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The Reaction with Red Cells of 7S Rabbit Antibody, its Sub-units and their Recombinants

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Summary. Univalent 3·5S antibody fragments obtained from 7S rabbit antibody specific for human group A red cells, have a very much smaller association constant in their reaction with human group A_1 red cells than have 7S or 5S antibody. Univalent 5S recombinants have a similarly low association constant, whereas bivalent 5S recombinants have an association constant similar to that of 7S antibody.

It is concluded that this supports the authors' hypothesis that rabbit anti-A 7S antibody is normally attached to red cells by both its specific antibody groups.

INTRODUCTION

We have shown (Greenbury, Moore and Nunn, 1963) for rabbit immune antibodies to human blood group A cells, that 19S antibody is a much more efficient haemagglutinating agent than 7S antibody. To produce '50 per cent agglutination' it is necessary for each cell to carry only twenty-five molecules of 19S antibody, whereas to produce the same degree of agglutination 19,500 molecules of 7S antibody are required on each cell. We have also shown (Greenbury *et al.*, 1963) that the mean distance between A-sites on the A_1 red cell is sufficiently small for several sites to lie within a circle whose radius is the length of a 7S antibody molecule. In these circumstances it would seem likely, if there were no steric impediment, that the free specific group of a 7S antibody molecule, already bound to the red cell by its other combining site, would attach itself to the same cell. Antibody molecules so attached would not be able to take part in agglutination. By contrast, if as we believe, the 19S antibody molecule is multivalent, it is improbable that all combining sites on a molecule could be attached to a single cell. We have therefore postulated that in the human group A cell/rabbit anti-A system that the usual mode of attachment of 7S antibody molecules to the red cell is a bivalent one; both the specific groups of the antibody being bound to a single cell.

It should be possible to throw further light on this question by studying the behaviour of univalent fragments produced by digestion of antibody with papain (Porter, 1959) or with pepsin followed by reduction with cysteine or 2-mercapthoethylamine (Nisonoff, Wissler, Lipman and Woernley, 1960). For instance, if 7S antibody is mostly attached to cells by both its specific groups, it would be expected that at saturation point cells would be able to accommodate on their surface twice as many univalent (3·5S) fragments as intact bivalent (7S) molecules.

However it was found that the association constant between 3·5S fragments and red cells was so low as to preclude the possibility of saturating cells with them. But this serendipitous finding of a large difference between the association constants of univalent and bi-

valent antibody (Greenbury, Moore and Nunn, 1964) is in itself evidence that univalent molecules differ from intact 7S antibody in their mode of attachment to the red cells.

Studies of the reaction with red cells of ^{131}I-labelled 7S antibody, its sub-units and their recombinants are reported below.

MATERIALS AND METHODS

Red Cells

Blood-bank group A_1 or O human red cells were used; they were washed three times in isotonic saline before use.

Raising of Immune Sera

Rabbits were immunized with five intravenous injections each of 5 ml. of a 5 per cent suspension of group A_1 red cells, given at 5 day intervals. They were bled a week after the last injection and two or three times during the following week.

Preparation of Antibody for Iodination

A crude γ-globulin fraction was precipitated from the antisera by adding one volume of serum to two volumes of 27 per cent w./v. Na_2SO_4 and leaving for 3 hours at 37°. The precipitate was washed in 18 per cent w./v. Na_2SO_4, dissolved in saline and then dialysed against saline until sulphate-free. The protein was then iodinated.

Iodination of γ-Globulin

The method of McFarlane (1963) was used (without pre-oxidation of sulphydryl groups), with preparation IBS-3 from the Radiochemical Centre, Amersham.

Preparation of 7S γ-Globulin by Chromatography

The method of Sober, Gutter, Wyckoff and Peterson (1956) was employed using DEAE-cellulose (Whatman DE50 powder) and eluting with five separate sodium phosphate buffers: 0·01 M, pH 8; 0·025 M, pH 7; 0·05 M, pH 6; 0·1 M, pH 5·5; 0·15 M, pH 4·6. The iodinated globulin was dialysed overnight against the starting buffer before application to the column. The fractions containing most protein eluted by the first buffer were pooled and dialysed against saline. These preparations are referred to as 7S γ-globulin.

