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A Fragment Comprising the Last Third of Bovine Serum Albumin Which Accounts for Almost All the Antigenic Reactivity of the Native Protein*

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The fragmentation of native bovine serum albumin by trypsin has been studied in aqueous solution under various conditions with regard to the yield and size of the fragments obtained. From a partial tryptic hydrolysate at pH 8.2 (40°, 1 hour), a homogeneous fragment was isolated in high yield by gel filtration on Sephadex G-100, followed by chromatography on DEAE-cellulose. The molecular weight of the fragment by gel filtration on calibrated Sephadex G-100 columns and by sodium dodecyl sulfate electrophoresis was 22,500. After reduction of the disulfide bonds followed by alkylation of the resultant thiol groups with iodoacetamide, the fragment retained homogeneity by disc electrophoresis and its molecular weight remained unchanged, indicating that it was composed of a single polypeptide chain. From its amino acid composition, sequence of the first 20 residues, and actions of carboxypeptidases A or B, it was unequivocally assigned to positions 377-571 in albumin. The inhibitory activity of the fragment was 90 to 93% towards the immune reaction of the protein with the IgG fraction of the antisera. The IgG fraction accounted for 96% of the total antibody activity in the antisera. An immunoabsorbent of fragment 377-571 removed 89 to 95% of the antibody to albumin. A fluorescent derivative of the fragment, which retained full immunochemical reactivity, was found to bind 2 mol of antibody/mol of peptide. The disulfides in peptide 377-571 were essential for its immunochemical reaction because the latter was entirely abolished upon reduction and S-alkylation of the disulfides. Since this fragment comprised only a third of the albumin molecule, but accounted for 90 to 95% of its antigenic reactivity, the results indicated that native albumin carries identical repeating antigenic reactive sites.

In previous studies we had reported the isolation and characterization of a large fragment from the first third of bovine serum albumin, which accounted for 84 to 90% of the total immune reaction of the native protein (1). The fragment was isolated by a novel approach which we had previously introduced (2) for obtaining fragments from disulfide-containing proteins without rupturing the disulfides. Reversible masking of the amino groups by citraconylation induces in these proteins conformational changes (3) which renders them accessible to tryptic hydrolysis at arginine peptide bonds. Following the removal of the protecting groups cleavage at the lysine peptide bonds may be continued, if desired. The aforementioned fragment, which was isolated after specific cleavage of bovine serum albumin at arginine peptide bonds (4), corresponded to positions 11-193 (less Arg 144) of albumin (1). However, for complete delineation of the antigenic reactive

sites of a protein antigen, the immunochemistry of a large variety of overlapping peptides from the parent protein (5, 6) must be studied. In order to elucidate the antigenic structure of albumin, one aspect of our strategy has been to find satisfactory procedures for fragmentation of the protein without rupturing the disulfide bonds and which yield peptides with good immunochemical reactivity. In the course of these studies we have found that albumin is partially hydrolyzable with trypsin and have studied its fragmentation pattern under various conditions. These studies, which are reported here, describe also the isolation and characterization of a homogeneous fragment from the protein, together with its detailed immunochemical properties.

EXPERIMENTAL PROCEDURE

Materials—Bovine serum albumin (crystallized three times) was obtained from Nutritional Biochemical Corp. Fluorescein isothiocyanate was from Calbiochem. Trypsin-TPCK (trypsin which had been treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone to eliminate chymotryptic activity, twice crystallized) was obtained from Worthington Biochemical Corp.

Tryptic Hydrolysis of Bovine Serum Albumin at 40°—Bovine serum albumin (1 g) was dissolved in 0.01 M sodium phosphate buffer (100 ml), pH 8, and the pH was adjusted to 8.2 and temperature brought to 40°. Tryptic hydrolysis was done at 40° with a trypsin/protein ratio of

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1/100 and the pH was maintained at 8.2 by the addition of 1 N NaOH. Hydrolysis periods of 30 min, 60 min, and 2 hours were investigated. After digestion, soybean trypsin inhibitor was added (14 mg). Inhibition of tryptic activity was confirmed by loss of action on *p*-tosyl-L-arginine methyl ester hydrochloride (7). The solution was then freeze-dried.

