

IMMUNOCHEMISTRY OF SERUM ALBUMIN—V.

A TIME-DEPENDENT EXAMINATION OF THE ANTIBODY RESPONSE TO BOVINE SERUM ALBUMIN BY THE ACTIVITY OF ITS THIRD DOMAIN*

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Abstract—Antisera against bovine serum albumin (BSA) were prepared in six rabbits. Serial bleedings were obtained from each rabbit at different times after immunization (from 3 to 398 days) and were kept and studied separately. The change of the reactivities of fragment 377–571, which corresponds to the last third of BSA, was investigated with the serial antisera from each rabbit. The inhibitory activity of fragment 377–571 exhibited a biphasic change with time after the first immunization and, in late antisera, this fragment inhibited by almost 90% the immunochemical reactivity of BSA. Immunoabsorbents of the fragment were studied with regard to their maximum ability to bind radioiodinated antibodies to albumin in two sets of serial bleedings from two individual rabbits. In immunoabsorbent studies the time-dependent changes of the immunochemical activity of this fragment were irregular and generally increased with time, but it did not bind all the BSA-antibodies, even in late antisera. The differences between the two methods are discussed. Thus, by using serial bleedings, we have shown time-dependent changes in the antigenic reactivity of fragment 377–571 and have been able to find a rational explanation for the discordant results from various laboratories obtained with unspecified pools of antisera.

INTRODUCTION

The antigenic structure of serum albumin has been of great interest for over two decades (Lapresle, 1955a,b; Porter, 1957). Several fragments from bovine serum albumin (BSA)‡ have been prepared by various workers and their immunochemistry studied. In studies from our laboratory (Habeeb *et al.*, 1974; Atassi *et al.*, 1976; Habeeb & Atassi, 1976, 1977) we have found that two fragments, one corresponding to sequence 11–193 and the other corresponding to sequence 377–571 of BSA, each accounted by itself for over 90% of the antigenic reactivity of the intact albumin molecule (Atassi *et al.*, 1976; Habeeb & Atassi, 1976) and each fragment cross-reacted with antisera to the other (Habeeb & Atassi, 1977). Similar findings of very high reactivities by BSA fragments of equal or even smaller size have been reported by other laboratories. A fragment (Mol. wt 13,700) has been found (Yuan & Sell, 1976) to remove all albumin antibodies from a rabbit antiserum to BSA, and two other fragments of albumin have been shown (Muckerheide *et al.*, 1977) to inhibit by 85–90% the reaction of intact BSA with its mouse antisera. In

contrast to these reports, other workers have obtained much lower reactivities with related and other BSA fragments (Porter, 1957; Teale & Benjamin, 1976; Peters *et al.*, 1977; Kamiyama, 1977).

Given that in each case the fragments have been purified to homogeneity and lacked immunochemically-detrimental scissions within disulfide loops, we have concluded that the differences in the manner by which the antisera were obtained were responsible for the discordant results, which obviously have a direct bearing on how the antigenic structure of BSA is visualized. It is noteworthy that each of the foregoing studies (except refs Habeeb & Atassi, 1977; Muckerheide *et al.*, 1977) employed a pool of antisera with bleedings combined from one or more rabbits and over an unspecified period. The purpose of this work was to determine whether the antibody response to BSA will exhibit time-dependent changes in reaction with a given part of the BSA molecule. Accordingly, to test this hypothesis, we have in this report studied antisera in single serial bleedings obtained at different periods after immunization (from 3 days up to 398 days) from six individual rabbits. The antigenic reactivity of fragment 377–571 with each of these serial antisera has been determined by two different methods. It was most significant that for each rabbit the antisera reacting with this fragment showed a time-dependent change, generally increasing with time after the initial immunization.

MATERIALS AND METHODS

Bovine serum albumin (4× crystallized) was obtained from ICN Pharmaceuticals. Hen egg-white lysozyme (3×

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‡Abbreviation used: BSA, bovine serum albumin.

crystallized) and rabbit hemoglobin ($2 \times$ crystallized) were purchased from Sigma Chemical Co. Trypsin-TPCK and soybean trypsin inhibitor were from Worthington Biochemicals Corp. Fragment 377-571 was isolated from a partial tryptic hydrolysate of bovine serum albumin as previously reported (Habeeb & Atassi, 1976) and was homogeneous by disc-electrophoresis. Sepharose CL-4B, Sephadex G-25 and Sephadex G-75 were from Pharmacia Fine Chemicals. Cyanogen bromide, *p*-benzoquinone and acetonitrile were from Eastman Organic Chemicals. Carrier-free ^{125}I was obtained from New England Nuclear. All other chemicals employed were analytical grade and were used without further purification.

