PEPSIN DIGESTION OF RABBIT AND SHEEP ANTIBODIES

THE EFFECT ON COMPLEMENT FIXATION

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Pepsin digestion of precipitating 7S rabbit antibodies yields a smaller 5S fragment which is still capable of precipitating homologous antigen (1). This digestion is thought to remove most, if not all, of Porter fragment III (1). Treating the 5S fragment with cysteine yields two fragments, which behave as Porter fragments I and II (1). Fragment III, when aggregated, can fix complement; fragments I and II cannot (2). The conclusion has been drawn that the complement-fixing ability of antibody is due to fragment III, and that the 5S fragment is, therefore, incapable of fixing complement (3).

Reinvestigation of this problem originally for other purposes, led to the finding that 5S rabbit, as well as 5S sheep antibody does retain certain complement-fixing abilities. However, as we intend to show, the complement-fixing abilities of 7S and 5S antibodies are both quantitatively and qualitatively different. A preliminary report of some of these findings has appeared (4).

Materials and Methods

Antiserum.—Adult New Zealand strain rabbits and adult sheep were immunized with human serum albumin (HuSA) in complete Freund's adjuvant. After a sufficiently high antibody titer was achieved, periodic booster injections and bleedings were made. Serum was stored at −20°C prior to use.

The 7S gamma globulin was isolated from pooled antiserum by a slight modification of the triple, solid sodium sulfate precipitation method of Kekwick (5). After the third precipitation, the antibody solution was extensively dialyzed against phosphate-saline buffer (pH 6.9, 0.17 M). The protein solution was then centrifuged in a Spinco model L preparative ultracentrifuge at 100,000 *g* for 18 hours at 4°C. The macroglobulin and aggregated gamma globulin sediment at the bottom of the tube and the top lipoprotein fractions were discarded. The 7S gamma globulin preparation, at 10 mg/ml, gave a single peak in a Spinco model E analytical ultracentrifuge, with a calculated (6) sedimentation constant ($s_{20,w}$) of 6.4 for both the rabbit and sheep gamma globulins. (There was occasionally a very small amount of 19S globulin in some of the sheep preparations.) The preparations were stored at −20° or 4°C prior to use, and repeated thawing and freezing were avoided.

Pepsin Digestion.—Portions of the 7S gamma globulins were dialyzed against acetate-sodium chloride buffer (pH 4.1, 0.12 M). Rabbit 7S gamma globulin was treated with 2 to 3 per cent pepsin (by weight protein) for 18 hours at 37°C (1). Sheep 7S gamma globulin

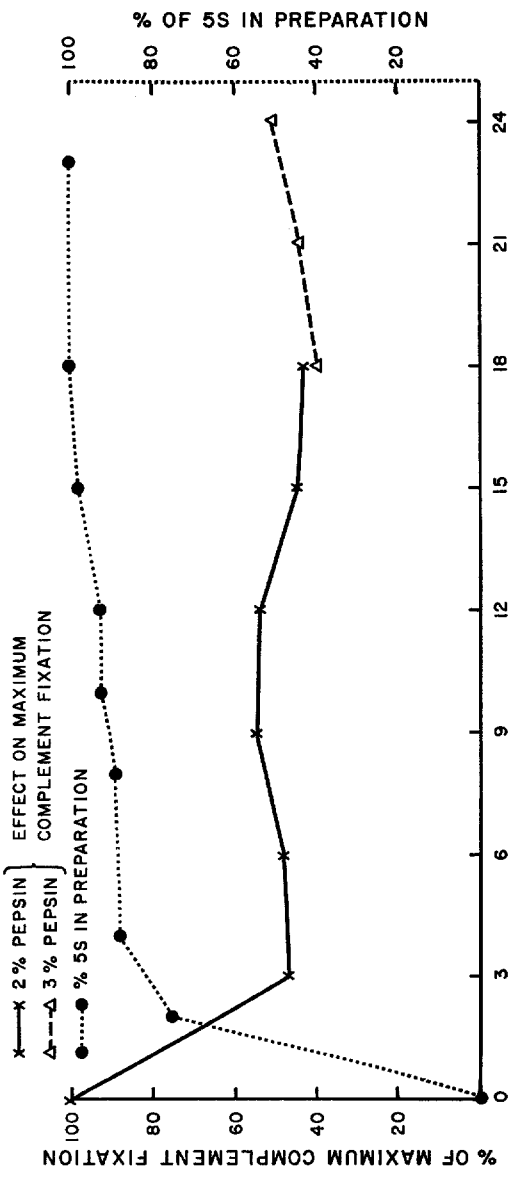

FIG. 1. The change with time of the proportion of 5S antibody produced by pepsin digestion (●·····●), and of the complement-fixing properties of the antibody (X—X,Δ---Δ).

