

IMMUNOCHEMISTRY OF SERUM ALBUMIN—IV

IMMUNOCHEMICAL CROSS-REACTION OF FRAGMENTS FROM THE FIRST THIRD AND THE LAST THIRD OF BOVINE SERUM ALBUMIN*

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Abstract—Antisera were raised in rabbits against fragment 11-193 (less Arg-144) or fragment 377-571 of bovine serum albumin (BSA). Several rabbits and bleedings at different time intervals from each rabbit were studied. Fragment 377-571, corresponding to the last third of BSA, was invariably more immunogenic than fragment 11-193. Antisera to fragment 377-571 showed, in quantitative precipitin analysis, appreciable cross-reactions with BSA and with fragment 11-193, which increased with time after immunization. Similar results were obtained with antisera to fragment 11-193. The reactivity of antisera to fragment 377-571 with BSA was removed almost completely by absorption with fragment 11-193. Conversely, absorption of the antisera with BSA removed their reaction with fragment 11-193 almost entirely. Joint absorption by BSA and fragment 11-193, each at equivalence, gave a reaction similar to that obtained by either fragment 11-193 alone or BSA alone in the region of antigen excess. After absorption of antisera to fragment 377-571 with BSA, addition of fragment 11-193 at equivalence (relative to the unabsorbed antiserum) gave no immune precipitate but inhibited 100% the precipitin reaction of the absorbed antisera with fragment 377-571. However, antibodies in these antisera were removed quantitatively on an immunoabsorbent of fragment 377-571. Similar findings were obtained in absorption studies on antisera to fragment 11-193. A BSA-immunoabsorbent removed quantitatively all the antibodies in whole antisera to fragment 377-571. Similarly, an immunoabsorbent of fragment 11-193 removed all these antibodies. Antibodies recovered from these immunoabsorbents did not react best in precipitin reaction with BSA or fragment 11-193, but with fragment 377-571. In this respect, they behaved exactly like the original whole antiserum and explained the observed differences in the precipitating ability of the antisera with fragments 11-193, 377-571 and BSA. These studies confirmed that BSA has repeating identical antigenic sites. Furthermore, fragments 377-571, 11-193 or BSA were essentially the same immunochemically but antisera directed against any of these as an immunogen differed only in their abilities to form insoluble immune complexes, and not in their abilities to bind with the other two cross-reacting antigens.

INTRODUCTION

In recent studies from our laboratories we have reported (Atassi *et al.*, 1976a) the isolation, characterization and immunochemistry of a fragment from the N-terminal third (sequence 11-193) of bovine serum albumin (BSA). Also, the preparation of another fragment from the C-terminal third (sequence 377-571) of BSA was reported (Habeeb & Atassi, 1976), together with similar detailed immunochemical studies. Neither fragment gave an immune precipitate with antisera to BSA but each by itself inhibited the reaction of BSA with its antisera almost completely (89-93%). Also, each fragment, on an immunoabsorbent, removed almost all (89-95%) of BSA antibodies from the antisera. On the basis of these findings we

advanced the concept that BSA carried identical repeating antigenic determinants (Atassi *et al.*, 1976a; Habeeb & Atassi, 1976). In the present work, this concept was subjected to further investigation by preparing antisera to each of the aforementioned fragments (11-193 and 377-571) and studying the cross-reactions of the two fragments as well as of BSA with these antisera.

MATERIALS AND METHODS

Materials

Bovine serum albumin (4× crystallized) was obtained from ICN Pharmaceuticals Inc. Trypsin-TPCK was from Worthington Biochemicals Corp. Fragment 11-193 was prepared by tryptic hydrolysis of citraconyl-BSA, followed by removal of the citraconyl masking-group and gel filtration on Sephadex G100 as recently described in detail (Atassi *et al.*, 1976a). Fragment 377-571 was isolated from a partial tryptic hydrolysate of BSA by gel filtration on Sephadex G-100, followed by chromatography on DEAE-cellulose (Habeeb & Atassi, 1976). Each of the fragments was shown to be homogeneous by disc-electrophoresis. Immunoabsorbents carrying fragment 11-193 or fragment 377-571 were prepared as previously described (Atassi *et*

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† Abbreviations used: BSA, bovine serum albumin.

al., 1976a; Habeeb & Atassi, 1976). Also, BSA or fragment were coupled onto sepharose by benzoquinone (Brandt *et al.*, 1975). Each immunoabsorbent contained 3.2 mg of the respective antigen/ml of packed sepharose.

Antisera

Antisera to fragment 377-571 or fragment 11-193 were raised in rabbits. Two different immunization schedules were employed:

Schedule (A). A rabbit was immunized with a given fragment by administering two injections intramuscularly (each, 5 mg protein in 1 ml of 1:1 emulsified mixture of phosphate-saline and Freund's complete adjuvant) 3 weeks apart. The rabbits were bled weekly starting 2 weeks after the last injection.

In order to prepare sufficient quantities of antisera for certain experiments from one rabbit, antisera of different bleedings were pooled as follows: (1) bleedings of weeks 7-10; (2) bleedings of weeks 11-13; (3) bleedings of weeks 14-16. Antisera from different rabbits were not mixed. The pools were freeze-dried and stored at -20°C . Portions of the freeze-dried materials were reconstituted to the original volume with distilled water immediately before use. Rabbits 487, 488 and 489 were injected with fragment 377-571, while rabbit 492 was injected with fragment 11-193.

