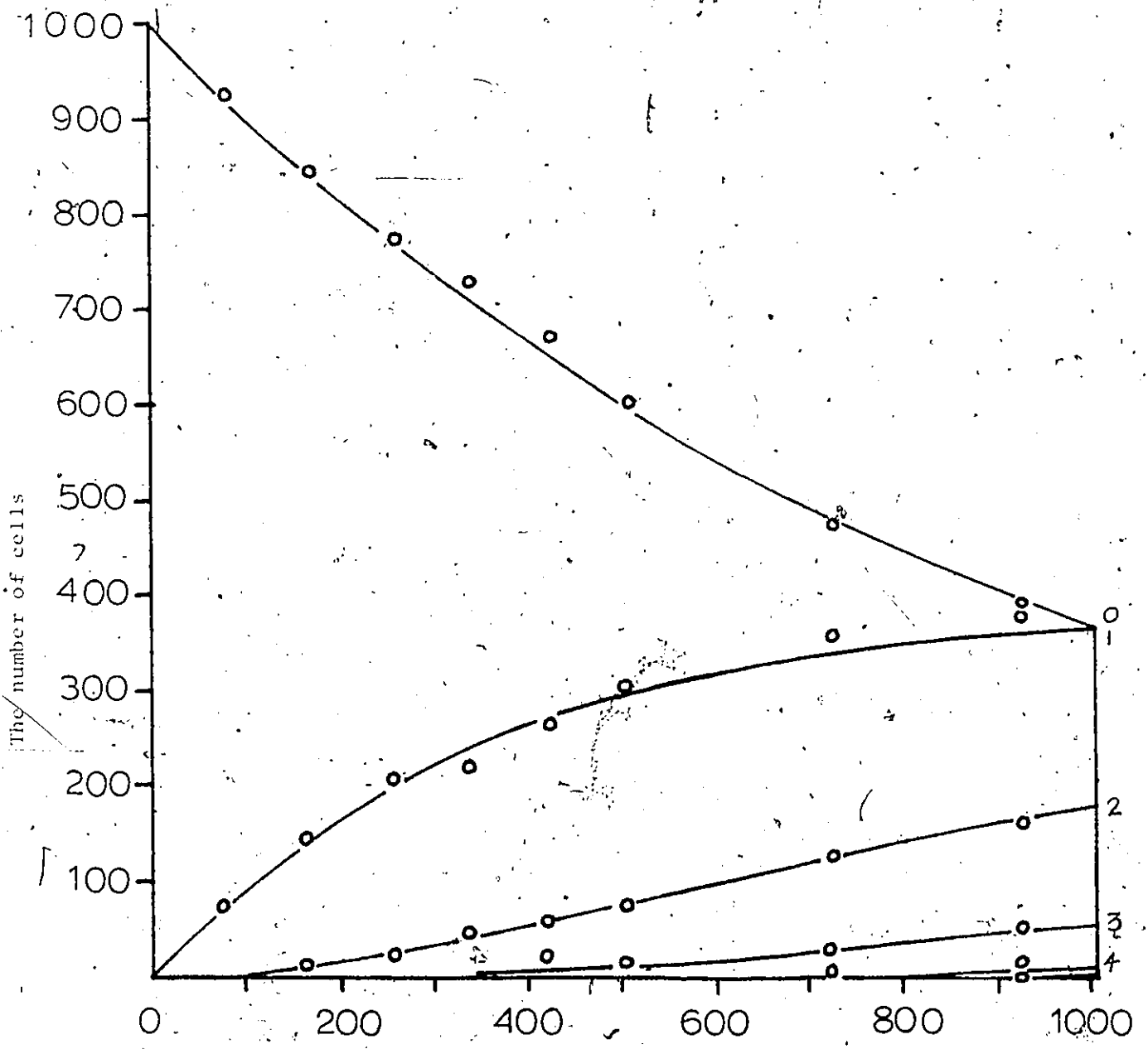
Abcissa : Binding-units per 1000 cells.

Right-hand scale: The number of binding units per cell represented by each of the lines.

Lines : Poissonian distribution of events, taken from the tables.

Circles : Values obtained by sampling from the table of random numbers.

FIGURE 5


1000
900
800
700
600
500
400
300
200
100
The number of cells
0
200
400
600
800
1000
Binding-units per 1000 cells
0
1
2
3
4

Technique: For each BU/C ratio; the erythrocytes with their associated bonds were laid out in the squares of a 50 x 20 square rectangle; the squares being numbered consecutively starting from 1 in the top left square and finishing at 1,000 in the bottom right square.

The pairing of a binding unit-free erythrocyte with another binding unit-free erythrocyte, being a non-event, was not considered in the coupling process. This considerably reduced the labour that the procedure entailed.

Taking them in numerical order, each sensitized erythrocyte was coupled with another erythrocyte selected at random by the use of the table of random digits. The coupling process was considered to consume one binding unit, and the magnitude of the aggregate and its associated binding units was calculated from the formula

$$A_x^{n_1} + A_y^{n_2} = A_{(x+y)}^{(n_1 + n_2) - 1}$$

where:

A_x is an aggregate of erythrocytes of magnitude x

A_y " " " " y

$A_{(x+y)}$ " " " " x + y

n_1 is the number of binding units associated with aggregate A_x

n_2 " " " " " " " A_y

$(n_1 + n_2) - 1$ is the number of binding units associated with the aggregate formed by the coupling of A_x and A_y; i.e., $A_{(x+y)}$.

For example:

A^4_2 would be a dimer with 4 associated free binding units, say in position 352 in the rectangle. From the tables, it is found that this is to be coupled with an aggregate in position 735. If this latter position is occupied by an A^0_3, which is a trimer with no associated free binding units, then the resultant aggregate becomes:

$$A^4_2 + A^0_3 = A^3_5$$

A^3_5 being an aggregate of five erythrocytes, with three associated free binding units.

Coupling was considered to occur at the higher of the two square values, the vacated square being blanked out. When a number was drawn from the tables corresponding to a blanked square, the number was disregarded and the next number drawn in its place. This process was continued until all the free binding units were consumed; the final population composition was then analyzed.

Figure 6 illustrates the variation in the total number of aggregates per 1,000 cells, the maximum aggregate magnitude, and the number of single cells per 1,000 cells with increasing BU/C ratios, whilst Figure 7 illustrates the variation in the numbers of each magnitude of aggregate with increasing BU/C ratio.

Two striking features are revealed by Figure 7:

1. The dimer theory is applicable only at BU/C ratios of and below, .016. This supports the view of Cameron and Bradish (1972) that the dimer hypothesis of Levine et al. (1953) is inappropriate.

LEGEND

Figure 6: Aggregation of an agglutinin-erythrocyte reaction mixture. Variation in the numbers of residual single cells, the total numbers of aggregates per 1000 cells, and the maximum aggregate magnitude with increasing BU/C ratio.

Ordinate: Count number.

Abcissa : BU/C ratio.

o ——— o = residual single cells per 1000

\+ ——— + = total aggregates per 1000 cells

⊙ ——— ⊙ = maximum aggregate magnitude

FIGURE 6

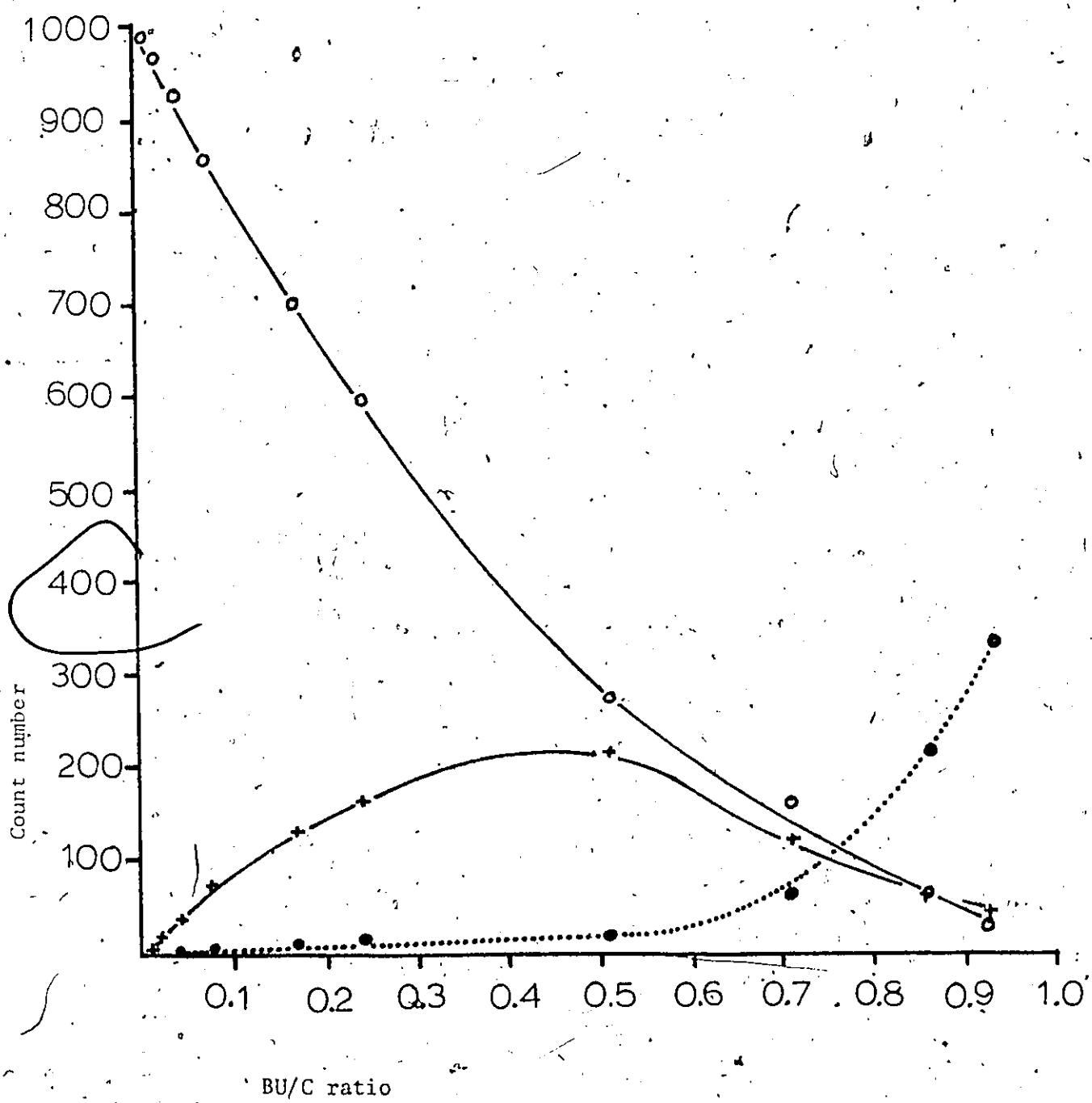

1000
900
800
700
600
500
400
300
200
100
Count number
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
BU/C ratio

FIGURE 7

LEGEND

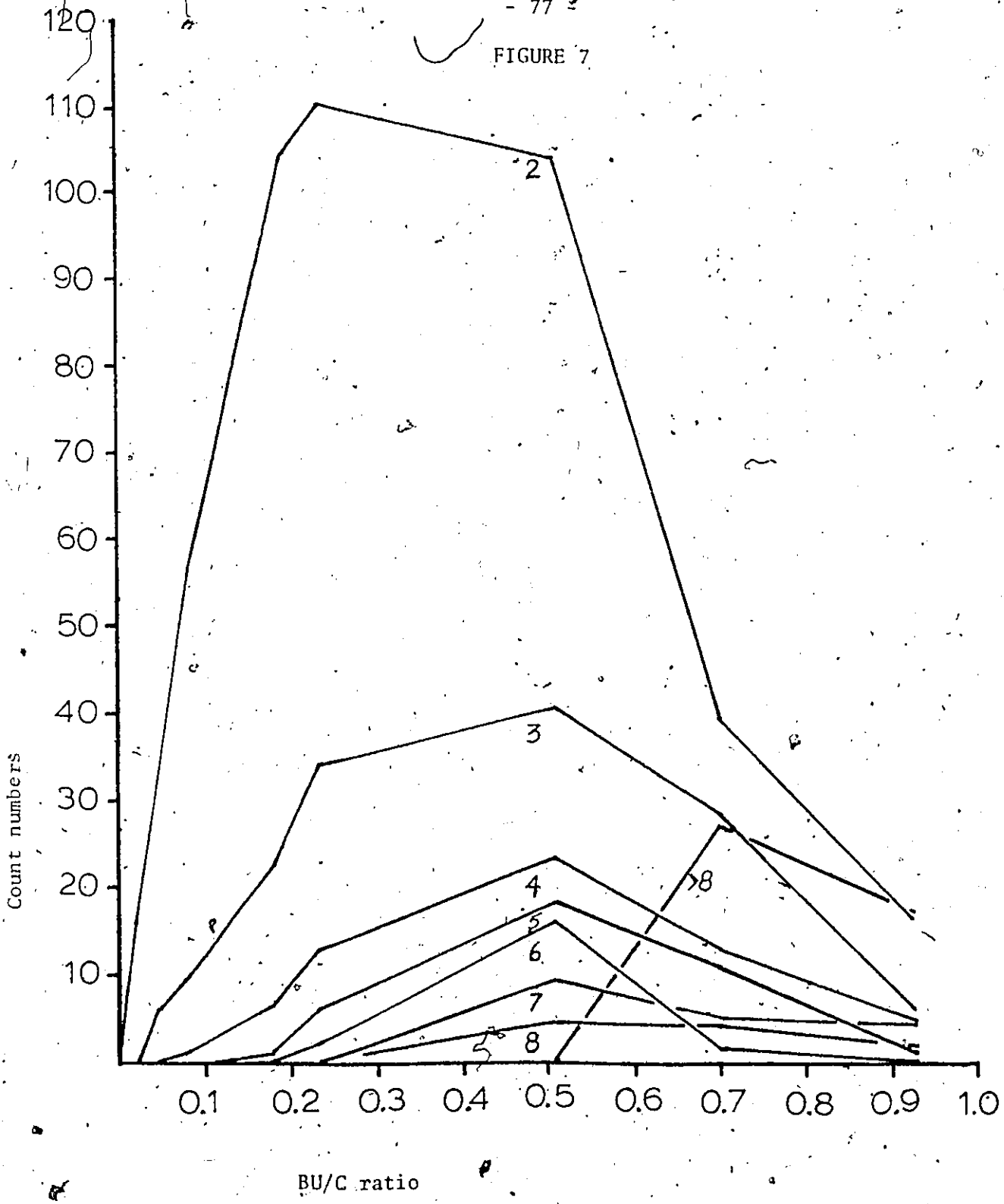
Figure 7: Aggregation of an agglutinin-erythrocyte reaction mixture. Variation in the numbers of each magnitude of aggregate with increasing BU/C ratio.