was digested with varying concentrations of pepsin for varying lengths of time (Fig. 1). Digestion with 2 to 3 per cent pepsin for 18 hours was found to be sufficient for complete conversion of the sheep 7S to 5S globulin. When the incubation was complete, insoluble sediment (representing Porter fragment III) was removed by centrifugation at 4°C. The pepsin was then inactivated by the dropwise addition of 1 N NaOH to a final pH of 8. The 5S fragment was isolated by precipitation with 18 gm per cent solid sodium sulfate, followed by extensive dialysis against phosphate-saline buffer. Both sheep and rabbit 5S preparations, at a concentration of 10 mg/ml, gave a single peak in the analytical ultracentrifuge with a calculated (6) sedimentation constant ($s_{20,w}$) of 4.7S; there was no material of greater sedimentation constant present. The 5S antibody solutions were stored at −20° or 4°C prior to use, and repeated freezing and thawing were avoided. Both 7S and 5S antibody preparations always were centrifuged at 1000 *g* for 30 minutes at 4°C, immediately prior to testing.

Antibody and Antigen Nitrogen (N).—These were determined by the Markham micro Kjeldahl method (7, p. 476).

Complement Fixation Using Guinea Pig Serum.—Complement fixation was performed with a large excess of complement using the sensitive modification of the method of Kabat and Mayer (7, p. 224) described by Barbaro and Becker (8). Fixation was carried out in a total volume of 1.0 ml for 1 hour at 37°C. The measurement of hemolysis was performed in a total volume of 1.5 ml. Complement fixation by immune aggregates (formed at equivalence) was studied both with washed preformed antigen-antibody aggregates (to be referred to as preformed aggregates in the rest of the text) and antigen-antibody aggregates formed in the presence of complement.

Antigen and antibody solutions were checked for anticomplementary activity with the following results: egg albumin and human serum albumin were not anticomplementary in the concentrations used: rabbit 7S antibody was anticomplementary at $\geq 5\mu g$ antibody N, rabbit 5S at ≥ 80 μg antibody N; sheep 5S was anticomplementary at ≥ 80 gm antibody N and sheep 7S was anticomplementary even at 1.5 μg antibody N.

The per cent of total complement fixed was calculated as:

$$\frac{\text{C'H}_{50}\text{[1] units fixed}}{\text{total C'H}_{50}} \times 100$$

When the antibody solution (Ab sol), by itself, had anticomplementary activity, the per cent of total complement fixed by the antibody-antigen aggregates formed in the presence of complement (Ab-Ag-sol) was calculated by:

$$\frac{(\text{C'H}_{50}\text{ fixed by Ab-Ag-sol}) - (\text{C'H}_{50}\text{-fixed by Ab sol})}{\text{Total C'H}_{50} - (\text{C'H}_{50}\text{-fixed by Ab sol})} \times 100$$

It was assumed that the preformed aggregates had no "anticomplementary" activity since they were washed free of unbound gamma globulin. The per cent of total complement ($C'H_{50}$) fixed was plotted against the concentration of the antibody nitrogen on semilog paper.

Absorption Studies.—Four ml of guinea pig serum was absorbed for 18 hours at 4°C with a washed preformed aggregate containing 2 mg of 5S antibody N. The precipitate was removed by high speed centrifugation, and then the absorbed guinea pig serum served as a source of complement for further experiments using fresh immune aggregates containing 7S and 5S antibody. Absorptions also were carried out in the presence of 0.001 M ethylenediaminetetraacetate acid (EDTA).

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[1] $C'H_{50}$ refers to the amount of complement that will hemolyze 50 per cent of a standard volume of sensitized sheep erythrocytes.