Schedule (B). Rabbits were immunized at weekly intervals up to the third injection. In each immunization, a rabbit received 3-4 mg of protein or fragment as an emulsion (1.5 ml) of 1:1 (v/v) of the protein solution in 0.01 M phosphate, pH 7.2 0.15 M NaCl and Freund's complete adjuvant. For the first immunization, the rabbit was injected in each of the hind foot pads (0.5 ml each) and subcutaneously in the neck (0.5 ml). Subsequent immunizations were given intramuscularly in each of the rabbit's hind flanks (0.5 ml each) and subcutaneously in the neck (0.5 ml). Test bleedings (about 50 ml) were taken from the ear four days after the first injection and then every 3-4 days thereafter up to one month. Following the first 3 immunizations, the rabbits were bled and boosted monthly up to 6 months, after which they were bled and boosted bi-monthly up to a year after the first injection. Antisera from individual bleedings were not pooled but were kept (-40°C) and studied separately. Rabbits B5 and B7 were immunized by this schedule with fragment 377-571.

Immunochemical methods

Agar double-diffusion and quantitative precipitin experiments were done as previously described (Atassi & Saplin, 1968). In the present work, quantitative precipitin reactions were carried out on aliquots (100 μl) of antiserum and equal volumes of varying amounts of antigen in 0.01 M phosphate buffer at pH 7.2 containing 0.15 M NaCl. The mixture was incubated at 38°C for 1 hr and then at 4°C for 2 days with occasional mixing. The precipitate was recovered by centrifugation and washed on the centrifuge 5 times with phosphate/saline buffer. The precipitates were

dissolved in 5% sodium dodecyl sulfate (100 μl). The solutions were diluted with water (0.5 ml) and then mixed with 1.5 ml of reagent C (Lowry *et al.*, 1951). After standing for 20 min, Folin reagent (150 μl) was added. The absorbance was read at 750 nm and converted to mg of antibody/ml of antiserum at equivalence by reference to a standard curve with known amounts of rabbit IgG treated in a similar manner and run simultaneously. No corrections were made for the antigen in the immune precipitate since it represented a very small fraction of the total absorbance. All quantitations were based on the mg per ml recovery. These values for the antisera used in the present studies are shown in Table 1.

Quantitative absorption experiments were carried out as previously described (Atassi & Saplin, 1968) by mixing an aliquot of a given antiserum with an amount of antigen necessary to achieve equivalence as determined from the precipitin curve. After removal of the precipitate, the absorbed antiserum was examined for its reactions with various antigens by quantitative precipitin analysis and the reactions were compared to those of the corresponding unabsorbed and appropriately diluted antiserum.

The amounts of specific antibody to a given fragment were also determined, in whole or absorbed antisera, by binding on an immunoabsorbent of the respective fragment as described elsewhere in detail (Atassi *et al.*, 1976a; Habeeb & Atassi, 1976). The antibody bound to the immunoabsorbent was eluted with 5 M guanidine hydrochloride and determined from the volume and absorbance of the eluent at 280 nm.

The IgG fraction was prepared from the antisera to fragment 11-193 by dialysis of the antiserum (3 ml) against several changes of 0.0175 M sodium phosphate buffer, pH 7.3, followed by application on a DEAE-cellulose column ($2 \times 25 \text{ cm}$) that had been pre-equilibrated with the same buffer. The unretarded peak corresponded to the IgG fraction of the antiserum. This step was necessary because low levels of antibody were obtained by immunization with fragment 11-193.

RESULTS

Reactions with antisera to fragment 377-571

In agar double-diffusion with these antisera, each of BSA and fragment 11-193 gave a single sharp line which fused completely with the single line of fragment 377-571, showing no spurs or intersections. With the 10-week bleeding of one antiserum (B7) to fragment 377-571, a spur was observed between fragment 377-571 and fragment 11-193 (as a continuation of the homologous-antigen line). This spur was very faint and must have accounted for a very small percentage of the total antibody. The spur was not observed in the earlier bleeding. Antisera to fragment 377-571 from 5 different rabbits at different

Table 1. Antibody titers of the antisera and the antigen employed in immunization

Rabbit	Antigen	18-day bleeding ^a	Antiserum titer (mg antibody/ml antiserum) ^a			
			10-week bleeding ^b	7-10-week pool ^c	11-13-week pool ^c	14-16-week pool ^c
B5 ^b	Fragment 377-571	0.71	3.45			
B7 ^b	Fragment 377-571	1.15	4.60			
487 ^c	Fragment 377-571			4.65	7.26	4.69
488 ^c	Fragment 377-571			0.71		
489 ^c	Fragment 377-571			5.73	9.30	
492 ^c	Fragment 11-193			0.72	0.41	0.25

^a Antibody content of the antiserum was determined from immune precipitation, at the equivalence point with the homologous antigen, as determined by the Folin reaction and comparison with a standard curve of rabbit IgG run simultaneously.

^b These represent single bleedings at the periods indicated after the first injections (immunization schedule B).

^c These represent pools of the indicated weekly bleedings after the first injection (immunization schedule A).

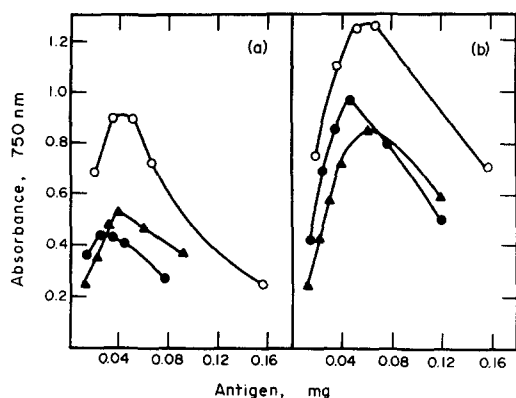


Fig. 1. Quantitative precipitin reactions with antisera (489) to fragment 377-571. (A) Antiserum from the pool of the 7-10-week bleedings; (B) antiserum from the 11-13-week pool; (O) fragment 377-571; (●) BSA; (▲) fragment 11-193.

