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# THE ELECTRIC CHARGES OF ANTIBODIES DIRECTED TO UNIQUE REGIONS OF HEN EGG-WHITE LYSOZYME[1]

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SUMMARY Two region specific antibody fractions were prepared using peptide-8 (sequence 57–107) and peptide-17 (sequences 1–27 and 122–129) of hen egg-white lysozyme (HL). The antibodies were obtained from three sheep immunized with HL. The association constants, $K_A$, of the bindings of anti-8 antibodies from two sheep with $^{125}$I-HL were $1.6\times10^5$ ($M^{-1}$) and $1.2\times10^5$ ($M^{-1}$), respectively. The $K_A$ of the bindings of anti-17 antibodies from the same two sheep with $^{125}$I-HL were $8.0\times10^5$ ($M^{-1}$) and $6.4\times10^5$ ($M^{-1}$), respectively.

Scatchard plots of the bindings of all four antibody preparations with $^{125}$I-HL gave straight lines. The results indicate that the range of $K_A$ values for the bindings of the region specific antibodies are restricted.

The isoelectrofocusing pattern of each region specific antibody preparation was also restricted. The numbers of discrete bands obtained on electrofocusing of each region specific antibody were always fewer than those of the mixture. There was also some selection of light chains depending on the kinds of determinant. Individual sheep had a minimum of three light chains of anti-8 antibodies.

## INTRODUCTION

There is great heterogeneity in the function (Karush, 1962) and structure (Lennox and Cohn, 1967) of antibodies. The association constant of anti-hapten antibody can vary 10,000 fold (Eisen and Siskind, 1964). But recently, some native antigens have been reported to give antibody populations with relatively restricted ranges of $K_A$ values (Haber et al., 1967; Fujio et al., 1968, 1969 and 1970; Pappenheimer et al., 1968; Isagholian and Brown, 1970).

When rabbit antibodies directed to HL are fractionated using two active peptides, peptide-8 (sequence 57–107) and peptide-17 (sequences 1–27 and 122–129), the fractions of antibodies obtained also have relatively restricted ranges of $K_A$ values for the binding with each peptide (Fujio et al., 1968) and with HL (Fujio et al., 1969 and 1970). The antibodies directed to each of the determinant groups with different specificities in a molecule

1 Parts of the work were reported at a Symposium on Immunochemistry in Japan (Fujio et al., 1969 and 1970) and at the 8th International Congress of Biochemistry (Amano et al., 1970).

seem to be produced in separate cells (Benjamin and Weigle, 1970). It is possible that the difference in the properties of each region specific antibody may become apparent if the latters are each produced in a completely different cell line. Therefore, it seems interesting to study the structural features of antibodies with specificities directed to different regions of a single molecule. Accordingly, two region specific antibody fractions were prepared from a single blood specimen of a sheep immunized with HL.

The electrofocusing patterns of the two region specific antibodies and of their light chains appeared different. This paper reports the charge differences in these antibodies and the characteristics of the bindings of the two antibody fractions.

## MATERIALS AND METHODS

### 1. *Immunization of sheep*

Three sheep (#10, #20 and #60) were immunized with highly purified HL as described previously (Fujio et al., 1971). Ten mg of HL were injected with complete Freund's adjuvant and 10 mg booster doses were given once every five weeks. Two hundred ml of blood were taken 7 days after each booster injection. Each blood sample was used separately.

The blood used in this series of experiments was taken from sheep #10 and #20 approximately 3 years after the first injection and from sheep #60 six month after the first injection. The antibody contents of the three sera were 4 to 6 mg per ml as estimated by the quantitative precipitin reaction (Fujio et al., 1971).

