

IMMUNOCHEMISTRY OF SERUM ALBUMIN—VIII.

THE ANTIGENIC REACTIVITY OF THE THIRD DOMAIN OF BOVINE SERUM ALBUMIN RESIDES IN THE LAST SUB-DOMAIN. A DYNAMIC EXAMINATION OF THE CHANGE OF ANTIBODY AFFINITY AND SPECIFICITY*

SHIGEKI SAKATA,¹ ROBERTA G. REED,² THEODORE PETERS, JR.²
and M. ZOUHAIR ATASSI^{1,†}

¹The Department of Immunology, Mayo Medical School, Rochester, MN 55901 and the

²Mary Imogene Bassett Hospital‡, Cooperstown, NY 13326, U.S.A.

(Received 7 December 1978)

Abstract—In a preceding paper, immunochemical and conformational studies on chemical derivatives of fragment 377–571 of bovine serum albumin (BSA) suggested that the immunochemical reactivity of this fragment (the third domain of the albumin molecule) resided entirely in the last sub-domain. In the present work, the immunochemical reactivities of fragments 377–571 and 504–581 with serial antisera to BSA were studied in detail. Antisera against BSA were raised in two rabbits and serial bleedings were obtained at different periods after the first immunization. The serial antisera were not mixed but were kept and studied separately. Immunoabsorbent titration studies showed that the plateau values of ¹²⁵I-antibody binding by the immunoabsorbents of the two fragments changed with time after the initial immunization but were, for a given antiserum, identical in all serial antisera from 15 days up to 398 days. However, in very early antisera (7 days) the larger fragment 377–571 possessed a higher immunochemical reactivity than the small fragment 504–581. The inhibitory activities of the two fragments towards the binding of ¹²⁵I-albumin and ¹²⁵I-fragment 377–571 with serial antisera to BSA in a Farr assay were compared. The two fragments exhibited comparable inhibitory activities and, in addition, fragment 504–581 was able to inhibit completely the binding of ¹²⁵I-fragment 377–571. Acid dissociation studies of complexes of serial ¹²⁵I-antibodies on immunoabsorbents revealed that the affinity of the antibodies increased while their heterogeneity decreased with time after immunization. The results with serial antisera from each of the two rabbits show that although in very early (7 days) antisera, the larger fragment appears to carry some additional antigenic sites, the shared antigenic sites become completely immunodominant relatively early (15 days) after the first immunization.

INTRODUCTION

In a recent communication we have examined (Sakata & Atassi, 1979) the antibody response to bovine serum albumin (BSA) in serial antisera taken from six rabbits from 7 to 398 days after the initial immunization. The reactivity of a fragment, previously isolated and characterized (Habeeb & Atassi, 1976) to correspond to sequence 377–571 (domain 3) of BSA, with the serial antisera from each of the rabbits was found to increase with time (Sakata & Atassi, 1979) so that with late antisera the fragment inhibited by as much as 90% the precipitin reaction of BSA. The small size of this fragment and its retention of such a large fraction of the immunochemical activity of BSA made it valuable for studies on the antigenic structure of BSA. Accordingly we focused our attention on narrowing down of the antigenic sites in domain 3 of BSA. In a preceding report (Kazim *et al.*, 1979) we suggested from the conformation and immunochemistry of

chemical derivatives of fragment 377–571, that all the antigenic sites of domain 3 lie in the last subdomain (ninth disulfide double loop) of BSA. If this were indeed correct then, barring any drastic conformational differences, a fragment corresponding to the last sub-domain should account for all the antigenic reactivity of the domain. We wish to report here a detailed analysis of the immunochemical reactivity of fragment 504–581 (corresponding to the last sub-domain) with antisera to albumin. Antisera from two rabbits, obtained at different periods after immunization (from 7 to 398 days), were kept and studied separately with regards to specificity as well as heterogeneity of antibody affinity.

MATERIALS AND METHODS

The materials used in these studies were obtained from the sources previously given (Sakata & Atassi, 1979). The preparation and purification of fragment 504–581 from BSA has been described (Peters & Hawn, 1967). Antisera from rabbits B8 and B9 were obtained from serial bleedings at different times after immunization and the immunization schedule has been described (Sakata & Atassi, 1979). Serial antisera from each rabbit were not mixed but kept separately in 5-ml aliquots in the frozen state (–40°C). Selected serial

*This paper is the 8th article in the series "Immunochemistry of Serum Albumin". The preceding article is by Kazim *et al.*, 1979.

†To whom correspondence should be addressed.

‡Affiliated with Columbia University, New York, NY.