periods during the immunization schedule were also studied by quantitative precipitin analysis. The antibody titers of the antisera from different bleedings of various rabbits, as determined from the equivalence points of quantitative precipitin reactions with the homologous antigen, are shown in Table 1. In quantitative precipitin analysis with BSA or with fragment 11-193 these antisera gave an immune precipitate. Figure 1 shows examples of the quantitative precipitin curves with antisera from the 7-10-week pool and the 11-13-week pool of rabbit 489. Table 2 summarizes extensive studies of the reactions of various bleedings from 5 different rabbits. It can be seen with rabbits B5 and B7, where single bleedings at 18 days and at 10 weeks were monitored, that the cross-reactions of the antisera with BSA was appreciable in the early bleedings (37 and 60%) and became even higher in the 10-week bleedings (63 and 85%). It was even more remarkable that antisera to fragment 377-571 from the C-terminal third of BSA exhibited a large cross-reaction with fragment 11-193 in early bleedings (about 60%) which was even further elevated in the 10-week bleedings (76-94%). Similar trends were exhibited by various antisera pools of rabbits 487, 488 or 489. Thus the cross-reactions of antisera 487 with BSA increased from 25% in the 7-10-week pool up to 61% in the 14-16-week pool, and for antiserum 489, these increased from 38 to 72%. The respective cross-reaction with fragment 11-193 increased from 18 to 40% for antisera 487

and from 46 to 60% for antisera 489. Antiserum 488 was tested with one 7-8-week pool and again showed considerable cross-reactions with BSA as well as with fragment 11-193.

The lack of spurring in agar double-diffusion, which appeared initially to be inconsistent with the results of quantitative precipitin analysis (Fig. 1 and Table 2), was afterwards completely rationalized by the findings subsequently described in this paper that the differences in the precipitin curves are due to the presence of soluble immune complexes.

Absorption studies with antisera to fragment 377-571

Absorption experiments were carried out in order to ascertain that the reactions discussed in the aforementioned section and summarized in Table 2, were true immune precipitates. These studies are also extremely valuable for the establishment of the specificity of the antibodies involved in these reactions and will enable the determination of whether or not BSA, fragment 377-571 and fragment 11-193 carry similar antigenic sites. Absorption of the antisera with fragment 377-571 (i.e. the homologous antigen), removed their reactivity entirely with any of these antigens. The antisera were therefore either absorbed with BSA alone, or with fragment 11-193 alone, or with both and the supernatant was examined in each case by quantitative precipitin analysis for its reactions with each of fragments 377-571, 11-193 and with BSA.

(a) *Absorption with BSA.* Absorption studies with BSA were performed on antisera from 2 bleedings of rabbit B7, 3 bleedings of rabbit 487 and 2 bleedings of rabbit 489. In each case, absorption with BSA at equivalence removed the reactivity of the antiserum supernatant with BSA. With antisera of rabbit B7 and the 11-13-week pool from rabbit 489, absorption with BSA removed all the reactivity with fragment 11-193 (e.g. see Table 3). In the case of other bleedings from rabbits 487 or 489, a very small amount of reactivity with fragment 11-193 remained after absorption with BSA. This was between 8-15% relative to reaction of the unabsorbed antiserum with fragment 377-571 at equivalence. The results of BSA-absorption of all the antisera are summarized in Table 3. Absorption also caused a substantial decrease in the reaction of the antisera with the homologous antigen and the equivalence point was at a lower (about half) antigen concentration. In most cases, the decrease was accounted for quantitatively by the amount of antibody removed by BSA in the absorption experiment.

Table 2. Quantitative precipitin reactions with antisera to fragment 377-571^a

Protein or fragment	Antiserum B5 ^b		Antiserum B7 ^b		Antiserum 487 ^c	Antiserum 488 ^c			Antiserum 489 ^c	
	18-day bleeding	10-week bleeding	18-day bleeding	10-week bleeding	7-10-week pool	11-13-week pool	14-16-week pool	7-8-week pool	7-10-week pool	11-13-week pool
377-571	100	100	100	100	100	100	100	100	100	100
11-193	62.3	93.6	59.0	75.8	17.8	30.9	39.1	47.9	46.4	60.1
BSA	37.1	59.8	62.5	84.5	24.6	60.0	61.1	36.6	38.4	72.0

^a Values are given in % precipitation at equivalence relative to reaction of fragment 377-571 with the respective antiserum as 100%. Results represent the average of 3-6 replicate determinations which varied $\pm 1.2\%$ or less. The % cross-reaction is based on mg of antibody precipitated at equivalence by each antigen as determined by the Folin reaction and comparison with a standard curve of rabbit IgG run simultaneously.

^b Single bleedings of these antisera, at the indicated periods after the first injections, were studied.

^c Antisera from weekly bleedings of the indicated periods after the first injection were pooled in groups, and these pools were studied.

Table 3. Reactions of antisera to fragment 371-571 after quantitative absorption with BSA

Protein or fragment	Reactions of absorbed antiserum relative to unabsorbed antiserum ^a						
	Antiserum B7 ^b			Antiserum 487 ^c 11-13-week pool	Antiserum 489 ^c		
	18-day bleeding	10-week bleeding	7-10-week pool		14-16-week pool	7-10-week pool	11-13-week pool
BSA	0	0	0	0	0	0	0
Fragment 11-193	0	0	8.0	15.2	15.2	11.0	0.77
Fragment 377-571	33.0	18.5	45.7	32.6	30.9	27.6	8.13
Ab absorbed by BSA ^d	67.1	83.3	23.9	63.4	61.7	37.2	72.5
Total Ab recovery ^e	100.1	101.8	69.6	95.7	92.6	64.8	80.6

^a Values were determined at equivalence from precipitin curves on each antigen with the antiserum supernatant obtained after absorption of the whole antiserum with BSA at equivalence and removal of the BSA-immune precipitate by centrifugation. Results are given in % reaction with the absorbed antiserum relative to reaction of unabsorbed antiserum (similarly diluted) with fragment 377-571 as 100%. Each value is the average of 3-6 replicate determinations which varied $\pm 1.1\%$ or less. Per cent reactions were based on mg antibody precipitated, determined as in footnote (c) of Table 1.