Antisera to BSA were obtained in six different outbred New Zealand White rabbits (B2, B3, B4, B8, B9, B10). Each rabbit was immunized according to the schedule as shown in Fig. 1 by injecting intramuscularly (each 3.75 mg albumin in 1.5 ml of an emulsion of equal volumes of phosphate buffered physiological saline at pH 7.2 and Freund's complete adjuvant). Rabbits were bled at different time intervals from 3 days after first immunization up to 398 days. Antisera from the serial bleedings obtained from each rabbit were not mixed but were kept and studied separately. Each antiserum was divided into 4.5-ml aliquots which were kept in the frozen state (-20°C) until use.

Quantitative precipitin and inhibition experiments were carried out with the whole antisera, i.e. not the IgG fraction, as previously described in detail (Atassi & Saplin, 1968). Purification of the IgG fraction from the antisera was performed as previously described (Lee & Atassi, 1977). Radioiodination of the IgG fractions by ^{125}I was done by the chloramine-T method (Hunter & Greenwood, 1962). Reagents and free ^{125}I were separated from radioiodinated IgG by gel filtration on a column of Sephadex G-25 (in phosphate-saline, pH 7.2 containing 0.1% rabbit hemoglobin) followed by dialysis at 0°C against phosphate buffered saline (0.01 M sodium phosphate buffer, pH 7.2 which was 0.15 M with respect to NaCl).

Sepharose CL-4B was activated by cyanogen bromide in 2 M sodium bicarbonate followed by coupling of BSA, lysozyme or fragment 377-571 in 0.2 M NaHCO_3 , pH 9.5 (March *et al.*, 1974). Activation of Sepharose by *p*-benzoquinone was done in 0.1 M phosphate buffer, pH 8.0 containing 20% ethanol (Brandt *et al.*, 1975). Coupling of albumin, lysozyme or fragment 377-571 onto activated Sepharose was done at pH 4.8. The adsorbents had 3.5-3.0 mg protein/ml of packed volume of Sepharose. Immunoabsorbent studies were performed in 0.1% rabbit hemoglobin solution in phosphate buffered saline, pH 7.2.

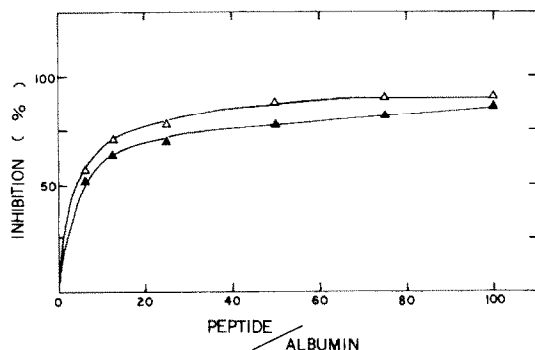


Fig. 1. Examples of the inhibition curves by fragment 377-571 towards the precipitin reaction of BSA with its antisera. (Δ) Antiserum of 95-day bleeding from rabbit B2; ($\blacktriangle$) antiserum of 80-day bleeding from rabbit B4. Each experimental point is the average of three determinations which varied $\pm 1\%$ or less. For results with other antisera see Table 1.

with shaking at 300 rev/min for at least 4 hr at room temperature. After shaking, immunoabsorbents were washed 6 times (3 ml each) with phosphate buffered saline and amounts of radioactive antibody bound to fragment 377-571, albumin or lysozyme immunoabsorbents were counted using a Packard well type α -counter. Results were expressed as per cent activity of the fragment relative to that of BSA as 100%.

Concentrations of protein solutions were determined from their absorbance at 280 nm and/or their nitrogen contents. Absorbance measurements were determined in a Zeiss PMQII spectrophotometer taking $E_{1\text{cm}}^{1\%}$ at 280 nm = 6.60 for BSA, 26.9 for hen egg-white lysozyme, and 13.8 for rabbit IgG. Nitrogen contents of protein solutions were determined as previously described (Atassi *et al.*, 1971).