Ordinate: Count numbers.

Abcissa : BU/C ratio.

The numerals on the curves are the aggregate magnitudes to which the curves correspond.

FIGURE 7


120
110
100
90
80
70
60
50
40
30
20
10
Count numbers
2
3
4
5
6
7
8
8
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
BU/C ratio

2. Aggregates of magnitude 8 are present at BU/C ratios of .277 and higher, and at ratios above .500, there is a very rapid rise in the numbers of aggregates of magnitudes >8, with a concurrent decrease in the numbers of aggregates of magnitude 8 and below. The practical implication of this result is that the sedimentation-enumeration method of Cameron and Bradish (1972) is probably only applicable at BU/C ratios below .500.

Inspection of Figure 6 suggests that it may be possible to establish the relationship of either the proportion of the initial cell population remaining as monomers after aggregation, or of the maximum aggregate size, with the BU/C ratio, as a tool for estimating the binding unit concentration in the reaction mixture.

The practical difficulty of counting the number of erythrocytes in aggregates of magnitudes >7, even when allowed to settle in formalin to be inspected as stationary systems, is such as to eliminate the practicability of this alternative. Attention was, therefore, given to the residual monomers.

Prediction by use of the model

A linear transformation was achieved by plotting the proportion of the initial concentration of erythrocytes in the reaction mixture remaining as monomers after agglutination, against the square root of the BU/C ratio in the mixture.

As shown in Figure 8, the relationship is linear for residual monomer proportions between .163 and .877.

FIGURE 8

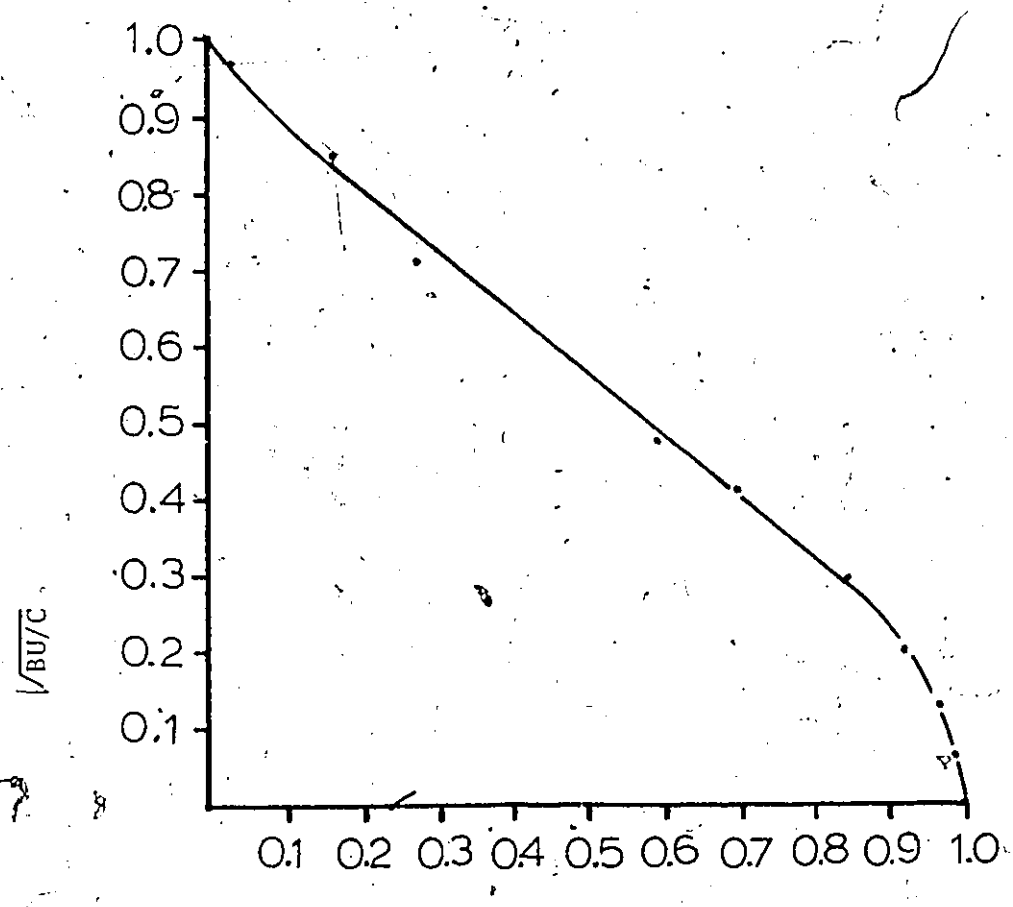
LEGEND

Figure 8: Aggregation of an agglutinin-erythrocyte reaction mixture. The linear relationship of the proportion of the erythrocytes remaining as monomers, after interaction, to the square root of the BU/C ratio.

Ordinate: $\sqrt{BU/C}$.

Abcissa : Proportion of cells remaining as monomers.

FIGURE 8


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
$\sqrt{BU/C}$
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Proportion of cells remaining as monomers

A regression calculated from the data for these five values gave a Y intercept value, (a) of .969 and a slope constant, (b) of -0.814.

We may say that:

The absolute value of the binding unit concentration in a reaction mixture may be calculated from the proportion of the initial erythrocytes remaining as monomers after agglutination has proceeded to completion.

This statement holds true so long as the proportion lies between the values .163 and .877.

The absolute BU/C ratio is given by the formula $BU/C = (a + bx)^2$, where: BU/C is the binding unit/cell ratio

a is the Y intercept = 0.969

b is the slope constant = -0.814

x is the proportion of the initial erythrocytes remaining as monomers after agglutination is complete.

Three things are apparent from consideration of this system:

1. It is necessary to know certainly that agglutination is complete in order to use the system.
2. There is no need to invoke a "saturation hypothesis" (Cameron & Bradish, 1972) to explain the presence of residual monomers when the cells are indeed in excess; they are present as a normal part of the population of aggregates.
3. These results could not be derived by empirical experiment.

Finally, we may say that the proposition stated at the beginning of this section has been supported by the theoretical results obtained. We may, therefore, proceed to attempt to obtain empirical evidence to support the two hypotheses on which the proposition rests.

EXPERIMENTAL - PART 3

Empirical Testing of the Theoretical Model

A. Evaluation of the technique

Two measures are needed in order to use the model.

1. The initial erythrocyte concentration used.
2. The monomer concentration after binding unit mediated interaction.

The empirical formula used is the BU/C ratio formula extended to give the binding unit concentration per ml. at zero dilution:

$$B/ml_o = \left(.969 - (.814 \times \frac{MC}{CC})\right)^2 \times CC/ml \times 2 \times df.$$

where: B/ml_o is the binding unit concentration per ml. at zero dilution

MC is the monomer count per unit volume after interaction

CC is the initial erythrocyte count per unit volume

CC/ml is the erythrocyte count per ml. in the reaction mixture

df is the dilution factor based on the agglutinin suspension added to the erythrocyte suspension

2 is the factor to correct for the addition of equal quantities of agglutinin suspension and erythrocyte suspension.

It is clear from consideration of the empirical formula that the lower limit of bond estimation is a direct function of the cell count per ml. of reaction mixture. It follows, therefore, that the initial cell count must be kept as low as possible to achieve maximum sensitivity; it is necessary to know the lowest counting range over which the response of cell count to dilution is linear, and the minimum number of samples which must be counted to achieve an acceptable level of precision.

A. Cont'd.

Linearity

A suspension of fresh human erythrocytes, thriced washed in PBS, was prepared in PBS.

When the suspension was diluted 1:2 in 5% formalin in PBS (to eliminate errors due to lysis of cells), the mean count of 14 x 0.1 mm^3 samples was 148.43. A series of 1.5-fold dilutions of the suspension were made in PBS. Each was diluted 1:2 with 5% formalin in PBS and 29 samples of 0.1 mm^3 counted, except for the first 1.5-fold dilution, when 22 samples were counted. This procedure yielded nine erythrocyte suspensions of known concentration relationships and with erythrocyte counts ranging from 148.43 to 6.10 per 0.1 mm^3.

When the cell counts in this series were plotted against the cell concentration relationships (Figure 9), the result indicated a slightly curvilinear relationship overall, with the obtained count falling increasingly below the predicted level with increasing cell concentration. By limiting the range of the counts treated, however, it is possible to consider the count/concentration relationship as linear over sufficient range to be used for testing the model. Figures 10-12 show the best-fit lines calculated for three ranges of counts; 0-17, 17-74 and 50-148. The response in each of these ranges is for all practical purposes linear, but the overall curvilinear nature of the response is indicated by the increase in the value of the Y-intercept (a) and the decrease in the value of the slope constant (b) as the cell concentration in the range increases. The model indicated a range of residual erythrocyte proportions of .163 to .877.. Considering the three linear ranges in Figures 10-12, then, we

FIGURE 9

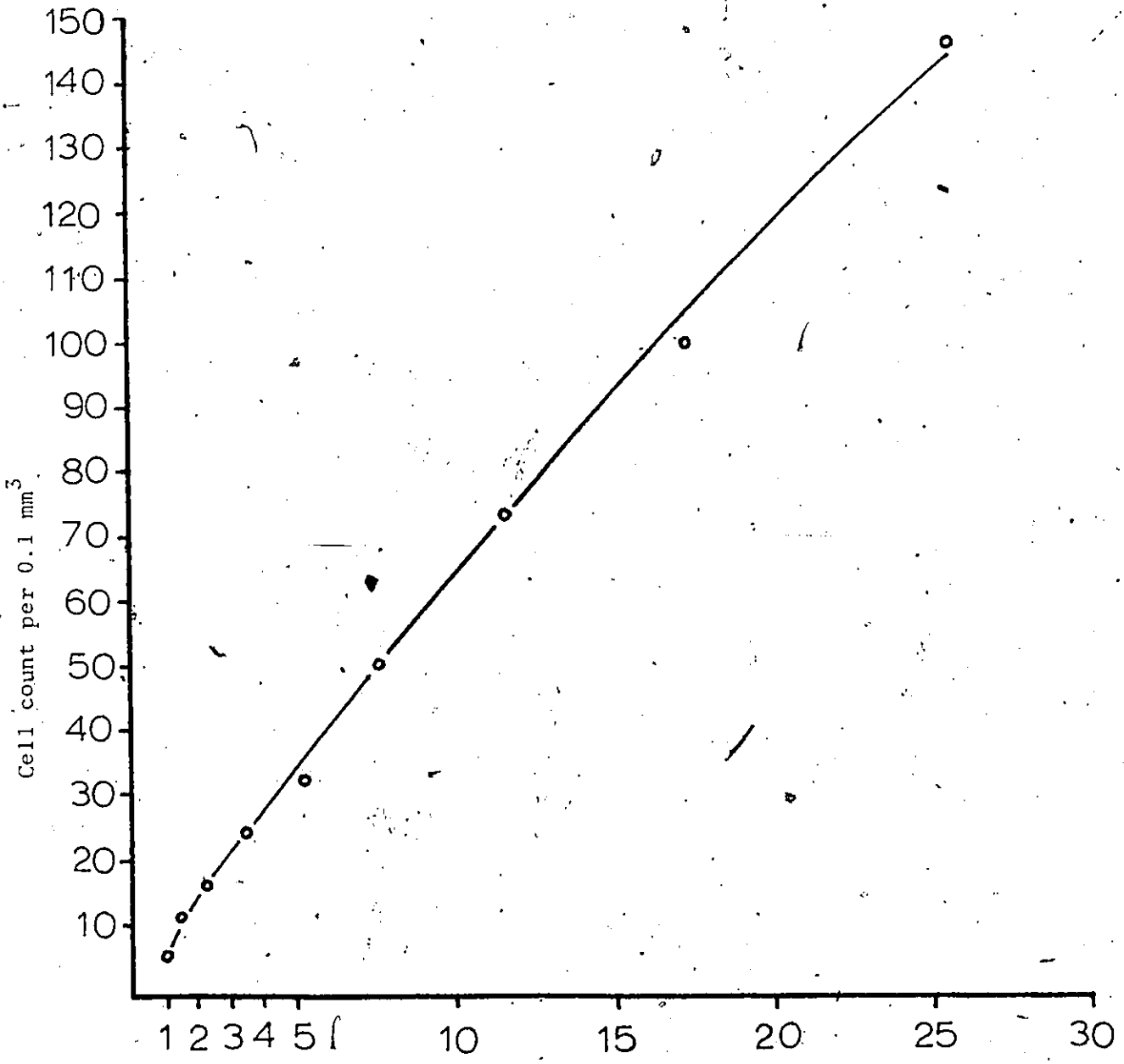
LEGEND

Figure 9: Erythrocyte counting in haemacytometer. Curvilinear response of the cell count to variation in cell concentration.

Ordinate: Cell count per 0.1 mm^3.

Abcissa : Cell concentration relationship.