Tryptic Hydrolysis of Protein at 60°—A solution of albumin (0.5 g) in 0.01 M NH_4HCO_3 (50 ml), containing 62 mg of calcium chloride (to make it 0.011 M with respect to CaCl_2) was brought to pH 8.2 and 60°. The solution was then treated with trypsin-TPCK¹ (5 mg) for 15 min, keeping the pH at 8.2 by the addition of 1 N NaOH. After cooling the solution to room temperature, soybean trypsin inhibitor (7 mg) was added and the hydrolysate was freeze-dried.

Gel Filtration of Tryptic Hydrolysates—A portion (0.3 g) of the freeze-dried hydrolysate in 0.01 M NH_4HCO_3 (10 ml) was applied to a column (2.7 × 80 cm) of Sephadex G-100 which was eluted with 0.01 M NH_4HCO_3 . The eluate was monitored for absorbance at 280 nm and fractions (7.5 ml) belonging to one peak were combined and freeze-dried. The relative amounts of protein in each peak and the total protein recovery were calculated from optical density as well as from dry weight recoveries.

Chromatography of Peptide on DEAE-cellulose—A portion (100 mg) of Peak 3 material in the aforementioned gel filtration experiment was dissolved in 0.005 M sodium phosphate buffer (2 ml) at pH 6.2 and dialyzed exhaustively against the same buffer. The solution was applied to a column (1.5 × 20 cm) of DEAE-cellulose which had been pre-equilibrated with the same buffer. The column was initially eluted with 0.005 M phosphate buffer, pH 6.2. Following emergence of the first protein peak, stepwise elution with salt concentrations at pH 6.2 was employed. Thus 0.005 M phosphate buffer was followed by 0.0175 M phosphate, then 0.0175 M phosphate + 0.077 M NaCl and finally 0.0175 M phosphate + 0.5 M NaCl. Column effluents were read at 280 nm and fractions (3 ml) under a given peak were pooled, dialyzed against water, and then freeze-dried.

Reduction and Alkylation of Peptide—To a solution (0.6 ml) of the peptide (30 mg) in Tris-glycine buffer, pH 7, containing 0.5 mg of EDTA/ml, was added 5 M guanidine hydrochloride at pH 8 (1 ml) containing 0.5 mg of EDTA/ml, followed by an aliquot (0.4 ml) of 0.25 M β -mercaptoethanol. The solution was allowed to stand at room temperature for 1 hour. A portion (0.15 ml) of the reduction mixture was removed, the peptide was precipitated and washed with 5% trichloroacetic acid, the free sulfhydryl content was determined by Ellman's reagent (8), and the number of reduced disulfides was calculated as described previously (9).

To the remainder of the reduced peptide was added iodoacetamide (30 mg) and the reaction was allowed to proceed at pH 8.0 for 30 min. Completeness of the alkylation was confirmed by reaction with 5,5'-dithiobis(2-nitrobenzoic acid), and subsequently by amino acid analysis. The reduced, S-alkylated peptide was desalted by gel filtration on a column (2 × 40 cm) of Sephadex G-25 in 0.01 M NH_4HCO_3 and the material was then freeze-dried.

Analytical Methods—Procedures for disc electrophoresis, peptide mapping, hydrolysis of protein, and peptide samples in constant boiling HCl or in 3 N *p*-toluenesulfonic acid (10), and amino acid analysis of the hydrolysates were carried out as previously described (1). Concentrations of protein and peptide solutions were derived from their nitrogen contents, which were determined by the method described elsewhere (11). The nitrogen contents of peptide 377–571 was calculated from its composition (Table II) and that of albumin was also taken as 16.3%. The molecular weight of the fragment was determined by gel filtration on a calibrated Sephadex G-100 column and also from its mobility in SDS-sodium phosphate disc electrophoresis relative to those of several standard proteins of known molecular weights by procedures already described (1). Sequence analyses were done on a Beckman 890C sequencer in 1 M Quadrol standard program No. 042772. The PTH-derivatives were identified by gas chromatography on a Hewlett Packard 7610A chromatograph with 2-mm (inside diameter), 4 feet long columns of DC 560 using the system of Pisano *et al.* (12), as well as by thin layer chromatography (13).

