

AN ABSTRACT OF THE THESIS OF

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Title: AN INVESTIGATION OF THE THERMAL STABILITY OF  
POLYGLYCYL BOVINE SERUM ALBUMIN

Abstract approved

  
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A heat stable derivative of bovine serum albumin containing 300 residues of glycine added to 30 sites on the surface of the protein has been prepared. This derivative may be heated to 100° C for prolonged periods of time without aggregation. By comparison, native BSA aggregates at 62° C under similar conditions. The spatial arrangement of the added glycine peptides is probably responsible for the observed stabilization of the protein. Two possible arrangements of the added glycyll chains have been suggested. The first considers the chains to be fully extended and projecting into the surrounding solvent. The second envisions the added chains folded back along the protein surface. The experimental evidence greatly favors the second suggestion.

Using trinitrobenzene sulfonic acid, it was determined that there were thirty reactive amino groups on the surface of native BSA. After denaturation of the protein with 8 M urea, 57 lysine groups

could be reacted. Since the preparation of the polyglycyl derivative is carried out under similar reaction conditions to those employed with trinitrobenzene sulfonic acid, it is most likely that the amino groups on the surface of the molecule react with the N-carboxy glycine anhydride.

The conformation characteristic of native BSA appears to be maintained in the derivative as indicated by various experimental measurements. The change in rotation associated with the acid expansion of BSA is paralleled by a similar but smaller change in the derivative. At pH 5.5 the specific rotation of the derivative was  $-47.7^{\circ}$  and the native was  $-64.6^{\circ}$ . The value for the polyglycyl BSA is several degrees lower than would be expected if it possessed the identical molar rotation as the native protein. A similar decrease in levorotation has been reported when ten moles of anionic detergent are bound to one mole of BSA. The ultraviolet difference spectra observed by perturbing the native and derivative protein by pH and heat indicated that the main matrix of each protein was undergoing a similar transition. In all cases observed the derivative showed less change in optical density than did the native protein. If the glycine chains were folded back along the protein surface shielding some chromophoric groups from contact with the solvent, this decrease could be explained.

The hydrodynamic measurements also support the suggestion

that the structure of the protein core of the derivative is retained. The derivative undergoes the same reversible acid expansion as the native protein. Polyglycyl BSA with an  $S_{20}$  value of 3.85 at pH 2.5 regained its native conformation which had a sedimentation coefficient of 5.45 by dialysis against phosphate buffer at pH 6.3. By approximation that may be made from the diffusion coefficient, it was shown that the degree of hydration of the derivative could have a large variation depending upon the spatial arrangement of the added peptides. If the added peptides were folded, the hydration would be less than the native protein and if they were extended the hydration would be greater than the native protein. The hydration was evaluated by the determination of the buoyant density of polyglycyl BSA in a series of salt solutions. The values of the hydration of the protein-salt complex determined are as follows: in CsCl, 0.42 gm  $H_2O$ /gm protein; in RbBr, 0.37 gm  $H_2O$ /gm protein; and in KBr, 0.33 gm  $H_2O$ /gm protein. The corresponding values of the hydration of the protein-salt complex for bovine mercaptalbumin reported by Ifft and Vinograd are as follows: in CsCl, 0.51 gm  $H_2O$ /gm protein; in RbBr, 0.46 gm  $H_2O$ /gm protein and in KBr, 0.37 gm  $H_2O$ /gm protein. The decrease in hydration found in the derivative may be adequately explained by the change in the surface to volume ratio that would be expected if the added peptides were folded back along the protein surface. An approximate calculation of the surface to volume ratio

of the derivative in this configuration gives a value of 0.83 that of the native protein. A similar calculation assuming that the chains are extended gives a ratio of 1.5. The decrease found is clearly more consistent with a molecule in which the added peptide chains are folded back along the protein surface. An increase of the activity of water accompanied by an increase in the hydration of the protein has been presented previously by Ifft and Vinograd, and was found to be valid for the hydration of the derivative.

Considering the experimental evidence that has been presented, it is felt that the added polyglycyl chains are folded back along the surface of the protein. When they are in this configuration they form hydrogen bonds with groups on the surface of the protein and would cover some of the hydrophobic regions on the surface of the protein. Both of these effects would cause the derivative to be more stable than the native protein.

An Investigation of the Thermal Stability of  
Polyglycyl Bovine Serum Albumin

by

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# AN INVESTIGATION OF THE THERMAL STABILITY OF POLYGLYCYL BOVINE SERUM ALBUMIN

## INTRODUCTION

Chemically modified proteins and synthetic polypeptides have been used as model compounds in the study of non-covalent forces involved in maintaining protein conformation. Of these forces hydrogen bonding and hydrophobic interactions are thought to exert the greatest influence. Hydrogen bonding itself has been rigorously defined and investigated in simple chemical systems and its application to protein structure has a solid theoretical background. Knowledge of hydrophobic interactions of non-polar regions in a protein, by comparison, is much more nebulous. These interactions result mainly from entropy gained by water by having the non-polar amino acid side chains buried within the interior of the protein molecule or conversely, the loss of entropy which results from the highly organized arrangement of water around exposed non-polar amino acids of the protein.

Synthetic polypeptides have been prepared as homopolymers, block polymers and copolymers. A substantial amount of useful information concerning the stability of the  $\alpha$ -helix in its relation to solvent-solute interaction and hydrophobic interaction has been reported. Fasman (16) has found the heat stability of the  $\alpha$ -helix of a

copolymer of L-glutamic acid and L-leucine is greater than that of the L-glutamic homopolymer indicating the stabilizing potential of the hydrophobic side chain. Studies in non-hydrogen bonding and hydrogen bonding solvents have shown the importance and strength of solvent-solute interaction in the helix. These studies of synthetic polymers have their inherent limitations. The solubility in aqueous medium of the synthetic polypeptides is one major consideration. Most non-polar amino acid polymers are insoluble in aqueous solutions. The solubility of glycine decreases rapidly with increasing chain length. The free amino acid is quite soluble (2.8 moles/liter), diglycine is less soluble (1.7 moles/liter) while triglycine is only slightly soluble (0.3 moles/liter). However, by attaching the polymer to a protein moiety, a peptide that would normally be completely insoluble is rendered soluble and its interactions may be studied.

The polypeptidylation of proteins by reaction of the protein with a N-carboxyamino acid anhydride has several advantages. The end product of the reaction gives addition to the  $\epsilon$ -amino groups of lysine. The degree of addition can be controlled by regulating the molar ratio of anhydride to protein. A reasonably homogeneous product is produced (12). Of major importance are the mild conditions of the reaction that allow the addition to take place when the protein is in the native state. It has been experimentally determined that the resulting protein retains its native conformation. The integrity of the native

structure of ribonuclease was maintained in polyalanyl ribonuclease derivative as shown by a study of the optical rotation and spectrophotometric measurements by Anfinsen, Sela and Cook (3). The extent of hydrolysis of a polyglycyl BSA with chymotrypsin was the same as the native protein with only the rate of hydrolysis varying. These studies by Konigsberg (37) also demonstrated that the tryptic activation of polyglycyl chymotrypsinogen was the same as the native protein. Sela found immunological activity of goat polyalanyl antibody was retained even after the addition of 600 alanine residues (11). A polyvalyl derivative of chymotrypsin prepared by Stracher and Becker (57) which had 50 moles of valine attached still retained 56% of the enzymatic activity of the native enzyme. These data provide evidence that the basic structural features of the native protein are preserved through the reaction and that the attached peptides, residing on the surface of the molecule, are primarily responsible for any observable changes.

Several recent studies have attempted to correlate protein shape or "asphericity" and hydration with the amino acid composition of the protein or more specifically with the ratio of polar to non-polar side chains. Fisher (17, 18) has proposed a limiting law relating the size and shape of the protein molecule to its composition. This concept is based on the premise that the non-polar residues of a protein, due to their limited solubility in aqueous media, will reside in the

interior of the protein giving a definite interior volume and the polar residues will form an outer shell, due to their limited solubility in non-polar media and high solubility in aqueous solution. The polar amino acids will also form a definite exterior volume. Fisher assumed that the average thickness of the outermost amino acid shell is  $4 \text{ \AA}$ . With these assumptions a function,  $\rho$ , was defined that related the total volume of the protein to the non-polar interior volume. The exterior volume or area of polar residues necessary to cover the interior volume was thus fixed and a surface to volume ratio established. A sphere is found to have the maximum ratio of volume to surface and in considering protein structure, a long thin rod the other extreme.

One of the greatest criticisms of this paper has been Fisher's classification of amino acids as polar or non-polar. One case of interest is the non-polarity or hydrophobic character of glycine. Fisher classifies glycine as non-polar while Tanford (58) and Waugh (63) regard it as an amino acid of low hydrophobic nature.

Tanford has published a systematic study of the amino acids to determine their hydrophobicity (58). His values for free energy of transfer were determined from the solubility data of the free amino acids. With these data, assuming that the non-polarity of the interior of a protein is similar to ethanol, he calculated the free energy of transferring one mole of an amino acid from an aqueous solution to an



ethanolic solution. The relation of  $\Delta F = -RT \frac{N_{\text{EtOH}}}{N_{\text{HOH}}}$  was used.  $N_{\text{HOH}}$  and  $N_{\text{EtOH}}$  represent the solubility expressed in mole fractions in the two solvents. To isolate the effects of the side chains, the free energy of transfer of glycine, which is used as a model of peptide backbone, was subtracted from the calculated value of each amino acid.

Bigelow (9) has taken the values of the hydrophobic nature of the various amino acids as calculated by Tanford and the general concepts from Fisher's limiting law and has attempted to correlate a new function, the average hydrophobicity of a protein, with the stability and structure of a given protein. A general relation was shown to exist in the 150 proteins that were examined in this study as to the function  $\rho$  of Fisher, and the new term the average hydrophobicity, with regard to the nature of the protein molecule.

With this background it was felt that a study of a highly modified polyglycyl protein would compliment and hopefully clarify existing thoughts. A highly modified polyglycyl bovine serum albumin (BSA) was chosen as a model system. Earlier investigation of a lightly modified BSA indicated that hydrodynamically it was identical to the native protein and the small shifts seen were due to the increased molecular weight of the added peptides. By the construction of Corey-Pauling-Koltun space filling molecular models, it was seen that a peptide chain of a minimum of five residues was necessary before any

possibility existed of interaction between the added peptide and the peptide backbone of the protein. The observation of this condition was made ignoring any interaction with adjacent amino acid side-chains. If the latter interaction was present it would necessitate an increase in the length of the added peptide before interaction with the protein backbone could take place.

Bovine serum albumin (BSA) was chosen as a model protein for several reasons. It may be obtained commercially in reasonably high purity essentially free of non-protein contaminants. It has been shown by a number of investigators that the molecule is a single peptide chain, thus eliminating non-covalent subunit interactions. Of major importance is the fact that the hydrodynamic properties of BSA have been as thoroughly investigated and characterized as those of any protein. An excellent review of the characteristics of BSA has been written by Foster (20). BSA exhibits a reversible molecular expansion in acid solution which arises largely from electrostatic repulsion, which can be studied with the modified derivative. Although it has definite molecular integrity between the pH extremes, it may be easily perturbed by a variety of denaturing conditions. In a study by Kauzmann et al. (36), the rate of the denaturation of BSA by urea was found to be effectively instantaneous as compared to the other proteins studied. Thus it may be assumed that the native conformation is a delicately poised system and the effect of the added peptides in a

derivative may be more easily detected in this system rather than one in which the protein conformation is more rigidly fixed.

