

## **The use of single-radial-haemolysis for rubella antibody studies**

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### SUMMARY

The use of a single-radial-haemolysis technique for the detection of antibody to rubella virus is described. The single-radial-haemolysis test was compared with the standard HI methods for the detection of antibody to rubella virus. A close correlation between the two methods was observed in a survey of over two thousand serum samples and the study indicated that single-radial-haemolysis was highly satisfactory as an assay method for IgG antibodies to rubella virus. It was found that the immuno-globulins active in SRH tests sedimented in the 7S range in sucrose rate gradients and were presumably immunoglobulins of the IgG class, but 19S immunoglobulins did not produce haemolysis.

### INTRODUCTION

The development of the single-radial-haemolysis (SRH) technique for the detection of antibody to influenza haemagglutinin (Schild, Oxford & Virelizier 1975*a*; Schild, Pereira & Chakraverty 1975*b*; Russell, McCahon & Beare, 1975), has led to the application of this method to other haemagglutinating viruses. It has been successfully used for rubella virus (Skaug, Ørstavik & Ulstrup, 1975; Strannegård, Grillner & Lindberg, 1975) and for mumps virus (Grillner & Blomberg, 1976; Väänänen, Hovi, Helle & Penttinen, 1976). We report here the advantages of the technique in screening large numbers of sera for rubella antibody and assess the validity of results in comparison with the haemagglutination-inhibition (HI) and complement fixation (CF) tests.

The SRH test is dependent on the ability of antiviral antibody in the presence of complement, to lyse erythrocytes which have been sensitized with rubella antigen. The SRH reaction is not affected by non-specific inhibitors present in human sera and its use eliminates the necessity for treatment of serum to remove such factors. Pre-treatment of serum for SRH tests requires only heat inactivation and microlitre volumes are sufficient for SRH assay. The method is simple and specific and large numbers of sera can be tested rapidly.

## MATERIAL AND METHODS

*Sera*

Serum samples from donors aged 18–30 years were obtained in 1976 from the North London Clinic of the National Blood Transfusion Service. Sera from children aged 10–11 years were collected as part of a measles vaccine surveillance study in 1974–5. Two human serum standards, the British Standard for anti-rubella serum 69/60 and a laboratory reference serum 66/161 together with a human serum negative in the HI test (titre < 1/8) were included in all SRH tests.

*Antigen*

Antigen was prepared in BHK-21 cell cultures using a wild strain of rubella virus GOS-10 obtained from Professor Dudgeon, Institute of Child Health, Great Ormond Street. The preparation of antigen was by the standard method for the extraction of haemagglutinin using Tween 80 and ether (Norrby, 1962). The antigen had a HA titre of 1/512 and was used undiluted in the SRH test.

*Erythrocytes*

Pigeon erythrocytes were used routinely for both the HI and SRH tests. However, similar results were obtained in the SRH test using erythrocytes from day-old chicks.

*SRH technique*

Erythrocytes were sensitized as described by Skaug *et al.* (1975) and suspended at a final concentration of 20% (v/v) in veronal buffer. The erythrocytes were incorporated in agarose by the method of Schild *et al.* (1975*b*); 0.3 ml of sensitized erythrocytes and 0.1 ml of fresh guinea-pig serum (complement) were mixed with 2.6 ml of molten agarose and poured onto a Hyland immunoplate. Wells of 2 mm diameter were punched in the agarose and 5  $\mu$ l of serum added to each well. Test sera were inactivated at 56 °C for 30 min before addition to the SRH plates. Plates were incubated in a moist chamber at 37 °C for 16 h. The diameter of the haemolysed zone was measured using a micrometer eye-piece scale.

Each serum sample was tested on a control plate containing 0.3 ml 20% normal (unsensitized) erythrocytes, 0.1 ml fresh guinea-pig complement and 2.6 ml agarose. In some cases inactivated sera were also added to a second control immunoplate in which the erythrocytes were treated with a 'mock' antigen made from control BHK-21 cultures by the same technique as the rubella antigen. All control plates were treated in the same way as the test plates.

