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COMPARATIVE STUDIES ON ANTIBODY FORMATION AGAINST HEN EGG WHITE LYSOZYME AND ITS SPLIT FRAGMENT

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SUMMARY A peptic fragment Fr. 17 (Lys^{1}–Cys–Asn^{27} Leu^{129}–Cys–Ala^{122}) of hen egg white lysozyme (HL) was previously found to retain at least one antigenic determinant region of the native protein. In this work a highly purified preparation of Fr. 17, contaminated with less than 0.01% HL and less than 1% of other fragments was found to be strongly immunogenic to rabbits.

The kinetic patterns of antibody formation against Fr. 17, assayed by passive hemagglutination (PHA), were quite different from those of antibody formation against HL.

The specificity of the antibody elicited to Fr. 17 was mainly directed to the Fr. 9–10-a region (Ala^{11}–Asn^{27}) while that of the antibody elicited to the Fr. 17 region in native HL was directed to the Fr. 15-b region (Lys^{1}–Cys–Ala^{10} Leu^{129}–Cys–Trp^{123}).

It is concluded that in the process of antibody formation, the recognition of the Fr. 17 region in native HL is different from that of fragment Fr. 17.

INTRODUCTION

Hen egg white lysozyme is a very suitable protein antigen to use in studies on the mechanism of antigen recognition in the process of the antibody formation, for the following reasons: (1) it has a low molecular weight, (2) its amino acid sequence has already been established by Jollès et al. (1963, 1964) and Jauregui-Adell et al. (1965) and independently by Canfield (1963) and Canfield and Liu (1965), (3) its three dimensional structure was elucidated in detail by Phillips (1967) and (4) some fragments which retain the antigenic determinant region of native protein have already been isolated and characterized by Shinka et al. (1967), Fujio et al. (1968) and Sakato et al. (1972).

Some of the antibody elicited to native HL reacts with fragment Fr. 17. This is one of the immunologically active fragments isolated after pepsin digestion and consists of 35 amino acid residues located at the N- and C-terminal regions of HL (Fujio et al. 1968). But it is uncertain whether this antibody is produced against fragment Fr. 17 after the latter is split

from HL in the process of antibody formation, or whether it is produced against the Fr. 17 region in native HL. This problem seems very important for elucidating the mechanism of antigen recognition.

Accordingly we purified Fr. 17 as completely as possible and obtained a preparation containing less than 0.01% HL and less than 1% of other fragments. Then the antibody responses to this purified Fr. 17 and to native HL in Freund's complete adjuvant (FCA) were compared. In analyses of antibodies, purified fragments of Fr. 17, Fr. 9–10-a and Fr. 15-b were used. The locations of these three fragments in HL are shown in Fig. 1.

The present paper describes the results of studies which indicate that in the process of antibody formation in rabbits the recognition of the Fr. 17 region in native HL is quite different from that of isolated fragment Fr. 17.

MATERIALS AND METHODS

1. *Preparation of fragment Fr. 17*

HL was subjected to limited digestion with pepsin by a slight modification of the method reported previously (Fujio et al., 1968). Six times recrystallized HL, from Seikagaku-kogyo Co. Ltd., was digested by pepsin at pH 1.1 by incubating 82.5 ml of 1.21% HL solution in 0.1 N HCl, with 1 ml of solution containing 1 mg of pepsin (Worthington Biochem. Corp.) at 37 C for 1 hr. Then the pH was adjusted to 5.0 with concentrated NaOH and the mixture was immediately applied to a Serva carboxymethyl (CM) cellulose column (3×32 cm) equilibrated with 0.05 M acetate buffer, pH 3.85. The column was washed with 2,000 ml of 0.1 M phosphate buffer, pH 6.0, to remove the unwanted fractions 2 to 15 of the previous report (Shinka et al., 1967). Then the column was eluted with 8,000 ml of a linear concentration gradient of 0.1 M to 0.3 M phosphate buffer, pH 6.0 at 25 C at a flow rate of 150 ml per hr. The material corresponding to fraction 17 was obtained with good resolution as described in the previous paper (Fujio et al., 1968).

For further purification, this fraction was diluted 1: 3 with water, adjusted to pH 3.85 with concentrated acetic acid and then applied to a CM-cellulose column (3×18 cm) equilibrated with 0.05 M acetate buffer, pH 3.85. The column was washed with 500 ml of 0.1 M phosphate buffer, pH 6.0, and then eluted with a linear concentration gradient obtained using 2,000 ml volumes of 0.1 M phosphate buffer, pH 6.0 and 0.3 M phosphate buffer, pH 6.0 at 25 C at a flow rate of 100 ml per hr. The fractions between the arrows in Fig. 2A were pooled and concentrated to a small volume in a rotatory evaporator. Then the material was subjected to gel filtration on a Sephadex G–25 column (4.4×146 cm) equilibrated with 0.05 M acetate buffer, pH 3.85 containing 0.3 M NaCl at room temperature.

The enzyme activity of HL in fractions eluted from the gel was measured with *M. lysodeikticus* as substrate, as described below. As shown in Fig. 2B, a trace of enzyme activity of HL was detected in the fractions eluted before those containing fragment Fr. 17. Therefore, the gel filtration was repeated twice more under the same conditions. In the last gel filtration no enzyme activity of HL was detected in any fractions. These fractions were pooled, desalted and lyophilized. The content of contaminating HL in this final preparation was found to be less than 0.01% based on the enzyme activity, and its contents of other fragments seemed to be less than 1% based on amino acid analysis.

2. *Preparation of fragments Fr. 9–10-a and Fr. 15-b*

One gram of HL was digested with pepsin as described above. Then the pH was adjusted to 3.85 with NaOH and the mixture was diluted 1: 30 with 0.05 M acetate buffer, pH 3.85 and applied to a CM-cellulose column (3×40 cm) equilibrated with the same buffer. The column was eluted at 25 C with 9,000 ml of a linear gradient of 0 to 0.15 M NaCl in 0.05 M acetate buffer, pH 5.0 at a flow rate of 100 ml per hr. Fractions of 13.5 ml were collected. As shown in Fig. 3, about 18 peaks were observed, and amino acid analysis showed that peaks L and Q contained Fr. 9–10-a and Fr. 15-b, respectively.