RESULTS

Inhibitory activity of fragment 377-571

The inhibitory activities of fragment 377-571 towards the precipitin reaction between BSA and every serial antiserum from 6 rabbits were examined. Figure 1 shows two inhibition curves given by fragment 377-571 with two such antisera. Since a large number (over 50) of such titrations is involved, Fig. 1 will suffice as a representative example of such titration curves. All the results are summarized in Table 1, which gives the values of the maximum inhibitory activity of the fragment with each serial antiserum as well as the antibody content (in $\mu\text{g}/0.1\text{ ml}$) of each antiserum. The latter is derived from the precipitin curves with native BSA at equivalence. The changes with time after the initial immunization of the amounts of specific antibody and maximum inhibitory activities of fragment 377-571 are shown diagrammatically for one rabbit (B8) in Fig. 2 and are summarized for all six rabbits in Table 1. In rabbit B8,

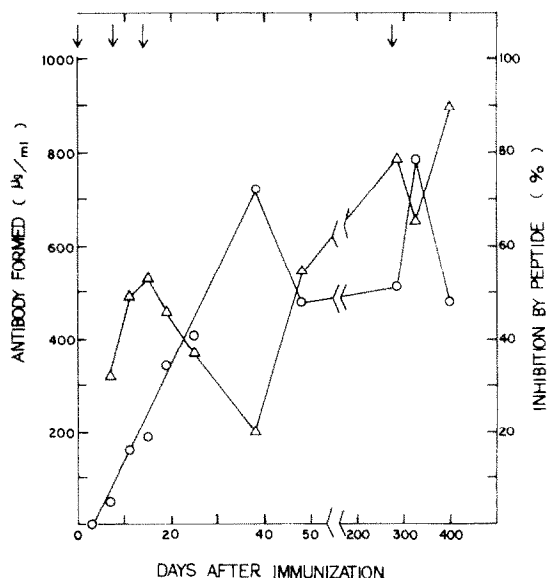


Fig. 2. A representation of time course changes of antibody contents and maximum inhibitory activities by fragment 377-571 in antisera of serial bleedings from rabbit B8. (○) Antibody content in $\mu\text{g}/100\text{ }\mu\text{l}$; (Δ) values of maximum inhibitory activity (%) by fragment 377-571. Arrows in figure indicate immunization with albumin. For results with six different rabbits, see Table 1.

Table 1. Changes of antibody content and inhibitory activity by fragment 377-571 in serial bleedings from six rabbits after immunization with albumin

Rabbit	Serial bleeding ^a (days)	Antibody content ^b ($\mu\text{g}/100\ \mu\text{l}$)	Inhibition by fragment 377-571 ^c (%)
B2	50	991	27
	55	968	36
	67	761	36
	80	769	32
	95	532	90
B3	50	611	36
	55	700	34
	67	666	41
	80	710	32
	95	587	21
B4	50	1086	52
	55	1198	35
	67	593	64
	80	517	86
B8	3	0	
	7	50	32
	11	162	49
	15	189	53
	19	345	46
	25	410	37
	38	722	20
	48	479	55
	286	513	79
	324	786	65
	398	479	89
B9	3	0	
	7	48	32
	11	171	31
	15	225	24
	19	193	38
	25	295	48
	38	462	16
	48	457	42
	286	382	86
	324	491	62
	398	429	83
B10	3	0	
	7	33	
	11	98	40
	15	161	39
	19	192	43
	25	432	50
	38	638	20
	48	761	18
	286	409	79
	324	665	64
	398	793	77

^aAll rabbits were immunized by the same immunization schedule. After the initial immunization, they were boosted at 7 days and 14 days. Rabbits B8, B9, B10 were again boosted at 250 days after the initial immunization.

^bValues determined from the equivalence point by quantitative precipitin reaction of native BSA with each antiserum.

