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Partial Modification of Bovine Serum Albumin with Dicarboxylic Anhydrides. Physical Properties of the Modified Species*

Ana Jonas† and Gregorio Weber

ABSTRACT: Bovine serum albumin was modified chemically with succinic, maleic, and citraconic anhydrides, and the properties of the derivatives were studied as to the nature and extent of the structural changes in the protein. Fluorescence polarization, rotational relaxation time, and sedimentation velocity measurements, as well as ionic strength, nature of salt, and pH dependence of fluorescence polarization experiments, led to the specification of conditions (*e.g.*, pH 7.0, water solution, 20°, for a 65% modified sample) under which the bovine serum albumin derivatives were shown to exist in an expanded form, very similar in its physical properties to bovine serum albumin expanded by electrostatic repulsion at pH 2. Modification of bovine serum albumin with citraconic anhydride was shown to be reversible; the citraconyl groups were easily removed under mild acidic conditions, without affecting the physical properties of the protein in relation to a control bovine serum albumin sample. The expansion of chemically modified serum albumin appeared to be a nonspecific process, depending on the gradual modification of about 80% exposed and 20% buried lysine residues in the protein.

Chemical modification with succinic anhydride was shown by Habeeb *et al.* (1958) to produce marked changes in the structure of bovine serum albumin. The conformational changes, detected by viscosity and sedimentation velocity measurements, were attributed to expansion resulting from the high charge density of the succinylated bovine serum albumin molecules. Similar results were obtained by Habeeb (1966), who determined the Stokes radii of chemically modified bovine serum albumin from elution volumes on Sephadex columns, and by Sun (1969), who measured the reduced viscosities of succinylated and partially succinylated bovine serum albumin as a function of pH.

In addition to bovine serum albumin many other proteins were reported to undergo structural changes on reaction with succinic anhydride, *e.g.*, γ-globulin and β-lactoglobulin (Habeeb *et al.*, 1958), transferrin, conalbumin, and orosomucoid (Bezkorovainy *et al.*, 1969), hemerythrin (Klotz and Keresztes-Nagy, 1963), and aldolase (Hass, 1964).

Butler *et al.* (1967, 1969) introduced maleic anhydride as a

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reversible blocking reagent for amino groups of peptides and proteins in amino acid sequencing experiments. Recently, Dixon and Perham (1968) used a similar amino group reagent, 2-methylmaleic anhydride (citraconic anhydride), which had the advantage over maleic anhydride of being very easily hydrolyzed under mild acidic conditions.

At low pH (from pH 4 to 2), bovine serum albumin is known to expand due to the electrostatic repulsion of the positive charges on its surface. The properties of the acid-expanded protein have been studied extensively (Weber, 1953; Harrington *et al.*, 1956; Foster, 1960), and subunit-like models for its tertiary structure were proposed on the basis of those investigations (Harrington *et al.*, 1956; Foster, 1960; Luzzatti *et al.*, 1961; Weber and Young, 1964; Bloomfield, 1966).

In view of the continued interest in the tertiary structure of bovine serum albumin, we undertook the study of conformational changes in this protein at different stages of chemical modification by dicarboxylic anhydrides, in an attempt to establish how related and how specific are the expansion processes by acid and by reaction with the anhydrides. The effect of the reversible reaction of citraconic anhydride on the structure of bovine serum albumin was also investigated.

Materials and Methods

Crystalline bovine serum albumin, Lot No. D 71309, was obtained from Armour Pharmaceutical Co. The molecular weight of the protein was taken as 67,000, and the presence of less than 5% dimer was ignored. DNS[1]-labeled bovine serum albumin[2] was prepared according to the method of Weber (1953). All the DNS-labeled bovine serum albumin preparations had a DNS:bovine serum albumin molar ratio of 2 to 3, as estimated from the absorbance of the conjugate at 340 mμ, using the molar extinction coefficient, ϵ 4.3×10^3 M^{-1} cm^{-1} (Weber, 1952b).

The succinic and maleic anhydrides were purchased from Eastman Organic Chemicals, while citraconic anhydride was obtained from Aldrich Chemical Co. All the salts, acids, and bases used in these experiments were commercially available, reagent grade chemicals. Sucrose, an Allied Chemical Co. product, was recrystallized once from ethanol. Sephadex gels were obtained from Pharmacia Fine Chemicals. Deionized and glass-distilled water was used throughout these experiments.