Much insight into the conformation of native BSA in solution has been gained by several recent studies. Luzzati et al. (39), studied its structure by small angle X-ray diffraction at pH 5.3 and 3.6. This study presents dimensions of possible shapes that represent the protein in solution. Both prolate and oblate ellipsoids of revolution were used as models. From these models, valuable information may be found concerning the surface to volume ratios and the molecular changes associated with the pH expansion.

A more recent paper by Bloomfield reports results of another study of BSA in solution by low angle X-ray analysis. In this presentation three possible molecular conformations are given. The model that is favored as being most probable consists of a "trimer" of two smaller units symmetrically and covalently attached to a larger inner unit. This concept of a "trimer" gains further substantiation from the work of Weber (64, 65) and that of Foster (1). Both of these studies utilized methods of cleavage of the peptides linking the subunits of the molecule followed by isolation of the fragments. Both workers conclude that the native protein is a "trimer." The values of the dimensions found by Bloomfield vary slightly from those of the earlier workers. Bloomfield feels that these differences are due to a significant amount of dimerized protein in the solutions used by the earlier

workers and the higher angle of scatter used, which is subject to greater experimental uncertainty. Completely independent of Bloomfield, Moser et al. (44), determined the hydrodynamic dimensions of BSA in solution by dielectric dispersion. The values obtained are essentially identical to those given by Bloomfield.

After the highly modified polyglycyl BSA had been prepared, one remarkable difference was noted. The polyglycyl derivative could be held at  $100^{\circ}\text{C}$  for prolonged periods of time without aggregation taking place, whereas the native protein, under similar conditions, would aggregate at  $62^{\circ}\text{C}$ . To determine what factors were giving the derivative its stability, a systematic study of the physical properties of the derivative was undertaken. The optical rotation was determined at varying acid pHs to determine if the helicity of the protein had been affected. Thermal and pH ultraviolet difference spectra were examined to investigate changes in the environment of the chromophoric residues. Sedimentation and diffusion coefficients were determined at varying pHs. With these values the Svedberg equation could be applied and approximations as to the degree of hydration or change in axial ratio of an assumed ellipsoid of revolution could be made. The hydration of a protein is a very difficult quantity to experimentally determine. The above method is only an approximation and is subject to large errors within the restricted confines of the assumptions that are made. A new method for determining

hydration using the measurement of the buoyant density of a protein in the analytical ultracentrifuge has been presented by Vinograd (31).

In this technique the protein or any macromolecular species is centrifuged in a solution containing a low molecular weight solute. The solution forms a density gradient at equilibrium by the opposing tendencies of diffusion and sedimentation. The initial solution is prepared such that the point at which the sum of the forces acting on the macromolecule is balanced and this equilibrium position of the protein is contained within the gradient. This effect, to concentrate the macromolecular species in a band at a given position in the centrifuge cell, is counterbalanced by Brownian motion. The opposing forces result in an inverse relation between the width of the band of the macromolecule in the density gradient and its molecular weight.

With the mathematical relation derived by Meselson, Stahl and Vinograd, the solvated molecular weight of the protein in the buoyant solution may be calculated. However, it is known that BSA binds anions. The solvated molecular weight determined by this method will include these bound anions and also the water that is necessary to buoy them. An independent measurement of the anion binding must be made in the solution in which the protein will band. With these data and using the theory and methods developed by Vinograd, the degree of hydration of a protein in normal aqueous solution may be determined. In his latest publication (33), Vinograd established a

definite relation between the activity of water and the degree of hydration of the protein in different salt solutions that would produce the same density but would be at different concentrations. The modified BSA was investigated under similar conditions to determine if this relationship would still be valid with the additional glycine residues exposed to the solvent.

This study was undertaken to determine in what manner the added glyceryl chains that have been attached to BSA are stabilizing the modified protein. Two possible explanations set the limits of the type of interaction that is responsible for the stabilization. The first is that the added peptides are extending, flagella like, into the surrounding aqueous media causing greater solvent-solute interaction. The second, extreme possibility is that the added peptides are folding back along the surface of the protein thereby causing stabilization by hydrophobic interaction. It is realized that neither condition will be fully met but the experimental evidence greatly favors the second hypothesis. In either case the interaction of the protein with the aqueous environment is of utmost importance.

In all experiments the native protein was run as a control and the values found could be compared with those in the literature to determine the accuracy of the experimental methods.

## METHODS

Preparation of N-Carboxy Glycine Anhydride, (Glycine NCA)

Glycine NCA was prepared according to the method of Berger *et al.* (8), with some modification. Glycine (5 gm) which had been dried over phosphorus pentoxide in vacuo at 100° C for one day, was suspended in anhydrous tetrahydrofuran (125 ml). The suspension was stirred, warmed to 40° C, and then phosgene was bubbled through the mixture for about one hour, or until the suspension had cleared. Dry nitrogen gas was then bubbled through the solution for two hours to remove excess phosgene. The solvent was removed by vacuum distillation at 25° C on a rotary evaporator and a whitish material was left in the flask. The glycine NCA thus obtained was dissolved in warm ethyl acetate and any insoluble material was filtered off. Petroleum ether was added dropwise to the filtrate to produce turbidity and the solution was kept in the cold for 12 hours to allow crystallization. The crystals were collected by filtration, washed with dry petroleum ether, and finally stored in vacuo over phosphorus pentoxide at -15° C. The crystalline glycine NCA began to decompose at 120° C.

### Preparation of Polyglycyl BSA

Native BSA (1.00 gm) was dissolved in 50 ml of 0.15 M sodium bicarbonate ( $\text{NaHCO}_3$ ) buffer at pH 8.2 and  $4^\circ\text{C}$ . Glycine NCA (500 mg) was added slowly with constant stirring and the reaction was allowed to continue for 24 hours at  $4^\circ\text{C}$ . The suspension obtained was centrifuged for 70 minutes at 17,000 g. The supernatant was collected, placed in Visking tubing (#18) and dialyzed against 0.15 M  $\text{NaHCO}_3$  for 18 hours to remove any small glycine peptides that might initiate polymerization during the second addition. The dialyzed material was returned to the reaction vessel and an additional 500 mg of glycine NCA added. After 24 hours at  $4^\circ\text{C}$  with constant stirring, the suspension was centrifuged as before and the supernatant fluid collected and dialyzed against 3 liters of distilled water for 48 hours. The dialyzed protein solution was lyophilized to dryness and the white fluffy powder obtained was crude polyglycyl BSA. Further purification was achieved by gel filtration.

### Purification of Polyglycyl BSA by Gel Filtration through Sephadex G 200

The crude polyglycyl BSA (970 mg) was dissolved in a small volume (5 ml) of 0.10 M NaCl. This solution was applied to the top of a Sephadex G-200 column (1.8 x 45 cm) that was previously equilibrated with the salt solution. The proteins were eluted with



the 0.10 M NaCl at constant flow rate of 15 ml/hr. maintained with an Accu-flow pump. Five ml fractions were collected. Protein in each fraction was estimated by determining the absorbancy at 279 m $\mu$ . A plot of absorbancy versus fraction number revealed two peaks. The first was small and contained any dimeric or highly polymeric material present in the native protein and any large glycine peptides formed during the modification reaction. The major peak was the second one which contained the monomeric polyglycyl BSA used in the remainder of this study.

Those fractions comprising the major peak were pooled and then exhaustively dialyzed against glass distilled water before being lyophilized. The purified polyglycyl BSA was stored in 100 mg lots in a desiccator at 4<sup>o</sup> C.

#### Amino Acid Analysis of Native and Polyglycyl BSA

The amino acid content of polyglycyl BSA was estimated using a Spinco Automatic Amino Acid Analyzer, Model 120B. Samples were prepared for analysis by acid hydrolysis as follows: Approximately 3 mg protein and 1 ml constant boiling HCl were placed in a 5 ml glass ampule. The ampule, containing the protein and acid, was alternately evacuated and sparged with nitrogen several times, then sealed under vacuum and placed in a boiling toluene bath to maintain a temperature of 110<sup>o</sup> C. Hydrolysis was continued for times ranging

from 25 to 60 hours. After hydrolysis the HCl was removed by flash evaporation and the residue was dissolved in 5 ml of a sample diluting buffer. A suitable aliquot (1 ml) was used for the amino acid analysis (54).

#### Dinitrophenylation of Native and Polyglycyl BSA

This method is a modification of the Sanger method of end group analysis. To approximately 3 mg of protein dissolved in 1 ml of water in a 5 ml ampule, 100 mg of  $\text{NaHCO}_3$  was added followed by 1.5 ml of absolute ethanol containing 5% fluordinitrobenzene (FDNB). The ampule was wrapped in aluminum foil to exclude light and shaken mechanically at room temperature for at least four hours. At the end of this period a yellow precipitate was obtained. The mixture was acidified with 6 N HCl and the precipitate collected by centrifugation. The yellow precipitate was washed with 95% ethanol and then with ether to remove unreacted dinitrofluorobenzene and dinitrophenol. The material, still in the aluminum foil coated ampule, was hydrolyzed using the previous method described above.

#### Sedimentation Velocity of Native and Polyglycyl BSA

Sedimentation velocity experiments were carried out in a routine manner in the Beckman Spinco Model E Ultracentrifuge equipped with schlieren optics. Normal runs were made in cells

with 12 mm centerpieces and standard and  $1^{\circ}$  positive wedge windows. When low protein concentration was required 30 mm centerpieces were used. Kodak Metallographic glass plates were used to record results. The photographic plates were read on a Nikon Model 12 Shadowgraph. These data were calculated following the procedure given by Schachman (50). Unless stated all  $S_{20}$  values were determined at 59,780 rpm and  $20.0^{\circ}$  C.

#### Diffusion Coefficients of Native and Polyglycyl BSA

Diffusion coefficients were determined in the Beckman Spinco Model E Ultracentrifuge using Rayleigh interference optics. A single sector synthetic boundary cell allowed the layering of approximately 0.15 ml of buffer onto 0.45 ml of the protein solution in the center chamber of the cell. The centrifuge was run at 19,160 rpm to allow diffusion across the boundary to take place. Photographs were taken at 16 minute intervals. The entire run lasted between 120 and 150 minutes. The photographic plates were read on a Nikon Shadowgraph Model 12. The data were treated by the general method of Schachman (50) and then corrected using the Fujita equation in the manner described by Baldwin (4).

### Optical Rotation of Native and Polyglycyl BSA

The determination of optical rotation at the sodium D line was carried out in the usual manner in a Perkin-Elmer 141 Automatic Polarimeter at room temperature ( $25 \pm 1^\circ \text{C}$ ). The cell had a path-length of 1.008 decimeters and a volume of 5 ml. The 589 m $\mu$  emission of a sodium light source was used.

Native BSA (62.51 mg) and polyglycyl BSA (42.42 mg) were dissolved in 7.0 ml of 0.20 M NaCl. The pH of the solution was determined with a Corning Model 12 pH Meter equipped with a combination electrode. The solution was placed in the polarimeter cell and the rotation noted, after which the solution was withdrawn from the cell and the pH redetermined. An aliquot of 1.0 N HCl was then added with an Alga micrometer syringe. The second pH was determined and the solution rotation was again measured. After the desired pH range had been covered in this manner the decrease in protein concentration due to addition of acid was determined and the  $\alpha]_D$  calculated.