FIGURE 9



Cell concentration relationship

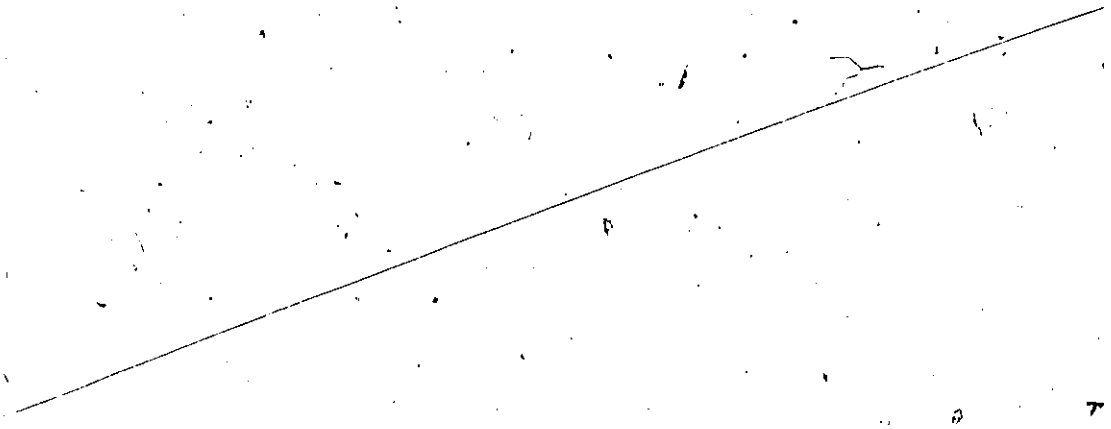


FIGURE 10

LEGEND

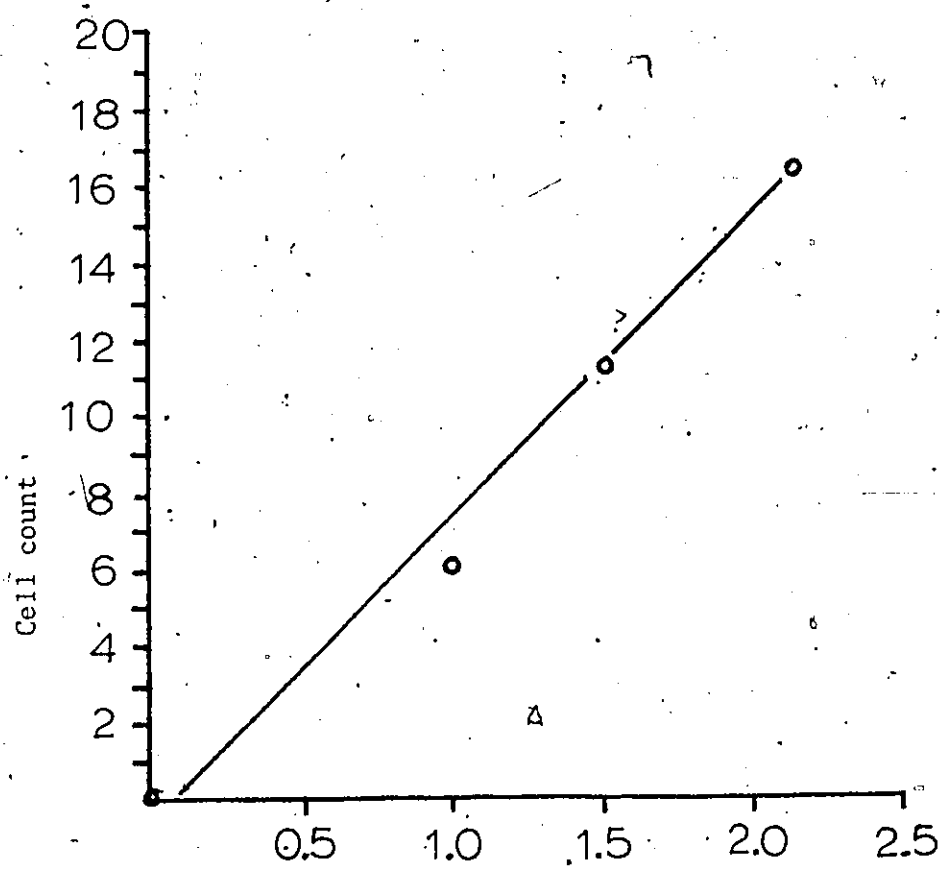
Figure 10: Erythrocyte counting in haemacytometer.

Best fit line (a = 0.51, b = 7.59) calculated for the count range 0-17 per 0.1 mm^3.

Ordinate: Cell count.

Abcissa : Cell concentration relationship.

FIGURE 10 .



cell concentration relationship

FIGURE 11.

LEGEND

Figure 11: Erythrocyte counting in haemacytometer.

Best fit line (a - 2.93, b = 6.23) for the count range 17-74 per 0.1 mm^3.

Ordinate: Cell count.

Abcissa : Cell concentration relationship.

FIGURE 11

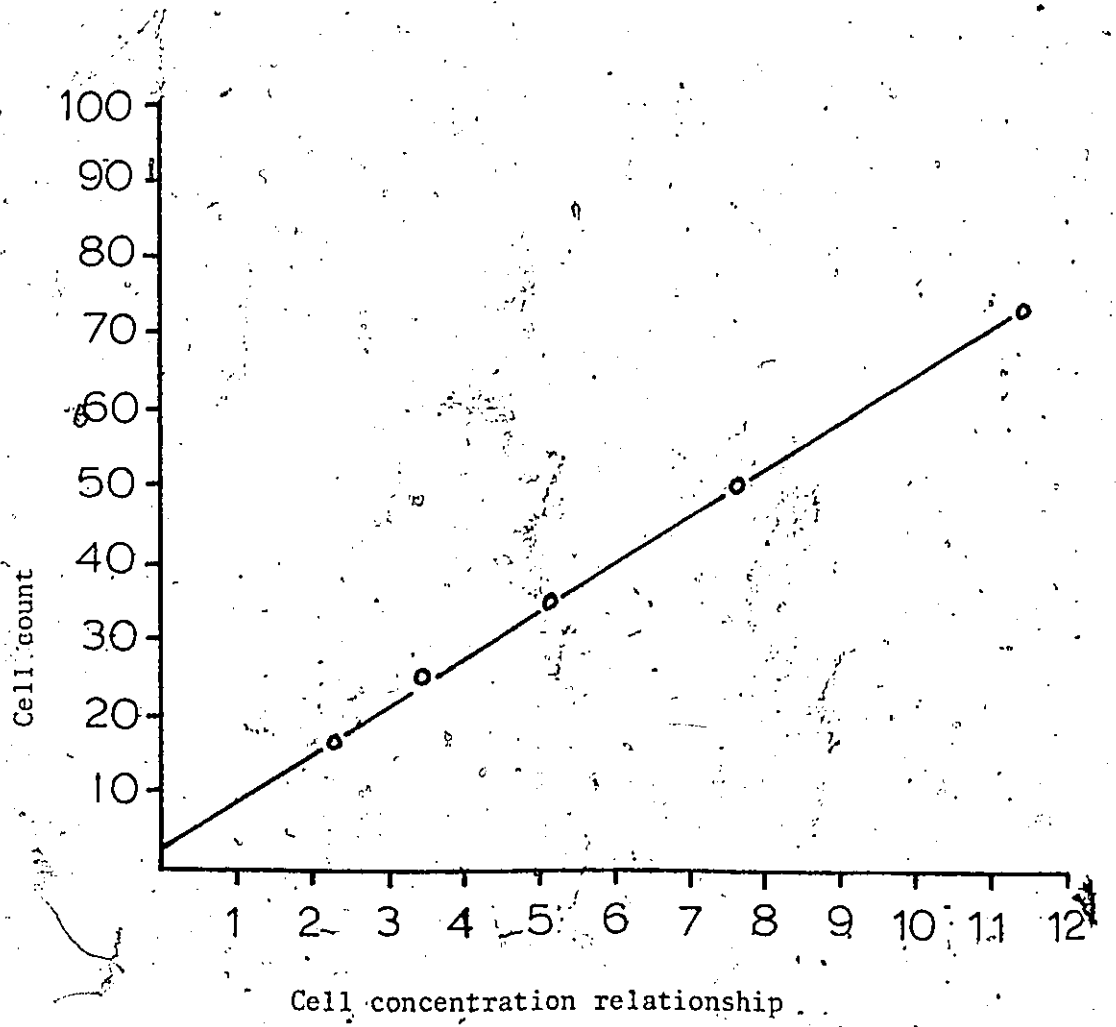

100
90
80
70
60
50
40
30
20
10
Cell count
1 2 3 4 5 6 7 8 9 10 11 12
Cell concentration relationship

FIGURE 12

LEGEND

Figure 12: Erythrocyte counting in haemacytometer.

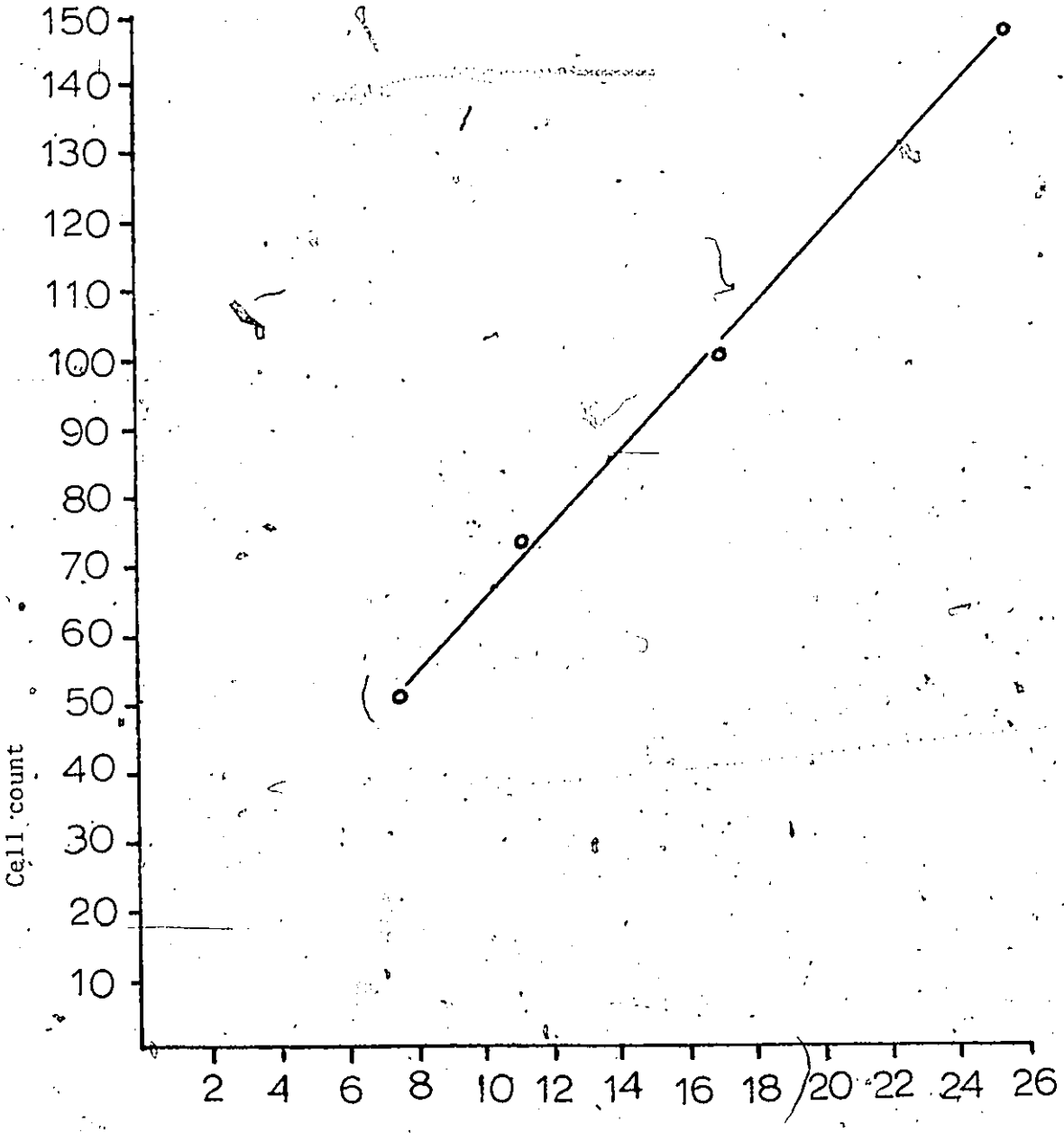
Best fit line (a = 10.94, b = 5.35)

calculated for the count range 51-148 per 0.1 mm^3.

Ordinate: Cell count.

Abcissa : Cell concentration relationship.

FIGURE 12



Cell concentration relationship

A. Cont'd.

find that the 50-148 allows for the use of a lower limit of .337 of the starting count, whereas, the 17-74 range allows for the use of a lower limit of .229 of the starting count. In the lowest count range treated (0-17), the zero was included to give the four points necessary for calculation of the regression but, in fact, it appears that the count of 6.10 is somewhat low and may indicate gross departure from linearity with decreasing cell count at this level. Since 6.1 is .359 of 17, the range of 17-74 would appear to be that of choice as this is the least constraining with respect to the range of counts which can be used for binding unit estimation.

Precision of the mean

Both the count magnitude and the sample multiplicity affect the precision of the ultimate binding unit estimate, and the effect of both of these factors on the Standard Error of the Mean was investigated. The Standard Error of the Mean was the subject since we are here concerned with the distribution of the population of the means derived from a predetermined sample multiplicity at a particular count magnitude, for on each occasion of the use of the model, the binding unit estimate is derived from the ratio of two population means. Table 12 is a presentation of the data from which Figures 9-12 were derived. It shows the effect of both sample magnitude and sample multiplicity on the Standard Error of the Mean expressed as a proportion of the mean. The shaded area indicates the approximate conditions which will yield $S_{\bar{x}} = .05$.