Absorption spectra were obtained with a Cary Model 15 recording spectrophotometer; a Zeiss spectrophotometer was used for absorbance (A) measurements. Sedimentation velocity coefficients were obtained on a Beckman analytical ultracentrifuge, Model E-359, using Schlieren optics.

Fluorescence spectra were recorded with the spectrofluorometer described by Weber and Young (1964), equipped with a high-gain current-detection preamplifier and electronic filter. Fluorescence polarization measurements were carried out on the instrument described by Weber (1956), having an amplifier unit with high-gain current-detection preamplifier, electronic filter, and analog sum and difference circuits, which in connection with a Dana digital voltmeter, Model 5403, is capable of displaying fluorescence polarization values directly. A Corning glass 7-60 filter was used to isolate the 366-mμ exciting line from the mercury lamp, and 3-72 Corning glass filters, plus 2 mm of a 1 M $NaNO_2$ solution were used to filter the DNS fluorescent light. The cross-correlation-phase fluorometer of Spencer and Weber (1969) was used in the measurement of absolute fluorescence lifetimes. The same emission filters were used as in the fluorescence polarization instrument.

Preparation of Bovine Serum Albumin and DNS-Labeled Bovine Serum Albumin Derivatives. The reactions of bovine serum albumin and DNS-labeled bovine serum albumin with succinic, maleic, and citraconic anhydrides were carried out at room temperature, with continuous stirring, and manual adjustment of pH with 1 M NaOH, to pH 6.0–7.5 for the succinic and maleic anhydrides, and to pH 7.0–8.5 for citraconic anhydride. Protein solutions were 0.5–1.0%, in water. The anhydrides were added to the protein solutions in small batches, and after each addition, when the reaction stopped as judged from the stationary pH of the solution, samples were removed for analysis. For maximum reaction of the amino groups of bovine serum albumin with succinic and maleic anhydrides, a 15:1 molar ratio of anhydride to amino group was required. The efficiency of the reaction with citraconic anhydride was approximately five times higher than with the other anhydrides. For a 70–80% modification of bovine serum albumin with citraconic anhydride a 1:1 molar ratio of anhydride to amino group was required, under the reaction conditions mentioned above. The unreacted material, which in water is largely hydrolyzed to the dicarboxylic acid, was removed, in the case of the succinylation reaction, by extensive dialysis against water, and in the other two reactions by gel filtration through a Sephadex G-25 column equilibrated with water. The succinylated proteins were very stable; the maleylated species remained stable under normal conditions during the course of the experiments; the citraconyl proteins hydrolyzed readily at pH values as high as 7 after several hours at room temperature, but when kept cold at pH 8, were stable for a few days. Removal of citraconyl groups from modified bovine serum albumin was effected by stirring water solutions of the protein at room temperature, pH 2.0–2.5, for 12 hr. The hydrolyzed groups were subsequently removed by gel filtration through a column of Sephadex G-25.

Protein Determinations. The protein content of solutions was determined by the Folin method (Folin and Ciocalteau, 1927). In the case of bovine serum albumin, DNS-labeled bovine serum albumin, and the succinylated samples, which have essentially the same absorption spectrum as the unmodified species, protein content was determined spectrophotometrically using a per cent extinction coefficient, at 280 mμ, of 6.6×10^2 g^{-1} cm^2. For DNS-labeled bovine serum albumin and succinyl DNS-labeled bovine serum albumin the contribution of the fluorescent dye to the absorbance at 280 mμ was taken into account (Weber, 1952b).

Extent of the Reaction of Amino Groups. The colorimetric ninhydrin assay (Harding and MacLean, 1916) at 570 mμ was used for the determination of free amino groups in succinyl bovine serum albumin derivatives. The extent of

[1] Abbreviation used in this work is: DNS, 1-dimethylaminonaphthalene-5-sulfonyl fluorescent label.