### Ultraviolet Difference Spectra of Native and Polyglycyl BSA

Ultraviolet difference spectra were measured in a Cary Model 14 recording spectrophotometer equipped with a thermostated cell compartment. For the studies of thermal difference spectra, the

sample cell compartment was connected to a thermostated bath controlled by a temperature programmer. This permitted the cell compartment to be heated and cooled at a predetermined rate. A copper strip, to which five thermocouples of copper-constantine were bonded, was attached to the side of the cuvette. The copper heat sink was insulated from the cuvette holder by thin cardboard. Another thermopile of five identical thermocouples in series was immersed in an ice bath and used as a reference. The leads of the thermopiles were connected to either a Model 2CD Moseley X-Y recorder or an Esterline Angus Model E1102E two channel recorder. The reference compartment of the spectrophotometer was thermostated independently at 20° C. A retransmitting potentiometer was attached to the Cary slide wire to permit direct correlation of optical densities on the Y axis of the Moseley recorder or one channel of the Esterline-Angus Recorder. All cuvettes were tightly sealed with Teflon stoppers which prevented evaporation.

For a typical thermal transition study, 8 ml of protein solution (2.5 mg/ml) was prepared in the appropriate buffer. Two matched silica cuvettes were filled with the same protein solution and placed in the reference and sample compartments of the spectrophotometer. The change in optical density was followed at 287 m $\mu$  as the temperature was increased from 20° C to 92° C or until aggregation occurred. The sample was then cooled in the sample chamber. The procedure

was repeated with a fresh sample at the same pH except the optical density change at 233 m $\mu$  was monitored.

The pH difference spectra was obtained in a similar fashion. Since the optical density shift was negative, the reference solution (pH 6.6) was placed in the sample compartment and the samples of varying pH in the reference compartment. The same method was followed when the spectra of the buffers were recorded. It was found that the buffers absorbed in the low wavelength (230-238 m $\mu$ ) region. To correct for this, the absorbance of the buffer solution was manually subtracted from the absorbance of the protein solution to obtain the final spectra.

#### Determination of Partial Specific Volumes of Native and Polyglycyl BSA

The method used was that of Martin et al. (42), combined with the modifications given by Gagen (23). This method involved determination of the sedimentation coefficients of a protein in several solutions of D<sub>2</sub>O with increasing density. From these data an extrapolation to the density where sedimentation no longer occurred was made. This extrapolated value has been designated the effective or hydrodynamic partial specific volume. Its value can be compared to the calculated partial specific volume determined by the amino acid composition of the protein. Since exchange of deuterium for hydrogen

occurs, the ratio of increased molecular weight was assumed to be proportional to the mole fraction of  $D_2O$ .

#### Buoyant Density Determination of Native and Polyglycyl BSA

The procedure followed in this study was essentially that described by Ifft and Vinograd (31). The solutions were prepared by mixing stock solutions of protein (5-10%) (water for the reference), 0.10 M sodium acetate (NaOAc) - acetic acid (HOAc) buffer, pH 5.3, and a concentrated solution of either cesium chloride, rubidium bromide, or potassium bromide. The final solutions contained between 0.5 to 1.0 mg of protein per ml and were 0.01 molar in the buffer. The density of the solutions were determined by refractive index measurements on a Bausch and Lomb Abby refractometer and adjusted and reread if necessary. After the run the density was again determined on the refractometer and checked by using a 500  $\mu$ l micropipet as a pycnometer. The density versus refractive index relation was taken from the paper of Ifft and Vinograd (33).

Two double sector,  $2\ 1/2^\circ$  filled-Epon synthetic boundary center-pieces with standard and  $1^\circ$  negative wedge windows were used in the Beckman Spinco Model E Analytical Ultracentrifuge. The negative window was needed to compensate for the high refractive increment of the salt solutions. Into one chamber of the double sector center-piece was placed 0.38 ml of the protein solution. The other chamber

was filled with 0.40 ml of reference solution of the same density. At a speed below 10,000 rpm a small quantity of the reference solution was transferred to the sample chamber to make the heights of the two chambers identical. The advantage of using the double sector synthetic boundary cell is that the base line of the reference solution is superimposed on the image of the sample solution and direct measurement is possible.

A number of initial experiments were necessary to establish the buoyant density of the protein in each salt solution. After the approximate buoyant density had been found, the solution density was adjusted until the center of the protein distribution was within 0.4 mm of the isoconcentration position, that point where the density was the same as the density at one atmosphere pressure. The samples were centrifuged at 56,100 rpm at 25° C between 24 and 32 hours. It was found that equilibrium was established after about 16 hours. Photographs were taken at the beginning of the run and after equilibrium had been established. The photographic plates were read on a Nikon Shadowgraph Model 12.

#### Anion Binding of Native and Polyglycyl BSA

The procedure followed in this study was that of Scatchard and Black (51). The pH values of each salt solution, at the concentration of the buoyant solution, the pH of a 1% isoionic protein solution, and



the pH of the protein solution in the presence of salt was measured using a Corning Model 12 pH meter. The difference in the pH of the protein in the salt solution and the protein in water was used to determine the anion binding,  $\nu$ . This quantity,  $\nu$ , is the average increase in negative charge per molecule. The initial protein concentration was determined by measuring the optical density at 279 m $\mu$  and using an  $E_{1\%}^{1\text{cm}}$  of 6.82. The determination of the number of anions bound to the protein requires a value for the electrostatic work factor,  $w$ , which in turn requires a value for the molecular radius of the protein. The radius of the polyglycyl BSA was assumed to be that of the native protein. This assumption was later shown to be reasonable.

## MATERIALS

Tetrahydrofuran, reagent grade, was purchased from Matheson, Coleman, and Bell. It was refluxed with lithium aluminum hydride for 12 hours and distilled just prior to use. The distillate in the fraction with a boiling point of 65-66° C was used.

Petroleum ether, B. p. 30°-60° C, reagent grade, was obtained from J. T. Baker Chemical Company. It was stored over CaCl<sub>2</sub> for two weeks, and was filtered prior to use.

Ethyl ether, anhydrous, was a J. T. Baker Chemical Company analyzed reagent. Unopened bottles were used without further purification.

Ethyl acetate, reagent grade, was obtained from Eastman Chemical Company. It was stored over calcium chloride for two weeks and was distilled just prior to use. The distillate obtained in the range of 76-77° C was used.

Phosgene gas was purchased from Matheson Company. It was passed through concentrated sulfuric acid before bubbling into the reaction flask.

Lithium aluminum hydride was purchased from Metal Hydrides, Incorporated.

Glycine, A grade, was obtained from Calbiochem.

Bovine Serum Albumin, Lots 11 and 12, recrystallized, was

purchased from Pentex, Incorporated.

Deuterium Oxide, Lot 261-51, was purchased from New England Nuclear Corporation at a stated purity of 99.7%.

Urea, reagent grade, was purchased from Sigma Chemical Company. It was purified by the method of Stein and Moore (56) before use.

Sodium chloride, sodium bicarbonate, sodium acetate, phosphorous pentoxide, all analytical reagent grade, were obtained from J. T. Baker Chemical Company.

Sephadex G-200 was purchased from Pharmacia Company.

Cesium chloride, and rubidium bromide, optical grade, were purchased from Gallard Schlessinger Manufacturing Corporation.

2, 4, 6, Trinitrobenzene sulfonic acid, white label grade, was purchased from Eastman Chemical Company.

## RESULTS AND DISCUSSION

### Preparation of Polyglycyl BSA

Polyglycyl BSA was prepared in a quantity such that all the physical characterization could be carried out on the same preparation. Unfortunately, some material was later lost and it was necessary to make a second preparation as similar to the first as possible. On the basis of amino acid analyses the two preparations were judged sufficiently similar for these studies (Table 1). It is recognized that the reaction of the N-carboxyamino acid anhydride with the protein will yield a distribution of product proteins whose added peptide chains have slightly varying length. When the preparations were purified by gel filtration the center of the elution peaks was used in the succeeding experiments. This process narrows the distribution of products yielding a more homogenous sample, but as later studies indicate, the material still is not completely homogeneous. The values in the table are normalized from micromoles present per sample to residue per mole by using a value of 56.6 residues aspartic acid in native BSA as reported by Stein and Moore (56). These data show that the product contained 296 added glycine residues at 30 sites. The average chain length of the added peptides was about ten residues. Inspection of molecular models indicates that chains

of this average length are sufficiently long to interact with the protein backbone. The calculated molecular weight of the derivative based on the number of added glycyI residues is 86,100 gm/mole using the accepted value of 69,000 gm/mole for the native protein.

Table 1. Extent of peptidylation of derivative

Material	Total moles glycine	Moles added glycine	Total moles lysine	DNP-lysine	Average chain length
native BSA	16.7	0	59.3	58.6	--
Prep I polyglycyl BSA	312.3	296	29.8	30.6	10
Prep II polyglycyl BSA	328.1	312	30.5	28.8	10.4

#### Trinitrophenylation of Native BSA

In the reaction of the BSA with the N-Carboxy-amino acid anhydride (NCA) only one half of the possible lysine residues that theoretically could react in the protein were acylated. The question as to whether those groups acylated were strategically located on the surface of the protein or whether the peptidylation reaction was non-specific, thus giving an average number of sites acylated of thirty of the possible sixty residues arises. The use of Sanger's method for determining the number of  $\epsilon$ -amino groups of lysine in a protein is a standard procedure. For determining available groups this method suffers from one serious limitation. The reaction solvent

50-60% ethanol, irreversibly denaturates the protein. Therefore a more suitable method was employed. A new reagent, trinitrobenzene sulfonic acid, TNBS, has been prepared and characterized by Satake (46, 48). The reaction conditions, an aqueous solution of pH 8.5, are such that the protein should maintain its native structure. This reagent made it possible to experimentally distinguish between exposed lysine residues in the native protein and those which were presumably "buried" or unreactive.

Figure 1 shows the results of the urea denaturation of BSA and the subsequent exposure of lysine residues. In the solution containing from zero to 2.5 molar urea 35 amino groups reacted. This number includes the N-terminal aspartic acid to yield 34  $\epsilon$ -amino groups of lysine reacted. The pH of this reaction is slightly higher than that used in the polypeptidylation and it has been shown by Konigsberg (37) that the number of sites acylated by the N-carboxyamino acid anhydride will increase with increasing pH. A plateau region between 4 and 6 molar urea solutions indicates a loosening of the molecular structure of the protein in this solution with the resulting exposure of 12 additional  $\epsilon$ -amino groups. This may be due to the expansion of the covalently bound subunits of the proposed "trimer" (10), but with some degree of structure retained within each unit. When the protein is dissolved in 8 molar urea, a total of 55 to 56  $\epsilon$ -amino groups react. This value is still 4 or 5 residues low for the total of 60 lysine