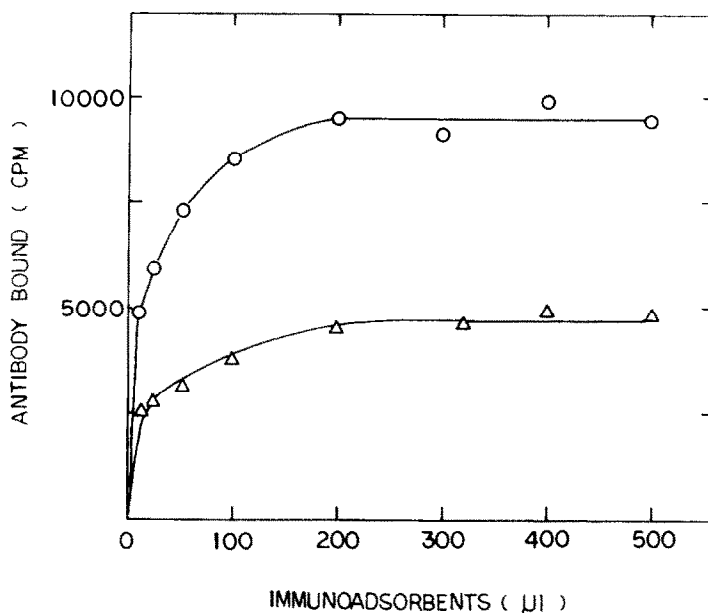


Fig. 1. An example of titration of fixed amounts of ^{125}I -antibodies, of a very late (398-day bleeding) antiserum against BSA from rabbit B8, with increasing amounts of immunoadsorbents. (○) BSA-immunoadsorbent; (△) fragment 504-581 immunoadsorbent. Values represent the average of triplicate determinations which varied $\pm 1.5\%$ or less. The results with other antisera are summarized in Table 1.

antisera were studied here. Purification of the IgG fraction from the serial antisera was performed by the procedure previously described (Lee & Atassi, 1977). Radioiodination of the IgG fractions and of proteins by ^{125}I was carried by the chloramine-T method (Hunter & Greenwood, 1962) and free ^{125}I was removed by gel filtration (Sakata & Atassi, 1979).

Proteins or peptide fragments were coupled onto CNBr-activated (March *et al.*, 1974) as well as onto *p*-benzoquinone activated (Brandt *et al.*, 1975) Sepharose CL-4B as previously described (Sakata & Atassi, 1979). The adsorbents carried 3.0–3.5 mg protein/ml of packed volume. Immunoadsorbent titration studies were done in 0.1% rabbit hemoglobin solution in 0.15 M NaCl–0.01 M sodium phosphate buffer, pH 7.2, by the procedure recently given (Sakata & Atassi, 1979). Lysozyme immunoadsorbents were used as controls to correct for non-specific binding. Dissociation studies were done in decreasing pH on ^{125}I -antibodies from selected serial bleedings bound to immunoadsorbents of albumin or fragment. The ^{125}I -antibody-immunoadsorbent complexes (in triplicates) were washed with solvents (5 times, each equivalent to 20-fold the volume of the immunoadsorbent) of decreasing pH intervals, starting with phosphate-saline at pH 7.2. Dissociation studies were done at room temp in phosphate buffers at pH > 5.6, in acetate buffers in the pH range 5.0–3.4, and in glycine-HCl buffers at pH < 3.4. The ionic strength of all buffers was kept constant (at $\mu = 0.16$) in order to avoid any dissociation effects due to ionic strength. A control (in triplicates) of the same immunoadsorbent-antibody complex, whose dissociation is under study, was washed simultaneously an equal number of times with phosphate-buffered saline at pH 7.2. The binding at a given pH is expressed in per cent relative to the radioactivity on the immunoadsorbent controls (that had been washed an equal number of times with phosphate-buffered saline) as 100%.

The binding of fixed amounts of ^{125}I -albumin or ^{125}I -fragment 377–571 with serially diluted (with 0.1% rabbit hemoglobin solution in phosphate-saline, pH 7.2) antisera was studied by the Farr assay (Farr, 1958) or by a 'double-antibody' method by the procedures recently described (Kazim & Atassi, 1978). For inhibition of the Farr assay, unlabelled protein or fragment was premixed with antiserum

(appropriately diluted to bind 50% of labelled material in the absence of inhibitor), followed after standing 2 hr at 22°C and overnight at 0°C, by the addition of radioiodinated albumin or radioiodinated fragment. Binding of radioiodinated protein was then examined by the usual procedure (Kazim & Atassi, 1978). Other immunochemical methods employed here were carried out according to previously described procedures (Atassi & Saplin, 1968).

