Conformational Change(s) Induced in Sheep Calcium-Dependent Antibody upon Interaction with Homologous Antigen. II. Analysis of Antibody (Fab Fragment) Hydrogens Blocked from Exchange upon Reaction with Homologous Antigen†

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ABSTRACT: The interaction of Fab from calcium-dependent sheep anti-$(Glu^{60}Ala^{30}Tyr^{10})_n$ and the homologous antigen has been studied by the tritium–hydrogen-exchange technique under a variety of experimental conditions. For Fab, which was tritiated from between 19 and 96 hr, interaction with antigen results in a trapping of 23–24 hydrogens/Fab molecule. This value is unaffected by variations in antigen levels and Fab concentrations. Based on data presented, these trapped hydrogens are believed to represent contact residues in the antibody combining site. Using exchange data obtained by EDTA dissociation of this reaction and the assumption that Fab exchange is additive, *i.e.*, consisting of exchange from the combining site independent of and unaffected by the remainder of the molecule, plus exchange of a background of indifferent hydrogens, the "exchange-out" kinetics of the trapped hydrogens are determined. These hydrogens are of two apparent classes. One class of 13 hydrogens has a half-time of exchange of about 1100 times greater than that for a free-surface peptide hydrogen, the other class, comprising 10–11 hydrogens, 130 times greater. From these values it appears that peptide residues of the antibody combining site are involved in strong hydrogen bonds suggesting that the combining site has much structure. The additivity assumption utilized appears to be valid based on the fact that the exchange kinetics of the trapped hydrogens so obtained are independent of the levels of unexchanged indifferent hydrogens of Fab. This is also consistent with the fact that experimental exchange curves can be constructed, if it is assumed that Fab–antigen interaction results only in a blocking phenomenon at the combining site. These data suggest that ligand binding by Fab does not result in conformational changes in Fab away from the combining site.

There is a great deal of interest in the nature of forces involved in the binding of antigen by antibody. Before detailed analysis of such forces can be performed, it would be advantageous to know the number and the nature of the structure of contact amino acids in both the antibody combining site and the antigenic determinant of the antigen. Much is known about the latter by virtue of extensive inhibition studies performed on many different systems (Kabat, 1956; Benjamini *et al.*, 1964, 1965; Schechter and Sela, 1965; Callahan *et al.*, 1971). It is also of importance to determine whether the antibody combining site can be conceived of as a rigid binding surface or as a site which can "close-on" the antigen. Some recent evidence suggests that certain polypeptide antigens may well fit themselves into the antibody combining site (Schechter *et al.*, 1971).

One approach (hapten inhibition) to determining the number of contact residues in the antibody combining site has suggested that 10–20 residues of the anti-dextran molecule would be required to form a binding surface or cavity complimentary in size with the hexaose maximum inhibitor for this system (Karush, 1962; Kabat, 1961). Two recent reports using hydrogen exchange appear to be relevant to this prediction: (1) in a Waldenstrom macroglobulin (γM_{wag}), which binds 4-nitrophenyl-ε-aminocaproic acid, about 17 hydrogens/combining site do not exchange or are blocked in the presence of the hapten (Ashman *et al.*, 1971). (2) With a calcium-dependent sheep anti-polypeptide system (Liberti *et al.*, 1970, 1971a,b; Maurer *et al.*, 1970), about 27 hydrogens/combining site do not exchange during the antibody-antigen reaction.

In this paper, we report the results of a hydrogen-exchange study of calcium-dependent sheep Fab reacting with its homologous antigen $(Glu,Ala,Tyr)_n$.[1] We have utilized the unique reversibility of this particular antigen–antibody reaction by EDTA to examine the nature of the hydrogens which do not exchange when this system reacts in the presence of Ca^{2+}. Further, in an effort to determine whether conformational changes occur in Fab on combination with antigen, we have studied the kinetics of this release of hydrogens at various times during the course of the reaction.

