Immunology, 1972, **22**, 747.

The use of Ammonium Sulphate Globulin Precipitation for Determination of Affinity of Anti-Protein Antibodies in Mouse Serum

M. W. STEWARD AND R. E. PETTY

Department of Immunology, Institute of Child Health, 30 Guilford Street, London WC1N 1EH

(*Received* 20*th July* 1971)

Summary. The use of a modification of the ammonium sulphate globulin precipitation technique for the determination of antibody affinity is reported. This method is shown to provide reproducible and valid measurements for the comparison of affinity of unselected and unpurified antibody to human serum albumin and transferrin in different mouse strains.

INTRODUCTION

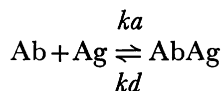
Antigen–antibody reactions are reversible and although antibodies show a marked heterogeneity of affinity, the average affinity at equilibrium is expressed by the equilibrium constant K.

Several techniques are available for the determination of K. These include equilibrium dialysis (Eisen and Karush, 1949), fluorescence quenching (Velick, Parker and Eisen, 1960), fluorescence polarization (Dandliker, Schapiro, Meduski, Alonao, Feigen and Hamricks, 1965) and fluorescence enhancement (Parker, Yeo, Johnson and Godt, 1967). The first three methods are applicable only to systems involving purified or partially purified antibody, and fluorescence enhancement may only be applied to antigens with certain fluorescence properties. Isolation and purification of antibody involves binding with antigen and the affinity of the isolated antibody is thus not representative of that of the total antibody present in the serum. Measurement of K of unselected and unpurified antibody to protein antigens in serum could not, therefore, be studied by these methods.

To study the immunopathological significance of the heterogeneity of antibody affinity (Soothill and Steward, 1971) we required a method which would allow measurement of K of unselected and unpurified antibody to protein antigens. We report and appraise a modification of the ammonium sulphate globulin precipitation technique of Farr (1958) for such measurements.

Principle of method

The quantitative relationship of the interaction between antigen and antibody at equilibrium is represented by the equation:



$$\mathrm{Ab} + \mathrm{Ag} \underset{kd}{\overset{ka}{\rightleftharpoons}} \mathrm{AbAg}$$

where Ab = free antibody
Ag = free antigen
AbAg = antibody–antigen complex.

From the law of mass action, the affinity of the antibody is represented by the equilibrium constant K:

$$K = \frac{ka}{kd} = \frac{[\mathrm{AbAg}]}{[\mathrm{Ag}]\,[\mathrm{Ab}]}$$

The higher the affinity of antibody, the greater the amount of antigen bound to antibody at equilibrium. The equilibrium constant, K, can be determined by measurement of antibody-bound antigen [Ag Ab] and free antigen [Ag] at equilibrium at several antigen concentrations as follows.

From the law of mass action the Langmuir Adsorption Isotherm may be derived:

$$\frac{[\mathrm{AbAg}]}{[\mathrm{Ab}]} = \frac{n\,K[\mathrm{Ag}]}{1+K[\mathrm{Ag}]}$$

where n = number of antibody-binding sites
K = equilibrium constant
[Ag] = free antigen concentration, moles/l.

The calculation of K from this equation requires a knowledge of total antibody concentration [Ab], bound antigen concentration [AbAg] or b, free antigen concentration [Ag] and number of combining sites, n. If K is determined with respect to total antibody binding sites [$\mathrm{Ab_t}$], rather than to [Ab] and n (Nisonoff and Pressman, 1958), the Langmuir equation becomes:

$$\frac{\mathrm{b}}{\mathrm{Ab_t}} = \frac{K[\mathrm{Ag}]}{1+K[\mathrm{Ag}]}$$

from which:

$$\frac{1}{\mathrm{b}} = \frac{1+K[\mathrm{Ag}]}{\mathrm{Ab_t}.K[\mathrm{Ag}]}$$

$$= \frac{1}{\mathrm{Ab_t}}\cdot\frac{1}{K}\cdot\frac{1}{[\mathrm{Ag}]}+\frac{1}{\mathrm{Ab_t}}.$$

Therefore, when $1/[\mathrm{Ag}] = 0$, $1/\mathrm{b} = 1/\mathrm{Ab_t}$.