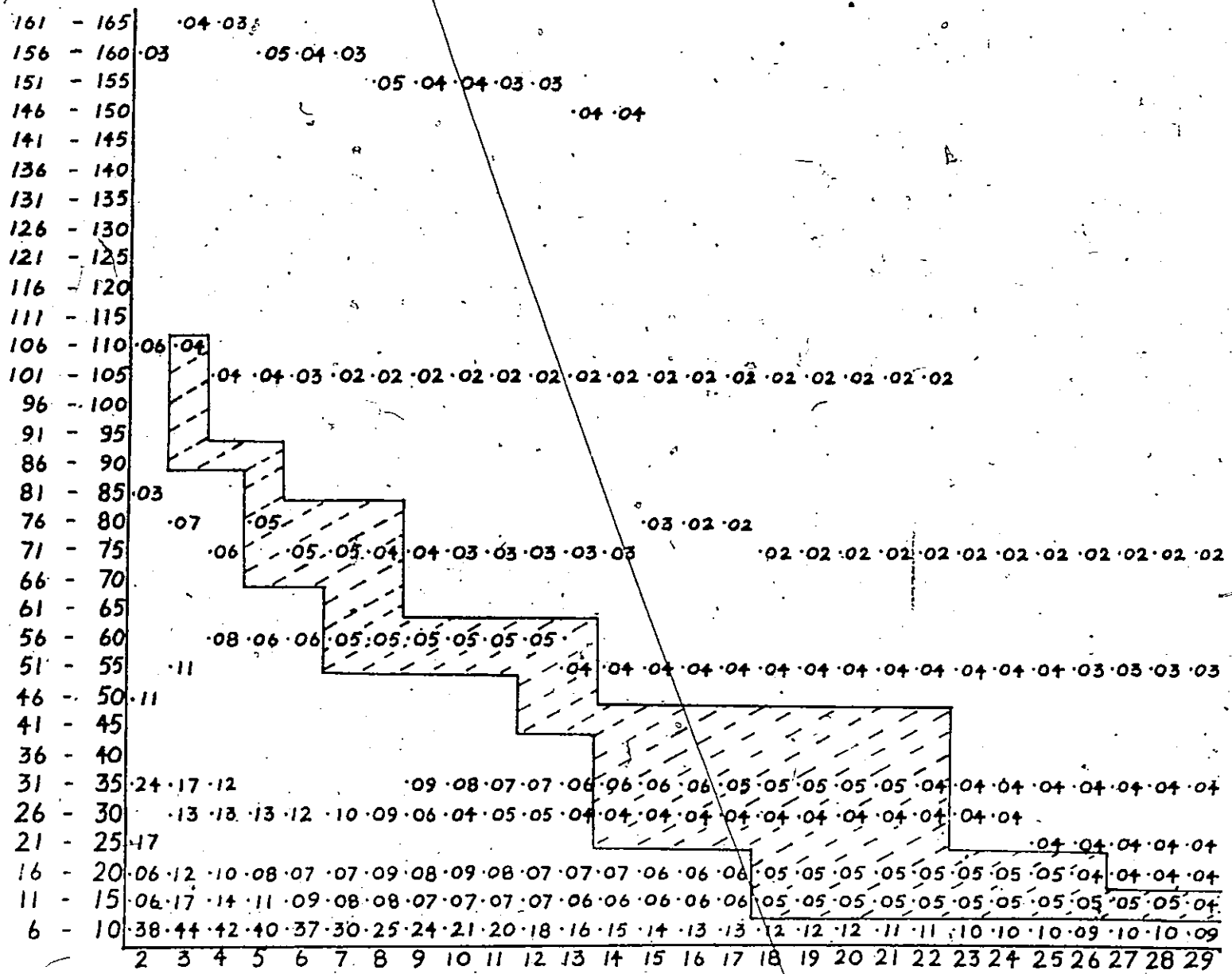
TABLE 12

LEGEND

Table 12: Variation in the value of the standard error of the mean with variation in both the count magnitude and the sample multiplicity.

For details, see text.

TABLE 12


161 - 165 ·04 ·03
156 - 160 ·03 ·05 ·04 ·03
151 - 155 ·05 ·04 ·04 ·03 ·03
146 - 150 ·04 ·04
141 - 145
136 - 140
131 - 135
126 - 130
121 - 125
116 - 120
111 - 115
106 - 110 ·06 ·04
101 - 105 ·04 ·04 ·03 ·02 ·02 ·02 ·02 ·02 ·02 ·02 ·02 ·02 ·02 ·02 ·02 ·02 ·02 ·02 ·02 ·02 ·02
96 - 100
91 - 95
86 - 90
81 - 85 ·03
76 - 80 ·07 ·05 ·03 ·02 ·02
71 - 75 ·06 ·05 ·05 ·04 ·04 ·03 ·03 ·03 ·03 ·03 ·02 ·02 ·02 ·02 ·02 ·02 ·02 ·02 ·02 ·02 ·02 ·02
66 - 70
61 - 65
56 - 60 ·08 ·06 ·06 ·05 ·05 ·05 ·05 ·05 ·05
51 - 55 ·11 ·04 ·04 ·04 ·04 ·04 ·04 ·04 ·04 ·04 ·04 ·04 ·04 ·04 ·04 ·03 ·03 ·03 ·03
46 - 50 ·11
41 - 45
36 - 40
31 - 35 ·24 ·17 ·12 ·09 ·08 ·07 ·07 ·06 ·06 ·06 ·05 ·05 ·05 ·05 ·05 ·04 ·04 ·04 ·04 ·04 ·04 ·04 ·04
26 - 30 ·13 ·18 ·13 ·12 ·10 ·09 ·06 ·04 ·05 ·05 ·04 ·04 ·04 ·04 ·04 ·04 ·04 ·04 ·04 ·04 ·04 ·04
21 - 25 ·17 ·04 ·04 ·04 ·04 ·04
16 - 20 ·06 ·12 ·10 ·08 ·07 ·07 ·09 ·08 ·09 ·08 ·07 ·07 ·07 ·06 ·06 ·06 ·05 ·05 ·05 ·05 ·05 ·05 ·05 ·05 ·04 ·04 ·04 ·04
11 - 15 ·06 ·17 ·14 ·11 ·09 ·08 ·08 ·07 ·07 ·07 ·07 ·06 ·06 ·06 ·06 ·06 ·05 ·05 ·05 ·05 ·05 ·05 ·05 ·05 ·05 ·05 ·05 ·04
6 - 10 ·38 ·44 ·42 ·40 ·37 ·30 ·25 ·24 ·21 ·20 ·18 ·16 ·15 ·14 ·13 ·13 ·12 ·12 ·12 ·11 ·11 ·10 ·10 ·10 ·09 ·10 ·10 ·09
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29

A. Cont'd.

The following conclusions can be drawn from this Table:

1. The lower the sample magnitude, the greater must be the sample multiplicity to achieve an acceptable precision of the estimate of the mean.
2. More than two samples must be counted regardless of the sample magnitude, to achieve precision stability.
3. With sample magnitudes >40 more than three samples must be counted to avoid gross variation in the estimate of the mean.
4. It is probably not possible in practice, using haemocytometer counting, to achieve sufficient precision with the 0-17 category of counts to use this range for bond estimation.
5. The range 17-74 yields means of reasonable precision with a sample multiplicity of 18 at the lower sample magnitude.
6. The upper limit of counting is probably in the region of 100-120 erythrocytes per 0.1 mm^3. At a count level of 150, both the mean and the standard error of the mean are erratic.

The considerations of both linearity and precision of the estimate of the mean, therefore, indicate the range 17-74 erythrocytes per 0.1 mm^3 to be the range of choice for binding unit concentration estimation.

Time required for complete aggregation

Any empirical test of the model can be valid only if the aggregation process has proceeded to completion, since this is one of the assumptions upon which the construction of the model was based. To determine the aggregation time, a PR8-virus reaction mixture was put on a rotator in

A. Cont'd.

such a way that the tube was inverted 66 times per minute. All equipment and materials were chilled to 4°C for the experiment. A series of identical materials, each being 4 ml. of reaction mixture in a 12 x 75 mm. tube fitted with a 00 rubber stopper were used. At intervals of time during rotation, 0.2 ml. of formalin was added to a tube which was then placed on a slow rotator to be inverted 8 times per minute until being counted. Inspection of Figure 13 shows that, under the conditions of the test, aggregation takes 2 1/2 hours to reach completion. This finding casts serious doubt on the effectiveness of the method of Cameron and Bradish (1972); here the material was "allowed to equilibrate for 15 minutes at room temperature", presumably stationary.

Confidence level

The confidence level must be determined, not upon statistical grounds, but on the basis of the relative costs of making Type I (false positive) and Type II (false negative) errors; (Hardyck & Petrinovich, 1969). It is not possible to make any decision that will be valid for all serological examinations carried out upon patients; screening of blood donors for evidence of Australia antigen in the plasma would, for example, demand that a low level of Type II errors is the objective and the confidence level for positive results should be relatively low. With rubella virus, the opposite attitude is appropriate. Since the work which led to the construction of the theoretical model was rubella virus serology, and since the objective is that this will be the first area of application, consideration was given to

FIGURE 13

LEGEND

Figure 13: Aggregation of a haemagglutinin-erythrocyte reaction mixture during gentle agitation. The mixture was inverted 66 times per minute at 4°C.

o — — o = residual monomer count

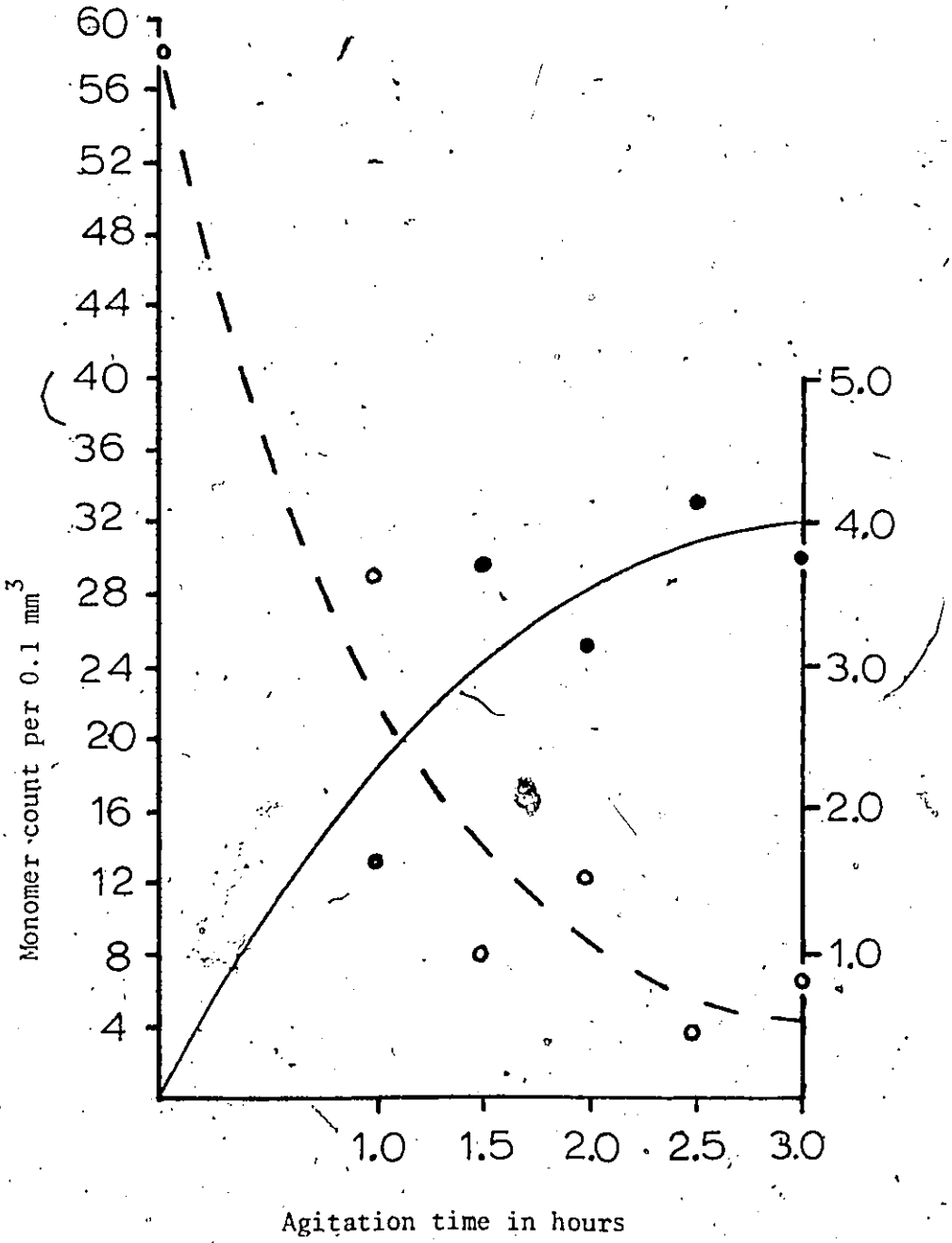
● —— ● = calculated BU concentration

Ordinate: Monomer count per $0,1\ mm^3$.

Abcissa : Agitation time in hours.

Right hand scale = $B/ml_o \times 10^{-8}$.

FIGURE 13


60
56
52
48
44
40
36
32
28
24
20
16
12
8
4
Monomer count per 0.1 mm³
5.0
4.0
3.0
2.0
1.0
1.0
1.5
2.0
2.5
3.0
Agitation time in hours

A. Cont'd.

the confidence level appropriate for rubella virus serology, and discussion was held with Dr. E. Rossier on the matter, since this decision must ultimately depend on clinical considerations.

Two situations exist in the rubella diagnosis field:

1. The diagnosis of active infection in a patient.
2. The determination of the rubella immune status of a patient in order to assess the person's risk level if in contact with a case, or consider the appropriateness of recommending immunization.

For the purpose of this exercise, the first of the two situations will be used as the decision-making basis. The costs of a false negative here will probably not be high since the response of the clinician will probably be 'no-action'; although the situation is not simple if one takes into account antibody titre rises of <4-fold, in combination with high antibody levels. Since the laboratory function is to supply data and guidance, any positive result must have a very high degree of certainty attached to it by the laboratory. The costs of a false positive are liable to be very high as it may involve the issue of abortion, with all the concommitant emotional overtones and value judgments, and committing the patient to high degrees of psychological trauma.

It is suggested, therefore, that a confidence level of 0.01 is appropriate. This enables the laboratory to place the necessary high degree of certainty on the positive results issued.

A. Cont'd.

Precision of the test

The direct way to assess the precision of the test is to calculate the mean value and 0.01 confidence limits for a series of binding unit concentration estimations, made independently on the same preparation. The result will include all sources of error and variation in the process of binding unit estimation.

Table 13, i-vi, shows a series of multiple binding unit estimates made in six experiments involving influenza-virus, PR8 strain. The starting counts vary from 41.08 to 87.9 and the number of counting levels varies from 3 to 6. The most precise estimate (6 samples) gives 0.01 confidence limits of $\pm$ 18% of the mean, the least precise estimate (3 samples) gives 0.01 confidence limits of $\pm$ 94% of the mean. It can be seen that examples 1 and 2 yield mean estimates that are very close, and the confidence limits would suggest that they are not different. However, a t-Test carried out on the data in the two examples gave a t-value of 8.427, significant for 7 degrees of freedom, P = <.005. The data, then, is sufficient to separate two haemagglutinin populations having a difference of 17% in HA concentration, at the <0.005 confidence level. The data also suggest that the mean binding unit concentration should be calculated from at least four independent estimates, when the precision of the estimate will probably be of the order of $\pm$ 30%-35% of the mean value at the .01 confidence level.