Materials and Methods

A complete description of the isolation of calcium-dependent sheep anti-$(Glu,Ala,Tyr)_n$ and its enzymatic hydrolysis to $(Fab')_2$ has been given in the preceding paper (Liberti *et al.*, 1972). Fab was prepared by very mild reduction and alkylation of $(Fab')_2$ by the method of Nisonoff (1964). After exhaustive dialysis against 0.05 M cacodylic acid (Matheson Coleman & Bell, E. Rutherford, N. J.)–0.15 M NaCl buffer at pH 6.00 ± 0.01, the preparation was concentrated to 10 mg/ml by pervaporation and frozen in aliquot samples for subsequent use. This fragment was characterized by the techniques used for the intact antibody and $(Fab')_2$ (Liberti *et al.*, 1972).

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[1] Abbreviations used are: $(Glu^{60}Ala^{30}Tyr^{10})_n$,$(Glu,Ala,Tyr)_n$; immunoglobulin nomenclature is in accordance with *Bull WHO 39*, 447 (1964).

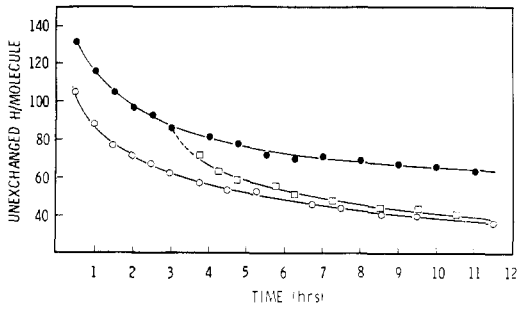

FIGURE 1: Exchange-out kinetics of Ca-dependent Fab in presence of 3.9 times equivalent concentration of $(Glu,Ala,Tyr)_n$ in cacodylate buffer (pH 6.00) at 6° (○), with 0.01 M Ca^{2+} added (●), with 0.01 M EDTA added (□). Reaction started on column (see text).

Hydrogen Exchange. Tritiation of Fab, column chromatography, rapid dialysis, and calculation of the number of hydrogens per molecule unexchanged for Fab were identical to the procedures detailed in the preceding paper (Liberti *et al.*, 1972). Because the essential features of these experiments were unaffected by tritium incubation times greater than 19 hr, the results given here are based on 19-hr incubations.

The system studied here was made to react either by adding $(Glu,Ala,Tyr)_n$ to the equilibrium mixture immediately before running it through the Bio-Gel column (equilibrated with 0.01 M Ca^{2+} in cacodylate–saline buffer) or by layering $(Glu,Ala,Tyr)_n$ into the bottom of the collecting vessel and thus starting the reaction immediately following column chromatography and mixing.

Results

Table I summarizes some of the physical parameters of the sheep calcium-dependent anti-$(Glu,Ala,Tyr)_n$ Fab. The molecular weight of this preparation as obtained graphically from the relationship between logarithm of molecular weight and relative electrophoretic mobility (5% polyacrylamide gel, 1% sodium dodecyl sulfate, and 0.05 M iodoacetamide) (Weber and Osborn, 1969) is 46,000. This value is, as expected, half that reported for the $(Fab')_2$ fragment.

Polyacrylamide electrophoresis (7.5% gel, 8 M urea) (Davis, 1964) of Fab yielded a single well-defined band. This is consistent with the apparent lack of heterogeneity observed for the parent macromolecules as shown by Liberti *et al.* (1972).

Figure 1 shows the hydrogen exchange-out kinetic curves of Fab reacting in 0.01 M Ca^{2+} with $(Glu,Ala,Tyr)_n$ at 3.9 times the equivalent concentration of $(Glu,Ala,Tyr)_n$[2] (upper curve), and the control curve performed in the absence of Ca^{2+} (lower curve). The results depicted were obtained by starting the interaction on a Ca^{2+} equilibrated Bio-Gel column by the procedures detailed in the accompanying paper (Liberti *et al.*, 1972). The concentration of Fab (0.11 mg/ml) is equimolar with respect to combining sites to that used for whole antibody and $(Fab')_2$ in the preceding paper (Liberti *et al.*, 1972). It is seen that these curves are essentially parallel and that $(Glu,Ala,Tyr)_n$ prevents about 27 hydrogens/molecule of Fab from exchanging; both of these results are very reproducible using the method described. The value of 27 trapped hydrogens is half that obtained previously with whole antibody and $(Fab')_2$ under similar conditions (Liberti *et al.*, 1972). Tritium exchange-in incubation times of up to 96 hr does not change the number of hydrogens which do not exchange. This indicates that all the hydrogens which can be blocked from exchange upon interaction are labeled in a 19-hr incubation. In addition, varying antigen concentrations from 1.5 to 5 times equivalent concentration of $(Glu,Ala,Tyr)_n$ have no significant effects. Another finding unique to Fab is that whereas with the parent immunoglobulin the number of hydrogens trapped is somewhat concentration dependent, here the number of unexchanging hydrogens is not affected by concentration. Thus, the results of Figure 1 obtain if the system is initially diluted to half-concentration.