RESULTS

Binding of ^{125}I -antibody from serial antisera by immunoadsorbents of fragment 504-581

The coupling of proteins or fragments onto Sepharose was effected by two different activation reactions (CNBr or *p*-benzoquinone) at two different pH conditions (pH 9.5 and pH 4.8). This was done as a precaution, in case coupling in a given reaction inadvertently involved side chains that are essential for immunochemical reactivity. Immunoadsorbent studies were designed to determine the maximum amount of antibody that could be bound by a given immunoadsorbent. This was done by adding fixed amounts of ^{125}I -antibody to increasing amounts of immunoadsorbents. As the volume of immunoadsorbent is increased, the amounts of antibody bound approach completion and the binding curves reach a plateau which represents the maximum amount of antibody that may be removed by a given immunoadsorbent. In all these studies, the binding was corrected for the non specific binding of radiolabelled albumin antibodies by an equal volume of lysozyme-Sepharose. Non-specific binding was less than 5% of the total ^{125}I -antibody bound to albumin-Sepharose. Figure 1 shows an example of two titration curves in which both BSA and fragment 504-581 immunoadsorbents achieved plateau (maximal antibody removal) at around 200 μl packed volume.

Table 1. Comparison of the antibody binding activities by immunoadsorbents of fragment 377-571 and fragment 504-581

Rabbit	Serial bleedings (days)	Per cent ^{125}I -antibody bound by immunoadsorbents ^a	
		Fragment 377-571	Fragment 504-581
B8	7	69	20
	15	21	21
	38	24	24
	48	19	14
	398	51	51
B9	7	58	17
	25	29	33
	38	40	37
	48	29	32
	398	54	57

^a Indicate the amounts of antibody bound in the plateau regions (see Fig. 1) by immunoadsorbents of the fragments relative to the plateau amount bound by BSA as 100%. Values represent the average of triplicate analyses which varied $\pm 1.5\%$ or less.

Table 2. Inhibition of the binding of antibody to ^{125}I -labelled albumin by albumin, fragment 377-571 or fragment 504-581^a

Rabbit	Bleeding time (days)	% Inhibition of ^{125}I -albumin binding by		
		albumin	fragment 377-571	fragment 504-581
B8	38	99.2 (3.5)	45.9 (5)	44.6 (8)
	398	97.7 (0.2)	54.0 (2)	52.2 (6)
B9	38	98.8 (3.0)	46.7 (5)	50.0 (8)
	398	98.1 (0.15)	54.0 (3)	54.8 (4)

^a Values are expressed in maximum inhibitory activities and represent the average of triplicate determinations which varied $\pm 1.3\%$ or less. The amount of ^{125}I -labelled albumin added was 2.5×10^{-5} mg. Values in parentheses represent the molar excess at 1/2 the maximum inhibition of unlabelled inhibitor relative to radioiodinated albumin. For fragment 377-571 maximum inhibition was achieved at the following molar excess: B8 or B9 (38 days), 150; B8 or B9 (398 days), 100. For fragment 504-581, the excess values at maximum inhibition were: B8 or B9 (38 days), 380; B8 or B9 (398 days), 100.

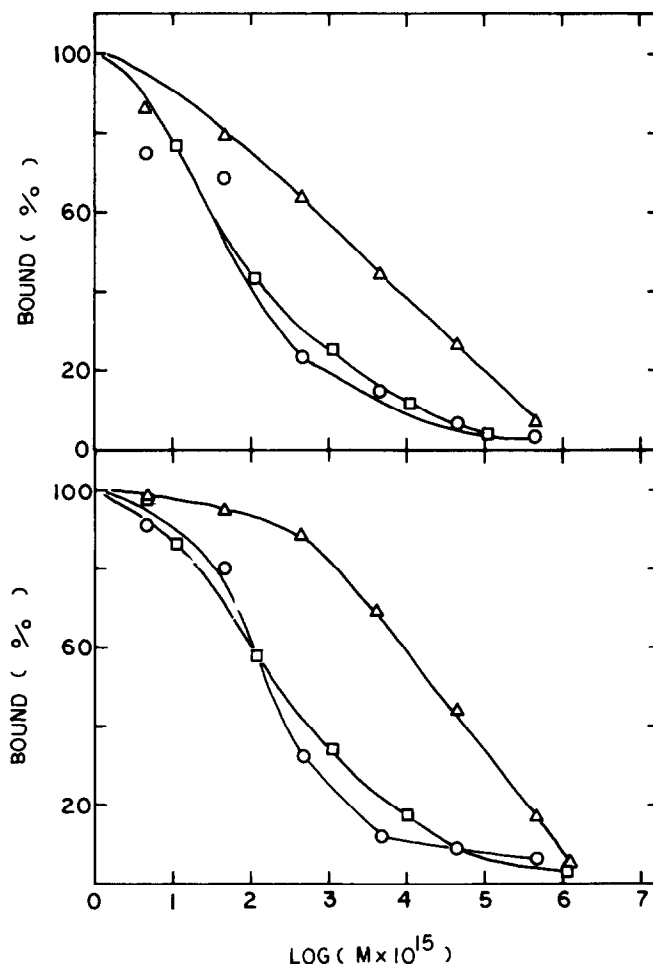


Fig. 2. Inhibition of the binding of ^{125}I -fragment 377-571 with antisera to BSA by (○) BSA, (□) fragment 377-571 and (△) fragment 504-581. Upper panel, 38-day antiserum from rabbit B8; lower panel 38-day antiserum of rabbit B9.