For further purification, the crude preparation of Fr. 9–10-a (peak L) was diluted 1: 8 with water and applied to a CM-cellulose column (2×20 cm). The column was eluted at 25 C with 4,000 ml of a linear gradient of 0 to 0.1 M NaCl in 0.05 M acetate buffer, pH 5.0, at a flow rate of 60 ml per hr. The same procedure was repeated twice more until a single symmetric peak was obtained. Finally the material was concentrated to 30 ml in a rotatory evaporator and subjected to gel filtration on a Sephadex G–25

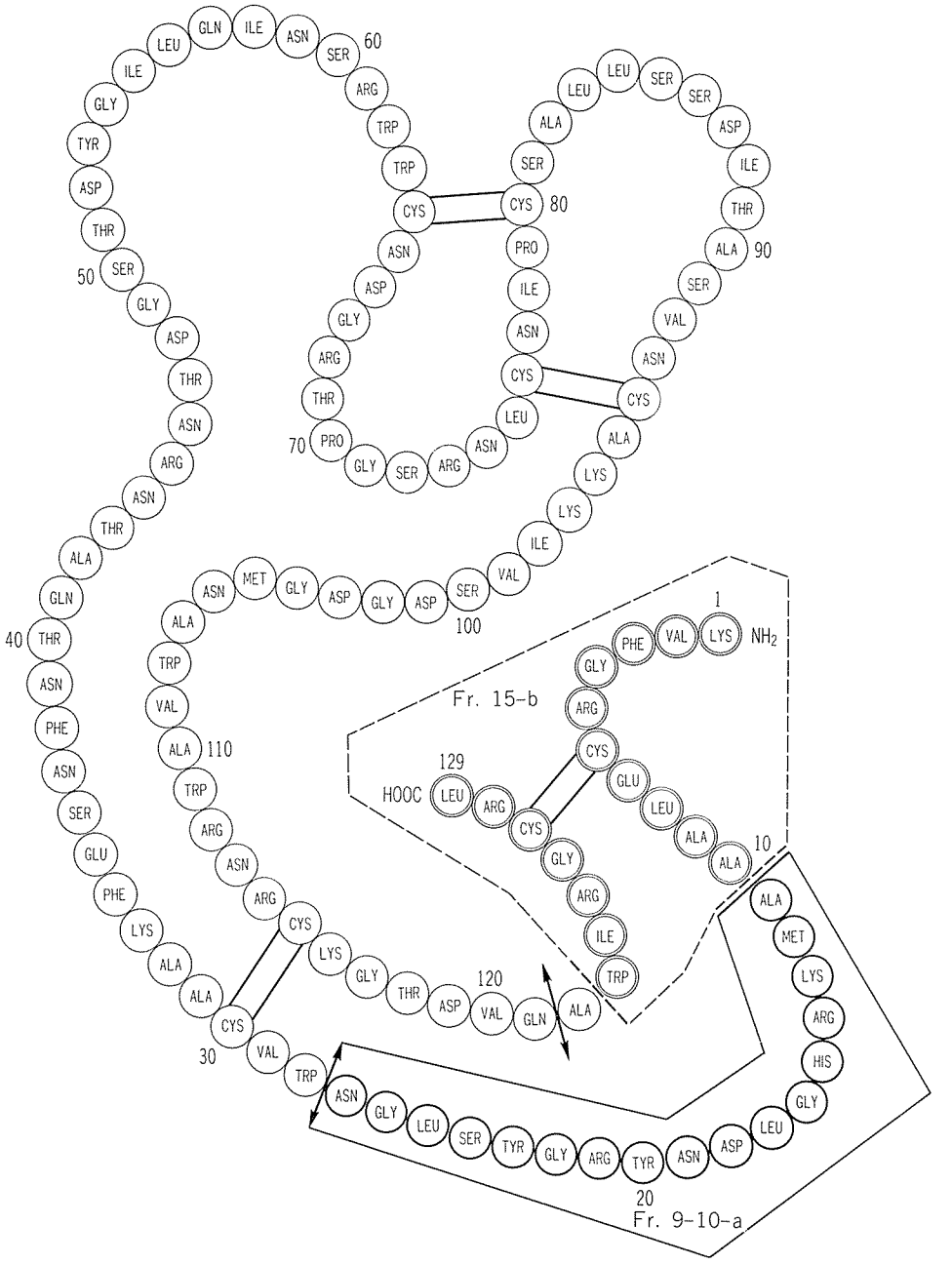


FIGURE 1. *Amino acid sequences of HL and fragments. Fr. 17, Lys*[1]*–Cys–Asn*[27] *Leu*[129]*–Cys–Ala*[122]*; Fr. 15-b, Lys*[1]*–Cys–Ala*[10] *Leu*[129]*–Cys–Trp*[123]*; Fr. 9–10-a, Ala*[11]*–Asn*[27]*. Arrows indicate the location of Fr. 17.*

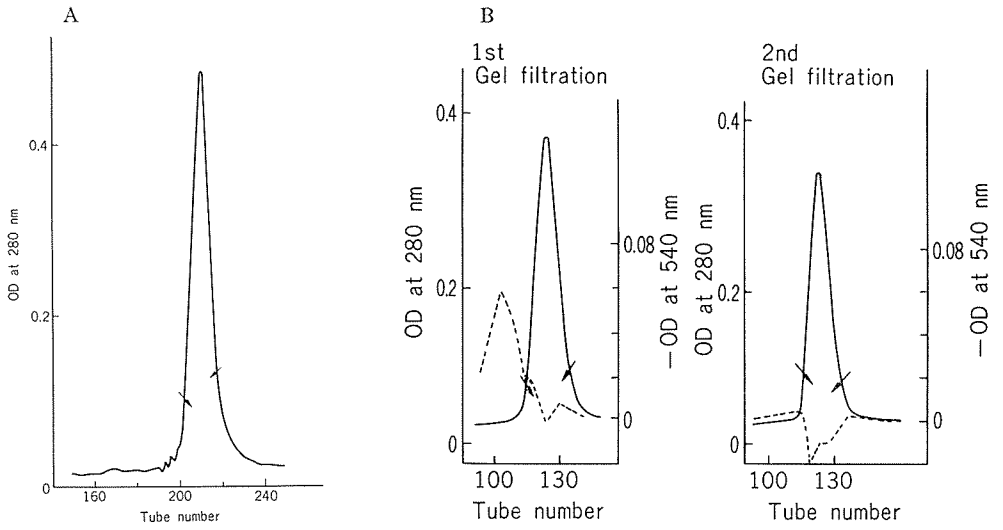


FIGURE 2. *The patterns of (A) CM-cellulose rechromatography of the crude preparation of fragment Fr. 17 (main component on 1st chromatography) and (B) its subsequent gel filtrations. A: column size, 3×18 cm; fraction volume, 14.5 ml; elution rate, 100 ml per hr; temperature, 25 C. B: column, Sephadex G-25 (4.4×146 cm); fraction volume, 10 ml; flow rate, 60 ml per hr; room temperature. (- - - -), reduction in turbidity of solution containing M. lysodeikticus by each fraction. Fractions between the two arrows in each pattern were collected.*

TABLE 1. *Amino acid composition of fragment Fr. 17*

Amino acid	Hydrolysis for		Lys[1]-Cys-Asn[27] [b] / Leu[129]-Cys-Ala[122]
	24 hr[a]	72 hr[a]	
Lys	1.9	1.9	2
His	0.9	0.9	1
Arg	5.0	5.2	5
Asp	3.0	2.8	3
Thr	0	0	0
Ser	0.6	0.6	1
Glu	1.2	1.2	1
Pro	0	0	0
Gly	5.0	5.0	5
Ala	3.3	3.5	4
½Cys	1.9	2.3	2
Val	1.0	1.0	1
Met	1.1	1.0	1
Ile	0.9	1.0	1
Leu	3.8	3.6	4
Tyr	1.7	1.7	2
Phe	1.0	1.1	1
Trp	ND[c]	ND	1

[a] Values are molar ratios, calculated assuming that there are 5 glycine residues per mole of Fr. 17.
[b] Moles of amino acids in the corresponding region of HL calculated from the data of Canfield and Liu (1965).
[c] Not determined.