TABLE I: Physical Parameters of Ca-Dependent Fab Fragment.

Molecular weight	46,000
$s_{20,w}$	3.5[a]
$A_{280nm}^{1\%}$	15.80

[a] Obtained at 4 mg/ml in cacodylate buffer.

The effect of stopping the reaction with EDTA after 3 hr is depicted by the middle curve in Figure 1. This curve falls to the level of the control curve 11–14 hr after EDTA additions (not shown). Effects of EDTA addition on the control system were negligible, *i.e.*, there was an accelerated release of one to two hydrogens from nonreacting Fab.

Figure 2 shows the results of an experiment similar to the one depicted in Figure 1 except that reaction was not started on the column. Instead, the antigen $(Glu,Ala,Tyr)_n$ was kept in the collecting vessel and Fab in Ca^{2+} was added to it and mixed after its passage through the Bio-Gel column. Relative to the experiment of Figure 1, the reaction here begins 60–70 sec after t_0, the time when exchange-out of Fab begins. The data of Figure 2 show that under these conditions the reacting and control curves diverge until a maximum difference of 20 hydrogens/molecule obtains at about 6–8 hr. Further, the difference is maintained for at least 30-hr reaction.

Figure 3 depicts the effects of EDTA on a Fab system identical to that of Figure 2. This reacting system was dissociated with EDTA at 3, 7, and 27 (see insert) hr. It is seen that these curves diverge from the reaction curve similarly. Further, it can be seen that qualitatively the exchange-out behavior with EDTA is essentially as for the system depicted in Figure 2 where 27 hydrogens/molecule do not exchange on reaction.

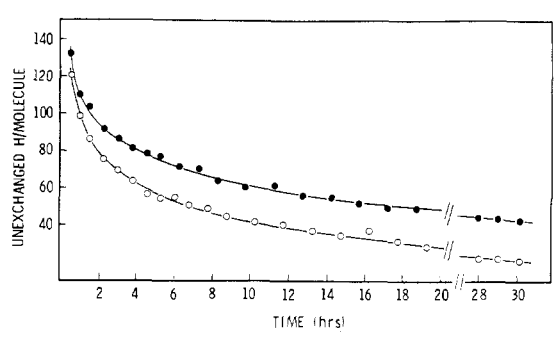

FIGURE 2: Exchange-out kinetics of Ca-dependent Fab in presence of 4.0 times equivalent concentration of $(Glu,Ala,Tyr)_n$. Reaction (upper curve) started after initial column chromatography (see text).

[2] An equivalent concentration of $(Glu,Ala,Tyr)_n$ is based on determinations with whole antibody and is the amount required for maximum precipitation.

Discussion

There are many complex factors which affect the kinetic exchange rate of a given protein hydrogen. To a good approximation, these factors fall into two broad categories, *viz.*, (a) those which are due to local environmental factors such as solvent effects, nearest-neighbor interactions, shielding, etc. (Berger *et al.*, 1959; Klotz and Frank, 1965; Scarpa *et al.*, 1967), and (b) those due to noncooperative segmental motion or "breathing" of the polypeptide chain (Rosenberg and Chakravarti, 1968; Rosenberg and Enberg, 1969; Englander and Mauel, 1972). When a macromolecule binds a specific ligand and the exchange rate of some particular class or classes of hydrogens is altered, this effect could be due to one of the above factors or combinations of both of the above. In the study by Ashman *et al.* (1971) where it was found that binding of 4-nitrophenyl-ε-aminocaproic acid by γM_{wag} results in a trapping of 16.6 ± 4 hydrogens/combining site, it was interpreted that this "kinetic alteration" was due largely to small configurational changes induced in γM_{wag} by hapten which result in changes or a partial freezing of the breathing of the macromolecule. Based on an estimate of the exchange kinetics of these blocked hydrogens, it was suggested that they are probably not associated with the combining site. Although this interpretation is feasible, we believe there is more suggestive evidence for an alternative explanation.