*HI test*

A microtitre system was used in which 25  $\mu$ l volumes of serum were diluted in twofold steps from 1/8 to 1/16,000. The diluent consisted of veronal buffer with 0.1% bovine albumen adjusted to pH 6.3  $\pm$  0.2 with 0.1 N-HCl. Before testing the sera were treated with MnCl<sub>2</sub> and heparin to remove non-specific inhibitors as described by Dold & Northrop (1968). Four haemagglutinating units of antigen were

employed in the test. Plates were held at 4 °C overnight and read after 1–2 h at room temperature next morning.

#### *CF test*

A microtitre system was used with overnight fixation at 4 °C. The antigen was a Tween-ether extract antigen made in BHK-21 cells.

#### *Fractionation of sera*

For identification of the sedimentation rates of immunoglobulins reacting in the SRH test, serum samples were layered on a 5–20 % linear sucrose density gradient, and centrifuged at 200,000 *g* (35,000 rev./min) for 18 h in a Beckman L5-50 with a SW-41 rotor. Sera were absorbed with pigeon erythrocytes and diluted 1/2 before sedimentation in the gradient. Fractions collected from gradients were tested for HI and SRH activities.

### RESULTS

#### *Comparison of results of SRH and HI tests*

Sera from 2092 blood donors and 120 children were tested for rubella antibody by both the SRH and HI techniques. In the SRH tests, sera were diluted 1/2 before inactivation. For screening sera by the SRH method, immunoplates were prepared with 20 or 28 wells of 2 mm diameter and zones of haemolysis recorded. With 28 sera per plate, it was not possible to measure zones of haemolysis as the outlines of zones merge, but positive and negative reactions were clearly distinguishable. A representative plate is shown in Plate 1. A visible zone of haemolysis of diameter  $\geq 2.2$  mm was accepted as a positive reaction although positive sera rarely gave zones of less than 4 mm diameter. There was very good agreement between the SRH and the HI test in detecting rubella antibody. Only 6 of 1918 serum samples tested with HI titres  $> 1/8$  failed to produce visible haemolysis. Only one of 294 sera tested which were negative by HI tests (titre  $< 1/8$ ) produced a SRH reaction (table 1). Similar findings have been reported by Grillner & Strannegård (1976). None of the sera produced non-specific zones of haemolysis on control plates containing normal unsensitized erythrocytes or on plates in which the erythrocytes were treated with 'mock' antigen.

To relate individual zone diameter to HI titre, 506 sera were tested on immunoplates containing a pattern of 14 wells of 2 mm diameter and zone diameters were measured with a micrometer eye piece. The scatter diagrams of the SRH zone diameter against the HI titre of these samples shows good correlation between the two methods of antibody measurement although there was considerable scatter of zone diameter for each HI titre (figure 1). The mean diameter of the haemolytic zones increased with the HI titre.

The two reference sera 66/161 and 69/60 and the HI negative serum were tested on each immunoplate in all the above experiments. The results obtained with the reference sera showed that the variation in zone diameter from plate to plate was small. The standard deviation within any one test was about 3% of the mean diameter. No haemolysis was produced by the known HI negative serum on any plate throughout the experiment.

Table 1. *Frequency of positive and negative sera in the SRH and HI tests for rubella antibody*

	No. sera	SRH	
		Pos.	Neg.
HI titre $\geq 1/8$	1918	1912	6
HI titre $< 1/8$	294	1	293

