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A Cl-Fixation Method for the Measurement of Chicken Anti-Viral Antibody

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Summary. Because of the failure of chicken antibody to activate the first component (C1) of guinea-pig complement a haemolytic system for the measurement of chicken C1 was developed. Chicken antisera to influenza and Rous sarcoma viruses were then assayed by the measurement of the fixation of chicken C1. Antibody titres obtained with this C1-fixation assay correlated well with those obtained by other methods.

The haemolytic system consisted of sheep erythrocytes sensitized with chicken antibody, partially purified preparations of chicken C1, and a guinea-pig serum reagent free of C1, but containing the remaining eight components of complement. Measurement of the residual chicken C1 by this method was profoundly influenced by ionic strength during both the fixation and the lytic phases, and by the order of addition of the reagents.

INTRODUCTION

The failure of chicken antibody to activate the first component (C1) of guinea-pig complement (Benson, Brumfield and Pomeroy, 1961; Brumfield, Benson and Pomeroy, 1961; Okazaki, Purchase, Fredrickson and Burmester, 1962; Rose and Orlans, 1962a), as well as the marked anticomplementary activity of heated chicken serum (Benson *et al.*, 1961; Brumfield *et al.*, 1961; Okazaki *et al.*, 1962; Rose and Orlans, 1962a, b; Rice, 1947; Orlans, Rose and Clapp, 1962), have hampered the development of a satisfactory complement-fixation test for the measurement of chicken antibody. For the titration of certain chicken antiviral sera, such as anti-Rous sarcoma virus, only time consuming and expensive neutralization methods are presently available.

The present communication describes the development of a sensitive method for the measurement of chicken C1 and its subsequent utilization in a C1-fixation test for titrating antibodies in chicken sera to influenza virus and to the Bryan strain of Rous sarcoma virus.

EXPERIMENTAL METHODS

Antisera

1. *Sensitizing antibody.* Adult Kimber chickens received eight equal intravenous injections containing a total of $1{\cdot}8 \times 10^{10}$ washed sheep erythrocytes over a 3-week period of time, and were bled 2 weeks after the last injection.

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2. *Antibody to PR8 virus.* 2-week-old Kimber chickens were inoculated on day 0 and on day 15 with 2 ml of A influenza virus strain PR8 (PR8 virus) which had been harvested as allantoic fluid, and adsorbed to and eluded from guinea-pig red cells. The chickens were bled at 5-day intervals beginning at day 0.

3. *Antibody to Rous sarcoma virus.* Antisera to the Bryan strain of Rous sarcoma virus (RSV–RAV 1) were obtained from Kimber chickens bearing Rous sarcomas induced by this virus.

Buffer solutions

1. *GVB*$^{++}$. Gelatin Veronal buffer with Ca^{++} and Mg^{++} consisted of isotonic Veronal buffer containing 0·1 per cent gelatin, 0·00015 M Ca^{++} and 0·0005 M Mg^{++}, pH 7·4 ($I = 0{\cdot}15$).

2. *DGVB*$^{++}$. Dextrose gelatin Veronal buffer with Ca^{++} and Mg^{++} was prepared by mixing equal volumes of 5 per cent dextrose in water and a gelatin Veronal buffer containing twice the usual amounts of Ca^{++}, Mg^{++} and gelatin ($I = 0{\cdot}075$).

3. *DG*$^{++}$. Dextrose gelatin with Ca^{++} and Mg^{++} consisted of 5 per cent dextrose in water with 0·1 per cent gelatin, 0·00015 M Ca^{++} and 0·0005 M Mg^{++}.

Sensitized sheep erythrocytes (EAb^{ch})

Washed sheep erythrocytes (1×10^9/ml) in 0·01 M isotonic sodium ethylenediaminetetraacetate (EDTA) in gelatin Veronal buffer without Ca^{++} and Mg^{++} were sensitized with an equal volume of unheated chicken antiserum diluted 1:60 in the same buffer (10 haemagglutinating doses) at room temperature for 20 minutes. The EAb^{ch} were then washed and resuspended to 1×10^8/ml in $DGVB^{++}$ for use, unless otherwise noted. One haemagglutinating dose is defined as the concentration of antibody which produces partial (2^+) agglutination of the concentration of sheep cells used for sensitization.