In the first place, 4-nitrophenyl-ε-aminocaproic acid blocks less hydrogens than the 20–27 for our system. This is what one might expect if these values reflect the number of contact residues of the respective antibodies since $(Glu,Ala,Tyr)_n$ as a macromolecular antigen would presumably occupy a larger volume of a combining site. Secondly, the values obtained for both systems are remarkably close to Karush's (1962) prediction for the number of contact residues in the antibody combining site. His values are based solely on considerations of a complimentary fit to a well-defined maximal inhibitor. Lastly, there is no convincing reported evidence of even small configurational changes in Fab following binding of ligand. With a system similar to that reported here (calcium-dependent sheep anti-$Glu^{60}Ala^{40})_n$, H. J. Callahan, P. A. Liberti, and P. H. Maurer (1972, unpublished results) were unable to show changes in antibody circular dichroism (250–310 nm) at large antigen excesses.

That these blocked hydrogens reflect contact residues in the combining site of intact antibody and $(Fab')_2$ has been presented in Liberti *et al.* (1972) where we showed that the number of blocked hydrogens varies in accordance with what might be expected under a variety of experimental conditions for a bivalent molecule. In this report, we show that the number of hydrogens blocked for reacting Fab, is essentially constant. For a univalent molecule this result is not surprising. More significant is the fact that the number of blocked hydrogens for Fab does not change employing preparations labeled by exchange-in incubations for from 19 to 96 hr. This shows that the blocked hydrogens are of a very restricted kind or that we are dealing with a very specific type of segmental freezing, suggesting involvement of only the antibody combining site. Based on the energy considerations of antibody–antigen combination ($\Delta G°$, −5 to −10 kcal $mole^{-1}$), it is conceivable that this kind of specific local segmental freezing could take place.[3]

[3] The molar free energy of denaturation for myoglobin is 16 kcal (Hermans and Acampora, 1967; see also Tanford, 1970). A reasonable maximal estimate for thermal segmental energy would be at most 10% of this value or 1.6 kcal $mole^{-1}$.

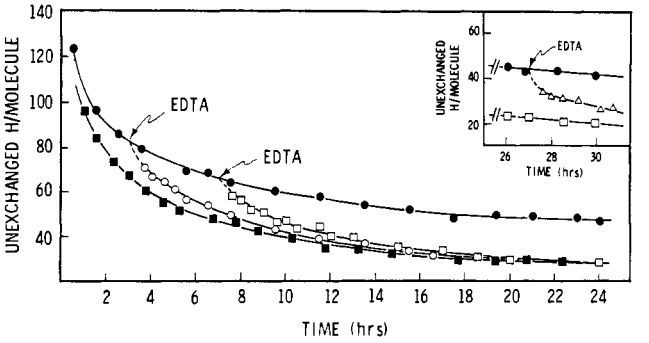

FIGURE 3: Exchange-out kinetics of Ca-dependent Fab in presence of 3.0 times equivalent concentration of $(Glu,Ala,Tyr)_n$. Reaction stopped with EDTA at 3 hr (○), 7 hr (□), and 27 hr (△) (see insert). Upper most curve with 0.01 M Ca^{2+} added, lower most, control.

We can give further suggestive evidence for this hypothesis by examining the differences observed between the system made to react at t_0 on the column in the presence of $(Glu,Ala,Tyr)_n$ (Figure 1), and the system where reaction begins 60–70 sec after t_0 (Figures 2 and 3). From the EDTA-dissociated reactions in both cases, it is apparent that 12–14 hr are required for the blocked hydrogens to exchange out. Further, because the blocked hydrogens remain trapped for as long as 30 hr we can reasonably assume they exchange with essentially zero rate while in the complexed state. That this is also reasonable is based on the fact that rate constants for antigen–antibody combination of 10^7 l. $mole^{-1}$ sec^{-1} (Hornick and Karush, 1969) have been measured as compared to 0.1 sec^{-1} for the exchange rate constant of surface peptide hydrogens (Hvidt and Nielson, 1966; Englander and Poulsen, 1969). Therefore, from a dynamic point of view antigen–antibody combination can be considered to occur with a frequency of such magnitude at the concentration of antibody used here that hydrogens of the combining site (which exchange with frequencies orders of magnitude lower) could escape only at very low and perhaps negligible rates. It is worth mentioning that the escape rate of these trapped hydrogens (when antigen and antibody are interacting) could provide a means of determining rate constants for antigen–antibody combination.

