chains should be defined as forming a group. Three such groups, to be called the V_κ group, the V_λ group, and the V_H group, have so far been characterized. The variable regions from the V_κ group and the V_λ group appear to be associated exclusively with constant regions from, respectively, κ-type and λ-type light chains. In contrast, the variable regions from the V_H group seem to occur in association with the constant regions from any of the heavy chain classes.

Within a group of variable regions it is possible to distinguish a number of subgroups. It is now clear that the nomenclature earlier proposed for subgroups[4] needs revision. Criteria for the differentiation of subgroups are being developed and will form the basis for future recommendations. Current information can be obtained from Dr. F. Putnam, Chairman of the Subcommittee on Human Immunoglobulins of the International Union of Immunological Societies.

Similarly to the proposal for the terms class and subclass, type and subtype, the terms group and subgroup may also be used to characterize the variable region of the immunoglobulin molecule.

Conformational Change(s) Induced in Sheep Calcium-Dependent Antibody upon Interaction with Homologous Polypeptide Antigen. I. Hydrogen-Exchange Studies of Immunoglobulin G and $(Fab')_2$ Fragment†

Paul A. Liberti,* William A. Stylos, and Paul H. Maurer

ABSTRACT: Hydrogen-exchange studies have been done on highly purified calcium-dependent sheep anti-poly($Glu^{60}Ala^{30}$-Tyr^{10}) and its $(Fab')_2$ fragment accompanying reaction with homologous antigen. These studies were performed on antigen–antibody soluble complexes formed in antigen excess. Levels of antigen varied from 2.4 to 10.5 times that required for maximum precipitation. At antigen levels greater than 3.5, there are 52–54 hydrogens/molecule of both antibody and $(Fab')_2$ fragment which do not exchange when these molecules react. It is believed that these hydrogens are from antibody combining site residues (27/site), and are prevented from exchange by interaction with antigen. This value exhibits some concentration dependence. At lower levels of antigen excess, two changes occur for both molecules which are (1) the number of hydrogens blocked decreases to about 40 hydrogens/molecule and (2) hydrogens which normally exchange very slowly in unreacted antibody experience an accelerated exchange. At the lowest levels of antigen studied, about 27 antibody hydrogens are released. Based on the extent of tritiation of the samples it is likely that a greater number may be involved.

From the absence of the release phenomenon in studies with Fab (Liberti *et al.*, 1972), it is likely that these hydrogens (which exchange slowly in unreacted antibody) are involved in significant structural changes near or at the hinge region of IgG and $(Fab')_2$ fragment. Because the release depends on antigen:antibody ratios it is suggested that conformational changes are induced in both IgG and $(Fab')_2$ by virtue of stresses imposed on them *via* the formation of large complexes. The probable relationship of this phenomenon with the Y-shaped model of antibody and the opening of the combining site–hinge region–combining site angle upon complex formation is discussed.

The biological significance of this phenomenon is unclear at this time since in at least two species (rabbit and guinea pig) considerable complement fixing ability has recently been demonstrated for $(Fab')_2$ fragment compared with intact antibody. The possibility that these changes are related to alterations at the complement binding site is considered.

One of the fundamental unresolved problems of immunochemistry is an understanding of the physical relation between primary union of antibody with antigen and various *in vitro* and *in vivo* sequelae such as complement fixation and passive cutaneous anaphylaxis. Additionally, there is considerable new interest in the existence of such a relationship because of recent findings of "antibody-like" receptors on immunocompetent cells (Mitchison, 1969). Currently, it is thought that antigen binding by cell receptors may result in some physical change(s) in the receptor which then triggers the cell to undergo other activities involved in antibody synthesis (Siskind and Benacerraf, 1969; Ada, 1970).

Based on accumulated data it is generally held that the 7S immunoglobulin molecule is "Y" shaped, the arms and base corresponding to the two Fab and Fc segments, respectively (Noelken *et al.*, 1965; Feinstein and Rowe, 1965; Valantine and Green, 1967; Cathou and O'Konski, 1970). Further, there is evidence from electron microscopy studies (Feinstein and Rowe, 1965) that interaction of antibody with antigen results in an increase in the angle between the arms of the "Y," *i.e.*, combining site–hinge region–combining site angle (cs–hr–cs angle). It has been suggested that this change possibly exposes a critical disulfide bond or the complement binding site (Edelman, 1970). Attempts to demonstrate such changes in gross conformation of the antibody molecule in solution as measured by a variety of techniques have been unsuccessful

† From the Department of Biochemistry, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107. *Received February 22, 1972.* This work was supported by National Institutes of Health Grant T1A1334 and A107825 and also in part by Public Health Service General Research Support Grant RR-5414.

(Cathou *et al.*, 1968; Green, 1969; Ashman *et al.*, 1971; Ashman and Metzger, 1971) or difficult to interpret in terms of current ideas (Warner and Shumaker, 1970). One plausible explanation for this inability to detect changes is that measurements capable of yielding unequivocal results have only been made with hapten–anti-hapten systems, which do not normally result in the pertinent biological effects. The only data with hapten systems which might indicate some conformational changes in antibody are from proteolysis studies of hapten-bound antibodies (Grossberg *et al.*, 1965).

Despite the above, there is still good reason to persist in the search for conformational change in antibody on interaction with antigen. Marrack and Richards (1971) very recently published an elegant study on the kinetics of turbidity and dyssymetry for albumin–anti-albumin interaction. Based on their data they believe that changes in the Fc region do in fact take place when antibody interacts with antigen.

Recently, we described a calcium-dependent antigen–antibody reaction (Liberti *et al.*, 1971; Maurer *et al.*, 1970) involving synthetic random polypeptide antigens of high glutamyl content. In the presence of Ca^{2+} these antigens, *e.g.*, $(Glu,Ala,Tyr)_n$,[1] undergo conformational changes which then create new and highly specific determinants. These calcium-dependent antigenic determinants react specifically with a particular population of anti-$(Glu,Ala,Tyr)_n$. Because of the reversibility of this reaction in the presence of Ca^{2+} complexing agents which affords both excellent purification of antibody and novel dissociation studies, as well as noninteracting controls, we have used the calcium-dependent $(Glu,Ala,Tyr)_n$ anti-$(Glu,Ala,Tyr)_n$ reaction as a model system for studying protein antigen–antibody interactions. The studies reported here are hydrogen exchange-out kinetics of sheep calcium-dependent anti-$(Glu,Ala,Tyr)_n$ and its $(Fab')_2$ fragment in soluble complexes with $(Glu,Ala,Tyr)_n$.

