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# The Kinetics of Antibody Binding to Membrane Antigens in Solution and at the Cell Surface

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The reaction kinetics of $^{125}$I-labelled mouse monoclonal antibodies binding to three cell-surface antigens of rat thymocytes (Thy-1.1, W3/13 and W3/25) were studied. The differences between bivalent and univalent interactions were determined by using antibody in the $F(ab')_2$ or Fab' form and by using antigen in polymeric or monomeric forms. Association rate constants ($k_{+1}$), dissociation rate constants ($k_{-1}$) and equilibrium constants were determined. Also, the dissociation kinetics of rabbit antibodies against rat Thy-1 antigen were studied. The major findings were as follows. (i) With $F(ab')_2$ antibody there was no simple relationship between antigen density at the cell surface and extent of bivalent binding. Extensive univalent binding was observed unless the antibody had a high $k_{-1}$ for the univalent interaction, in which case all binding was bivalent. (ii) $k_{+1}$ values were similar for $F(ab')_2$ or Fab' antibody, and for the different antibodies were in the range $0.8 \times 10^5$–$1.1 \times 10^6$ $M^{-1} \cdot s^{-1}$. These differences were sufficient to affect the interpretation of serological assays with the different antibodies. (iii) Antibody bound bivalently dissociated much more slowly than that bound univalently. However, the $k_{-1}$ values for the univalently bound antibody were sufficiently low in most cases that the lifetime of the univalent complex was similar to or greater than the time needed for the assay. Thus the results could be interpreted on the basis of irreversible reactions. The overall conclusion from the study is that for an understanding of the binding of antibody to cell-surface antigens the kinetics of the interaction are of major importance and theories based on equilibrium binding are inappropriate.

Antibodies are finding increasing use as the primary tools for identification, assay and purification of membrane molecules (reviewed by Williams, 1977). This approach has become much more versatile, since it is now possible to produce pure monoclonal antibodies against membrane molecules whose identity was previously unknown (Köhler & Milstein, 1976; Williams *et al.*, 1977).

In studying membrane molecules with conventional or monoclonal antibodies it is important that quantitative assays are used, yet the traditional assays that measure haemagglutination or complement-mediated cytotoxicity (reviewed by Klein, 1975) allow only qualitative interpretations. More recently indirect radioactive binding assays (alternatively called cellular radioimmunoassays) have been used to measure the binding of antibody to cell surfaces. In these assays cells are first incubated with a test antibody, washed, incubated with $^{125}$I-labelled anti-immunoglobulin antibody, washed again and counted for bound $^{125}$I radioactivity in a $\gamma$-radiation counter. Early studies (Harder & McKhann, 1968; Sparks *et al.*, 1969) did not lead to wide adoption of binding assays, possibly because of high background values. Non-specific binding was diminished when affinity-chromatography-purified anti-immunoglobulin antibody was used in the second incubation (Goldstein *et al.*, 1973; Acton *et al.*, 1974), and for quantitative studies aggregates should be removed from the purified antibody preferably by preparation of $F(ab')_2$ (Jensenius & Williams, 1974). Non-specific binding due to the first reagent is virtually absent when monoclonal antibodies are used.

In alternative assay procedures $^{125}$I-labelled staphylococcal protein A can be used as the second reagent (Welsh *et al.*, 1975), and tissue homogenates can be used instead of intact cells (Barclay, 1977).

Abbreviations used: IgG, immunoglobulin G; $F(ab')_2$, pepsin-digest fragment of IgG; Fab', $F(ab')_2$ reduced from dimeric to monomeric form; SDS, sodium dodecyl sulphate; DAB buffer, Dulbecco buffer A and B, pH 7.3.



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Indirect binding assays with $^{125}$I-labelled anti-immunoglobulin can be used in a variety of ways (Morris & Williams, 1975; Williams, 1977). If both first and second antibodies are at saturation, an estimate of antigenic site number can be obtained and the distribution of antigen among a cell mixture determined by radioautography or more easily by using a fluorescence-activated cell sorter, with fluorescein–anti-immunoglobulin as the second reagent. Alternatively antibody can be first incubated with antigen and after centrifugation the residual unbound antibody assayed by using the indirect binding assay. In this way the relative amounts of antigen can be measured in different tissue homogenates. These absorption or inhibition assays can also be extended to studying antigens solubilized in detergents if glutaraldehyde-fixed target cells are used to assay residual antibody (Williams, 1973; Letarte-Muirhead *et al.*, 1974).

The quantitative application of indirect binding assays to studies on membrane antigens has been mainly evaluated in the analysis of rat Thy-1 antigens (Letarte-Muirhead *et al.*, 1974, 1975; Morris & Williams, 1975), and these assays proved of major importance in the eventual purification of Thy-1 antigen. More recently indirect binding assays have been used in analysing a variety of other complex antisera (Fabre & Williams, 1977; Morris & Williams, 1977; Mason & Gallico, 1978; MacDonald *et al.*, 1978; Dalchau & Fabre, 1979), and this technique has been adopted as the main assay for the detection of monoclonal antibodies produced by hybridomas (Koprowski *et al.*, 1977; Williams *et al.*, 1977).

With cell-surface antigens serological assays are complicated, since antibody may bind bivalently to membrane-bound antigen but only univalently to antigen solubilized in detergent. This difference in valency of binding results in a more rapid dissociation of antibody from soluble antigen when compared with that from cells. Inhibition assays with detergent-solubilized antigens are unsatisfactory if the complex of antibody and soluble antigen dissociates significantly during the time that the target cells take to bind the uncomplexed free antibody. In these circumstances the target cells not only take up free antibody initially present when the cells are added but acquire further antibody as it dissociates from the soluble antigen. As a consequence the amount of free antibody is overestimated. In contrast, if the dissociation rate for univalent binding is sufficiently small, meaningful results can be obtained. This situation has appeared to hold in a number of cases where the absorptive capacity of membrane (polymeric antigen) has not been affected by solubilization in deoxycholate to produce monomeric antigen (Letarte-Muirhead *et al.*, 1974; Morris & Williams, 1977; Fabre & Williams, 1977). However, this is not always so, since one type of anti-Thy-1.1 antibody was found to be absorbed with polymeric but not monomeric antigen (Letarte-Muirhead *et al.*, 1974; Williams *et al.*, 1976).

In a theoretical paper Reynolds (1979) has challenged previous interpretations of serological studies on cell-surface antigens. The criticisms were applied particularly to studies that used the indirect binding assay, but hold for any assay where antibodies are used. Reynolds (1979) derived equations for the interaction of antibody with membrane antigens in which it was assumed that equilibrium binding is achieved in the assays and thus that bivalent interactions are of major importance.

In the present paper studies are reported on the kinetics of association and dissociation of antibody interacting with cell-surface antigens in polymeric or monomeric forms. In most cases monoclonal antibodies were used, since these make possible experiments that cannot be done with conventional antibodies, which are heterogeneous and present at low concentrations in serum. The binding of monoclonal anti-Thy-1.1 antibody and rabbit anti-Thy-1 antibody to cells and soluble antigen was analysed, as was binding to cells of two other monoclonal antibodies called W3/13 and W3/25. The latter two antibodies appeared to present a paradox in that rat thymocytes bound 2–3 times more W3/13 antibody than W3/25 antibody at saturation, yet more thymocytes were needed to absorb W3/13 antibody than W3/25 antibody (Williams *et al.*, 1977).

The results of these studies show that a theory based on interactions at equilibrium is inappropriate for the analysis of the binding of antibodies to membrane antigens. The kinetics of association and dissociation are of primary importance.

## Experimental

### *Animals*

Wistar rats and Balb/c, C3H and AKR mice were from Olac 1976 Ltd., Bicester, Oxon, U.K. AO and PVG/c rats were bred, specific-pathogen-free, in the M.R.C. Cellular Immunology Unit.

### *Experimental conditions*

All procedures were at 5°C or on ice unless stated otherwise.

### *Determination of protein concentration*

The concentrations of immunoglobulin fractions were calculated from the absorbance read at 280nm on a Gilford 250 spectrophotometer with $A^{1cm}_{1\%} = 14$ as standard.

### *Rabbit anti-(rat Thy-1) antibody*

Two intramuscular injections of 100$\mu$g of pure rat Thy-1L+ antigen in complete Freund's adjuvant were given at an interval of 1 week (Letarte-

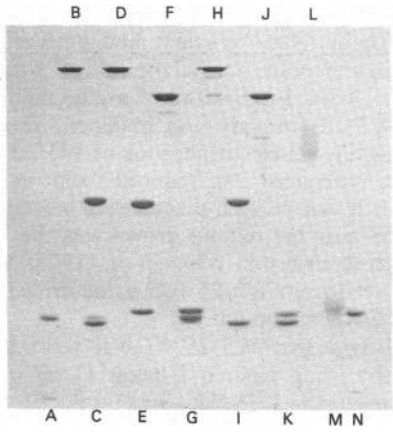


Fig. 1. *Analysis of antibodies by SDS/polyacrylamide-gel electrophoresis*

Discontinuous polyacrylamide gels were used on a Bio-Rad slab gel apparatus (Bio-Rad Laboratories, Watford, Herts., U.K.) as described by Laemmli (1970). The gels were 1.5mm thick and consisted of a 10% acrylamide separating gel and 3% stacking gel. Immunoglobulin samples (50$\mu$l) at 400$\mu$g/ml were added to 50$\mu$l of sample buffer containing 5% (w/v) SDS and 25% (w/v) glycerol, with 2% (w/v) dithiothreitol added when samples were to be reduced. The samples were boiled for 5min and alkylated by addition of 20$\mu$l of 1M-iodoacetamide. A 30$\mu$l portion (50$\mu$g) was loaded on the gels, which were stained with Coomassie Blue after electrophoresis. In slots A, C, E, G, I, K, M and N samples were reduced, whereas those in slots B, D, F, H, J and L were unreduced. The samples were: A and N, $\kappa$-chain from X 63 myeloma (identical with that of the NSI myeloma); B and C, W3/13 HLK IgG; D and E, MRC OX 7 HL IgG (anti-Thy-1.1); F and G, MRC OX 7 $F(ab')_2$; H and I, W3/25 HL IgG; J and K, W3/25 $F(ab')_2$; L and M, $F(ab')_2$ from rabbit anti-Thy-1 IgG.

Muirhead *et al.*, 1975). The Thy-1 antigen had been cross-linked in the oligomeric form (Kuchel *et al.*, 1978) with 0.125% glutaraldehyde. After 3 months the rabbit was boosted intravenously with 100$\mu$g of Thy-1 antigen and the serum used was taken 2 weeks later. The anti-Thy-1 antibody content was 1mg/ml when assayed as described by Morris & Williams (1975). Immunoglobulin in the serum was precipitated with 16% (w/v) $Na_2SO_4$ and degraded with pepsin at pH4.5 in 50mM-sodium acetate buffer for 20h at 37°C (Nisonoff *et al.*, 1960). $F(ab')_2$ was isolated by gel filtration on Sephadex G-200, and SDS/polyacrylamide-gel electrophoresis thereof in unreduced and reduced forms is shown in Fig. 1 (L and M). The $F(ab')_2$ was labelled with $^{125}I$ (1mCi/140$\mu$g) by method 2 (see below), and 8% of the $^{125}I$-labelled $F(ab')_2$ was bound by excess rat thymocytes compared with 1% background binding with rabbit thymocytes.

*Monoclonal anti-Thy-1.1 antibody (MRC OX 7 antibody)*

A hybridoma secreting anti-Thy-1.1 antibody was prepared by W. R. McMaster (unpublished work) by using the techniques of Köhler & Milstein (1976). Balb/c mice were immunized twice with 25$\mu$g of rat thymocyte Thy-1 antigen subcutaneously in complete Freund's adjuvant and boosted intravenously with 10$\mu$g of Thy-1 antigen without adjuvant 4 days before fusing of the mouse spleen cells with NSI/1-Ag4-1 myeloma cells. The selection of hybrids, cloning and screening for hybrids making anti-thymocyte antibody was as by McMaster & Williams (1979). One cloned hybrid made antibody that bound to thymocytes expressing the Thy-1.1 determinant (rat and AKR mouse) but not to those with the Thy-1.2 determinant (Balb/c or C3H mouse). Binding of this antibody was inhibited by pure rat brain Thy-1 antigen, thus establishing it as an anti-Thy-1 antibody specific for the Thy-1.1 determinant. The hybridoma and the antibody have been coded MRC OX 7.