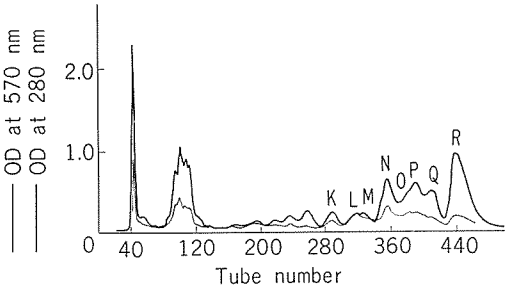


FIGURE 3. *Isolation of Fr. 9-10-a and Fr. 15-b from a peptic digest of HL by CM-cellulose chromatography. Column size, 3×40 cm; fraction volume, 13.5 ml; flow rate, 100 ml per hr; temperature, 25 C; ——, OD at 280 nm; ——, OD at 570 nm (ninhydrin reaction). Peak L contained Fr. 9-10-a and Q contained Fr. 15-b.*

column (4.4×146 cm) equilibrated with 0.05 M acetate buffer, pH 3.85 at room temperature to remove minor contaminations. Fractions of 10.5 ml were collected at a flow rate of 60 ml per hr. This procedure was repeated once more, and then the material was concentrated to 30 ml and desalted and lyophilized. This final preparation of fragment Fr. 9–10-a contained less than 0.02% HL based on assay of enzyme activity, and less than 1% of other fragments judging from the data of amino acid analysis (Table 2).

The crude preparation of Fr. 15-b (peak Q) was purified in the same way as Fr. 9–10-a, except that for elution a linear gradient was achieved using 2,000 ml volumes of 0.05 M acetate buffer, pH 5.0, and of the same buffer containing 0.15 M NaCl. After two further chromatographies on CM-cellulose under the same conditions, gel filtration were performed twice on a Sephadex G–25 column (4.4×146 cm) equilibrated with 0.05 M acetate buffer, pH 3.85 containing 0.3 M NaCl at room temperature. Finally, the peak component was desalted and lyophilized. This final preparation of Fr. 15-b was calculated to contain less than 0.02% HL from its enzyme activity and less than 10% of other fragments from data of amino acid analysis (Table 3).

3. *Measurement of the enzyme activity of HL*

The enzyme activity of HL in the preparation of Fr. 17 could not be measured directly using *M. lysodeikticus* as substrate, because Fr. 17 inhibits the enzyme activity of HL on this substrate (Amano

TABLE 2. *Amino acid composition of fragment Fr. 9–10-a*

Amino acid	Hydrolysis for 24 hr[a]	Hydrolysis for 72 hr[a]	Ala^{11}–Asn^{27} [b]
Lys	0.9	0.9	1
His	0.9	0.9	1
Arg	2.0	2.1	2
Asp	3.0	2.9	3
Thr	0	0	0
Ser	0.8	0.8	1
Glu	0	0	0
Pro	0	0	0
Gly	3.0	3.0	3
Ala	1.0	1.1	1
½Cys	0	0	0
Val	0	0	0
Met	1.0	0.9	1
Ile	0	0	0
Leu	1.9	1.8	2
Tyr	1.6	1.5	2
Phe	0	0	0
Trp	ND[c]	ND	0

[a] Values are molar ratios, calculated assuming that there are 3 glycine residues per mole of Fr. 9–10-a.
[b] Moles of amino acids in the corresponding region of HL calculated from the data of Canfield and Liu (1965).
[c] Not determined.

TABLE 3. *Amino acid composition of fragment Fr. 15-b*

Amino acid	Hydrolysis for 24 hr[a]	Lys^{1}–Cys–Ala^{10} [b] | Leu^{129}–Cys–Trp^{123}
Lys	0.98	1
His	0.03	0
Arg	3.24	3
Asp	0.09	0
Thr	<0.01	0
Ser	0.03	0
Glu	1.10	1
Pro	0.00	0
Gly	2.00	2
Ala	2.14	2
½Cys	1.76	2
Val	0.84	1
Met	0.00	0
Ile	0.81	1
Leu	1.84	2
Tyr	0.05	0
Phe	0.87	1
Trp	ND[c]	1

[a] Values are molar ratios, calculated assuming that there are 2 glycine residues per mole of Fr. 15-b.
[b] Moles of amino acids in the corresponding region of HL calculated from the data of Canfield and Liu (1965).
[c] Not determined.

and Fujio, 1968). Therefore, the presence of enzyme activity in the preparation of Fr. 17 was deduced from the activities in neighboring fractions eluted during the final gel filtration. Three ml of a suspension of 0.25 mg of *M. lysodeikticus* in 6.8 ml of 1/15 M phosphate buffer, pH 6.0 was mixed with 0.2 ml of the sample solution and incubated at room temperature for 4 hr. Then the reduction in turbidity of the mixture was measured at 540 nm. In this assay system, concentrations of 1×10^{-3} and 1×10^{-4} mg of HL per ml of 0.05 M acetate buffer, pH 3.85 containing 0.3 M NaCl caused 0.073 and 0.025 reduction in turbidity, respectively.

The enzyme activity of HL in preparations of Fr. 9–10-a and Fr. 15-b was measured as described before (Shinka et al., 1962).

4. *Determination of fragment concentration*

The concentration of each fragment was determined from the absorption at 280 nm, and at 570 nm after the ninhydrin reaction.

The ninhydrin reaction was carried out by the method of Cocking and Yemm (1955).

5. *Desalting*

The solution containing a fragment was concentrated to a small volume in a rotatory evaporator, subjected to gel filtration on a Sephadex G–25 column (3.9×85.5 cm) equilibrated with acetic acid (15% v/v) and then lyophilized.

6. *Amino acid analysis*

The amino acid compositions of fragments were determined by the method of Spackman et al. (1958), using a Yanagimoto amino acid analyzer, Model LC–5. Fragments were hydrolyzed by treatment with constant boiling 5.7 N HCl at 105± 1 C for 24 and 72 hr. The molar ratios of amino acids were calculated taking glycine as a standard.

7. *Immunization of rabbits*

Three rabbits (no. 177, no. 176 and no. 175) were immunized with 2 mg of Fr. 17. As controls two rabbits (no. 1711 and no. 1710) were immunized with 2 mg of six times crystallized HL and one rabbit (no. 178) was immunized with 1 μg of same HL. All antigens were emulsified in FCA. Booster immunizations with each antigen were given in the same way 5 weeks and 12 weeks after the first immunization. Blood was taken from each rabbit just before the first immunization and 2 weeks (a), 4 weeks (b), 6 weeks (c), 8 weeks (d), 11 weeks (e) and 14 weeks (f) after the first immunization.

8. *Neutralizing activity of antisera*

Inhibition of the enzyme activity of HL by anti-Fr. 17 sera was tested with *M. lysodeikticus* as described before (Shinka et al., 1962).