^b See footnote ^b of Tables 1 and 2.

^c See footnote ^c of Tables 1 and 2.

^d This represents the amount of antibody removed upon absorption of the whole antiserum by BSA at equivalence.

^e Total antibody was the sum of antibody removed by BSA and the residual reaction of the supernatant (absorbed antiserum) with fragment 377-571.

In a few cases the decrease of reaction with fragment 377-571 was greater than the amount of antibody removed by BSA in the absorption step (see the 7-10-week pools from rabbits 487 and 488 in Table 3). This discrepancy may have been due to the presence of non-precipitating antibodies giving soluble antigen-antibody complexes and it will be shown subsequently in this report that these are removed quantitatively on an immunoabsorbent of the homologous antigen or of BSA.

(b) *Absorption with fragment 11-193.* The results of absorption studies of 5 different bleedings from antisera 487 and 489 are summarized in Table 4. Absorption of the antisera with fragment 11-193 at equivalence removed their reactivity with BSA almost completely, leaving a very small amount (3-7%) of reaction. Reactions of the absorbed antisera with fragment 377-571 decreased by an amount which corresponded quantitatively to the amount of antibody removed in absorption by fragment 11-193 (see total antibody recovery in Table 4). The equivalence point

shifted to a lower (about half) concentration of homologous antigen. In one case, the 7-10-week pool of antiserum 489, the decrease in reaction with the homologous antigen was greater than the amount of antibody removed by absorption. This may have been caused by the presence of soluble antigen-antibody complexes which were recovered by immunoabsorbent experiments described in a subsequent section.

(c) *Joint absorption with BSA and with fragment 11-193.* In these studies, BSA and fragment 11-193 were added together, to the same aliquot of antiserum, each in an amount that was calculated to achieve equivalence for that particular antigen with the whole antiserum. It was found that little or no immune precipitate was obtained by this joint absorption. The small amount of immune precipitate, if obtained by the joint absorption of an antiserum, was quantitatively comparable with the amounts obtained in absorption by either BSA alone or fragment 11-193 alone when it was used in *twice* the antigen concentration required to reach equivalence.

Table 4. Reactions of antisera to fragment 377-571 after quantitative absorption with fragment 11-193

Protein or fragment	Reactions of absorbed antiserum relative to unabsorbed antiserum ^a				
	7-10-week pool	Antiserum 487 ^b		Antiserum 489 ^b	
		11-13-week pool	14-16-week pool	7-10-week pool	11-13-week pool
Fragment 11-193 ^b	0	0	0	0	0
BSA ^b	6.9	6.3	6.9	5.6	3.1
Fragment 377-571 ^b	86.4	60.9	58.8	45.0	34.0
Ab absorbed by 11-193 ^d	21.5	38.7	44.8	38.1	66.1
Total Ab recovery ^e	107.9	99.6	103.6	83.1	100.1

^a Values are in % reaction with the absorbed antiserum relative to the reaction of the unabsorbed antiserum (similarly diluted) with fragment 377-571 as 100%. Results are based on mg antibody precipitated (see footnote ^a of Table 1).

^b Values were determined at equivalence from precipitin curves on each antigen with the absorbed antiserum and each represents the average of 3 or 6 replicate determinations which varied $\pm 1.2\%$ or less.

^c See footnote ^c in Table 1.

^d This represents the amount of antibody removed upon absorption of the whole antiserum by fragment 11-193 at equivalence.

^e Value represents the sum of antibody removed by fragment 11-193 and the residual reaction of the supernatant (absorbed antiserum) with fragment 377-571.

Table 5. Inhibition of the reaction of BSA-absorbed anti-fragment 377-571 with fragment 377-571 by an excess of fragment 11-193

Stepwise operations ^a	Fraction of antibody obtained relative to total antibody in antiserum ^a				
	Antiserum 487		Antiserum 489		
	7-10-week pool	11-13-week pool	14-16-week pool	7-10-week pool	11-13-week pool
(1) Antibody removed by BSA in absorption at equivalence	20.8 (0.97)	64.5 (4.68)	64.4 (3.02)	37.7 (2.16)	65.9 (6.13)
(2) Ab precipitated in antigen excess of 11-193	5.4 (0.25)	1.93 (0.14)	0	0	0
(3) Reaction with fragment 377-571 (precipitin curve)	0	0	0	0	0
(4) Ab removed on immunoabsorbent of 377-571	74.6 (3.47)	37.6 (2.73)	34.8 (1.63)	82.5 (4.73)	34.0 (3.16)
Total antibody recovered	100.8 (4.69)	104.0 (7.55)	99.2 (6.65)	120.2 (6.89)	99.9 (9.29)

^a Values are given in % antibody recovered relative to total antibody content (in mg/ml) of the whole antiserum (see Table 1). Values in parentheses represent antibody recovery (in mg/ml) from the antiserum by a given operation.

^b Operations 1-4 were carried serially on the same aliquot of antiserum.

(1) Antiserum was first absorbed with BSA at equivalence and the values here give the amounts of antibody removed by this step.