[2] It should be noted that the physical properties of bovine serum albumin and DNS-labeled bovine serum albumin are essentially identical (Harrington *et al.*, 1956; Weber, 1952b; Weber and Young, 1964); therefore, the results obtained in this work for the DNS-labeled protein also apply to the unlabeled species.

reaction of maleic anhydride with the amino groups of bovine serum albumin was determined from the ultraviolet absorption spectra of the derivatives (after subtraction of the protein contribution), using the molar extinction coefficients given by Butler *et al.* (1969) for ε-maleyllysine. ε 8.6 × 10^2 M^{-1} cm^{-1}, at 270 mμ, was used in the calculations under the assumption that the absorbance values for ε-maleyllysine are additive on bovine serum albumin. The approximate number of amino groups reacted with citraconic anhydride was estimated from fluorescence polarization or sedimentation velocity measurements on the bovine serum albumin citraconyl derivatives, by using the correlation of the extent of reaction of succinyl and maleyl bovine serum albumin derivatives with their fluorescence polarization and sedimentation velocity properties. In all cases the extent of the reaction of the amino groups was expressed as a percentage, relative to native bovine serum albumin which was taken to be 0%.

Fluorescence Polarization (p) Measurements. Rotational relaxation times and qualitative indications of conformational changes in the DNS-labeled proteins, were obtained from polarization measurements in connection with the equation derived by Perrin (1926) and extended by Weber (1952a) to globular macromolecules carrying randomly oriented fluorescent oscillators. For plane polarized exciting light the Perrin equation is: $(1/p) - (1/3) = [(1/p_0) - (1/3)][1 + (3\tau/\rho_h)]$, where p is the observed fluorescence polarization, p_0 is the limiting polarization observed in the absence of Brownian rotations, τ is the lifetime of the excited state of the fluorescent label, and ρ_h is the harmonic mean of the principal rotational relaxation times of the prolate ellipsoid model of the macromolecule.

Since ρ_h is directly proportional to η/T (viscosity of the solvent divided by the temperature in degrees Kelvin) plots of $1/p$ against T/η give straight lines. Rotational relaxation times can be calculated from the $1/p$ intercept, $1/p_0$, and the slope of the straight line using the expression

$$\frac{\text{slope}}{\frac{1}{p_0} - \frac{1}{3}} = \frac{3\eta\tau}{\rho_h T}$$

Experimentally, rotational relaxation times were obtained by measuring the fluorescence polarization of protein solutions containing varying amounts of sucrose, at a constant temperature of 25.0°. The relationship of sucrose added, to the viscosity of the solution, was determined from the data by Bingham and Jackson (1918).

The structural changes in DNS-labeled bovine serum albumin, on chemical modification with the dicarboxylic anhydrides, were followed by observing the changes in the fluorescence polarization of the solutions. It was determined that the variations in polarization were due, in effect, to changes in ρ_h (proportional to the rotational volume of the protein), since the p_0 and τ parameters of DNS-labeled bovine serum albumin did not change significantly after reaction with the anhydrides.

The lifetime (τ) of DNS in DNS-labeled bovine serum albumin solutions is essentially independent of pH and ionic strength. The variation of τ in the pH range from 2 to 9 does not exceed 7%, and an increase of ionic strength from 0 to 0.2 has no effect on τ (Harrington *et al.*, 1956; Steiner and McAlister, 1957).

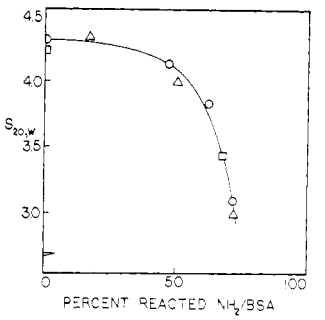

FIGURE 1: Sedimentation velocity coefficients ($s_{20,w}$) of bovine serum albumin derivatives, as a function of the percentage of amino groups reacted per protein molecule (percent reacted NH_2/bovine serum albumin), obtained in 0.1 M sodium phosphate buffer, pH 7.0: (○ ○) succinyl bovine serum albumin; (△ △) maleyl bovine serum albumin; (□ □) citraconyl bovine serum albumin.

Results

Extent of Reaction and Structural Changes in Succinyl, Maleyl, and Citraconyl Bovine Serum Albumin Derivatives. The change in structure of bovine serum albumin, with the extent of reaction of its amino groups, was followed by analytical ultracentrifugation. Under the conditions of these experiments (0.1 M sodium phosphate buffer, pH 7.0, 0.5% protein solutions, at 20°) the sedimentation velocity coefficient ($s_{20,w}$) remained constant at 4.30–4.23 S until 40% of the amino residues of bovine serum albumin had been modified. Further reaction of the lysine residues caused a gradual decrease in $s_{20,w}$, a value of 3.0 S being reached at 75% acylation (Figure 1). In the calculation of $s_{20,w}$ values a partial specific volume ($\bar{v}$) of 0.734 was used for bovine serum albumin and all the modified protein samples. According to Habeeb (1966), completely succinylated bovine serum albumin has $\bar{v} = 0.716$, but substitution of this value in the calculations resulted in a 0.5% change in the final result, insignificant by comparison with the 1–2% error in $s_{20,w}$ values.