Immunochemical Methods—Antisera to albumin were raised in three rabbits (14) and were mixed and freeze-dried. Antisera were reconstituted to the original volume with water to contain 1.7 mg of anti-albumin/ml. Quantitative precipitin and inhibition experiments

were carried out as previously described in detail (15). All immunochemical reactions were carried out in 0.01 M phosphate buffer at pH 7.0 containing 0.15 M NaCl. Methods for the preparation of the IgG fraction of anti-albumin (which accounted for 96% of the total antibody reactivity of the whole antiserum), the preparation of immunoabsorbents, the determination of antibody binding by immunoabsorbents, the reaction of the peptide with fluorescein isothiocyanate, and the determination of binding of the fluorescein-peptide conjugate to anti-albumin antibody have been described (1).

Preparation of Pure Anti-albumin Antibody by Sepharose-Albumin Immunoabsorbent—A suspension (2 ml) of Sepharose-albumin conjugate was mixed gently, in a small chromatographic column (1 × 10 cm), with antiserum to albumin (10 ml) for 2 hours at room temperature. The protein was then run out and the column was eluted with 0.01 M phosphate, pH 7.0/0.15 M NaCl until absorbance at 280 nm reached 0.005. The absorbed antibody was eluted with 5 M guanidine hydrochloride and the solution was desalted on a column (1.5 × 35 cm) of Sephadex G-25 by elution with 0.01 M NH_4HCO_3 . Fractions from the first peak were pooled and concentrated with a PM-30 Diaflo membrane and the antibody content was determined by quantitative precipitin reaction.

RESULTS

Tryptic Hydrolysis of Bovine Serum Albumin—Hydrolysis of albumin with trypsin at 40° for 30, 60, and 120 min resulted in similar products as shown by disc electrophoresis in Tris-glycine (Fig. 1) and in SDS gels. Also, the relative amounts of the fragments, as determined by gel filtration (Fig. 2 and Table I), were very similar. Tryptic hydrolysis at 60° gave a much lower yield of the 22,500-dalton fragment (Fig. 3). As will be subsequently shown, this fragment carried very high immunochemical reactivity and its preparation in good yield was desirable. A hydrolysis time of 1 hour at 40° was therefore chosen for all subsequent preparations.

Gel Filtration of Tryptic Hydrolysate—A gel filtration pattern of a 60-min tryptic hydrolysate is shown in Fig. 2. Five components were obtained. The first component corresponded to an aggregate of albumin and was not investigated. Component 2 corresponded to native protein and its quantitative precipitin curve with antisera to albumin was superimposable with that of the native protein. Component 3 possessed inhibitory activity, while the fourth and fifth components had little or no inhibitory activity. The fourth component corresponded to a fragment of $M_r = 8000$. The fifth component was composed of small peptides and gave at least 46 spots by peptide mapping. The recoveries of each component, by weight, are shown in Table I.

Chromatography and Characterization of Third Component from Gel Filtration—Examination of component 3 from gel

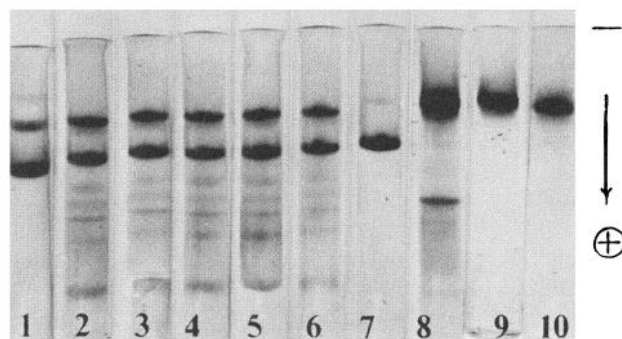


FIG. 1. Disc electrophoresis patterns in Tris-glycine buffer of: 1, bovine serum albumin; 2, tryptic hydrolysate at 40° and 2 hours; 3, 4, and 5, tryptic hydrolysates at 40° and 1 hour; 6, tryptic hydrolysate at 40° and 30 min; 7, Peak 2 in Fig. 2A; 8, Peak 3 in Fig. 2A; 9 and 10, Peak 3 from Fig. 2A, after chromatography on DEAE-cellulose (Fig. 2B).

¹ The abbreviations used are: TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate.