Preparation of 5S Fragments

7S γ-globulin was buffered at pH 4·2 with sodium acetate and acetic acid. Pepsin (twice crystallized, L. Light & Co. Ltd., Colnbrook, Bucks.), weight 2–3 per cent that of the γ-globulin was added, the mixture was kept at 37° for 6 hours and then dialysed against saline. This process degrades 7S γ-globulin to 5S protein (Nisonoff *et al.*, 1960) and smaller peptides, some of which were dialysable. The 5S antibody was still bivalent as it had an agglutinating efficiency similar to 7S antibody.

Preparation of 3·5S Fragments

(a) *Papain digestion of 7S-globulin.* Papain (crystalline mercuripapain, Nutritional Biochemical Corporation, Cleveland, Ohio), 1 per cent of the weight of globulin, was added to 7S γ-globulin in 0·01 M cysteine and 0·002 M EDTA, buffered at pH 7 with sodium phosphate. The mixture was kept at 37° over night and then dialysed against saline. This

produces three fragments from an antibody molecule, two with antibody activity and molecular weight 50,000, and a third without antibody activity, molecular weight 80,000 (Porter, 1959). Chromatography on carboxymethyl cellulose (Whatman CM70 powder) gave results quantitatively similar to those of Stelos, Radzimski and Pressman (1962) but the unfractionated papain-digest was used in our red cell experiments.

(b) *Reduction of 5S pepsin-digested γ-globulin.* The 5S protein solution was adjusted to pH 5 with acetic acid and sodium acetate, and 2-mercaptoethylamine (MEA) (0·01 M) was added. The mixture was kept at 37° for 3 hours and then the sulphydryl groups were alkylated with iodoacetate at pH 7 in sodium phosphate buffer, at a final concentration 0·05 M. After 1 hour at room temperature the mixture was dialysed against saline. In earlier experiments reduction was performed for 1½ hours at 37°, or with cysteine at pH 7 in phosphate buffer, but this frequently left about 5 per cent of the 5S protein unreduced, and was unsatisfactory for recombination experiments. The 3-hour reduction time did not affect the association constant or the ability to recombine of the 3·5S fragments. The 3·5S antibody was devoid of agglutinating activity.

Recombination of 3·5S Fragments

5S protein reduced with MEA as stated but not treated with iodoacetate was dialysed against several changes of saline; oxygen was bubbled through the dialysis fluid for 1–2 days. Separation of recombined 5S from non-recombined 3·5S protein was achieved by gel filtration on Sephadex G-100.

Chromatography on Sephadex G-100 (Pharmacia, Uppsala, Sweden)

Samples of 2 ml. or less were applied to a 125 × 1·2 cm. (140 ml.) column of Sephadex G-100, and eluted in saline, with a flow rate of 8 ml. per hour. A typical chromatogram is shown in Fig. 1.

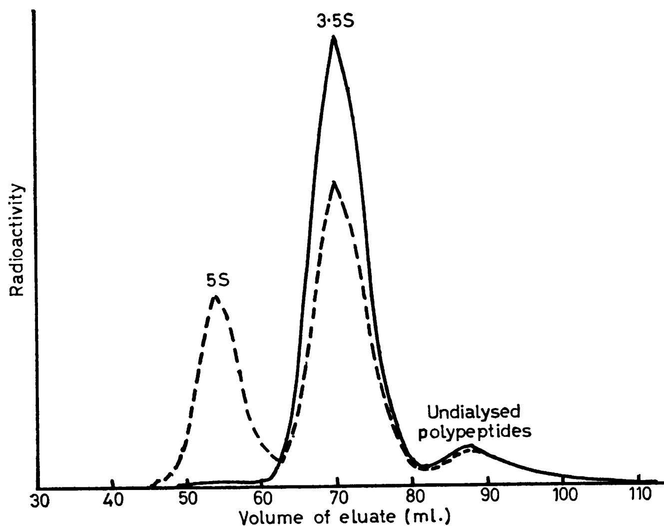

FIG. 1. Chromatograms on Sephadex G-100 of reduced alkylated (———) or recombined (- - -) pepsin digest of 7S γ-globulin.

Protein Estimation

γ-Globulin concentrations were estimated by measuring the optical density at 280 mμ and using a factor of 0·7 to convert $O.D.^{1\,cm.}_{280}$ to mg./ml. of protein.

Measurement of Radioactivity

All measurements were made in a well-type scintillation counter.