^cMaximum percentage inhibition by fragment 377-571 of the precipitin reaction of BSA with each antiserum. Each value is the average triplicate determinations which varied $\pm 1\%$ or less.

the titer of specific antibody at 7 days after first immunization with BSA was $50\ \mu\text{g}/100\ \mu\text{l}$ and following two booster immunizations at 7 and 15 days with the same amounts of albumin the titer increased up to $722\ \mu\text{g}/100\ \mu\text{l}$ in 38 days. Inhibitory activities of fragment 377-571 varied from 20 up to 89% in the same rabbit. As shown in Fig. 2, the change in the inhibitory activity of fragment 377-571 showed a biphasic pattern. Its increase paralleled the rise in amounts of specific antibody up to 15 days after first immunization. However, after the second booster immunization (15 days), in contrast to the increase of specific antibody, the inhibitory activity gradually decreased from 53 to 20%. In late antisera this phenomenon was also observed. After booster immunization at 286 days an increase in specific antibody titer was observed, but the inhibitory activity of fragment 377-571 decreased from 79 to 65%. With decreasing antibody titer from $786\ \mu\text{g}/100\ \mu\text{l}$ to $479\ \mu\text{g}/100\ \mu\text{l}$, the inhibitory activity of this fragment again increased from 65 to 80%. Except for rabbit B3, this phenomenon was observed in all the rabbits examined (Table 1). The inhibitory activity of this fragment changed from 27 to 90% in rabbit B2; 21-41% in rabbit B3; 35-86% in rabbit B4; 20-89% in rabbit B8; 16-86% in rabbit B9 and 19-79% in rabbit B10. Therefore, except for rabbit B3 (which expired and could not be followed beyond day 95), in late antisera this fragment represented almost all the immunochemical activity of the albumin molecule.

Binding of radiolabelled antibodies to immunoabsorbents of fragment 377-571

Ten different IgG fractions of serial antisera from two rabbits (B8 and B9) were purified and iodinated with ^{125}I and kept at 0°C in 0.1% rabbit hemoglobin solution in phosphate buffered saline, pH 7.2. These radioiodinated preparations retained the full immunochemical reactivity of the respective parent antiserum, as determined by a micro-precipitin reaction. The immunochemical reactivity of fragment

Table 2. Comparisons of the inhibitory activities of fragment 377-571 and antibody binding abilities by immunoabsorbents of the fragment

Rabbit	Serial bleeding (days)	Maximum inhibitory activity (%) ^a	^{125}I -Antibody bound by immunoabsorbents (%) ^b
B8	7	32	69
	15	53	21
	38	20	24
	48	55	19
	398	89	51
B9	7	32	58
	25	48	29
	38	16	40
	48	42	29
	398	83	54

^aValues are from Table 1.

^bIndicate amounts of antibody bound in the plateau regions (see Fig. 3) by immunoabsorbents of the fragment relative to amounts bound by BSA-Sepharose as 100%. Values represent the average of triplicate analyses which varied $\pm 1.5\%$ or less.

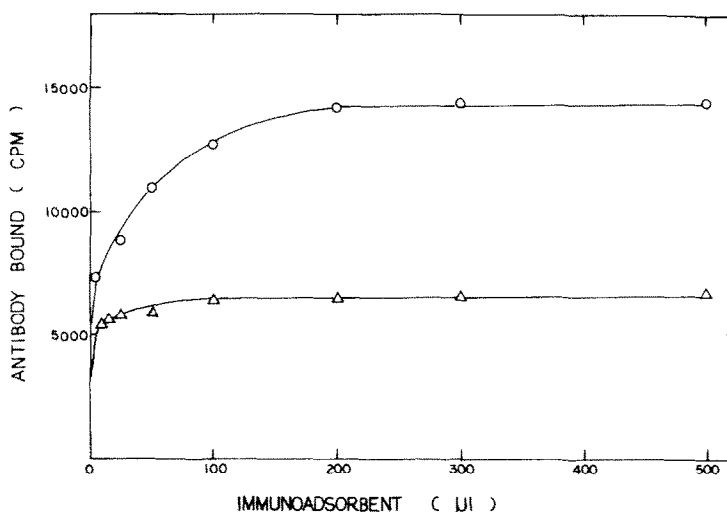


Fig. 3. An example of titration of constant amounts of radioiodinated antibody (antiserum of 398 day-bleeding from rabbit B8) with increasing amounts of immunoadsorbents. (○) BSA immunoadsorbent; (△) fragment 377-571 immunoadsorbent. Values represent the average of three determinations which varied $\pm 1.5\%$ or less. The results with other antisera are summarized in Table 2.