Guinea-pig supernatant I (Sup. I)

A reagent containing eight components of guinea-pig complement was prepared by precipitation and removal of C1 from guinea-pig serum by lowering the ionic strength to $I = 0{\cdot}04$ with water at pH 7·5 (Nelson, 1961; Nelson, Jensen, Gigli, and Tamura 1966). The resultant supernatant fluid (Sup. I) contained less than 1 per cent of the C1 activity of whole serum, but retained greater than 99 per cent of the activity of each of the remaining eight components of guinea-pig complement.

Sucrose density gradient ultracentrifugation

The procedure described by Lefkowitz, Williams, Howard and Sigel (1966) was employed for sucrose density gradient fractionation.

Haemagglutination inhibition (HI) test

Standard procedures using guinea-pig erythrocytes were followed. Four haemagglutinating doses of PR8 virus were used in the HI test. One haemagglutinating dose of virus is defined as the amount of virus in the said volume, which produces partial (2^+) agglutination of a 1 per cent suspension of guinea-pig erythrocytes.

Titration of antibody to Rous sarcoma virus.

The methods for *in vivo* tests are described by Sigel, Fugmann and Stolfi (1969) and for *in vitro* tests by Rubin, Cornelius and Fanshier (1961).

RESULTS

TITRATION OF CHICKEN C1

Preliminary microtitrations of C1 in whole chicken serum were performed in the presence of guinea-pig Sup. I in order to determine the optimal ionic strength for this measurement. One drop of dilutions of chicken serum in buffers of varying ionic strength (prepared by mixing appropriate volumes of GVB^{++} and DG^{++}) were reacted (30° for 30 minutes) with one drop of the corresponding buffer and one drop of EAb^{ch} (1×10^8/ml) in the corresponding buffer. One drop of guinea-pig Sup. I diluted in the corresponding buffer, or one drop of buffer alone was then added and haemolysis was allowed to proceed at 37° for 60 minutes. Visual estimation of 50 per cent haemolytic titres (CH^{50}/ml) showed that an ionic strength of 0·075 was optimal for the measurement of chicken C1 (Table 1) under the conditions of test.

TABLE 1

IMMUNE HAEMOLYSIS BY CHICKEN C1 AT VARIOUS IONIC STRENGTHS IN THE PRESENCE AND ABSENCE OF GUINEA-PIG SUP. I

Ionic strength of buffer (I)	Haemolytic titre*	
	Chicken serum	Chicken serum + Guinea-pig Sup. I
0·200	<20	<20
0·150	<20	30
0·075	30	5120
0·050	<20	1280
0·040	<20	960

* Reciprocal of dilution producing approximately 50 per cent haemolysis in microtitre assay.

Although C1 activity was maximal at $I=0{\cdot}075$, a rapid loss of activity occurred if diluted chicken serum (or partially purified chicken C1, to be described below) was allowed to stand at this ionic strength before reaction with EAb^{ch}. Inasmuch as a period of standing is required in the fixation phase of a CF test, it was necessary to determine the ionic strength at which chicken C1 would remain stable. It was found that chicken C1 was stable upon standing at $I = 0{\cdot}15$ for as long as 18–20 hours at 4° and so this ionic strength was used during the fixation phase.

It was also noted that the order of addition of the reagents was critical. Maximal haemolysis with chicken C1 resulted only if it was reacted with EAb^{ch} for at least 30 minutes at 30° before the addition of Sup. I.

Chicken C1 was measured by an endpoint dilution titration incorporating the conditions delineated by the above findings. The data from these titrations were plotted according to the von Krogh transformation (Mayer, 1961) and yielded a straight line

between 20 and 80 per cent haemolysis. Titres of chicken C1 obtained from these plots ranged between 8000 and 10,000 CH_{50}/ml.