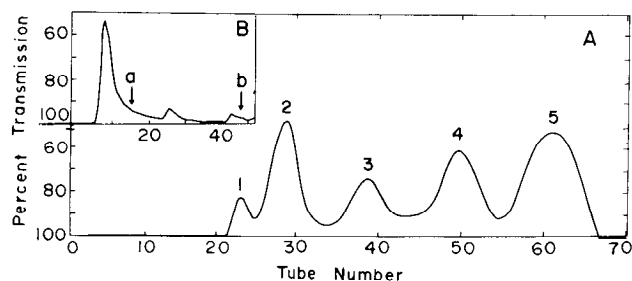


FIG. 2. A, gel filtration pattern of a 40° tryptic hydrolysate (1 hour) of albumin. The hydrolysate (0.3 g) was applied onto a column (2.7 × 80 cm) of Sephadex G-100 which was eluted with 0.01 M NH_4HCO_3 . Fractions (7.5 ml each) were analyzed continuously by an ultraviolet monitor. B, chromatographic pattern on DEAE-cellulose of Peak 3 from A. The material (100 mg) was applied on column (1.5 × 20 cm) which was subjected to stepwise elution. Initial elution was with 0.005 M sodium phosphate buffer at pH 6.2. At Position a the column was eluted with 0.0175 M phosphate, pH 6.2, and at Position b the eluent was changed to 0.0175 M phosphate, pH 6.2, + 0.077 M NaCl. Fractions (3 ml) were read continuously by an ultraviolet monitor.

TABLE I

Recovery of fractions from tryptic hydrolysates of albumin under various conditions

Recoveries are given in per cent of total and are based on the weight recovery of each fraction in gel filtration on Sephadex G-100 (see Figs. 2 and 3)

Conditions of tryptic hydrolysis	Per cent recovery				
	Fraction 1	Fraction 2	Fraction 3 ^a	Fraction 4	Fraction 5
I. Hydrolysis at 40°					
30 min ^b	1.7	15.3	21.8	19.6	41.0
1 hr ^c	2.2	14.7	23.7	20.4	39.5
2 hr ^d	1.9	14.0	20.9	18.3	44.2
II. Hydrolysis at 60°					
15 min ^e	1.6	15.8	9.1	25.9	46.7

^a Note that a maximum weight recovery of 33% constitutes a 100% yield, since this fragment is one-third the size of the albumin molecule.

^b Average of 4 experiments.

^c Average of 8 experiments.

^d Average of 12 experiments.

^e Average of 2 experiments.

filtration by disc electrophoresis revealed considerable heterogeneity (Fig. 1). Chromatography on DEAE-cellulose was therefore carried out and the pattern is shown in Fig. 2. The major fraction, which emerged unretarded from the column, was homogeneous in disc electrophoresis (Fig. 1). Determination of its molecular weight on calibrated Sephadex columns gave a value of 22,500. In SDS electrophoresis, it gave a molecular weight of 23,000. After reduction of the disulfides and S-alkylation of the resultant thiol groups, component 3 was still electrophoretically homogeneous and in SDS electrophoresis showed no change in its molecular weight. This indicated that component 3 comprised a single continuous polypeptide chain which was cross-linked by disulfides. The fragment had no free thiol groups. Amino acid analysis of the fragment (Table II) showed it to correspond to positions 377–571 of albumin. In the sequence of albumin (16) positions 400, 401, and 402 have not been identified. Therefore the theoretical composition lacks three unknown residues. In our composition (Table II) there seemed to be an additional residue each of aspartic acid, lysine, and histidine. The molec-

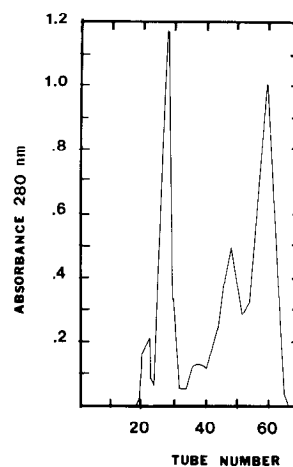


FIG. 3. Gel filtration pattern of a 60° tryptic hydrolysate (15 min). The hydrolysate (0.5 g) was applied onto a column (2.7 × 80 cm) of Sephadex G-100 which was eluted with 0.01 M NH_4HCO_3 . Fractions (7.5 ml each) were read at 280 nm.

ular weight of the fragment, calculated from its amino acid composition by assuming these three additional residues, was 22,145. Sequence determination of the first 20 residues showed them to correspond to positions 377–396 of the protein (Fig. 4). Carboxypeptidase A had no action on the fragment, whereas carboxypeptidase B yielded only lysine, which was identified by peptide mapping of the digest. From the results of amino acid analysis, sequence determination and behavior with the two carboxypeptidases the fragment corresponded with excellent agreement to positions 377–571 of albumin.