Large amounts of antibody were prepared by growing MRC OX 7 cells as an ascites tumour in mice injected 3 weeks earlier with pristane (2,6,10,14-tetramethylpentadecane) (Potter, 1972). Immunoglobulin was prepared from 150ml of fluid by precipitation with 16% (w/v) $Na_2SO_4$. The IgG was purified on DEAE-cellulose (Whatman DE-52), equilibrated with 25mM-Tris/HCl buffer (pH7.5)/50mM-NaCl/3mM-$NaN_3$. Approx. 70mg of IgG came straight through the column, but this lacked anti-Thy-1.1 antibody activity. The anti-Thy-1.1 IgG (930mg) was eluted with 70mM-NaCl, and SDS/polyacrylamide-gel electrophoresis thereof in unreduced and reduced forms is shown in Fig. 1 (D and E). It can be seen that the IgG was pure and that NSI myeloma $\kappa$-chain was absent (see Fig. 1, A). This is usually secreted by hybridomas along with the antibody L-chain. Thus the MRC OX 7 antibody can be designated MRC OX 7 HL, in the nomenclature of Köhler & Milstein (1976), where L designates the antibody L-chain.

MRC OX 7 IgG was degraded with 2% (w/v) pepsin at pH4 in sodium acetate buffer for 20h at 37°C. $F(ab')_2$ was obtained in good yield and purified by gel filtration on Sephacryl S-200. The product was completely devoid of any IgG, as shown by SDS/polyacrylamide-gel electrophoresis of unreduced and reduced material (Fig. 1, F and G).

The MRC OX 7 $F(ab')_2$ was labelled with $^{125}I$ (1mCi/280$\mu$g) by method 2 (see below), and with six different preparations 88–95% of the $^{125}I$-labelled $F(ab')_2$ bound to rat thymocytes compared with a

background on C3H mouse or rabbit thymocytes of 0.4% with two washes or 1–2% with one wash only. One month after labelling the percentage of $^{125}$I-labelled F(ab')$_2$ bound by excess rat thymocytes was decreased from 95% to 85%.

To prepare $^{125}$I-labelled Fab' from $^{125}$I-labelled F(ab')$_2$, 0.3 ml of 20 mM-mercaptoethanol in DAB buffer (Dulbecco buffer A and B, pH 7.3; Oxoid, London E.C.4, U.K.), was added to 0.3 ml of $^{125}$I-labelled F(ab')$_2$ at 160 $\mu$g/ml in DAB buffer containing 0.5% bovine serum albumin and incubated at 27°C for 60 min. Iodoacetamide (60 $\mu$l at 0.15 M) was added and after 10 min the mixture was either diluted 5-fold in DAB buffer containing 0.5% bovine serum albumin or loaded on to a Sephacryl S-200 column (1.2 cm × 50 cm). After gel filtration one peak of $^{125}$I radioactivity was obtained at an elution volume greater than that for bovine serum albumin, as expected for $^{125}$I-labelled Fab'. The leading fractions of the peak were discarded, and the rest were pooled and stored at 5°C. The composition of the $^{125}$I-labelled Fab' was analysed by SDS/polyacrylamide-gel electrophoresis without reduction, and the gels were sliced for counting of $\gamma$-radioactivity. Without gel filtration 2–3% of the radioactivity ran with F(ab')$_2$, and 55–65% was at the Fab' position with the rest at the position of the L-chain, indicating that about 40% of the H-chain–L-chain disulphide bonds had been reduced. After gel filtration the $^{125}$I-labelled Fab' contained no $^{125}$I-labelled F(ab')$_2$, and this preparation was used in most experiments. The maximum proportion of $^{125}$I-labelled Fab' that bound to rat thymocytes was 60–70%, with a background of 1.5% on C3H mouse or rabbit thymocytes with one wash.

*W3/13 and W3/25 monoclonal antibodies*

These antibodies are the products of hybridomas prepared by fusing NSI myeloma cells with spleen cells from a Balb/c mouse immunized with rat thymocyte membrane. Both recognize antigens of rat thymocytes and T-lymphocytes (Williams *et al.*, 1977). W3/13 IgG and W3/25 IgG were prepared as described above for the MRC OX 7 antibody. For W13/13 antibody the IgG came straight through the DEAE-cellulose column, with 560 mg of IgG being obtained from 350 ml of ascitic fluid. The W3/13 IgG contains the NSI-cell antibody $\kappa$-chain, but this appears to be present in lower amounts than the antibody L-chain [compare Fig. 1 (C), which shows reduced W3/13 IgG after SDS/polyacrylamide-gel electrophoresis, with Fig. 1 (A), which shows the pattern for myeloma L-chain; Fig. 1 (B) shows the pattern for unreduced W3/13 IgG]. The antibody is thus designated W3/13 HLK.

The $Na_2SO_4$ precipitate of W3/25 antibody was applied to DEAE-cellulose in Tris/HCl buffer (see above) without 50 mM-NaCl. The antibody was eluted with an NaCl gradient and emerged as a sharp peak after some normal mouse IgG was eluted from the column. From 80 ml of ascitic fluid 60 mg of pure W3/25 antibody was obtained. The SDS/polyacrylamide-gel electrophoresis of W3/25 IgG is shown, in unreduced and reduced form, in Fig. 1 (H and I). It can be seen that there is no myeloma L-chain because the tumour grown was the W3/25 HL variant described in White *et al.* (1978). F(ab')$_2$ was produced from W3/25 IgG as described above for MRC OX 7 antibody.

W3/13 IgG and W3/25 F(ab')$_2$ were labelled with $^{125}$I by using method 1 below (1 mCi/40 $\mu$g), and $^{125}$I-labelled W3/25 Fab' was produced without contaminating $^{125}$I-labelled F(ab')$_2$ by reduction and gel filtration as described for MRC OX 7 antibody. The maximal binding of the labelled antibodies to rat thymocytes was 60% for W3/13 IgG, 80% for W3/25 F(ab')$_2$ and 65% for W3/25 Fab'. Backgrounds were 3% after three washes when antigen was in excess, but <0.5% when antibody was in excess.

*Rabbit F(ab')$_2$ anti-(mouse IgG) antibody*

This was as used by Williams *et al.* (1977) with antibody cross-reacting with rat immunoglobulin removed by passage through a (rat IgG)–Sepharose 4B column.

*Labelling of antibodies with $^{125}$I*

The chloramine-T method of Hunter & Greenwood (1962) was used in two ways.

*Method* 1 (*Jensenius & Williams*, 1974). To 5 $\mu$l of [$^{125}$I]iodide (100 mCi/ml, carrier-free) was added 10 $\mu$l of chloramine-T at 2 mg/ml in 0.3 M-sodium phosphate buffer, pH 7.4, and then 20 $\mu$l of antibody at 1 mg/ml in 25 mM-Tris/HCl buffer, pH 7.4. After 2 min incubation at room temperature (approx. 20°C) 25 $\mu$l of saturated tyrosine was added followed 1 min later by 50 $\mu$l of foetal calf serum. The mixture was immediately loaded on to a 5 ml Sephadex G-50 (fine grade) column poured in a disposable pipette and the radiolabel in the excluded fraction was collected. Where a more-concentrated reagent was required, the amount of antibody was increased together with the amount of [$^{125}$I]iodide. Radiolabelled antibody was stored at 4°C in DAB buffer containing 10 mM-$NaN_3$.

When measurements of absorbance were to be made on the product, the tyrosine, foetal calf serum and $NaN_3$ were omitted from the preparation.

*Method* 2 (*adopted to minimize any possible damage to antibody; based on the method of Stanley et al.*, 1975). [$^{125}$I]Iodide (1 mCi, 10 $\mu$l), 50 $\mu$l of KI (8 $\mu$g/ml in 0.3 M-sodium phosphate buffer, pH 7.4), 100 $\mu$l of chloramine-T (0.2 mg/ml in 0.3 M-sodium phosphate buffer, pH 7.4) and 10 $\mu$l of dimethyl

sulphoxide were mixed together. Antibody (100 $\mu$l at 2.8 mg/ml) was then added, and the mixture was incubated for 2.5 min at room temperature followed by gel filtration on a 5 ml Sephadex G-50 (fine grade) column (prewashed with DAB buffer containing 10% bovine serum albumin and DAB buffer again until the eluate was protein-free). The absorbance of radiolabelled fractions was read at 280 and 260 nm, and the recovery of antibody was calculated to be 80–85%. The $^{125}$I-labelled antibody was stored (at 5°C) at 160 $\mu$g/ml in DAB buffer containing 0.5% bovine serum albumin and 10 mM-$NaN_3$. The KI was added to ensure a molar ratio of 1:1 for I and antibody.

W3/13 and W3/25 antibodies were labelled by method 1, and MRC OX 7 (anti-Thy-1.1) $F(ab')_2$ and rabbit $F(ab')_2$ were labelled by method 2. In all cases the labelled antibody was shown to be undenatured by binding to rat thymocytes under saturating conditions with $^{125}$I-labelled antibody mixed in known ratios with unlabelled antibody. The binding obtained was not significantly different from the theoretical value. Other details of the $^{125}$I-labelled antibodies can be found above.

*Cells*

Thymocytes from AKR and C3H mice, from rabbits and from AO, PVG/c and Wistar rats were used. Rat bone-marrow and spleen cells were from AO rats. Cells were prepared in DAB buffer containing either 0.5% bovine serum albumin or 2% foetal calf serum. Standard methods were used (Ford, 1978; Hunt, 1978), and included removal of erythrocytes from bone-marrow cells with $NH_4Cl$ and removal of dead cells from spleen preparations by using Isopaque/Ficoll. Viabilities as assessed with the Trypan Blue-exclusion method were >95% for thymocytes, 85% for bone-marrow cells and 95% for spleen cells. Cells were counted in a Coulter model FN counter with saponin added to lyse erythrocytes.

Glutaraldehyde-fixed cells were prepared as described by Williams (1973) with glutaraldehyde aged by storage at room temperature. The cells were stored frozen in 25 mM-Tris/HCl buffer, pH 8, containing 0.14 M-NaCl and 5% bovine serum albumin.

*Binding of $^{125}$I-labelled antibody to cells*

In most cases this involved direct interaction of $^{125}$I-labelled antibody with cells at 0–5°C in 2.5 ml plastic tubes or 1.5 ml Beckman Microfuge tubes or micro-titre plates with 96 U-bottom wells (volume 0.25 ml). Cells (including in many cases glutaraldehyde-fixed sheep erythrocytes to allow formation of a clearly visible pellet on centrifugation) and antibody were diluted in DAB buffer containing 10 mM-$NaN_3$ and either 0.5% bovine serum albumin or 2% foetal calf serum and added in the amounts given in the Figure legends. During incubation cells were regularly mixed by shaking tubes every 15 min or rotating stoppered tubes on a wheel at 5°C or agitating plates on a micro-shaker. At the end of the incubation buffer (as above) was added in volumes of 1.2–1.5 ml for tubes and 0.15 ml for micro-titre plates. The cells were pelleted by centrifugation: plastic tubes in an MSE 4L centrifuge at 400$g$ for 5 min or 1500$g$ for 2.5 min; Beckman tubes in a Beckman Microfuge at 6000$g$ for 1 min; micro-titre plates is an MSE 6L centrifuge at 280$g$ for 1.5 min. One wash was usually done for assays in tubes, and the time for adding buffer and beginning centrifugation was as short as 15 s when Beckman tubes were used. With micro-titre plates cells were washed three times. Controls for specificity of binding were the use of glutaraldehyde-fixed C3H mouse or rabbit thymocytes instead of rat thymocytes or the preincubation of rat cells with excess of unlabelled monoclonal antibody before the addition of $^{125}$I-labelled antibody.

In some cases indirect binding assays were done by using micro-titre plates with one wash after the first binding step with unlabelled antibody and three washes for the second where $^{125}$I-labelled anti-(mouse IgG) antibody was used. Controls in these experiments involved incubation in the first step with W6/32 antibody, a monoclonal anti-HLA antibody that does not bind to rat cells (Barnstable *et al.*, 1978).

In all experiments the radioactivity (c.p.m.) bound to the pelleted cells was recorded by using a $\gamma$-radiation counter.

By using these assay methods the following parameters were determined.

*Molecules of antibody bound/cell at saturation*

This was determined by measuring binding at antibody concentrations where a 2-fold increase in antibody concentration gave <5% increase in binding. The number of molecules bound/cell was calculated from the radioactivity (c.p.m.) bound, the specific radioactivity of labelled antibody and the cell number. The number of molecules of Fab' bound/cell at saturation was used to calculate antigen concentration for use in the following experiments.