### 2. *Preparation of region specific antibody fractions from individual sheep*

Two peptides, peptide-8 (sequence 57–107) and peptide-17 (sequence 1–27 and 122–129), were coupled to Sepharose 4B separately by the method of Omenn, Ontjes and Anfinsen (1970). Details of method were given in our previous report (Fujio et al., 1971). Six tenths to 0.9 mg of anti-8 antibody were eluted per ml of anti-HL antiserum from the peptide-8-Sepharose column with 0.1 N acetic acid and 1.0 to 1.5 mg of anti-17 antibody were eluted per ml of anti-HL antiserum by the same procedures. The eluted antibody fractions were dialyzed extensively against 0.01 M acetate, pH 5.5, at 4 C for 3 days and then against 0.02 M sodium phosphate, 0.15 M NaCl, pH 6.0 (PBS, pH 6.0) (Björk and Tanford., 1971).

### 3. *Equilibrium dialysis*

A micro-dialysis cell (50 $\mu$l capacity) like that described by Eisen (1971) was used. Samples of 50 $\mu$l of various concentrations of $^{125}$I-HL were dialyzed against an equal volume of antibody solution (3–5 mg per ml) at 10 C for 24 hours. Triplicate samples were prepared for each concentration of $^{125}$I-HL. The solvent was PBS, pH 6.0. Details of the method were described previously (Fujio et al., 1971). HL was labelled with $^{125}$I by the method of McFarlane (1958). Radioactivity was counted in a Packard Auto-Gamma Spectrometer, Model 3002. Approximately one iodine atom per mole of HL was introduced.

As a control, normal sheep globulin (NSG) was prepared by ammonium sulfate precipitation (half saturation at 0 C) and then separation of the 7S fraction on a Sephadex G-150 column. The $^{125}$I-HL was dialyzed against NSG in PBS, pH 6.0 at 10 C. No appreciable binding of $^{125}$I-HL with NSG was observed.

### 4. *Isoelectrofocusing in polyacrylamide gel*

The method of Wrigely was followed (1968).

First, the photopolymerization and chemical polymerization method were compared, and the patterns were found to be essentially the same. The stability of the gels in chemical polymerization were better than in photopolymerization. Therefore chemical polymerization was used in the following experiments. The ampholytes, pH 3–10 and pH 5–8, were purchased from LKB (Sweden).

Samples of 200 $\mu$g of antibody protein or light chain were applied on the top of a column (gel layer: $0.52 \times 6.5$ cm) in 10% sucrose. Before application of the sample, ampholytes in 5% sucrose were introduced to the top of the column and 200 v were applied between the two electrodes for 1 hr. After applying the sample, the voltage was kept at 200 v for 2 hr. Then the voltage was raised to 300 v and maintained for another 2 hr. All runs were made at 4 C. The locations of protein bands were visualized by staining with a solution of 0.2% bromophenol blue in ethanol: water: acetic acid (50: 45: 5, v/v/v). The gel was destained with a solution of

ethanol: water: acetic acid (30: 65: 5, v/v/v) by the method of Awdeh (1969). The optical densities of protein bands were traced using a Gilford Spectrophotometer with a linear transport (type 2410) at 600 mμ.

5. *Miscellaneous methods*

The concentration of each protein was determined by the biuret reaction (Kabat and Mayer 1961) on a micro-scale (200 μl sample). The biuret reaction of each protein was standardized with a solution of each protein in which the nitrogen had been determined by the Kjeldahl-Nessler method (Yokoi and Akashi, 1955).

The sedimentation velocity of the antibody preparation was examined with a Hitachi analytical ultracentrifuge at 47,000 rev/min and at 20 C. The solvent was PBS, pH 6.0.

Immunoelectrophoresis was also used routinely following the method of Scheidegger (1955). One per cent agarose in diethylbarbiturate buffer ($\mu$= 0.025), pH 8.6 was used. Anti-sheep whole serum antisera were prepared in rabbits.

## RESULTS

1. *Bindings of $^{125}$I-HL by the region specific antibody fractions*

The two region specific antibody fractions from two sheep were tested by analytical centrifugation and also by immunoelectrophoresis.