Immunochemistry of Fragment 377–571—In quantitative precipitin analysis, the fragment failed to give an immune precipitate with antisera to albumin when tested over a wide range of peptide concentration. However, peptide 377–571 inhibited the reaction of albumin with its antisera. The inhibitory activity of the peptide was $91.5 \pm 1.5\%$ towards the reaction of albumin with the IgG fraction of the antisera (Fig. 5). The IgG fraction, as mentioned earlier, accounted for 96% of the total antibody activity of the whole antiserum. The molar excess of peptide required to achieve half the maximum inhibition was 11. After reduction at the disulfides followed by S-alkylation the peptide had no inhibitory activity, even at several hundred fold-molar excess of peptide relative to albumin. Since at this level of broad delineation of antigenic structure variability should not be expected (17), antisera from three rabbits were mixed for the purpose of quick screening. Differences will appear at the stage of final delineation (18, 19) and when the investigation approaches that stage, antisera should not be mixed (6).

Binding of Antibody by Peptide-Immunoabsorbent—The results of binding studies with immunoabsorbents are summarized in Table III. The albumin-immunoabsorbent absorbed out quantitatively all the antibodies in the IgG fraction. With the Sepharose-peptide conjugate, the first column bound 62% of the total antibody. Residual antibody to peptide 377–571 was removed by subsequent passage of effluent fractions through a series of peptide-immunoabsorbent columns. The amount of antibody removed by peptide-immunoabsorbent leveled off in three absorption experiments at 89% of total antibody in the IgG fraction. The total amount of bound antibody which was elutable by 5 M guanidine hydrochloride was 95%. These results are in excellent agreement with the

Antibody Binding to Peptide-Fluorescein Derivative—The fluorescein derivative of peptide 377–571 had a fluorescein/peptide ratio of 0.65, and a fluorescence intensity of 51,500 units/mg. The peptide derivative retained its full inhibitory activity. After mixing of this derivative with antibody solution, fluorescence eluted with the antibody peak on gel filtration (Fig. 6). The amount of fluorescein derivative bound to different amounts of anti-albumin antibody is shown in Table IV, together with antibody/peptide molar ratio. Experiments were carried out at different concentrations of peptide and

Amino acid composition of fraction 3 of the tryptic hydrolysate^a

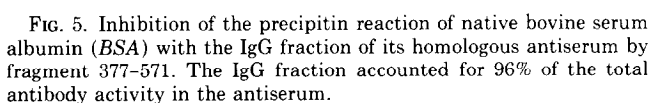
Amino acid	Calculated ^a for positions 377-571	Found
Aspartic acid	15	16.2
Threonine	17	17.0
Serine	8	7.95
Glutamic acid	26	25.8
Proline	12	11.6
Glycine	4	4.15
Alanine	11	10.9
½ Cystine	12	12.0
Valine	17	16.3
Methionine	2	1.56
Isoleucine	5	4.96
Leucine	19	18.5
Tyrosine	4	3.86
Phenylalanine	8	7.76
Tryptophan	0	0
Lysine	20	20.6
Histidine	4	4.82
Arginine	8	7.93
Total No. ^a residues		195
M_r^a		22,145
% N		16.37

FOUND SEQUENCE

[illegible]

antibody, but in the region of peptide excess. It can be seen in Table IV that 2 mol of antibody were bound/mol of peptide. Similar results were obtained when either purified specific antibody to albumin or the IgG fraction of the antiserum was employed. In the latter case, results were calculated on the basis of the specific antibody content in the IgG fraction as determined by quantitative precipitin analysis.