*Association rate constant ($k_{+1}$)*

This was usually determined from initial reaction rates of binding measured at cell concentrations and time points that were appropriate (see Figure and Table legends). $k_{+1}$ was calculated from the classical second-order kinetic equation:

$$k_{+1} = \frac{\text{initial rate}}{[A_0][B_0]} \quad (1)$$

where $[A_0]$ is initial antigen concentration and $[B_0]$ is initial antibody concentration.

In some cases, where dissociation of binding was negligible, the reaction was followed to completion and the data were plotted as $\log[a/(a-x)]$ (where $a$ is maximum binding and $x$ is binding at time $t$) versus time ($t$). An excess of antigen was used and the reaction followed pseudo-first-order kinetics:

$$k_{+1} = \frac{2.303 \times \text{slope of the semi-log plot}}{\text{concn. of antigen}} \quad (2)$$

*Dissociation rate constant ($k_{-1}$)*

Two methods were used. In the first cells were preincubated with $^{125}$I-labelled antibody for a time such that a doubling of the incubation time gave no detectable increase in binding. Then a large excess of unlabelled antibody was added to saturate any remaining antigenic sites and compete for rebinding with any $^{125}$I-labelled antibody that dissociated. The cells were washed at successive time points and the radioactivity of the residual $^{125}$I-labelled antibody was counted. The binding occurring when labelled and unlabelled antibodies were pre-mixed was subtracted, and the results were plotted as percentage radioactivity bound versus log time. The time for 50% dissociation ($t_{\frac{1}{2}}$) was read from this graph and used to calculate $k_{-1}$:

$$k_{-1} = \frac{0.693}{t_{\frac{1}{2}}} \quad (3)$$

In the second method cells and $^{125}$I-labelled antibody were preincubated under conditions of large antigen excess until equilibrium was achieved, and the mixture was then diluted to a known volume. The rate of relaxation to a new equilibrium was measured by determining radioactivity (c.p.m.) bound at various times. From the dissociation curve $k_{-1}$ was calculated according to the equation:

$$k_{-1} = \frac{1}{\left(\frac{K_1[A_0]}{F} + 1\right) \cdot t} \cdot \ln\left(\frac{\text{c.p.m.}_{\text{initial}} - \text{c.p.m.}_{\text{final}}}{\text{c.p.m.}_{(t)} - \text{c.p.m.}_{\text{final}}}\right) \quad (4)$$

and $K_1$, the equilibrium constant, by:

$$K_1 = \frac{\text{c.p.m.}_{\text{final}} \cdot F - \text{c.p.m.}_{\text{initial}}}{[A_0](\text{c.p.m.}_{\text{initial}} - \text{c.p.m.}_{\text{final}})} \quad (5)$$

where $[A_0]$ is the antigen concentration in initial suspension, $\text{c.p.m.}_{\text{initial}}$ is the radioactivity (c.p.m.) bound before dilution, $\text{c.p.m.}_{\text{final}}$ is the radioactivity (c.p.m.) bound after relaxation to new equilibrium after dilution, $\text{c.p.m.}_{(t)}$ is the radioactivity (c.p.m.) bound at time $t$, and $F$ is the factor by which the original suspension was diluted.

*Equilibrium constant ($K_1$)*

Cells and $^{125}$I-labelled antibody were incubated at appropriate concentrations, and binding at equilibrium was determined by washing cells at a time such that a further 60 min incubation gave no increase in binding. From radioactivity (c.p.m.) bound the concentration of bound antibody, $[AB_e]$, could be calculated and, given a knowledge of initial antigen and antibody concentrations, the equilibrium concentrations of antigen, $[A_e]$, and antibody, $[B_e]$, could be calculated. The equilibrium constant, $K_1$, was then determined from:

$$K_1 = \frac{[AB_e]}{[A_e][B_e]} \quad (6)$$

See also determination of the equilibrium constant, $K_1$, by eqn. (5).

*Measurement of rate of dissociation with soluble Thy-1 antigen and construction of inhibition assays*

$^{125}$I-labelled antibody was incubated with thymocyte Thy-1 antigen purified as described by Letarte-Muirhead *et al.* (1975) or with brain Thy-1 antigen as described by Barclay *et al.* (1975), with the modification that lipid was removed by chloroform/methanol (2:1, v/v) extraction and that an affinity column of MRC OX 7 monoclonal antibody was used rather than lectin affinity chromatography (A. F. Williams, unpublished work). The pure Thy-1 antigen was precipitated with ethanol to remove deoxycholate and solubilized in 3 mM-$NaN_3$. Thy-1 antigen was diluted in 10 mM-Tris/HCl buffer, pH 8, containing 0.25% bovine serum albumin and 10 mM-$NaN_3$ or the same buffer medium containing 0.5% deoxycholate also, and 25 $\mu$l portions were incubated for 1 h with 25 $\mu$l of $^{125}$I-labelled antibody diluted into the above buffer without deoxycholate. Glutaraldehyde-fixed thymocytes ($3 \times 10^6$ or $5 \times 10^6$) were then added, and binding of antibody was measured by incubation at increasing time intervals. From these measurements the percentage of antibody remaining in soluble complexes was calculated as given in the Results section, and this was plotted for the determination of $t_{\frac{1}{2}}$ and $k_{-1}$ as described above (eqn. 3).

To construct inhibition curves with increasing amounts of Thy-1 antigen, $^{125}$I-labelled antibody and antigen were diluted in buffers as above, incubated together and assayed for residual antibody by addition of fixed thymocytes (details in Figure legends).

*Labelling of cells with fluorescent antibody*

Thymus, spleen and bone-marrow cells ($2.5 \times 10^6$) suspended in DAB buffer containing 0.5% bovine serum albumin and 10 mM-$NaN_3$ were incubated for 40 min with 100 $\mu$l of F(ab')$_2$ MRC OX 7 (anti-

Thy-1.1) antibody at 28 $\mu$g/ml or normal mouse IgG at 30 $\mu$g/ml (controls). After two washes the cells were then incubated with 100 $\mu$l of fluorescein-conjugated rabbit $F(ab')_2$–anti-(mouse IgG) at 20 $\mu$g/ml. The cells were again washed twice and suspended for quantitative determination of fluorescent antibody bound by using the fluorescence-activated cell sorter (Loken & Herzenberg, 1975).

## Results

### (1) *Binding of monoclonal anti-Thy-1.1 antibody to different lymphoid cells*

Rat thymocytes, bone-marrow cells and spleen cells were incubated with $^{125}$I-labelled $F(ab')_2$ or $^{125}$I-labelled Fab' anti-Thy-1.1 antibody at saturating concentrations and the radioactivity (c.p.m.) bound was measured after addition of buffer and immediate sedimentation of the cells. The numbers of molecules of antibody bound/cell were calculated by using the known specific radioactivities of the antibodies, which were identical for $^{125}$I-labelled $F(ab')_2$ and $^{125}$I-labelled Fab' since the latter was prepared from the former. The experimental details and the results are given in Table 1.

Rat thymocytes bound $1.04 \times 10^6$ molecules of $^{125}$I-labelled Fab' antibody and $7.04 \times 10^5$ of $^{125}$I-labelled $F(ab')_2$, giving an Fab'/$F(ab')_2$ binding ratio of 1.48:1. This indicates that 52% of the $F(ab')_2$ was involved in univalent binding, with 48% showing bivalent interaction. With glutaraldehyde-fixed thymocytes the binding per cell was diminished by about one-third for both Fab' and $F(ab')_2$, but the Fab'/$F(ab')_2$ binding ratio was as for fresh cells. Binding to bone-marrow cells and spleen cells was about 10% and 5% respectively of that to thymocytes on a per-cell basis, but not all cells in the latter tissues carry Thy-1 antigen. This can be seen in Fig. 2, which shows the various lymphoid-cell populations labelled with $F(ab')_2$ anti-Thy-1.1 anti-

Table 1. *Saturating binding of $^{125}$I-labelled $F(ab')_2$ and $^{125}$I-labelled Fab' anti-Thy-1.1 to lymphoid cells*

For binding to rat cells, thymocytes at $10^6$ per assay and bone-marrow cells and spleen cells at $5 \times 10^6$ were incubated in duplicate with 40 $\mu$l of $^{125}$I-labelled $F(ab')_2$ at 16 $\mu$g/ml and 8 $\mu$g/ml and with 75 $\mu$l of $^{125}$I-labelled Fab' at 8 $\mu$g/ml and 4 $\mu$g/ml for 60 min on ice, after which 1.5 ml of DAB buffer containing 0.5% bovine serum albumin and 10 mM-$NaN_3$ was added and the cells were pelleted by centrifugation at 1500 *g* for 2.5 min. Before the incubations with $^{125}$I-labelled antibody the cells had been preincubated for 45 min with 10 $\mu$l, at 30 $\mu$g/ml, of unlabelled $F(ab')_2$ anti-Thy-1.1 (to give blocking for control binding) or normal mouse IgG (in assays for specific binding). The ratios of specific to control binding of either Fab' or $F(ab')_2$ at the two concentrations were for thymocytes 20:1 and 38:1, for bone-marrow cells 9:1 and 14:1, and for spleen cells 4:1 and 8:1. The binding did not increase with antibody concentration, and the values shown are the means of the four assays for each cell type minus the control values. For AKR-mouse thymocytes and some of the experiments with rat thymocytes $2.5 \times 10^5$ or $5 \times 10^5$ cells with $5 \times 10^6$ glutaraldehyde-fixed sheep erythrocytes were incubated with 30 $\mu$l of antibody at saturating concentrations of $^{125}$I-labelled $F(ab')_2$ (4–8 $\mu$g/ml) or $^{125}$I-labelled Fab' (5–11 $\mu$g/ml) for 60 min on ice. Controls involved incubations with $2.5 \times 10^5$ or $5 \times 10^5$ glutaraldehyde-fixed rabbit thymocytes and $5 \times 10^6$ sheep erythrocytes. The cells were washed with 1.2 ml of buffer in a Beckman Microfuge. Each value used in the calculation of means ± S.D. was taken from at least three assays with control values subtracted.

| | Fresh rat cells* | | | | | |
|---|---|---|---|---|---|---|
| | Thymocytes | Bone-marrow cells | Spleen cells | Fresh rat thymocytes† | Glutaraldehyde-fixed rat thymocytes‡ | Fresh AKR-mouse thymocytes§ |
| $10^{-3}$ × No. of molecules of antibody specifically bound per cell | | | | | | |
| $F(ab')_2$ | 704 | 75 | 38 | 704 ± 43 | 462 ± 59 | 318 ± 28 |
| Fab' | 1050 | 112 | 54 | 1040 ± 75 | 686 ± 55 | 637 ± 79 |
| Fab'/$F(ab')_2$ binding ratio | 1.49 | 1.49 | 1.42 | 1.48 | 1.48 | 1.98 |
| Relative amounts of Thy-1 antigen on the basis of Fab' binding to fresh rat thymocytes | 100 | 10.7 | 5.2 | | 66 | 63 |
| Percentage of cells specifically labelled | 96 | 31 | 16 | | | |
| $10^{-3}$ × No. of molecules of Fab' bound per labelled cell | 1090 | 360 | 340 | | | |

* Results from one experiment; two other experiments gave similar results.
† Means ± S.D. for seven experiments.
‡ Means ± S.D. for three experiments.
§ Means ± S.D. for four experiments.