Haemagglutination Titrations

Two-fold dilutions of the antibody were made in saline in Perspex agglutination trays and an equal volume (0·4 ml.) of a 2 per cent red cell suspension was added to each cup. After 1–2 hours at room temperature the end point was estimated by observation of the pattern formed by the sedimented cells.

Bovine Albumin

In all red cell absorption experiments a 50-fold excess of bovine albumin (Bovumin, Ortho Pharmaceutical Ltd., Saunderton, Bucks.) was added to the antibody preparation. This procedure reduced to a minimum the non-specific absorption of labelled protein to red cells and test tubes.

Preabsorption of Antibody Preparation

Immediately before use all antibody preparations were absorbed with a large excess of packed human group O red cells.

Measurements of Association Constants of Antibody Preparations

If, in an antigen–antibody system which obeys the law of mass action, the concentration of antibody is sufficiently low to leave uncombined the major portion of the antigen, such that [Ag – AbAg] = [Ag], then the law of mass action can be written:

$$K = \frac{[AbAg]}{[Ag][Ab - AbAg]} \tag{1}$$

When half the antibody is combined with antigen, i.e. when [Ab – AbAg] = [AbAg] this equation becomes

$$K = \frac{1}{[Ag]} \tag{2}$$

where [Ag] and [Ab] are the initial concentrations of antigen and of antibody respectively and [AbAg] is the concentration of antigen–antibody complex at equilibrium.

Thus in these conditions the association constant can be defined as the reciprocal of the antigen (red cells) concentration required to absorb half the available antibody.

Aliquots of suitably diluted ^{131}I-labelled antibody preparation were added to test tubes containing varying quantities of centrifuged group A_1 red cells, such as to provide a range of known final concentrations of red cells in antibody. The red cells were resuspended in the antibody by means of a vortex shaker. After 30 minutes at bench temperature the test tubes were centrifuged, the supernatant fluid was removed and the radioactivity of the tube and its cells was counted. The cells were then washed out of the tube and the activity (always very small) remaining in the tube was counted. The difference between

the two counts represented the sum of the protein adhering to the cells and that contained in the unremovable supernatant. The amount of antibody on the cells was arrived at by further subtracting the counts of group O cells treated in the same way as group A cells. It might appear that it would have been preferable to wash the cells before counting, but in the case of unavid preparations this would have involved a large and unknown error due to elution of antibody. By using small diameter (Widal) tubes for the high concentrations of cells and a fine capillary pipette attached to a suction pump it was possible with care to remove all but a very small amount of supernatant before counting. This can be seen in the values for O cells in the figures.

Percentage uptake of antibody was plotted against cell concentration, and from this 'avidity curve' the concentration of red cells ($1/K$) absorbing half the antibody was determined. Methods of calculating the total antibody in each preparation are given in the descriptions of individual experiments.

RESULTS

EXPERIMENT 1

The Association Constants of 7S, 5S and 3·5S Antibody

Fig. 2 shows 'avidity curves' for 7S antibody, peptic digest (5S), reduced peptic digest (3·5S) and papain digest (3·5S) preparations derived from a single immune rabbit serum. The results obtained with other rabbit sera are shown in Table 1.

TABLE 1

ASSOCIATION CONSTANTS OF 7S, 5S AND 3·5S ANTIBODY

Serum	$1/K_{7S}$*	$1/K_{5S}$	$1/K_{3\cdot5S}$ (*Papain*)	$1/K_{3\cdot5S}$ (*Pepsin* +*MEA*)	$K_{5S}/K_{3\cdot5S}$ (*Pepsin* +*MEA*)
86	0·14	0·15	16·3	24·2	161
88	0·032	0·028	10·9	12·3	440
101†	0·016	0·016	7·8	7·4	460
101†		0·037	—	13·0	350
101†	0·032	0·036	—	14·5	400
112	0·024	0·026	—	8·9	340
115	0·038	0·035	—	15·5	440

* $1/K$ = [RBC] at 50 per cent antibody absorption.

† Each line represents a separate preparation made from serum 101. Percentages of anti-A antibody in the 7S globulin preparations were as follows: Serum 86, 38 per cent; 88, 40 per cent; 101, 46 per cent; 112, 41 per cent; 115, 18 per cent.