Similar titrations were carried out on all other serial ^{125}I -antibodies and Table 1 summarizes the plateau binding values of immunoabsorbents carrying fragment 377–571 or fragment 504–581. It can be seen that in serial bleedings from both rabbits, the amounts of antibodies bound by each fragment on immunoabsorbent changed with time of bleeding after immunization. In very early (7 days) antisera, fragment 377–571 bound a higher amount of antibodies than fragment 504–581. The difference between the reactivities of the two fragments disappeared relatively rapidly (15 days) after immunization. In fact the per cent of antibodies bound by the two fragments remained equal with a given antiserum, but increased with time, from the 15-day up to the 398-day bleedings (Table 1).

In immunoabsorbents that were prepared by *p*-benzoquinone activation under two different pH conditions, identical results were obtained (not shown). In these studies the amounts of ^{125}I -antibody bound to albumin-Sepharose or a fragment-Sepharose were higher than with those obtained on CNBr-prepared immunoabsorbents. However, the non-specific binding (lysozyme-Sepharose) was also elevated and, after correction for this, the per cent of ^{125}I -antibody bound by a fragment-Sepharose was essentially unchanged by the two methods.

Inhibition of antibody binding to radioiodinated albumin or fragment 377–571 by fragment 504–581

The abilities of unlabelled free fragment 377–571 and fragment 504–581 to inhibit the binding of ^{125}I -albumin with selected serial antisera from rabbits B8 and B9 were compared. The results with these antisera are summarized in Table 2. The two fragments possessed comparable inhibitory activities with a given serial antiserum from 38 days to 398 days. These results were in complete agreement with the findings from immunoabsorbent studies (Table 1). Additional

studies were then carried out to double check whether the immunochemical reactivity of fragment 504–581 accounts for the entire reactivity of the larger fragment 377–571. This was done by determining the ability of unlabelled free fragment 504–581 to compete with radiolabelled fragment 377–571 in binding with antisera from rabbits B8 and B9. The results (Fig. 2) clearly show that fragment 504–581 was able to inhibit the binding of radioiodinated fragment 377–571 completely, but required a larger molar excess than that required by unlabelled fragment 377–571 to inhibit the binding of its radioiodinated derivative.

Acid dissociation studies of serial ^{125}I -antibodies on immunoabsorbents

The acid dissociation profiles of radiolabelled antibody, bound on albumin-immunoabsorbents, from three different antisera (7 days, 36 days and 398 days after first immunization) from rabbit B9 are shown in Fig. 3. About 1×10^4 counts/min of each iodinated antibody was bound to 500 μl of albumin-Sepharose. After washing immunoabsorbents 5 times (each equal to 20 volumes of packed adsorbent) with buffers of different pH ($\mu = 0.16$), the percent bound was expressed relative to radioactivity remaining in identical reference immunoabsorbents which were washed similarly with phosphate-saline (pH 7.2) at the same time. In antibody bound to BSA, pH-dissociation curves of early antisera (7 days, 38 days) were almost identical, however late antisera showed different profiles. At pH 4.0 15% of the antibody dissociated in early antiserum and 8% in late antiserum. At pH 3.0, 63% of the antibody dissociated in early antisera while 40% in late antiserum. Thus, the immune complexes of late antisera are more resistant to acid dissociation than those of early antisera. The pH values of 50% dissociation in early and late antisera are summarized in Table 3. Early antisera (7 days and 38 days) reached this point at a higher pH