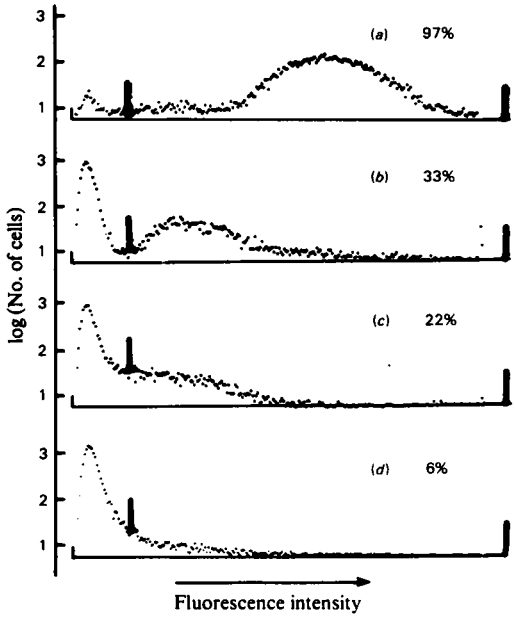


Fig. 2. *Analysis of rat lymphoid cells labelled with anti-Thy-1.1 antibody by the fluorescence-activated cell sorter*

Thymocytes (*a*), bone-marrow cells (*b*) and spleen cells (*c*) were incubated with $F(ab')_2$ anti-Thy-1.1 antibody and then with fluorescein-labelled $F(ab')_2$ anti-(mouse IgG) antibody and washed again. The details are given in the Experimental section. The fluorescein–antibody bound was measured quantitatively with a Becton Dickinson fluorescence-activated cell sorter, and the percentage of cells labelled is shown for each cell type. Controls involved incubation of cells with normal mouse IgG instead of anti-Thy-1.1 antibody, and that for spleen cells, which gave the most non-specific labelling, is shown in (*d*). Percentages of cells labelled in the other controls were: thymocytes, 1%; bone-marrow cells, 2%.

body followed by fluorescein-labelled rabbit anti-(mouse IgG) antibody. Virtually all thymocytes were labelled, compared with 31% of bone-marrow cells and 16% of spleen cells. If binding per labelled cell is calculated, this comes to about $3 \times 10^5$ Fab' molecules for bone-marrow cells and spleen cells compared with $10^6$ Fab' molecules for thymocytes. This 3-fold difference is in accord with the mode positions for fluorescence intensity of labelled cells in Fig. 2. The different antigen density had little effect on the Fab'/$F(ab')_2$ binding ratio, since this was close to 1.5 for bone-marrow cells, spleen cells and thymocytes.

All the data in Table 1 are in good agreement with results obtained with conventional antibodies (reviewed by Williams *et al.*, 1976). Previously it was shown that about 600000 molecules of bivalent antibody from various types of anti-Thy-1 sera were bound per thymocyte at saturation, and this agrees well with $7 \times 10^5$ molecules of $F(ab')_2$ monoclonal antibody bound/cell (Table 1). For an estimate of total Thy-1 determinants a radioimmunoassay calibrated with pure Thy-1 antigen was used previously (Williams *et al.*, 1976). Assuming a mol.wt. of 24000 as determined by SDS/polyacrylamide-gel electrophoresis it was calculated that there were $6.4 \times 10^5$ molecules of Thy-1 antigen at the surface of each cell. However, this value requires recalculation, since the correct molecular weight for brain Thy-1 antigen is 17500 (Kuchel *et al.*, 1978), and, if this is used, a value of $8.8 \times 10^5$ Thy-1 antigen molecules per thymocyte is obtained. This agrees well with the binding of $^{125}$I-labelled Fab', which should also estimate the total number of antigenic determinants per cell. The relative amounts of Thy-1 antigen on thymocytes, bone-marrow cells and spleen cells also agree with previous estimates based on absorption analysis with these cells. By absorption the average relative values were about 100, 10 and 3.5 respectively for thymocytes, bone-marrow cells and spleen cells, and on the basis of binding of $^{125}$I-labelled Fab' monoclonal antibody the relative amounts are 100, 10 and 5 (Table 1). Thus it is clear that in the absorption analysis the uptake of antibody has been in direct proportion with the amount of antigen, and not with the square of the number of sites/cell as was suggested by Reynolds (1979).

The binding of $^{125}$I-labelled $F(ab')_2$ and $^{125}$I-labelled Fab' monoclonal anti-Thy-1.1 antibody to AKR-mouse thymocytes was also measured. At saturation the average binding was $6.4 \times 10^5$ molecules of $^{125}$I-labelled Fab' antibody/cell, suggesting that the number of Thy-1 antigen molecules on AKR-mouse thymocytes is about two-thirds that of rat thymocytes. The Fab'/$F(ab')_2$ binding ratio on AKR-mouse thymocytes was 1.98:1, showing that virtually all the antibody was bound in a bivalent interaction. This observation suggests that the ratio of 1.48:1 obtained for rat thymocytes is not due to technical problems. The difference in binding between AKR-mouse and rat thymocytes is almost certainly due to the fact that $k_{-1}$ for binding to AKR-mouse thymocytes is 10 times that for binding to rat thymocytes (see below). Thus on AKR-mouse thymocytes antibody-binding sites are rapidly dissociating and re-associating, and in this situation binding would equilibrate to be all bivalent given sufficient mobility of antigen in the membrane.

(2) *Measurement of the association rate constant ($k_{+1}$) for the binding of monoclonal anti-Thy-1.1 antibody to thymocytes*

The association constant $k_{+1}$ was calculated from

initial reaction rates measured by incubating $^{125}$I-labelled F(ab')$_2$ or $^{125}$I-labelled Fab' anti-Thy-1.1 antibody with fresh or glutaraldehyde-fixed thymocytes from the rat or AKR mouse. In Fig. 3 the time course of the initial reaction is shown for F(ab')$_2$ and Fab' with fresh rat thymocytes under conditions of antigen excess. For both F(ab')$_2$ and Fab' the reaction had the characteristics of single-hit kinetics, with the rate of reaction for F(ab')$_2$ being about 30% faster than for Fab' in the experiment shown. Overall six determinations of $k_{+1}$ were done, in which F(ab')$_2$ binding and Fab' binding were compared. The means ± S.D. for $k_{+1}$ were $4.6 \times 10^5 \pm 0.3 \times 10^5$ M$^{-1}\cdot$s$^{-1}$ for $^{125}$I-labelled F(ab')$_2$ and $3.6 \times 10^5 \pm 0.3 \times 10^5$ M$^{-1}\cdot$s$^{-1}$ for $^{125}$I-labelled Fab', and thus on average the $k_{+1}$ was 28% higher for F(ab')$_2$ than for Fab'.

The data for all experiments are summarized in Table 2, and it is clear that the $k_{+1}$ values are very similar for the interaction of antibody with fresh or fixed rat thymocytes and AKR-mouse thymocytes.

### (3) *Measurement of the rate of dissociation of cell-bound monoclonal anti-Thy-1.1 antibody*

In most cases this was done by preincubating cells with $^{125}$I-labelled antibody and then observing its dissociation by adding excess unlabelled antibody to prevent re-association of the $^{125}$I-labelled antibody. Dissociation of $^{125}$I-labelled Fab' was studied with fresh rat thymocytes at 4°C, fixed rat thymocytes at 4°C and 18°C (Fig. 4) and AKR-mouse thymocytes at 4°C (Fig. 5). The rate of dissociation

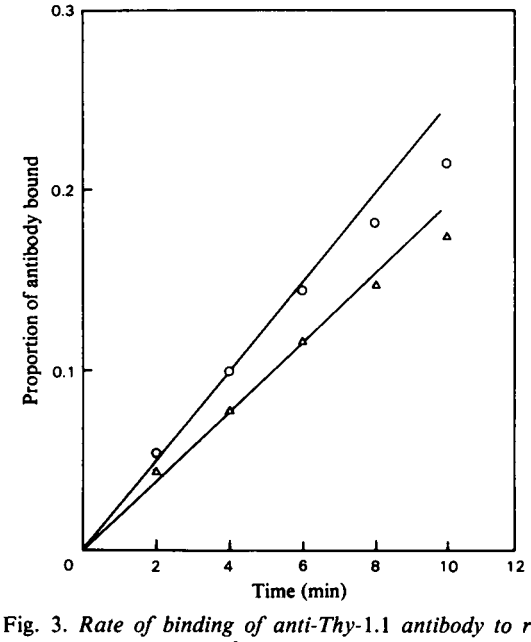


Fig. 3. *Rate of binding of anti-Thy-1.1 antibody to rat thymocytes*

$^{125}$I-labelled F(ab')$_2$ (○) or $^{125}$I-labelled Fab' (△) monoclonal anti-Thy-1.1 antibody was added to Beckman tubes in 25 μl portions. The amounts and radioactivities of bindable antibody were: $^{125}$I-labelled F(ab')$_2$, 16.4 ng, $6.4 \times 10^4$ c.p.m.; $^{125}$I-labelled Fab', 8.2 ng, $3.2 \times 10^4$ c.p.m. Then 0.975 ml of DAB buffer with 0.5%, bovine serum albumin and 10 mM-$NaN_3$, containing $5 \times 10^5$ fresh rat thymocytes and $5 \times 10^6$ glutaraldehyde-fixed sheep erythrocytes, was added to duplicate assays at 2 min intervals up to 8 min. At 10 min all cells were pelleted in a Beckman Microfuge and radioactivity (c.p.m.) bound was measured. In control incubations $5 \times 10^5$ fixed rabbit thymocytes replaced the rat cells, and the binding, which was 1.4% of the radioactivity (c.p.m.) added, has been subtracted from all values. The concentration of Thy-1 antigen was 0.81 nM and that of bindable $^{125}$I-labelled F(ab')$_2$ or $^{125}$I-labelled Fab' 0.16 nM.

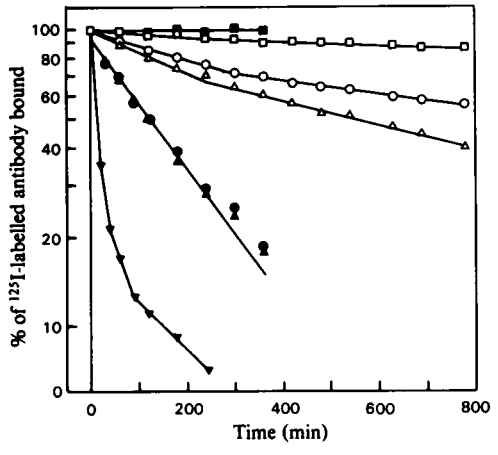


Fig. 4. *Dissociation of monoclonal $^{125}$I-labelled F(ab')$_2$ and $^{125}$I-labelled Fab' anti-Thy-1.1 antibody from rat thymocytes*

All assays were in duplicate and the mixtures contained $5 \times 10^5$ fixed rat thymocytes and $5 \times 10^6$ fixed sheep erythrocytes, which were pelleted before addition of 25 μl of $^{125}$I-labelled F(ab')$_2$ (□, ○ and △) or $^{125}$I-labelled Fab' (■, ●, ▲ and ▼) at 4 μg/ml. The mixtures were incubated for 60 min followed by addition of buffer only (□ and ■), anti-Thy-1.1 IgG at 50 μg/ml (○ and ●) or anti-Thy-1.1 IgG at 1 mg/ml (△, ▲ and ▼). All assays were on ice except for the ▼ points, where incubations were at 18°C. At various times 1.5 ml of buffer was added and the cells were pelleted for determination of radioactivity (c.p.m.) bound. In control incubations $^{125}$I-labelled antibody and unlabelled antibody were pre-mixed and incubated with cells for 180 min before the washing. The control radioactivities (c.p.m.) were subtracted from the specific-binding values, which were then expressed as percentages of the radioactivity (c.p.m.) bound minus controls for assays washed at the end of the preincubation period.

was not affected by the concentration of unlabelled antibody added, and this is shown in Fig. 4 for experiments with glutaraldehyde-fixed rat thymocytes. Similar results were obtained with fresh cells, and in both cases the dissociation of $^{125}$I-labelled Fab' initially followed the kinetics of a first-order reaction by giving a straight-line plot of log [radioactivity (c.p.m.)] versus time. However, as dissociation progressed the rate became lower. This was not a major effect in the experiments shown at 4°C, but it has been consistently observed and was more prominent at 18°C with rat thymocytes (Fig. 4) and at 4°C with AKR-mouse thymocytes (Fig. 5). The $k_{-1}$ values determined from the dissociation curves in experiments at 4°C are summarized in Table 2, and the values refer to the initial dissociation rates. The conclusions from these data and Figs. 4 and 5 are that $^{125}$I-labelled Fab' dissociates from AKR-mouse thymocytes 10–15 times faster than from rat thymocytes at 4°C ($t_{\frac{1}{2}}$ averages about 120min for rat thymocytes and 8min for AKR-mouse thymocytes) and that by increasing the temperature to 18°C dissociation from rat thymocytes is increased about 10-fold.

The $k_{-1}$ for $^{125}$I-labelled Fab' dissociating from rat thymocytes at 4°C was also measured by observing the kinetics of relaxation when thymocytes incubated with $^{125}$I-labelled Fab' were diluted 10-fold with buffer. The result is plotted in Fig. 6, and gave a $K_1$ value of $3.8 \times 10^9 M^{-1}$ and 89min for the $t_{\frac{1}{2}}$ dissociation time (see the Experimental section for details of calculations). The $t_{\frac{1}{2}}$ is in reasonable agreement with the above values, and the $K_1$ value with the determinations discussed below.