(2) To the supernatant from (1) was added an amount of fragment 11-193 which corresponded to its equivalence point with the whole and *not* the absorbed antiserum. This will be in the region of antigen excess towards the absorbed antiserum from (1). Any trace of immune precipitate was removed and the amounts are indicated.

(3) The supernatant from (2) was examined by quantitative precipitin reaction with varying dilutions of fragment 377-571. No immune precipitate was obtained.

(4) The sample from (3) was passed on an immunoabsorbent of fragment 377-571. The amounts of antibody removed are indicated.

Serial reaction of antisera with the three antigens

The following series of studies were performed on the same aliquot of a given antiserum:

(1) Antiserum was absorbed with BSA at equivalence and the immune precipitate was removed and quantitated. The amount of antibody removed at this stage (Table 5) compared quite well with the expected reactivity of BSA (see Table 2) with that antiserum.

(2) The supernatant was mixed with an amount of fragment 11-193 that was calculated to correspond to the equivalence point of the peptide with the whole (unabsorbed) antiserum (i.e. *not* the equivalence amount for the absorbed antiserum). This will be in the region of antigen excess for fragment 11-193 and therefore little or no immune precipitate was obtained [see section (c) above]. At any rate, any trace of immune precipitate was removed and quantitated.

(3) The supernatant was then examined for its reaction with fragment 377-571 by quantitative precipitin reaction over a wide range of antigen concentration. No immune precipitate was obtained at this stage. Taking into account that BSA-absorbed antisera show a shift in the equivalence with respect to their reaction with the homologous antigen, then the amount of fragment 11-193 added in step 2 corresponded to 6-8-fold molar excess relative to the expected equivalence point of fragment 377-571 with the absorbed antiserum.

(4) The antibody that did not give an immune precipitate with fragment 377-571 in step 3 was recovered on an immunoabsorbent carrying fragment 377-571.

These results are summarized in Table 5. It is clear from Table 5 that the lack of an immune precipitate with absorbed antiserum and fragment 377-571, in the presence of a 6-8-fold excess of fragment 11-193, was not due to the absence of antibodies with appropriate specificities. The latter could be recovered quantitatively on an immunoabsorbent and, together with the antibodies removed in absorption by BSA,

accounted for 99-104% of the total antibody content of the antiserum. In one case (the 7-10-week pool of antiserum 489), the amount of antibody removed by the immunoabsorbent of fragment 377-571 (83%) was greater than that expected from the difference (about 62%) between the reactions of BSA and fragment 377-571 with the unabsorbed antiserum (see Table 2). This indicates the presence of non-precipitating antibodies even for the homologous antigen at equivalence that cannot be detected by quantitative precipitin analysis. Also, the excellent antibody recoveries here (Table 5) with the 7-10-week pool of antiserum 487 and the 11-13-week pool of antiserum 489 which could not be achieved by quantitative precipitin analysis (Table 3) indicate that the latter method precluded the detection of some soluble antigen-antibody complexes.

Reactions with antisera to fragment 11-193

This fragment was a poor immunogen as determined from testing the antisera by quantitative precipitin reactions as well as by an immunoabsorbent carrying fragment 11-193 to detect any non-precipitating antibodies. Of the 3 rabbits immunized only one gave antibody titers of sufficient levels to be employed in meaningful study. Even with this rabbit, an unusual decrease in amount of antibody titer occurred relatively rapidly with progress of immunization. Antisera to fragment 11-193 showed a considerable cross-reaction (28-37%) with fragment 377-571. Also, its cross-reaction with BSA which was appreciable (16%) in the 7-10-week pool was further elevated (to about 57%) in the later 14-16-week pool. The results of quantitative precipitin analysis with pools of three different bleedings from rabbit 492 are shown in Table 6.

Absorption studies on antiserum to fragment 11-193

Only the 7-10-week pool from rabbit 492 was subjected to absorption studies because the other antisera

Table 6. Quantitative precipitin reactions with antisera to fragment 11-193

Protein or fragment	Reactions with antisera 492 ^a		
	7-10-week pool	11-13-week pool	14-16-week pool
11-193	100	100	100
377-571	30.3	36.6	28.0
BSA	16.2	17.1	56.0

^a Results are given in % precipitation at equivalence relative to reaction of fragment 11-193. Values are based on mg antibody precipitated at equivalence which were determined as described in footnote ^a, Table 1.

were too weak for meaningful investigations. The results are summarized in Table 7. After absorption with BSA at equivalence, the antiserum did not react with BSA, had little reaction with fragment 377-571, while its reaction with the homologous antigen (fragment 11-193) decreased by a corresponding amount. Absorption of the antiserum with fragment 377-571 (whose cross-reaction is higher than that of BSA) removed all the antiserum reactivity, both against fragment 377-571 as well as against BSA. Reaction of the absorbed antiserum with the homologous antigen was also lower than that of the whole anti-

serum. A better recovery of this antibody was achieved by an immunoabsorbent of 11-193 than by quantitative precipitin reaction (Table 7), again indicating the presence of a small amount (about 6%) of non-precipitating antibodies giving soluble complexes between the absorbed antiserum and the homologous antigen (fragment 11-193).

Immunoabsorbent studies with antisera to fragment 377-571

These studies were carried out on the 7-10-week pool and the 11-13-week pool from rabbit 489. An aliquot of the antiserum was passed through either an immunoabsorbent of BSA or of fragment 11-193 or of the homologous antigen.