PREPARATION OF CHICKEN C1

Chicken C1 could not be isolated from whole serum by precipitation at $I = 0{\cdot}04$ (pH 7·5), the procedure used to obtain guinea-pig C1 (Nelson, 1961; Nelson *et al.*, 1966), for neither the precipitate nor the supernatant fraction was reactive. Ultimately, it was found that diluting chicken serum 1:8 with 0·02 M acetate buffer, pH 5 (final relative NaCl concentration = 0·02 M, pH 6), resulted in a precipitate containing 60 per cent of the C1

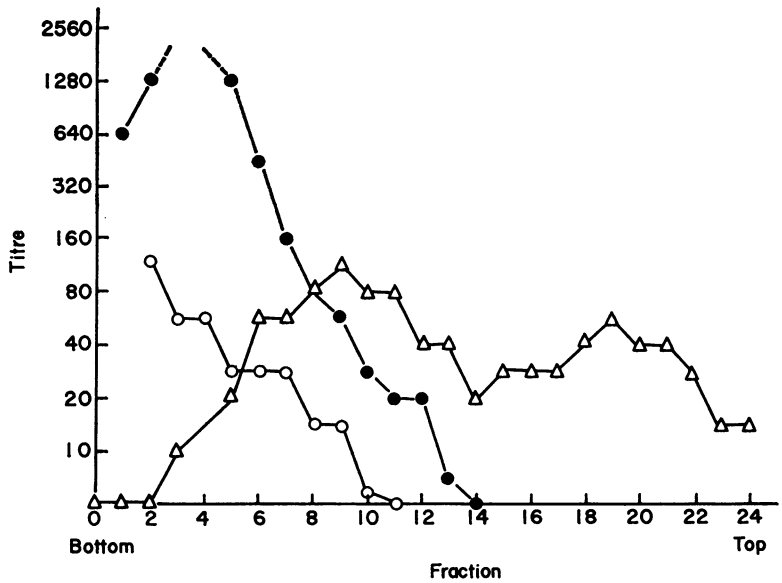

FIG. 1. Sucrose density gradient analysis of C1 activity in whole chicken serum (○) and in a fraction of serum precipitated at 0·02 *I*, pH 6·0 (●), and of C1-inhibitory activity in the fraction of chicken serum which was soluble at 0·02 *I*, pH 6·0 (△).

activity of whole serum. The supernatant fraction was complement-inhibitory. The precipitate was redissolved in ten times the original serum volume of GVB^{++} containing 1 per cent gelatin and was stable upon storage at −70°.

SUCROSE DENSITY GRADIENT ULTRACENTRIFUGATION

Whole chicken serum, chicken C1 (precipitated fraction), and the complement-inhibitory supernatant fraction of chicken serum were subjected to sucrose density gradient ultracentrifugation. The sedimentation of chicken C1 (Fig. 1) indicated that, like C1 from guinea-pig (Borsos and Rapp, 1965) and from human (Naff, Pensky and Lepow, 1964) sera, chicken C1 is a macromolecule. The lower reactivity of whole serum in relation to that of partially purified C1 (Fig. 1). suggests the presence of an inhibitor in, or near, the same area.

The ultracentrifuged supernatant fraction was tested for inhibition of chicken C1 (Tamura and Nelson, 1967) and at least two areas of inhibition could be detected (Fig. 1).

ANTICOMPLEMENTARY ACTIVITY OF CHICKEN ANTISERA AND ANTIGENS

Heated chicken antiserum was anticomplementary at dilutions as high as 1:1200. A major portion of this activity could be precipitated from the antiserum along with the Cl by diluting unheated antiserum with water to $I = 0{\cdot}03$. Antisera treated in this manner were only slightly anticomplementary at dilutions of 1:50 to 1:100 and no antibody activity was lost from the supernatant fraction as measured by haemagglutination inhibition in the PR8 system. All chicken antisera were treated in this manner prior to assay in the Cl-fixation test.