$\mathrm{Ab_t}$ may thus be determined by extrapolation of the line resulting from the plot of 1/b *vs* 1/[Ag] which is theoretically a straight line. However, because of the heterogeneity of antibody affinity, the plot deviates from linearity. This deviation at antibody excess may be due to proportionately greater binding of antigen by high affinity antibody. Presumably deviation from linearity at antigen excess occurs, and may be due to proportionately lower binding by low affinity antibody. However, for practical purposes, in the range of antigen levels used in this assay (100 $\mu\mu$ moles to 1000 $\mu\mu$ moles), the line is linear.

The value of K, the reciprocal of [Ag] at which half the antibody binding sites are occupied by antigen, is obtained from a plot of the logarithmic transformation of the Sips equation (Sips, 1948; Karush, 1962).

$$\frac{r}{n} = \frac{(K\,\mathrm{Ag})^{a}}{1+(K\,\mathrm{Ag})^{a}}$$

Thus:

$$\log \frac{r}{n-r} = a \log K + a \log [Ag]$$

where: r = moles of antigen bound per mole of antibody
n = valency of antibody
a = heterogeneity index.

Since: $n = 2$

$$\text{Moles of antibody} = \frac{Ab_t}{2}$$

and hence: $r = \frac{b}{Ab_t/2}$

thus: $\log \left(\frac{r}{n-r}\right)$ becomes $\log \left(\frac{b}{Ab_t - b}\right)$.

Therefore, in a plot of $\log \frac{b}{Ab_t - b}$ *vs* $\log [Ag]$

when: $\log \frac{b}{Ab_t - b} = 0$,

$$K = \frac{1}{[Ag]}$$

K has units of litres/mole (l/m).

Appraisal of the validity of measurements of the kinetics of reaction between a protein antigen and antibody directed to it must take into consideration many factors. Measurement of affinity to protein antigens is influenced by aggregation, non-specific association of antigen with antibody, and by heterogeneity of the antibody population. Of much greater importance in the measurement of affinity to protein antigen is consideration of the influence of antigenic heterogeneity. Thus values of K for protein antigen–antibody reactions represent the summation of the reactions between a heterogeneous antigen and a heterogeneous antibody population. Such values are comparable with, but not identical to, affinity determinations using purified (but still heterogeneous) antibody to single haptenic antigens, and direct comparisons between the two cannot be made. Nonetheless, valid comparisons of K as determined in the manner outlined above can be made with different antibodies to the same protein antigen. We have measured K of antibodies in the serum of different inbred strains of mice and have shown consistent interstrain differences.

MATERIALS AND METHODS

Mice

CBA, Simpson and C/A inbred mouse strains were maintained in the animal house of the Institute of Child Health.

Immunization

Mice 2–3-month old of either sex were immunized by intraperitoneal injection of 1·0 mg of antigen in 0·1 ml sterile saline once weekly for 4 weeks. Serum was obtained from blood drawn by intracardiac puncture under anaesthesia 2 weeks after the 4th injection.

Antigens

Antigens used were human serum albumin (HSA) kindly supplied by the Blood Products Laboratory (Lister Institute), human serum transferrin (HST) (Sigma Chemical Co. Ltd.) and 2,4-dinitro phenyl-HSA (DNP-HSA). DNP-HSA was prepared from 2,4 dinitrobenzene sulphonic acid (Eastman Chemical Co.) by the method of Eisen (1967). The DNP substituted HSA was separated from unreacted 2,4 dinitrobenzene sulphonic acid by gel filtration through a column of Sephadex G-25 equilibrated in 0·1 M potassium phosphate buffer pH 6·8. The degree of substitution was determined spectrophotometrically.

HSA and HST were labelled with ^{125}I by the method of McFarlane (1958). They were soluble (>95 per cent) in half-saturated $(NH_4)_2SO_4$ and precipitable (>98 per cent) by 10 per cent trichloracetic acid. No aggregation was detected by polyacrylamide disc-gel electrophoresis of ^{125}I-labelled HST and less than 5 per cent of the [^{125}I]-HSA was aggregated.