Conformational changes in chemically modified DNS-labeled bovine serum albumin were determined by measuring the fluorescence polarization, at 23°, of solutions in sodium phosphate buffer, pH 7.0, and in water, adjusted to pH 7.0 with NaOH. Figure 2 summarizes the results for succinyl and maleyl derivatives of DNS-labeled bovine serum albumin. In 0.1 M buffer the fluorescence polarization of the solutions is constant to about 45% reacted amino groups; at higher degrees of chemical modification the polarization decreases with constant slope. In 0.01 M buffer and in water, the changes in polarization are parallel, but the decrease in polarization starts at 30% and 10% acylation, respectively. The lowest polarization value, 0.20–0.21, is reached at 65–90% acylation in water solutions.

Maleyl derivatives of bovine serum albumin appear to undergo structural changes when the number of modified lysine residues is some 7% lower than for succinyl derivatives under the same experimental conditions.

Samples of citraconyl DNS-labeled bovine serum albumin, where the extent of reaction of the amino groups was not known, had $s_{20,w}$ and polarization values corresponding to the same number of reacted amino groups (within ±5%) in Figure 1 and Figure 2. It was concluded, therefore, that

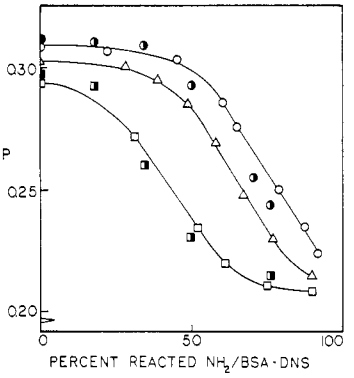

FIGURE 2: Fluoroescence polarization (p) of DNS-labeled bovine serum albumin derivatives, as a function of the percentage of amino groups reacted per protein molecule (per cent reacted NH_2/DNS-labeled bovine serum albumin. Succinyl DNS-labeled bovine serum albumin in: (○ ○) 0.1 M sodium phosphate buffer, pH 7.0; (△ △) 0.01 M sodium phosphate buffer, pH 7.0; (□ □) water. Maleyl DNS-labeled bovine serum albumin in: (◑ ◑) 0.1 M sodium phosphate buffer, pH 7.0; (◨ ◨) water.

a reasonable estimate of the extent of reaction can be obtained either from $s_{20,w}$ or, more conveniently, from polarization measurements under controlled experimental conditions.

Rotational relaxation times for the modified DNS-labeled bovine serum albumin samples were obtained from $1/p$ against T/η plots, which indicate a decrease in ρ_h with increasing modification of the amino groups, as was expected from the polarization measurements reported above. The slight decrease in p_0 for extensively modified samples, may indicate some local rotational freedom of the chromophore (Weber, 1952a,b). At the same degree of acylation, the ρ_h values calculated for succinyl, maleyl, and citraconyl DNS-labeled bovine serum albumin derivatives were very similar; therefore, the results for the succinyl samples, given in Figure 3 and Table I, are also representative of the other derivatives.

Absolute fluorescence lifetimes were measured for a citraconyl DNS-labeled bovine serum albumin sample, having

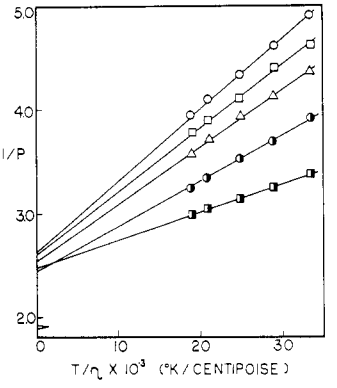

FIGURE 3: $1/p$ against T/η plots for the determination of rotational relaxation times of DNS-labeled bovine serum albumin derivatives. The measurements were made in water solutions adjusted to pH 7.0, at 25.0°. Succinyl DNS-labeled bovine serum albumin samples: (◑ ◑) 31% modified, (△ △) 55% modified, (◧ □) 74%: modified, (○ ○) 80% modified, control DNS-labeled bovine serum albumin (◨ ◨).