9. *Sensitization of sheep red blood cells (SRBC)*

Sensitization was performed by the method of Onkelinx et al. (1969). Ten ml of 0.1 M glutaraldehyde in phosphate buffered saline, pH 7.2 (PBS–7), were added to a mixture of 10 ml of a 5% suspension of SRBC which had been washed twice with PBS–7, and 1 ml of a 0.1% solution of a fragment or of 0.125% HL solution in PBS–7. After incubation at 37 C for 15 min with gentle stirring, 1 ml of 1% BSA solution in PBS–7 was added and the mixture was kept for 15 min under the same conditions. The sensitized SRBC were then washed 3 times with PBS–7 and suspended in PBS–7 containing 0.1% gelatin and 0.1% NaN_3 to make a 0.5% suspension.

10. *Passive hemagglutination (PHA)*

PHA was performed with 0.025 ml portions of serial dilutions of antisera. The diluent was PBS–7 containing 0.1% gelatin and 0.1% NaN_3. Each serum dilution was mixed with 0.025 ml of a 0.5% suspension of HL or fragment coated SRBC (HL–SRBC, Fr. 17–SRBC, Fr. 15-b–SRBC and Fr. 9–10-a–SRBC) in the same buffer. Preimmune sera were used as serum controls and rabbit gammaglobulin coated SRBC and uncoated SRBC were used as antigen controls. The end titers of agglutination were read after 20 hr of incubation at room temperature.

Inhibition of hemagglutination was tested by adding 0.025 ml of PBS–7 containing the inhibitor to each serum dilution 30 min before addition of antigen-coated SRBC. The percentage inhibition was calculated from the formula: $\{(A-B)/A\}\times100$ where A and B represent the dilution factors of the end titers of PHA without and with inhibitor, respectively.

11. *Labelling of Fr. 17*

Fr. 17 was labelled with $Na^{125}I$ by the method of McConahey and Dixon (1966). Unbound iodine was removed from the preparation of ^{125}I–Fr. 17 by chromatography on a CM-cellulose column (1×5 cm) equilibrated with 0.05 M acetate buffer,

pH 3.85. The column was eluted with 0.05 M acetate buffer, pH 3.85 containing 0.15 M NaCl and then ^{125}I–Fr. 17 was eluted with 0.2 M $NaHCO_3$.

^{3}H–Acetyl–Fr. 17 was prepared by the method of Spragg et al. (1966). The ^{3}H–acetyl–Fr. 17 recovered, with a specific activity of 13,575 cpm/μg, was used in inhibition studies. An average of approximately one mole of acetyl group was introduced per mole of Fr. 17.

12. *Antigen binding experiment*

Individual serum was diluted with borate buffered saline (BBS), pH 8.0. For dilutions of more than 1 : 20, saline containing 1 : 10 dilution of normal rabbit serum was used as diluent. A mixture of 50 μliters of each serum dilution and 50 μliters of a 0.5 μg/ml solution of ^{125}I–Fr. 17 in 0.1 M $NaHCO_3$ was incubated at 20 C for 2 hr and precipitated by 50 μliters of hyperimmune goat antiserum to rabbit gamma-globulin (obtained from Kanonji Institute. The Research Foundation for Microbial Diseases of Osaka University), which had not been absorbed with rabbit gamma-M-immunoglobulin. After incubation at 0 C overnight, the precipitate was washed twice with cold BBS. The radioactivity of the precipitate was counted in a Packard, Model 3002, Tricarb Scintillation Spectrometer. The percentage of antigen bound was calculated from the formula: X–(Y–X)K/Y where X, Y and K represent counts precipitated, total counts added and the fraction of the antigen precipitated by normal serum under comparable conditions (i.e., background), respectively (Scibienski, 1973). The antigen binding capacity (ABC–33) was determined by plotting the average of the percentages of antigen bound in two experiments against the log of the serum dilution. From the curve, the dilution of antiserum which bound 33% of the added antigen was determined (Scibienski, 1973). From this value, the μ moles of antigen bound by 1 ml of undiluted antiserum were calculated. When the binding was too low to assess the 33% point, the ABC was calculated directly from the serum, diluted 1 : 10 in BBS.

For inhibition assay, the inhibitor at an appropriate concentration in 100 μliters of PBS–7 was first mixed wih 50 μliters of no. 1711 (f) antiserum diluted 1 : 5 in PBS–7 or 25 μliters of no. 177 (f) antiserum diluted 1 : 5 in PBS–7. Then 2 hr later 25 μliters of a solution of 20.5 μg of ^{3}H–acetyl–Fr. 17 per ml was added. The percent inhibition was calculated from the formula: $\{(A-B)/A\}\times 100$ where A and B represent the percentages of antigen bound in the absent and present of inhibitor, respectively. In this experiment, the radioactivity was measured in a Beckman Liquid Scintillation Counter, LS200B.

13. *Gel filtration of antisera*

No. 1711 (b) and no. 1711 (f) anti-HL sera (1 ml) were fractionated on a Sephadex G–200 column (2 × 135 cm) equilibrated with PBS–7 and fractions of 1.7 ml were collected.

No. 177 (b) and no. 177 (f) anti-Fr. 17 sera (3 ml) were also fractionated on a Sephadex G–200 column (4 × 135 cm) equilibrated with PBS–7 and fractions of 5.3 ml were collected.

The antibody activity of fractions containing 19S and 7S immunoglobulins was assayed by PHA, using Fr. 17–SRBC, Fr. 9–10–a–SRBC, Fr. 15–b SRBC and HL–SRBC, as described above.

RESULTS

1. *Kinetic study of antibody formation against HL by PHA*

In the rabbits (no. 1711 and no. 1710) which received 2 mg of HL per injection, the agglutinin titer of HL–SRBC increased with time during immunization and antibodies reacting with Fr. 17–SRBC were formed, as shown in

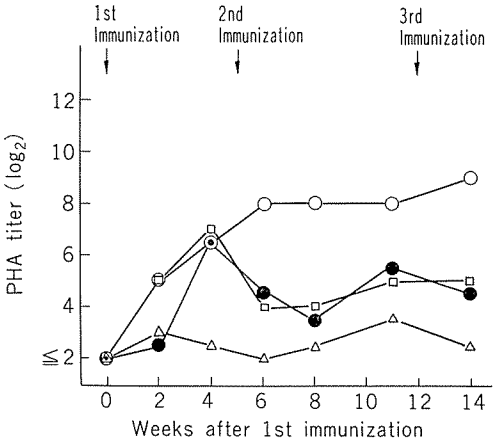
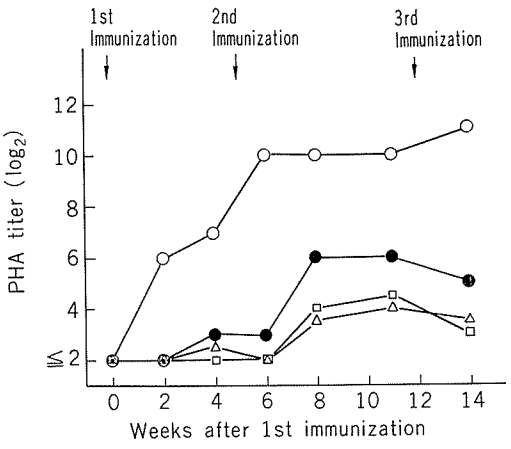