Treatment of Immune Precipitates with Cysteine.—A number of samples of washed preformed aggregates containing 7S or 5S antibody were dialyzed first against 1000 volumes of 0.01 M cysteine in phosphate-saline buffer at 35°C for 2 hours, then against 1000 volumes of 0.02 M iodoacetamide in phosphate-saline buffer at 4°C for 24 hours, and then further dialyzed against phosphate-saline buffer. Control aggregates were treated in a similar manner, except that dialysis against phosphate-saline buffer was substituted for that against cysteine.

Complement Fixation Using Rabbit Serum.—A number of experiments using fresh rabbit serum as a source of complement were done in the same manner as with guinea pig serum. However, because of the lower total complement level in rabbit serum (20 to 60 $C'H_{50}$ units/ml), at the end of the 1 hour period of fixation, 6.5 ml of TBS at 4°C was added, instead of the 19 ml of TBS used in the guinea pig system (8).

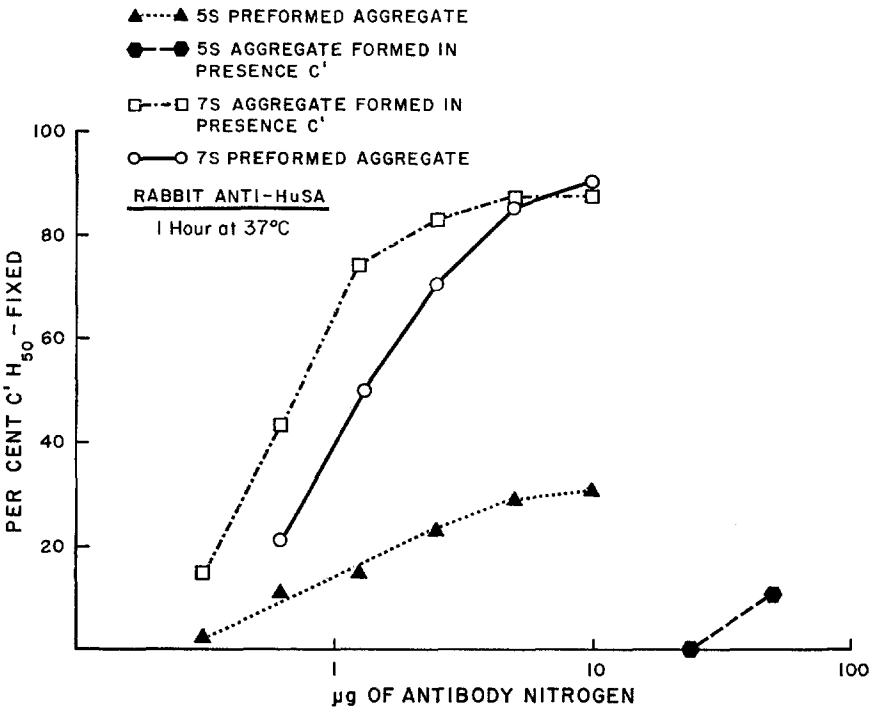

FIG. 2. Comparison of the complement-fixing abilities of 7S and 5S rabbit anti-HuSA antibody, including antibody-antigen aggregates formed in the presence of complement and washed preformed antibody-antigen aggregates.

RESULTS

Complement Fixation with Guinea Pig Complement.—

(*a*) *Rabbit anti-HuSA:* Fig. 2 depicts the relative complement-fixing properties of 7S and 5S rabbit anti-HuSA washed preformed antibody-antigen aggregates and antibody-antigen aggregates formed in the presence of comple-

ment. The results were representative of over 8 experiments on 3 preparations of 2 different pools of rabbit antisera to HuSA. The results are identical with those reported for 7S and 5S rabbit antibodies to egg albumin (4). Here, the logarithm of the concentration of the 7S antibody contained in the immune aggregates is plotted against the per cent of the total complement fixed. The 7S immune aggregates formed sigmoid-shaped curves. Comparing the middle, straight parallel portions of the curves, the 7S antibody-antigen aggregates formed in the presence of complement were up to 2 times more active in fixing complement than were 7S preformed aggregates.

The amount of complement fixed by the 5S preformed aggregates when plotted, as above, formed a hyperbolic curve. The 5S preformed aggregates were approximately 100 times more active in fixing complement than were 5S antibody-antigen aggregates formed in the presence of complement.