TABLE I: Rotational Relaxation Times of DNS-Labeled Bovine Serum Albumin Derivatives.[a]

% NH_2 Reacted/ Bovine Serum Albumin[b]	$1/p_0$	ρ_h (nsec)
31	2.49	57
55	2.62	48
74	2.68	43
80	2.75	39

[a] Rotational relaxation times were calculated using the $1/p$ against T/η plots, shown in Figure 3. [b] Per cent of amino groups reacted per protein molecule, taking unmodified bovine serum albumin as 0%.

70% of its amino groups modified. The results obtained in 0.1 M sodium phosphate buffer, pH 7.0, 25.0°, with the phase and modulation modes of the lifetime instrument were 10.9 nsec and 17.3 nsec, respectively, indicating a marked inhomogeneity of the DNS chromophore on bovine serum albumin (Spencer and Weber, 1969). A parallel measurement on unmodified DNS-labeled bovine serum albumin gave identical results, 11.0 and 17.1 nsec. Since the same lifetimes were obtained in both cases, and the fluorescence spectra of the DNS labels for all three types of bovine serum albumin derivatives were the same, it was assumed that the control DNS-labeled bovine serum albumin and all the modified DNS-labeled bovine serum albumin samples have the same average lifetime for the DNS label, which was taken to be 12 nsec, a value also obtained by Steiner and McAlister (1957).

Effect of Ionic Strength, Nature of Salt, and pH on the Expansion of Bovine Serum Albumin Derivatives. Ionic strength, as indicated in Figure 2, has a marked effect on the expansion of modified bovine serum albumin. The amount of LiCl necessary to produce complete reversal of the structural changes in the protein (as estimated from fluorescence polarization measurements in water solutions adjusted to pH 7.0, at 23°), was determined for succinyl DNS-labeled bovine serum albumin samples having 43, 65, 85, and 91% reacted amino groups. The results, shown in Figure 4, indicate that the expansion is completely reversed at an ionic strength of 0.5 for the 43 and 65% samples; the 85 and 91% samples, however, reach 93 and 85% reversal, respectively, at an ionic strength of 0.8. At 65% and lower modification of the amino groups, reversal of the expansion occurs readily as soon as some of the negative charges are shielded, while at 85% and higher modification there is no indication of refolding below a critical ionic strength.

Various salts were tested for their effectiveness in reversing the expansion of the bovine serum albumin derivatives. To 43% succinyl DNS-labeled bovine serum albumin samples in water, adjusted to pH 7.0, and having an initial polarization of 0.253, chloride salts of several cations were added until a polarization value of 0.295, at 23°, was reached. Cations have a reversing effect on the expansion in the order: $Ca^{2+} \approx Mg^{2+} \gg NH_4^+ \approx Na^+ \approx Li^+$, with the divalent, more polarizable ions, being about ten times more effective in shielding

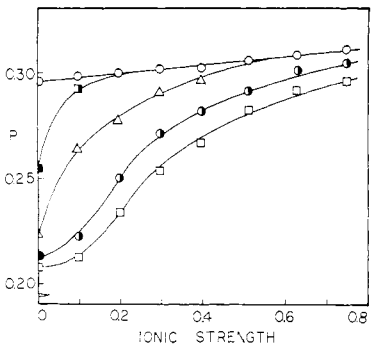

FIGURE 4: Reversibility of the expansion of DNS-labeled bovine serum albumin derivatives. Effect of ionic strength on the expansion, in terms of fluorescence polarization (p), of succinyl BSA-DNS derivatives at pH 7.0: (○ ○) control DNS-labeled bovine serum albumin, (◨ ◧) 43% modified sample, (△ △) 65% modified sample, (◑ ◑) 85% modified sample, (□ □) 91% modified sample.

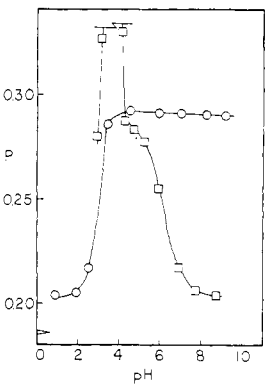

FIGURE 5: Effect of pH on the expansion, in terms of fluorescence polarization (p), of a 48% modified DNS-labeled bovine serum albumin sample: (○ ○) control DNS-labeled bovine serum albumin, (□ □) 48% succinyl DNS-labeled bovine serum albumin.

the negative charges on the surface of succinyl bovine serum albumin, than the monovalent cations.