Fragmentation of bovine serum by trypsin at 40° released mainly the COOH-terminal peptide 377-571 and a lower (about 8000) molecular weight peptide and the rest of the molecule was extensively degraded. On the other hand, in tryptic hydrolysis at 60° the amount of peptide 377-571 was considerably decreased and, in addition to more undigested albumin, smaller molecular weight fragments were mainly produced. Recently, milder (at 25°) digestion of albumin in solution at pH 8.15 with a much smaller amount of trypsin (1 mg/g of albumin) was reported (20) to give two fragments of molecular weights 39,000 and 9,000. Tryptic hydrolysis of



*Absorption of anti-albumin antibodies by albumin-immunoabsorbent
or by immunoabsorbent carrying fragment 377-571*

Protein or peptide on immuno- absorbent	No. of column	Amount antibody applied ^a	Amount antibody bound		Amount antibody eluted ^{a, b}	
		mg	mg	%	mg	%
Albumin	1	1.1	1.1	100	1.13	103
Peptide 377-571	1	1.030	0.64	62.1	0.67	65.1
	2		0.15	14.6	0.18	17.5
	3		0.13	12.6	0.13	12.6
	4		0	0	0	0
Total			0.92	89.3	0.98	95.2

^a Determined by quantitative precipitin reaction.

^b Eluted with 5 M guanidine HCl, pH 8.5.

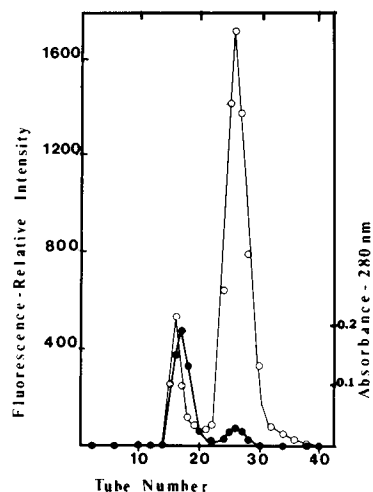


FIG. 6. Elution pattern of a mixture of the IgG fraction of anti-albumin and the fluorescent derivative of fragment 377-571 on Sephadex G-100 (1.5 × 86 cm). Fractions (3.3 ml) were monitored for absorbance at 280 nm (●), and for fluorescence at 520 nm (○).

TABLE IV

Binding of fluorescein-peptide derivative with anti-albumin antibody

Anti-albumin antibody		Fluorescein-peptide	Fluorescence in antibody peak	Peptide bound		Antibody/peptide molar ratio
mg	μmol	mg		mg	μmol	
I. Antibody in IgG fraction ^a						
1.035	0.00647	0.385	3704	0.072	0.0032	2.02
1.035	0.00647	0.385	4192	0.0814	0.0036	1.80
0.517	0.00323	0.900	1870	0.0363	0.0016	2.02
II. Pure antibody from immunoabsorbent						
0.40	0.0025	0.20	1380	0.0268	0.00120	2.08
0.40	0.0025	0.40	1555	0.0302	0.00134	1.87
0.40	0.0025	0.60	1488	0.0290	0.00129	1.94
0.40	0.0025	0.80	1453	0.0282	0.00125	2.00

^a Determined by quantitative precipitin analysis.

albumin coupled to palmityl-aminoethylaminoagarose gave two fragments of molecular weights 23,000 and 9,000 (20). The 23,000-dalton fragment corresponded to positions 377-581 (20). By contrast, tryptic hydrolysis of citraconylated albumin at arginine peptide bonds yielded an NH₂-terminal fragment (11-193), with extensive digestion of the COOH-terminal part of the molecule (1). The pathways of tryptic hydrolysis indicate different regions of susceptibility under various conditions of hydrolysis.

The fragment 377-571 studied here differs somewhat from that of Peters and Feldhoff (sequence 377-581, Ref. 20). It is also noteworthy that our fragmentation procedure is simpler since it is performed directly on the native protein in solution and does not require the preparation of an affinity column. Furthermore, the recovery of the fragment (which is 21 to 24% of the total weight) corresponds to 64 to 73% of the possible theoretical yield. Peters and Feldhoff (20) performed no biological or immunochemical studies on fragment 377-581 and, therefore, its immunochemical behavior cannot be compared directly with our shorter fragment 377-571. However, the two fragments should be expected to have identical immunochemical properties.