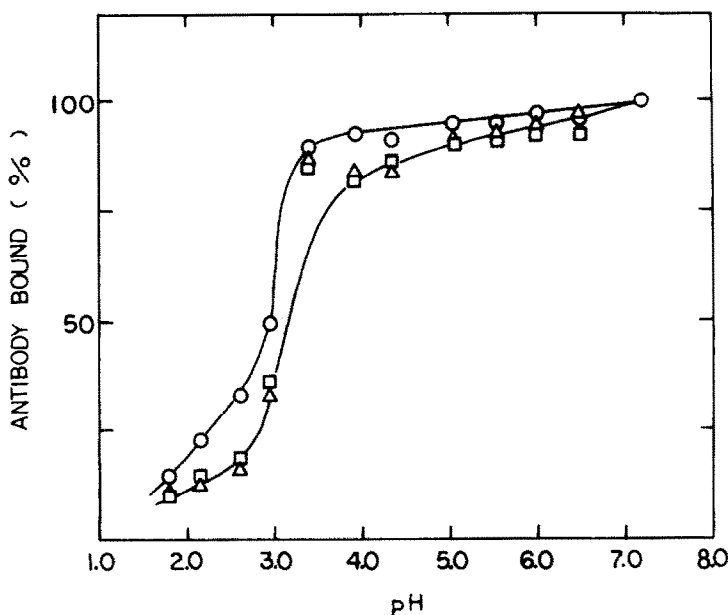


Fig. 3. Acid dissociation profiles of three serial ^{125}I -antibodies from rabbit B9 that are bound to BSA-immunoabsorbents. (Δ) 7-day antiserum; ($\square$) 38-day antiserum; ($\circ$) 398-day antiserum.

Table 3. Summary of acid dissociation parameters of selected serial antibodies*

Rabbit	Bleeding (days)	Antibody complex with albumin-immunoabsorbent		Antibody complex with immunoabsorbent of fragment 504-581	
		$\Delta\text{pH}_{20}^{90}$	pH at 50% dissociation	$\Delta\text{pH}_{20}^{90}$	pH at 50% dissociation
B9	7	2.32	3.14	3.40	2.92
	38	2.32	3.14	1.34	2.68
	398	1.40	2.91	— ^b	2.22
B8	398	1.40	2.91	— ^b	2.22

* The $\Delta\text{pH}_{20}^{90}$ value is the pH span between 90–20% binding (10%–80% dissociation) of ^{125}I -antibody on immunoabsorbents of BSA or fragment 504–581. It serves as an index for the heterogeneity of binding affinity of antibodies. The value of pH at 50% dissociation indicates the relative resistance of the immune complex to acid dissociation. For details see text.

^b Antibody which was bound to immunoabsorbent of fragment 504–581 did not reach 80% dissociation even at pH 1.78.

than late (398 days) antisera which indicated that the late antisera are more resistant to acid dissociation. We did not study dissociation profiles below pH 1.78, but at this pH about 10% of the antibody in early antisera and 14% in late antisera remained bound. To express more quantitatively the degree of heterogeneity of antibody populations in antisera, we have introduced (Sakata & Atassi, 1979) the term $\Delta\text{pH}_{20}^{90}$ as an index of affinity heterogeneities of antibodies. This denotes the pH span between 10% dissociation (90% remains bound) to 80% dissociation (20% binding) and reflects the population affinity of antibody molecules. The $\Delta\text{pH}_{20}^{90}$ values are summarized in Table 3. This value was higher in early antisera (15 days and 38 days) than in late antisera (398 days) which clearly indicates that early antisera were more heterogeneous, in terms of affinity, than late antisera. A late antiserum (398 days) of rabbit B8 gave almost identical dissociation profiles to the 398-day antiserum from rabbit B9.

The acid dissociation profiles of three serial ^{125}I -antibodies (from rabbit B9) on the adsorbents of fragment 504–581 are shown in Fig. 4. The

dissociation parameters (pH 50% dissociation and $\Delta\text{pH}_{20}^{90}$ are summarized in Table 3. Acid dissociation profiles of antibody directed against this fragment clearly changed with time. At pH 4.0, 22.5% of the antibody dissociated in the 7-day antiserum whereas almost no dissociation occurred in the 39-day and 398-day antisera. At pH 3.0 45% of the antibody dissociated in the 7-day antiserum, while only 25% in the 38-day and 11% in the 398-day antisera, respectively. The pH of the 50% dissociation was higher for early antisera than for late antisera. Also, the $\Delta\text{pH}_{20}^{90}$ values were considerably higher for early antisera than for late antisera. The results with a late antiserum (398 days) of rabbit B8 were almost identical with the results of the 398-day antiserum of rabbit B9 (see Table 3). The results clearly indicate that late antisera to this part of BSA are more restricted in terms of affinity, and more resistant to acid dissociation than early antisera.