While native bovine serum albumin has a compact structure, insensitive to pH changes from pH 4 to pH 10.5, chemically modified bovine serum albumin undergoes marked structural changes in this pH region. Fluorescence polarization values were measured at 23°, on a water solution of a succinyl DNS-labeled bovine serum albumin derivative, having 48% of the amino groups reacted; pH was adjusted with NaOH and HCl. The sample at pH 4.8 was diluted five times, and its fluorescence polarization was measured, in order to determine whether the increase in polarization from pH 9.0 to pH 4.8 was due to aggregation of protein or to reversal of expansion. The fluorescence polarization value was identical with that of the undiluted sample, excluding aggregation as the cause of the increase in polarization.

Figure 5 shows the effect of pH on the structure of native and modified bovine serum albumin. The conformation of the modified protein changes throughout the entire pH range covered in the experiment (*i.e.*, pH 3–9), except at pH 9, where the lowest polarization value is attained, and close to pH 4.5, where the excess negative charges appear to be neutralized. From pH 4.0 to pH 3.0, around the isoelectric point of the bovine serum albumin derivative, the protein precipitates, but redissolves readily at pH values below 3. The maximum expansion, in terms of fluorescence polarization, that is attained by native and modified bovine serum albumin, at pH 2 and pH 9, respectively, is 0.205.

Reversible Modification of Bovine Serum Albumin by Citraconic Anhydride. Samples of citraconyl bovine serum albumin, at various extents of reaction of the amino groups, had the same properties as the corresponding samples of succinyl and maleyl bovine serum albumin.

A parallel series of experiments was run on a citraconyl DNS-labeled bovine serum albumin sample, having 70% modified amino groups, and on a control DNS-labeled bovine serum albumin sample. Measurements of fluorescence polarization, rotational relaxation time, sedimentation velocity, absorption and fluorescence spectra, and pH dependence of fluorescence polarization were carried out under the same conditions as the corresponding experiments which have been described above. Starch-gel electrophoresis was run in 0.1 M sodium phosphate buffer, pH 7.0 for 8 hr, at 5°. The results, summarized in Table II, indicate that after acid treatment and removal of free citraconyl groups, the previously modified protein has the same properties as native bovine serum albumin. The acid treatment, however, changes somewhat the properties of the DNS-labeled bovine serum albumin itself. The lifetime of the DNS label increases by 3 nsec and the maximum fluorescence wavelength is shifted to the blue by 9 mμ, indicating that the environment of the chromophore has become less polar. In addition, the decrease in ρ_h and in $s_{20,w}$ points to a slight structural change in the protein. Since it has been reported that crystalline bovine serum albumin upon standing at pH 3.3 for 2–3 days at 5°, releases fatty acids with a concomitant increase in the degree of microheterogeneity and an "unknown irreversible alteration of the protein" (Williams and Foster, 1960; Sogami and Foster, 1968), it is reasonable to assume that the slight structural changes in DNS-labeled bovine serum albumin, observed here, are due to defatting of the protein.

Discussion

From the results presented in this paper it is clear that the change in the structure of bovine serum albumin as its amino groups react with dicarboxylic anhydrides is due essentially to the electrostatic repulsion of added negative charges. The attachment of the acylating groups themselves causes very little or no effect on the protein structure, as indicated by the complete reversal of the expansion of a 65% modified sample at ionic strength 0.5, and nearly 93% reversal, at ionic strength 0.8, of an 85% modified protein.

The physical properties of succinyl, maleyl, and citraconyl bovine serum albumin derivatives are almost identical, but they vary with the extent of amino group modification and with ionic strength, pH, and nature of salts present in solution. A 65% modified bovine serum albumin sample has ρ_h 52 nsec (obtained in water solution, pH 7.0, 20°) and $s_{20,w}$ = 3.60 S (determined at pH 7.0, ionic strength 0.2, protein concentration 0.5%), in close agreement with the corresponding properties of bovine serum albumin expanded at pH 2. The acid expanded form of bovine serum albumin has ρ_h 54 nsec (determined at pH 2, in water solution, 20°) (Weber, 1953;

TABLE II: Reversible Modification of DNS-Labeled Bovine Serum Albumin by Citraconic Anhydride.[a]