FIGURE 4. *Kinetic study of antibody formation in a rabbit (no. 1711) immunized with native HL by PHA with SRBC sensitized with various antigens.* ○, *HL–SRBC;* ●, *Fr. 17–SRBC;* □, *Fr. 15–b–SRBC;* △, *Fr. 9–10–a–SRBC.*



Figs. 4 and 5. The pattern of the agglutinin titer of Fr. 17–SRBC was superimposed on that of Fr. 15-b–SRBC (Fig. 4) except in the early phase in one rabbit, and was parallel to the latter in the other (Fig. 5). On the other hand, the pattern of Fr. 17–SRBC was quite different from that of Fr. 9–10-a–SRBC in one rabbit (Fig. 4), and fairly parallel to the latter

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FIGURE 5. *Kinetic study of antibody formation in a rabbit (no. 1710) immunized with native HL by PHA with SRBC sensitized with various antigens.* ○, *HL–SRBC;* ●, *Fr. 17–SRBC;* □, *Fr. 15-b–SRBC;* △, *Fr. 9–10-a–SRBC.*

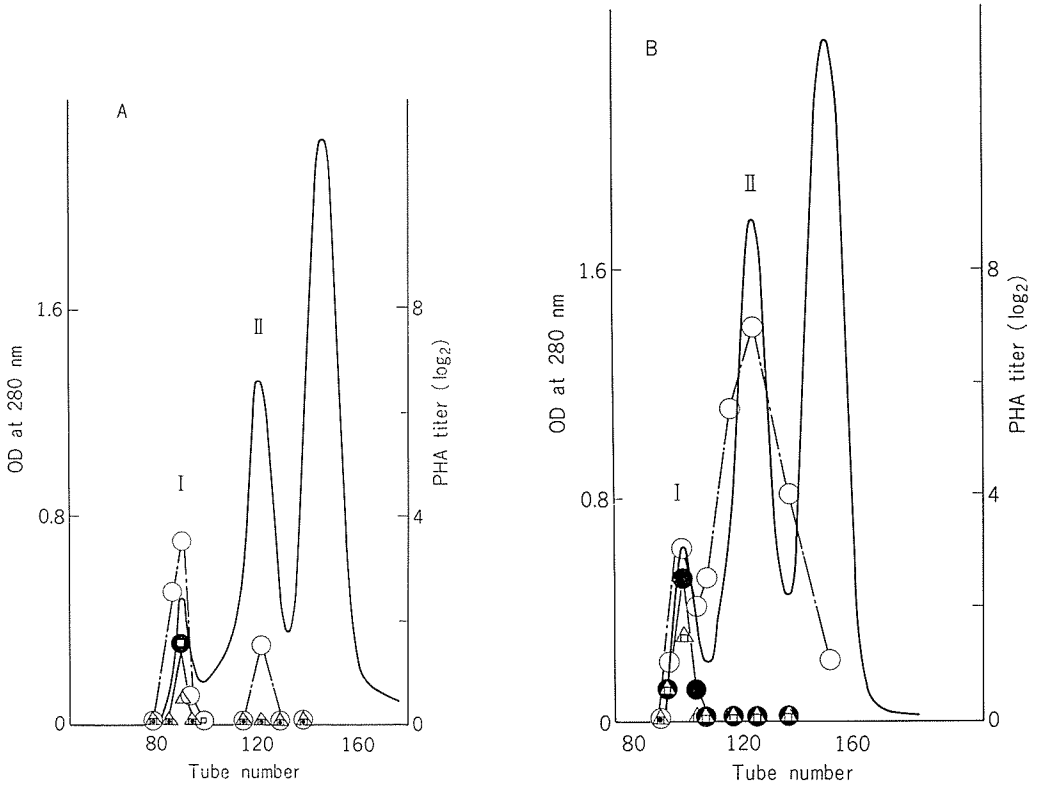

FIGURE 6. *Fractionation of two anti-HL antisera on a Sephadex G–200 column (2×135 cm) using PBS–7 at room temperature. Peak I contained 19 S immunoglobulin and Peak II contained 7 S immunoglobulin. The effluent was collected in 1.7 ml fractions at a flow rate of 20 ml per hr. A, b serum of no. 1711 rabbit; B, f serum of no. 1711 rabbit. Antibody titers were assayed by PHA with SRBC sensitized with various antigens. Hemagglutination titers are means of values in two parallel titrations.* ○, *HL–SRBC;* ●, *Fr. 17–SRBC;* △, *Fr. 9–10-a–SRBC;* □, *Fr. 15-b–SRBC.*

TABLE 4. *Inhibitory effects of various fragments on Fr. 17–SRBC agglutination by anti-HL antiserum (no. 1710(e))*

	Inhibitor (μg/ml)	Inhibition[a] (%)
	0	0
Fr. 17	125	100
Fr. 15-b	125	75
Fr. 9–10-a	125	0

[a] Percentage inhibitions were calculated from values in four parallel titrations.

in the other (Fig. 5). From the fact that in both rabbits the patterns of Fr. 17–SRBC and Fr. 15-b–SRBC were superimposable or parallel it seemed that the main antibody fraction which reacted with Fr. 17–SRBC also reacted with Fr. 15-b–SRBC.

To investigate this, experiments on inhibition of hemagglutination were carried out using Fr. 17, Fr. 9–10-a and Fr. 15-b as inhibitors with no. 1710(e) antiserum, in which the agglutinin titers of Fr. 15-b–SRBC and Fr. 9–10-a–SRBC were both high and were very similar. As shown in Table 4, agglutination of Fr. 17–SRBC was inhibited completely by homologous Fr. 17 and 75% by Fr. 15-b but was not inhibited by Fr. 9–10-a.

To examine the class distribution of hemagglutinating antibodies evoked by HL, early (no. 1711(b)) and late (no. 1711(f)) antisera were fractionated on columns of Sephadex G-200. As shown in Fig. 6, agglutinins of Fr. 17–SRBC, Fr. 9–10-a–SRBC and Fr. 15-b–SRBC were found exclusively in the 19S fractions of both antisera.

2. *Kinetic study of antibody formation against Fr. 17 by PHA*

Three rabbits (nos. 175, 176 and 177) were immunized with Fr. 17 in FCA (2 mg per injection). As shown in Figs. 7, 8 and 9, Fr. 17 had very strong immunogenicity in these rabbits, but the antisera did not give any precipitin line with homologous Fr. 17 or HL in agarose gel. This strong antibody response could not be due to contamination with HL, because as shown in Fig. 10, when a rabbit (no. 178) was immunized with HL at 5 times the maximum possible amount contaminating 2 mg of the preparation of Fr. 17, it did not show a quick antibody response but only a slight response after the third immunization. The maximum agglutinin titer of Fr. 17–SRBC was attained at an early stage of immunization and gradually decreased after the second immunization. Moreover the pattern of the agglutinin titer of Fr. 9–10-a–SRBC was almost superimposable on that of Fr. 17–SRBC while that of Fr. 15-b–SRBC, though significant, was quite different from the latter.