We shall thus consider the exchange of Fab as follows: indifferent hydrogens which are associated with all parts of the molecule except the combining site, and a group of 20–27 hydrogens which exchange or can be blocked independently of the rest of Fab. Based on this assumed model, subtraction of hydrogens per molecule of the EDTA dissociated reactions from their reacting counterparts yields the number of hydrogens per molecule which have exchanged from the combining site. When these values are subtracted from the total number of blocked hydrogens (*i.e.*, 20 or 27), and expressed as a function of time, the kinetics of the trapped hydrogens are obtained in a convenient form. These data are summarized in Table II.

Figure 4 shows a semilogarithmic plot of the data obtained for the 3-hr dissociation experiment of Figure 1, where 27 hydrogens were trapped and the data from Figure 3 where 20 hydrogens were trapped. From Figure 4 and Table II it is seen that the difference of 7 hydrogens obtained in the two procedures involves fast-exchanging hydrogens. Note also from Table II that the kinetics of the four dissociation experiments presented are the same and yet the number of indifferent hydrogens at the start of each dissociation are not. This finding is important to the question of whether ligand binding induces conformational change(s) in Fab (away from the combining

TABLE II: Time Dependence of Exchange of Antigen-Blocked Fab Hydrogens.

	Reaction after Column Chromatography[a]			Reaction on Column[a]
Period (hr) of *Rx* before start of dissociation:	3	7	27	3
Hydrogens/molecule[b]:	82	66	44	87
Time	Blocked hydrogens/molecule unexchanged[a]			
0	20	20	20	27
0.5	13.5	15	14	16.5
1.0	11	12	10	10.5
1.5	9.8	10	10	9
2.0	8.5	9	9	8
2.5	7.3	7.5	7.5	7
3.0	6.8	7	7	6
3.5	6	6	6	5.5
4.0	5	5	5.5	5
5.0	4.5	4		4
6.0	3.8	3		3
8.0	2	2		2
10.0	1.5	2		1.5
12.0	0.5	1		1
13.0	0	0		0

[a] See text for explanation. [b] Numbers of indifferent plus blocked hydrogens at start of dissociation.

site). Were such changes taking place, some fraction of the total indifferent hydrogens would be expected to have an altered exchange rate constant. The data of Table II do not appear to support this possibility, since, were the exchange reactions not separable into two parts or were a secondary phenomenon such as configurational changes occurring elsewhere in the molecule, the exchange-out kinetics of these hydrogens (obtained by a subtractive method) would contain a component due to the changes in the rest of the molecule. Because the number or level of indifferent hydrogens depends on when dissociation begins, they would contribute a time-dependent component to the data of Table II. This is apparently not the case.

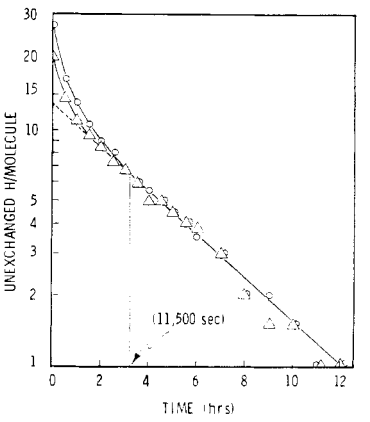

FIGURE 4: Exchange-out kinetics of antigen blocked Fab hydrogens, 27 hydrogens/molecule blocked (○), 20 hydrogens/molecule blocked (△).

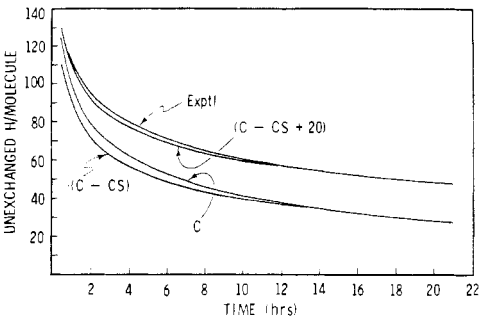

FIGURE 5: Theoretical exchange-out kinetics of Ca-dependent Fab curve [(c − cs + 20)] compared to experimental curve (exptl). See text for assumptions and method.