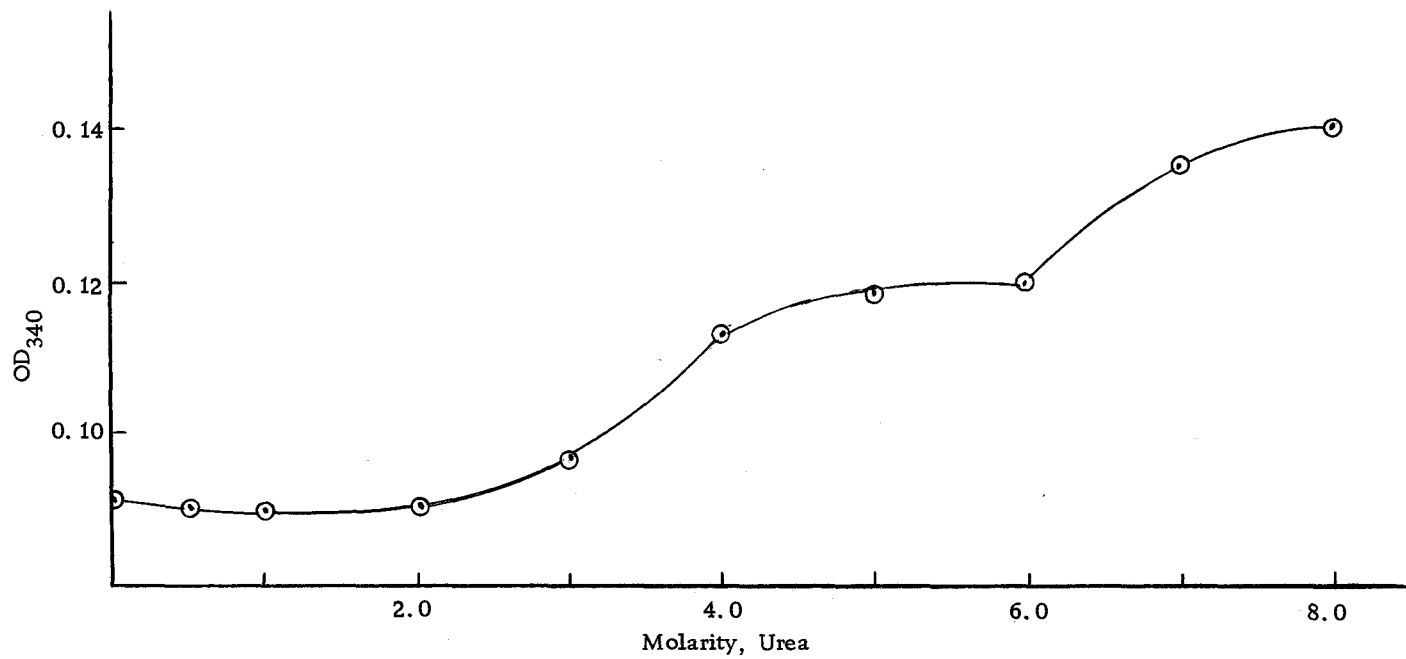


Figure 1. Trinitrophenylation of native BSA in urea solutions.

residues that are found by amino acid analysis. It is thought that although the molecule is essentially in a random coil configuration, the 17 cystine bridges cause a retention of some order in the molecule and that the remaining lysine residues are buried in a highly non-polar region or regions.

The question asked, that is whether the  $\epsilon$ -amino groups of lysine that react in the polypeptidylation reaction are those residues found on the surface of the protein or whether those sites reacted represent a non-specific distribution of the total possible, has been answered. The results can best be interpreted on the basis that a specific number of  $\epsilon$ -amino groups are found at the surface of the protein and it is these that react with the N-carboxyamino acid anhydride.

#### Optical Rotation of Polyglycyl BSA and Native BSA

The optical rotation of polyglycyl BSA when it is compared to that of the native protein may furnish useful information as to the manner in which the stabilization of the derivative is accomplished by the added peptide chains. Native BSA has been shown to contain approximately 40% helix (20). If this type of secondary structure has been perturbed by the addition of the glycine chains it should be reflected in a change in rotation. A second type of interaction that can be detected by optical rotation in the derivative is one in which



the added peptides are interacting with themselves or parts of the native protein giving rise to a dissymmetric structure that would be optically active. The polyglycyl derivative is especially suited to detect these interactions, if any exist, as the glycine residue itself is optically inactive. It is realized that the underlying physical principles that govern the optical rotation of a protein are complex and Kauzmann (35) has concluded that presently it is not possible to interpret the changes observed in terms of precise structural alteration of the protein.

Table 2 shows the values for the specific and molar rotation of native and polyglycyl BSA. The specific rotation of native BSA is  $-64.6^{\circ}$  in a solution at slightly acidic pH where the native conformation is found; that of the derivative under identical conditions is  $-47.7^{\circ}$ . These are values of the specific rotation in which the concentration is expressed in grams of solute per 100 ml of solution. The values for the molar rotation and the specific rotation at pH 5.5 are given in Table 2. There is a slight variation in the molar rotation of the polyglycyl derivative when it is compared to the native BSA. If the molar rotation of the two proteins were identical, the derivative would have a specific rotation of  $52^{\circ}$ . A similar decrease in levorotation has also been noted by Markus (41) when native BSA binds ten moles of sodium dodecyl sulfate. It is thought that this is a local effect on the protein surface. It is evident that there are no

major changes in the degree of helicity and that the added glycine chains are not forming any optically active structure.

Table 2. Optical rotation of native and polyglycyl BSA

Protein	Molecular weight	$-\alpha]_D$	$-[M]_D \times 10^{-5}$
Native BSA	69,000	64.6	4.76
Polyglycyl BSA	86,100	47.7	4.25

The optical rotation of native and the polyglycyl derivative of BSA as it varies with pH is shown in Figure 2. The change of the levorotation, an increase followed by a decrease, seen in both the native protein and the derivative below pH 4 is typical of the acid expansion of the protein followed by further loss of structure to a random coil configuration. These changes are small in the experiment shown but are comparable to the changes reported by Williams and Foster (67) who measured the optical rotation of BSA as a function of pH and also of ionic strength of the medium. The size of the levorotation is extremely sensitive to ionic strength. Typical of the values they reported are: at pH 2.5 the specific rotation of zero ionic strength is  $-86^\circ$ , at 0.02 molar sodium chloride  $-77^\circ$ , and at 0.10 molar sodium chloride  $-67^\circ$ . Since the present study was designed to determine the effect of added peptides on BSA, this and all other experiments were conducted at an ionic strength of 0.10.

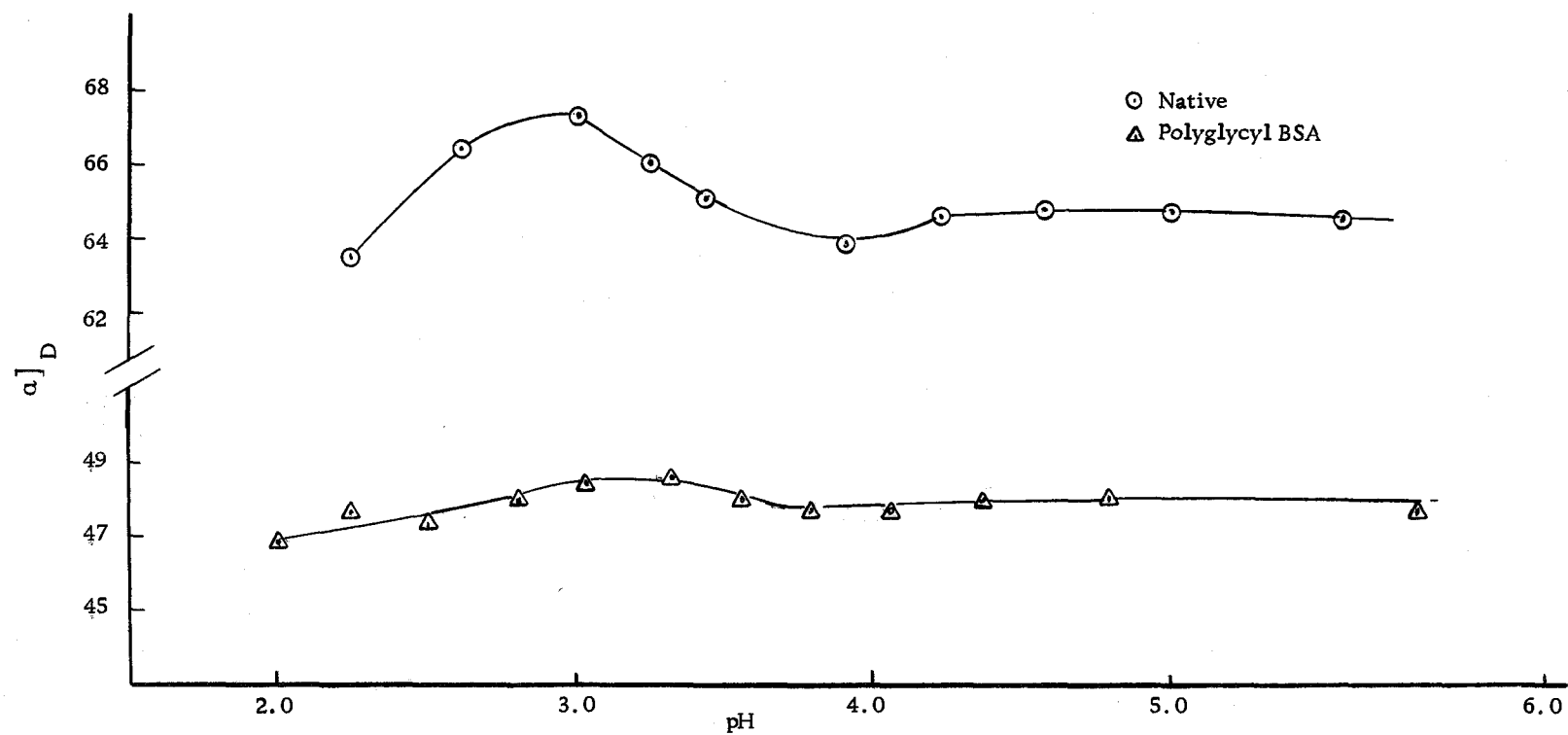


Figure 2. Optical rotation of native and polyglycyl BSA as a function of pH. The protein was in 0.10 M NaCl at 25° C.

The optical rotation determination was therefore carried out at this ionic strength.

### Ultraviolet Difference Spectra of Native and Polyglycyl BSA

The technique utilizing ultraviolet difference spectra has been well established as a useful probe in investigating protein structure. Very small changes in optical density, that would be difficult to measure by normal spectral methods, may be magnified by using high protein concentrations so they may be studied with a minimum of experimental uncertainty. It has been shown that the aromatic amino acids do not have identical spectral properties when they are contained as residues within the protein structure as they do when they are present as the free amino acids (66). Mainly by the method of difference spectra, it has been found that the differences can be explained by environmental effects on the chromophoric groups. In the case of proteins, these differences reflect changes in the character of the interior of the molecule where most aromatic residues reside. Scheraga (11) has shown that by changing the pH of a protein solution and denaturing the protein the spectral changes at 287 m $\mu$  may be explained by the changing environment of the tyrosyl chromophores of the protein. Similar studies have been reported by a number of laboratories, all reaching the same conclusion.

A second large absorbance peak is usually found by comparison

of native to denatured proteins using differential spectrophotometry in the 230-240  $m\mu$  region. Glazer and Smith (24) suggested, since the size of this absorbance is larger than the peak at 287  $m\mu$  that it must be due to the peptide bond absorptivity. Eisenberg and Edsall (15) have proposed that this peak is due mainly to the tyrosyl chromophores as in the 287  $m\mu$  peak. Lynn (40), in a study of poly-L-tyrosyl acetamidated ribonuclease, felt that this large peak reflected a joint phenomenon of both the peptide and tyrosyl absorptivity.

By attempting to perturb the environment of the chromophoric residues in the native and then the polyglycyl BSA, it should be possible to determine if the added glycyl residues are stabilizing the derivative in a manner which would involve the chromophores. The proteins were perturbed by lowering the pH, causing the molecule to expand, and by heating, which in the extreme case causes thermal aggregation of the native protein. It is thought that the thermal aggregation of BSA is caused when the molecule expands and the non-polar residues are exposed to the aqueous medium. It is then thermodynamically favorable for these non-polar residues to aggregate, effectively forming a micelle, and thus removing themselves from the aqueous environment.

### pH Difference Spectra

Figures 3 and 4 show the difference spectra of native and polyglycyl BSA produced by lowering the pH of the sample solution and maintaining the reference at pH 6.55 where the native conformation exists. It is seen that in both proteins the spectra show a major peak at 287  $m\mu$  and another in the region between 230-240  $m\mu$ . A minor but distinct shoulder of the 287  $m\mu$  peak is evident at 280  $m\mu$ .