The 7S preformed aggregates always fixed more complement than did 5S immune aggregates. However, as seen from Fig. 2, there were other differences between 7S and 5S preparations which were at least as striking. The ascending slopes of the straight portion of the lines for the 5S and 7S washed aggregates were markedly different. This of course, made it impossible to quantitatively compare the complement-fixing abilities of the two antibodies. Moreover, all of the complement was fixed with the addition of sufficient 7S immune aggregate; this was not true of the 5S immune aggregates. With the 5S preformed aggregate pictured in Fig. 2 no more than 31 per cent of the 100 $C'H_{50}$ units added was fixed, no matter how much 5S aggregate was added.

In different experiments, this maximum which the 5S was capable of fixing varied from 20 to 40 per cent of the total complement added when different 5S antibodies and different guinea pig sera were used.

Since apparently not all of the complement added was capable of being fixed by 5S antibody containing aggregate, it was of interest to calculate the proportion of complement which was fixed by a given amount of 5S antibody, not in terms of the total complement added, but rather in terms of the maximum amount of complement which the 5S preformed aggregate was capable of fixing. This was done by calculating the ratio of the number of the units of complement fixed by a given quantity of 5S preformed aggregate to the maximum amount fixable by the 5S preformed aggregates; *i.e.*,

$$\frac{\text{per cent total } C'H_{50}\text{-fixed}}{\text{maximum } C'H_{50}\text{-fixed}} \times 100 \text{ per cent.}$$

This was plotted against the antibody N concentration in the usual manner. In Fig. 3 it is seen that the curve for the 5S preformed aggregate could be superimposed on the curve for per cent total complement fixed by 7S preformed aggregate.

(*b*) *Sheep anti-HuSA:* Fig. 4 depicts the relative complement-fixing of 7S

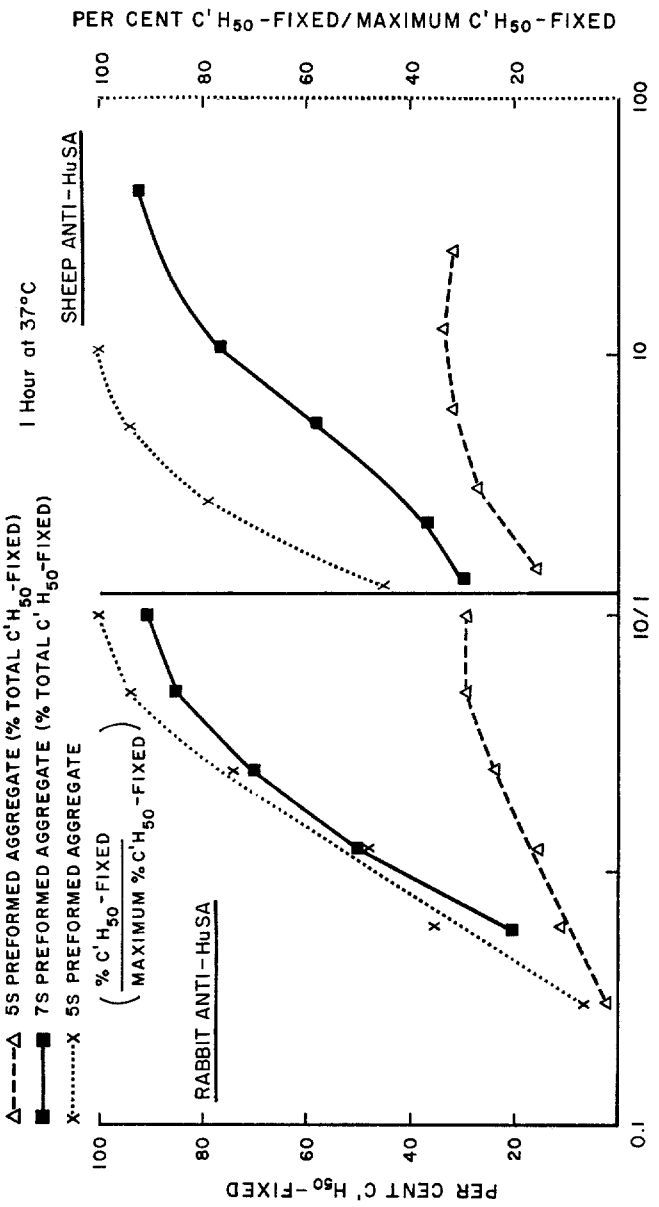

FIG. 3. Comparison of the complement-fixing abilities of 7S and 5S washed preformed aggregates in terms of total complement added (Δ----Δ), and maximum fixable complement (X·····X).

and 5S sheep anti-HuSA preformed aggregate, and antibody-antigen aggregates formed in the presence of complement. These results were representative of over 40 experiments on 20 preparations of 4 different pools of sheep anti-HuSA antiserum.