DISCUSSION

In a preceding communication we have shown

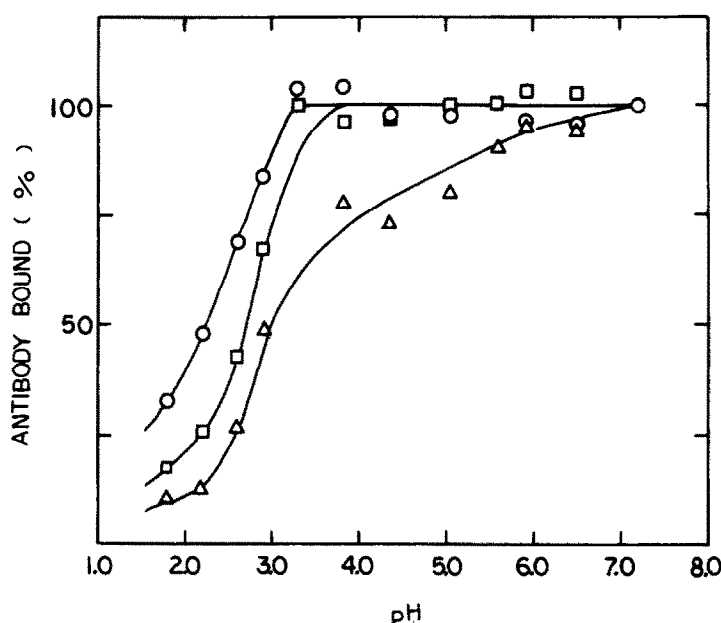


Fig. 4. Acid dissociation profiles of three serial ^{125}I -antibodies from rabbit B9 that are bound to immunoabsorbents of fragment 504–581. (Δ) 7-day antiserum; ($\square$) 38-day antiserum; ($\circ$) 398-day antiserum.

(Kazim *et al.*, 1979) by conformational and immunochemical studies on derivatives of fragment 377–571 nitrated at the tyrosine residues or modified at the arginine residues by cyclohexanedione, that all the tyrosine and most likely all the arginine residues are not located within the antigenic sites in this part (domain 3) of the BSA molecule. In the primary structure (Brown, 1976) of this part (sequence 377–581) of BSA the four tyrosine and the eight arginine residues are all located within the sequence 377–494 (double loops 7 and 8). In view of the manner in which these twelve residues are distributed within this sub-domain, it was suggested (Kazim *et al.*, 1979) that the antigenic reactivity of fragment 377–571 resides entirely within the sequence 495–571 (i.e. the double loop 9). Therefore, isolation and immunochemical investigation of double loop 9 (subdomain 6) should be extremely valuable for providing an independent verification or otherwise of this conclusion. This would be especially useful if the isolated subdomain retained the conformation it has within fragment 377–571 and within native BSA, in view of the fact that the immune response to native proteins is directed against their native three-dimensional structure (Atassi, 1967; Atassi & Thomas, 1969). Fragment 504–581, which corresponds almost exactly to double loop 9 of BSA had been previously isolated (Peters & Hawn, 1967) and CD studies have shown (Reed *et al.*, 1975) that it has retained in the free form, a remarkably highly ordered structure relative to fragment 377–581 or to native BSA. Accordingly, a detailed investigation was undertaken here of the immunochemical reactivity of fragment 504–581 in comparison to that of the larger fragment 377–571.

We have recently shown (Sakata & Atassi, 1979) with serial antisera from six different rabbits that the amount of antibodies reacting with fragment 377–571 in antisera to BSA is not static, but rather greatly dependent on the time antiserum is obtained after the first immunization. Therefore, the proper testing of whether or not the smaller fragment 504–581 carries the entire antigenic reactivity of the larger fragment 377–571, will have to be performed with different antisera from serial bleedings and not with an antiserum from a single bleeding nor with a pool of antisera. In this study, the immunochemical investigations were carried out with antisera to BSA from two different rabbits, obtained at different times after immunization (from 7 to 398 days).

The present immunoabsorbent studies show that fragment 377–571 binds at maximum (plateau value) a much higher proportion of the BSA antibodies in very early antisera (7 days) than fragment 504–581. However, this difference rapidly disappears within 15 days, and the immunoabsorbents of the two fragments bind identical plateau amounts of antibodies from a given serial antiserum from 15 days up to 398 days. Clearly, in very early antisera (7 days), a fraction of the antibodies is directed against additional sites on fragment 377–571 that are not present on the smaller fragment 504–581. However, the shared antigenic sites become immunodominant very rapidly after immunization and antibodies directed to both fragments increase with time in an identical manner throughout the period examined (up to 398 days). We have recently shown (Sakata & Atassi, 1979) that the

inhibitory activity of fragment 377–571 towards the precipitin reaction of BSA with its serial antisera from six different rabbits, also steadily increased with time after the initial immunization reaching as much as 90% with late antisera. The inhibitory activity of fragment 504–581 was tested with one such antiserum (rabbit B2 95 days) and it was identical to that of fragment 377–581, except that larger excess of the smaller fragment was required to reach the plateau.