(a) *Immunoabsorbent of fragment 377-571.* The results are summarized in Table 8. A solution emerging after passage of an antiserum through the immunoabsorbent showed no reaction, by quantitative precipitin analysis and by immunoabsorbent studies, neither with fragment 377-571 nor with BSA. The bound antibody was then eluted from the immunoabsorbent with 5 M guanidine-hydrochloride quantitated, dialysed against 0.01 M phosphate buffer (pH 7.4) containing 0.15 M NaCl and then examined by

Table 7. Absorption studies on antiserum to fragment 11-193

Test antigen and experiment	Whole antiserum	Reactions with antiserum 492 (7-10-week pool) ^a	
		Antiserum absorbed with BSA	Antiserum absorbed with 377-571
Ab absorbed out by antigen		15.1	26.3
BSA (precipitin curve) ^b	16.2	0	2.40
377-571 (precipitin curve) ^b	30.3	6.07	0
11-193 (immunoabsorbent) ^c	—	72.7	73.7
(precipitin curve) ^b	100		65.7
Total Ab recovery	100	93.9	102.4 ^d
			94.3 ^e

^a Results are given in % reaction relative to the reaction of fragment 11-193 with the unabsorbed (whole) antiserum as 100%, and represent the average of triplicate determinations which varied $\pm 1.5\%$ or less. Values are based on mg antibody recovered in each experiment determined as in footnote ^a, Table 1.

^b These values were derived from quantitative precipitin analysis with the antiserum and varying amounts of the antigen indicated, and refer to the amount of antibody precipitated at equivalence in each precipitin curve.

^c Values represent the average of the amount of antibody bound on, and the amount of antibody recovered from, an immunoabsorbent carrying fragment 11-193. These two amounts differed by 2-4%.

^d This value is the sum of antibody recovered with BSA, fragment 377-571 and then an immunoabsorbent of fragment 11-193.

^e This value is the sum of antibody recovered with BSA, fragment 377-571 and then with 11-193 at equivalence by quantitative precipitin analysis.

Table 8. Immunoabsorbent studies on antisera (489) to fragment 377-571

Antigen	Whole antiserum	Immunoabsorbent, antibody recovered and its reactions ^a					
		Immunoabsorbent of fragment 377-571		BSA-immunoabsorbent		Immunoabsorbent of fragment 11-193	
		Effluent ^c	Ab eluted ^d	Effluent ^c	Ab eluted ^d	Effluent ^c	Ab eluted ^d
I. Antiserum of the 7-10-week pool							
Ab recovered (mg) ^b		0	7.8	0	7.9	0	7.7
BSA	39	0	40	0	49	0	29
Fragment 377-571	100	0	100	0	100	0	100
II. Antiserum of the 11-13-week pool							
Ab recovered (mg) ^b		0	11.7	0	11.9	0	11.3
BSA	72	0	75.4	0	77	0	N.D.
Fragment 377-571	100	0	100	0	100	0	N.D.

^a Values are given in % quantitative precipitin reaction at equivalence relative to reaction of fragment 377-571 as 100%. Results represent the average of triplicate analyses which varied $\pm 1.5\%$ or less.

^b Amount of antibody recovered from 1 ml of whole antiserum applied on the immunoabsorbent.

^c This fraction represents the amount and reactivity of any antibody that is *not* bound on the immunoabsorbent.

^d This represents the amount and reactivity of the antibody fraction that was displaced from the immunoabsorbent by 5 M guanidine-hydrochloride. After dialysis against 0.01 M phosphate buffer at pH 7.4 containing 0.15 M NaCl, its quantitative precipitin reactions with BSA and fragment 377-571 were determined.

quantitative precipitin analysis for its reactions with fragment 377-571 and with BSA. The antibody recovered by elution from the immunoabsorbent had an equal reactivity (in terms of mg antibody precipitated at equivalence) to that of the original whole antiserum with fragment 377-571. Also, this antibody fraction exhibited (Table 8) a reaction with BSA which was similar to that of the original whole antiserum.

(b) *Immunoabsorbent of BSA.* The solution emerging after passage of antiserum 489 (see Table 8) on a BSA-immunoabsorbent failed to react, in quantitative precipitin analysis and by immunoabsorbent studies, not only with BSA but also with fragment 377-571. The antibody recovered by displacement from the immunoabsorbent, behaved in terms of reactivity very much like the original antiserum. It was quite surprising, therefore, that pure antibody which was originally removed by a BSA-immunoabsorbent, reacted in quantitative precipitin analysis at equivalence, only partially with BSA. The extent of this partial reaction (49 and 77% with the 7-10-week and the 11-13-week pools, respectively, of antiserum 489) compared well with those of the original whole antisera (40 and 72%) relative to the homologous reaction. The original whole antiserum and its antibody fraction from the BSA-immunoabsorbent showed in each case equal reactivities with the homologous antigen (in terms of mg antibody precipitated at equivalence by fragment 377-571). Furthermore, for a given aliquot of antiserum, equal amounts of total antibody were recovered on an immunoabsorbent of BSA or an immunoabsorbent of fragment 377-571 (see Table 8). Therefore, not only did antibodies obtained from the antisera possess identical reactivities but were in addition recovered in equal amounts regardless of whether the immunoabsorbent carried BSA or fragment 377-571.

(c) *Immunoabsorbent of fragment 11-193.* The results from these immunoabsorbents were highly significant. It was remarkable that an immunoabsorbent of fragment 11-193 removed *all* antibodies in antisera to fragment 377-571 (see Table 8). The effluent solution (of unbound material) showed *no* reaction by precipitin analysis and by immunoabsorbent studies, not only with fragment 11-193 but also with BSA and with fragment 377-571. The amount of antibody recovered from a given antiserum by this immunoabsorbent was equal (per ml of antiserum) to the amounts displaced from an immunoabsorbent of BSA or of fragment 377-571 (Table 8). Not only were the amounts of bound antibody similar, but also their immunochemical reactivities were quite comparable. Therefore, fragment 11-193 removed quantitatively *all* antibodies from antisera to fragment 377-571 even though it did not form equal amounts of insoluble complexes to those of the homologous antigen in quantitative precipitin reaction. Again, like those from the BSA-immunoabsorbent, antibodies isolated from an immunoabsorbent of fragment 11-193 reacted best in precipitin analysis with fragment 377-571 and not with the material used for their isolation on the immunoabsorbent (Table 8).