In these measurements care must be taken to insure that the protein concentration is identical in each solution of the given protein investigated. It was attempted to prepare solutions of concentration of the native and derivative protein. This latter attempt was not completely realized as the concentration of the native protein was 1.81 mg/ml and that of the derivative 1.76 mg/ml. The necessary corrections for this difference in concentration were made so that the values shown in the figures are those which would be found if the protein concentration were identical.

Figure 5 summarizes the pH effects on the proteins by indicating the changes associated with the two major absorbance peaks. For both the native protein and the derivative considerable change is found, but the polyglycyl derivative shows less change. The increase in optical density at 235  $m\mu$  shows a greater difference between the native and the polyglycyl BSA than that at 287  $m\mu$ . This is probably due to the fact that at 287  $m\mu$  the observed changes are due to the

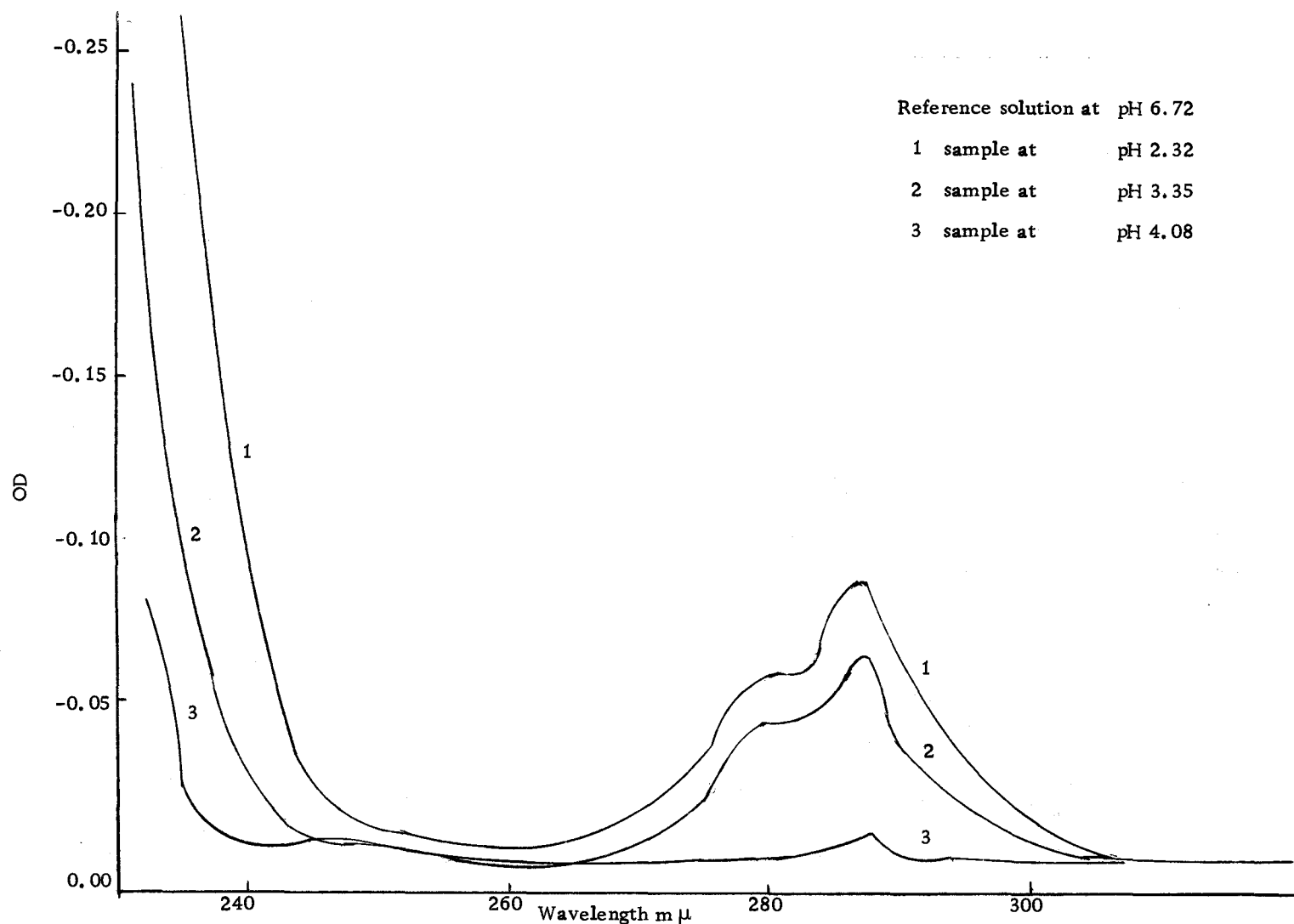


Figure 3. pH Difference spectra of polyglycyl BSA. The protein concentration was  $2.6 \times 10^{-5}$  M in 0.10 M HOAc-NaOAc buffers at 25° C.

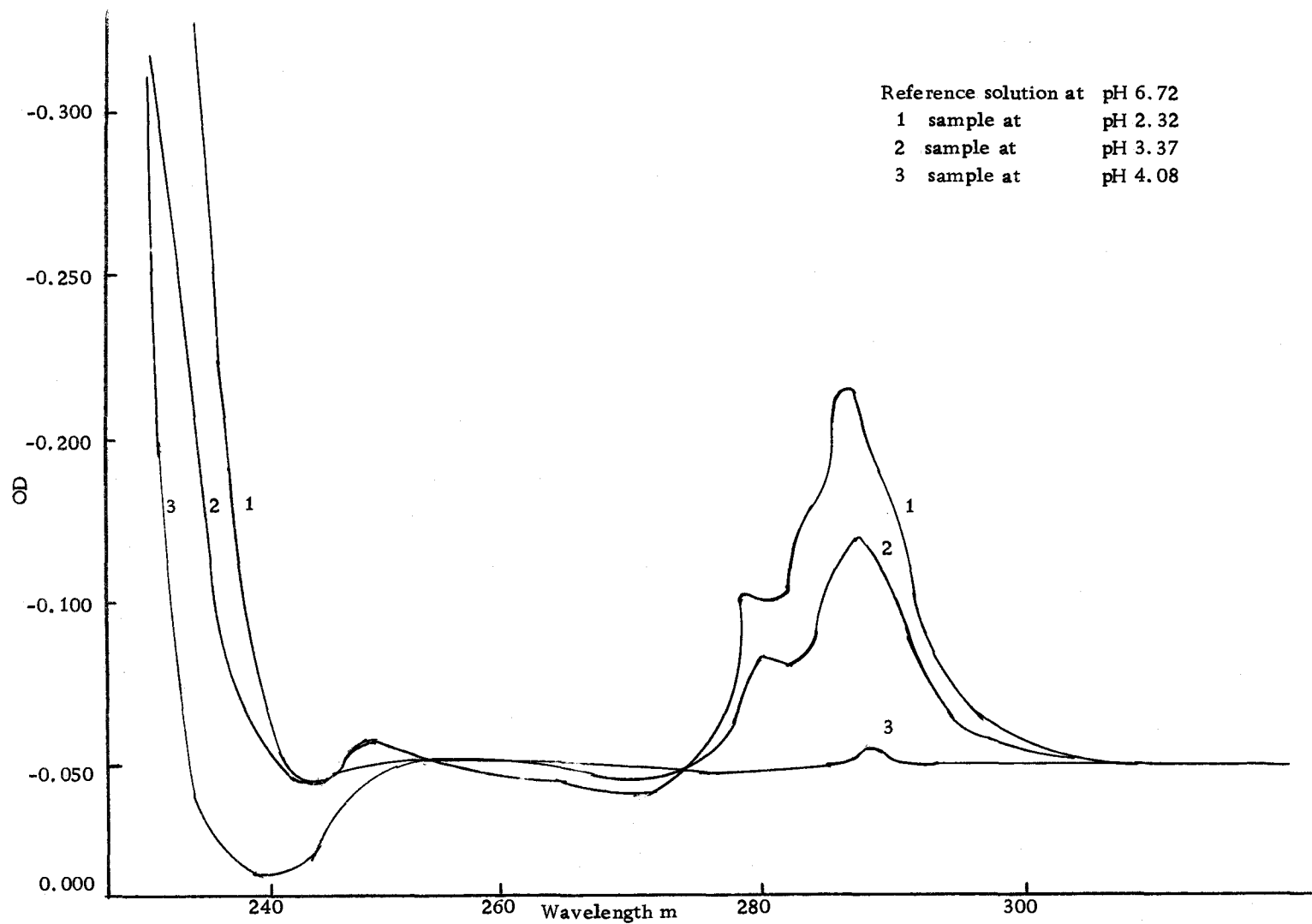


Figure 4. pH Difference spectra of native BSA. The protein concentration was  $2.6 \times 10^{-5}$  M in 0.10 M HOAc-NaOAc buffers at 25° C.



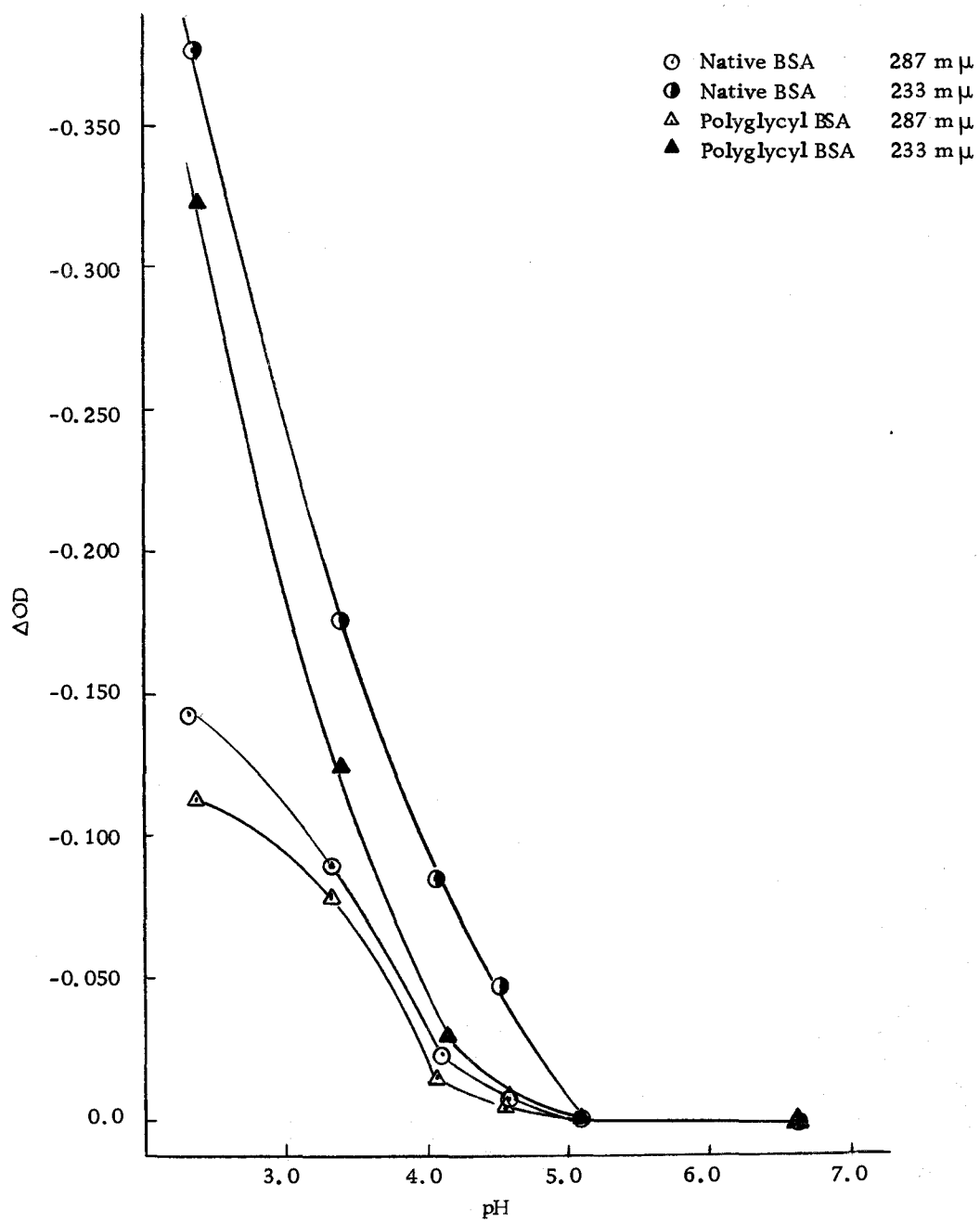


Figure 5. Summary of the pH difference spectra of native on polyglycyl BSA at 287 m $\mu$  and 233 m $\mu$ .

tryosyl residues alone while that change seen at 235  $m\mu$  may have both the aromatic residues and peptide bonds contributing to the increase.