For the assay of anti-DNP antibodies, [^3H]-ε-DNP-lysine was prepared from [^3H] dinitrofluorobenzene (Radiochemical Centre, Amersham) and N-t-butyloxycarbonyl-L-lysine (Sigma Chemical Co. Ltd.) by the method of Eisen, Simms and Potter (1968).

Methods of separation of bound and free antigen

1. *The ammonium sulphate globulin precipitation technique.* Manipulations were carried out at room temperature. Equal volumes of mouse serum, usually 50 μl, made up to a volume of 0·1 ml with 0·1 M phosphate buffered saline pH 7·3 were incubated for 1 hour at room temperature with 0·1 ml ^{125}I-labelled antigen at five concentrations. The conical tubes used were of 0·4 ml capacity (Gelman Hawksley Ltd.) and were previously coated with a 1 per cent solution of bovine serum in phosphate buffered saline and dried to minimise non-specific binding of radiolabelled protein. After incubation, 0·2 ml saturated $(NH_4)_2SO_4$ was added to each tube, mixed immediately and the tubes were incubated for a further 1 hour at room temperature, before centrifuging at 15,000 rev/min for 5 minutes in a Beckman 152 Microfuge. 0·1 ml of the supernatant was removed and the precipitate was washed twice with half-saturated $(NH_4)_2SO_4$. Radioactivity in the supernatants and precipitates from experiments using ^{125}I-labelled antigens were counted in a Panax Gamma 160 spectrometer. Precipitates from experiments with [^3H]ε-DNP-lysine were solubilized in NCS solubilizer (Amersham-Searle, Des Plaines, Illinois, U.S.A.) and transferred to 6 ml of scintillation fluid; this consisted of 0·1 g dimethyl POPOP and 4·0 g PPO dissolved in 1 l of toluene. Supernatants were transferred to 6·0 ml of the scintillation fluid containing the solubilizer. Counting was performed in a Nuclear Chicago β counter. Quenching corrections were made for both precipitate and supernatant containing vials by recounting after the addition of a standard amount of [^3H] hexadecane. Total radioactivity in the supernatant represents free antigen; radioactivity in the precipitate, after correction for non-specific precipitation using values obtained by similar treatment of serum from unimmunized animals, represents bound antigen. Serum from a pool obtained from 100 mice of different strains immunized with antigen in adjuvant was included at intervals as a positive control of the method. Data which produced poor lines in the 1/b *vs* 1/c plot (less than three points on a straight line) were rejected and, where possible, repeated.

2. *Polyacrylamide disc gel electrophoresis.* Separation of bound and free ^{125}I-labelled antigen using polyacrylamide disc gel electrophoresis was performed as described by Steward *et al.* (1969). Equal volumes of mouse serum were incubated with ^{125}I-labelled antigen at five

antigen concentrations for 1 hour at room temperature. Aliquots of the five reaction mixtures were electrophoresed on polyacrylamide gels. The gels were cut into slices and the radioactivity in each slice was determined. Bound and free antigen values were obtained by addition of counts in the gel slices.

3. *Anti-IgG precipitation of bound antigen.* The K of rabbit anti-HSA antibody was determined by the ammonium sulphate technique and by the use of sheep anti-rabbit IgG to precipitate antibody–antigen complexes (Minden, Anthony and Farr, 1969). The amount of antiserum required to precipitate all the IgG present in the test serum was first determined by construction of a precipitin curve. Equal volumes of rabbit anti-HSA antiserum were incubated at room temperature with five [^{125}I] HSA concentrations for 1 hour. The determined volume of anti-IgG antiserum was then added to each tube and incubation continued for 2 hours at 37° then overnight at 4°. After centrifugation aliquots of the supernatants were removed and the precipitates washed with cold saline. The radioactivity in both precipitates and supernatants was counted.

Calculation of antibody levels

The levels of antibody in μg/ml serum were calculated from Ab_t values assuming (a) all antibody produced by the animals with our immunization procedure was IgG, (b) there are two binding sites per IgG molecule of molecular weight 160,000, (c) that under the conditions used in this assay, that is in antigen excess, a ratio of one antibody-binding site per antigen molecule is approached.