The dissociation of $^{125}$I-labelled F(ab')$_2$ was measured by the method involving preincubation and addition of unlabelled antibody. The results were more complex than for $^{125}$I-labelled Fab', owing to bivalent binding, and the amount of unlabelled antibody added influenced the dissociation rate. This is clearly seen for AKR-mouse thymocytes in Fig. 5, where $t_{\frac{1}{2}}$ was 150min with unlabelled antibody added at 50$\mu$g/ml and 44min with antibody added at 1mg/ml. This presumably is due to the fact that the unlabelled antibody competes with the $^{125}$I-

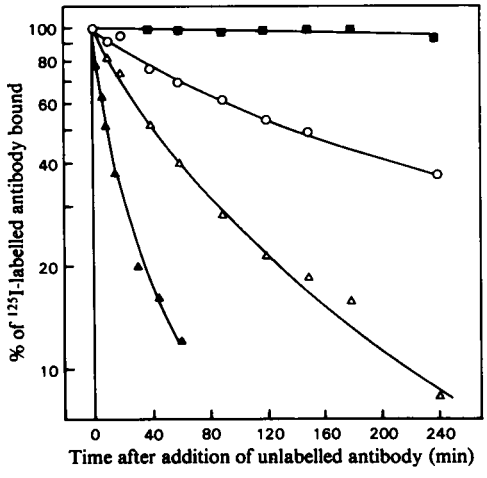


Fig. 5. *Dissociation of monoclonal $^{125}$I-labelled F(ab')$_2$ and $^{125}$I-labelled Fab' anti-Thy-1.1 antibody from AKR-mouse thymocytes*

First $5 \times 10^5$ AKR-mouse thymocytes and $5 \times 10^6$ fixed sheep erythrocytes were pelleted, and then 25$\mu$l of $^{125}$I-labelled F(ab')$_2$ (4$\mu$g/ml) or 10$\mu$l of $^{125}$I-labelled Fab' (8$\mu$g/ml) was added to the pellet. The mixtures were incubated for 60min, and this was followed by addition to $^{125}$I-labelled F(ab')$_2$ assay mixtures of 25$\mu$l of: buffer only (■), anti-Thy-1.1 IgG at 50$\mu$g/ml (○) or anti-Thy-1.1 IgG at 1mg/ml (△). To $^{125}$I-labelled Fab' assay mixtures was added 10$\mu$l of buffer only or anti-Thy-1.1 IgG at 1mg/ml (▲). At various times 1.5ml of buffer was added and the cells were pelleted for determination of radioactivity (c.p.m.) bound. Control incubations were done where $^{125}$I-labelled antibody and unlabelled antibody were pre-mixed and incubated with the cells for 60min before the washing, and the radioactivity (c.p.m.) bound was subtracted from the experimental values, which were then expressed as percentages of the radioactivity (c.p.m.) bound minus controls for assays washed at the end of the preincubation period.

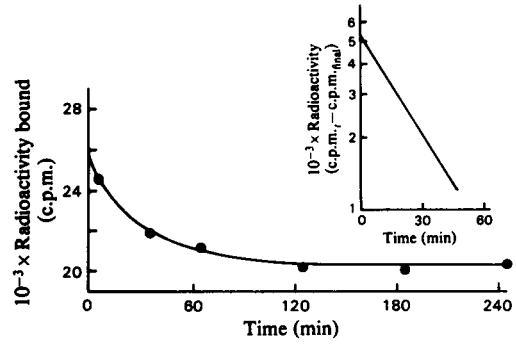


Fig. 6. *Dissociation rate for $^{125}$I-labelled Fab' anti-Thy-1.1 antibody by dilution*

Fresh PVG/c-rat thymocytes, at $0.5 \times 10^6$/ml, were incubated for 2h with $^{125}$I-labelled Fab' anti-Thy-1.1 antibody at 0.125$\mu$g/ml in a total volume of 100$\mu$l in 2.5ml plastic tubes. At various times thereafter 900$\mu$l of buffer was added to each tube, the last addition being made 4h after the first. Immediately after this last addition all cells were pelleted, washed promptly once and assayed for radioactivity (c.p.m.) bound. The times at which the dilutions were made are shown in the Figure. All determinations were in triplicate.

labelled $F(ab')_2$ for re-binding when one binding site dissociates. This means that with $^{125}$I-labelled $F(ab')_2$ the true rate of dissociation was not being measured, and the $t_{\frac{1}{2}}$ would be much greater than 150 min.

With $^{125}$I-labelled $F(ab')_2$ and rat thymocytes the situation is even more complicated, since about 50% of the antibody gives bivalent binding, with the other 50% bound univalently. A biphasic dissociation would have been expected, with the slower phase extrapolating back to 50% antibody bound. Also, the concentration of unlabelled antibody should affect the lower dissociation rate. Both a biphasic dissociation and an effect of concentration of unlabelled antibody were observed (Fig. 4), but the lower rate, regardless of concentration of unlabelled antibody, extrapolated back to 84% antibody bound at zero time. The $t_{\frac{1}{2}}$ for the rapid phase of dissociation was about 100 min, and thus consistent with the dissociation of $^{125}$I-labelled Fab'. When buffer only was added, there was also some decrease in the amount of $^{125}$I-labelled $F(ab')_2$ bound (Fig. 4), but this did not occur in experiments with $^{125}$I-labelled Fab'. The experiments shown in Fig. 4 were done with glutaraldehyde-fixed rat thymocytes, and similar results were obtained when fresh rat thymocytes were used in experiments lasting 6 h.

The data for dissociation of $^{125}$I-labelled $F(ab')_2$ from rat thymocytes are difficult to interpret, and the results possibly hinge on what happens when a binding site dissociates. A free Thy-1 determinant could be competed for by the dissociated binding site, by a free binding site of univalently bound antibody or by unlabelled antibody. The relative rates of these interactions are likely to determine the nature of the dissociation curves, but it is not obvious how these rates can be measured. The possibility that an antibody bound by a univalent interaction may subsequently become bivalent is supported by the fact that some antibody slowly dissociates when buffer only is added to cells pre-incubated with $^{125}$I-labelled $F(ab')_2$. This may partially explain why 84% rather than 50% of the $^{125}$I-labelled $F(ab')_2$ antibody dissociates as if it were bivalently bound.

(4) *Determination of the equilibrium constant for the binding of the $^{125}$I-labelled Fab' anti-Thy*-1.1 *antibody to rat thymocytes*

The equilibrium constant for the binding of

Table 2. *Kinetic parameters for $^{125}$I-labelled $F(ab')_2$ and $^{125}$I-labelled Fab' anti-Thy*-1.1 *antibody binding to thymocytes*
All data are for studies at 0–4°C. $k_{+1}$ values were calculated from initial reaction rates measured as indicated in Fig. 3. The antibody concentrations were 0.16–0.25 nM and the cell numbers were varied to give Thy-1 antigen concentrations from 0.4 to 2.6 nM. Change in antibody or antigen concentration had no significant effect on the $k_{+1}$ values observed. The means ± S.D. were from the following numbers of observations: fresh rat thymocytes, $F(ab')_2$, six values, Fab', 13 values; glutaraldehyde-fixed rat thymocytes, Fab', three values; fresh AKR-mouse thymocytes, $F(ab')_2$, four values. The $k_{-1}$ values were for $^{125}$I-labelled Fab' dissociating from cells, as indicated in Figs. 4 and 5. The numbers of determinations were: fresh rat thymocytes, four values; glutaraldehyde-fixed rat thymocytes, five values; fresh AKR-mouse thymocytes, three values. The calculated $K_1$ values were from $k_{+1}$ and $k_{-1}$ values for $^{125}$I-labelled Fab' with rat thymocytes, whereas for AKR-mouse thymocytes the $k_{+1}$ value for $^{125}$I-labelled $F(ab')_2$ multiplied by 0.7 was used because $k_{+1}$ for $^{125}$I-labelled Fab' could not be easily determined because of the high $k_{-1}$ value. Experimental values for $K_1$ were determined by incubating thymocytes with mixing by rotation in a volume of 1 ml in numbers to give Thy-1 antigen concentrations of 0.2–1.8 nM for rat thymocytes and 1.0–6.0 nM for AKR-mouse thymocytes. $^{125}$I-labelled Fab' concentration was usually 0.25 nM of bindable antibody. Six assays in Beckman Microfuge tubes were set up at any one antigen and antibody concentration, with three being pelleted at 5 h and three at 6 h incubation at 5°C. At these times the radioactivities (c.p.m.) bound were not significantly different, and the radioactivities (c.p.m.) for the six assays were averaged and divided by the total radioactivity (c.p.m.) of bindable antibody to give the proportion bound at equilibrium for calculation of $K_1$. Controls involved incubation of glutaraldehyde-fixed rabbit or C3H-mouse thymocytes. The means shown were from the following numbers of observations: fresh rat thymocytes, ten values; glutaraldehyde-fixed rat thymocytes, three values; fresh AKR-mouse thymocytes, four values.

| | Rat thymocytes | | AKR-mouse thymocytes |
|---|---|---|---|
| Parameter | Fresh | Fixed | Fresh |
| $10^{-5}k_{+1}$ ($M^{-1}\cdot s^{-1}$) | | | |
| $F(ab')_2$ | 4.6 ± 0.3 | | 5.7 ± 0.9 |
| Fab' | 3.7 ± 0.4 | 4.9 ± 0.5 | |
| $10^{3}k_{-1}$ ($s^{-1}$) | | | |
| Fab' | 0.098 ± 0.02 | 0.094 ± 0.02 | 1.5 ± 0.5 |
| $10^{-9}K_1$ ($M^{-1}$) | | | |
| Fab' (calculated) | 3.8 | 5.2 | 0.27 |
| Fab' (experimental) | 2.7 ± 0.4 | 2.8 ± 0.3 | 0.29 ± 0.03 |

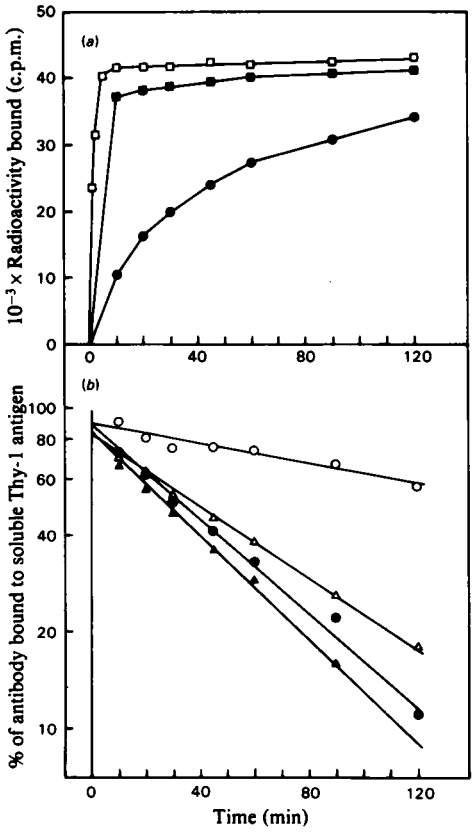


Fig. 7. *Interaction of $^{125}$I-labelled F(ab')$_2$ and $^{125}$I-labelled Fab' anti-Thy-1.1 antibody with soluble Thy-1 antigen*
(*a*) Time course of binding of $^{125}$I-labelled F(ab')$_2$: □, when 25 μl of antibody (12 ng; $5 \times 10^4$ c.p.m.) and 25 μl of 0.5% deoxycholate were incubated with $5 \times 10^6$ fixed rat thymocytes (50 μl in 5% bovine serum albumin); ■, when 25 μl of antibody was incubated with a mixture of 25 μl of Thy-1 antigen (12.5 ng) in 0.5% deoxycholate plus $5 \times 10^6$ rat thymocytes; ●, when 25 μl of antibody was pre-incubated for 60 min with 25 μl of brain Thy-1 antigen (12.5 ng) in 0.5% deoxycholate and then incubated with $5 \times 10^6$ fixed rat thymocytes. At various times 1.5 ml of buffer was added and cells were pelleted for determination of radioactivity (c.p.m.) bound. Control binding involved replacing the rat cells with $5 \times 10^6$ fixed rabbit thymocytes and binding was 2000 c.p.m. (*b*) Percentage of antibody complexed with Thy-1 antigen plotted versus time, determined from data as in (*a*). Control binding was subtracted from all points, and the radioactivity (c.p.m.) of $^{125}$I-labelled antibody bound after pre-incubation with Thy-1 antigen was expressed as a percentage of the average binding of all values where antibody was incubated with pre-mixed Thy-1 antigen and thymocytes. This value was subtracted from 100 to give the percentage still in the complex. ● and ○, Dissociation of $^{125}$I-labelled F(ab')$_2$ from Thy-1 antigen in the presence (●) and absence (○) of deoxycholate; ▲ and △, dissociation of $^{125}$I-labelled Fab' from Thy-1 antigen in the presence (▲) and absence (△) of deoxycholate. In all cases the experiments were set up as shown in (*a*) and each point is from duplicate assays.