It can be seen that, in each case where the starting count is approximately 50, the estimate yielded by a monomer count below 0.5 of the starting count appears to be lower than the estimates from residual

TABLE 13 i-vi

LEGEND

Table 13 i-vi: Binding-unit estimations on six preparations of PR8 virus by the monomer counting technique.

Each of the multiple estimates made in each experiment was calculated from the counts obtained using a dilutio of virus different from those used for the other estimates within that experiment. Human erythrocytes were used in i-v, chick erythrocytes in vi. The virus concentration relationships (VCR) were calculated from the virus dilutions used.

SC = starting count

$\bar{x}$ = arithmetic mean of the BU/ml_o estimates

$S_{\bar{x}}$ = standard error of the mean of the BU/ml_o estimates

Confidence limits are at the 0.01 level.

TABLE 13 i

VCR	Monomer count	BU/ml_o estimates ($\times 10^{-8}$)
1.61	23.42	1.08
1.46	24.37	1.10
1.33	28.21	0.86
1.21	27.88	0.97
1.10	30.04	1.07
1.00	34.08	1.30

SC = 41.08

$\bar{x}$ = 1.063

$S_{\bar{x}}$ = 0.047

99% Confidence limits = $0.873 \longleftarrow 1.063_8 \longrightarrow 1.253$ ($\pm$ 18%)
$\times 10^8$ per ml.

TABLE 13 ii

VCR	Monomer count	BU/ml_o estimates ($\times 10^{-8}$)
0.4	25.39	1.34
0.6	30.61	1.46
1.0	41.00	1.05

SC = 51.1

$\bar{x}$ = 1.283

$S_{\bar{x}}$ = 0.122

99% confidence limits = 0.073 $\longleftarrow$ 1.283 $\longrightarrow$ 2.493 ($\pm$ 94%) $\times 10^8$ per ml.

TABLE 13 iii

VCR	Monomer count	BU/ml$_o$ estimates ($\times 10^{-9}$)
5.06	23.71	1.7
3.38	26.92	2.4
2.25	34.38	1.6
1.50	41.96	3.5
1.00	50.39	3.9

SC = 87.9

$\bar{x}$ = 2.62

$S_{\bar{x}}$ = 0.77

99% confidence limits = 1.804 $\longleftarrow$ 2.62 $\longrightarrow$ 3.436 ($\pm$ 31%)
$\times 10^9$ per ml.

TABLE 13 iv

VCR	Monomer count	BU/ml_o estimates ($\times 10^{-8}$)
3.38	21.72	8.81
2.25	28.28	9.37
1.50	35.17	9.06
1.00	37.78	11.20

SC = 53.11

$\bar{x}$ = 9.61

$S_{\bar{x}}$ = 0.542

99% confidence limits = 6.443 $\longleftarrow$ 9.61 $\longrightarrow$ 12.777 ($\pm$ 33%)
$\times 10^8$ per ml.

TABLE 13 v

VCR	Monomer count	BU/ml$_o$ estimates ($\times 10^{-8}$)
4.00	18.78	0.94
2.00	27.06	1.22
1.33	35.94	1.00
1.00	38.89	1.04

SC = 51.22

$\bar{x}$ = 1.05

$S_{\bar{x}}$ = 0.06

99% confidence limits = 0.7 $\longleftarrow$ 1.05 $\longrightarrow$ 1.4 ($\pm$ 33%)
$\times 10^8$ per ml

TABLE 13 vi

VCR	Monomer count	BU/ml_o estimates ($\times 10^{-8}$)
4.00	4.00	0.80
2.00	2.00	0.97
1.33	1.33	0.97
1.00	1.00	1.05

SC = 53.89

$\bar{x}$ = 0.95

$S_{\bar{x}}$ = 0.05

99% confidence limits = 0.66 $\longleftarrow$ 0.95 $\longrightarrow$ 1.24 ($\pm$ 31%)
$\times 10^8$ per ml.

A. Cont'd.

monomer count proportions above 0.5. The reason for this is that counts on the lower part of any portion of the curve shown in Figure 9 are high relative to a count in the upper portion. The structure of the formula for BU/C estimation is such that a relatively high obtained count will yield a relatively low binding unit estimate. With a starting count of 50, it is probably wise to use a series of dilutions giving residual monomer proportions between 0.8 and 0.5 of the starting count for binding unit estimation.

Results using chick embryo and human erythrocytes

The estimates shown in Table 13 (iii) are of the same virus suspension, but carried out using human erythrocytes (#5) and chick embryo erythrocytes (#6). The two estimates of $1.05 \pm 0.35 \times 10^8$/ml. and $0.95 \pm 0.29 \times 10^8$/ml. are not significantly different ($P = 0.25$, 6 df) since a t-Test yields a t-value of 1.2422.

It appears, therefore, that PR8 virus will agglutinate chick and human cells with equal effectiveness and that erythrocytes of either species may be used in the test.

B. Testing the model

Test conditions. The technique to be used in order to obtain a binding unit concentration estimate on an influenza haemagglutinin suspension can now be stated:

All reagents and equipment are chilled to 4°C.

B. Cont'd.

A series of 1.2-fold or 1.5-fold dilutions are made in PBS to cover the appropriate range. Two ml. of each dilution is placed in a 12 x 75 mm. tube and two ml. of PBS in a further, control, tube. To each tube is added two ml. of a suspension of fresh human or chick erythrocytes (human erythrocytes are the easier to count), at a level of 8.5×10^5 to 1.1×10^6 per ml. in PBS. As the erythrocytes are added to a tube, it is stoppered and immediately inverted twice to smoothly mix the cells and agglutinin. When all tubes have been prepared, they are placed on a rotator and inverted 66 times a minute for 2 1/2 hours at 4°C. Each tube then receives 0.2 ml. of formalin; again the tubes are treated individually and the contents mixed by inverting two or three times. After being formolized, the tubes are placed on a slow rotator at 4°C to be inverted eight times a minute. Counting is started immediately after formolization; the aim is to count four dilutions giving residual monomers of between 0.8 and 0.5 of the starting count. Each count is the arithmetic mean of 18 samples.

The binding unit concentration is calculated, using the empirical formula, and the final value is the mean of those calculated for each dilution. The confidence limits can be calculated for any desired confidence level.

Construction of an empirical equivalent of the theoretical model

In order to determine whether the results obtained by counting at different dilution levels support the BU/C. residual monomer proportion relationship predicted by the Random Distribution and Interaction Model, the following experiment was carried out.

B. Cont'd.

Method. Infected allantoic fluid was diluted to give a series of dilutions from which counts covering the range of proportions in the theoretical model could be obtained. The technique used was as described under 'Test Conditions'. A low starting count was used as it was considered that there may be less deviation from linearity at the lower residual proportion levels than if a high (>75.0) count were used. The binding unit concentration for each dilution level was estimated.

It was found (Table 14) that all of the dilutions which gave residual monomer proportions of <0.8 gave similar bond estimates. The mean of these estimates was calculated and used as the basis for determining the BU/C ratio at the starting level for the experiment. From this figure, the BU/C ratio at each dilution level was calculated and the square root of each was plotted against the appropriate residual monomer proportion.

Results. There was a very close correspondence between the value of the binding units per cell in the reaction mixture, calculated from the dilution used and the starting binding unit concentration, and the value predicted by the model for the different values of MC/cc. Figure 14 shows the plot of the values obtained in the empirical experiment superimposed on the curve derived in the theoretical model. We may say, then, that the empirical data are in agreement with the theoretical model and, therefore, support the hypotheses, proposition, and assumptions on which the model was based. As was predicted, the lower starting count of 37.0 appears to have given consistent BU/ml_o estimates over a wider range of residual monomer proportions than was found when using a starting count of 50.

TABLE 14

LEGEND

Table 14: Estimations of the BU/ml. values for an influenza virus (PR8) suspension.

The estimations were made independently using the dilutions shown in the table.

The BU/C ratio at 1:1 to 1:67 ($\bar{x}$ = 5.32 x 10^5/ml.) and the erythrocyte concentration.

The BU/C ratios at the other dilutions are calculated from the BU/C ratio at 1:1 and the dilution.

Starting erythrocyte count = 37.00 per 0.1 mm^3.

TABLE 14

Dilution	Erythrocyte count/0.1 mm^3	Erythrocyte count as proportions of starting count (M^c/cc)	BU/C ratio	BU/C	BU/ml $_{o_5}$ (x 10^{-5})
1:80	36.94	0.998	0.004	0.063	14.83
1:40	33.94	0.914	0.018	0.134	15.00
1:15	33.33	0.900	0.048	0.219	6.36
1:6.7	28.83	0.779	0.107	0.327	5.70
1:4	26.61	0.719	0.180	0.424	4.47
1:2.5	18.83	0.509	0.288	0.537	5.83
1:1.8	16.00	0.432	0.399	0.632	5.20
1:1.1	8.22	0.222	0.654	0.809	5.19
1:1	5.28	0.142	0.719	0.848	5.52

FIGURE 14

LEGEND

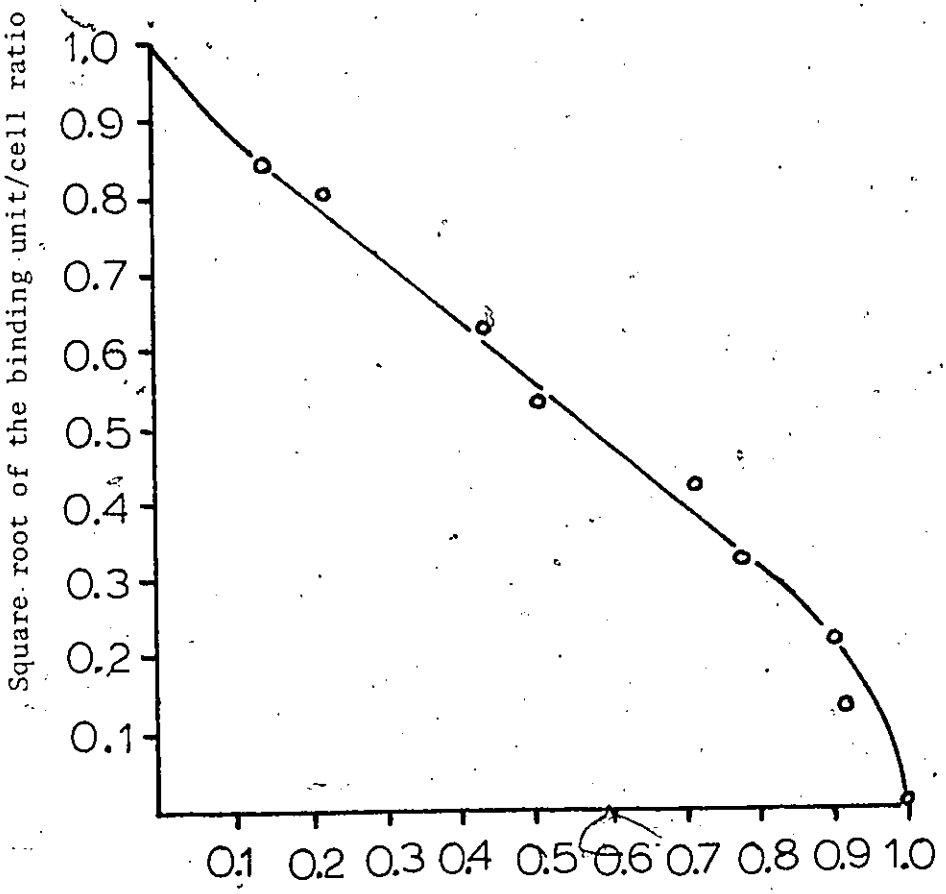
Figure 14: Binding-unit concentration estimates for a series of influenza-virus (PR8) dilutions.

Plot of the points derived from the empirical data super-imposed upon the relationship line derived from the theoretical model and illustrated in Figure 8.

Ordinate: Square root of the binding unit/cell ratio.

Abcissa : Proportion of the initial erythrocytes remaining as monomers after completion of interaction.

FIGURE 14



Proportion of the initial erythrocytes remaining as monomers after completion of interaction

EXPERIMENTAL - PART 4

Relationships between the Dimensions of Influenza-Virus Suspensions

A. Binding-units and infectivity

At this stage, a binding-unit may be defined as 'that quantity of agglutinin which will cause two erythrocytes to aggregate'. This is what the model is designed to measure so that the definition is a circular one.

The estimates of binding-unit concentration shown in the examples in Table 13 (i-vi), however, are made upon infected allantoic fluid. The values obtained suggest that, under appropriate circumstances, one binding-unit may equal one infectious particle. A series of experiments were designed to test this hypothesis.

Experiment No. 1. Influenza-virus (PR8) strain at a dilution of 10^{-7}, was inoculated into the allantoic sac of several eggs at the 10th day of development. The eggs were incubated for 44 hours at 37°C. For test, a sample of allantoic fluid was harvested from a non-chilled egg in a single action to avoid contamination by chick erythrocytes. The sample was assayed for HA by monomer count and for infectivity by inoculating into 96 wells of human embryo lung cells on serum-free medium, .025 ml. per well.

Problems were encountered with the cell cultures. They reacted badly to the change to serum-free medium and did not produce haemadsorbing plaques until four days after inoculation. The results are, therefore, probably not reliable.

A. Cont'd.

An HA estimate of $1.06 \pm .19 \times 10^8$ BU/ml_0 (.01 confidence limits) was obtained. This is 2.27 times the infectivity estimate of 4.4×10^7 infective units per ml. This may be due to both insensitivity of the cultures and the fact that only one plate was readable, the 2×10^{-7} dilution. This plate showed $23/_{96}$ wells positive which, from the Poissonian distribution, indicates a mean count of .22 in .025 ml.

Experiment No. 2. A second assay was attempted in the same manner as the first, except that infectivity was assayed by inoculating dilutions of virus, 10^{-7}, $.5 \times 10^{-7}$, and $.33 \times 10^{-7}$ into six eggs per dilution, 0.1 ml. per egg by the allantoic route. The eggs were chilled and tested for haemagglutinin in the allantoic fluid 48 hours after inoculation.