### Thermal Difference Spectra

Several proteins studied exhibit a reversible thermal transition that can be studied by ultraviolet difference spectra. A thermal transition of ribonuclease has been particularly well characterized (11, 19, 62). The midpoint of the optical density change with temperature, is defined as the transition temperature,  $T_m$ . Von Hippel and Wong (62) and Lynn (40) have used this quantity to measure the effect of a variety of salts and denaturing agents on the protein's conformation. Since the thermal transition is a reversible process, the variation of the equilibrium constant with temperature may be used to evaluate the change in enthalpy. If a similar thermal transition could be found in BSA, the effect of the added glycyl peptides could be measured and a direct estimation of the stabilization determined.

Changes in the optical density were observed at both the 233  $m\mu$  and at the 287  $m\mu$  peak for BSA and polyglycyl BSA. Plots of  $\Delta A_{233}$  and  $\Delta A_{287}$  versus  $T$  showed no definite transition. The observed changes in absorption increased throughout the temperature range investigated, 20° to 92° C. In the case of the native protein near its

isoelectric point, aggregation occurred.

There are several plausible explanations for this behavior. BSA contains 21 tyrosyl residues. Herskovits and Laskowski (30), using the solvent perturbation technique, have shown that 70% of the tyrosyl residues, 15 residues, are buried within the protein interior. Ribonuclease, which has a distinct transition, contains only 6 tyrosyl residues of which 3 are buried within the protein interior. The increase in the complexity of a transition involving 15 residues as compared to 3 residues may be easily envisioned. The second explanation relates to the concept of the microheterogeneity of BSA that has been presented by Foster (47) and also by Anderson (2). The microheterogeneity suggested by Foster may be the result of a slight difference in the folding of some portion of the peptide chain, variation in hydrogen bonding within the molecule, or some limited substitution of amino acids in the peptide backbone. Either explanation should be sufficient to explain the lack of a discrete thermal transition in BSA.

Finding a lack of a discrete thermal transition it was then hoped that the evaluation of the rate of increase in optical density at different temperatures could be utilized to determine the Arrhenius activation energy. It was found that this was not possible as the rate of increase of optical density, once the temperature where the increase began was found, appeared to be independent of temperature.

There was a slight increase of initial rates, but this could not be accurately evaluated as the increase in temperature throughout the cell was not instantaneous. For these reasons the data on the thermally induced ultraviolet difference spectra is much more qualitative and less quantitative than desired.

A composite of the differences in optical density between the cool reference solution and the heated sample solution found at 287  $m\mu$  and at 233  $m\mu$  for temperatures of 50° C and 92° C for native and polyglycyl BSA is shown in Figure 6. These temperatures were chosen as points at which representative changes were observed. As in the difference spectra obtained by perturbing the protein by acid, the polyglycyl derivative shows less change in the environment of the chromophores than does the native protein. The decrease in the change of optical density of the polyglycyl BSA at pH 5.3 may be associated with the fact that the solution is near the isoelectric point of the protein where the protein is least soluble. By comparison the native protein aggregates at 62° C at this pH and ionic strength. The decrease of optical density at the acid extreme of pH investigated is thought to be due to the fact that the protein is in the random coil configuration and it may be possible for some of the non-polar residues to again associate. The lack of aggregation of the native protein at low pH is primarily due to intermolecular electrostatic repulsion as all the carboxyls are protonated giving the protein a

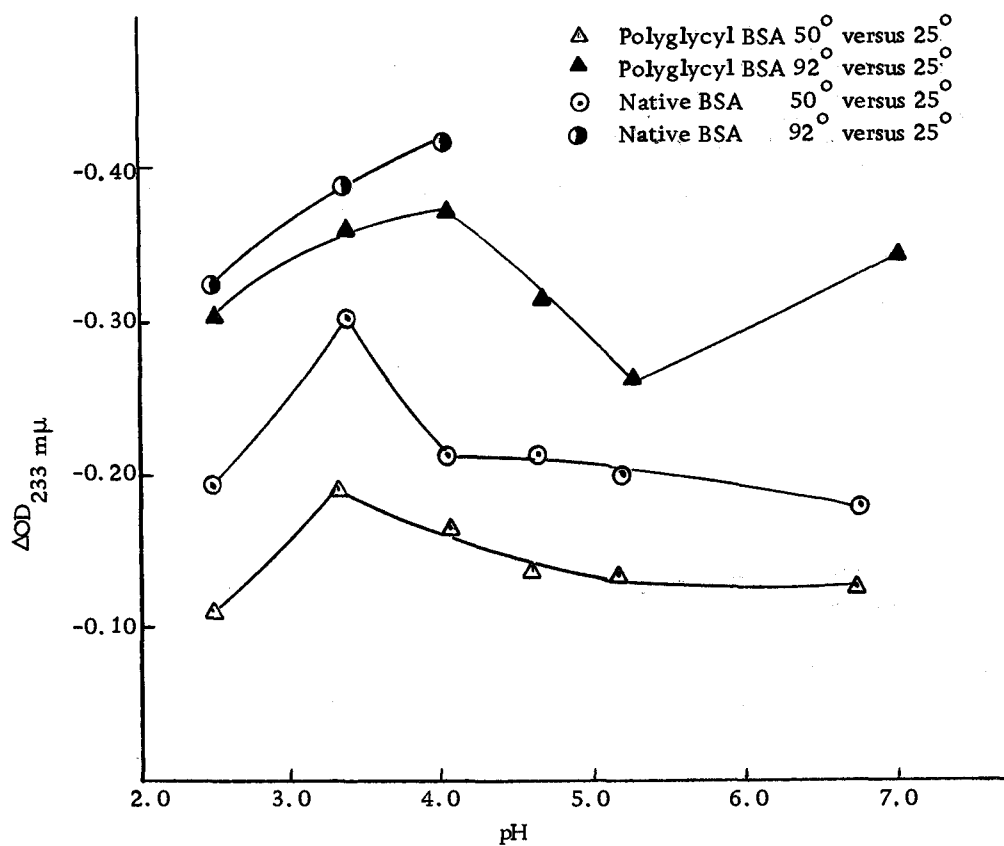


Figure 6. Composite of the thermal difference spectra of native and polyglycyl BSA at different pH values at 233 m $\mu$ . The protein (concentration  $2.58 \times 10^{-5}$  M) was in 0.10 M HOAc-NaOAc buffers.

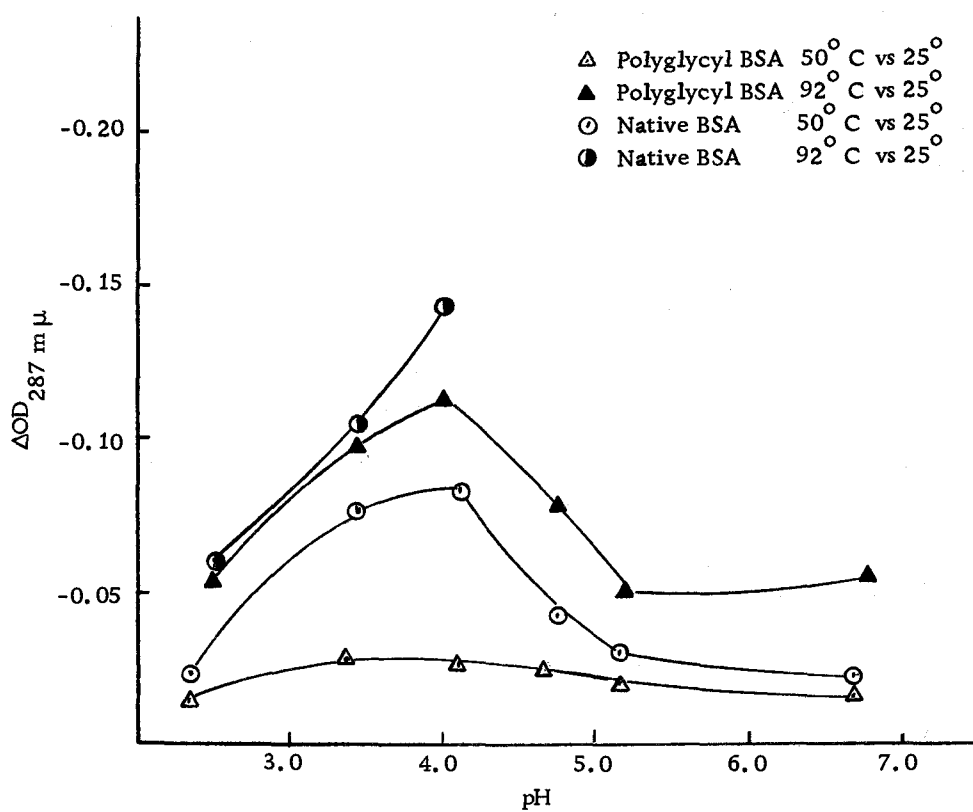


Figure 7. Composite of the thermal difference spectra of native and polyglycyl BSA at different pH values at 287 m $\mu$ . The protein (concentration  $2.58 \times 10^{-5}$  M) was in 0.10 M HOAc-NaOAc buffers.

large positive charge. The increase in optical density between pH 3 and 4 may be due to the general expansion of the protein subunits which exposes the chromophores but will not allow them the freedom to interact with each other.

In all the cases shown the changes observed in the native protein are paralleled by similar changes in the polyglycyl derivative. This indicates that the matrix of the BSA structure found in the derivative is encountering the same disruptive forces as the unmodified protein.

#### Sedimentation Velocity of Native and Polyglycyl BSA

The sedimentation coefficient,  $S_{20}$ , of a macromolecule is affected by both the molecular weight and the hydrodynamic shape of the molecule. Since BSA and the polyglycyl derivative consist of a single peptide chain there is no possibility of subunit dissociation during acid expansion which would result in a change in molecular weight. The changes observed in the sedimentation coefficient are therefore indicative of changes in the protein's conformation.