$^{125}$I-labelled Fab' anti-Thy-1.1 monoclonal antibody to rat and AKR-mouse thymocytes was measured by incubating known quantities of cells and antibody together and determining the equilibrium composition as described in Table 2 and the Experimental section. The data are summarized in Table 2, and show that the affinity of binding was the same for fresh and fixed rat thymocytes at a value of about $2.7 \times 10^9 M^{-1}$. The $K_1$ for binding to AKR-mouse thymocytes was about 10-fold lower, as would be expected from the $k_{-1}$ measurements. In all cases the experimental $K_1$ values agreed quite well with the values calculated from the association and dissociation rate constants.

### (5) *Dissociation of $^{125}$I-labelled F(ab')$_2$ and $^{125}$I-labelled Fab' anti-Thy-1.1 antibody from soluble complexes with Thy-1 antigen*

Pure Thy-1 antigen exists as a soluble oligomer in the absence of detergent or as a monomer in deoxycholate (Kuchel *et al.*, 1978). Similarly membrane vesicles provide a polymeric form of Thy-1 antigen that is not sedimented at low speeds of centrifugation, and the antigen can again be rendered monomeric by solubilization in deoxycholate.

The interaction of $^{125}$I-labelled F(ab')$_2$ and $^{125}$I-labelled Fab' anti-Thy-1.1 antibody with these forms of Thy-1 antigen was studied by preincubating antibody with excess antigen to form complexes and then adding rat thymocytes to bind any free antibody. As antibody dissociates it is bound mainly by the thymocytes, since these were added in 6–10-fold excess over the soluble or membrane Thy-1 antigen. The experimental system worked because in the conditions used the antibody was bound very rapidly, as shown in Fig. 7(*a*), where binding in the absence of soluble Thy-1 antigen was almost complete in 5 min and was virtually unchanged from 10 to 120 min. When soluble Thy-1 antigen was mixed with the thymocytes, there was a small inhibition of binding in comparison with the considerable inhibition seen when antibody and soluble Thy-1 antigen were preincubated (Fig. 7*a*). The difference in radioactivity (c.p.m.) between these two curves represents antibody still complexed with Thy-1 antigen, and this can be expressed as a percentage by dividing by the radioactivity (c.p.m.) bound when Thy-1 antigen and thymocytes were pre-mixed. The only difficulty in doing this is that this latter binding increases somewhat with time because of re-equilibration of antibody from the small number of soluble complexes to the thymo-

cytes from which the antibody dissociates more slowly. Because of this the binding at any time point is not the perfect high-binding control for the points where antibody was preincubated with Thy-1 antigen. For simplicity an average of the points where Thy-1 antigen and thymocytes were pre-mixed was used in the calculations.

Fig. 7(*b*) shows the data for dissociation of $^{125}$I-labelled F(ab')$_2$ and $^{125}$I-labelled Fab' from complexes with brain Thy-1 antigen. With $^{125}$I-labelled F(ab')$_2$ antibody and no deoxycholate the time for 50% dissociation was about 200 min, compared with 40 min in the presence of deoxycholate. This latter value was very similar to that obtained in the presence or absence of deoxycholate for $^{125}$I-labelled Fab'. This establishes that the effect of deoxycholate on the dissociation of $^{125}$I-labelled F(ab')$_2$ is due to the fact that the antigen has been made univalent.

Table 3 summarizes the results from a variety of experiments of the type shown in Fig. 7. The pattern was similar for all forms of Thy-1 antigen including thymocyte membranes, although the $^{125}$I-labelled F(ab')$_2$ dissociated much more slowly from membrane in the absence of deoxycholate than from the pure oligomeric Thy-1 antigen in the absence of deoxycholate. The average $t_{\frac{1}{2}}$ for the univalent dissociation was about 40 min, and thus the dissociation is about 3 times as fast as that for $^{125}$I-labelled Fab' dissociating from intact thymocytes ($t_{\frac{1}{2}} = 120$ min).

In assays where soluble antigens were determined quantitatively by measuring their ability to inhibit the binding of antibody to glutaraldehyde-fixed target cells, the experimental system was very similar to that used for Fig. 7. With an antibody like the monoclonal anti-Thy-1.1 antibody used in the bivalent form, one would expect the inhibition with antigen to be influenced by the presence or absence of deoxycholate, since this affects the dissociation time. An experiment to illustrate this is shown in Fig. 8, where $^{125}$I-labelled F(ab')$_2$ anti-Thy-1.1 antibody has been incubated with various amounts of Thy-1 antigen in the presence or absence of deoxycholate. Free antibody was measured by incubation with $5 \times 10^5$ or $2 \times 10^6$ thymocytes. With low numbers of thymocytes (10 ng of cellular Thy-1 antigen) the presence of deoxycholate did not greatly affect the amount of antigen needed for 50% inhibition, whereas with the higher number of target thymocytes (40 ng of cellular Thy-1 antigen) twice as much antigen was needed in the presence of deoxycholate for inhibition of binding. The binding step in this assay was 60 min, and in this time in the presence of deoxycholate much of the antibody will have dissociated once from soluble antigen. With low numbers of target cells much of this must rebind to soluble Thy-1 antigen, whereas with more thymocytes added the free antibody is substantially bound by the cells. With this antibody a useful assay would result if numbers of target cells were minimized and if the incubation time were decreased to as short a period as was possible.

### (6) *Dissociation of $^{125}$I-labelled rabbit F(ab')$_2$ anti-Thy-1 antibody from soluble Thy-1 antigen*

In previous studies it was observed that deoxycholate had no effect on the inhibition curve of rabbit anti-Thy-1 antibody with rat Thy-1 antigen (Williams *et al.*, 1976). Thus it would be expected that the $t_{\frac{1}{2}}$ for rabbit antibody would be longer than

Table 3. *Dissociation of $^{125}$I-labelled F(ab')$_2$ or $^{125}$I-labelled Fab' anti-Thy-1.1 antibody from rat Thy-1 antigen in the presence or absence of deoxycholate*

The $t_{\frac{1}{2}}$ values for dissociation of complexes of soluble Thy-1 antigen with $^{125}$I-labelled F(ab')$_2$ or $^{125}$I-labelled Fab' anti-Thy-1.1 antibody were measured as indicated in Fig. 7. In each case the results are from one experiment, except for brain Thy-1 antigen, where mean ± S.D. values for at least three experiments are given.

| | $t_{\frac{1}{2}}$ values (min) for dissociation of: | | | |
|---|---|---|---|---|
| | $^{125}$I-labelled F(ab')$_2$ | | $^{125}$I-labelled Fab' | |
| Deoxycholate ... | Absent | Present | Absent | Present |
| Purified Thy-1 antigen | | | | |
| Brain Thy-1 antigen | 226 ± 31 | 39 ± 5 | 44 ± 7 | 31 ± 6 |
| Thymocyte Thy-1L+ antigen | 200 | 20 | 38 | |
| | | | 48 | 30 |
| Thymocyte Thy-1L− antigen | 280 | 38 | 38 | |
| | | | 49 | 33 |
| Thymocyte membrane* | | | | |
| Thy-1 antigen | 850 | 88 | 55 | 43 |

* Thymocyte membrane was prepared by the Tween 40 method (Letarte-Muirhead *et al.*, 1975), homogenized extensively and centrifuged at 1500 *g* for 30 min to remove any material that would be sedimented in the binding assays. It was then used in the same way as purified Thy-1 antigen.

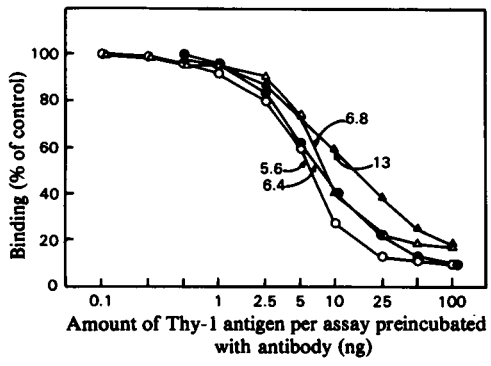


Fig. 8. *Titration curve for inhibition of binding of $^{125}$I-labelled F(ab')$_2$ anti-Thy-1.1 antibody with brain Thy-1 antigen*

$^{125}$I-labelled F(ab')$_2$ anti-Thy-1.1 antibody (25 $\mu$l; 13 ng; $6 \times 10^4$ c.p.m.) was incubated for 60 min with 25 $\mu$l containing the amounts of brain Thy-1 antigen shown, in the presence (● and ▲) or absence (○ and △) of 0.5% deoxycholate. Then 50 $\mu$l containing $5 \times 10^6$ fixed sheep erythrocytes and either $5 \times 10^5$ (● and ○) or $2 \times 10^6$ (▲ and △) fixed rat thymocytes in 5.0% bovine serum albumin was added. After 60 min incubation on ice, 1.5 ml of buffer was added and the cells were pelleted for determination of radioactivity (c.p.m.) bound. The binding was expressed as a percentage of that occurring in the absence of Thy-1 antigen, and this was $4.6 \times 10^4$ c.p.m. and $5.3 \times 10^4$ c.p.m. respectively with $5 \times 10^5$ and $2 \times 10^6$ thymocytes added. The arrowed numbers show ng of Thy-1 antigen needed for 50% inhibition, and this point was taken to be midway between the plateaus of maximal binding (100%) and of maximal inhibition, which was to 10% with $5 \times 10^5$ thymocytes and to 18% with $2 \times 10^6$ cells added.

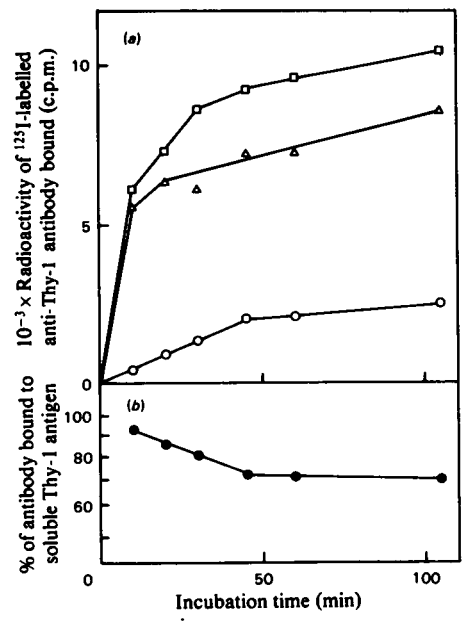


Fig. 9. *Interaction of $^{125}$I-labelled rabbit F(ab')$_2$ anti-Thy-1 antibody with Thy-1 antigen in the presence of deoxycholate*

(*a*) Time course of binding of $^{125}$I-labelled rabbit F(ab')$_2$ (□) when 25 $\mu$l of antibody (18 ng; $1.5 \times 10^5$ c.p.m.) (of which 6.5% was anti-Thy-1 antibody) and 25 $\mu$l of 0.5% deoxycholate were incubated with $3 \times 10^6$ fixed rat thymocytes (30 $\mu$l in 5% bovine serum albumin), or (△) when 25 $\mu$l of antibody was incubated with a mixture of 25 $\mu$l of thymocyte Thy-1L+ antigen (5 ng) in 0.5% deoxycholate plus $3 \times 10^6$ thymocytes, or (○) when the antibody and Thy-1 antigen were preincubated for 60 min before the addition of thymocytes. At various times cells were washed twice with 1.5 ml of buffer and the radioactivity (c.p.m.) bound was measured. Controls involving incubation with fixed rabbit thymocytes gave 1500 c.p.m. bound and have been subtracted. (*b*) Percentage of antibody remaining complexed with soluble Thy-1 antigen; these values were calculated from the formula: $100 - 100 \times$ (c.p.m. bound after preincubation with Thy-1 antigen)/(c.p.m. bound at the same time point when Thy-1 antigen and thymocytes were pre-mixed).