Plotting the per cent of total complement fixed by the 7S immune aggregates against the logarithm of the concentration of 7S antibody gave the typical sigmoid-shaped curves. Using the middle, straight, parallel portion of these curves one could estimate that the 7S preformed aggregates were 3 times

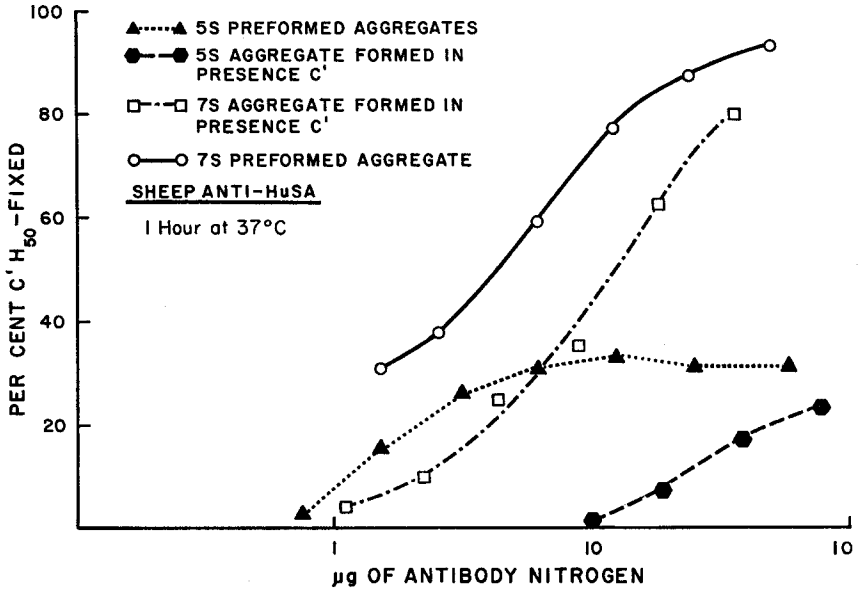

FIG. 4. Comparison of the complement-fixing abilities of 7S and 5S sheep anti-HuSA antibody, including antibody-antigen aggregates formed in the presence of complement and washed preformed antibody-antigen aggregates.

more active in fixing complement than were the 7S antibody-antigen aggregates formed in the presence of complement.

The 5S preformed aggregates were 30 times more active in fixing complement than were the 5S antibody-antigen aggregates formed in the presence of complement. The 7S preformed aggregate fixed more complement than did 5S preformed aggregate. The slopes of the lines were parallel at low antibody N concentrations, but at higher concentrations the slopes diverged. Therefore, it was difficult to compare quantitatively the relative complement-fixing abilities of the sheep 7S and 5S antibodies. However, as with rabbit 5S, the sheep 5S preformed aggregates fixed complement up to a maximum, which was well below the total complement added. This maximum complement fixation

varied from 20 to 40 per cent of the total complement added, when different 5S antibody preparations, and/or guinea pig sera were used. When one then calculated the

$$\frac{\text{per cent total } C'H_{50}\text{-fixed}}{\text{maximum } C'H_{50}\text{-fixed}} \times 100 \text{ per cent}$$

for preformed aggregates, the plotted curve (Fig. 3) was parallel, but shifted to the left on the abscissa, to the curve of per cent total complement fixation