Materials and Methods

The preparation of sheep antisera and its analysis for calcium-dependent antibody have been previously described (Maurer *et al.*, 1970). The preparation and properties of $(Glu,Ala,Tyr)_n$ have been reported (Blout and Idelson, 1956; Katchalski and Sela, 1958) as well as some physical characterization of the sample used in this study (Liberti *et al.*, 1971). Radioactive $(Glu,Ala,Tyr)_n$ was prepared as was $(Glu,Ala,Tyr)_n$ using uniformly labeled γ-benzyl [^{14}C]glutamic acid *N*-carboxyanhydride. Before use, both preparations were exhaustively dialyzed against distilled water at 5°. Radioactive $(Glu,Ala,Tyr)_n$ was further purified of lower molecular weight components by passage thru G-25 Sephadex (Pharmacia); polymer trailing behind the main peak (about 5%) was discarded.

Calcium-Dependent Antibody and Its Fragments. High-avidity antibody (greater than 90% precipitable at 1 mg/ml) was isolated by precipitating 100–200 ml of heat decomplemented sheep anti-$(Glu,Ala,Tyr)_n$ (1.5 mg/ml of antibody of which 90% was calcium dependent) with an amount of $(Glu,Ala,Tyr)_n$ which gives optimal precipitation. After 24- to 48-hr incubation at 5°, the antiserum was centrifuged, the supernatant was discarded, and the precipitate was washed at 5° three times with calcium-buffered saline (0.05 M Tris-HCl (pH 7.8)–0.01 M $CaCl_2$–0.15 M NaCl); each wash volume was equal to 30% of the original serum volume. The precipitate was dissolved in an amount of 0.5 M EDTA (adjusted to pH 8.0) such that addition of Tris-HCl–saline buffer gave a final EDTA concentration of 0.008 M and an antibody concentration of 1 mg/ml. The resultant solution was centrifuged to remove insoluble calcium-independent complexes. Calcium-dependent antibody and $(Glu,Ala,Tyr)_n$ were reprecipitated by making the EDTA antigen–antibody mixture 0.02 M in $CaCl_2$. The above cycle of dissolution and precipitation was repeated (sometimes up to six cycles) until more than 90% of the antibody was precipitated. Antigen was removed using DEAE-Sephadex A-50 (Pharmacia) as described previously (Maurer *et al.*, 1970). The antibody preparation was then dialyzed against buffer (0.05 M cacodylic acid (Matheson, Coleman and Bell, E. Rutherford, N. J.)–0.15 M NaCl) at pH 6.00 ± 0.01. After testing an aliquot of the antibody preparation for calcium-independent antibody, by measuring light scattered at 90° on addition of an equivalent concentration of $(Glu,Ala,Tyr)_n$; and for antigen contamination, by scattered light on addition of calcium (final concentration 0.02 M) to a second aliquot; calcium-dependent antibody was concentrated at room temperature to 10 mg/ml by pervaporation, dialyzed against cacodylate buffer, and kept frozen in aliquot samples.

Calcium-dependent $(Fab')_2$ was prepared by 18-hr pepsin treatment of the purified antibody as described by Nisonoff *et al.* (1960). After inactivation of pepsin with alkali, the hydrolysate was dialyzed overnight at 5° *vs.* Tris-HCl–saline (pH 7.8) buffer (three changes). The protein concentration was adjusted to 1 mg/ml and calcium was added to a final molarity of 0.01 M. Because some loss in precipitating activity of chromatographically isolated $(Fab')_2$ was observed, precipitating $(Fab')_2$ was isolated by addition of the optimal amount of $(Glu,Ala,Tyr)_n$. The cycling process described above was used to obtain $(Fab')_2$ of greater than 90% precipitability. The removal of $(Glu,Ala,Tyr)_n$ and test for same, as well as concentration and dialysis, were done as above.

Physical Characterization. All preparations used were assayed by sedimentation analysis in the usual manner and by polyacrylamide gel electrophoresis (Davis, 1964). Additionally, homogeneity and molecular weight determination were done by disc electrophoresis in the presence of 1% sodium dodecyl sulfate (Weber and Osburn, 1969). Iodoacetamide (0.05 M) was added to the media where indicated, to prevent disulfide interchange, while 2-mercaptoethanol (1%) was used for reduction when desired. The following were used as molecular weight markers: chymotrypsinogen A, ovalbumin, bovine serum albumin (Mann Research Laboratories, New York, N. Y.), bovine hemoglobin, and twice-recrystallized rabbit γ-globulin (Pentex, Kankakee, Ill.).

Extinction coefficients for both preparations were determined from optical density (Hitachi-Perkin Elmer Model 139) and quadruplet microKjeldahl analyses (Markham, 1942). Based on a nitrogen to protein weight conversion factor of 6.25, the $A_{280}^{1\%}$ obtained are: 13.9 for purified calcium-dependent sheep anti-$(Glu,Ala,Tyr)_n$ and 15.8 for its $(Fab')_2$ fragment.

Hydrogen Exchange. Hydrogen exchange-out kinetics were measured by the tritium isotope method of Englander (1968) which utilizes column chromatography followed by rapid dialysis for measuring unexchanged tritium of a protein. Exchanges were done at 6 ± 0.25° in a novel and convenient cooling arrangement constructed as follows: A 6 × 18 in. cylindrical battery jar containing buffer for the exchange was placed inside a similar 8.75 × 18 in. jar into which had first been placed a spacer such that the top of the inner cylinder

[1] Abbreviations used are: $(Glu^{60}Ala^{30}Tyr^{10})_n$, $(Glu,Ala,Tyr)_n$; the angle between the combining site, hinge region, combining site of the IgG molecule is denoted cs–hr–cs angle. The immunoglobulin nomenclature is in accordance with *Bull. WHO 39*, 447 (1964).

stood 3 in. higher than the outer jar. Two such arrangements were placed into an Aminco constant-temperature laboratory bath (Scientific Instruments Co., Silver Spring, Md.); cooling water from the bath's circulating pump (about 40% of the output) was circulated around the annular space created by the two concentric cylinders. Rapid-dialyzing apparati which were modified versions of the Englander–Crowe device (1965) were suspended into the buffer jar (6.5-l. capacity) from stirring motors (110 rpm) mounted on a rack affixed to the water bath. The improved rapid-dialyzing apparati have a greater dialyzing surface to volume ratio because the dialysis tubing can be stretched to a greater degree and afford more rapid and convenient sampling (P. A. Liberti, 1970, unpublished results).