That the two fragments 377–571 and 504–581 possess identical immunochemical reactivities was then further confirmed by their comparable inhibitory activities towards the binding of ^{125}I -albumin with its antisera and by the ability of fragment 504–581 to fully displace radioiodinated fragment 377–571 from binding with selected serial antisera to albumin. The finding that the smaller fragment fully inhibited the binding of the larger fragment with both the 38-day or the 398-day antisera from rabbits B8 and B9 was in full agreement with the results of the immunoabsorbent studies discussed above. The larger fragment 377–571 was proposed (Habeeb & Atassi, 1976) to carry two antigenic sites and the smaller fragment 504–581 has been suggested (Peters *et al.*, 1977) to carry at least one of these sites. The present findings indicate that both sites on the larger fragment are fully retained on the smaller fragment.

In view of the change in the amount of antibodies directed against fragment 377–571 with the time antiserum is obtained after immunization, it was extremely important to determine whether the change in specificity or at least in immunodominance of antibodies to certain antigenic sites was also accompanied by a change in heterogeneity of antibody affinity. The heterogeneity in the antibody response is of course well known, even against simple haptens (Karush, 1957; Nisonoff & Pressman, 1958, 1959; Onodera & Shiokawa, 1976). The heterogeneity of the antibody response against protein antigens would be much more complex. Karush (1957) and Nisonoff and Pressman (1958, 1959) have estimated heterogeneity quantitatively through direct binding measurements by the method of equilibrium dialysis. Such a method however cannot be applied when the antigen is a protein molecule. For antigens that are immobilized on a solid support, we have recently introduced (Sakata & Atassi, 1979) the acid dissociation profile to investigate antibody heterogeneity, using the pH of 50% dissociation and the ΔpH_{20}^0 as convenient parameters in such studies. Although we have not yet correlated these parameters with other physicochemical parameters of antibody heterogeneity, the method nevertheless provides a simple and convenient approach to study antibody heterogeneity and binding affinity to immobilized antigens. The pH at which 50% of the antibody molecules dissociate will express the average stability of the antibody–antigen complex against acid dissociation. The data shown in Figs. 3 and 4 clearly indicate that the BSA antibodies and the fraction therein directed against fragment 504–581 became more resistant to acid dissociation with time. In fact even at pH 1.78 with late antisera (398 days) on BSA-immunoabsorbents, about 15% of the antibody still resisted dissociation. More strikingly, at this low pH with the late antisera on immunoabsorbents of fragment 504–581, 33% of the antibody remained

bound to the immunoadsorbents. These findings are highly significant in view of the fact that acid dissociation after binding antibodies to immunoadsorbent is one of the routine techniques used to isolate specific antibodies and has been applied (Teale & Benjamin, 1977; Kamiyama, 1977) to BSA and some of its fragments. It should be realized that not only are these methods not quantitative, but lead unfortunately to the exclusion and loss of the fraction of antibodies with highest affinity. Therefore, any subsequent studies on the antibodies thus recovered suffer from the shortcoming that only low and intermediate affinity antibodies are represented while a large fraction of higher affinity is completely lost. For example, recently albumin antibodies were dissociated from fragment immunoadsorbents at pH 2.0 (Teale & Benjamin, 1977) or at pH 2.3 (Kamiyama, 1977). Although in each case the ionic strength was a little higher than that used in Figs. 3 and 4, our studies (S. Sakata & M. Z. Atassi, unpublished results) have shown that the dissociation profile is virtually unaltered throughout the range ($\mu = 0.05$ –1.1). Examination of Fig. 4 shows that at pH 2.0 and 2.3, about 43% and 55%, respectively, remain bound and are therefore not recovered. Thus, obviously studies involving only half the antibody population and of the lower affinity type at that must be interpreted with caution.

In conclusion, using serial antisera obtained from bleedings at different times after immunization, we have shown that the immunochemical reactivity of domain 3 (sequence 377–581) of BSA resides entirely in its last subdomain (sequence 504–581) in bleedings from 15 days up to 398 days. Although, in very early (7 days) bleedings the larger fragment carries some additional antigenic sites, the common sites become immunodominant relatively rapidly (15 days) after immunization. The heterogeneity of the antibodies decreases while their affinity increases with time after immunization.

Acknowledgements—This work was supported in part by grants from the National Institute of Arthritis and Metabolic Diseases (AM 18920), the National Institute of Allergy and Infectious Diseases (AI 13181) and the National Heart, Lung and Blood Institute (HLB 02751), National Institutes of Health, U.S. Public Health Service, and the Stephen C. Clark Research Fund of the Mary Imogene Bassett Hospital.