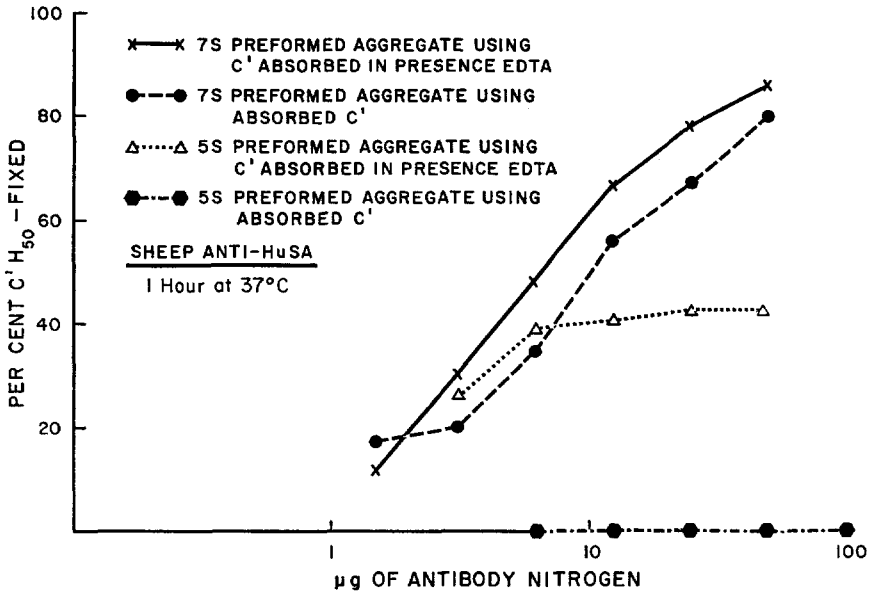

FIG. 5. Effect of prior absorption of guinea pig serum with sheep anti-HuSA washed preformed 5S antibody-antigen aggregate, on the complement-fixing ability of fresh 5S and 7S sheep immune aggregates.

for 7S preformed aggregates. Therefore, it appeared that the sheep 5S preformed aggregate fixed that portion of complement which it was capable of fixing with actually greater ability than the 7S fixed total complement.

Absorption Studies (Fig. 5).—

A. Sheep antibody: In the experiment pictured in Fig. 5, when guinea pig serum was absorbed with 5S washed preformed aggregates, 33 per cent of the total hemolytic complement was removed. The 67 per cent remaining was found to have lost all of its ability to fix with a fresh 5S immune aggregate. In other experiments most, but not all, of the "5S fixable complement" was removed by absorption. However, the 7S immune aggregate fixed this absorbed complement to the same degree as it would unabsorbed guinea pig serum. When the

absorption was carried out in the presence of 0.001 M EDTA, and then an equimolar concentration of Ca^{++} was added, 12 per cent of the total complement activity was lost, but the 7S and 5S immune aggregates fixed the 88 per cent remaining complement to the same respective degrees as they did using the unabsorbed guinea pig serum.

B. Rabbit antibody: The same general results were obtained with absorption of guinea pig serum with 5S rabbit preformed aggregates as with sheep 5S.

TABLE I

Effect of 0.01 M Cysteine Treatment on the Complement-Fixing Properties of Washed 7S and 5S Preformed Aggregates

Antiserum	Antibody N	$C'H_{50}$-fixed					
		5S			7S		
		Untreated	Control	Cysteine	Untreated	Control	Cysteine
	μg	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Rabbit anti-HuSA	100	42	45.5	0			
	50	42.5	44.5	0			
	25	41	44.5	5			
	10	31	34	6	90.5	92	77.5
	5	29	—	0	85.5	79.5	66
	2.5	23	—	—	70	66	54.5
	1.2	15	—	—	50	54.5	43.5
	0.62	11	—	—	21	—	—
	0.31	2	—	—	0	—	—
Sheep anti-HuSA	200	50		6.5			
	100	50		0			
	50	50		1	83		83
	25	50		7	71		74
	12.5	50		5	55		61
	6.2	45		0	53		49

Cysteine Treatment of Washed Preformed Antibody-Antigen Aggregates (Table I).—

Washed preformed aggregates, containing 5S rabbit antibody, treated with cysteine for 1 hour lost about 50 per cent of their complement-fixing ability. However, 2 hours of treatment with cysteine destroyed essentially 100 per cent of the complement-fixing abilities of sheep and rabbit 5S, but only ≤ 20 per cent of rabbit 7S and 0 per cent of sheep 7S antibody.

Complement Fixation with Different Sources of Complement.—

The same 5S rabbit anti-HuSA preformed aggregate fixed only a quantitatively identical portion of complement, as depicted in Fig. 2, using either (*a*)

fresh guinea pig serum, (*b*) that same guinea pig serum after it had been frozen at −20°C for 24 hours and then thawed, or (*c*) reconstituted lyophilized guinea pig serum. A preparation of 7S rabbit preformed aggregate fixed total complement in the same quantitative manner as depicted in Fig. 2, no matter which of the above 3 preparations of guinea pig serum was used.