Experiments on inhibition of hemagglutination of Fr. 17–SRBC were performed using Fr. 17, Fr. 9–10-a, Fr. 15-b and HL as inhibitors with early (no. 177(b)) and late (no. 177(f)) antisera. As can be seen in Figs. 11 and 12, Fr. 9–10-a inhibited the hemagglutination of Fr. 17–SRBC with both antisera, while Fr. 15-b only inhibited that of late antiserum. These results indicate that the main antibodies produced by Fr. 17, are directed to the Fr. 9 10-a portion of Fr. 17.

To investigate this further, experiments on inhibition of the hemagglutinations of Fr. 9–10-a–SRBC and Fr. 15-b–SRBC with no. 177 (b) antiserum were performed using Fr. 9–10-a and Fr. 15-b as inhibitors. As shown in Table 5, no inhibition of the hemagglutination of Fr. 9–10-a–SRBC by Fr. 15-b or of that of Fr. 15-b–SRBC by Fr. 9–10-a was observed. This means, that an early antiserum, no. 177 (b), contained two kinds of antibodies directed to the Fr. 9–10-a portion and the Fr. 15-b portion of Fr. 17, respectively, although the main antibody fraction evoked by Fr. 17 was directed to the Fr. 9–10-a portion of Fr. 17, as described above.

To examine the class distribution of hemagglutinating antibodies evoked by Fr. 17, early (no. 177(b)) and late (no. 177(f)) antisera were fractionated on columns of Sephadex G–200. As can be seen in Fig. 13, the early antiserum contained only 19S hemagglutinins to Fr. 17–

SRBC and Fr. 9–10-a–SRBC. No hemagglutinin to Fr. 15-b–SRBC was detected in either the 19S or 7S fraction, due to dilution during the procedure and the low titer of hemagglutinin. The late antiserum contained 19S antibodies to Fr. 17–SRBC, Fr. 9–10-a–SRBC, Fr. 15-b–SRBC and also 7S antibodies to Fr. 17–SRBC and Fr. 9–10-a–SRBC.

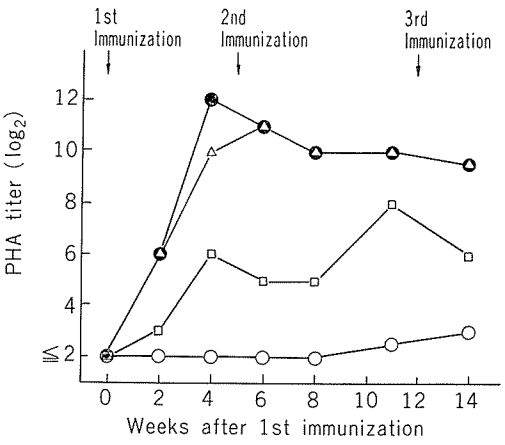

FIGURE 7. *Kinetic study of antibody formation in a rabbit (no. 177) immunized with Fr. 17 by PHA with SRBC sensitized with various antigens.* ○, *HL–SRBC;* ●, *Fr. 17–SRBC;* □, *Fr. 15-b–SRBC;* △, *Fr. 9–10-a–SRBC.*

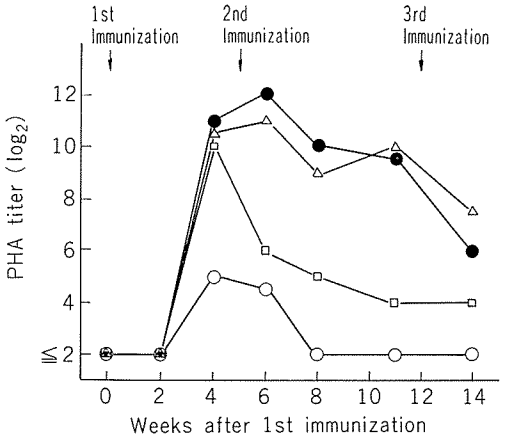

FIGURE 8. *Kinetic study of antibody formation in a rabbit (no. 176) immunized with Fr. 17 by PHA with SRBC sensitized with various antigens.* ○, *HL–SRBC;* ●, *Fr. 17–SRBC;* □, *Fr. 15-b–SRBC;* △, *Fr. 9–10-a–SRBC.*

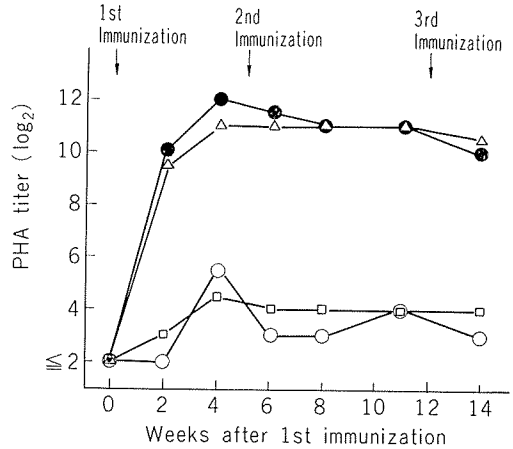

FIGURE 9. *Kinetic study of antibody formation in a rabbit (no. 175) immunized with Fr. 17 by PHA with SRBC sensitized with various antigens.* ○, *HL–SRBC;* ●, *Fr. 17–SRBC;* □, *Fr. 15-b–SRBC;* △, *Fr. 9–10-a–SRBC.*

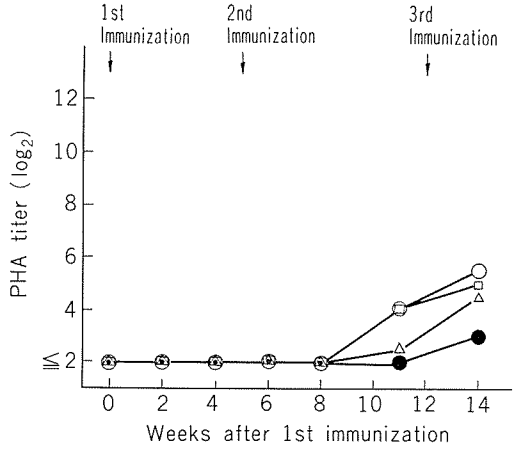

FIGURE 10. *Kinetic study of antibody formation in a rabbit (no. 178) immunized with a low dose of native HL by PHA with SRBC sensitized with various antigens.* ○, *HL–SRBC;* ●, *Fr. 17–SRBC;* □, *Fr. 15-b–SRBC;* △, *Fr. 9–10-a–SRBC.*

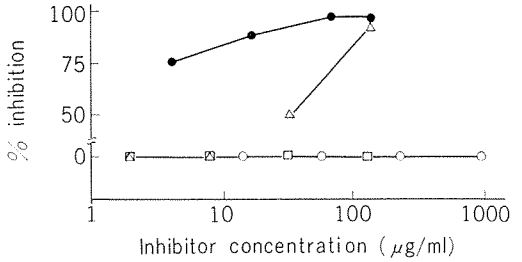

FIGURE 11. *Inhibition of Fr. 17–SRBC agglutination of no. 177 (b) serum by various doses of Fr. 9–10-a (△), Fr. 15-b (□), Fr. 17 (●) and HL (○). percentage inhibitions were calculated from the means of the titers in three parallel titrations.*