REFERENCES

- Atassi M. Z. (1967) Immunochemistry of sperm-whale myoglobins prepared with various modified porphyrins and metalloporphyrins. *Biochem. J.* **103**, 29–35.
- Atassi M. Z. & Saplin B. J. (1968) Immunochemistry of sperm-whale myoglobin. I. The specific interaction of some tryptic peptides and of peptides containing all the reactive regions of the antigen. *Biochemistry* **7**, 688–698.
- Atassi M. Z. & Thomas A. V. (1969) Immunochemistry of sperm-whale myoglobin. IV. The role of the arginine residues in the conformation and differentiation of their roles in the antigenic reactivity. *Biochemistry* **8**, 3385–3394.
- Brandt J., Anderson L.-O. & Porath J. (1975) Covalent attachment of proteins to polysaccharide carriers by means of benzoquinone. *Biochim. biophys. Acta* **386**, 196–202.
- Brown J. R. (1976) Structural origins of mammalian albumin. *Fedn Proc. Fedn Am. Socs exp. Biol.* **35**, 2141–2144.
- Farr R. S. (1958) A quantitative immunochemical measure of the primary interaction between I* BSA and antibody. *J. infect. Dis.* **103**, 239–262.
- Habeeb A. F. S. A. & Atassi M. Z. (1976) A fragment comprising the last third of bovine serum albumin which accounts for almost all the antigenic reactivity of the native protein. *J. biol. Chem.* **251**, 4616–4621.
- Hunter W. M. & Greenwood F. C. (1962) Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature* **194**, 495–496.
- Kamiyama T. (1977) Immunological cross-reactions and species-specificities of cyanogen bromide cleaved fragments of bovine, goat and sheep serum albumins. *Immunochemistry* **14**, 91–98.
- Karush F. (1957) The interaction of purified anti- β -lactoside antibody with haptens. *J. Am. chem. Soc.* **79**, 3380–3384.
- Kazim A. L. & Atassi M. Z. (1978) Production of autoantibodies by immunization with rabbit myoglobin. *Immunochemistry* **15**, 67–70.
- Kazim A. L., Habeeb A. F. S. A. & Atassi M. Z. (1979) Immunochemistry of serum albumin—VII. Effects of chemical modification of tyrosine and arginine residues on the conformation and immunochemistry of a fragment corresponding to the last third of bovine serum albumin. *Molec. Immunol.* **16**, 457–464.
- Lee C.-L. & Atassi M. Z. (1977) Enzymic and immunochemical properties of lysozyme. Accurate definition of the antigenic site around the disulfide bridge 30–115 (site 3) by 'surface-simulation' synthesis. *Biochem J.* **167**, 571–581.
- March S. T., Parikh I. & Cuatrecasas P. (1974) A simplified method for cyanogen bromide activation of agarose for affinity chromatography. *Analyt. Biochem.* **60**, 149–152.
- Nisonoff A. & Pressman D. (1958) Heterogeneity and average combining constants of antibodies from individual rabbits. *J. Immun.* **80**, 417–428.
- Nisonoff A. & Pressman D. (1959) Heterogeneity of antibody sites in their relative combining affinities for structurality related haptens. *J. Immun.* **81**, 126–135.
- Onodera M., Shiokawa H. & Takagi T. (1976) Fluorescent probes for antibody active sites. I. Production of antibodies specific to the *N*-methyl-2-anilino-naphthalene-6-sulfonyl group in rabbits and some fluorescent properties of the hapten bound to the antibodies. *J. Biochem.* **79**, 195–202.
- Peters T., Jr. & Hawn C. (1967) Isolation of two large peptide fragments from the amino- and carboxyl-terminal positions of serum albumin. *J. biol. Chem.* **242**, 1566–1573.
- Peters T., Jr., Feldhoff R. C. & Reed R. G. (1977) Immunochemical studies of fragments of bovine serum albumin. *J. biol. Chem.* **252**, 8464–8468.
- Reed R. G., Feldhoff R. C., Clute D. L. & Peters T., Jr. (1975) Fragments of bovine serum albumin produced by limited proteolysis, conformation and ligand binding. *Biochemistry* **14**, 4578–4583.
- Sakata S. & Atassi M. Z. (1979) Immunochemistry of serum albumin. VI. A dynamic approach to the immunochemical cross-reactions of proteins using serum albumins from various species as models. *Biochim. biophys. Acta* **576**, 322–332.
- Sakata S. & Atassi M. Z. (1979) Immunochemistry of serum albumin—V. A time-dependent examination of the antibody response to bovine serum albumin by the activity of its third domain. *Molec. Immunol.* **16**, 451–456.
- Teale J. M. & Benjamin D. C. (1977) Antibody as immunological probe for studying refolding of bovine serum albumin. *J. biol. Chem.* **252**, 4521–4526.