These analyses suggested that all the region specific antibody fractions belonged to the $\gamma$ G-class.

These antibodies did not give any measurable precipitate when mixed with HL.

Samples of 50 μl of anti-8 or anti-17 antibody (3–5 mg per ml in each case) from individual sheep were put into one compartment of dialysis cells and an equal volume of various concentrations ($1\times10^{-5}$ M to $4.8\times10^{-4}$ M) of $^{125}$I-HL were put into the other compartment. The solvent was PBS, pH 6.0. The cells were rotated at 10 C for 24 hours. The specific activity of the $^{125}$I-HL used in this experiment was approximately $10^4$ count/min per $10^{-9}$ mole of HL. Twenty five μl aliquots were taken from each compartment and radioactivity was counted in a Packard Auto-Gamma counter. At least 1,000 counts were recorded on each sample. The amounts of bound antigen were calculated by substracting the counts in the cell compartment containing free antigen from that containing antibody.

The results of the binding experiment, showed that sheep anti-8 antibody is easily purified by one step adsorption and elution of the antibody using a peptide-8-Sepharose column. However, the purity of some anti-17 antibody preparations decreased 25% as estimated by the "r" value in the binding experiment. In such cases, the adsorption and elution of antibody using the peptide-17-Sepharose column were repeated. The difference in $K_A$ values of the anti-17 antibody from the first and second columns were within the limits of experimental error of the method used.

The bindings of four preparations of anti-8 and anti-17 antibodies, from sheep #10 and #60 were examined and results are shown in Fig. 1 and 2. The association constants of the anti-8 and anti-17 antibodies of sheep #10 with $^{125}$I-HL were $1.6\times10^5$ ($M^{-1}$) and

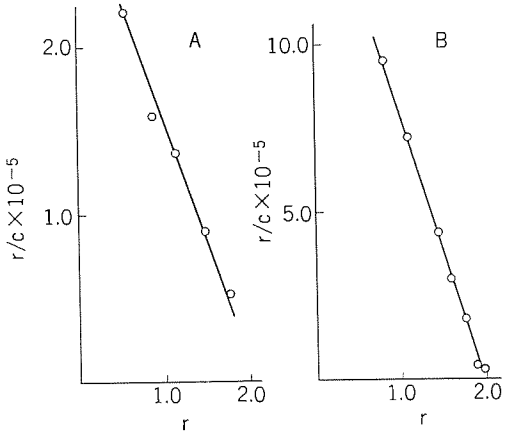


FIGURE 1. *Bindings of region specific antibodies (Sheep #10) with $^{125}$I-HL at 10* C.
*A: Anti-8 antibody. B: Anti-17 antibody. r: Moles of bound $^{125}$I-HL per mole of antibody (M.W. of antibody taken as 150,000). c: Concentration of free $^{125}$I-HL (M).*

$8.0\times10^5$ ($M^{-1}$), respectively. Those of the anti-8 and anti-17 antibodies of sheep #60 with $^{125}$I-HL were $1.2\times10^5$ and $6.4\times10^5$ ($M^{-1}$), respectively. Scatchard plots of the bindings of these four preparations of antibodies with $^{125}$I-HL gave straight lines. These facts indicate that the range of $K_A$ values of the region specific antibodies of sheep are also restricted as found with rabbit antibodies (Fujio et al., 1971).