The same sheep and rabbit 5S washed preformed aggregates fixed from 0 to 100 per cent, usually about 20 per cent, of the complement from many different rabbit sera. This marked variation was not found with guinea pig sera, and is probably related to the very low total amount of complement found in rabbit sera (20 to 60 $C'H_{50}$ units/ml) in contrast to guinea pig serum (800 to 1100 $C'H_{50}$ units/ml).

Complement fixation was also tested in a total volume of 10 ml (7, p. 214), using the same proportions of reagents as when fixation was carried out in 1 ml (8). The titration of complement left was then performed both by the method of Barbaro and Becker in 1.5 ml (8), and by that of Mayer in 7.5 ml (7, p. 149). Both methods showed that it required 10 times as much antibody nitrogen in the 10 ml system to get the same amount of complement fixation as was found in the 1 ml system. This was true for both 7S and 5S rabbit antibody.

DISCUSSION

It is evident from the results just presented that the 5S antibody preparations, from pepsin-digested 7S rabbit and sheep antibody, retained a small but definite ability to fix guinea pig complement. This is contrary to the conclusions of others (3, 9, 10). However, Amirian and Leikhim (11) found that pepsin-digested rabbit antibody to sheep erythrocytes still retained some complement-fixing ability, while a pepsin-digested rabbit antibody to pertussis did not. Baxter and Small (12) found "little, if any," complement-fixing *in vivo* with pepsin-treated rabbit anti-rat kidney antibody.

Pepsin digestion of 7S rabbit antibody removes most if not all, of Porter fraction III (1). Fraction III is insoluble in phosphate-saline buffer, and was removed from the 5S preparation by centrifugation. It was highly unlikely that contamination of the 5S preparation by fraction III could have accounted for the complement-fixing ability of the 5S antibody. No 3.5S material (*i.e.* Porter fraction III) was found in ultracentrifugal analysis of our 5S preparations. More important, Taranta and Franklin have shown that rabbit fraction III added to 5S antibody confers no added complement-fixing ability on the latter (3).

Analysis of each 5S preparation in the ultracentrifuge also gave no evidence of a component with a sedimentation coefficient of 7S. Because of the much greater sensitivity of complement fixation as compared to sedimentation analysis, this evidence suffices only to demonstrate that there was no gross

contamination of the 5S preparations by 7S antibody; it cannot answer the question of whether the complement fixation demonstrated by the 5S antibody was due to contamination with trace amounts of 7S antibody.

In this regard, it is of interest that at 18 hours when the pepsin-digested sheep 7S antibody had reached a minimum in complement-fixing ability, the same digest showed only 5S material by ultracentrifugal analysis (Fig. 1). Digestion of either sheep or rabbit antibody with 3 to 5 per cent pepsin for as long as 24 hours did not cause a further decrease in complement-fixing ability of the 5S antibody.

With respect to the question of possible contamination of the pepsin-digested antibody with 7S antibody, the decisive point, however, is that the complement-fixing activity of the pepsin-digested antibody is qualitatively different from that of the undigested 7S antibody. When one considers only the complement-fixing ability of the preformed aggregates as pictured in Figs. 2 and 4, it is evident that the slopes of the curves for 5S antibody are distinctly less than the slopes of the 7S curve. If the fixation of the 5S preparation were due to contamination by a small amount of 7S antibody, the method of plotting is such that the 5S curves would have the same slope as the 7S, but would merely be shifted to the right on the abscissa by a distance reflecting the degree of contamination.

In addition, immune complexes made with 5S antibody are incapable of fixing all of the complement which was added; as seen in Figs. 2 and 4, increasing the amount of preformed 5S antibody-antigen aggregates beyond a certain level did not increase the complement fixation. This behavior was in vivid contrast to that of 7S antibody, where there was a progressive increase in the amount of complement fixed, until upon the addition of sufficient 7S immune aggregates, all of the complement added was fixed. Thus the "ceiling" on the amount of complement fixable by the 5S preformed aggregate would make it appear that the 5S antibody is responsible by itself for the fixation of complement. Moreover, this behavior of the 5S antibody discloses a heretofore unrecognized heterogeneity of guinea pig complement, there being at least two portions of complement, one capable of being fixed by 5S antibody, and the other not. This heterogeneity was confirmed by the finding that only the former portion could be absorbed from guinea pig serum by prior treatment with performed 5S antibody-antigen aggregates. Such absorbed guinea pig serum served to further define the difference between 7S and 5S immune aggregates, since the complement remaining after absorption could be fixed by 7S immune aggregates but not by 5S aggregates.