Although it may be argued that the number of eggs in each group is small, the results in the groups are consistent with each other (Table 15). Calculation of the infectivity concentration from the mean for 0.1 ml. estimated from the Poissonian distribution gives infectivity unit counts of 1.0×10^8, 1.32×10^8, and 1.5×10^8 infective units per ml., mean count 1.27×10^8 ml., this count being almost identical with the BU/ml_0 observed mean of 1.28×10^8 per ml.

Experiment No. 3. In a further experiment, allantoic fluid was obtained as previously and the binding-unit concentration assayed at 1.05×10^9 BU/ml_0. A dilution was prepared to give 10 binding-units per ml. and 0.1 ml. was inoculated allantoically into each of 12 x 11 day-old fertile eggs.

TABLE 15

LEGEND

Table 15: Egg infectivity, infective unit concentration, and BU/ml estimation comparison.

Influenza virus (PR8) allantoic fluid.

Each egg received 0.1 ml. of virus dilution.

TABLE 15

Virus dilution	Eggs positive/ eggs inoculated	± (from tables of Poisson Distribution)	Infectivity count/ml_o
10^{-7}	3/5	0.83	0.83×10^8
0.5×10^{-7}	3/6	0.66	1.32×10^8
0.33×10^{-7}	2/6	0.42	1.27×10^8

$BU/ml_o = 1.28 \times 10^8$ per ml.

A. Cont'd.

The tables of Poissonian distribution predict that, for $\bar{x} = 1$, the distribution will be .3679 negative and .6321 positive. The predicted number of infected eggs of the 12 inoculated is, therefore, 7.5852. The observed number of eggs positive out of the 12 inoculated was 7. Again, as in the previous two experiments, the results are consistent with the hypothesis that one binding-unit is equal to one infectious unit.

Experiment No. 4. Two influenza-virus suspensions were assayed for haemagglutinin by monomer count and for infectivity by inoculating 28 eggs per suspension with 0.1 ml. of virus dilution by the allantoic route. The dilution inoculated was determined from the BU/ml_o estimation, and calculated to give one binding unit per 0.1 ml. The eggs were chilled after 48 hours of incubation and checked for HA activity by the allantoic fluid.

The results are as follows:

The first virus suspension was assayed as:

Binding units/ml_o	2.36×10^8
Infective units/ml_o	4.11×10^8
Infective units/binding unit	1.74

The second suspension was assayed as:

Binding units/ml_o	1.02×10^8
Infective units/ml_o	1.58×10^8
Infective units/binding unit	1.55

For these four experiments, the mean value of egg infective units (EIU) per binding unit is: 1.29 EIU = 1BU.

B. Binding-units and HA units

Experiment No. 1. Two virus suspensions were assayed by monomer count to determine their binding unit concentration using human erythrocytes. The same suspensions were evaluated to determine their HA titres, using the pattern method, with .2% bovine albumin in PBS as the diluent, and mixing 0.025 ml. quantities of the virus dilutions, which had been prepared in tubes in 1 ml. quantities, and 0.3% chick embryo erythrocytes in round bottomed wells of micro-titre plates. The tests were allowed to settle for two hours at room temperature.

The results (Table 16), yielded values of 5.6×10^5 and 8.6×10^5 binding units per HA unit.

Experiment No. 2. Three virus suspensions were assayed by monomer count to determine their binding-unit concentrations and by the plate method to determine their conventional HA titres. The dilutions for the conventional test were doubling dilutions in 0.025 ml. quantities using standard droppers and 0.025 ml. Takatsy loops. Diluent, cells, temperature and settling were as in experiment No. 1. Each titration was in triplicate and the arithmetic mean of the reciprocals of the three dilutions was taken as the activity level of the original suspension, expressed as HA units (HAU).

TABLE 16

LEGEND

Table 16: Estimation of binding units per tube HA unit (BU/HAU). Binding unit estimation was by monomer counting using human erythrocytes. Tube HA was by microtitre technique using 0.3% chick erythrocytes and 0.2% bovine albumin in PBS as diluent.

TABLE 16

	BU/ml$_o$		HA titre	BU/HAU
1.	2.17×10^9)			
	2.50×10^9)	2.24×10^9	1:4000	5.6×10^5
	2.04×10^9)			
2.	5.53×10^8)			
	4.41×10^8)	4.42×10^8	1:512	8.6×10^5
	3.31×10^8)			

B. Cont'd.

The results are as follows:

First virus suspension:

Binding units/ml_o	2.36×10^8
HAU at zero dilution	133
BU/HAU	1.77×10^6

Second virus suspension:

Binding units/ml_o	1.02×10^8
HAU at zero dilution	133
BU/HAU	7.67×10^5

Third virus suspension:

Binding units/ml_o	8.54×10^5
HAU at zero dilution	1.33
BU/HAU	6.42×10^5

Mean BU/HAU for the three suspensions:

1.06×10^6

C. Binding unit assay by monomer count and by aggregate evaluation

An infected allantoic fluid was assayed by monomer count at a dilution of 1:2,400.

Estimate of $BU/ml_o = 9.06 \times 10^8$

During the counting process, the numbers and values of the aggregates present in the same preparation were recorded and the BU/C ratio calculated according to the method of Cameron & Bradish (1972), that is, assuming n-1 binding units per aggregate. From this value,

C. Cont'd.

multiplied by the starting cell concentration used to add to the virus suspension, the binding unit concentration was derived.

$$\text{Estimate of } BU/ml_o = 6.6 \times 10^8$$

This finding, that the binding unit estimate by aggregate evaluation is slightly below that obtained by monomer count, has been found on each occasion that the two methods have been compared and may reflect a true difference in the results obtained by them.

D. Relationship between unit characteristics of a virus

Consider the population parameters of physical particle count, infective unit count, and binding unit count. A number of workers have estimated the equivalence between physical particle and infectious unit count: Levine et al. (1953) found a mean of 4.05 physical particles equivalent to 3.0 absolute haemagglutinin particles (as defined by these authors) and that 1.6 - 4.8 ($\bar{x}$ = 2.88) haemagglutination particles were equivalent to 1 infective unit, so that it follows that the ratios of physical particles: haemagglutinating particles: infective units are 3.8:2.8:1. Ping-Yao Cheng (1961), following the dimer-forming unit hypothesis of Levine et al. (1953), but working with SFV, found ratios (the means of two assays) of dimer-forming units: plaque-forming units: physical particles of 1:1:1.4. Cameron and Bradish (1972), also working with SFV, found varying ratios of RBC-RBC bonds: infective units: physical particles as follows:

9.8:1:0.27 (36.3:3.7:1)

11.9:1:0.87 (13.68:1.15:1)

9.4:1:8.83 (1.13:0.11:1)

26.0:1:1.42 (18.31:0.70:1)

D. Cont'd.

If the physical particle to infective unit ratios are considered, since these are the most likely to be comparable, it can be seen that there is considerable variation between the values obtained by different workers. The results of the comparisons of binding-units estimated by monomer counting and egg infectivity reported above, imply a near 1:1 ratio, which agrees with the dimer-unit: plaque-forming unit ratio obtained by Ping-Yao Cheng; yet the empirical results of Cameron and Bradish (1972) and those reported in this work, and the Random Distribution and Interaction Model all imply that the dimers-only hypothesis is inappropriate as an explanation of the haemagglutination process. Clearly, a question needing to be resolved is whether under ideal conditions, it is reasonable to expect that a 1:1:1 ratio will exist between physical particles, infective units, and HA particles, or whether it is possible that some other ratio is applicable which may satisfactorily represent the absolute conditions which exist. It is possible to propose a number of conditions which may represent the true population conditions of a virus suspension:

1. Proportional equivalence

The logical consequences of this proposition are complex. Suppose that x physical particles ≡ 1 binding unit. Then it must be postulated that only 1 out of x physical particles is capable of primary adsorption, that the remaining x-1 out of x particles are not capable of primary adsorption, but adsorb secondarily, that any association of a number of particles <x is not capable of agglutinating two erythrocytes, and that the primary particle activates exactly x-1 sites in addition to its own.

D. 1. Cont'd.

If it is postulated that <u>any one</u> of the x physical particles is capable of adsorbing to an erythrocyte, then it is necessary to postulate random distribution of all the particles among the available erythrocytes. This would confound the Random Distribution Model on theoretical grounds when it has not, so far, been confounded empirically. Table 17 illustrates this argument. Column A shows the Poissonian distribution for $\bar{x} = 0.5$. Column C shows the multiplicity of events when $\bar{x} = 6$ if 12 physical particles = 1 binding unit. Since any adsorbed multiplicity of <12 will be ineffective, only multiplicities of 12-23 can be effective as single binding units, and it follows that there will be a negligible number of double effective binding-units on the erythrocytes. Also, the proportion of erythrocytes having effective binding units adsorbed is 0.02009, so that if 12 physical particles = 1 binding unit, then it logically follows that, with Poissonian distribution, 600 physical particles = 1 binding unit, which is nonsense. The distribution of the physical particles then, is not Poissonian and, for the condition of 12 = 1, the distribution of physical particles must be as shown in column B. Hence, both primary adsorbing particles and dependent secondarily adsorbing particles must be postulated.

It is then necessary to postulate:

a) A replication mechanism to account for the presence of two types of particle.

b) A genetic code on the RNA to give rise to the production of the appropriate proportions of primary and secondary adsorption particles.

TABLE 17

LEGEND

Table 17: The non Poissonian distribution of particles in an equivalence situation when the ratio of physical particles to binding units is 12:1.

Column A is the Poissonian distribution of events when $\bar{x} = 0.5$.

Column B is the distribution of physical particles when 12 physical particles = 1 HA particle and the HA particles are distributed in a Poissonian manner.

Column C shows the Poissonian distribution when $\bar{x} = 6$.

TABLE 17

x	A $\bar{x} = 0.5$	B $12 = 1, \bar{x} = 0.5$	C $\bar{x} = 6$
0	.606531	.606531	.002479
1	.303265		.014873
2	.075816		.044618
3	.012636		.089235
4	.001580		.133853
5	.000158		.160623
6	.000013		.160623
7	.000001		.137677
8			.103258
9			.068838
10			.041303
11			.022529
12		.303265	.011264
13			.005199
14			.002228
15			.000891
16			.000334
17			.000118
18			.000039
19			.000012
20			.000004
21			.000001
22			
24		.075816	
36		.012636	
48		.001580	
60		.000158	
72		.000013	
84		.000001	

D. 1. Cont'd.

c) Monovalent HA characteristic of all individual particles acting in multiplicities of <x.

d) A mechanism involving the erythrocyte surface to render x-1 sites functional once one has been occupied.

e) A limiting mechanism to avoid activation of more than x-1 sites on the erythrocyte otherwise random distribution of all the particles must result.

Although all of the above is possible, it must be considered whether it is probable in the light of current knowledge. The situation where one multiplicity of physical particles is equivalent to an infective unit and yet another is equivalent to a binding unit, leads to a number of necessary mechanisms that is so large that acceptability is doubtful.

2. Aggregate equivalence

If this is considered as a characteristic of the virus population not necessary for either infectivity or HA, then it is an empirical variable and may be postulated as a variable feature of the virus, possibly dependent on the physical conditions. If, however, it is postulated that the aggregation is a necessary condition for infectivity and agglutination, then it is also necessary to postulate a mechanism, by virtue of which the aggregate is effective, whereas the individual physical particles are not. Since the aggregation is a feature of the particles, not of the cell, then the mechanism must be a feature of the particles.

D. 2. Cont'd.

To each particle must be assigned an increment of adsorbing capacity, which only becomes effective when the increments total 1. If the aggregate size, and therefore the physical particle:infective unit or the physical particle:binding unit ratio is to be standard value (following logically from an ascribed increment per physical particle), then there must also be postulated a limiting mechanism that is a feature of the physical particle. If the physical particle:infective unit ratio is different from the physical particle:binding unit ratio, there are only two possible explanations.

a) The different aggregate sizes exist simultaneously with common physical particles as their sub-units.

b) Two different aggregate sizes exist simultaneously with different particles as their sub-units. The aggregates then constitute separate populations, one of which is infective and one of which is haemagglutinating.

The first explanation is not logically possible and the second carries with it the necessity of further postulations:

a) Two types of particle must be produced, one possessing the appropriate increment of HA capacity, the other possessing the appropriate increment of infective capacity. To ascribe both factors to each particle entails the difficulty that the aggregate form requiring the lesser number of increments would be the limiting one and the population would be either infective or haemagglutinating; it could not be both.

D. 2. Cont'd.

b) As with 'proportional equivalence', it must be postulated that there are replication mechanisms and the appropriate genetic code available for the production of two types of particle in the appropriate proportions.

c) The minimum ratio of physical particles to infective units and of physical particles to binding units must be 2:1 since the population will be 1:1 + 1:1. This requires a 1:1 ratio of infective units to binding units. Once this ratio departs from 1:1, then the ratio of physical particles to either infective units or binding units will be >2:1, although the other ratio will simultaneously fall to some value between 2:1 and 1:1. A ratio pattern of 1;1:1;, i.e., 'total identity', is not logically possible.

3. Variable equivalence

This, if it is a valid hypothesis, must be a variant of either 'proportional equivalence' or of 'aggregate equivalence', and in either case, entails the appropriate complications with, in addition, that of having a separate set of constant values for each virus under consideration and/or for every different set of conditions. With the current motivation in science being to search for unity of pattern and function, a hypothesis which defines the characteristics as being infinitely variable is contrary to current attitudes. This does not, of course, invalidate the hypothesis, but it does suggest that there may be more acceptable explanations.

D. Cont'd.

4. Total identity

Each individual physical particle invariably displays HA and infectivity characteristics. This is the simplest of all the hypotheses and there is, at this point, little logical argument that can be put forward to sustain it. It must stand, however, as an available refuge if all other hypotheses are not supported. The only study to support this hypothesis is that of Ping-Yao Cheng (1961), in which the premises of 'dimers-only' on which the bonding unit calculations depends is open to question. Being simple, the hypothesis avoids internal inconsistencies or the necessity for postulating further hypothetical mechanisms to avoid logical inconsistencies or to make it feasible. Yet its very simplicity may be the foundation of its invalidity.