Calcium-dependent antibody, its fragments as well as nonspecific sheep IgG (Pentex, Kankakee, Ill.) were tritiated by adding 5 μl of 1-Ci/g tritiated water (New England Nuclear, Boston, Mass.) to 0.15 ml of protein (10–12 mg/ml) giving a final tritium concentration of 32 mCi/ml. These equilibrium mixtures were incubated for either 19 or 96 hr at 6°. Tritiated protein was separated from the equilibrium mixture on columns of Bio-Gel P-2, 100–200 mesh (Bio-Rad Laboratories, Richmond, Calif.), under pressure (7 lb of nitrogen). Jacketed Fisher-Porter columns (Warminister, Pa.), 1.5 × 15 cm fitted with top and bottom stopcock adaptors, were used; the gel column was 13 cm high before applying pressure. The buffer column from the top of the gel through the stopcock to the buffer reservoir was free of air bubbles. The column jackets were serially connected to the Aminco bath; column effluents were within 1.1° of the temperature of the buffer jar contents. Separations were done as follows. After compressing the gel by running it under pressure, the lower and upper column stopcocks were closed and the upper stopcock adaptor was removed. Sample, into which 30 mg of sucrose had been dissolved, was layered on the surface of the gel, the stopcock was replaced, and reapplication of pressure and elution into a temperature-controlled collecting flask was commenced immediately. The elapsed time between introduction of sample and collection of tritiated protein never exceeded 60 sec. After removal of aliquot samples for ^{14}C-labeled (Glu,Ala,Tyr)$_n$ counting and absorbance measurements, antigen-antibody mixtures were rapidly transferred to the rapid-dialyzing apparati for subsequent sampling.

Radioactive counting was done with a Nuclear-Chicago Mark II liquid scintillation counter. The instrument was set for two-channel, ^{3}H and ^{14}C, dual-label counting. Quenching and spectral curves were obtained by measuring toluene standards (New England Nuclear, Boston, Mass.) either ^{3}H or ^{14}C labeled. Aquasol (10 ml; New England Nuclear, Boston, Mass.) was used as the counting cocktail throughout. All measurements were made in quadruplet aliquots, generally 50 μl of protein solution/vial; in this fashion quench corrections for an entire experiment are usually constant.

For the tritium technique, calculations of unexchanged hydrogens/molecule of protein at any time, t, are made using the equation developed by Englander (1963)

$$\frac{H_t}{\text{molecule}} = \frac{110.8}{1.21}\frac{\epsilon_m}{A_t}\frac{T_t}{T_0} \quad (1)$$

where 110.8 is mg of H/g of H_2O, 1.21 is a constant correcting for a protein's slight preference for ^{3}H over ^{1}H, ϵ_m is the molar extinction coefficient, T_t and A_t are disintegrations per minute of ^{3}H and absorbance of the protein sample, respectively, and T_0 is the disintegrations per minute of ^{3}H in the equilibrium mixture. T_0 and T_t must apply to the same volume of undiluted equilibrating mixture and sample. In these experiments, ^{14}C activity from (nondialyzable) (Glu,Ala,Tyr)$_n$ was used to follow changes in A_t. Since counts per minute of ^{3}H counted in one channel for dual-channel counting contain counts due to ^{14}C, which is counted independently in a second channel, corrections must be made. For two-channel dual-label counting, disintegrations per minute of ^{3}H are obtained from

$$T = x[B - z(C \cdot y)] \quad (2)$$

where C is counts per minute of ^{14}C; y, a quench factor, converting C to disintegrations per minute of ^{14}C; z is a quench-dependent factor giving the fraction of disintegrations per minute of ^{14}C which must be subtracted from B, the total counts per minute in the channel counting both ^{3}H and ^{14}C. The term in brackets (outer) is then counts per minute of ^{3}H, and x is the quench factor which converts counts per minute of ^{3}H to T. Combination and rearrangement of eq 1 and 2 yield

$$\frac{H_t}{\text{molecule}} = K(x_t/y_t)(B_t/C_t) - K(x_t z_t) \quad (3)$$

which gives H/molecule in terms of a dual-channel ratio. This equation is generally applicable to dual-label technique and affords very simple calculation when quench terms (x_t/y_t and $x_t \cdot z_t$) are constant for an experiment. The term K is

$$K = \frac{110.8}{1.21}\frac{\epsilon_m}{T_0}\left(\frac{y_n C_n}{A_n}\right) \quad (4)$$

where the bracket term is the ratio of disintegrations per minute of ^{14}C to absorbance for any aliquot sample (generally the first) which requires the only absorbance measurement. In these experiments absorbance measurements were corrected for scattering where necessary in the usual manner from plots of log A *vs.* log λ.

Results

Polyacrylamide disc gel (7.5%) electrophoresis at pH 8.5, 8 M urea (Davis, 1964) of calcium-dependent anti-(Glu,Ala,Tyr)$_n$ resulted in a single well-defined band indicating little heterogeneity. For the (Fab′)$_2$ fragment similar results were obtained; however, a faster moving band estimated to be less than 2% of the total was evident. This component which may be Fab′ could not be detected by ultracentrifugal analysis.

Molecular weights of the H and L chains, of reduced calcium-dependent anti-(Glu,Ala,Tyr)$_n$, as determined from the linear relation between the logarithms of molecular weights of marker proteins and their respective electrophoretic mobilities in polyacrylamide gels, were found to be 49,500 and 24,500, respectively. These data yield a molecular weight of 148,000 for the intact antibody. Conditions used were 10% acrylamide, 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol, and 0.01 M phosphate buffer (pH 7.0) (Weber and Osburn, 1969). Under these conditions a single band was observed for both the H chain and L chain of calcium-dependent anti-(Glu,Ala,Tyr)$_n$. For 10% gels, the limits of molecular weight reliability are approximately from 20,000 to 100,000.

The molecular weight obtained for the calcium-dependent (Fab′)$_2$ fragment by this method is 92,000 (average of triplicate determination). Here 5% gels, 1% sodium dodecyl sulfate, and 0.05 M iodoacetamide were used. The molecular weight of this fragment was also estimated by a second

TABLE I: Physical Parameter of Calcium-Dependent Sheep IgG and $(Fab')_2$ Fragment.