RESULTS

REPRODUCIBILITY OF RESULTS

The significant difference of K of anti-HSA antibodies in two inbred strains of mice (Fig. 1) illustrates the use of the technique appraised in this paper for determining K. Values for K were determined in the range from low 10^5 l/m to high 10^6 l/m. The range of values for K within the inbred strains CBA and Simpson was clearly restricted. In addition to the differences between strains of mice there is heterogeneity of K within each mouse strain. That this is true heterogeneity of antibody response rather than a methodological artefact is shown in Fig. 2. Values of K and of total antibody of individual sera showed a much greater range than the repeated determinations on a pool of comparable sera, the latter representing methodological error. The values of K of a positive control pool of serum determined on different days are shown in Fig. 3. The values are clearly within the range of experimental error.

RELATIONSHIP OF K TO ANTIBODY LEVEL (Ab)

That there is no association between affinity and amount of antibody is shown in Table 1. Pools of mouse serum, one containing high affinity antibody ($K = 10^6$ l/m) and one containing low affinity antibody ($K = 10^5$ l/m) to HSA, were diluted with non-immune serum from the same mouse strain to make a series of antibody preparations of different antibody concentrations. The Ab_t ($\mu\mu$moles/50 μl serum) observed correlated well with that anticipated by dilution with non-immune serum. Variation in K was within the range of methodological error indicated in Fig. 2 and the values of affinity are clearly independent of amount of antibody over a wide range of antibody concentrations; additional evidence for

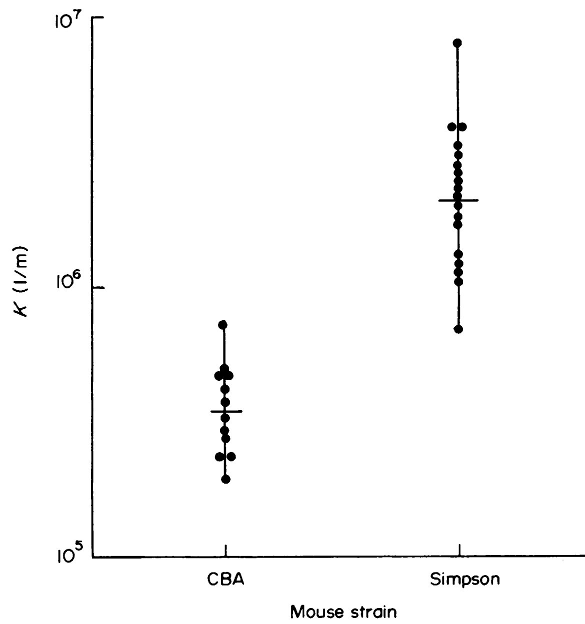


Fig. 1. Affinity of anti-HSA antibodies in the sera of two inbred strains of mice.

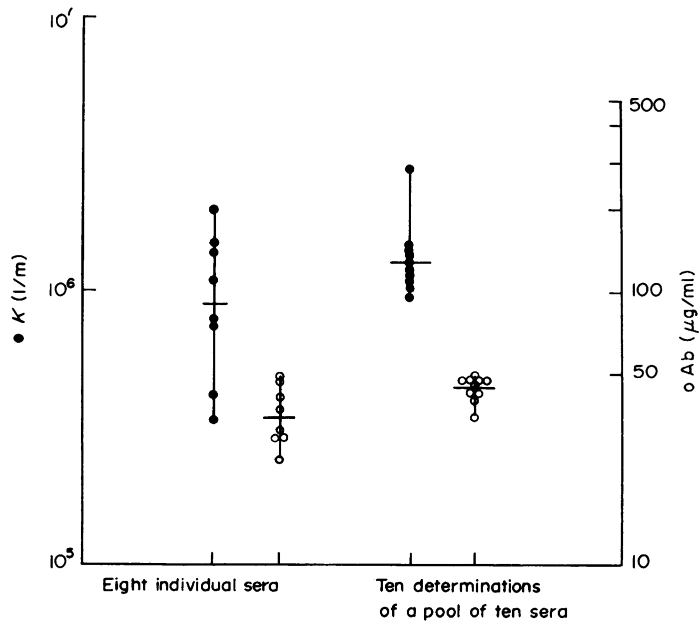


Fig. 2. Affinity and antibody levels of mouse anti-HSA sera.