377-571 with a given antiserum was determined from the amount of radioiodinated antibody that can be maximally bound to this fragment immunoadsorbent relative to the amount that can be maximally bound by the albumin immunoadsorbent. A lysozyme immunoadsorbent was used to determine the extent of nonspecific binding. The amount of radioactivity bound to a given volume of lysozyme immunoadsorbent was subtracted from that bound to an equal volume of BSA or fragment 377-571 immunoadsorbents. Such nonspecific binding was less than 5% of the total radioactivity bound to albumin immunoadsorbents. To determine the maximum amount of antibody that can be bound by an immunoadsorbent, constant amounts of radioactive antibody were titrated with increasing amounts of the immunoadsorbent. Figure 3 shows an example of two such titration curves in which both BSA and fragment 377-571 achieved maximal antibody removal (plateau) at 200 μ l of packed volume of each immunoadsorbent. All the 10 radiolabelled antibodies were studied in this manner and the plateau binding values by fragment 377-571 are given in Table 2. It can be seen that the immunochemical reactivity of fragment 377-571 on immunoadsorbent changed with time: from 19 to 69% in rabbit B8, and from 29 to 58% in rabbit B9. It was hard to demonstrate any correlations between the values of direct binding by fragment immunoadsorbent and the results of inhibition studies mentioned above.

Sephacrose CL-4B was also activated using *p*-benzoquinone followed by coupling at pH 4.8 of albumin, fragment 377-571 or lysozyme. This was done because of the possibility that different amino acid side chains would be involved in the two coupling procedures. Comparison of the binding activities was done using both immunoadsorbents. Although the radioactivity bound by fragment 377-571 immunoadsorbent became higher than a CNBr-activated immunoadsorbent, the amount of radioactivity bound by albumin-Sephacrose, as well as the nonspecific binding by the lysozyme-Sephacrose,

were also higher. In fact, for a given antiserum, the per cent of labelled antibody bound (after correction for nonspecific binding) by the fragment immunoadsorbent was essentially the same by the two methods (results are not shown). We also carried out the coupling of albumin in the N form and the F form, namely BSA was coupled to Sepharose at pH 3.5 (F form) and pH 4.8 (N form) acetate buffer. However, the attachment of albumin in its different isomers had no effect on its immunochemical reactivity (results are not shown).

DISCUSSION

The present findings clearly reveal that the immunochemical reactivity of fragment 377-571 with antisera to albumin is not static but rather changes with time of bleeding after the first immunization. Thus, the inhibitory activity of the fragment varied from 20 up to 90%. In late antisera (later than 286 days) from each of the rabbits examined, the inhibitory activity of the fragment accounted for almost all the antigenic reactivity of native BSA. The results become even more compelling when it is pointed out that the inhibitory activities were determined with whole antisera and not with the IgG fractions of these antisera, and we have previously shown (Atassi *et al.*, 1976; Habeeb & Atassi, 1976) that when the IgG fraction is employed the inhibitory activity of the fragment is improved by about 10%. Thus, the inhibition by the fragment most likely accounts for the entire precipitin reactivity of albumin with its late antisera. As already mentioned in the Introduction, previous reports, including those from our own laboratory, on the immunochemical activities of different BSA fragments were all based on pooled antisera (Habeeb *et al.*, 1974; Atassi *et al.*, 1976; Habeeb & Atassi, 1976, 1977; Teale & Benjamin, 1976; Peters *et al.*, 1977; Kamiyama, 1977). Thus, for example, the inhibitory activity of fragment 377-581 by the Farr assay was reported by Teale & Benjamin (1976) to be 30%, with one antiserum pool, whereas

Peters *et al.* (1977) found that the same fragment inhibited by 43% the precipitin reaction of albumin with a pool of antisera to albumin. Results from our laboratory (Habeeb & Atassi, 1976) revealed that fragment 377–571 inhibited the precipitin reaction of albumin better than 90%. With the present investigations, carefully performed on separate serial bleedings from individual rabbits, we are now able to render a rational explanation to the various different values from different laboratories concerning the inhibitory activity of domain 3 of BSA.