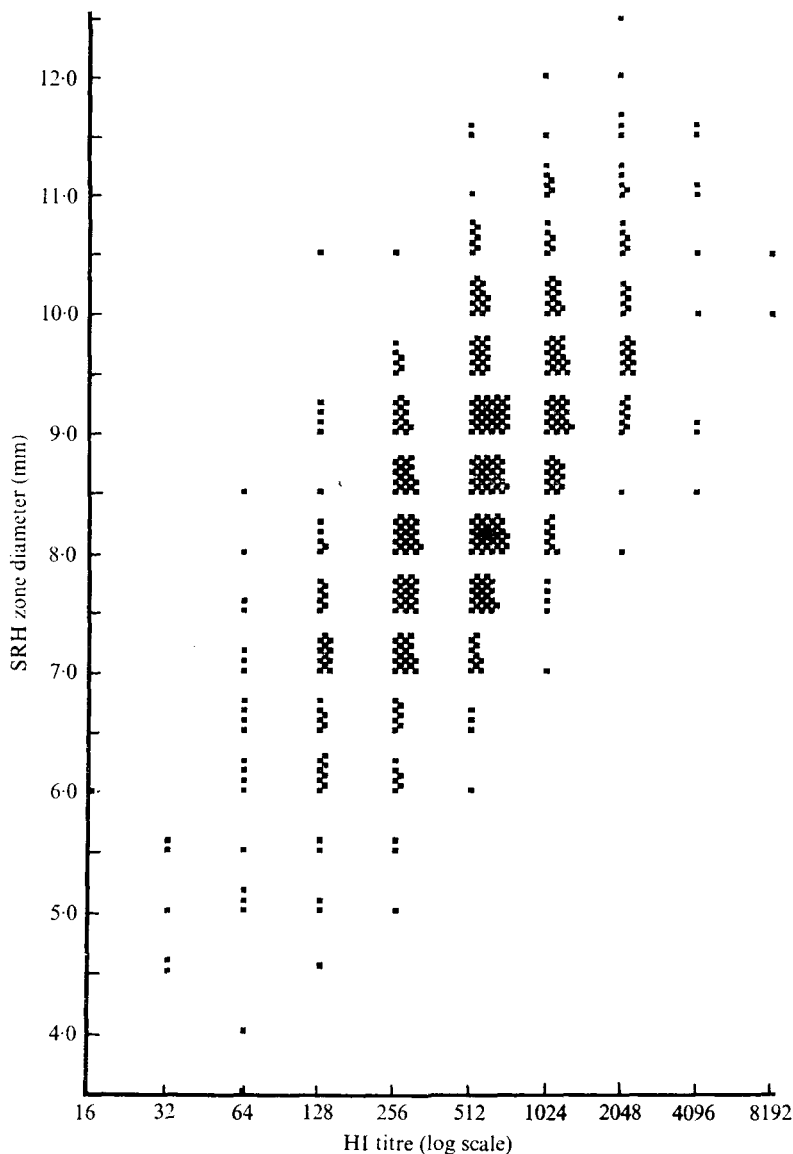


Figure 1. Relation between HI titre and SRH zone diameters obtained on 506 SRH positive sera. Each point of the chart represents the results obtained with an individual serum sample. HI titres are recorded as the reciprocal of the highest dilution inhibiting haemagglutination.

Table 2. Comparison of positive results obtained using the SRH and CF tests

CF titre	Number	SRH zone diameter	
		Median	Range
2	2	- ve*	-
4	5	6.5	6.5-8.5
8	15	8.0	6.0-9.5
16	22	9.0	7.8-11.0
32	18	9.75	9.0-11.5
64	6	10.75	9.0-12.0

\* - ve, no haemolysis.

#### Correlation with CFT

A selected number of human sera with known CF antibody titres for rubella antigen were tested by SRH. A close correlation was noted between the results of the two tests. The median and range of the haemolytic zone diameters in relation to the CF titres are given in table 2. Only two sera with low CF antibody (1/2) failed to produce a zone of haemolysis.

#### Preliminary studies into the use of SRH as an assay method

Human sera of varying HI titres were selected for study. Serial  $\log_2$  dilutions of sera were added to wells and the zone diameters measured.

Figure 2 shows the results of four replicate titrations of a serum sample with an HI titre of 1/4096 which was tested over a range of dilutions from 1/1 to 1/4096 by SRH. A plot of zone diameter against  $\log_2$  dose gave a linear relationship in the range of SRH zone diameters 4.0-12.5 mm. Haemolysis was detected at a dilution of 1/1024 but not at 1/2048.

The reference human serum 66/161 routinely used in this laboratory was compared with the British Standard anti-rubella serum 69/60 and the diameters of the haemolytic zones recorded. A linear relationship was found between diameter and  $\log_2$  serum dilution and the two lines were reasonably parallel giving a valid parallel line assay. The assay was repeated and two more human serum samples with HI titres of 1/1024 and 1/512 were included in the comparison. All sera were titrated in duplicate. The results obtained gave a satisfactory multiple parallel line assay as shown in figure 3.