5. Partial identity

The inference here is that the values for physical-particle count, infective-unit count, and binding-unit count are measures of dimensions of the population of particles, that there is some proportion of the particles which have total identity, some physical particles which are effective in infectivity or HA, and some proportion which possibly is not effective in either of these categories. The hypothesis does not postulate a fixed proportion, but may do this under precisely specified conditions; it can, however, accommodate variations in any dimension either between preparations or over time within one preparation. The key feature is, that it is postulated that some proportion of the particles in a population, under normal conditions, will individually display physical particle, infectivity, and HA characteristics.

D. Cont'd.

6. Spurious identity

The essence of this hypothesis is, that there is no a priori reason for ascribing identity to two measures of a population which are equal, or partial identity to two measures of a population which are not equal, they should be treated as independent populations.

There are two possible logical consequences of this approach.

1. A virus population contains three entities, one seen by EM examination, one not seen, but having infective characteristics and carrying the code for the production of all three entities, and one not seen and not infective, but having HA characteristics.

2. A virus population consists of three types of particle, one seen by EM, but lacking infectivity or HA characteristics, one seen and having infective capacity, and one seen and having HA capacity.

The first consequence is virtually a spontaneous generation doctrine. The second results in the necessity to postulate a genetic code and mechanisms to produce multiple particles, although there is no necessity to postulate constant proportions, merely two, or perhaps three, types of particle.

The choice between available alternative explanations can be made on philosophical grounds, or on the basis of key experiments designed to eliminate some of the alternatives, or both.

D. Cont'd.

Philosophically, a choice can be made on the basis of the Principle of Parsimony, or Occam's Razor. This may be paraphrased in modern English as "given that two hypotheses save all of the appearances, the more simple is the more likely to be true". This is not to say that an explanation will be true because it is simple, but that this is the formal criterion to use in the absence of others being available.

Considering the six hypotheses on this basis:

'Total identity' must be eliminated on the grounds that the available data lend it no support; however, it may be a special case of 'Partial identity'.

All of the 'Equivalence' hypotheses, and the 'Spurious identity' hypothesis entail postulations of multiple particle types and complex mechanisms to account for their presence. There is no data to support the idea of two types of particle being coded for and produced by a cell under normal conditions.

The only acceptable hypothesis then, would appear to be that of 'Partial identity'. This allows for the presence of all types of particle without postulating special mechanisms for their production, accommodates the idea of decay of one characteristic without prejudice to the others, e.g., decay of infectivity in influenza-virus in allantoic fluid without loss of HA titre (unpublished data, 1955); and it is consonant with the varying ratios of physical particles: infective units: HA activity published in the literature since the results of any study can be so presented, it is purely a mathematical exercise.

D. Cont'd.

In addition to the philosophical considerations, we are now in a position to plan definitive experiments; these must be so designed that they test key necessary consequences of the hypotheses. Table 18 illustrates a programme to determine which hypothesis is the acceptable one. The key experiment is that of EM examination of dimers for this differentiates the "Identity' hypothesis from the 'Equivalence' ones. The von Magnus phenomenon, particularly as analyzed by Fazekas de St. Groth (1955), implies that the 'Total identity' hypothesis can be eliminated immediately. The only other experiment required is to determine the infectivity of virus eluted from erythrocytes.

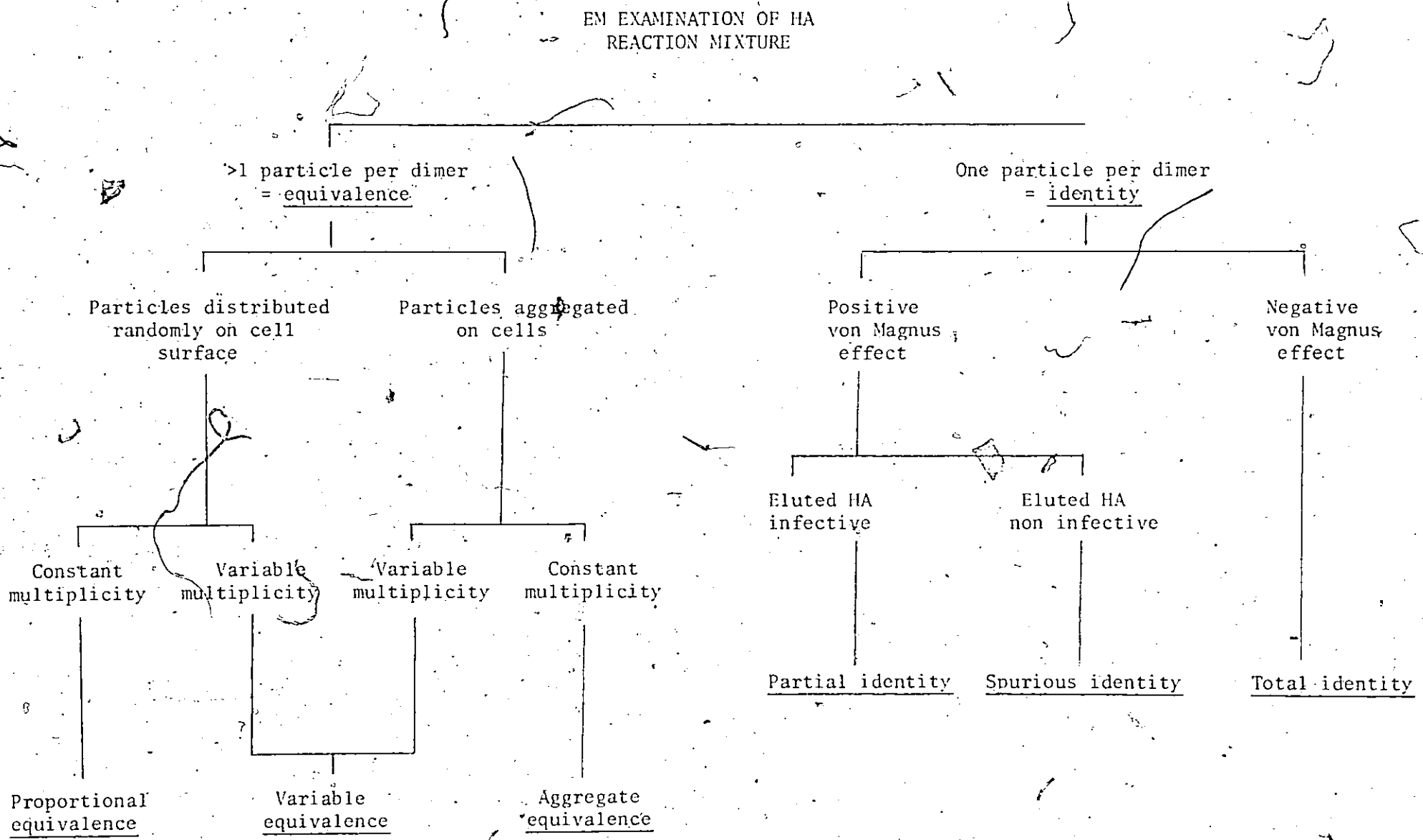
A freshly prepared influenza-virus allantoic fluid, (0), was held at 4°C to be assayed. Concurrently, an aliquot was absorbed at 4°C for 2 1/2 hours on a rotator using 1/10th the allantoic fluid volume of packed, washed, human erythrocytes. The preparation was centrifuged in the cold to deposit the erythrocytes and the supernatant fluid, (ASN), removed. The erythrocytes were thrice washed and resuspended in fresh, normal allantoic fluid and this preparation was heated at 37°C in a water-bath for three hours. The erythrocytes were deposited by centrifugation at room temperature and the supernatant fluid (E), was removed. Fluids O, E, and ASN were assayed by monomer count and tube-HA for haemagglutinin concentration, and fluids O and E by inoculating into fertile eggs, 0.1 ml. allantoically at an estimated inoculum of 1 BU/egg, 28 eggs per fluid, for infectivity. The eggs were chilled after 48 hours of incubation at 37°C and the allantoic fluids tested for haemagglutinating activity.

TABLE 18.

LEGEND

Table 18: A programme design to determine the true relationship between two dimensions of a virus particle suspension.

TABLE 18


EM EXAMINATION OF HA REACTION MIXTURE
>1 particle per dimer = equivalence
One particle per dimer = identity
Particles distributed randomly on cell surface
Particles aggregated on cells
Positive von Magnus effect
Negative von Magnus effect
Eluted HA infective
Eluted HA non infective
Constant multiplicity
Variable multiplicity
Variable multiplicity
Constant multiplicity
Partial identity
Spurious identity
Total identity
Proportional equivalence
Variable equivalence
Aggregate equivalence

D. Cont'd.

The data presented in Table 19 indicate that:

1. The proportion of agglutinin adsorbed by the erythrocytes was 99.6% of the initial population.
2. The proportion of adsprbed agglutinin eluted from the erythrocytes during three hours of incubation at 37°C was 48%.
3. There was a difference of 10% between the infective activity of the native and that of the eluted virus. This difference is probably not significant.
4. The pooled results derived from the three materials tested yielded a value of 1.06 BU/HAU.
5. When the mean of the infective activity figures is applied to the mean BU/HAU, the figure of 1.74×10^6 infective units/HAU is derived. This figure compares favourably with the results of Channock and Sabin (1954) from three sets of whose data can be derived a mean value of 3.96×10^6 infective units of WEE-virus per HAU, but is approximately 10 times the mean of five values given by Levine et al. (1953) for influenza-virus, 0.15×10^6 infective units per HAU.

The absence of a significant difference between native and eluted virus in terms of infectivity indicates that binding units and infective units are not only equal, they are probably identical.

Now the data of Levine et al. (1953) gives a mean of 3.8 influenza-virus physical particles per infective unit, Ping-Yao Cheng (1961) found 1.4 physical particles of SFV per plaque-forming unit, and Cameron & Bradish (1972) found a mean of 0.7 physical particles per infective unit, also using SFV. The influenza-virus result, then implies an 'equivalence' relationship, which infers two types of particle etc., whilst the results

TABLE 19

LEGEND

Table 19: Comparison of native influenza-virus (PR8) and HA eluted from erythrocytes.

Infectivity and binding-unit: HAU ratios of native, eluted, and residual agglutinin after absorption of the native material with erythrocytes.

O = native material

E = HA eluted from erythrocytes during 3 hours of holding at 37°C

SNF = residual material after absorption of the native material with erythrocytes

BU = binding units

HAU = tube haemagglutinating units

IU = infective units

TABLE 19

	Dilution	BU/ml	Mean BU/ml	% of 0	Tube HA titre	Mean HA titre	BU/HAU	1 BU/egg eggs neg/ eggs inoc.	$\bar{x}$ from Poisson
	1:1000	1.36×10^8			1:160				
	1:2000	1.58×10^8			1:160				
0			2.11×10^8	100		1:133	0.63×10^6	5/28	1.74
	1:4000	2.77×10^8			1:80				
	1:6000	2.74×10^8							
	1:500	8.99×10^7			1:160				
E	1:1000	1.26×10^8	1.02×10^8	48	1:80	1:133	1.30×10^6	6/28	1.55
	1:2000	8.89×10^7			1:160				
	1:10	4.40×10^5			1:1				
SNF	1:20	6.13×10^5	8.54×10^5	0.4	1:2	1:133	1.56×10^6		
	1:40	1.51×10^6			1:1				

D. Cont'd.

with SFV imply an 'identity' relationship, in which case it follows from the von Magnus effect and the infectivity of eluted virus results that the 'Partial identity' hypothesis is probably the appropriate one. It may also be argued that the influenza results fit the 'Partial identity' paradigm and that it is tradition that has produced the habit of thinking in terms of 'Equivalence'.

DISCUSSION

The technique of assaying Rubella-virus antibody by means of the HI test has found one of its most important applications in the determination of the immune status of women before, and during the first trimester of pregnancy.

Some of the problems encountered by clinicians in the management of such cases stem from inadequacies inherent in the HI test. In common with most serological tests, the HI is an end-point-of-reaction determination. As such, the sensitivity is a function of the technique used, and test-retest stability is a function of the degree of standardization achieved in the laboratory situation. This type of test is extremely difficult to standardize across two different laboratory situations, and the problems entailed in standardizing a test on a country-wide basis for epidemiological studies are enormous. A problem common to all end-point tests is that of deciding at what level the serum dilutions will start. A common starting dilution is 1:4, another is 1:10. Why one or the other? In fact, why either? It is easy to make a case for either one when the concern is the diagnosis of acute infection with, for example, respiratory and enteroviruses, for the objective here is to demonstrate a rise in antibody titre, although here the arbitrary minimum diagnostic rise of 4-fold is a function of the historical experience that a serum titre can vary by 2-fold in the test-retest situation.

In the case of determining whether a woman is at risk with respect to rubella-virus infection, there appears to be no clear-cut answer as to why a particular serum dilution is used as the lower level, unless it is that this is the dilution which is produced by the pre-test serum treatment favoured by the particular laboratory.

Whatever the reason, there is currently no way of determining the absolute immune status of a patient. This raises the question of what it is that is meant by the terms 'immune' and 'non-immune'. Since the minimum serum test level used is arbitrary, these terms themselves are arbitrary; and studies on the correlation of infection risk or infant abnormality with the immune status of the mother are bereft of some of their value.

A further complicating factor is the antigen dose used in the test. In the complement fixation test, there is little room for arbitrary decision-making, since many antigens show a more-or-less clear-cut optimum concentration which will yield the highest serum antibody titre. In the HI test, in common with virus-neutralization tests, the sensitivity of the test is a direct function of the concentration of the antigen in the system. Theoretically, the absolute reactivity of a serum would be known if this could be stated in terms of the neutralizing equivalent of some absolute quantity of test antigen, but there is currently no known assay system which will give an absolute quantification of HA antigen. The current method of pre-titration gives a rubella antigen titre, or dose, which bears an inverse relationship to the erythrocyte concentration. In the test situation, the assessed reactivity of a patient's serum is a function of the interaction of the concentration of erythrocytes and the number of antigen doses selected for the test. A negative result is also dependent

upon the starting dilution used for the serum. When there is added to this the pH sensitivity (Holmes and Warburton, 1967; Stewart et al. 1967 and Lieberhaber, 1970) and temperature dependence (Stewart et al. 1967) of rubella haemagglutination and the thermo-stability problems liable to be encountered with the antigen, probably the only way to achieve test-retest stability is absolute standardization of every facet of the test situation.