In constructing Fig. 2 and the 'avidity curves' from which the data in Table 1 are derived, the total amount of antibody in the preparations has been taken, in the case of 7S and 5S antibody, as the amount absorbed by 16 per cent cells. It is clear from the asymptotic shape of the curves, that the error in this assumption is small. For 3·5S (pepsin and MEA) antibody it has been assumed that reduction has not destroyed any antibody and that the reduced preparations contain the same proportion of antibody radioactivity as the unreduced digest. The maintained steep rise of the curve is compatible with this assumption. For 3·5S (papain) antibody we have assumed that the inert fragment III (Porter, 1959) is the same as the fragments split off by pepsin and that the proportion of antibody radioactivity in the preparation is the same as that in whole, undialysed, peptic digest.

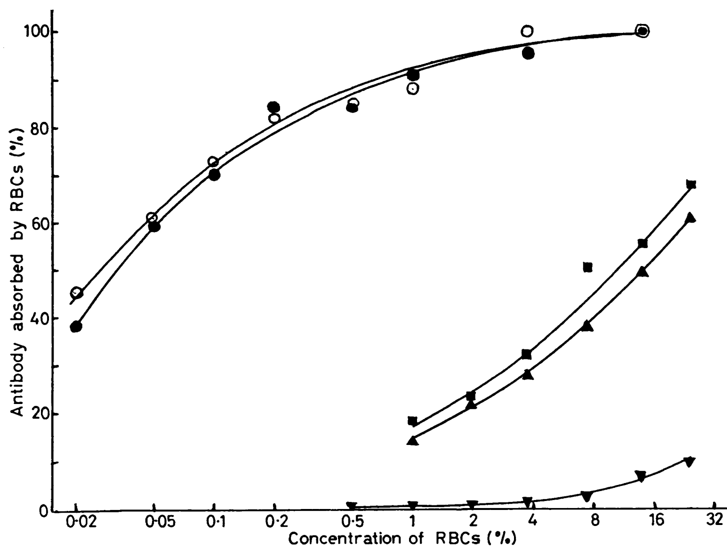

FIG. 2. Avidity of 7S antibody and its sub-units (Serum 88). ●, 7S antibody; ○, 5S pepsin-digested antibody; ■, 3·5S antibody (papain digest); ▲, 3·5S antibody (pepsin + MEA); ▼, mean O cell uptake.

EXPERIMENT 2

Association Constant of Univalent 5S Antibody

Nisonoff & Mandy (1962) have shown that if the reducing agent is removed and the pH is adjusted to 8, the 3·5S fragments produced by reduction of peptic digest will recombine in a random manner to re-form 5S molecules. If the low combining constant of 3·5S fragments is due to their univalence, 5S recombinants consisting of one antibody and one non-antibody moiety, being univalent, will have the same K as 3·5S antibody.

A 25-fold excess of unlabelled normal rabbit 7S-globulin was added to ^{131}I-labelled 7S globulin from an immune rabbit serum. The mixture was digested with pepsin and reduced with MEA, at which stage it contained three classes of 3·5S fragments: (1) labelled antibody (^{131}Ab.); (2) labelled non-antibody (^{131}nonAb), since the immune globulin was only partly antibody; (3) unlabelled non-antibody (nonAb). Part of the reduced mixture was treated with iodoacetate in order to demonstrate by fractionation on Sephadex G-100 that no significant portion of 5S protein remained unreduced. The remainder was allowed to recombine and the newly formed 5S protein was separated from the unrecombined 3·5S protein by fractionation on Sephadex G-100.

The new 5S fraction should consist of six classes of molecules of the following constitution (1) ^{131}Ab–nonAb; (2) ^{131}Ab–^{131}nonAb; (3) ^{131}Ab–^{131}Ab; (4) ^{131}nonAb—^{131}nonAb; (5) ^{131}nonAb–nonAb; (6) nonAb–nonAb. Assuming random recombination, the distribution of radioactivity among these recombinations is derived by expansion of the expression:

$$\{x\ ^{131}\text{Ab} + (1 - x)\ ^{131}\text{nonAb} + y\ \text{nonAb}\}^2 \qquad (3)$$

where x is the proportion of immune 7S globulin which is antibody, and y is the ratio of unlabelled 7S globulin to immune 7S globulin.