Although a large (about 30) molar excess of the peptide

relative to albumin was seemingly required to achieve maximum inhibition, the peptide possessed in fact a high binding efficiency as indicated from the small molar excess required to achieve 50% of the maximum inhibition. This is several times smaller than the excess required of even intact whole antigenic-reactive regions of myoglobin in inhibition of their homologous immune system (18, 19, 21-23). The efficiency of the present peptide is similar to that shown by disulfide-containing antigenic reactive sites of lysozyme (2, 24-26). Accordingly, by comparison with other systems, the molar excess required of the present peptide to achieve maximum inhibition is quite low. We had previously shown (15) that a large molar excess of an inhibitory peptide is necessary because of conformational factors. Since the immune response to native protein antigens is directed against their native three-dimensional structure (27), for proper binding with antibody, an antigenic reactive site must assume a mode of folding which approximates the one it had in the native protein (15, 28-30). The probability of finding such a conformational state will improve with increase in peptide concentration (15). The high efficiency of the present peptide is due to the disulfides which prevent complete unfolding. In fact rupture of the disulfides resulted in complete loss of the inhibitory activity, indicating their importance in bringing the parts of the antigenic site(s) in the conformational proximity necessary for binding with antibody.

The high inhibitory activity of fragment 377-571 is in excellent quantitative agreement with its behavior in immunoabsorbent studies. Immunoabsorbent studies, unlike the inhibition studies, would also account for any antibodies that may form soluble complexes with the intact antigen. It is significant, therefore, that the amount of total antibody removed by the peptide was equivalent to the peptide's inhibitory activity. However, a single immunoabsorbent did not remove all the albumin antibody, but removal of the latter leveled off at 89 to 95% in three immunoabsorbent experiments. This may have been mostly caused by a decrease in the binding efficiency of the peptide due to its covalent modification and conformational change in the process of cross-linking onto the Sepharose.

The present fluorescent binding studies, which showed that peptide 377-571 was able to bind with 2 mol of antibody molecules, do not reveal whether the peptide carried two antigenic sites or four antigenic sites. However, it is more likely that only two antigenic sites are present. With these antisera, 6 antibody molecules were bound/mol of albumin by extrapolation to zero antigen concentration. Clearly, this peptide which comprises a third of the parent protein would also be expected to carry a third (*i.e.* two) of its total sites. Furthermore, it is sterically very difficult for a single antibody molecule to bind with two sites on the same peptide antigen. Therefore, the binding sites of the antibody will be filled by two different peptide molecules and the presence of four antigenic sites would favor the formation of an immune precipitate, which is not the case.

The immunochemical reactivity of peptide 377-571 can be seen in proper perspective when it is compared to the reported behavior of other albumin fragments. Fragments from human serum albumin, isolated from prolonged chymotryptic hydrolysates, react only partially with antisera to human serum albumin (31, 32). Furthermore, the recovery of these fragments is extremely low (0.038 to 0.3% of the starting material). Another human serum albumin fragment, prepared (33-35) from cathepsin digests, had a molecular weight of 11,000 and

inhibits the homologous immune reaction only 15 to 40%. The present peptide, which was obtained in high yield, possesses a remarkably high immunochemical reactivity accounting virtually for all (89 to 95%) of the antigenic reactivity of the native protein.

Recently we reported (1) the preparation and immunochemistry of another fragment from an entirely different part of the molecule (*i.e.* positions 11 to 193, less Arg 144) which also accounted for almost all (89%) of the antigenic reactivity of albumin. The fact that two fragments, one comprising the first third and the other the last third of the protein molecule, can each by itself account for the entire antigenic reactivity is unique in protein immunochemistry and was entirely unexpected. The structure of albumin has been suggested (16) to comprise three repeating units or domains. It is not yet possible to determine if this suggested structural repetition can rationalize the immunochemical findings, since the antigenic sites may be made up of residues that are distant in sequence but close in three-dimensional structure (25, 26, 36). At any rate, as we have previously suggested (1), the immunochemical results can only be explained by the concept that albumin carries repeating and immunochemically identical antigenic reactive sites. Further understanding of this somewhat strange phenomenon will require the elucidation of the total antigenic structure, and work is now being carried out in our laboratories towards this goal.

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