	IgG	$(Fab')_2$
Mol Wt	148,000[a]	92,000[b]
$s^0_{w,20}$[c]	6.6	5.0
$A^{1\%}_{280nm}$	13.9	15.8

[a] Calculated from disc gel electrophoretic results of H and L chains. [b] From disc gel experiment and precipitin method described in text. [c] Measured in cacodylate buffer at pH 6.0.

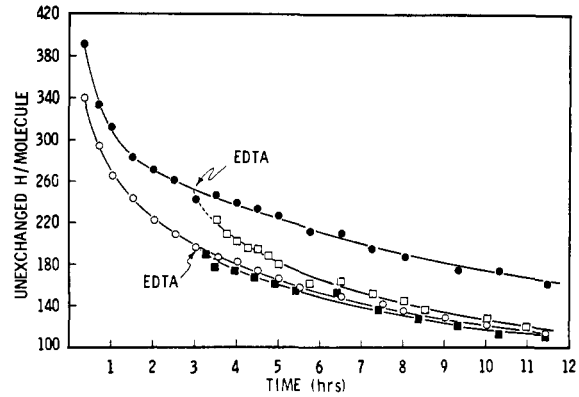

FIGURE 1: Exchange-out kinetics of calcium-dependent anti-$(Glu,Ala,Tyr)_n$ at 3.4-times Ag excess in cacodylate buffer (pH 6.00) at 6°, with 0.01 M Ca^{2+} added (●); with 0.01 M EDTA added (□) and (■); with cacodylate buffer alone (○).

method. This is based on the assumption that high avidity antibody and its $(Fab')_2$ fragment will combine with equal amounts of ^{14}C-labeled $(Glu,Ala,Tyr)_n$ in an equivalent fashion at the region of maximum precipitation which is a narrow zone for this system. Thus, relative masses were determined by precipitin and radioactive analysis. It was found that $(Fab')_2$ has 62% of the mass of intact antibody. This leads to a molecular weight which is in excellent agreement with the 92,000 value given above. The above data, along with sedimentation coefficients and extinction coefficients, are summarized in Table I.

Hydrogen Exchange of Calcium-Dependent Antibody. Control experiments designed to determine if Ca^{2+} or $(Glu,Ala,Tyr)_n$ perturb hydrogen exchange-out kinetics of nonspecific sheep IgG showed these reagents to be without effect except where noted. Similarly, exchange kinetics of calcium-dependent anti-$(Glu,Ala,Tyr)_n$ were not affected by addition of Ca^{2+}.

Because we wish to compare the exchange kinetics of antibody and fragments when perturbed by antigen specifically and nonspecifically under conditions of similar degrees of tritiation, it is desirable to commence interactions in a very reproducible fashion. This could be done conveniently on the Bio-Gel column since both components cochromatograph. To effect this, experiments were done as follows. Immediately before a tritiated antibody–sucrose sample in cacodylate buffer was layered on the Bio-Gel column (equilibrated with cacodylate buffer containing 0.01 M $CaCl_2$), $(Glu,Ala,Tyr)_n$ in the required amount was added and mixed into the sample to which sucrose had been previously added. In this fashion, with the polypeptide antigen present, initial antigen–antibody interaction takes place on the column just as soon as the high sucrose content (20%) is removed and Ca^{2+} interacts with the antigen. Because use of pressurized columns gives excellent separation of tritiated protein from ^3H_2O by virtue of reducing the void volume (Hanson, 1969) fixed volumes of eluate were always collected; hence, predetermined antibody concentrations and antibody:antigen ratios in the exchanging mixtures could easily be achieved. We note by this method that there are apparently some nonspecifically trapped hydrogens in the first few hours of exchange for the interacting systems. This effect is discussed in detail in the companion paper. It does not, however, effect the significant parts of these results.

Figure 1 shows the hydrogen exchange-out curves for calcium-dependent anti-$(Glu,Ala,Tyr)_n$ (0.18 mg/ml) in the presence of 3.4 times the concentration of antigen required for maximum precipitation (3.4-times Ag excess). These experimental curves are highly reproducible and have minimal scatter in the data points. The average deviation found in the quadruplet analysis used to obtain each point was generally less than 3%; this is due largely to the use of ^{14}C antigen as a means for following changes in antibody concentration during the exchange.

The uppermost curve of Figure 1 was obtained in the *presence* of 0.01 M Ca^{2+}, *i.e.*, under reactive conditions. The smooth lower curve obtains for calcium-dependent anti-$(Glu,Ala,Tyr)_n$ and $(Glu,Ala,Tyr)_n$ at 3.4-times Ag excess in the *absence* of Ca^{2+} and will be referred to as a noninteracting control. From these essentially parallel curves it is seen that antigen–antibody reaction prevents or blocks a number of hydrogens from exchanging. The numbers of hydrogens blocked increases slightly with time to 52–54/molecule after 3-hr reaction and remains constant thereafter. We have found that this difference is maintained for as long as 24 hr. For calcium-dependent anti-$(Glu,Ala,Tyr)_n$, the number of hydrogens blocked is somewhat concentration dependent. Thus, if exchange-out at 3.4 times Ag excess is performed at an antibody concentration of 0.09 mg/ml (half the concentration of the experiment depicted in Figure 1), the number of hydrogens blocked drops to 40/molecule.

The effect on the hydrogen-exchange kinetics of reversing the calcium-dependent reaction with EDTA, and the latter's effects on the noninteracting control are depicted by the curves of Figure 1 beginning at 3 hr. These results were obtained by removing aliquots from both the reacting system and noninteracting control and rapidly dialyzing them *vs.* 0.010 M EDTA in cacodylate buffer. Under these conditions, there are no pH changes due to EDTA–Ca^{2+} complex formation. Furthermore, interaction ceases immediately as judged by light-scattering measurements, which showed turbidity decreases 97% by the time a measurement can be made (3 sec) after EDTA addition, and to the turbidity of noninteracting controls within three minutes.

From the figure it can be seen that EDTA has a small effect on the control system, causing a release of three to five hydrogens. This difference obtains to about 15 hr after EDTA treatment and thereafter the two control curves converge (not shown). Nonspecific sheep IgG behaves similarly, suggesting that divalent ions may play a small role in IgG structure (higher concentrations of EDTA have greater effects).