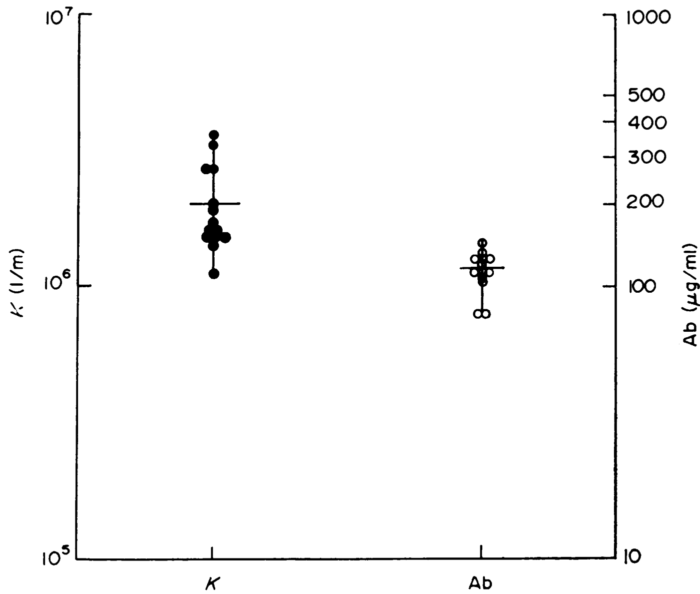


FIG. 3. Affinity and antibody levels of a positive control anti-HSA mouse serum determined on different occasions.

TABLE 1

THE EFFECT OF ANTIBODY CONCENTRATION, EXPRESSED AS Ab_t, $\mu\mu$moles/50 μl SERUM, ON THE VALUE OF $\bar{K}$ DETERMINED FOR TWO POOLS OF ANTIBODY

Ab_t ($\mu\mu$moles/50 μl)		$\bar{K} \times 10^6$
Expected	Observed	l/m
High affinity pool		
154	154	6·7
123	127	5·3
93	95	8·7
77	78	5·6
62	66	6·4
46	47	5·0
12	20	5·0
Low affinity pool		
77	77	0·7
38	32	1·1
15	18	0·8
8	12	0·9

this conclusion is given in Fig. 4. Values of K and the corresponding antibody levels for a large number of sera are shown. No relationship between these two parameters is demonstrated.

COMPARISON OF THE AMMONIUM SULPHATE GLOBULIN PRECIPITATION METHOD WITH OTHER METHODS FOR DETERMINATION OF AFFINITY ON UNFRACTIONATED SERUM

In order to validate further the Farr technique as a method of determining affinity to HSA and HST, comparison was made with values of K determined by polyacrylamide disc gel electrophoresis and anti-immunoglobulin precipitation of globulin-bound

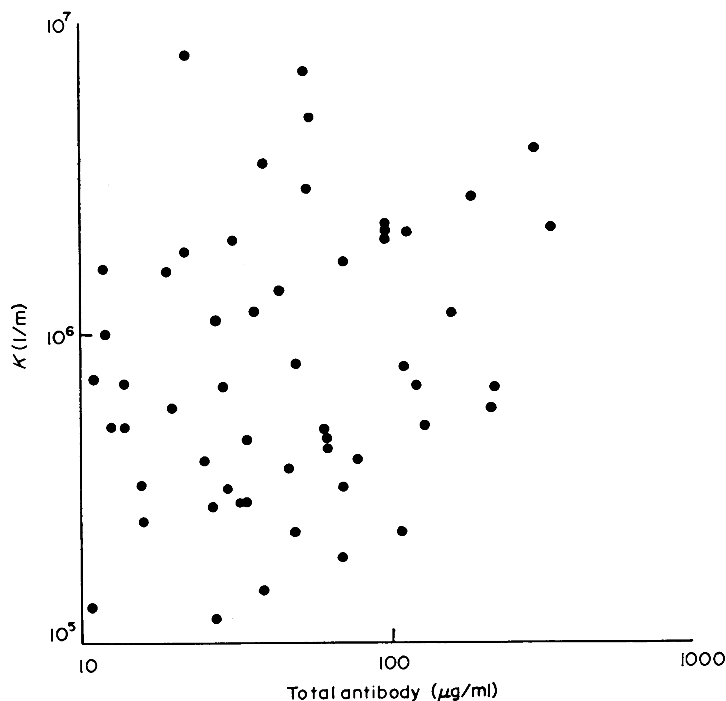


FIG. 4. Scattergram test of the relationship between K and antibody levels in serum of mice of several strains.