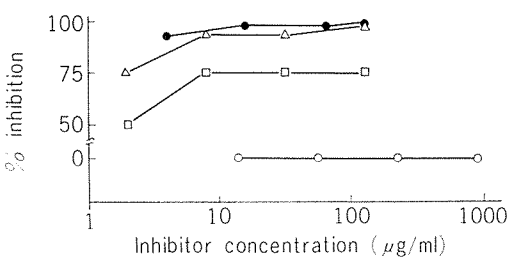

FIGURE 12. *Inhibition of Fr. 17–SRBC agglutination of no. 177 (f) serum by various doses of Fr. 9–10-a (△), Fr. 15-b (□), Fr. 17 (●) and HL (○). Values were calculated in the same way as in Fig. 12.*

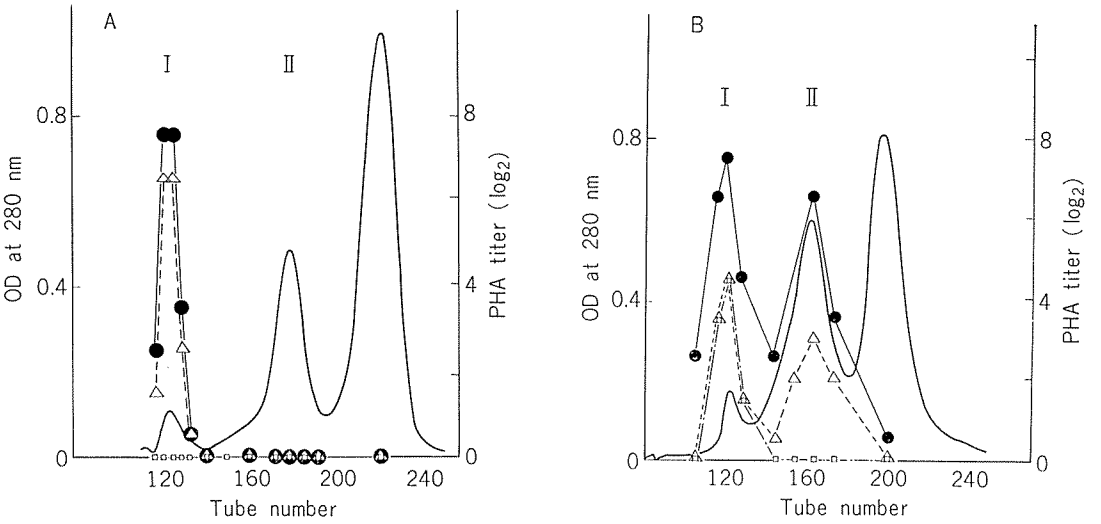

FIGURE 13. *Fractionation of anti-Fr. 17 antisera on a Sephadex G–200 column (4×135 cm) using PBS–7 at room temperature. Peak I contained 19 S immunoglobulin and Peak II contained 7 S immunoglobulin. The effluent was collected in 5.3 ml fractions. The flow rate was 32 ml per hr. A, b serum of no. 177 rabbit; B, f serum of no. 177 rabbit. Antibody titers were assayed by PHA with SRBC sensitized with various antigens. Hemagglutination titers are means of values in two parallel titrations. ●, Fr. 17–SRBC; △, Fr. 9–10-a–SRBC; □, Fr. 15-b–SRBC.*

3. *Kinetic study on antibody formation against Fr. 17 by direct binding of ^{125}I–Fr. 17*

The antibody response of rabbits (no. 176 and no. 177) immunized with Fr. 17 was investigated by estimating the antigen-binding capacity of ^{125}I–Fr. 17. As controls, a rabbit (no. 178) was immunized with a small dose of HL, and two others (no. 1710 and no. 1711) with 2 mg of HL per injection. The results are shown in Fig. 14. In rabbits no. 176 and no. 177, the antigen binding capacity to ^{125}I–Fr. 17 only increased after the secondary immunization. Thus the observed patterns were quite different from those of hemagglutinin to Fr. 17–SRBC, which showed a quick response after the first immunization, as shown in Figs. 7, 8 and 9. The rabbits which re-

TABLE 5. *Inhibitory effects of two fragments on agglutination of Fr. 9-10-a-SRBC or Fr. 15-b-SRBC by anti-Fr. 17 antiserum (no. 177 (b))*

Sensitized SRBC	Inhibitor	(μg/ml)	Inhibition[a] (%)
Fr. 9-10-a-SRBC		0	0
	Fr. 9-10-a	100	100
	Fr. 15-b	100	0
Fr. 15-b-SRBC		0	0
	Fr. 9-10-a	100	0
	Fr. 15-b	100	100

[a] Percentage inhibitions were calculated from values in four parallel titrations.

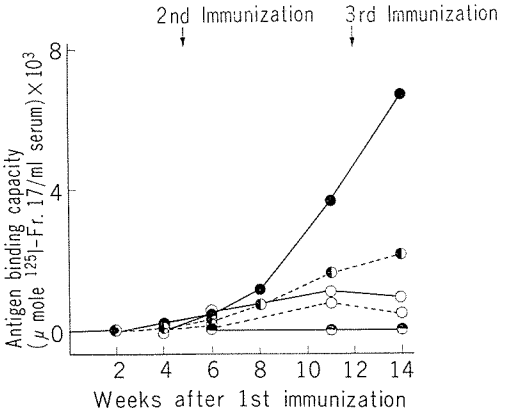

FIGURE 14. *Kinetic study of antibody level against Fr. 17 by direct binding of ^{125}I-Fr. 17.* ●——●, *no. 177 rabbit immunized with Fr. 17;* ◐- - -◐, *no. 176 rabbit immunized with Fr. 17;* ○——○, *no. 1711 rabbit immunized with native HL;* ○- - -○, *no. 1710 rabbit immunized with native HL;* ◓——◓, *no. 178 rabbit immunized with a low dose of native HL.*

ceived a large dose of HL also showed only a slight increase after the second immunization. The rabbit, which was immunized with a small dose of HL did not show any increase. This result excludes the possibility that the antibody response in rabbits immunized with Fr. 17 was due to contamination of the preparation with a very small amount of HL.