#### The immunoglobulin class active in the SRH test

Sucrose rate gradient fractionation of human and monkey sera showed that the antibody active in the SRH test had a sedimentation coefficient of 7S and was presumably immunoglobulin of the IgG class. For a human serum known to contain rubella antibodies of IgG and IgM class (kindly supplied by Professor Banatvala, St Thomas' Hospital), the sucrose gradient fractions positive in the SRH test were in the 7S region of the gradient only and no SRH activity was found in the 19S region.

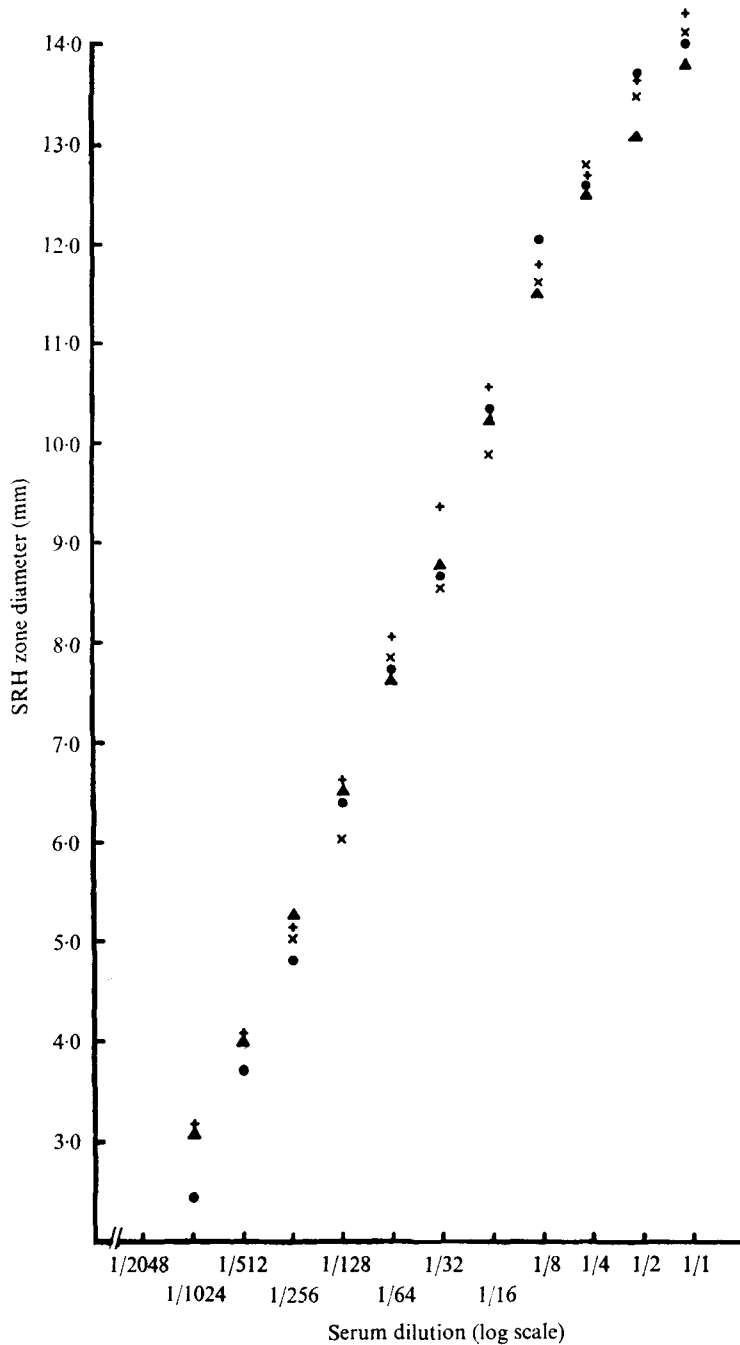


Fig. 2. A dose response curve for a human serum with a HI titre 1/4096. An approximately linear relationship was found when haemolysis zone diameters were plotted against  $\log_2$  serum dilutions. The symbols + x ● ▲ represent the results of four independent replicate SRH tests.