Property[b]	Samples before Acid Treatment		Samples after Acid Treatment	
	Citraconyl DNS-Labeled Bovine Serum Albumin	DNS-Labeled Bovine Serum Albumin	Citraconyl DNS-Labeled Bovine Serum Albumin	DNS-Labeled Bovine Serum Albumin
p (0.1 M Na phosphate buffer, pH 7.0)	0.282	0.311	0.295	0.292
p (H_2O)	0.229	0.295	0.288	0.285
τ phase (nsec)	10.9	11.0	14.0	13.6
τ modulation (nsec)	17.3	17.0	20.3	19.9
τ average (nsec)[c]	12	12	15	15
$\rho_{h,25}$ (nsec)	42	90	78	81
$s_{20,w}$ (S)	3.34	4.20	4.00	4.06
Absorption spectra	DNS-labeled bovine serum albumin + citraconyl	DNS-labeled bovine serum albumin	DNS-labeled bovine serum albumin	DNS-labeled bovine serum albumin
DNS fluorescence maximum (mμ)	511	505	496	496
DNS fluorescence intensity with respect to DNS-labeled bovine serum albumin	0.8	1.0	1.0	1.0
pH dependence of p in the neutral region	Marked	None	None	None
Electrophoresis	Higher mobility than DNS-labeled bovine serum albumin	DNS-labeled bovine serum albumin	DNS-labeled bovine serum albumin	DNS-labeled bovine serum albumin

[a] Parallel experiments were carried out on a 70% modified citraconyl DNS-labeled bovine serum albumin sample and on a control DNS-labeled serum bovine albumin sample, before and after acid treatment for the removal of citraconyl groups. [b] Physical properties and experiments carried out on the samples. [c] The average τ of 12 nsec was taken from Steiner and McAlister (1957); 15 nsec is an interpolation between the τ's obtained by phase and modulation.

Harrington *et al.*, 1956) and $s_{20,w}$ = 3.58 S (obtained at pH 2, ionic strength 0.2, protein concentration 0.3%) (Harrington *et al.*, 1956).

It appears that from 60 to 80% modification of amino groups, conditions could be found under which each bovine serum albumin derivative could exist in an expanded form very similar to the acid expanded protein. The 60–80% range of protein modification was chosen by reference to Figure 2, which shows that in water, 60–90% modified samples have fluorescence polarization values close to the fluorescence polarization of acid-expanded bovine serum albumin in water (Figure 5). The expansion of samples modified to a larger extent than 80% was not completely reversible (Figure 4).

The fact that very similar expanded forms of the protein are produced by changing the charges on bovine serum albumin in opposite directions, points to a nonspecific expansion process. That the intermediate steps in the expansion, by added negative charges, are also nonspecific, is indicated by the gradual change in structure of the bovine serum albumin derivatives, as the number of reacted amino groups increases beyond the initial charges necessary to overcome the secondary forces which stabilize the native structure of the protein.

At about 70% modification of the amino groups on bovine serum albumin, reversal of the expansion occurs readily as soon as some of the negative charges are shielded by added ions; at 85% and higher modification, however, a critical ionic strength has to be reached before the refolding starts (Figure 4). A possible explanation for these observations is that native bovine serum albumin may have about 20% buried lysine residues, which would be attacked by the anhydrides only after the surface residues were modified and the protein was sufficiently expanded. In the refolding process, the protein derivatives having most of their amino groups modified, including the "buried" groups, would require an initial shielding of the charge on the exposed internal surfaces, before these surfaces could approach and start re-forming the short-range interactions of the native protein.

There is supporting evidence for the heterogeneous nature of lysine residues on serum albumin. Goldfarb (1970) reported the presence, in human serum albumin, of lysine groups having three distinct velocity constants for the reaction with trinitrobenzenesulfonic acid. In addition, the most

extensively modified samples of bovine serum albumin, in this study, had only 90–95% reacted amino groups, when compared with the native protein, indicating that a few amino groups do not react with the anhydrides even in the most expanded state of the protein.

Regeneration of bovine serum albumin from citraconyl bovine serum albumin, in terms of physical properties, was easy and complete; the amino acid composition, however, was not determined after the series of blocking and unblocking reactions. Recently, a report on the reaction of citraconic anhydride with aldolase (Gibbons and Perham, 1970) indicated that partial destruction of thiol groups occurred during the reversible modification of the amino groups. Therefore, the reversible reaction of proteins with citraconic anhydride must be used with caution in studies of functional properties, which depend on the three dimensional, as well as the primary structure of proteins. In physical studies of the tertiary and subunit structure of proteins, however, this reagent could find considerable use.

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