The anticomplementary activity of PR8 virus in allantoic fluid was removed by haemadsorption to guinea-pig erythrocytes at 0°, centrifugation, and elution at 37° into phosphate buffered saline.

Reduction of the anticomplementary activity of RSV-RAV 1 proved to be difficult. Absorption with kaolin, treatment with formalin, heating at 56° for up to 60 minutes, as well as purification of the virus by differential centrifugation, washing, sedimentation through 40 per cent sucrose and fractionation on a sucrose density gradient by ultracentrifugation were equally unsatisfactory for removing the inhibitory action from the virus. However, it was found that reacting the virus with undiluted chicken serum reduced the anticomplementary activity of the virus, and fresh virus destroyed the Cl activity of the serum. Therefore, 3 ml of purified virus were mixed with 3 ml of undiluted chicken serum which was free of RSV-RAV 1 neutralizing antibody and incubated at 30° for 30 minutes. Twice in succession, the virus was then pelleted by centrifugation, resuspended in 1 ml of fresh serum and incubated at 30° for 30 minutes. The virus was then washed, resuspended in 3 ml of GVB^{++} and tested for inhibition of Cl activity. The inhibitory activity of the virus preparation was reduced from 1:2048 to 1:24 after the three exposures to normal chicken serum. Conversely, the Cl activity of the first sample of serum was reduced in titre from 1:10,000 to 1:12 after exposure to the virus. Virus treated in this manner was then evaluated for use as antigen in Cl-fixation tests.

Cl-FIXATION TESTS

Cl-fixation tests were performed with antigens, chicken antisera and chicken Cl treated or prepared as described above. The detailed method is outlined in Table 2.

1. *PR8 virus and chicken antiserum.* A representative antigen–antibody checkerboard assay with PR8 virus and chicken antiserum to the virus (Table 3) showed that a 1:16 dilution of the virus was optimal for the system and resulted in an antibody titre of between 1:800 and 1:1600. The antiserum was only slightly anticomplementary at dilutions of 1:50 to 1:100.

Sera from a series of bleedings of chickens which had been immunized with the PR8 virus were compared in Cl-fixation and HI activity. On each day sera from approximately 100 chickens were pooled. Each pool was tested on two occasions with identical results. The results, represented in Fig. 2, show that there was good correlation between the two types of test with the exception of the serum from the earliest bleeding (5 days) which showed significantly lower Cl fixation than HI activity. This finding is believed to be a reflection of the predominance of IgM in the 5 day serum.

2. *RSV-RAV 1 and chicken antiserum.* Table 4 shows the results in Cl-fixation with chicken antiserum and two dilutions of RSV-RAV 1 which had been reacted with normal

TABLE 2

METHOD FOR Cl-FIXATION ASSAY

GVB^{++} (I 0·15)	0·2 ml Antigen 0·2 ml Cl (5 ClH_{50} units) 0·2 ml Antibody dilution
	18–20 hours at 4°
	0·6 ml DG^{++} (to lower ionic strength to I 0·075)
$DGVB^{++}$ (I 0·075)	0·2 ml EAb^{ch} (1×10^8/ml) 30 minutes at 30° 0·2 ml guinea-pig Sup. I (C4,2,3,5,6,7,8,9) 60 minutes at 37°
	Centrifuge and read

TABLE 3

TITRATION OF CHICKEN ANTIBODY TO PR8 VIRUS IN Cl-FIXATION TEST*

Dilution of antigen (PR8 virus)	Antibody dilution (chicken anti-PR8 virus)							
	1/50	1/100	1/200	1/400	1/800	1/1600	1/3200	None
1/8	0	0	0	0	2	3	4	4
1/16	0	0	0	0	0	3	4	4
1/32	0	0	0	0	2	3	4	4
None	2	3	4	4	4	4	4	4

* Cl_{50} units used in test.
0 = no lysis; 4 = complete lysis; 2 = 50 per cent lysis (endpoint).