One approach which may be used to try to overcome the above problems is to investigate the conventional test; to try to pin-point and correct inadequacies in the test and in the reagents used, and to try to improve the stability of the test in order that advantage may be taken of an improved sensitivity of antibody assay. In this way, low-level reactors who would normally show a negative result may become included in the 'immune' category. This effect may be enhanced by devising methods of lowering the dilution threshold. However, the threshold will still exist, and the placing of a borderline reactor above or below it will be a function of the antigen dose used in the test. These two factors, the antigen dose and the dilution threshold, are the ultimate determinants in the clinical evaluation of immunity to foetal infection during pregnancy. An alternative approach is to attempt to produce a method which will assay antibody concentration in absolute terms. This would facilitate clinical studies having an absolute frame of reference and would enable those in the clinical field to give a clearer meaning to 'immunity'.

Standardization of Rubella HAI Test

Temperature of the test

For a number of years, the practice has been to carry out all rubella HI tests in a cold-room at 4°C. All reagents and equipment are stored in the room to ensure that there can be no confusion about when and where reagents should be cold. This is not entirely clear in the CDC Standard Rubella HAI Test Procedures (1970) where the instruction is that 'All reagents must be cooled to 4°C before use in the haemagglutination-inhibition test'. There is no instruction that the four steps of serum dilution and addition of antigen should be done at other than room temperature. However, the test is incubated for one hour at 4°C, after which cold RBC suspension is added. The instruction to 'add RBC's to each plate separately to avoid warming the reagent' when a large number of plates are included in the test implies that the procedure is being carried out at room temperature. This technique has standardization problems in that the upper limit of temperature tolerance for rubella-virus HA reaction is not known and variations due to differing technical staff and work pressures are impossible to define. This problem is acknowledged by the Flow Laboratories (1973) manual where the suggestion is made to work with the plates in ice-trays. Carrying out the entire test in the cold room avoids all uncertainties in this area.

Chick cells

The instruction issued by the CDC (1970), to bleed one to three-day old chicks, is followed by Flow Laboratories (1973). This procedure is in accord with the recommendations of workers since the original publication by Stewart et al. (1967).

However, the system has inherent disadvantages:

a) New chicks must be hatched or bought in at intervals.

b) The cells must be stored in Alsever's solution so that they vary in quality over time, although it is alleged that they can be stored for up to six weeks for use.

c) The task of bleeding one to three day-old chicks is one which is intensely disliked by many technologists.

The practice of bleeding 14-16 day-old embryos in-shell has eliminated all chick erythrocyte supply problems. The fertile egg is the ideal storage vehicle for chick erythrocytes; they are available fresh on any day, and the technical staff seem to be unaffected by bleeding an egg into the allantoic sac and harvesting the fluid. Rubella HI tests are performed in the laboratory two or three times each week. No variations in test results have been experienced during the several years that embryo erythrocytes have been used and, even when stored sera have been re-tested for some diagnostic purpose up to two years after the initial test, the titre has been reproduced. This suggests that there is little, if any, variation in sensitivity among the erythrocytes of individual, or groups of, embryos.

Antigen stability

The impression gained from the literature is that the Tween-80-diethyl-ether treatment of Norby (1962), when applied to rubella-virus, produces enhancement of the HI titre (Holmes and Warburton, 1967), and an agglutinin which is stable for up to one hour at room temperature and up to 12 hours at 4°C (Lieberhaber, 1970).

Antigen instability, experienced over a period of time, and examined in detail in the experimental situation, is not in accord with these workers' results; this discrepancy in findings may be due to the techniques used. Holmes and Warburton (1967) did not report stability problems so presumably these were not encountered; this may be due to the fact that they eluted their virus at 37°C before treating it with Tween-80/Ether. Since they do not report otherwise, it can be presumed that the treatment was carried out at, or near, room temperature as in the original technique for measles virus (Norby, 1962). Lieberhaber (1970) diluted his TE-HA 'in HSAG, pH 6.0, at 25°C to contain 8 HAU/0.025 ml'. This material was then chilled to 4°C. A similar preparation in HSAG, pH 6.2, was prepared in the same way and held at room temperature. The procedures of both these workers would probably ensure that most of the low stability (LS) component, which is postulated as a result of the work in this paper, would have degraded by the time that the material came to be tested, and only the more stable HS component would remain. Workers using the CDC rubella HI method, routinely would also not encounter stability problems since, for antigen titration, an initial dilution of antigen is allowed to stand at 4°C for 15 minutes; it is then diluted and again allowed to stand at 4°C for 15 minutes after which the cells are added. Reference to Figure 4 in the text shows that the postulated LS component has reached zero concentration after 35 minutes at 4°C, so that it would not be detected by the CDC titration technique. This corresponds to our own practice of titrating the antigen under the conditions of the test.

A question which still remains is whether the decayed antigen present in a preparation which has degraded to some 5-10% of its original titre can cause interference by blocking antibody, even though it is not capable of causing effective haemagglutination. Lieberhaber (1970), in considering the effect of undetected antigen at high pH values, concludes that the test will be rendered insensitive. The stability of the agglutination pattern at temperatures at which the free agglutinin is unstable (Figure 4, Table 2) offers not only an alternative antibody titration system, but also some indication of the rate of adsorption of agglutinin to erythrocytes. It was seen (Figure 4) that the degradation of the LS component is complete within 35 minutes, yet the concentration shows no detectable fall during this time when it is adsorbed on erythrocytes (Figure 2). It must be concluded, therefore, that the rate of adsorption is far greater than the rate of degradation. If the adsorption time were much more than virtually zero, a far higher starting level than 575 in Figure 2 would need to be postulated. When the same type of analysis was carried out on this data as on that used in Figure 4, the slope of the lines for the LS and HS components indicated 575 to be the true zero time activity level.

Haemagglutination-Inhibition and Haemadsorption Reversal

It was seen that there was an 8-fold improvement in antibody titre by HAR over HI. There are a number of possible explanations for this. The two-fold increase in antibody titre achieved at 4°C by omitting kaolin treatment may have been due to removal of globulin by the kaolin (Lieberhaber, 1970); the same effect was not observed when the test was carried out at room temperature, however, where the serum titre is marginally higher than at 4°C with both serum treatments giving the same results. It is unlikely that the effect was due to residual non-specific inhibitors or reversal factors since there was no evidence of inhibition or reversal by the negative serum. The marginally higher antibody titre at room temperature may have been due to slight inactivation of antigen at this temperature, but the inactivation cannot be severe or a 'tail' of partial reaction would have been apparent as in Table 6 at the 1.0 and .67 unit levels. In the test in Table 8, there was the normally rapid - ⟶ + transition over one dilution step.

Another factor in increasing assay sensitivity may have been the lower antigen dose used in the HAR test, 2.3 units as against 6.0 units in the HI test. If this were so, the 4-fold increase in test sensitivity was achieved by means of a 2.6-fold reduction in antigen concentration. The results may be substantiation for the suggestion by Lieberhaber (1970) that the presence of undetected antigen may lead to test insensitivity; in the HAR test, there is little or no superfluous or degraded antigen present if the cells are sensitized immediately the antigen is reconstituted. The individual contributions of the above factors would probably be difficult to determine, but the overall effect has been an 8-fold increase in test sensitivity.

The results of the attempts to use goat anti-human IgG suggest that a more complex situation may exist in the area of non-specific inhibitors and reversal factors than is implied by the work of Lieberhaber (1970). It is clear that the data can only be satisfied by postulating a 2-part factor in goat serum which is capable of acting, not only to inhibit agglutination, but also to reverse the effect. The nature of the complex is not known, nor is it known whether it is, indeed, a complex, or two factors acting sequentially. This feature of the work is important if it is necessary to use non-human sera in the test, where it is not known what specific differences exist. It is clear that each species would require careful preliminary investigation before being used in a rubella HI or HAR test.

Lastly, it would appear that it is possible, under the right conditions, to use anti-human globulin sera to identify the reacting globulin in the HI or HAR test. If these are to be useful, however, they must be functional with sera diluted only 1:10 or 1:20, whereas, the Hyland product was found to be effective against only 1/23rd of its own concentration of human serum. The use of an antiserum of such low potency in a routine test situation would be impractical as the serum could not be treated and used at a high enough concentration, and the cost would be prohibitive. The need here is for hyperimmune serum giving at least a 1:1 ratio for complete neutralization of the globulin present in normal human serum.

Binding Unit Estimation by Monomer Counting

An alternative to preparing hyperimmune anti-human IgG serum would be to make the HI or HAR test even more sensitive, but the impression gained from the work so far is that the limit has probably been reached in terms of a useable test for day-to-day work. The work of Cameron and Bradish (1972) is frustrating in that it uses an estimate of the binding-unit concentration, yet ultimately yields a sophisticated end-point titration method. This is probably due to several factors: the estimate obtained is dilution dependent, it is by no means certain that agglutination has proceeded to completion and, therefore, the results are relative and dependent upon rigid test standardization, and the underlying assumption of RBCs in excess has no valid foundation. The theoretical model that has been constructed in this work is unrefined. For instance, only one point is estimated at each level for Figure 8 showing the relationship of binding-unit concentration and residual monomers after reaction to completion. Clearly, for this figure to show the absolute relationship, there should be some 30 points at each level, but this is not reasonably possible by hand-calculation and would need the use of an appropriately programmed computer. Nevertheless, there is a high probability that the figure is a reasonable representation of reality. The results of work on cell concentration, counting range, and time of reaction indicate that the conditions must be carefully predetermined. However, once the conditions have been established, the constraints are minimal. The starting count needs to be in the order of 40 to 70, but preferably the lower level; this needs to be precisely estimated, not absolutely fixed. The counts after reaction need only to fall between the level determined by the

lowest counting level (17) and the upper limit of linear response (0.8 of the starting count). The overall effect is to give a very flexible assay technique, which is capable of measuring the absolute concentration of HA binding units, and gives results which are not substantially different from those obtained by direct aggregate evaluation.

Relationship of Binding Units to other Parameters

Although it has been shown that, under the right conditions, there may be 1:1 relationship between HA binding units and infective units, there is no a priori reason to suppose that this always is, or needs to be, so. Levine et al. (1953) estimated values varying from 1.6 to 4.5 Influenza HA 'particles' per egg infective unit, although the premise of their method of estimating HA particles was probably inappropriate, i.e., that the aggregates are dimers only. For NDV, their estimates were 1.8 and 3.2 HA particles per egg infective unit. Ping-Yao Cheng (1961) reported 1.05 and 0.94 dimer-forming units per plaque-forming unit of Semliki Forest Virus, and Cameron and Bradish (1972) working with the same virus, gave bond: plaque-forming unit ratios varying from 3.8 to 0.12, using concentrations of plaque-forming units varying from 1.0×10^9 to 0.62×10^{12} per ml. This probably says very little. For an HA unit: infective unit ratio to be meaningful in a particular situation, it is necessary to know that:

a) The preparation is such that infectivity is not likely to have appreciably degraded during storage, no matter how short this time.

b) The method of assaying the HA concentration is giving an absolute value.

None of the above workers satisfy the second condition and there are no criteria by which to judge the first.

The empirical work reported in this paper appears to confirm the hypothesis upon which the theoretical model was predicted so that it is probable that the monomer counting method is assaying the absolute concentration of binding units.

When the allantoic fluids were prepared for the infective-unit: binding-unit ratio estimations, they were inoculated with approximately 10 infective doses of virus and harvested at a time in the virus-production cycle when virus from the final step was probably just being released into the allantoic fluid. It was reasoned that, at this stage, there would be as high a proportion of the virus population as possible showing both infectivity and HA activity. In the two experiments which showed a 1:1 ratio of HA binding units to infective units, the freshly harvested fluid was held at 4°C only long enough for the B/ml_o estimate to be made, approximately 3 hours, before being inoculated into eggs. In the third experiment in which this ratio was assayed, the time at 4°C from harvesting to egg inoculation was approximately 9 1/2 hours due to washing the aggregates and eluting the virus from them. The first experiments yielded IU/BU ratios of 0.99:1 and 0.87:1. The third experiment with the long holding time gave IU/BU ratios of 1.74:1 and 1.55:1.

Experience with storing influenza-virus infected allantoic fluid indicates that infectivity decays more rapidly than haemagglutinin, so the change from <1 to >1 in the infective-units equivalent to one binding-unit is probably due to experimental variation rather than decay of

haemagglutinin. The four results being considered cover exactly a two-fold range; if this is considered as due to experimental variation, the mean of the values may be taken and calculated as 1.29, standard deviation 0.423. The value of 1.0, then, is 0.686 standard score units below the mean and is not significantly different from 1.29. In other words, these results are compatible with a 1:1 ratio of infective units to binding units within the variation of the exporimental results.

If the five ratios of physical particles:infective particles given by Cameron and Bradish (1972) are treated in the same manner as above, it is found that one physical particle is equivalent to 1.33 infective particles, standard deviation 1.41, when the value of 1 is 0.234 standard score units below the mean. Again, the data are compatible with a 1:1 ratio within experimental variation.

The conclusion that must emerge from the above data, when considered in conjunction with the von Magnus effect and the approximately equal infectivity of native and eluted influenza-virus discussed previously, is that it is quite possible to have 1:1:1 ratios of physical particles: infective particles:binding units. However, the very variable results obtained by workers, infers that this is the exception and that the actual result probably will always be a function of the conditions of the particular experiment under consideration. The final word on this must await the outcome of investigation of the number of physical particles that are associated with a dimer and the distribution of physical particles on erythrocytes at various calculated BU/C ratios. Although there is a fair

amount of evidence to support the view that 'partial identity' is the true representation of reality, it is only by demonstrating that one physical particle is capable of agglutinating two erythrocytes that the concept of 'equivalence' can be eliminated from the available hypotheses. Also, this is the only empirical work that can establish unequivocally that one binding-unit, as estimated by the monomer-counting technique, is one agglutinating particle. It is of paramount importance that the EM studies be carried out.

If, as appears likely, the monomer counting technique estimates what the Random Distribution and Interaction Model predicts that it should, then there will be available a method of absolute quantification of agglutinin which will be the basis for a method of absolute quantification of antibody. If this can be achieved and applied to individual fractions of serum γ-globulin, then serology will take its rightful place in the armoury of the diagnostic laboratory.

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