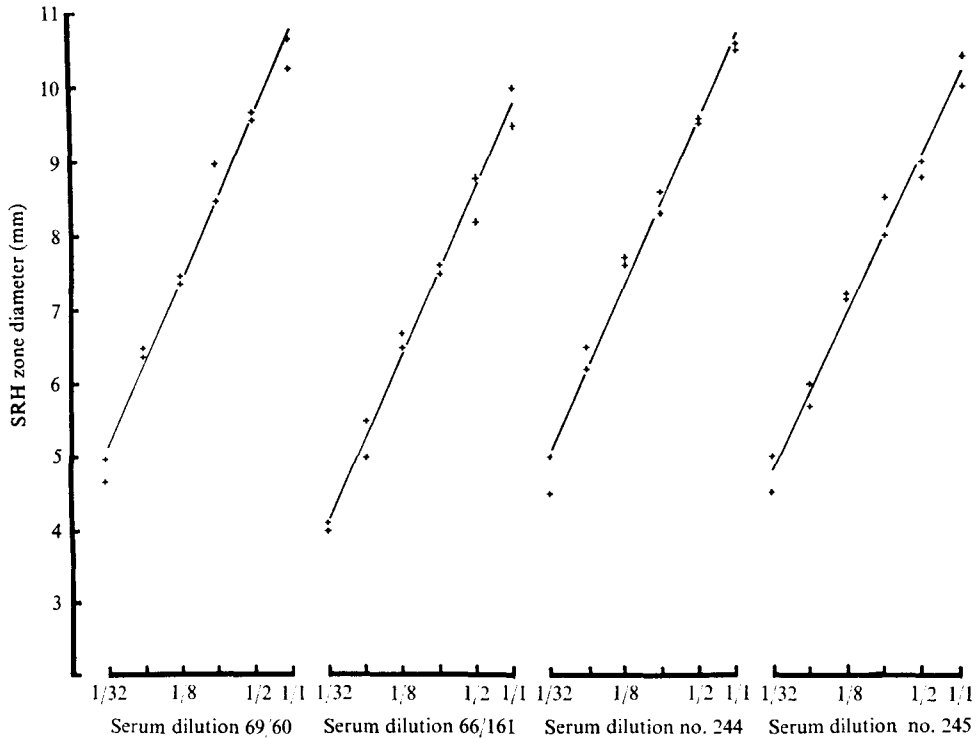


Fig. 3. The regression lines for the four sera: the British Standard for anti-rubella serum 69/60, reference serum 66/161 and randomly chosen human sera (244 and 245) tested in twofold dilutions.

Table 3. Antibody response in monkeys injected intravenously with  $10^{6.7}$  TCD<sub>50</sub> of GOS-10 rubella virus

Day Post-inoc.	Monkey A		Monkey B	
	HI*	SRH†	HI	SRH
7	< 8	0	< 8	0
10	64	0	< 8	0
14	2048	> 10.0	258	0
21	1024	12.6	512	10.3

\* HI titre, reciprocal of the dilution.

† SRH, zone diameter in millimetres.

Two monkeys were inoculated intravenously with live rubella virus. Blood samples were collected at days 3, 7, 10, 14 and then weekly up to 10 weeks. The results of HI and SRH tests on non-fractionated serum samples are given in Table 3. Antibody to rubella virus was first detected at days 10 and 14 in monkeys A and B respectively by the HI test only. The SRH test on these serum samples was negative. Subsequent samples collected four and seven days later respectively gave positive reactions by both tests. With fractionated sera, HI activity in the early serum samples was found only in the 19S region of the gradient; however