In the case of a labelled immune 7S globulin containing 40 per cent antibody (x = 0·4) and a 25-fold excess of normal unlabelled globulin (y = 25), only about 1·5 per cent of the antibody radioactivity is in the bivalent form, ^{131}Ab–^{131}Ab, which can be ignored.

Thus the avidity curve for the mixed recombinants can be taken as that for univalent 5S antibody.

Fig. 3 shows the results of a typical experiment of this nature. 'Avidity curves' are

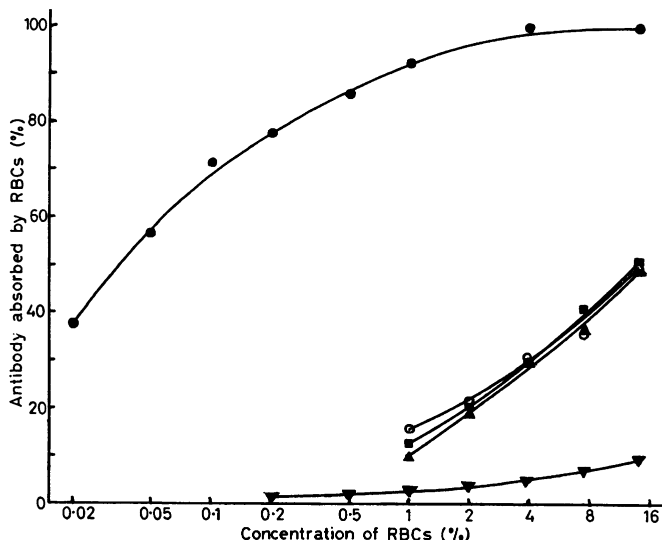

Fig. 3. Avidity of bivalent and univalent 5S and 3·5S antibody (Serum 101). ●, bivalent 5S; ○, univalent 5S; ■, 3·5S (reduced 5S which did not recombine); ▲, 3·5S (reduced alkylated 5S); ▼, mean O cell uptake.

drawn for peptic digest, reduced alkylated peptic digest and for the material contained in each of the first two peaks obtained by Sephadex filtration of the recombined mixture: respectively newly formed univalent 5S antibody and 3·5S antibody which remained uncombined. There is no significant difference in association constants between 3·5S antibody and univalent 5S antibody.

EXPERIMENT 3

Association Constant of Recombined Bivalent 5S Antibody

If the low association constants of 3·5S and univalent 5S antibody are due to their univalence and not to an injury to the molecule caused by reduction, and irreversible on recombination, then bivalent recombinants, Ab–Ab, should have the same association constant as the original 5S antibody. In order to demonstrate conclusively the behaviour of bivalent recombinants it is necessary to work with purified antibody. We were unsuccessful in achieving any useful degree of recombination of 3·5S antibody absorbed and subsequently eluted from red cells, or of 3·5S fragments derived from 5S antibody which had been eluted from red cells. The reason for our lack of success was not clear, although it may have been due to the presence of red cell protein in the eluates.

In view of the failure with purified antibody, recombination experiments were done with our two best antisera in which 41 per cent and 46 per cent respectively of the 7S γ-globulin consisted of anti-A antibody. Labelled 7S γ-globulin was digested with pepsin and reduced. It was then allowed to recombine and was chromatographed on Sephadex-G100. Avidity curves were prepared for the original 7S γ-globulin and the first two peaks obtained from the Sephadex column, i.e. new 5S and unrecombined 3·5S protein, see Fig.

4. The 5S avidity curve is a resultant of the association constants of its two constituents, univalent and bivalent recombinants. From expression (3) it can be calculated (when $y = 0$) that in the recombined 5S fraction 17 per cent and 21 per cent of the radioactivity in the respective sera will be associated with bivalent antibody, 48 per cent and 50 per cent with univalent antibody and the remainder with non-antibody. If the radioactivity corresponding to the expected uptake of univalent antibody is subtracted from the actual uptake from whole recombined 5S, the resulting curve should represent the uptake of the bivalent recombinants.