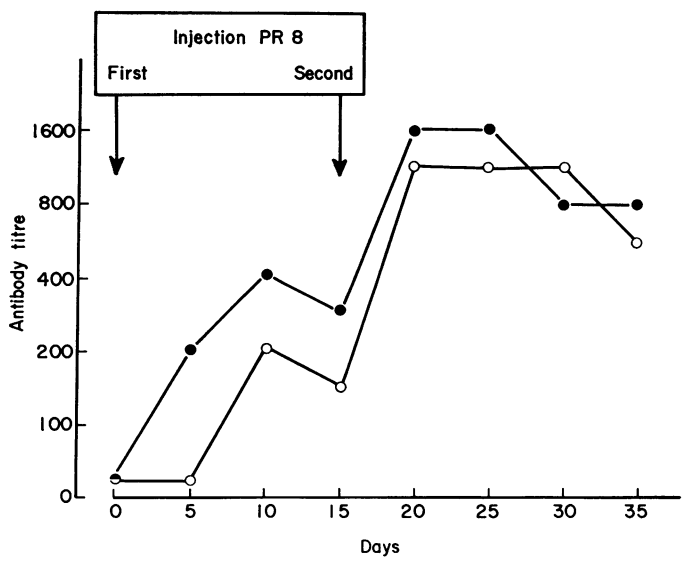

FIG. 2. Cl-fixation (○) and haemagglutination inhibition (●) assays of anti-PR8 virus antibody in chicken sera collected on various days after immunization.

chicken serum prior to its utilization as antigen in the test. It may be seen that only the 1:50 dilution of virus was still slightly anticomplementary. An antibody titre of 1:2560 was obtained in the presence of the virus at a 1:100 dilution. This Cl fixation titre correlated well with that of 1:2000 found by *in vitro* and *in vivo* neutralization titrations with the same antiserum.

TABLE 4

TITRATION OF CHICKEN ANTIBODY TO RSV-RAV 1 IN Cl-FIXATION TEST*

Dilutions of antigen (RSV-RAV 1)	Antibody dilutions (chicken anti-RSV-RAV 1)							
	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	None
1/50	0	0	0	0	0	0	1	<4
1/100	0	0	0	0	0	2	3	4
None	4	4	4	4	4	4	4	4

* 5 ClH_{50} units used in test.
0 = no lysis; 4 = complete lysis; 2 = 50 per cent lysis (endpoint); <4 = nearly complete lysis.

DISCUSSION

Several factors which have limited the usefulness of a complement fixation test for the measurement of chicken antibodies have been eliminated in the described Cl fixation test. The incompatibility of mammalian Cl and chicken antibody was avoided by using chicken Cl as well as chicken sensitizing antiserum. The use of a guinea-pig serum reagent (Sup. I) containing all of the components of complement except the incompatible Cl increased the sensitivity of measurements of chicken Cl. The use of partially purified Cl avoided any natural antibodies as well as any complement inhibitors present in whole chicken complement.

Other experimental parameters needed to be carefully controlled successfully to employ this method. These included the ionic strength of 0·15 for stability of the chicken Cl during the fixation phase, the ionic strength of 0·075 during the lytic phase, and the reaction of chicken Cl with EAb^{ch} prior to the addition of Sup. I.

The marked anticomplementarity of heated chicken serum necessitated the development of a method for removing Cl from unheated antisera. In addition, both the antisera and the antigens had to be treated to remove anticomplementary activity. In this regard the Rous sarcoma virus (RSV-RAV 1) proved of particular interest, and ultimately only a rather tedious absorption procedure using normal chicken serum was successful. The inhibitory effect of this virus did not seem to be enzymatic in nature in that it was heat and formalin resistant and in that it could be overcome by the addition of chicken Cl. Further study is necessary to determine whether this Cl-inactivating activity is an intrinsic property of the virion itself.