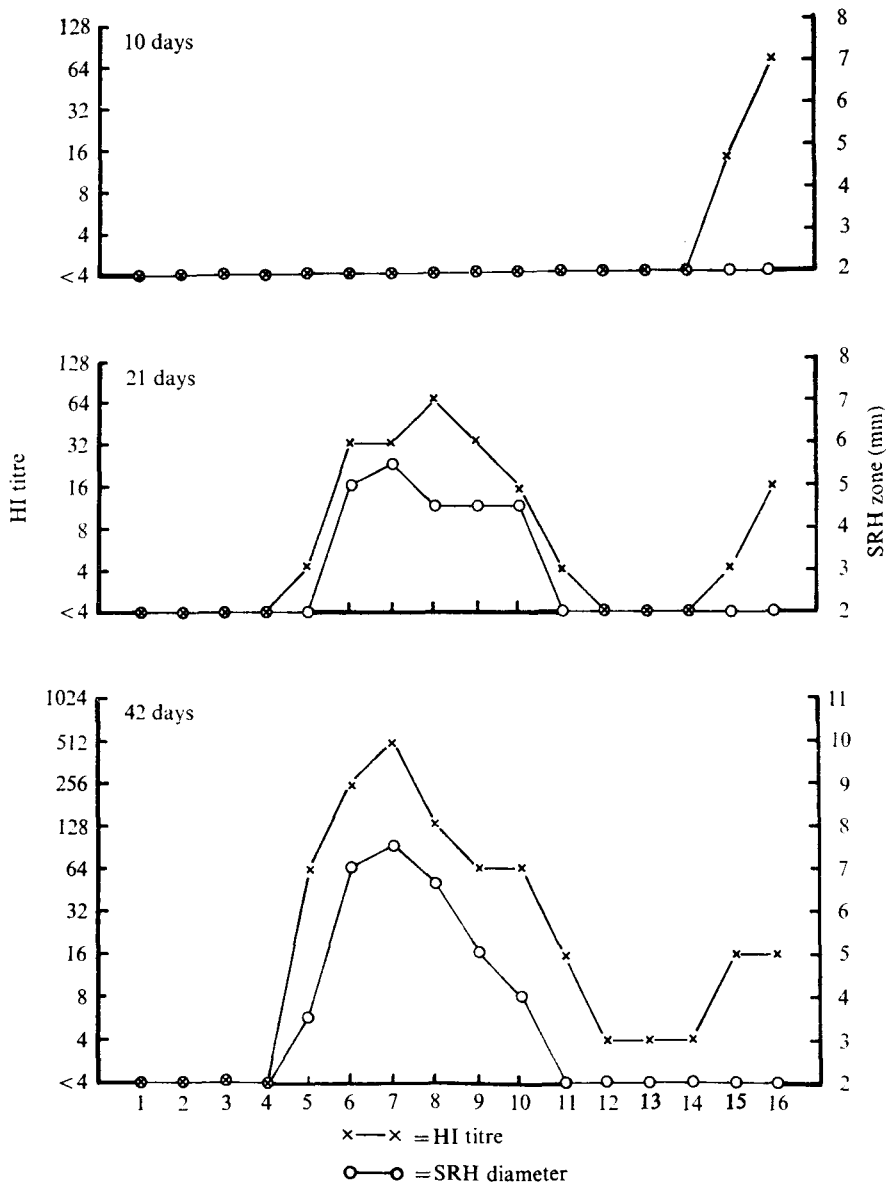


Fig. 4. HI titres and SRH zone diameters of fractions of monkey sera obtained on sucrose density gradient fractionation on days 10, 21 and 42 after inoculation. Fractions 1–16 were counted from the top to the bottom of the gradient. Fractions 6–8 represent the 7S region of the gradient and fractions 15 and 16 the 19S region. The HI titres (x—x) are recorded as the reciprocal of the dilution and SRH zone diameters (o—o) are in mm.

these fractions were consistently negative in the SRH test. With the development of HI antibody in the 7S region of the gradient, the SRH reactions of fractions from the 7S region of the gradient became positive. Figure 4 illustrates the SRH and HI activity detected in the sucrose density gradient fractions of representative monkey sera collected at 10, 21 and 42 days after infection of the animal.



## DISCUSSION

Evidence is rapidly accumulating to support the value of the SRH test in assays of anti-viral antibodies. In influenza virus, it is antibody to haemagglutinin which produces efficient SRH reactions (Schild *et al.* 1975*b*; Russell *et al.* 1975). It has also been shown that antibody to neuraminidase can be detected (Callow & Beare, 1976) but anti-neuraminidase antibody gives haemolysis only if high concentrations of antigen are added to erythrocytes (Schild *et al.* 1975*b*). Antibody to the internal antigens of influenza virus nucleoprotein and matrix protein do not give haemolysis. For rubella, it has not been clearly established which antigen is involved in SRH but the antibodies which give radial-haemolysis are closely correlated with HI antibody of sedimentation rate 7S and it would seem most likely that for rubella too, the SRH reaction is dependent on antibodies to the surface antigens of the virus and probably anti-haemagglutinin antibody.