These derived curves for recombined bivalent 5S antibody are shown in Fig. 4 in broken

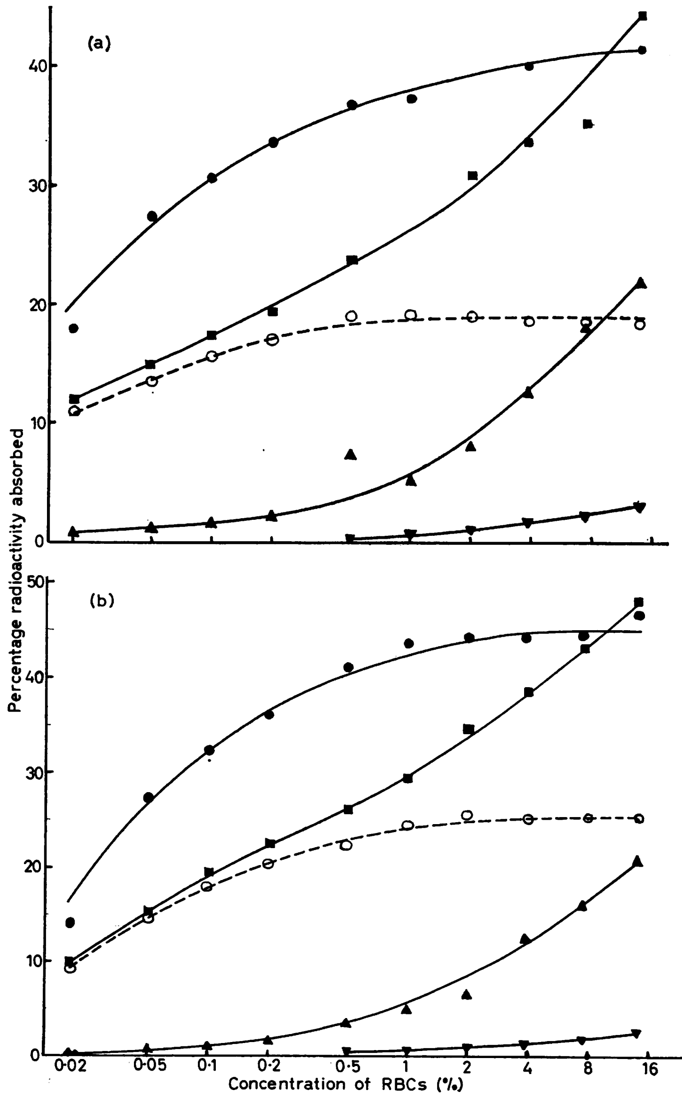

Fig. 4. Avidity of recombined bivalent 5S antibody; (a) serum 112, (b) serum 101. ●, 7S antibody; ■, recombined 5S antibody; ○, calculated uptake of bivalent 5S antibody; ▲, 3·5S antibody (reduced 5S which did not recombine); ▼, mean O cell uptake.

line. They resemble closely the curves for 7S antibody and give a comparable figure for the association constant. The broken-line curves are slightly higher than they should be according to expression (3); this may be accounted for by the underestimate involved in taking as the value for 7S antibody the uptake by 16 per cent cells. The small amount of unreduced 5S protein, 4 per cent and 2 per cent respectively in the two preparations, does not significantly alter the shape or position of the derived curves.

The recombined 5S fractions had agglutination titres of about half that of the original 7S γ-globulin. This was in accordance with the calculated expectation that the two preparations, 41 per cent and 45 per cent respectively of the original antibody, would, after splitting and recombining, appear in the form of bivalent 5S antibody.

DISCUSSION

If it is physically possible for 7S and 5S antibody to combine with red cells so that both the specific groups of an antibody molecule are attached to the same cell, then for reasons given in the introduction and enlarged upon below, it is most probable that antibody molecules will be attached to red cells in this manner. If this is in fact the mode of attachment of 7S antibody to red cells, then it would be expected that 7S antibody would be an inefficient agglutinating agent—the starting point of the present investigation—and that univalent antibody would have a much lower association constant than bivalent antibody. The results of our experiments are in accordance with this expectation and it remains to examine with what justification the decreased K of univalent antibody can be attributed solely or mainly to its univalence.

The experimental findings may be summarized as follows:

1. 5S antibody which is known to be bivalent, agglutinates as efficiently as intact 7S antibody and has the same association constant.
2. 3·5S antibody, which is univalent (Fudenberg, Mandy and Nisonoff, 1963) has an association constant 1/150 to 1/450 that of 7S antibody. There is no difference between 3·5S antibody fragments whether derived from papain digestion or reduction of peptic digest.
3. Univalent 5S antibody, formed by the combination of 3·5S antibody with 3·5S non-antibody has the same association constant as 3·5S antibody.
4. 5S antibody formed by the recombination of two 3·5S antibody fragments and therefore bivalent, has a much greater association constant than 3·5S or univalent 5S antibody. Within the limits of our technique it appears to have the same association constant as 7S or unreduced 5S antibody.