antigen (Table 2). There was close agreement between values for K and Ab_t of mouse anti-HSA and mouse anti-HST antibodies obtained by the Farr technique and by polyacrylamide disc gel electrophoresis. Using the anti-immunoglobulin precipitation method, a K value twice as high as that determined by the Farr technique was obtained in the rabbit anti-HSA system. The Farr technique would include contribution to affinity made by all classes of immunoglobulin, some of which may contain antibody of lower affinity; the anti-IgG-specific antiglobulin technique excludes the contribution of other immunoglobulin classes. Stupp, Yoshida and Paul (1969) reported close agreement between values for K of mouse anti-DNP antibodies obtained by the Farr technique and by equilibrium dialysis.

Table 2
Comparison of the values obtained for affinity and total antibody by various methods

Antibody	Method	Ab (μg/ml)	$K \times 10^6$ l/m
Mouse anti-HSA	Farr	109	1·33
	Disc gel	97	1·92
Mouse anti-HST	Farr	105	6·90
	Disc gel	99	6·50
Rabbit anti-HSA	Farr	1330	2·80
	Anti-immunoglobulin	1600	6·20

Table 3
The affinity of anti-DNP antibodies produced in inbred mouse strains shown to produce antibody of high K and low K antibody to HSA

Mouse Strain	DNP_8 HSA		DNP_{14} HSA	
	No.	Average K l/m	No.	Average K l/m
Low K	3	$1{\cdot}85 \times 10^5$	6	$2{\cdot}5 \times 10^5$
High K	6	$1{\cdot}45 \times 10^6$	5	$3{\cdot}1 \times 10^6$

DETERMINATION OF THE AFFINITY OF ANTI-DNP ANTIBODIES

In order to validate the results obtained with protein antigens the ammonium sulphate precipitation technique was applied to the anti-DNP system (Stupp *et al.*, 1969). The results, shown in Table 3, indicate that the values of K determined for anti-hapten antibodies are comparable to the values obtained by Stupp *et al.* (1969) and that the interstrain difference, seen for antiprotein antibodies (Fig. 1) is also shown for anti-hapten antibodies.

DISCUSSION

The data presented in this paper demonstrate the reliability and reproducibility of the ammonium sulphate method of precipitation of immunoglobulin-bound antigen for the determination of the affinity of antibodies in unfractionated mouse serum to the antigens HSA, HST and DNP. The values of K determined by this method are independent of the antibody concentration over a wide range and are in agreement with values obtained by antiglobulin and disc gel electrophoresis separation of bound and free antigen.

The reaction between a protein antigen and the corresponding antibody is complex as a result of the heterogeneity of antigen-antibody and their binding characteristics. Affinity describes the summation of interactions between antigen and antibody. Included in these interactions are those resulting from antigenic stimulation, i.e. specific antigen–antibody interactions, and those inherent in reactions between any two proteins, i.e. non-specific interactions. It is becoming apparent (Eisen, Little, Osterland and Simms, 1967; Haimovich, Tarrab, Sulica and Sela, 1970) that measurable interaction between antigen and immunoglobulin is present in the serum of unimmunized animals and in myeloma proteins. This activity may represent natural antibody. The impossibility of precisely defining 'antibody' is circumvented in the measurement of affinity by using whole serum. Thus the

only selection of antibody that we have made is that involving subtraction of the level of antigen which is bound to immunoglobulin in an unimmunized animal. Imposition of this parameter may have eliminated the contribution by natural antibody to the value of K.

The calculation of K of anti-HSA and anti-HST antibodies has been made assuming that one molecule of bivalent antibody binds two molecules of antigen. For a complex protein antigen, there are several antigenic sites per molecule and more than one antibody molecule will be bound per molecule of antigen. The calculated Ab_t levels are thus lower than the actual levels present. The affinity of anti-protein antibodies determined on the basis of these levels, although further from absolute values than those determined for antibodies to haptens, are valid for comparative purposes for antibody raised to the same antigen in different animals or strains. Indeed, we have shown that the affinity of anti-protein antibodies is of the same order as the affinity of antibodies to the simpler antigenic determinant DNP in the different strains of mice studied. Thus, in spite of the problems of measuring the affinity of antibody to complex protein antigens, we have shown reproducible interstrain differences of such measurements (Soothill and Steward, 1971). As such, these measurements will form the basis of our studies of the immunopathological significance of the heterogeneity of antibody affinity.