The pH profile of the sedimentation coefficients shown in Figure 8 indicates that the polyglycyl derivative undergoes the same low pH expansion as the native protein. The transition appears to be less sharp in the derivative than it is in the native. This observation may be explained by considering the known heterogeneity of

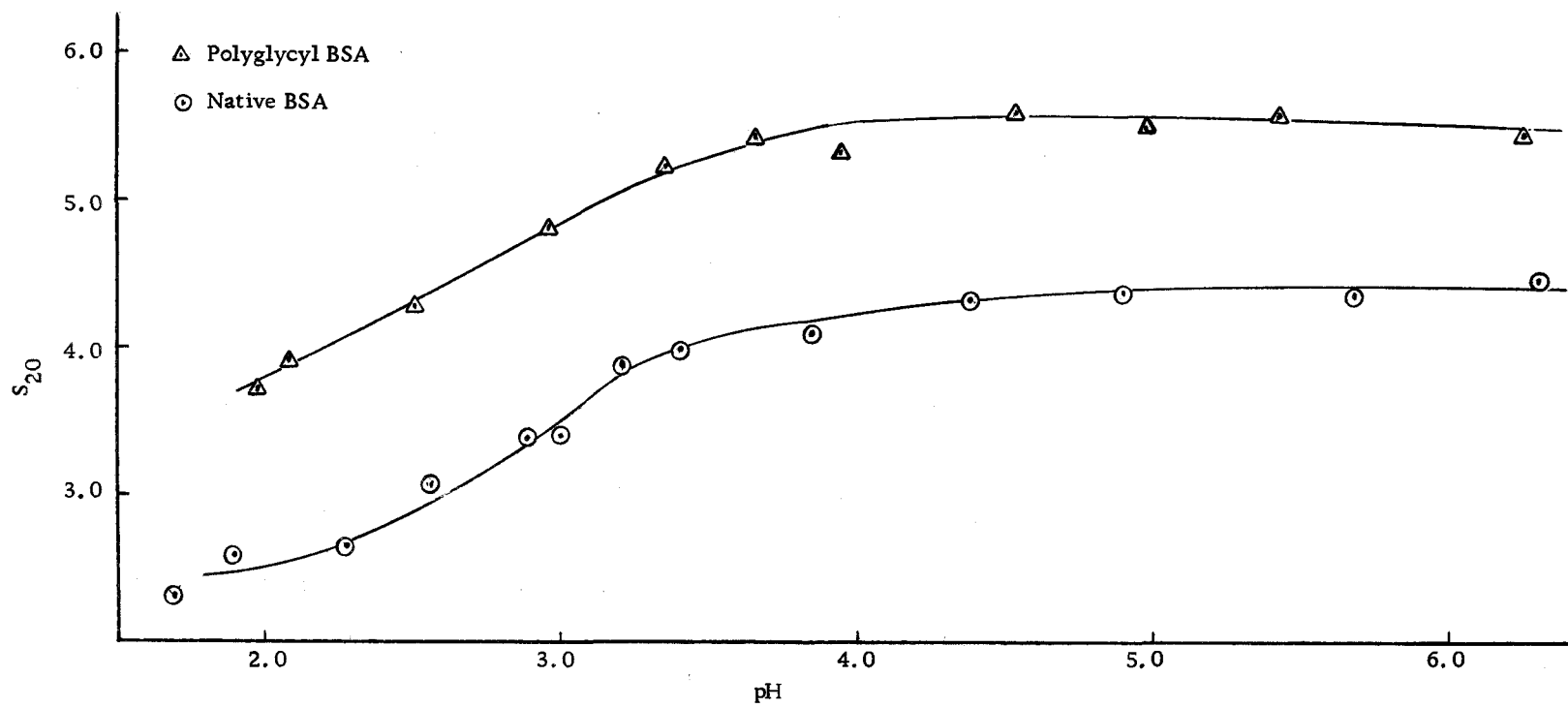


Figure 8. Sedimentation velocity of native and polyglycyl BSA as a function of pH.



polypeptidyl proteins. Scatter in the values of the sedimentation coefficients at low pH are especially noticeable in the study of the native protein. Slater (53) has shown, by electron microscopy of BSA at pH 2.0, a large variation in the random coil form ranging from very loose globular forms to extended and folded chains. This evidence indicates that the low pH conformation is not a discrete hydrodynamic particle and scattering of  $S_{20}$  values is to be expected.

Of great importance in this study was the determination of the reversibility of the acid expanded form to the native conformation of polyglycyl BSA. Tanford (60, 61) and Foster (20) have shown that the acid expansion of native BSA is fully reversible as judged by hydrodynamic characterization. These results were confirmed in the present study in that native BSA in the acid expanded form could be reverted to the native conformation by dialysis of the sample against an appropriate buffer. BSA with an  $S_{20}$  value of 2.63 at pH 2.25 could be reversed to the more compact native structure with a  $S_{20}$  value of 4.31 by dialyzing the sample against phosphate buffer of pH 6.2. Aggregation of the protein occurred when direct neutralization of the acid solution was attempted. Local effects of the addition of the base to the solution creating a situation where the protein could not adjust to the rapid pH change may account for this observation. Dialysis is a more gentle treatment, allowing the protein to gradually adjust to the changing conditions. The acid expansion of polyglycyl

BSA was also found to be reversible. Polyglycyl BSA with an  $S_{20}$  value of 3.85 regained its native conformation with a sedimentation coefficient of 5.45 by dialysis against the same phosphate buffer used in the native case.

The reversibility of the low pH conformation change of the native protein is not unexpected. Anfinsen (3) has denatured ribonuclease and then reduced the four S-S bridges. Following slow reoxidation, a protein with the same hydrodynamic characteristics and enzymatic activity of the untreated protein was isolated. This experiment demonstrated that the forces influencing protein conformation reside in the amino acid sequence of the peptide chain. BSA, under the conditions described earlier, would be expected to behave in a similar fashion, for although it is a more complex molecule, the 17 S-S bridges were not cleaved. In the case of the polyglycyl derivative, it might be energetically favorable for the derivative to assume a different conformation that would include some of the added glycyl residues. It appears, however, that the native conformation of the derivative is more desirable than any other possibility. It is recognized that this type of hydrodynamic measurement is not accurate enough to detect subtle changes that might occur. The ultraviolet spectra of the derivative after it had been expanded and then reversed could not be distinguished from an untreated sample at the same pH providing further substantiation to the suggestion that

the native conformation is regained by the derivative.

### Partial Specific Volumes of Native and Polyglycyl BSA

The partial specific volumes of polyglycyl BSA and native BSA, run as a control, were determined by the method of Martin et al. (42), with the modifications of the technique presented by Gagen (23). The method involves the determination of the sedimentation coefficient of a protein in a series of solutions of increasing density. With these sedimentation values it is possible to extrapolate to the density at which sedimentation will no longer occur. Schachman (49) has described the sedimentation behavior of a macromolecule by the relation

$$f\eta S = M_1(1 - \bar{V}_1\rho) + \alpha M_2(1 - \bar{V}_2\rho)$$

In this equation  $f$  is the frictional coefficient,  $\eta$  is the viscosity,  $S$  the sedimentation coefficient,  $\bar{V}$  the partial specific volume,  $M$  the molecular weight,  $\rho$  the density, and  $\alpha$  is a correction for interaction between solvent and solute. The subscripts 1 and 2 stand for the water or solvent solution and the macromolecule respectively. At the density where sedimentation will no longer occur, the left side of the equation will reduce to zero and the density related to the effective partial specific volume is found by the following relation:

$$V_h = \frac{1}{\rho} = \frac{M_1 \bar{V}_1 + \alpha M_2 \bar{V}_2}{M_1 + \alpha M_2}$$

The term the effective partial specific volume which is calculated by the above equation is equivalent to the thermodynamic partial specific volume only if the interaction term  $\alpha$  is zero. Therefore it is necessary to choose a solvent system which will not cause any preferential interaction between it and the protein. Gagen has found that deuterium oxide will meet these requirements in most cases. The correction of the sedimentation coefficients for the increase in molecular weight attributed to the deuteration of the groups with exchangeable hydrogens and for viscosity effects has been given in the methods section.

The results of this type of determination for polyglycyl BSA and for native BSA are shown in Figure 9. A value of  $0.722 \pm 0.002$  ml/gm is found for the derivative. This value is in reasonable agreement with a value of 0.718 ml/gm which was calculated from the weight fraction of the added glycine residues and their partial specific volumes. Native BSA gave a value of 0.732 ml/gm which is within the experimental error of the expected value of 0.734 ml/gm (20).

#### Diffusion Coefficient of Native and Polyglycyl BSA

The diffusion coefficient of a protein may be used in a series of approximations to determine protein conformation by considering

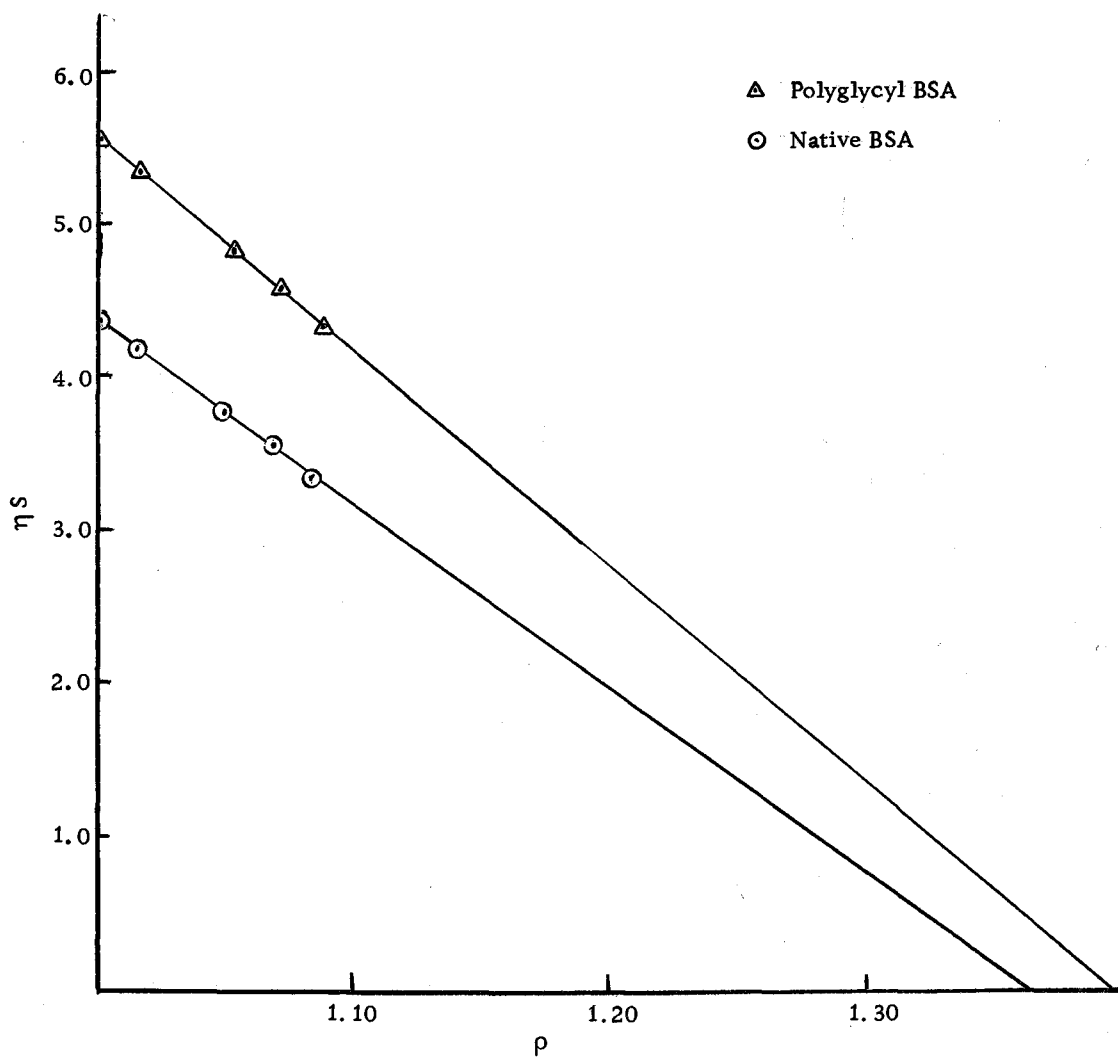


Figure 9. The viscosity corrected sedimentation velocity of native and polyglycyl BSA as a function of solution density.

the protein as a hydrodynamic particle. Theoretically, the diffusion coefficient of a particle at infinite dilution has been considered to depend only on the temperature and the frictional coefficient. The frictional coefficient reflects two inseparable parameters of the protein, the hydration and the shape of the protein in solution. The difference in frictional ratio between a sphere and an ellipsoid of revolution is described by the shape factor. In the approximations, the hydration and the shape factor may be minimized and maximized for the evaluation of the other unknown.