It is possible that 3·5S antibody is less avid than 7S antibody on account of its small molecular size. This seems unlikely, because reduction in molecular size from 7S to 5S is unaccompanied by any reduction in association constant, and 5S univalent antibody is no more avid than 3·5S antibody.

It might be argued that univalent antibody is unavid because the specific group has been damaged directly by the reducing agents (cysteine or MEA) or by a distortion of the rest of the molecule which might occur and indirectly affect the specific group; if so, this distortion is not remedied by recombination of 3·5S fragments. Neither of these explanations seems probable however in view of the findings of Nisonoff and Woernley (1959). These workers using antibody to a small hapten, p-iodobenzoate, showed by equilibrium

dialysis that the association constant remained unaltered after treatment of the antibody with papain. No diminution of K would be expected in this instance, since with a low molecular weight free hapten, there is no possibility of bivalent attachment of an antibody molecule to a single molecule of hapten.

However, the antigenic sites on group A substance and therefore the corresponding specific antibody sites are probably larger than those of the antibodies investigated by Nisonoff and Woernley. The anti-A groups may on this account be more vulnerable to distorting influences. Irremediable damage to the antibody combining site can only be finally excluded by showing that re-formed 5S antibody (Ab–Ab) has the same association constant as unreduced 5S antibody, which for its unequivocal demonstration requires the reduction and recombination of purified 5S antibody. However we have shown in experiment 3 that the recombinants from a mixture containing 3·5S antibody and 3·5S non-antibody are much more avid than 3·5S or monovalent 5S antibody. Moreover calculations show that the increase in avidity agrees well with what would be expected if the bivalent recombinants had the same association constant as unreduced 5S antibody.

We therefore propose that the low association constant of 3·5S and univalent 5S antibody is due to their univalence, and conversely, that bivalent 5S and 7S antibody combine avidly with red cells because they do so with both combining sites.

In the interpretation of these findings we rely on the assumption that the molecular fragments which we have produced correspond to those investigated by other workers. There is no reason to doubt the validity of this premise, since our materials do not differ in chromatographic, agglutinating or recombining properties from those described by others. Moreover anti-ovalbumin, which we have submitted to the same treatment as our anti-A antibodies retains its ability to inhibit precipitation of the antigen by intact homologous antibody (Porter, 1959). We cannot, in the absence of detailed knowledge of the properties of their reagents, interpret the interesting finding of Fudenberg *et al.* (1962) that red cells sensitized with 3·5S anti-O cell antibody had after three washings a Coombs titre similar to that of the agglutinating titre of their 7S antibody. But it cannot be said on the available evidence that our results are contradictory to theirs.

Making certain assumptions it is possible to arrive at a plausible estimate of the relationship of association constants of univalent and bivalent antibody. If 7S antibody is a flexible rod-shaped molecule 250 Å long with a combining site at each end (Almeida, Cinader and Howatson, 1963) and if the second specific group of an antibody molecule attached by one specific group to a red cell, is unrestricted in its ability to combine with any of the antigen sites (Aǵ) within a circle of radius 250 Å, then (see Appendix)

$$K_2 = K_1(1 + \tfrac{1}{2}K_1[\text{Aǵ}]) \simeq \tfrac{1}{2}(K_1)^2[\text{Aǵ}] \qquad (4)$$

where K_2 and K_1 are respectively the association constants for bivalent and univalent antibody and [Aǵ] is the antigen concentration within hemispheres of radius 250 Å around the antigen sites.

If the A-antigen site distribution on the red cell is uniform then:

$$[\text{Aǵ}] = \left(\frac{n}{A}\cdot\pi r^2 - 1\right)\cdot\frac{10^3}{\frac{2}{3}\pi r^3 N} \simeq \frac{3\times 10^3 n}{2ArN} \text{ moles/litre} \qquad (5)$$

where n is the number of A-antigen sites per A_1 red cell, A the surface area of the red cell, r the length of an antibody molecule and N Avogadro's number.