As shown in Figure 1, dissociation of the antigen–antibody complex results in a marked accelerated exchange rate of calcium-dependent anti-$(Glu,Ala,Tyr)_n$ for about the first hour after EDTA, and thereafter at a slower rate. After 11–13 hr (not shown) the number of hydrogens per molecule for this

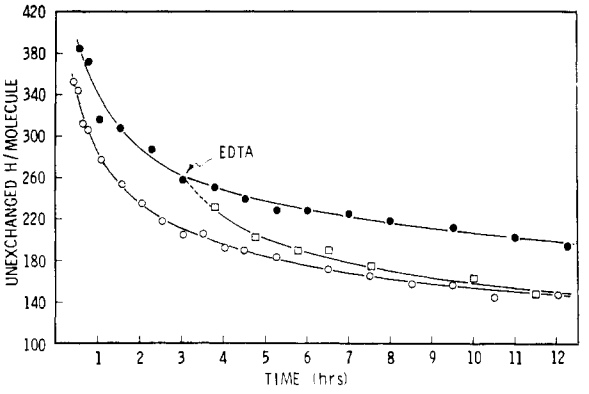

FIGURE 2: Exchange-out kinetics of calcium-dependent anti-(Glu,-Ala,Tyr)$_n$ at 10-times Ag excess, with 0.01 M Ca^{2+} added (●); with 0.01 M EDTA added (□); with cacodylate buffer alone (○).

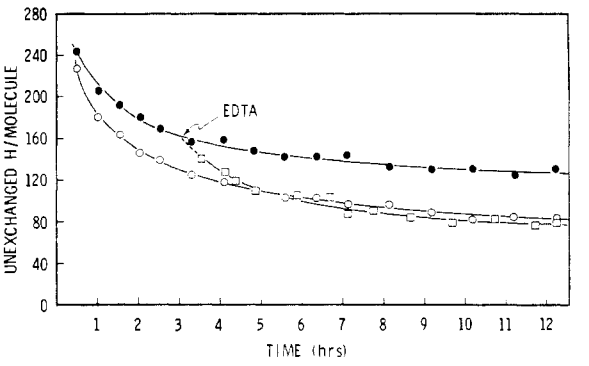

FIGURE 4: Exchange-out kinetics of (Fab')$_2$ fragment of calcium-dependent anti-(Glu, Ala,Tyr)$_n$ at 3.2-times Ag excess, with 0.01 M Ca^{2+} added (●); with 0.01 M EDTA added (□); with cacodylate buffer alone (○).

system is identical with that of the noninteracting control. This is a demonstration of the reversibility of this Ca-dependent reaction, since dissociation results in a complete exchange of the blocked hydrogens. By comparing the value of hydrogens per molecule at 11 hr (8 hr after EDTA) for the Ca^{2+} and EDTA curves, it will be seen that 47 of the total 52–54 blocked hydrogens, or about 92%, have exchanged out by this time.

Data used for the above were obtained for antibody tritiated for 19 hr. Exchange-in incubation periods of up to 96 hr do not affect the essential features of the curves shown here except for higher background levels (indifferent hydrogens), as expected. Hence, all further results given are for 19-hr incubations.

Figure 2 shows a similar experiment for calcium-dependent anti-(Glu,Ala,Tyr)$_n$ at 10-times Ag excess. Here 54 hydrogens/molecule are blocked from exchange upon interaction. The effect of EDTA on the reacting system (similar to that already shown) is again depicted by the curve beginning at 3 hr. The effect of EDTA on the control (not shown) is similar to that of Figure 1. It is worth noting that the noninteracting control curve at this level of antigen is different from that of Figure 1, apparently because the high levels of antigen nonspecifically retard exchange. This indicates the value of the noninteracting control and the unique nature of this system.

Figure 3 shows the exchange-out curves for calcium-dependent anti-(Glu,Ala,Tyr)$_n$ at 2.6 times Ag excess. It can be seen that the curve for the reacting system is initially identical with the noninteracting control and begins to diverge from it after 2–3 hr. This is quite different from the results obtained at the higher antigen excess levels. Dissociation of the reaction with EDTA at 3 hr results in a release of blocked hydrogens similar to the previous results. However, only 39 are released after 8 hr as compared to 47 and 49 by that time for the experiments at higher antigen levels as shown in Figures 1 and 2, respectively. Note also, at 8 hr after dissociation, the curve for the EDTA treated reaction is 20 hydrogens/molecule below the noninteracting control curve.

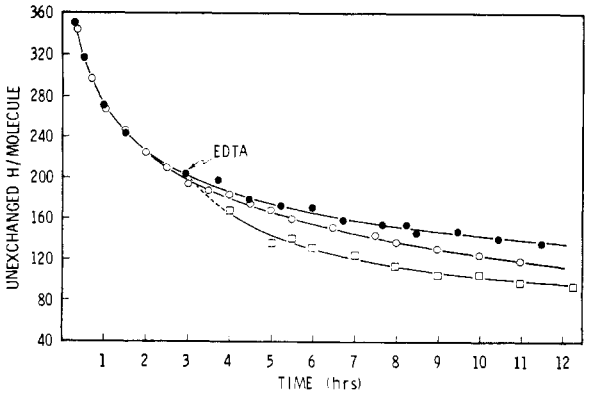

FIGURE 3: Exchange-out kinetics of calcium-dependent anti-(Glu,-Ala,Tyr)$_n$ at 2.6-times Ag excess, with 0.01 M Ca^{2+} added (●); with 0.01 M EDTA added (□); with cacodylate buffer alone (○).

Figure 4 shows the hydrogen-exchange results for the (Fab')$_2$ fragment of calcium-dependent anti-(Glu,Ala,Tyr)$_n$ at 3.2 times Ag excess. These experiments were done at an (Fab')$_2$ concentration of 0.11 mg/ml which is equivalent to the concentration of IgG (0.18 mg/ml) used above. Note from the noninteracting control curve there are about 220 and 80 hydrogens per molecule at 1 and 12 hr, respectively. These values are, as might be expected, very close to two-thirds of those for the similarly treated intact antibody noninteracting control at these respective times. As seen for the reacting and noninteracting control curves after 11-hr exchange, there is a 44-hydrogen/molecule difference. At 8 hr after EDTA dissociation, 49 hydrogens are released and the EDTA-exchange curve goes slightly below that of the noninteracting control. The effect of EDTA (not shown) on the noninteracting control is similar to that of whole antibody.