4. *Effects of various inhibitors on the binding of ^{3}H-acetyl-Fr. 17 with antibody elicited to Fr. 17 or HL.*

Tests on inhibition of the binding of ^{3}H-acetyl-Fr. 17 with antibodies of late antisera to Fr. 17 (no. 177(f)) and HL (no. 1711(f)) were performed using Fr. 17, Fr. 15-b, F. 9-10-a and HL as inhibitors. Fig. 15 shows the data obtained with late HL antiserum. Using an approximately equimolar concentration of Fr. 17, the binding of labelled Fr. 17 was inhibited by 40%, and at similar concentrations the degree of inhibition decreased in the order, HL>Fr. 15-b. Fr. 9-10-a was a very weak inhibitor of the antibody reacting with Fr. 17 evoked by HL. The degree of inhibition increased with increase in the con-

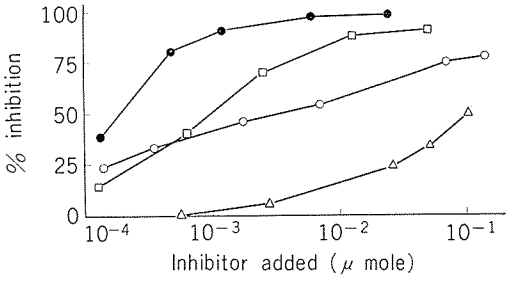

FIGURE 15. *Inhibition of binding of ^{3}H-acetyl-Fr. 17 with 50 μliters of no. 1711 (f) anti-HL antiserum, diluted 1: 5 in PBS-7, by HL* (○), *Fr. 17* (●), *Fr. 15-b* (□) *and Fr. 9-10-a* (△). *Values are means of values in two parallel experiments.*

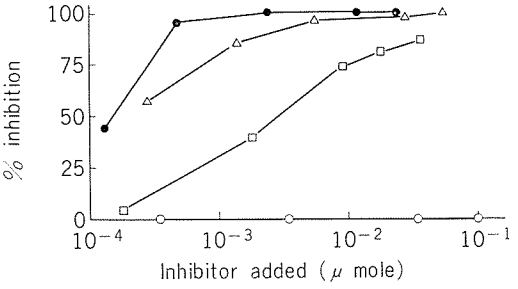

FIGURE 16. *Inhibition of binding of ^{3}H-acetyl-Fr. 17 with 25 μliters of no. 177 (f) antiserum, diluted 1: 5 in PBS-7, by HL* (○), *Fr. 17* (●), *Fr. 15-b* (□) *and Fr. 9-10-a* (△). *Values are means of values in two parallel experiments.*

centration of Fr. 17. The degree of inhibition by Fr. 15-b also increased with increase in concentration but inhibition was observed at a higher concentration range. The slope of the curve for increase in inhibition with increase in concentration of HL was not so steep as that of Fr. 17 or Fr. 15-b. The reason for this is unknown.

With late Fr. 17 antiserum (Fig. 16), HL did not inhibit the binding at all. The curve of Fr. 9–10-a was very similar to that of homologous Fr. 17. In this serum Fr. 15-b caused some inhibition of the binding, but definitely less than Fr. 9–10-a. These data indicate that antibodies produced by Fr. 17 have higher affinity to the Fr. 9–10-a portion of Fr. 17 than to the Fr. 15-b portion.

DISCUSSION

A prerequisite in studying the antigenicity of Fr. 17, an enzymic product of HL, is the purity of the preparation. Accordingly in the present work extensive efforts were made to remove contaminating HL from the preparation. For this purpose, after isolating the fraction from the peptic digest of HL it was rechromatographed on a CM-cellulose column and subjected to gel filtration on a Sephadex G–25 column equilibrated with 0.05 M acetate buffer (pH 3.85) containing 0.3 M NaCl. The final preparation of Fr. 17 obtained in this way contained less than 0.01% HL and less than 1% of other fragments. This purity seemed adequate to allow use of the preparation as an immunogen, because only a very weak antibody response was observed in a rabbit immunized with 1 μg of HL, which corresponds to 5 times the maximum contamination of the amount of Fr. 17 (2 mg) administered to rabbits in a single dose. The Fr. 9–10-a preparation was as pure as the Fr. 17 preparation judging from the results of amino acid analysis and measurement of enzymic activity of HL on *Micrococcus lysodeikticus*. The preparation of Fr. 15-b contained as little HL as Fr. 17, but was contaminated with traces of other fragments, as shown in Table 3. Nevertheless, this preparation seemed to be sufficiently pure for use in assay of antibodies.

The pattern of the antibody response to immunization with Fr. 17 assayed by PHA was different from that assayed by antigen binding experiments. The reason of this difference could be explained by a difference in class distribution of antibodies: namely, in the early antiserum to Fr. 17 only 19S hemagglutinin of Fr. 17–SRBC was detected while in the late antiserum both 7S and 19S hemagglutinins were demonstrated. The pattern of the antibody response to Fr. 17 assayed by PHA seemed to be mainly due to 19S hemagglutinin since on molecular basis 19S antibodies showed a higher efficiency of hemagglutination than 7S antibodies (Onoue et al., 1965; Hornick and Karush, 1972). On the other hand, 19S antibodies produced in the early stage of immunization showed a lower affinity than 7S antibodies produced in the later stage (Jerne and Avegno, 1956; Eisen and Siskind, 1964; Siskind and Benacerraf, 1969), so data on the antigen binding capacity were due to 7S antibodies.

Antibodies in early antisera to Fr. 17 were heterogeneous. The major population only reacted with Fr. 9–10-a (Fig. 11) while a minor population only reacted with Fr. 15-b (Fig. 7, 11). The latter seem to be distinct from the antibodies which also react with Fr. 15-b in HL antisera, because they did not agglutinate HL–SRBC strongly (Fig. 7). The late antisera to Fr. 17 may contain another antibody population which reacts with both Fr. 9–10-a and Fr. 15-b, in addition to the two kinds of antibodies described above, found in early antisera (Fig. 12, 16).

In an experiment on inhibition of ^{3}H-acetyl-Fr. 17 binding with HL antiserum, HL was less inhibitory than Fr. 17. The reason for this is unknown, but it is interesting in this connection that it is reported that HL antisera produced with the aid of FCA contained antibody which reacted with denatured (reduced and carboxymethylated) HL but not with native

HL (Scibienski, 1973).

The kinetic pattern of the PHA antibody response to Fr. 17 was different from that to HL, when both were assayed using Fr. 17–SRBC, Fr. 15-b–SRBC and Fr. 9–10-a–SRBC. These data seem to suggest that antigen recognition to the two immunogens in the process of the antibody formation was different. Results on the inhibition of binding of labelled Fr. 17 with anti-Fr. 17 and anti-HL antisera by Fr. 9–10-a and Fr. 15-b also support this possibility. In connection with this problem, the results of Sachs, et al. (1972) on staphylococcal nuclease are consistent with our data about the difference in the immunogenicities of native protein and its split fragment, while on the contrary Arnon and Sela (1969) reported that an HL fragment (sequence 64–83) bound to synthetic branched polypeptide elicited antibodies which reacted with the corresponding portion of native HL. The latter results could be explained by supposing that peptide 64–83 retains the native steric structure, because a disulfide bond between Cys^{65} and Cys^{80} may keep the native structure of the sequence Cys^{65}–Cys^{80}.

In this study no strong cross reactivity between anti-Fr. 17 antisera and native HL was seen. This was thought to be due to the drastic change of the three dimensional structure of fragment Fr. 17 during its isolation from native HL, even though fragment Fr. 17 retains some of its native α-helical structure, as reported by Matthyssens and Kanarek (1974).

Matthyssens and Kanarek (1974) also recently reported that the Tyr^{20}–Tyr^{23} region was the determinant group of Fr. 17 which combined with antibodies evoked by native HL. However, in their work HL was nitrated and this procedure may denature other determinant groups. No evidence in support of their conclusion was obtained in our studies.

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