ACKNOWLEDGMENTS

The authors thank Professor J. F. Soothill for helpful criticism of the manuscript, Mr A. Weir, Chief Animal Technician, Institute of Child Health Animal House and his staff for invaluable assistance with the mice, Mr N. A. West for skilled technical assistance and the Canadian Arthritis and Rheumatism Society for a Research Fellowship (R.E.P.).

REFERENCES

DANDLIKER, W. B., SCHAPIRO, H. C., MEDUSKI, J. W., ALONSO, R., FEIGEN, G. A. & HAMRICKS, J. R. JR (1965). 'Application of fluorescence polarization to the antigen–antibody reaction.' *Immunochemistry*, **1**, 165.

EISEN, H. N. (1967). In *Methods in Immunology and Immunochemistry* (Ed. by C. A. Williams and M. W. Chase), Vol. I. Academic Press, London.

EISEN, H. N. & KARUSH, F. (1949). 'The interaction of purified antibody with homologous hapten. Antibody valence and binding constant.' *J. Amer. chem. Soc.*, **71**, 363.

EISEN, H. N., LITTLE, J. R., OSTERLAND, C. K. & SIMMS, E. S. (1967). 'A myeloma protein with antibody activity.' *Cold Spr. Harb. Symp. quant. Biol.*, **32**, 75.

EISEN, H. N., SIMMS, E. S. & POTTER, M. (1968). 'Mouse myeloma proteins with antihapten antibody activity. The protein produced by plasma cell tumor MOPC 315.' *Biochemistry*, **7**, 4126.

FARR, R. S. (1958). 'A quantitative immunochemical measure of the primary interaction between I* BSA and antibody.' *J. infect. Dis.*, **103**, 239.

HAIMOVICH, J., TARRAB, R., SULICA, A. & SELA, M. (1970). 'Antibodies of different specificities in normal rabbit sera.' *J. Immunol.*, **104**, 1033.

KARUSH, F. (1962) 'Immunologic specificity and molecular structure.' *Advances in Immunology*, **2**, 1.

MCFARLANE, A. S. (1958). 'Efficient trace labelling of proteins with iodine.' *Nature (Lond.)*, **182**, 53.

MINDEN, P., ANTHONY, B. F. & FARR, R. S. (1969). 'A comparison of seven procedures to detect the primary binding of antigen by antibody.' *J. Immunol.*, **102**, 832.

NISONOFF, A. & PRESSMAN, D. (1958). 'Heterogeneity and average combining constants of antibodies from individual rabbits.' *J. Immunol.*, **80**, 417.

PARKER, C. W., YOO, T. J., JOHNSON, M. C. & GODT, S. M. (1967). 'Fluorescent probes for the study of the antibody-hapten reaction. 1. Binding of the 5-dimethylaminonaphthalene-1-sulphonamido group by homologous rabbit antibody.' *Biochemistry*, **6**, 3408.

SIPS, R. (1948). 'On the structure of a catalyst surface.' *J. Chem. Phys.*, **16**, 490.

SOOTHILL, J. F. & STEWARD, M. W. (1971). 'The immunopathological significance of the heterogeneity of antibody affinity.' *Clin. exp. Immunol.* **9**, 193.

STEWARD, M. W., TODD, C. W., KINDT, T. J. & DAVID, G. S. (1969). 'Low molecular weight mercaptoethanol sensitive antibody in rabbits.' *Immunochemistry*, **6**, 649.

STUPP, Y., YOSHIDA, T. & PAUL, W. E. (1969). 'Determination of antibody-hapten equilibrium constants by an ammonium sulphate method.' *J. Immunology*, **103**, 625.

VELICK, S. F., PARKER, C. W. & EISEN, H. N. (1960). 'Excitation energy transfer and the quantitative study of the antibody hapten reaction.' *Proc. nat. Acad. Sci. (Wash.)*, **46**, 1470.