At higher levels of antigen, (10.5 times Ag excess) the exchange of the (Fab')$_2$ fragment is almost identical with the results obtained for whole antibody at similar conditions and hence is not shown. The dilution effect mentioned above for whole antibody also obtains for (Fab')$_2$. Thus, for example, at 4.2 times Ag excess and an (Fab')$_2$ concentration of 0.06 mg/ml, only 42 hydrogens/molecule are blocked.

The hydrogen-exchange results for the (Fab')$_2$ fragment of calcium-dependent anti-(Glu,Ala,Tyr)$_n$ at 2.4 times Ag excess are shown in Figure 5. At this level of antigen, the curves are almost identical with those of the parent molecule under similar conditions (compare Figures 5 and 3). After 11-hr exchange, the reaction curve is 20 hydrogens/molecule above the noninteracting control. At 8 hr after dissociation, 37 hydrogens/molecule have been released and the curve for the EDTA-dissociated reaction is about 18 hydrogens/molecule below its noninteracting control curve.

Discussion

The calcium-dependent antigen–antibody reaction studied here is unique and has many qualities which make it an ideal

TABLE II: Blocked Hydrogens of Antibody and $(Fab')_2$ under Various Conditions.

System	Antigen Excess Level[a]	Δ_{max}(Hydrogens/Molecule) Ca–EDTA
IgG	2.6	42[c]
IgG	3.4	52–54
IgG	10.0	54
IgG	3.5 dilute[b]	43[c]
$(Fab')_2$	2.4	40
$(Fab')_2$	3.2	52[c]
$(Fab')_2$	4.2	54
$(Fab')_2$	10.5	54[c]
$(Fab')_2$	4.2 dilute[b]	42[c]

[a] Number of times the concentration of $(Glu,Ala,Tyr)_n$ which gives maximum precipitation. [b] At half-concentration, see text. [c] Calculated from differences at 8 hr after EDTA dissociation which are 92% of maximum.

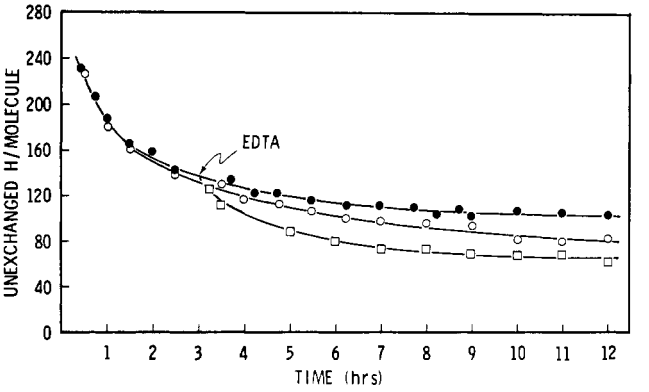

FIGURE 5: Exchange-out kinetics of $(Fab')_2$ fragment of calcium dependent anti-$(Glu,Ala,Tyr)_n$ at 2.4-times Ag excess, with 0.01 M Ca^{2+} added (●); with 0.01 M EDTA added (□); with cacodylate buffer alone (○).

model system for the study of anti protein–antibody interaction. Firstly, antigenic determinants of $(Glu,Ala,Tyr)_n$ should be similar to those of proteins since this antigen is a linear polypeptide having some ordered structure and about 20 antigenic determinants/mol wt 50,000 (Liberti *et al.*, 1971). Secondly, random-sequence polypeptides form soluble antigen–antibody complexes more readily than anti-protein systems (Clark *et al.*, 1968; Maurer *et al.*, 1970), thus interaction not too far from equivalence can be studied in soluble form. This may be particularly true of sheep antibody systems (Zimmering *et al.*, 1965). The most unique aspect of this system is, however, the ability to manipulate interaction *via* Ca^{2+} and EDTA. In addition to reversibility, this allows assessment of "nonspecific" effects of antigen on antibody which, as we have seen, at high antigen levels can be substantial.

Besides the above reasons which make this a convenient model system for study, the results on the apparent lack of obvious heterogeneity, as demonstrated by the disc gel results and preliminary isoelectric focusing studies lend obvious advantages which were not anticipated. Considering "the avidity fractionation" done on these samples *via* the multiple reprecipitation procedure described, some restriction would be expected since these preparations represent only 40–50% of the calcium-dependent antibody initially precipitable. Further, the fact that we are selecting antibody against a calcium-dependent determinant must surely represent a substantial restriction since only about ten such determinants are available per 50,000 molecular weight of $(Glu,Ala,Tyr)_n$ (Liberti *et al.*, 1971). Consequently, in view of the above and the findings of Roholt *et al.* (1970) on the limited heterogeneity of antibody these results are not surprising.

From preliminary hydrogen-exchange studies of calcium-dependent $(Glu,Ala,Tyr)_n$–anti-$(Glu,Ala,Tyr)_n$ interactions (Liberti *et al.*, 1970), we suggested that antibody reaction with macromolecular antigen results in two measurable phenomena which are (1) blocking of hydrogens and (2) a simultaneous release of hydrogens under certain conditions. We felt that blocked hydrogens represented or reflected the numbers of contact residues of the antibody combining site, while released hydrogens were involved in changes at the antibody hinge region since no release was apparent in studies with the Fab fragment of calcium-dependent anti-$(Glu,Ala,Tyr)_n$. To gain further insight into these possibilities, we utilize in this more extensive study the reversibility of this calcium-dependent reaction with EDTA.

In order to fully appreciate the EDTA manipulation, let us briefly consider the following situation. Reacting antibody exchanges in the presence of antigen and Ca^{2+} and is compared to its control exchange done in the absence of Ca^{2+}. After some period of time, it is seen that some hydrogens do not exchange from the reacting calcium-dependent anti-$(Glu,Ala,Tyr)_n$ as determined from the difference between the hydrogen-exchange curves. Before ascribing this difference to trapping or blocking, it must be asked whether the difference obtained is wholly blocking or a combination of blocking plus a release of hydrogens from reacting antibody. A convenient way of determining this is to dissociate the reaction and allow the blocked hydrogens to exchange out. If only blocked hydrogens are involved, the exchange curve for the dissociated reaction will eventually merge with that of the control, *i.e.*, all the blocked hydrogens will have been released. On the other hand, if reaction is accompanied by an accelerated release of a group of hydrogens as well, then the exchange curve for the EDTA-dissociated reaction will eventually fall to the level of the control curve and go below it since reacted antibody will be deficient in those hydrogens released relative to the control.