To test whether the independence between combining site hydrogens and indifferent hydrogens found later in the course of reaction (Table II) obtains at the onset of interaction, we can attempt to construct an experimental reacting curve based on additivity. This is shown in Figure 5. The smooth curve (c) of Figure 5 is obtained directly from Figure 2 and is the exchange-out curve of noninteracting Fab. The curve which is initially below it (c − cs) is the curve for the Fab indifferent hydrogens which is obtained by subtracting data taken from the 20 blocked hydrogens curve of Figure 4 from this control curve. If 20 H/molecule (blocked) are added to the indifferent curve, the curve (c − cs + 20) is obtained which should be the reaction curve if additivity and independence obtained at the onset of interaction. It is seen that the reaction curve (Exptl) from Figure 2 which is reproduced here is within experimental error of the constructed curve.

Based on the above, it appears that parallel curves obtained for reactions started on the column (Figure 1) contain an artifact in the early part of the reaction. Although it is reasonable to expect more trapping by starting the reaction before any hydrogens have exchanged out of Fab, nevertheless, there is every reason to believe that the curves should diverge as occurs when the reactions are started after column chromatography.

To understand first the difference between 20 and 27 hydrogens per molecule trapped under the two experimental conditions, we performed an experiment designed to determine if $(Glu,Ala,Tyr)_n$ during its brief exposure to the equilibrating mixture before column chromatography had exchanged in any ^3H which might subsequently be trapped by antigen–antibody reaction. It was found that when $(Glu,Ala,Tyr)_n$ is added to an equilibrating mixture (^3H_2O, sucrose, and buffer) and chromatographed in a manner analogous to experimental conditions, it is eluted from the Bio-Gel free of tritium. This is so despite the fact that for reactions started in this manner $(Glu,Ala,Tyr)_n$ is in contact with the equilibrium mixture for about 40 sec and column elution times were all close to 60 sec. It is possible, however, that $(Glu,Ala,Tyr)_n$ could exchange in some hydrogens on contact with the equilibrium mixture which antigen–antibody reaction on the column traps before they could have exchanged out. A more likely explanation is that the 7 hydrogens in question are from both components of the reaction, *i.e.*, some hydrogens which exchanged into $(Glu,Ala,Tyr)_n$ and are trapped by reaction yielding the 27 value, and some which exchanged-out of antibody before reaction starts lowering the number to 20 trapped. Thus, an upper limit of 23–24

trapped antibody hydrogens appears maximal for this system.

The discrepancy between the initial shape of the interacting curve for the reaction started on the column (Figure 2) and that obtained when interaction takes place after chromatography appears to be due to an initial trapping of a group of very fast hydrogens (about 17) which are eventually exchanged out after 3–6 hr (compare Figures 1 and 2). This can be explained by several possibilities which are: (1) Some tritiated water could have been physically trapped between the surfaces created by antigen and antibody near the combining site, with segmental motion of both antibody and antigen outside the combining site this tritiated water could be freed. (2) $(Glu,Ala,Tyr)_n$ could have exchanged in some tritium on exchangeable sites which weakly interact with antibody or sterically are retarded from exchange but not blocked. (3) There is an initial retardation of some very fast hydrogens which are involved in a conformational change in Fab upon interaction. The last possibility seems most unlikely as it is difficult to conceive of a significant conformational change either in the combining site or away from it which would be solely reflected in very fast hydrogens. If one of the first two possibilities are correct, then the number of trapped hydrogens (23–24) which remain blocked over long periods of time are very meaningful. In other words, there is an implication that segmental motion around both the antibody combining site and the antigenic determinant is sufficient to free those exchangeable hydrogens which are nonspecifically trapped in early part of the exchange. From this we feel that the 23–24 trapped hydrogens represent exchangeable contact hydrogens which for this system is probably their upper limit.

At the pH used in these experiments, side-chain hydrogens exchange out with a half-time of about 0.1 sec (see, *e.g.*, Englander and Mauel, 1972). Because about five half-times are required for a class to completely exchange out, it is doubtful that reaction started either during or after column chromatography could have trapped any side-chain hydrogens Thus, the 23–24 hydrogens which are trapped here most likely represent peptide hydrogens, or as we have interpreted them, contact residues.