When the ceiling on the complement fixation by the 5S antibody was first observed, it was attributed to the combination of an antigenic component of the 5S immune aggregate with a natural antibody contained in guinea pig serum, with a resultant fixation of complement. The absence of complement-fixation

of absorbed guinea pig serum with 5S immune aggregates could then be explained by the removal of this supposed natural antibody. This explanation was rendered untenable, or at least extremely unlikely, when it was found that no decrease of the 5S fixable complement occurred when the absorption by the 5S preformed aggregates took place in the presence of 0.001 M EDTA.

The action of cysteine on the complement-fixing ability of the 5S preformed aggregate is characteristic of the action of sulfhydryl compounds on 5S antibodies (1), and is distinctly different from their effect on 7S antibody (13). Cysteine destroyed essentially all of the complement-fixing ability of the 5S complexes by the production of the non-complement-fixing fractions I and II (2). Cysteine did have some effect on the rabbit 7S preformed aggregate, but this was qualitatively and quantitatively different from the effect on the 5S preformed aggregates.

The accumulation of evidence just reviewed leaves little doubt that the complement-fixing activity of the 5S immune aggregates is not due to contamination with unaltered 7S antibody, and is thus presumably due to the 5S antibody itself. Numerous questions are obviously raised by these observations, which for the present must remain unanswered. The nature of the portion of complement, which is capable of being fixed by 5S antibody, or which of the components of complement is being fixed, is completely unclear. The 5S fixable complement appears in a relatively constant amount in guinea pig serum which has been freshly drawn, freshly frozen, or lyophilized. It also appears to a highly variable degree in rabbit serum. Whether it is present *in vivo* in either species is not now known. There is no good explanation for the difference in complement-fixing abilities of washed preformed aggregates and antibody-antigen aggregates formed in the presence of complement.

The complement-fixing site on the 7S antibody molecule has obviously been changed by pepsin treatment, if not completely eliminated. The nature of this change is not known, although the results pictured in Fig. 1 make it possible that this change might be stepwise. From our own results as well as those of Amirian and Leikhim (11) and Baxter and Small (12) already referred to, it would appear that different 5S antibodies vary somewhat in their ability to fix complement. The results pictured in Fig. 1 make it unlikely that this is related to the completeness of digestion by pepsin, but whether this is related to slight variation in procedure from experiment to experiment, or is inherent in the 7S antibody used for digestion, is unclear. It is also unclear whether the action of pepsin and papain on the complement-fixing site of 7S antibody is the same, and thus whether Porter fraction III contains all or only a portion of the complement-fixing site(s).

The finding of residual complement-fixing capacity in 5S antibody, although opening further areas of investigation of the nature of complement and the complement-fixing site of antibody, unfortunately makes difficult the un-

ambiguous interpretation of experiments designed to use 5S antibody for the investigation of the biological role of complement (9, 14).

SUMMARY

7S sheep antibody is similar to 7S rabbit antibody, insofar as it too can be digested with pepsin to yield a 5S fragment still capable of precipitating homologous antigen. The 5S fragment from rabbit as well as sheep antibody is capable of fixing guinea pig complement. However, this fixation differs from the fixation by 7S antibody, since preformed antibody-antigen aggregates containing either sheep or rabbit 5S antibody fix only a maximum of 20 to 40 per cent of the total complement. The portion of complement which is fixable by the 5S preformed aggregates seems to be different from the remainder of the complement. Prior treatment of guinea pig serum with 5S washed preformed aggregates removes that portion of complement fixable by 5S antibody, and will leave the complement remaining unable to be fixed by further addition of 5S immune aggregates. Such absorbed complement, however, is as capable as is unabsorbed complement of being fixed by 7S antibody. The 5S sheep and rabbit antibody fixes the portion of complement which it is capable of fixing, as well as or better than the corresponding 7S antibody fixes the total complement. 5S preformed aggregates are broken down by treatment with cysteine, and their complement-fixing ability is lost.

The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

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