The maximum values of blocked hydrogens obtained from $\Delta_{max}(hydrogens/molecule)_{Ca-EDTA}$ for the reactions studied are given in the third column of Table II. Several of these values are calculated from Δ values obtained 8 hr after EDTA dissociation based on the fact that 92% of the trapped hydrogens have exchanged out by this time (Liberti *et al.*, 1972). From the table, it may be seen first that the values of hydrogens trapped in antibody and $(Fab')_2$ are very similar where antigen:antibody ratios and/or concentration are the same. Note that these values decrease from values of about 52–54 hydrogens blocked per molecule at antigen excess greater than 3.2 times to 40–42 at levels of about 2.5 times. Dilution of the antigen–antibody reaction has a similar effect.

The value of 52–54 blocked hydrogens obtained for intact antibody and $(Fab')_2$ fragment at the higher levels of antigen excess are twice that obtained for the Fab′ fragment at the same conditions. Further, the number of blocked hydrogens (27/molecule) of calcium-dependent anti-$(Glu,Ala,Tyr)_n$

TABLE III: Release of IgG and $(Fab')_2$ Hydrogens upon Interaction with Antigen at Low Levels of Antigen Excess.

	IgG			$(Fab')_2$		
	Δ(Hydrogens/Molecule) Ca^{2+}–NIC[b]		Hydrogens	Δ(Hydrogens/Molecule) Ca^{2+}–NIC[b]		Hydrogens
Time (hr)	Predicted[a]	Exptl	Released	Predicted[a]	Exptl	Released
3	31	4	27	31	6	25
4	32	6	26	32	8	24
5	34	8	26	34	10	24
6	36	10	26	36	12	24
8	37	14	23	37	16	21
10	38	18	20	38	18	20
12	40	22	18	40	22	18

[a] Predicted differences between interacting and noninteracting antibody for 40 blocked hydrogens in both cases. [b] NIC represents noninteracting control.

Fab', as shown in the accompanying paper (Liberti *et al.*, 1972), is not antigen concentration dependent, or for that matter, concentration (Fab) dependent (within the limits studied in these experiments). From this observation we believe the blocked hydrogens are in the antibody combining site and are trapped from exchange upon antigen–antibody interaction.

Although this contention is explored in much greater detail in the companion paper (Liberti *et al.*, 1972), we note here some features which give support to this interpretation. As shown in Table II, the decrease in blocked hydrogens as antibody–antigen equivalence is approached or the antibody concentration diluted represents a 26% change. Were blocked hydrogens reflecting combining site hydrogens in contact with antigens several consequences might be expected. In the case of dilution, the fact that antibody is bivalent could lead to a decreased blocking since less combining sites might be expected to be filled. But, such an effect should reasonably be absent with monovalent Fab, which is the case. For blocked hydrogens decreasing as antigen:antibody ratios decrease, several interpretations relating them to combining site hydrogens are feasible. Perhaps the most plausible explanation would derive from a theoretical treatment due to Talmage (1959). Using the Goldberg model (1952) for antigen–antibody complex formation, Talmage showed that under the conditions where antibody concentration is low relative to its dissociation constant the percentage of antibody combining sites binding antigen is an increasing function of antigen concentration in the region of moderate antigen excess. This would suggest that the result demonstrated here might be a general one. There is, however, also the possibility that this phenomenon is due to steric restrictions on the antibody molecule within a lattice such that at maximum complex size not all combining sites need be filled. A third possible explanation is that this result is a peculiarity of our system, since we deal with highly fractionated antibody against a random-sequence polypeptide. Hence, it is possible that an equivalent concentration of $(Glu,Ala,Tyr)_n$ may contain determinants which do not interact maximally with the combining sites of the antibody, whereas at higher concentrations of antigen, this condition is achieved.

Regarding the release of hydrogens which is readily apparent in three cases (see Figures 3, 4, and 5), more information about them can be obtained by considering antibody exchange as separable into parts, *viz.*, a group of combining site hydrogens which can be blocked by interaction with antigen, a group of hydrogens which can undergo accelerated exchange upon reaction and a group of presumably indifferent or background hydrogens. In the companion paper we show that those hydrogens which can be blocked by antigen-exchange independently of the remainder of Fab hydrogens. Consequently, we can assume the same condition should apply to the Fab portion of intact antibody. Further, the exchange kinetics of these hydrogens in the unblocked state have been determined (Liberti *et al.*, 1972). Hence, for antibody or $(Fab')_2$ in the case where the number of blocked hydrogens which obtain upon interaction has been determined (using the EDTA reaction), we can reasonably predict how the antibody-interacting and control-exchange curves should diverge (*i.e.*, $\Delta(\text{hydrogen/molecule})_{Ca^{2+}\text{–noninteracting control}}$) as a function of time if only blocking occurs. Note that the curves will diverge in accordance with the unperturbed exchange kinetics of the hydrogens which are blocked by antigen because the only difference between reacting and control antibody is that these hydrogens are permitted to exchange for the latter. Application of the exchange-out kinetics of combining site hydrogens which are given in the accompanying paper (Liberti *et al.*, 1972) to the 40 antigen-blocked hydrogens found for calcium-dependent anti-$(Glu,Ala,Tyr)_n$ at 2.6 times Ag excess (Figure 3) and its $(Fab')_2$ fragment at 2.4 times Ag excess (Figure 5) gives these differences, *i.e.*, $\Delta(\text{hydrogen/molecule})_{Ca^{2+}\text{–noninteracting control}}$, which are listed twice for convenience in the second and fifth columns of Table III. In the third and sixth columns are listed the experimental values of blocked hydrogens which are obtained from the Ca^{2+} and the noninteracting control curves of the respective systems (Figures 3 and 5). The difference between the experimental and predicted values are given in the fourth and seventh columns. These differences arise because interacting antibody and $(Fab')_2$ are deficient in hydrogens by virtue of accelerated exchange of some hydrogens. It can be seen that at least 26–27 antibody and $(Fab')_2$ hydrogens have experienced accelerated exchange upon interacting relative to their controls. Note also that these "releasable" hydrogens exchange from the control since by 12 hr their numbers reduce by about 11. Since only 11 of these 26 released hydrogens are normally exchanged out by 12 hr, it is apparent that they are very slowly exchanging in unreacted antibody and are involved in molecular structure.