Finally, it has recently been reported (Heinzel *et al.*, 1976) that non-specific binding of proteins to albumin-Sepharose may take place when the Sepharose

is activated by CNBr for coupling. Accordingly, BSA or a fragment were coupled onto Sepharose by benzoquinone (Brandt *et al.*, 1975) and the aforementioned studies were repeated. The results obtained were identical regardless of the method of preparation of the immunoabsorbent and ruled out any interference by non-specific protein binding.

DISCUSSION

The results of quantitative precipitin reactions with antisera to fragment 377-571 indicated that there was in these antisera a substantial antibody population that cross-reacted with BSA. This was not entirely unexpected, since antibodies directed against antigenic sites of a major fragment from the molecule can recognize those antigenic sites when they are encountered on intact BSA. Fragment 377-571, which is cross-linked by 6 disulfide bonds, should retain a highly folded structure as reported for fragment 377-581 (Reed *et al.*, 1975; Teale & Benjamin, 1976). The proportion of antibodies that would recognize sites on the intact BSA molecule increased, also as expected, with the duration of immunization. This is somewhat analogous to the observations, for example, that antisera to amidinated BSA or guanidinated BSA (Habeeb, 1968) or glutaraldehyde-treated BSA (Habeeb, 1969) showed increasing reactivity with BSA upon prolongation of the immunization schedule.

The reaction of antisera to fragment 377-571 with an entirely different part of the BSA molecule (i.e. fragment 11-193) is quite remarkable. As with BSA, antibodies that recognized sites on the N-terminal third of the molecule also increased with time. Since in absorption experiments, BSA removed almost all the reactivity of the antiserum with fragment 11-193, and conversely absorption with fragment 11-193 again removed almost all the reactivity with BSA, it became evident that these two antigens reacted essentially with the same antibody population. This conclusion was supported by the finding that when a given antiserum was mixed simultaneously both with BSA and with fragment 11-193, each at equivalence, the reaction mixture behaved as if it were in antigen excess of either BSA or fragment 11-193.

Clearly both BSA and fragment 11-193 were competing for the same antibody population. It is not unusual to expect the reactive sites on fragment 11-193 to be completely expressed on the intact BSA molecule. However, the reverse is totally unexpected and the intact molecule would normally carry additional antigenic sites that are not present on the N-terminal third part of the molecule, especially in view of the fact that the immunogen represented the C-terminal third part of the molecule. This could imply that BSA reacts through the *same* antigenic sites that are present on the N-terminal fragment 11-193 with *no* additional sites elsewhere on the intact BSA molecule. However, since the immune response was directed in fact against the C-terminal fragment 377-571, then it would be expected that BSA should cross-react *in addition* through antigenic sites present on the C-terminal third of the molecule. Since little or no such additional reactivity is observed on BSA over that exhibited by fragment 11-193, the results suggested that BSA and fragment 11-193 and fragment 377-571 carry identical antigenic sites.

Further support for this conclusion was derived from studies with antisera to fragment 11-193. These antisera exhibited appreciable cross-reactions with BSA as well as with fragment 377-571. Again, with these antisera, absorption with BSA left little or no reaction with fragment 377-571. Conversely, absorption with the latter fragment left no reaction with BSA in the antiserum supernatant. It is, therefore, evident that BSA carries no new antigenic sites that are not expressed on fragment 377-571 despite the fact that the immune response was directed against the N-terminal part 11-193.

If this conclusion was correct then it may be expected that BSA or fragment 11-193 should have equal antigenic reactivity to that of fragment 377-571 with antisera to the latter. Although with some antisera, a cross-reaction went over 85-90%, a more representative cross-reaction was between 50-70%. Another matter of concern which seemed to contradict the concept of identical antigenic sites on the three antigens, is that neither absorption with BSA nor with fragment 11-193 removed all the reactivity with the homologous antigen (fragment 377-571). This apparent contradiction could be eliminated by postulating the presence of soluble immune complexes. This postulate is, of course, amenable to experimental verification which was obtained. Thus when both BSA and fragment 11-193 were added simultaneously, each at equivalence, to an aliquot of a given antiserum, the mixture behaved as if it were in the region of antigen excess of either of the two antigens alone. More convincingly, however, since the addition of an excess of fragment 11-193 to a BSA-absorbed antiserum of fragment 377-571 inhibited completely its reaction with the homologous antigen (fragment 377-571), it was evident that the immunogen (fragment 377-571) carried no new antigenic sites that will not react with fragment 11-193 from the N-terminal third of the molecule. That a fragment representing a third of the molecule should elicit an immune response that was recognized totally and quantitatively by an entirely different part of the molecule is extremely unusual. Further, the aforementioned reasoning has obviously led to the inescapable conclusion that a portion of a protein representing only a third of the molecule has given essentially the same immune response as that derived from immunization with the whole protein.

These concepts are without precedence in protein immunochemistry and required exhaustive investigation, especially in view of the finding that with a given antiserum to a fragment, the highest precipitin reaction was obtained with the homologous antigen (see Tables 2 and 6). Therefore, we resorted to investigating immunochemical specificities by immunoabsorbent studies which provided strong evidence that BSA, fragment 11-193 and fragment 377-571 were immunochemically identical.