The change in the values of the inhibitory activity of fragment 377–571 is most likely due to the presence of non-precipitating antibodies. These give soluble immune complexes with albumin and have long been known (Farr, 1958). The amounts of these soluble complexes are also known to change with time after immunization (Farr, 1958; Talmage & Maurer, 1953; Habeeb & Atassi, 1977). The inhibition of soluble immune complexes will not be detectable by the precipitin reaction. Therefore, for this system, immunochemical studies should employ additional quantitative techniques. We have used in this work a quantitative immunoabsorbent titration approach which would in principle entrap all antibodies, i.e. precipitating and non-precipitating. Furthermore, titration of fixed and minute amounts of radiolabelled antibodies with increasing amounts of immunoabsorbent would ensure that a plateau is reached indicating that all antibodies that can potentially bind with the immunoabsorbents had been depleted. However, the process of covalent coupling may take place via amino acid residues that are essential for immunochemical reactivity and the latter may thus be impaired. The fragment was therefore coupled on to Sepharose by two different activation reactions in view of the possibility that coupling may proceed via different amino acid side chains in each reaction and could avoid or minimize the involvement of antigenically essential side chains in cross-link formation. The results of antibody binding by the two immunoabsorbents were essentially the same, except that higher nonspecific binding (lysozyme–Sepharose) was obtained with the immunoabsorbents prepared by *p*-benzoquinone activation.

As in the inhibition of the precipitin reaction, the binding of serial ^{125}I -antibodies with the immunoabsorbent of fragment 377–571 also varied with time after the initial immunization, but there was no apparent quantitative relationship with the results of the inhibition studies. In the 7-day antisera higher amounts of antibodies were bound by fragment immunoabsorbent than would be expected from the inhibition data. With later antisera to BSA, the activity of the fragment on immunoabsorbent was about half its inhibitory activity. Because the two techniques rely on different principles and even though they often yield similar results with some protein systems (Lee & Atassi, 1977; Atassi, 1978*a,b*), different values should not be unexpected and have in fact been described (Kazim & Atassai, 1977). The differences may be explained on the basis that inhibition studies failed to measure non-precipitating antibodies that are readily detected by direct binding studies particularly in early antisera. In addition, the coupling of the fragment to Sepharose may have taken

place through immunochemically essential amino acids in such a way that the reactivity of one (out of two) antigenic sites was preferentially obliterated. Preferential coupling of a protein via selective side chains is not unusual and has been well documented (Cuatrecasas, 1969) in the coupling of insulin to Sepharose.

It is essential to comment here on our new immunoabsorbent approach to antibody binding and compare the results reported here with those derived by more 'traditional' immunoabsorptions which we had previously reported (Habeeb & Atassi, 1976, 1977). In those earlier studies (Habeeb & Atassi, 1976, 1977) our determinations relied on absorbance at 280 nm for measurements of amounts of antibody bound. Accordingly, huge amounts of antibody (1.0–1.1 mg) relative to immunoabsorbent (1 ml) had to be employed in those studies. One arbitrary volume of immunoabsorbent was employed and the fragment adsorbent bound 95% relative to absorbance bound by BSA–Sepharose as 100%. In the present work, the amount of radioiodinated antibody employed was less than 1 μg and was titrated with increasing amounts of immunoabsorbent to find the maximum (plateau) binding values which were achieved at 300–500 μl of immunoabsorbent. Therefore, in the previous studies which employed 1 ml of immunoabsorbent, the amounts of antibodies removed are far from saturation and would be comparable to values obtained at the extreme left (1 μl of immunoabsorbent) of Fig. 3, i.e. greater than 90% relative to 1 μl of BSA-adsorbent as 100%. In fact, Fig. 3 shows that the fragment immunoabsorbent approaches complete antibody removal in lower immunoabsorbent volume than the BSA–Sepharose. In the present work, the plateau values were carefully established. These precautions have been generally overlooked by workers engaged in protein immunoabsorbent studies.