60 min, which was the incubation time used in the assays. This was tested by preincubating $^{125}$I-labelled rabbit F(ab')$_2$ anti-Thy-1 antibody with Thy-1 antigen in deoxycholate and observing dissociation by adding thymocytes and washing cells at increasing time points. The binding of rabbit antibody was less ideal than the monoclonal antibody in that binding without competing Thy-1 antigen increased throughout the assay. However, it is clear from Fig. 9(*a*) that most of the antibody did not dissociate from preformed complexes in 100 min. In Fig. 9(*b*) the data have been converted into percentages of antibody bound to soluble Thy-1 antigen, and this shows that about 30% of the antibody dissociates with a $t_{\frac{1}{2}}$ of about 20 min but that for the rest the $t_{\frac{1}{2}}$ is >100 min. With this antibody, inhibition curves with increasing amounts of Thy-1 antigen were constructed in deoxycholate, and the effect of adding different numbers of thymocytes in the binding step was examined as for the monoclonal antibody in Fig. 8. The amount of Thy-1 antigen needed for 50% inhibition was independent of the number of target cells and of the presence or absence of deoxycholate (results not shown), as would be expected from the data in Fig. 9.

### (7) *Determination of the number of antigenic sites on thymocytes recognized by W3/13 and W3/25 monoclonal antibodies*

These monoclonal antibodies recognize different antigenic determinants on thymocytes and peripheral T-lymphocytes. Their derivation is given in

brief outline in the Experimental section and in more detail in Williams *et al.* (1977).

The number of W3/25 antigenic sites on rat thymocytes and the fraction of $F(ab')_2$ W3/25 antibody that binds bivalently to cells was determined by measuring the binding of $^{125}$I-labelled $F(ab')_2$ and $^{125}$I-labelled Fab' W3/25 antibody under saturating conditions. From the known specific radioactivity of the $^{125}$I-labelled Fab' the number of antigenic sites per thymocyte was calculated to be $1.5 \times 10^4$ (Table 4), in good agreement with the results obtained by Williams *et al.* (1977). The number of molecules of $^{125}$I-labelled Fab' bound per thymocyte was on average 1.29 times that for $^{125}$I-labelled $F(ab')_2$ W3/25 antibody (Table 4), showing that 29% of the $F(ab')_2$ antibody bound to the cell membrane via both binding sites.

In the absence of a suitable W3/13 monoclonal antibody lacking the myeloma $\kappa$-chain, direct quantitative measurement of the density of antigenic sites for W3/13 antibody was not attempted. However, when thymocytes were incubated with saturating concentrations of W3/13 or W3/25 antibody followed by $^{125}$I-labelled rabbit $F(ab')_2$ anti-(mouse Ig) antibody, the radioactivity bound with W3/13 antibody was 2.2 times as great as with W3/25 antibody, again confirming the earlier result (Williams *et al.*, 1977). For the calculation of the kinetic parameters the number of molecules of antigen for W3/13 antibody per thymocyte was taken to be $3.75 \times 10^4$.

### (8) *Association rate constants for W3/13 and W3/25 monoclonal antibodies*

Association rate constants for both antibodies were measured by using unlabelled monoclonal antibody in the first stage of the assay and $^{125}$I-labelled rabbit $F(ab')_2$ anti-(mouse Ig) in the second. The experimental results, shown in Fig. 10, indicate that W3/25 antibody has a $k_{+1}$ of $1.1 \times 10^6 M^{-1} \cdot s^{-1}$, which is 14 times that for W3/13 antibody (Table 4).

### (9) *Dissociation rate constants for W3/13 and W3/25 antibodies*

Dissociation rates were measured by incubating thymocytes with saturating amounts of $^{125}$I-labelled antibodies and then adding a large excess of unlabelled antibody to block the re-binding of dissociated label. For neither antibody was the dissociation rate influenced by the concentration of unlabelled antibody added, even when, in the case of W3/13 antibody, this was increased to 670 $\mu$g/ml. Results are shown in Fig. 12 and summarized in Table 4.

The failure to influence the dissociation rates by increasing the amount of unlabelled antibody added contrasts with the result obtained with the anti-Thy-1.1 monoclonal antibody. It may be that the dissociation observed is that due to antibody bound by only one binding site [for W3/25 antibody about 70% of antibody is so bound (section 7)] and that bivalently bound antibody dissociation is too slow to be observed in the 4h period of the assay. This hypothesis derives support from the observation that in two independent experiments, in which 69% of the $^{125}$I-labelled $F(ab')_2$ reagent bound univalently, the $k_{-1}$ value for $^{125}$I-labelled Fab' W3/25 antibody was 1.42 times that for $^{125}$I-labelled $F(ab')_2$ antibody. This ratio is close to the expected value of 1.45, if it is assumed that the dissociation of bivalently bound antibody is negligible.

### (10) *Binding of W3/13 and W3/25 monoclonal antibodies to thymocytes at different antibody concentrations*

In the earlier study (Williams *et al.*, 1977) the number of antigenic sites per thymocyte recognized by these monoclonal antibodies was determined

Table 4. *Parameters for the binding of W3/13 and W3/25 monoclonal antibodies to rat thymocytes*
All results refer to measurements made between 1.5°C and 6°C unless otherwise stated in the Table. Values for means ± S.D. are based on at least three separate determinations.

| Reagent | $10^{-5}k_{+1}$ ($M^{-1} \cdot s^{-1}$) | $10^5 k_{-1}$ ($s^{-1}$) | $10^{-4}$ × No. of molecules of antibody bound/cell |
|---|---|---|---|
| W3/13 antibody | | | |
| W3/13 IgG | 0.81 ± 0.06 | — | — |
| $^{125}$I-labelled W3/13 IgG | — | 1.5, 2.2 | — |
| $^{125}$I-labelled W3/13 $F(ab')_2$ | 0.78 | 3.3, 3.5 | — |
| W3/25 antibody | | | |
| W3/25 HLK | 10.6, 11.0 | — | — |
| $^{125}$I-labelled W3/25 $F(ab')_2$ | 10.7 ± 1.3 | 1.67 ± 0.2 | 1.17 ± 0.15 |
| $^{125}$I-labelled W3/25 $F(ab')_2$ at 24°C | 20.8, 21.1 | See the text | — |
| $^{125}$I-labelled W3/25 Fab' | — | 2.3, 2.4 | 1.51 ± 0.2 |
| $^{125}$I-labelled W3/25 Fab' at 24°C | — | 59, 61* | — |

* These values are for the initial dissociation shown in Fig. 12.

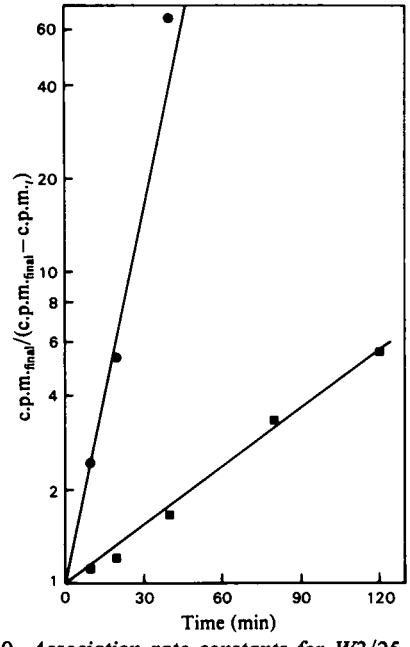


Fig. 10. *Association rate constants for W3/25 (●) and W3/13 (■) monoclonal antibodies binding to thymocytes*

Thymocytes obtained from PVG/c rats were made to $10^8$/ml. A 50$\mu$l portion of cells was incubated on ice with 50$\mu$l of antibody in 2.5 ml tubes for timed intervals between 5 and 120 min. Antibodies used were tissue-culture supernatants diluted to approx. 0.1$\mu$g of antibody/ml. At the end of the incubation period the cells were washed, and a second incubation was carried out with $^{125}$I-labelled rabbit F(ab')$_2$ anti-(mouse Ig) at 20$\mu$g/ml. After 1 h the cells were washed three times and the bound radioactivity was determined. All assays were in triplicate. Controls consisted of using monoclonal anti-HLA (W6/32) antibody, which does not react with rat thymocytes, in the first incubation. Association rate constants were obtained from the relationship:

$$k_{+1}[A_0]t = \ln\left(\frac{\text{c.p.m.}_{\text{final}}}{\text{c.p.m.}_{\text{final}} - \text{c.p.m.}_t}\right)$$

where c.p.m.$_{\text{final}}$ is the radioactivity bound at the plateau of the binding curve, c.p.m.$_t$ is the observed radioactivity bound at time $t$, $t$ is the incubation time (s) and $[A_0]$ is the antigen concentration (M).

by measuring the number of molecules of antibody bound at saturation. The number of thymocytes required to absorb a known quantity of antibody was also assayed. These two techniques produced the paradoxical result that, whereas in binding at saturation there was 2½ times as much W3/13 antibody bound per cell as W3/25 antibody, about 3 times as many thymocytes were required to absorb W3/13 antibody compared with W3/25 antibody. The observation that W3/25 antibody binds much more rapidly to thymocytes than does W3/13 antibody provides a straightforward explanation for this discrepancy, in that in the absorption assays the reaction of W3/13 antibody with thymocytes may not have gone to completion before the supernatant was assayed for residual unbound antibody. In this way the absorption experiments would underestimate the amount of antigen for W3/13 antibody present on thymocytes. To confirm this conclusion the numbers of thymocytes required to absorb 50% of W3/13 and W3/25 antibodies were determined at different antibody concentrations. With higher concentrations of antibodies it was expected that the binding of W3/13 antibody should go more nearly to completion in the fixed incubation period chosen. Fig. 11(*a*) shows the experimental results. For the W3/25 antibody the number of thymocytes required to bind 50% of the antibody was directly proportional to the concentration of antibody used, whereas for W3/13 antibody binding appeared to be more efficient as the concentration of antibody employed in the assay was increased. At the higher antibody concentration the absorbing capacity of thymocytes for W3/13 antibody exceeded that for W3/25 antibody by a 1.6:1 ratio, whereas at the lower concentration the corresponding ratio is only 0.43:1. From a knowledge of the association constants of W3/13 and W3/25 antibodies and the number of antigenic sites per thymocyte (see sections 7 and 8), these absorption findings were compared with theoretical predictions based on the assumption of single-hit kinetics and irreversible binding. Experimental details are given in the legend to Fig. 11(*b*), which compares the observed and calculated absorption values. In deriving the theoretical results account has to be taken of the fact that one molecule of bivalent antibody binds to more than one antigenic site. Thus, if $f$ is the average number of sites covered per antibody molecule bound, it is readily shown that:

$$\text{Fraction of antibody bound} = \frac{e^{-k_{+1}(f[B_0]-[A_0])t} - 1}{e^{-k_{+1}(f[B_0]-[A_0])t} - f\dfrac{[B_0]}{[A_0]}}$$

where $[A_0]$ is the concentration of antigen site (M), $[B_0]$ is the concentration of antibody (M), $t$ is time (s) and $k_{+1}$ is the association constant ($M^{-1}\cdot s^{-1}$).