The finding that an immunoabsorbent carrying BSA or fragment 11-193 removed quantitatively all the antibodies in antisera to fragment 377-571, clearly demonstrated that no *new* antigenic specificity was generated when fragment 377-571 was used as an immunogen. In fact whether the immunoabsorbents carried BSA or fragment 11-193 or fragment 377-571, they possessed equal efficiency in binding antibodies

since the amounts of antibodies (in equal aliquots of a given antiserum) that could be displaced from the three immunoabsorbents were identical. The behaviors of the eluted antibody fractions in quantitative precipitin reactions towards BSA or fragment 377-571 compared well with those of the respective original antisera. Thus, for example, an antibody fraction isolated from a BSA-immunoabsorbent gave in quantitative precipitin analysis only a partial reaction with BSA and a complete reaction with fragment 377-571. The relative extent of the two reactions were virtually identical to those exhibited by the original whole antiserum (see Table 8). Similarly, the antibody fraction isolated on an immunoabsorbent of fragment 11-193 reacted only partially with fragment 11-193 or with BSA. This is unusual, because the antibody fraction isolated on an immunoabsorbent of a particular antigen would have been expected to react best with that antigen which was not the case, and these antibodies behaved exactly like those isolated on an immunoabsorbent of fragment 377-571. The behavior of these antibodies isolated on specific immunoabsorbents is vital for the understanding of the behavior of the original whole antisera in quantitative precipitin reactions. The studies clearly indicate the presence of non-precipitating antisera, the precipitating efficiency of which is best with the homologous antigen. Even though they do not precipitate efficiently with BSA or fragment 11-193, they can be removed entirely by their respective immunoabsorbents and when they are thus recovered they still do not precipitate efficiently with BSA but behave exactly like the original antiserum. It has long been known (Farr, 1958) that BSA-antisera contain substantial amounts of non-precipitating antibodies which give soluble immune complexes even at equivalence in the precipitin reaction. The amounts of these soluble complexes vary with time after immunization (Talmage & Maurer, 1953; Farr, 1958). Clearly, on the cross-reacting fragment 11-193 are expressed all the antigenic sites that are present on the immunogen itself (fragment 377-571) and these are also entirely expressed on BSA.

Previously we have shown (Atassi *et al.*, 1976a) that fragment 11-193 inhibited the reaction of BSA with its antisera almost completely (88%) and an immunoabsorbent of the fragment removed almost all (89%) the antibodies in BSA-antisera. Soon afterwards we showed (Habeeb & Atassi, 1976) that the C-terminal fragment 377-571 also inhibited almost completely ($91.5 \pm 1.5\%$) the reaction of BSA with its antisera. An immunoabsorbent of fragment 377-571 removed 89-95% of the antibody from BSA-antisera. It is relevant here to mention that fragment 377-581 was recently found (Teale & Benjamin, 1976) to inhibit the reaction of radioiodinated BSA with its antibodies by the Farr assay at a much lower level (35%) than the extents of reactivities which we have found (90-95%). This is most likely a function of the limitation of determining inhibition by the Farr assay which they employed. The procedure determines inhibition by measuring the amount of free radiolabeled antigen liberated by an inhibiting peptide. In such studies, the method will yield erroneously low results because inhibition by a monovalent, divalent (or even higher) fragment is not expected to liberate much if

any free-labeled antigen. The reason for this is that the competing fragment will displace antibodies from antigenic sites on the protein antigen that are represented on the fragment. However, other antigenic sites on the protein antigen would remain bound to their respective specific antibodies and these complexes will be precipitated by ammonium sulfate. The inhibiting peptide is expected to liberate little or no free antigen but will liberate free antibodies by displacing them only from the corresponding sites on the protein antigen in the immune complex. The fact that so much (35%) free BSA was liberated, contrary to expectation, attests to remarkable potency of the competing fragment 377-581.

Since the two fragments (one comprising the first third and the other the last third of the protein) could each by itself account for the entire antigenic reactivity, the results were explained by the concept that BSA carries repeating and immunochemically related antigenic sites (Atassi *et al.*, 1976a; Habeeb & Atassi, 1976). This concept was unique in protein immunochemistry and demanded an in-depth investigation.

The present work clearly shows that antisera to one fragment are recognized totally by the other fragment as well as by intact BSA. They only showed differences in their abilities to form immune precipitates with the three antigens but not in their ability to bind. The aforementioned reports and the results described in this communication strongly establish that BSA has repeating immunochemically equivalent antigenic sites. It is not implied that one site is repeating six times but at least two sites are repeating three times each and the sites may have differences in binding energy. Furthermore, no particularly unique antigenic specificity that is peculiar to the immunogen is generated regardless of whether the immunogen is BSA, the first third fragment 11-193, or the last third fragment 377-571. These conclusions are evidently not dependent on the animal or on the immunization schedule or on the duration of immunization. It is not apparent at present whether the antigenic sites are made up of residues that are directly linked by peptide bonds like in myoglobin (Atassi, 1975) or are constructed from conformationally-adjacent residues

that are otherwise distant in sequence like in lysozyme (Atassi *et al.*, 1976b, c, d; Lee & Atassi, 1976). Examination of the primary structure of BSA determined by Brown (1975) does not show an obvious repetition of a sequence feature which has to comprise 6 or 7 residues as in myoglobin (Atassi, 1975). Furthermore, the intactness of the disulfides in fragment 11-193 (Atassi *et al.*, 1976a) and fragment 377-571 (Habeeb & Atassi, 1976) is essential for their immune reaction. This is analogous to the structural requirement in lysozyme for covalent maintenance of the conformational proximity of the residues constructing the antigenic sites (Atassi *et al.*, 1976b, c, d; Lee & Atassi, 1976). At any rate, it is best not to speculate at the present time on the nature of the antigenic sites in BSA but await the determination of its total antigenic structure.

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