The advantage of the test lies in the ease of operation. Only microlitre quantities of serum are required. The preparation of samples requires inactivation at 56 °C for 30 min and non-specific inhibitors of rubella haemagglutination do not interfere with the test, hence the treatment of sera to remove such inhibitors is not needed for SRH tests. In addition, non-specific agglutinins of pigeon erythrocytes sometimes found in human sera which might influence HI reactions do not interfere with passive haemolysis, thus there is no need to absorb sera with erythrocytes as is routine practice in HI tests. Strannegård *et al.* (1975) suggested that the reason agglutinins do not interfere in SRH may be that they are 'natural antibodies' to erythrocytes of IgM class. We have confirmed their finding that the IgG antibody is the active component in the SRH test. One of the deficiencies of the SRH test is that it does not detect the early IgM antibody response in the acute phase of illness. It was suggested by Strannegård *et al.* (1975) that this could be an aid to diagnosis in which a result HI positive and SRN negative would indicate exclusively IgM antibody. This suggestion requires further study. In the present study a very small proportion of human sera with low titre HI antibody gave no haemolysis. These sera were from adults and the nature of the antibodies present is under investigation.

Our preliminary study indicates that SRH is an acceptable assay method in estimating antibody content. Our results correlate very well with those of Grillner & Strannegård (1976). Further work is necessary to judge how reproducible are the results obtained within laboratories and between laboratories. To establish the SRH technique as a standard test for the estimation of rubella antibody, an anti-rubella serum is being prepared in guinea-pigs and together with the British Standard anti-rubella serum (human) will constitute a suitable basis for a collaborative study.

We would like to thank Dr T. E. Cleghorn and Dr John Barbara and his staff of the North London Blood Transfusion Centre for their co-operation in the collection of sera. We would also like to thank Dr Frank Taffs of this Institute for immunizing the monkeys.

Note added in proof:

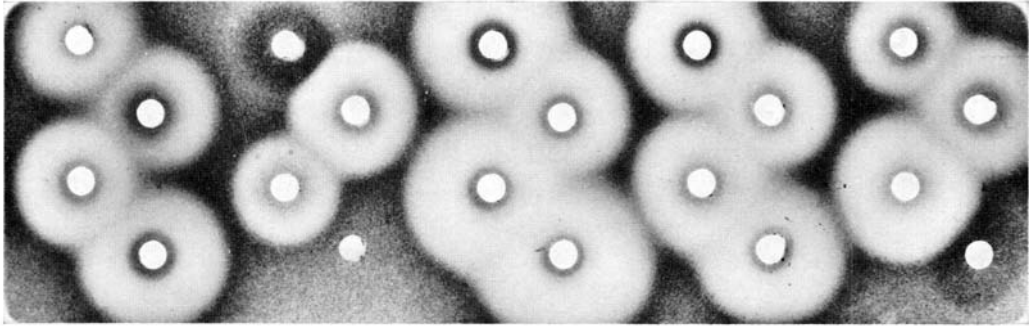
The use of chromium chloride as a coupling agent for the attachment of rubella antigen to red cells (Väänänen *et al.* 1976) has made it possible to replace pigeon cells by sheep cells in the SRH test (Clarke, M., Boustred, J. and Schild, G. C. unpublished).

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#### EXPLANATION OF PLATE

A single-radial-haemolysis immunoplate on which 20 serum samples have been tested. Wells contain 5  $\mu$ l of serum diluted 1/2. The first well on the left on the top row contains the standard human sera 69/60 and the second well a negative control serum. The remaining wells contain human serum samples in a screening test for antibody to rubella. On the bottom row the serum placed in well 2 (from left) and well 5 gave no SRH reaction. The remaining sera are positive.



MAIRIN CLARKE AND OTHERS

*(Facing p. 364)*