The Cl-fixation test described here was found to be reproducible and sensitive for the detection of chicken antibody. In addition, the results correlated well with those of certain other methods ordinarily employed to measure antibodies in chicken serum. Owing to its complexity, this test may not be the one of choice when other, simpler means are available. However, when the alternative is an expensive and time-consuming method, this test

should prove of particular value. Once the reagents have been prepared, it offers a relatively fast and simple procedure for the routine measurement of complement fixing antibodies of chicken serum.

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REFERENCES

BENSON, H. N., BRUMFIELD, H. P. and POMEROY, B. S. (1961). 'Requirement of avian C'1 for fixation of guinea pig complement by avian antibody–antigen complexes.' *J. Immunol.*, **87**, 616.

BORSOS, T. and RAPP, H. J. (1965). 'Estimation of molecular size of complement components by sephadex chromatography.' *J. Immunol.*, **94**, 510.

BRUMFIELD, H. P., BENSON, H. N. and POMEROY, B. S. (1961). 'Procedure for modified complement fixation test with turkey, duck, and chicken serum antibody.' *Avian Dis.*, **5**, 270.

LEFKOWITZ, S. S., WILLIAMS, J. A., HOWARD, B. E. and SIGEL, M. M. (1966). 'Adenovirus antibody measured by the passive hemagglutination test.' *J. Bact.*, **91**, 205.

MAYER, M. M. (1961). *Experimental Immunochemistry* (Ed. by E. A. Kabat and M. M. Mayer). Charles C. Thomas, Springfield, Illinois.

NAFF, G. B., PENSKY, J. and LEPOW, I. H. (1964). 'The macromolecular nature of the first component of human complement.' *J. exp. Med.*, **119**, 593.

NELSON, R. A. (1961). 'Mechanisms of cell and tissue damage produced by immune reactions.' *2nd International Symposium on Immunopathology* (Ed. by P. Grabar and P. Miescher), p. 819. Schwabe, Basel.

NELSON, R. A., JENSEN, J. J., GIGLI, I. and TAMURA. N. (1966). 'Methods for the separation, purification and measurement of nine components of hemolytic complement in guinea-pig serum.' *Immunochemistry*, **3**, 111.

OKAZAKI, W., PURCHASE, H. G., FREDRICKSON, T. N. and BURMESTER, B. B. (1962). 'Modification of complement fixation test for estimation of Rous sarcoma virus antibodies in turkey and chicken serums.' *Proc. Soc. exp. Biol. (N.Y.)*, **111**, 377.

ORLANS, E., ROSE, M. E. and CLAPP, K. H. (1962). 'Fowl antibody. V. The interactions of fresh and heated fowl serum and of guinea-pig complement measured by the lysis of sensitized red cells.' *Immunology*, **5**, 649.

RICE, C. E. (1947). 'Atypical behaviour of certain avian antisera in complement fixation tests.' *Canad. J. comp. Med.*, **11**, 236.

ROSE, M. E. and ORLANS, E. (1962a). 'Fowl antibody. III. Its haemolytic activity with complements of various species and some properties of fowl complement.' *Immunology*, **5**, 633.

ROSE, M. E. and ORLANS, E. (1962b). 'Fowl antibody. IV. The estimation of haemolytic fowl complement. *Immunology*, **5**, 642.

SIGEL, M. M., FUGMANN, R. A. and STOLFI, R. L. (1970). 'Immunity and Tolerance in Oncogenesis.' *IVth Int. Conf. on Cancer. Perugia.* (Ed. by L. Severi), p. 117. Div. of Cancer Res., Univ. of Perugia.

RUBIN, H., CORRELIUS, A. and FAUSHIER, L. (1961). 'The pattern of congenital transmission of an avian leukosis virus.' *Proc. nat. Acad. Sci. (Wash.)*, **47**, 1058.

TAMURA, N. and NELSON, R. A. (1967). 'Three naturally-occurring inhibitors of components of complements in guinea pig and rabbit serum.' *J. Immunol.*, **99**, 582.