From the curves of Figure 4, it is seen that the trapped hydrogens can be considered to belong to two classes: one class of about 13 (note the extrapolation of the linear position of the curves of Figure 4) which exchange with a half-time of 11,500 sec, and a faster class of 10 or 11 having a half-time of exchange of about 1300 sec. Based on the difference in trapped hydrogens by the two procedures as discussed above, this faster group probably contains 3 or 4 hydrogens which exchange with a half-time of about 10–15 sec, indicating that they are free peptide hydrogens. The two classes of hydrogens have half-times which are respectively about 1100 and 130 times greater than one would expect for surface peptide hydrogens under these conditions (Englander and Poulsen, 1969).

It is of interest to compare these results to those obtained by Ashman *et al.* (1971). They find for the 17 blocked hydrogens an approximate rate constant of 6×10^{-2} min^{-1} or a half-time of exchange of 600 sec. At our pH and temperature this latter value is equivalent to about 40,000 sec which indicates that in both cases the same kinds of hydrogens are being affected.

The magnitude of these half-time values are somewhat puzzling for surface residues, as we have interpreted them. Nonetheless, we are dealing with a very specific surface in the case of the antibody combining site. There is good reason to believe that the remarkable antibody specificity must be due not only to the presence of certain functional groups, but also critical and perhaps rigidly fixed spatial arrangement of these groups. Considering the fact that the antibody combining site is on a protein surface, the values we obtain for half-times for these residues would indicate a highly hydrogen bonded and structured combining site. If this is so one could also envision the trapped hydrogens as being unexchangeable largely on a steric or shielded basis with a lesser freezing contribution due to reaction with antigen as was found in hydrogen-exchange studies of ligand-bound staphylococcal nuclease (Schechter *et al.*, 1969).

As for the question of whether conformational changes are induced in Fab away from the combining site upon interaction with antigen, and the more specific question concerning the possibility of the combining site closing on the antigen, our data at this point show no evidence for either possibility. For a combining site as structured as our half-times of exchange indicate, it is somewhat difficult to conceive of its being able to close on the antigen. From the fact that we are able to construct the reaction curve (Figure 5) on the assumption that the only phenomenon taking place involves blocking at the combining site, it would appear that no conformational changes occur in Fab away from the combining site on combination with antigen. Further, the fact that the exchange-out kinetics of the combining site hydrogens are independent of the number of unexchanged indifferent hydrogens as demonstrated in Table II lend weight to the contention that ligand binding does not effect other parts of the Fab molecule. These conclusions are, of course, subject to the limitation that only about 25% of the hydrogens outside of the combining site have been measured in this study.

Despite our belief that we have interpreted these experiments correctly a certain degree of caution must be exercised since these results were obtained by a single technique, *viz.*, tritium–hydrogen exchange. Although this method has a very broad spectrum of sensitivity, we are currently attempting to confirm these results through spectral and calorimetric methods which can be used to probe in a more specific fashion. Nonetheless, some speculation on the interpretation of these results is in order. First, the apparent independence of the combining site from the remainder of the molecule could well explain the lack of successes obtained in earlier studies where attempts were made to find conformational changes in antibody or Fab on ligand binding. The only significant study reported concerns the effect of hapten binding on the enzymatic hydrolysis of homologous antibody (Grossberg *et al.*, 1965). Here it would appear that their results are probably due to ligand-shielded susceptible bonds as has been suggested by Metzger (1970). Lastly, we feel at this time that if any changes are to be detected in other parts of the antibody molecule such as the hinge region and Fc′ fragment they will be due perhaps to tortional forces arising from interaction with macromolecular antigen. We have shown in the accompanying paper (Liberti *et al.*, 1972) that under certain conditions specific changes take place in the antibody molecule upon interaction. Unfortunately they can not as yet be unequivocally related to biologically relevant phenomena. That demonstration, however, might prove to be the first step in this direction.