Expressing K_1 in litres per mole,

$$K_1 = \frac{NV}{10kn} \text{ litres/mole} \tag{6}$$

where V is the volume of a red cell in ml., and k is the reciprocal of the association constant for univalent antibody, expressed as a percentage of cells. Then from (4), (5) and (6)

$$\frac{K_2}{K_1} = \tfrac{1}{2}K_1[\text{Ag}'] = \frac{300\ V}{4Ark} \tag{7}$$

If $V = 10^{-10}$ ml., $A = 1{\cdot}65 \times 10^{-6}$ cm² (Ponder, 1948), $r = 2{\cdot}5 \times 10^{-6}$ cm., then the ratio $K_2/K_1 = 1800/k$. Taking values of k('$1/K_{3.5S}$') from Table 1, this corresponds to an expected range of K_2/K_1 from 75 to 240, i.e. about half the experimental values.

The derivation of K_2/K_1 is independent, within the terms of reference and the values under consideration, of the number of sites per red cell, provided they are evenly distributed. If there are $1{\cdot}6 \times 10^6$ A-antigen sites per group A_1 red cell (corresponding to $8{\cdot}3 \times 10^5$ bivalent 7S antibody molecules, Greenbury *et al.*, 1963) then there would be twenty antigen sites per circle of radius 250 Å. It may well be, however, that the antigen sites are not evenly distributed: if in this area there were as many as 100 effective sites, which is probably a maximum if the diameter of the antibody molecule is taken as 40 Å (Almeida *et al.*, 1963), then the ratio K_2/K_1 would be five times as great.

It will prove interesting to see whether human isoantibodies to red cell antigens behave similarly.

APPENDIX

Derivation of a Theoretical Relationship between the Association Constants of Monovalent and Bivalent Antibody for Antigen

Assuming the law of mass action, in the case of monovalent antibody, and antigen, we have:

$$\text{Rate of association} = k_A\ [\text{Ag}]\ [\text{Ab}] \tag{1}$$

$$\text{Rate of dissociation} = k_D\ [\text{Ag.Ab}] \tag{2}$$

At equilibrium

$$k_A\ [\text{Ag}]\ [\text{Ab}] = k_D\ [\text{Ag.Ab}] \tag{3}$$

or

$$K_1 = \frac{[\text{Ag.Ab}]}{[\text{Ag}][\text{Ab}]} = \frac{k_A}{k_D} \tag{4}$$

where [Ag], [Ab] and [Ag.Ab] are the equilibrium concentrations of antigen, antibody and complex, k_A and k_D the association and dissociation rate constants and K_1 the equilibrium constant.

In the case of bivalent antibody the initial attachment of antibody by one combining site would be similar to the attachment of monovalent antibody:

i.e.

$$K_1 = \frac{k_A}{k_D} = \frac{[\text{Ag.Ab}_1]}{[\text{Ag}][\text{Ab}]} \tag{5}$$

[Ag.Ab_1] being the concentration of antigen–antibody complex in which antibody is attached by one end. The two-fold increase in k_A expected because antibody could attach by either end would be approximately cancelled because of the slower rate of diffusion of the larger molecule.

The free combining site of a monovalently attached antibody would then obey the law of mass action with respect to association with other antigen sites within its reach:

thus $$k_A\,[\mathrm{Ag.Ab_1}]\,[\mathrm{Ag'}] = 2\,k_D\,[\mathrm{Ag.Ab_2}] \qquad (6)$$

where [Ag′] is the average concentration of antigen sites within the volume available to one combining site of an antibody whose other site is attached to antigen, and $[\mathrm{Ag.Ab_2}]$ is the concentration of antigen–antibody complex in which antibody is attached by both sites.

By definition the association constant for bivalent antibody

$$K_2 = \frac{[\mathrm{Ag.Ab_1} + \mathrm{Ag.Ab_2}]}{[\mathrm{Ag}][\mathrm{Ab}]} \qquad (7)$$

from (5), (6) and (7),

$$K_2 = \frac{[\mathrm{Ag.Ab_1}]}{[\mathrm{Ag}][\mathrm{Ab}]}\cdot\left(1+\frac{k_A}{2k_D}\cdot[\mathrm{Ag'}]\right) = K_1(1+\tfrac{1}{2}K_1[\mathrm{Ag'}]) \qquad (8)$$

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