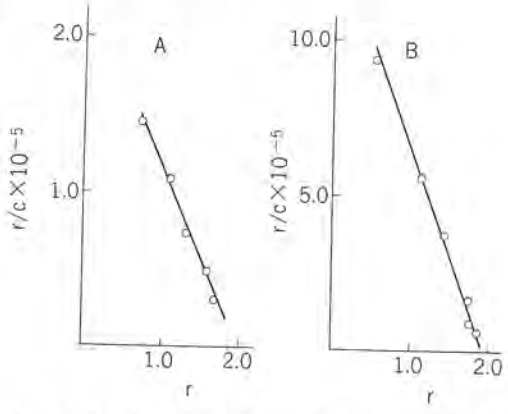


FIGURE 2. *Bindings of region specific antibodies (sheep #60) with $^{125}$I-HL at 10 C.*

*A: Anti-8 antibody. B: Anti-17 antibody. r: Moles of bound $^{125}$I-HL per mole of antibody (M.W. of antibody is taken as 150,000). c: Concentration of free $^{125}$I-HL (M).*

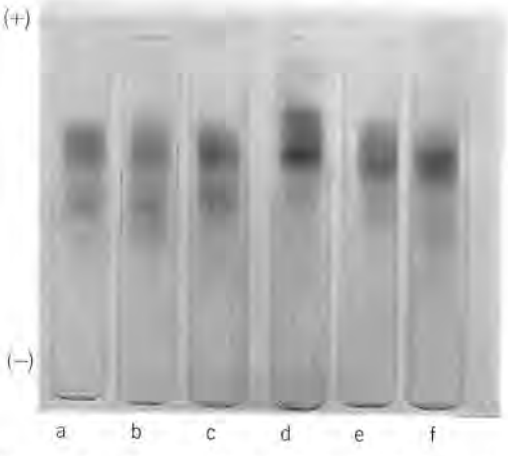


FIGURE 3. *Isoelectrofocusing patterns of the region specific antibody fractions from three sheep.*

*a: Anti-8 antibody of sheep #10. b: Anti-8 antibody of sheep #20. c: Anti-8 antibody of sheep #60. d: Anti-17 antibody of sheep #10. e: Anti-17 antibody of sheep #20. f: Anti-17 antibody of sheep #60. 200 μg of each sample were applied. Ampholyte pH 5–8 was used. The runs were made in 4% acrylamide in 6 M deionized urea.*

## 2. *Isoelectrofocusing of the region specific antibodies and of their light chain preparations*

Since the range of $K_A$ values of the region specific antibodies was shown to be relatively restricted, the electric charges of these antibodies were tested by isoelectrofocusing.

Fig. 3. shows the electrofocusing patterns of the three antibody preparations of anti-8 and anti-17 antibodies. An example of the densitometric scanning pattern of a set of antibody fractions is shown in Fig. 4.

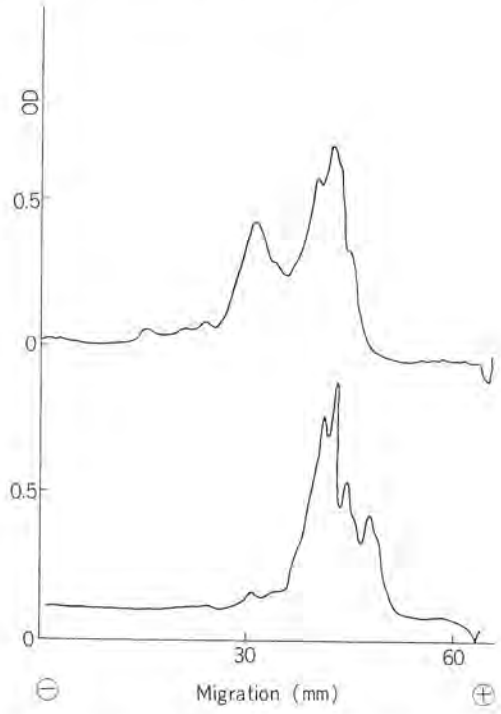


FIGURE 4. *Isoelectrofocusing patterns of two region specific antibody fractions from sheep #60.*

*Upper: Anti-8 antibody. Lower: Anti-17 antibody. The experimental conditions were as for Fig. 3 Optical density were recorded at 600 mμ.*

The electric charges of both kinds of antibody fractions were heterogeneous. Nevertheless, the overall band distribution of the anti-8 antibodies are quite similar. Some similarity among anti-17 antibodies can also be noticed. For comparison, both antibody fractions of sheep #20 and a mixture of equal amounts of each antibody are compared (Fig. 5). Anti-8, anti-17 and the mixture contained 10, 15 and 24 discrete bands, respectively. Therefore it is quite possible that there is a certain degree of selection of antibody populations depending on the determinant groups within a molecule.