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References

Ashman, R. F., and Kaplan, A. P., and Metzger, H. (1971), *Immunochemistry 8*, 627.
Benjamini, E., Young, J. D., Peterson, W. J., Leung, C. Y., and Shimizu, M. (1965), *Biochemistry 4*, 2081.
Benjamini, E., Young, J. D., Shimizu, M., and Leung, C. (1964), *Biochemistry 3*, 1145.
Berger, A., Lowenstein, A., and Meiboon, S. (1959), *J. Amer. Chem. Soc. 81*, 62.
Callahan, H. J., Maurer, P. H., and Liberti, P. A. (1971), *Biochemistry 10*, 3467.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Englander, S. W., and Mauel, C. (1972), *J. Biol. Chem. 247* (in press).
Englander, S. W., and Poulsen, A. (1969), *Biopolymers 7*, 379.
Grossberg, A. L., Markus, G., and Pressman, D. (1965), *Proc. Nat. Acad. Sci. U. S. 54*, 942.
Hermans, J., and Acampora, G. (1967), *J. Amer. Chem. Soc. 89*, 1547.
Hornick, C. L., and Karush, F. (1969), *Israel J. Med. Sci. 5*, 163.
Hvidt, A., and Nielson, S. (1966), *Advan. Protein Chem. 21*, 286.
Kabat, E. A. (1956), *J. Immunol. 77*, 377.
Kabat, E. A. (1961), Experimental Immunochemistry, Springfield, Ill., C. C Thomas, p 243.
Karush, F. (1962), *Advan. Immunol. 2*, 1.
Klotz, I. M., and Frank, B. H. (1965), *J. Amer. Chem. Soc. 87*, 2721.
Liberti, P. A., Maurer, P. H., and Clark, H. G. (1971b), *Biochemistry 10*, 1632.
Liberti, P. A., Stylos, W. A., and Maurer, P. H. (1970), 160th National Meeting of the American Chemical Society, Chicago, Ill., Abstract BIOL42.
Liberti, P. A., Stylos, W. A., and Maurer, P. H. (1971a), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, Abs. No. 878.
Liberti, P. A., Stylos, W. A., and Maurer, P. H. (1972), *Biochemistry 11*, 3312.
Maurer, P. H., Clark, L. G., and Liberti, P. A. (1970), *J. Immunol. 105*, 567.
Metzger, H. (1970), *Annu. Rev. Biochem. 39*, 889.
Nisonoff, A. (1964), Methods in Medical Research, Chicago, Ill., Yearbook Medical Publishers, Inc., p 139.
Rosenberg, A., and Chakravarti, K. (1968), *J. Biol. Chem. 243*, 5193.
Rosenberg, A., and Engberg, J. (1969), *J. Biol. Chem. 244*, 6153.
Scarpa, J. S., Mueller, D. D., and Klotz, I. M. (1967), *J. Amer. Chem. Soc. 89*, 6024.
Schechter, A. N., Maravek, L., and Anfinsen, C. B. (1969), *J. Biol. Chem. 244*, 4981.
Schechter, B., Conway-Jacobs, A., and Sela, M. (1971), *Eur. J. Biochem. 20*, 321.
Schechter, I., and Sela, M. (1965), *Biochim. Biophys. Acta 104*, 298.
Tanford, C. (1970), *Advan. Protein Chem. 24*, 1.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Studies on Antibodies Directed toward Single Antigenic Sites on Globular Proteins†

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ABSTRACT: We have isolated an antibody population which reacts with hemoglobin S but not with hemoglobin A. These proteins differ by a single amino acid substitution. We have isolated an antibody population which reacts with human cytochrome *c* but not with monkey cytochrome *c*. These proteins also differ by a single amino acid residue. These antibody populations react with their specific antigens with a stoichiometry of one antibody combining site per asymetrical antigenic unit. A second, different antibody population which also reacts with human cytochrome *c* with a stoichiometry of unity has been isolated by virtue of its excess production. The kinetics of reaction of these site specific antibody populations indicates considerable homogeneity. This study represents the first isolation of antibodies specific to single chemical modifications of globular proteins and the first demonstration of a one to one binding stoichiometry between such an antigen and an antibody population.

From classical studies in immunology we know that antisera to proteins show great specificity. Interspecies immunochemical differences between homologous proteins are the rule; and genetically controlled intraspecies differences are frequently detectable (Margoliash *et al.* (1970) and Sarich and Wilson (1967). The immunochemical differences are related to differences in the amino acid sequences of the homologous proteins and can be viewed as modifications of the haptenic

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