Because this release phenomenon is absent in Fab but exists both in antibody and $(Fab')_2$ fragment, it seems plausible to ascribe this change to alterations in Fab which take place at or near the "hinge region." Further, since this change only becomes apparent as lower antigen:antibody ratios are approached, it seems reasonable to conclude that antibody and $(Fab')_2$ fragment undergo a conformational change dependent on antigen:antibody ratios. Recent high-resolution circular dichroism studies (H. J. Callahan, P. A. Liberti, and P. H. Maurer, unpublished results) on a calcium-dependent anti-$(Glu^{60}Ala^{30})_n$ system appear to confirm this ratio-dependent phenomenon.

It appears that this phenomenon which is observed in solution is related to that found in electron microscopy studies of ferritin–anti-ferritin complexes by Feinstein and Rowe

(1965). From their data, they concluded that the cs–hr–cs angle varies with ferritin–antibody ratios, *i.e.*, increases as equivalence is approached. Based on their findings and ours, which indicate that there is an apparent barrier to exchange of the released hydrogens in relatively unstressed antibody (*i.e.*, at high antigen levels or unreacted), it appears that unreacted antibody molecule has a preferred configuration. In view of this, it must be asked how the changes which are observed occur. Our data show no evidence for a "click open" hypothesis, *i.e.*, a filling of the combining site resulting in a spontaneous opening of the cs–hr–cs angle since the release of hydrogens is dependent upon antigen:antibody ratios. Also, some physical changes would have been apparent in solution studies of hapten antibody systems and none have been reported (Warner and Schumaker, 1970; Metzger, 1970). It seems reasonable to suggest that the perturbation we see in the antibody molecule as equivalence is approached is due to torsional forces on the antibody molecule due to intermolecular bridging in complex formation. In this connection, the average sedimentation coefficient of calcium-dependent anti-$(Glu,Ala,Tyr)_n$ interacting with antigen at 10 times antigen excess is about 9 S. Thus, it is probable that antibody and/or $(Fab')_2$ is involved in little or no intermolecular bridging, *i.e.*, IgG–antigen–IgG, under these conditions since such complexes would have an S value of at least 11. This is also reasonable since we have shown that there are about ten calcium-dependent antigenic determinants per molecule for this polypeptide (Liberti *et al.*, 1971). In the case of the antigen levels where the effect obtains, we have found that these mixtures will eventually give some precipitin (about 25%) after 48-hr incubation and have an initial average sedimentation coefficient of about 20 S. Thus, under conditions where hydrogens are released upon interaction, antibody is definitely involved in complex formation. Hence, restriction of rotational and translational motion of macromolecular antigen and antibody would be imposed *via* cross-linking and could conceivably give rise to the torsional forces mentioned above. This would suggest that the energy barrier keeping antibody in a preferred configuration might not be very large.

It is of interest to attempt to relate our observations that hydrogens are released when antibody is interacting in large complexes to some current thoughts on the Y configuration of the antibody molecule. Assuming our findings are a manifestation in solution of the observations of Feinstein and Rowe (1965) by electron microscopy then it is conceivable that the cs–hr–cs angle is normally less than 180° (Cathou and O'Konski, 1970) and possibly opens upon interaction, as has been suggested, given the appropriate conditions. Although our results can give no clue as to which direction the cs–hr–cs angle might change, it is quite possible that the releasable hydrogens could either be "buried" *via* Fab segment contact near the hinge region or involved in some intersegment noncovalent bonding, such that they become accessible to solvent when the cs–hr–cs angle is made to change. In either case these hydrogens appear to be involved with forces which give antibody a preferred configuration in the unreacted state. Although antibody does appear to exhibit segmental flexibility based on recent measurement (Yguerabide *et al.*, 1970), our findings plus the apparent difficulty of locating a loosely structured (low electron density) hinge region from X-ray studies (Sarma *et al.*, 1971) may indicate some restriction of this flexibility. Although we are able to see an involvement of only 26 residues out of a total of 1300 (for intact antibody), which is indeed a small effect, we do not know presently if this number would increase were our preparations fully tritiated. Attempts to perform such studies have not been successful since purified sheep antibodies lose considerable precipitating activity on standing at 5° for more that 5 days. Maximum exchange-in requires from 8 to 10 days.

Regarding the biological significance of released hydrogens, the fact that antibody and $(Fab')_2$ behave similarly would at first seem to exclude any meaningful association of these changes with the biologically active Fc portion of antibody. In fact, it appears from these studies as if Fc enjoys a good deal of independence from the rest of the antibody molecule. Nevertheless, there are many reasons why this effect could be relevant. As mentioned above, we do not know if all the hydrogens which might undergo release are labeled. In this connection, there is the possibility that those which are labeled in antibody and $(Fab')_2$ are not identical. For example, $(Fab')_2$-sensitive hydrogens may be easier to label, *i.e.*, exchange-in with a greater rate constant. Thus, Fc hydrogens might be involved. Another concern is the lack of adequate characterization of sheep antibodies. We have assumed that pepsin digestion of sheep IgG destroys complement fixing ability, yet neither intact sheep antibody nor the $(Fab')_2$ fragment are very efficient in complement-fixing capacity. In this connection, rabbit $(Fab')_2$ fragment is known to fix complement. However, it does not quantitatively do so to the extent of intact antibody and does so through a slightly different mechanism (Reid, 1971). Sanberg and Osler (1971) report similar observations for guinea pig immunoglobulins. If sheep antibodies behave similarly and there is Fc involvement as mentioned above, the released hydrogens might be a demonstration of changes at the complement binding site which Kehoe and Fougereau (1969) showed is a 60-residue fragment in the amino-terminal end of Fc. Hence, an absolute assignment of our observation to biologically relevant or irrelevant function may at this time be premature.

We are currently in the process of studying rabbit calcium-dependent antibody interactions using techniques similar to these. The use of rabbit antibodies should provide a greater manipulative latitude, which as stated was found to be limited for sheep, as well as the advantage of working with a system which is well characterized. In any event, the ready availability of sheep calcium-dependent antibodies have allowed us to begin probing what appear to be some very interesting phenomena.

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