Values of $f$ for W3/25 antibody were derived from the data of section (7) and the relationship:

$$f = 2 \times \frac{\text{radioactivity (c.p.m.) bound with } ^{125}\text{I-labelled Fab' W3/25}}{\text{radioactivity (c.p.m.) bound with } ^{125}\text{I-labelled F(ab')}_2\text{ W3/25}}$$

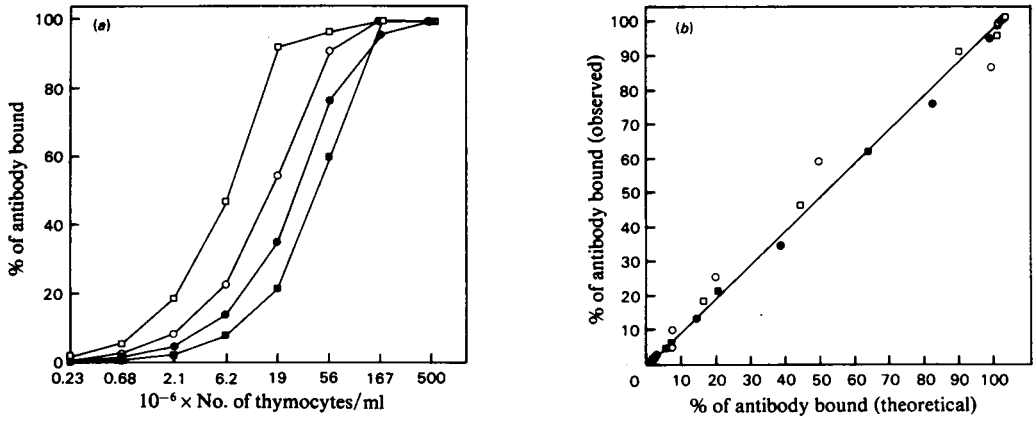


Fig. 11. *(a) Binding of W3/13 and W3/25 monoclonal antibodies to thymocytes at different antibody concentrations, and (b) comparison of observed and calculated absorption data*

In (*a*) PVG/c-rat thymocytes were made to a concentration of $10^9$/ml, and 3-fold serial dilutions were made from them. Monoclonal W3/13 antibody was diluted 1:200 (●) and 1:1640 (○), and W3/25 antibody 1:440 (■) and 1:3600 (□) (these dilutions were chosen on the basis of a preliminary experiment that established that at these dilutions the concentrations of W3/13 antibody were the same as those for W3/25 antibody). For the assay 20 $\mu$l of thymocytes were incubated at 4°C with 20 $\mu$l of antibody for 2 h in micro-titre plates with constant agitation. The cells were then washed once and incubated for 1 h with $^{125}$I-labelled rabbit F(ab')$_2$ anti-(mouse Ig) at 4°C. After three washes the cell-bound radioactivity was determined. Control assays were performed with W6/32 antibody at 1:200 and 1:1640. All determinations were in triplicate. A second experiment gave a similar result. (*b*) The '% of antibody bound (observed)' is that shown in (*a*). The '% of antibody bound (theoretical)' is derived from the equation given in the main text with the following values inserted in the equation:

$k_{+1(W3/25)} = 1.1 \times 10^6 M^{-1} \cdot s^{-1}$ (Table 4)
$k_{+1(W3/13)} = 0.84 \times 10^5 M^{-1} \cdot s^{-1}$ (Table 4)
$[A_0]_{(W3/25)} = 1.25 \times 10^{-8} M$ at $5 \times 10^8$ thymocytes/ml (equivalent to $1.5 \times 10^4$ antigenic sites/cell) (Table 4)
$[A_0]_{(W3/13)} = 3.12 \times 10^{-8} M$ at $5 \times 10^8$ thymocytes/ml (equivalent to $3.75 \times 10^4$ antigenic sites/cell) (Williams *et al.*, (1977)
$f = 1.28$
$t = 7200$ s
$[B_0] = 1.8 \times 10^{-9} M$ at the higher concentration of antibody

For the purposes of the calculation it was assumed that the value of $f$ for W3/13 antibody was the same as that for W3/25 antibody.

It is evident from Fig. 11(*b*) that the theory quite accurately predicts the experimental results. From the $k_{-1}$ values of $1.9 \times 10^{-5} s^{-1}$ for W3/13 antibody and $1.7 \times 10^{-5} s^{-1}$ for W3/25 antibody (Table 4), it was expected that for the 2 h incubation period used in the absorption experiment dissociation could be neglected. This expectation was confirmed by substituting these values of $k_{-1}$ in the expression for a second-order reversible reaction (Day *et al.*, 1963). The calculated values derived from this equation differed by only a few percent from those obtained from the assumption of irreversibility.

(11) *Effect of temperature on reaction kinetics for W3/25 antibody*

The association constant $k_{+1}$ for $^{125}$I-labelled W3/25 antibody was determined at 1.5°C and at room temperature. In two independent experiments the values of $k_{+1}$ at 24°C were 1.97 and 1.94 times those obtained at 1.5°C (Table 4).

In contrast with the moderate effect of temperature on $k_{+1}$, the dissociation of W3/25 antibody from the thymocyte membrane was markedly altered. Details are given in the legend to Fig. 12, which shows the effect on the $k_{-1}$ values for $^{125}$I-labelled W3/25 Fab' and $^{125}$I-labelled F(ab')$_2$ of increasing the reaction temperature from 6°C to 26°C. The results at 6°C are uncomplicated and have been described in section (9). The results obtained at 26°C are not so readily interpreted. In particular, the dissociation of $^{125}$I-labelled W3/25 Fab' does not follow first-order kinetics, as the initial rapid dissociation with a $k_{-1}$ value of $6.0 \times 10^{-4} s^{-1}$ is followed by a slower one with $k_{-1}$ equal to $1.5 \times 10^{-4} s^{-1}$. This effect became even more pronounced when the temperature was raised to 37°C (results not shown), as the initial $k_{-1}$ increased to over $1.4 \times 10^{-3} s^{-1}$ and

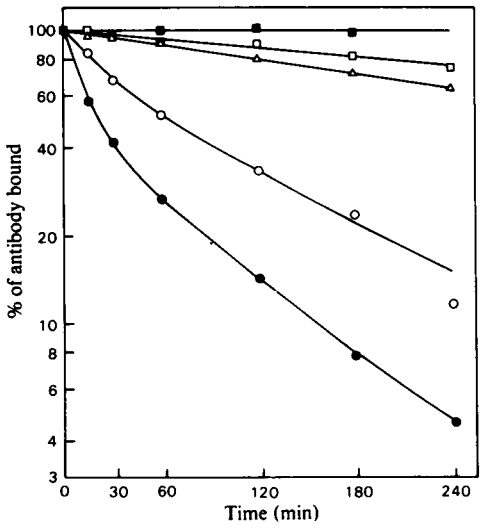


Fig. 12. *Effect of temperature on the dissociation of W3/25 antibody from the thymocyte membrane*
Thymocytes were obtained from PVG/c rats and adjusted to a concentration of $3.5 \times 10^7$ cells/ml. A 50$\mu$l portion of thymocytes was incubated at 6°C (□ and △) or 26°C (○ and ●) with 50$\mu$l of $^{125}$I-labelled F(ab')$_2$ W3/25 antibody (□ and ○) or $^{125}$I-labelled Fab' W3/25 antibody (△ and ●) at 1.4$\mu$g/ml for 1h in micro-titre plates. At various intervals thereafter 20$\mu$l of unlabelled W3/25 antibody at 260$\mu$g/ml was added, and the incubation was continued until a total interval of 5h had elapsed from the start of the experiment. The micro-titre plates were then spun at 4°C for 1½min and the pellets were washed twice. As controls (■) 20$\mu$l of DAB buffer containing 2% foetal calf serum was used in place of the unlabelled antibody in an incubation at 6°C. To determine the maximum degree of inhibition of binding achievable by adding the unlabelled antibody, 20$\mu$l of this reagent was premixed with 50$\mu$l of $^{125}$I-labelled antibody before addition of this mixture to the cells. All assays were in triplicate.

the $k_{-1}$ for the slower component became $1.9 \times 10^{-4}$s$^{-1}$. These results are similar to those obtained with $^{125}$I-labelled Fab' anti-Thy-1.1 (section 3), in that with this reagent also the dissociation from the thymocyte membrane was biphasic at 18°C. The explanation for these observations is not clear.

## Discussion

The association of antibody with cell-membrane antigens followed single-hit kinetics for all the antibodies studied, and in this respect resembles the reaction between antibody and haptenated virus (Gopalakrishnan & Karush, 1974). Association rate constants were in the range $10^5$–$10^6$M$^{-1}$·s$^{-1}$, and thus of similar magnitude to $k_{+1}$ values for antibody–protein antigen interactions (Karush, 1978) or for the interaction immunoglobulin E with its receptor on mast cells (Kulczycki & Metzger, 1974). All these values are about two orders of magnitude smaller than those determined for antibody–hapten reactions (Day *et al.*, 1963; Karush, 1978). Part of this difference may be due to differences in diffusion coefficients, which are approximately proportional to (molecular weight)$^{-1}$. However, it is clear that other factors must also be important in determining association rate constants of antibody–antigen interactions. Even for the same hapten there can be a wide range of $k_{+1}$ values (Karush, 1978), and differences in diffusion rate cannot explain the 14-fold difference between $k_{+1}$ values for W3/13 and W3/25 antibodies interacting with cell surfaces. The practical consequences of low and variable $k_{+1}$ values for the interpretation of absorption analyses are illustrated by the results obtained with W3/13 and W3/25 antibodies (see section 10).

As expected, the dissociation of bivalently bound antibody from the thymocyte membrane was much slower than that for univalently bound antibody, and it may be expected that, given long enough, virtually all antibody would become bivalently bound, i.e. the system would relax to its most stable state. However, because of the low $k_{-1}$ values observed for single-site binding and the predominance of univalent over bivalent binding, the time to reach this final equilibrium was in general much longer than the time required to allow the initial forward reaction to go to completion. When the dissociation of univalently bound antibody was rapid, as with anti-Thy-1.1 monoclonal antibody binding to AKR-mouse thymocytes, all the antibody became bivalently associated with the membrane. It may be concluded, for practical purposes, that theoretical analyses based on the assumption of reaction equilibrium with a predominance of bivalent binding (Reynolds, 1979) are inappropriate in discussing the interaction of antibodies with cell-membrane antigens.

The dissociation of antibody from the cell membrane showed a departure from first-order kinetics, even when the Fab' fragment was used. The reason for this result is not known, but it may reflect heterogeneity of the antigen, either in its mobility in the cell membrane or in spatial relationships with other membrane molecules. Another possibility is heterogeneity in the antibody resulting from differences in the number of tyrosine residues that are iodinated. A related problem, that is also unexplained, was the difference in the dissociation rate constant for binding of $^{125}$I-labelled Fab' anti-Thy-1.1 to antigen in solution ($t_{\frac{1}{2}} = 40$min) com-

pared with that to antigen on intact cells ($t_{\frac{1}{2}}$ = 120 min).

Dissociation rate constants showed a greater degree of variability (range $1.5 \times 10^{-5}$–$1.6 \times 10^{-3}$ $s^{-1}$) than did association rate constants (range $0.8 \times 10^{5}$–$1.1 \times 10^{6}$ $M^{-1} \cdot s^{-1}$), in keeping with the conclusion of Froese (1968) that differences in antibody affinities arise mainly through variations in dissociation rates. This conclusion was also supported by the observation that the differences in the affinity of $^{125}$I-labelled Fab′ anti-Thy-1.1 in its reaction with AKR-mouse and rat thymocytes was entirely a consequence of its more rapid dissociation from the mouse cells.

The studies on the ratio of the number of antibody molecules that bind to the cell membrane via two binding sites rather than one showed that, regardless of the density of antigenic sites on the cell membrane, a high proportion of molecules bind by one site only. This result held over a range of almost two orders of magnitude in site density ($1.5 \times 10^{4}$ to $10^{6}$ determinants/cell) and illustrates that single-site binding is of major significance. An exception to this rule, to which reference has already been made, was the binding of the $F(ab')_2$ fragment of monoclonal anti-Thy-1.1 to AKR-mouse thymocytes. It is evident that it is the dissociation rate of the complex of univalently bound antibody with antigen, rather than the density of antigenic sites on the cell membrane, that determines whether or not only bivalency of binding is obtained.

Experiments in which antigen was used in monomeric or polymeric forms to inhibit the subsequent binding of antibody to target cells illustrated the importance of the dissociation rate constant in such absorption assays. For such assays to be strictly quantitative it is essential that preformed antigen–antibody complexes do not dissociate significantly during the second stage of the assay. When membrane antigen is converted into its monomeric form by solubilization in detergents, this condition implies a low dissociation constant for univalent binding. Whether or not this condition is satisfied may best be established by testing whether solubilization of antigen affects the inhibition curve. Even if this condition were not rigorously fulfilled, assays measuring relative antigenic activities might still be possible if the antigen being assayed were made monomeric in all cases. Thus the relative amount of antigen on different cell types could be compared in the presence of non-ionic detergents and antigenic activity assayed throughout the purification of antigen.

Temperature effects were not extensively studied, but it was shown that, as expected, an increase in temperature had a much greater effect on dissociation rates than on association rates. The association rate constant of $^{125}$I-labelled $F(ab')_2$ W3/25 antibody increased 2-fold on going from 2°C to 22°C. A similar result has been obtained with antibody–hapten interactions (Day *et al.*, 1963). Dissociation rate constants increased by more than 10-fold for W3/25 and anti-Thy-1.1 antibodies with assays at room temperature compared with those on ice. As discussed above, dissociation of univalent binding is undesirable in absorption assays, and it is clear that these should be carried out on ice.

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