In conclusion, the present studies clearly show that the differences between the immunochemical reactivities of the third domain of BSA (sequence 377–571), reported from various laboratories, are caused by time-dependent changes in the specificity of the antibody response to this part of BSA. It should also be expected that similar time-dependent changes in antibody specificity and/or immunodominance towards other parts of the albumin molecule would take place. It is quite likely that in late antisera the immune recognition becomes more tolerant and the antibodies more accommodating to minor structural differences between antigenic sites in this domain and elsewhere in the molecule, thus permitting the reported cross-reactions (Habeeb & Atassi, 1977) and the apparent increase in the antigenic reactivity of a given domain of albumin. Further studies are now in progress to elucidate differences in affinity and specificity (if any) between early and late antisera.

REFERENCES

- Atassi M. Z. (1978*a*) The precise and entire antigenic structure of lysozyme: Implications of surface-simulation synthesis and the molecular feature of proteins antigenic sites. In *Immunobiology of Proteins and Peptides* (Edited by Atassi M. Z. & Stavitsky A. B.) Vol. 1, pp. 41–100. Plenum Press, New York.

- Atassi M. Z. (1978*b*) Precise determination of the entire antigenic structure of lysozyme: Molecular features of protein antigenic sites and the potential of 'surface-simulation' synthesis, a powerful new concept for protein binding sites. *Immunochemistry* **15**, 909–936.
- 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., Perlstein M. T. & Habeeb A. F. S. A. (1971) Conformational studies on modified proteins and peptides. *J. biol. Chem.* **246**, 3291–3296.
- Atassi M. Z., Habeeb A. F. S. A. & Lee C.-L. (1976) Immunochemistry of serum albumin—II. Isolation and characterization of a fragment from the first third of bovine serum albumin carrying almost all the antigenic reactivity of the protein. *Immunochemistry* **13**, 547–555.
- 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.
- Cuatrecasas P. (1969) Interaction of insulin with the cell membrane: The primary action of insulin. *Proc. natn. Acad. Sci. U.S.A.* **63**, 450–457.
- 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.
- Habeeb A. F. S. A. & Atassi M. Z. (1977) Immunochemistry of serum albumin—IV. Immunochemical cross-reaction of fragments from the first third and the last third of bovine serum albumin. *Immunochemistry* **14**, 449–457.
- Habeeb A. F. S. A., Atassi M. Z. & Lee C.-L. (1974) Peptides with strong immunochemical inhibitory activity from bovine serum albumin. Application of a novel approach. *Biochim. biophys. Acta* **342**, 389–395.
- 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 bovine, goat and sheep serum albumins. *Immunochemistry* **14**, 85–90.
- Kazim A. L. & Atassi M. Z. (1977) Antibodies against protein antigenic sites that are identical in the homologous protein of the immunized animal. Autoreactivity in rabbits of antibodies to sperm-whale myoglobin. *Biochim. biophys. Acta* **494**, 277–282.
- Lapresle C. (1955*a*) Etude de la dégradation de la sérumalbumine humaine par un extract de rate de lapin. *Annls Inst. Pasteur, Paris* **89**, 654–665.
- Lapresle C. (1955*b*) Degradation of human serum albumin by extract of rabbit spleen. I. Optimum conditions for activity and mode of action of the proteases of rabbit spleen. *Bull. Soc. Chim. biol.* **37**, 969–975.
- 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.
- Muckerheide A., Pesce A. J. & Michael J. G. (1977) Immunosuppressive properties of a peptic fragment of BSA. *J. Immun.* **119**, 1340–1345.
- Peters T., Jr., Feldhoff R. C. & Reed R. G. (1977) Immunochemical studies of fragments of bovine serum albumin. *J. biol. Chem.* **252**, 8464–8468.
- Porter R. R. (1957) The isolation and properties of a fragment of bovine serum albumin which retains the ability to combine with rabbit antiserum. *Biochem. J.* **66**, 677–686.
- Talmage D. W. & Maurer P. H. (1953) I¹³¹-labelled antigen precipitation as a measure of quantity and quality of antibody. *J. infect. Dis.* **92**, 288–300.
- Teale J. M. & Benjamin D. C. (1976) Antibody as an immunological probe for studying the refolding of bovine serum albumin. *J. biol. Chem.* **251**, 4609–4615.
- Yuan L. & Sell K. W. (1976) Miniature BSA in immune responses. *Fedn Proc. Fedn Am. Soc. exp. Biol.* **35**, 753.