Next, we studied the selection in light chains of region specific antibodies. The light chains of both antibody fractions were prepared essentially by the method of Fleischmann, Pain and Porter (1962), but the free sulfhydryl groups were alkylated by treatment with recrystallized iodoacetamide. The heavy and light chains were separated on a Sephadex G-100 column saturated with 1 N acetic acid at 4 C. The yields of light chains from anti-8 antibody and anti-17 antibody were 29.5% and 30% respectively on the basis of the optical density at 280 mμ. The electrofocusing patterns of the light chains of anti-8, anti-17 and a mixture of the two (1: 1) are shown in Fig. 6. The bands of the light chain preparations are much less complex than those of the whole molecules of antibodies. Anti-8, anti-17 and the mixtures had 5 to 7, 7 to 9 and 11 to 12 discrete bands, respectively. The mixture consistently had more discrete bands than the light chains of either of the antibody fractions alone. Some components of the light chains of anti-17 antibody were not found in the light chains of anti-8 antibody, and vice versa. The results again indicate that there may be some selection of antibody population depending on the kind of determinant group.


FIGURE 5. *Comparison of the isoelectrofocusing patterns of anti-8, anti-17 and a mixture of both (1: 1) of sheep #20. a: Anti-8 antibody. b: Anti-17 antibody. c: Mixture of both antibodies.*
*The experimental conditions were as for Fig. 3.*

## DISCUSSION

The restricted range of $K_A$ values of region specific antibodies has already been noticed in material from rabbits (Fujio et al., 1971). This phenomenon was also found in antibody fractions from sheep. The association constants of the region specific antibody fractions with HL were in the order of $10^5$ ($M^{-1}$) in both rabbits and sheep. It seems likely that


FIGURE 6. *Isoelectrofocusing patterns of the light chains of anti-8, anti-17 and a mixture of both (1: 1) of sheep #10. 200 μg. of each sample were applied. Ampholyte 3–10 was used.*
*The run was made in 4% acrylamide in 6 M deionized urea. a: Light chain of anti-8. b: Light chain of anti-17. c: Mixture of the light chains of both.*

the narrow range of $K_A$ values of these antibody fractions is controlled by the nature of the antigenic determinant. As a matter of fact, genetic factors are actually known to exert strong control on the immune response in general. But if the genetic relationship between antigen and the host is apart beyond some extent, the influence of genetic factors on the immune response may be minimized.

The elctric charges of those antibody fractions were tested and all the antibody fractions were found to be heterogeneous. At least, two explanations of this phenomenon can be considered. First, the heterogeneity of the electric charges of the antibody may merely reflect structural differences which do not involve the active site of antibody. Second, the structures responsible for the observed heterogeneity are actually located in the active site of each antibody, but only involve minor differences in amino acids, so that the $K_A$ values stayed within the limits of experimental error.

Neverthless, a certain degree of selection of antibody populations seems to occur because the numbers of discrete bands of each region specific antibody fraction observed by electrofocusing are always less than that of the mixture. In general, the heterogeneity in light chains of anti-8 antibodies seems to be less than that of anti-17 antibodies. A minimum of three light chains were found in one sheep when the isoelectrofocusing pattern of light chains were compared using 200 $\mu$g of material. Maron et al. (1971) independently reported similar selection of antibody populations depending on the kind of determinant group from studies on the anti-"loop" (sequence 60–83) antibody in goat anti-HL antiserum.

Isoelectrofocusing experiments were also carried out on the region specific antibody fractions of rabbits. The results were essentially the same as those reported in this paper on sheep.

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