The system in which the diffusion coefficient was determined was considered to be comprised of two components, the protein and the water, even though low molecular weight salt, sodium chloride or buffer, was present to minimize electrostatic interaction of the protein. Since this third component was present in equal concentration throughout the experimental system, its effect has generally been ignored.

The diffusion coefficient of the protein may be determined directly by studies of the spreading of a boundary, artificially created in the centrifuge cell, by optical methods or it may be calculated from the Svedberg equation,  $M = S_{20}^0 RT / D_{20}^0 (1 - \bar{V}\rho)$ . Both methods were applied to the derivative. The Svedberg equation makes use of the fact that the frictional coefficient for the protein is identical in both the process of sedimentation and that of diffusion.

Using the sedimentation coefficient extrapolated to zero concentration, a reasonable diffusion coefficient was obtained. The apparent diffusion coefficient was also measured using the analytical ultracentrifuge equipped with interference optics. With the available equipment, these values could not accurately be determined by this method. For this reason the value measured is included only as a comparison. The diffusion coefficient determined at pH 5.5 using the Svedberg equation was  $5.67 \text{ cm}^2/\text{sec}$ . The apparent diffusion coefficient determined in the ultracentrifuge was  $5.1 \pm 0.2 \text{ cm}^2/\text{sec}$ .

With a value for the diffusion coefficient it was possible to make approximations as to the possible effects that the added glycol chains had on the native protein. It is emphasized that at this stage of the investigation these are only crude approximations, but by the comparison between the native and the derivative protein, they may give possible indications as to the reason for the stabilization and indicate the direction to be taken in further investigations.

The minimum frictional coefficient for a particle, assuming it has a spherical shape and no hydration, can be calculated with the following equation:

$$f_{\min} = \frac{KT}{D_{\max}} = 6\eta \left[ \frac{3M\bar{V}}{4N} \right]^{1/3}$$

where  $\eta$  is viscosity expressed in poises,  $M$  the molecular

weight of the polymer, and  $\bar{V}$  the partial specific volume of the polymer (59). After the minimum frictional coefficient had been obtained the relation between its value and the actual coefficient, which includes the shape and hydration factors, can be obtained. The minimum frictional coefficient will correspond to the maximum possible diffusion coefficient. The relation between these quantities, the shape factor, and the hydration is shown below.

$$\frac{f}{f_{\min}} = \frac{D_{\max}^0}{D^0} = \frac{f}{f_0} \left[ \frac{\bar{V}_2 + \delta_1 \bar{V}_1^0}{\bar{V}_2} \right]^{1/3}$$

The same notation is used as in the preceding equation. For native BSA the ratio of the maximum to observed diffusion coefficient was 1.322 while the ratio for the derivative was 1.316. This indicated that the combination of the two factors of hydration and shape were very similar for the two proteins.

As a first approximation to determine the maximum solvation, the shape factor  $f/f_0$  was taken to be one. The native protein yields a maximum solvation of 1.07 gm H<sub>2</sub>O/gm protein while the derivative had a value of 0.919 gm H<sub>2</sub>O/gm. Methods for evaluation of the shape factor,  $f/f_0$ , are given by Tanford (59). For prolate ellipsoids the following equation may be used to approximate this quantity



$$\frac{f}{f_0} = \frac{(1 - b^2/a^2)^{1/2}}{(b/a)^{2/3} \ln \frac{1 + (1 - b^2/a^2)^{1/2}}{b/a}}$$

where  $b$  is the minor axis and  $a$  is the major axis.

With the dimensions available for BSA based on low angle X-ray experiments, this quantity can be evaluated. To evaluate the maximum effect the added peptides may exert on the derivative two possible spatial arrangements were calculated. The first assumed that the added peptides extend out from the protein into the solvent. The length of the fully extended chains,  $36 \text{ \AA}$  can be calculated from the Corey-Pauling-Koltun model. The second assumed that the peptides were folded back along the protein giving a molecule essentially the same dimensions as that of the native. It was further presumed that the added peptides are evenly distributed over the surface of the protein.

The calculated shape factor for the native protein was 1.108, while it was 1.048 for the derivative in the extended peptide arrangement. Using these values a recalculation of the hydration of the protein gave 0.51 gm  $\text{H}_2\text{O}$ /gm protein for native BSA, 0.71 gm  $\text{H}_2\text{O}$ /gm protein for the derivative with maximum extension of the added peptides, and 0.48 gm  $\text{H}_2\text{O}$ /gm protein for the derivative if the added peptides were folded back along the protein.

It was evident that the derivative may have a large variation in

hydration depending on the spatial arrangement of the added glycines. A study to determine the hydration by a more direct method should eliminate one of the unknowns in the frictional coefficient, thus indicating the spatial arrangement of the added peptide. The model, assuming that the peptides are folded back along the protein, was favored because it was easier to envision the stabilization against thermal aggregation by the additional glycines shielding some of the non-polar residues against the aqueous environment, than it was if they were extending into the solvent.

It was possible to determine whether there was a large probability of the added glycine chains overlapping by using the dimensions of the models of BSA that have been published (10, 39, 44). Two assumptions were necessary before the calculation could be made. It was assumed that the original sites of acylation were evenly distributed over the surface of the protein, and the second assumption was that the added chain was flexible. Using a surface area of  $18,000 \text{ \AA}^2$  from Bloomfield's paper (10) and a chain length of  $36 \text{ \AA}$ , it was found that the surface area of BSA was large enough to exclude overlapping of the added chains. Since the distribution of the sites was not known, there may be several chains that overlap but most will not.

Buoyant Density of Native and Polyglycyl BSA

The determination of the buoyant density of macromolecular species has only recently been applied to proteins. It has been an established procedure using the preparative ultracentrifuge in the study of viruses and nucleic acids. Ifft and Vinograd (31) have published a study of the applicability of this technique to protein work using the analytical ultracentrifuge. Since the buoyant density depends both on the anhydrous composition and the hydration of the material studied, the behavior of bovine mercaptalbumin (BMA) in a series of salts to determine the effect of the activity of water upon the hydration of the protein was examined. It was also established that a homogeneous protein would produce the necessary Gaussian distribution at its equilibrium position in the solution. Their studies present a method for determining the solvated molecular weight of the protein in the buoyant solution. The basic equation used is

$$M_s = \frac{RT\rho_0}{r_0 \left[ \frac{d\rho}{dr} \right]} \omega^2 \sigma^2$$

in which  $R$  is the gas constant,  $T$  the temperature,  $\rho_0$  the buoyant density of the material investigated,  $\sigma$  the standard deviation of the band, which is proportional to the molecular weight,  $\omega$  the angular velocity expressed in radians per second, and  $(d\rho/dr)$  is the density gradient that exists in the centrifuge cell. The

subscripts indicate that the values are taken at the band center.

There are four experimentally determined quantities: the buoyant density, the standard deviation of the distribution, the equilibrium position in the ultracentrifuge cell, and the density gradient. A brief explanation as to the evaluation of each will be given.

The equilibrium position  $r_0$  is measured directly from the schlieren pattern recorded on the photographic plate.

The buoyant density is assessed with a knowledge of the initial density of the solution at atmospheric pressure and the equilibrium position. The distance the equilibrium position is removed from the position in the cell at which point the density is that of the initial solution, the isoconcentration position, times the density gradient will determine the buoyant density of the protein, or mathematically  $\rho_0 = \rho_e + \Delta r(dp/dr)$ . The isoconcentration position is determined as the root mean square of the distance between the meniscus of the solution and the bottom of the cell.

The standard deviation,  $\sigma$ , may be evaluated by several methods. The method used in this study involved the determination of the slope of the plot of  $\ln K(n-n_0)$  versus  $(r-r_0)^2$  which may be

used in the expression  $\frac{\ln K(n-n_0)}{(r-r_0)^2} = -\frac{1}{2\sigma^2}$ . The quantity  $K$  is an

instrument constant obtained from the magnification of the image

through the optics of the ultracentrifuge,  $\eta$  is the refractive index of the solution,  $\eta_0$  is the refractive index of the solvent,  $r_0$  is the equilibrium position of the protein band and  $r$  is the distance at which the refractive indices are measured. This method for evaluating  $\sigma$  is the most accurate of those presented by Vinograd.

Several density gradients that describe the redistribution of the solutes have been presented by Vinograd (33). The buoyant density gradient is calculated from the equation

$$(d\rho/dr)_{\text{buoy}} = \left[ \frac{1}{\beta} + \frac{(k-k_s)}{1-\alpha} \rho_0^2 \right] \omega^2 r$$

where  $k$  is the compressibility of the salt solution,  $k_s$  the compressibility of the polymer, and  $\alpha$  has been defined as the measure of the change in preferential solvation of the polymer with distance in the centrifuge cell. The function  $\beta$  has been defined as  $(d \ln a / dP) RT / (1 - \bar{V}_p)$ , "a" being the activity,  $\bar{V}_p$  the partial specific volume of the solute,  $\rho_0$  the density of the salt solution, and  $P$  the hydrostatic pressure in the cell. Numerical values of  $\beta$  as a function of density for the salts used in this study have been tabulated (32).

Vinograd has presented a method to determine the buoyant density gradient experimentally by running two separate cells in a single equilibrium run. The cells contain solutions of slightly different densities in which the protein will band on either side of the

isoconcentration point of the cell. If these band centers of the proteins are within a millimeter of each other, the compression, activity of water and hydration may be assumed identical in each case and the separation of the modes of the bands are a measure of the buoyant density that may be computed with the relation

$$(dp/dr)_{\text{buoy}} = \frac{\rho_{e2} - \rho_{e1}}{r_{01} - r_{02}}$$

where  $\rho_e$  is the isoconcentration density,  $r_0$  the equilibrium position and subscripts 1 and 2 denote the two cells used in the experiment. The final gradient which is used in the equation to determine the solvated molecular weight is the effective density gradient which is expressed as

$$(dp/dr)_{\text{Eff}} = \left[ \frac{(1-a)}{\beta} + (k-k_s)\rho_{02} \right] \omega^2 r$$

This is based on the assumption that the solvated species in the band has an invariant density except for the effects of pressure and the activity of the water associated with it.

A typical schlieren pattern obtained using two cells, one with a  $1^\circ$  negative wedge window, containing the polyglycyl BSA in CsCl is shown in Figure 10. The integral curve of the concentration distribution obtained by plotting  $K(\eta - \eta_0)$  verses  $(r - r_0)$  is shown in Figure 11 and the semi-log plot of  $K(\eta - \eta_0)$  verses  $(r - r_0)^2$  is

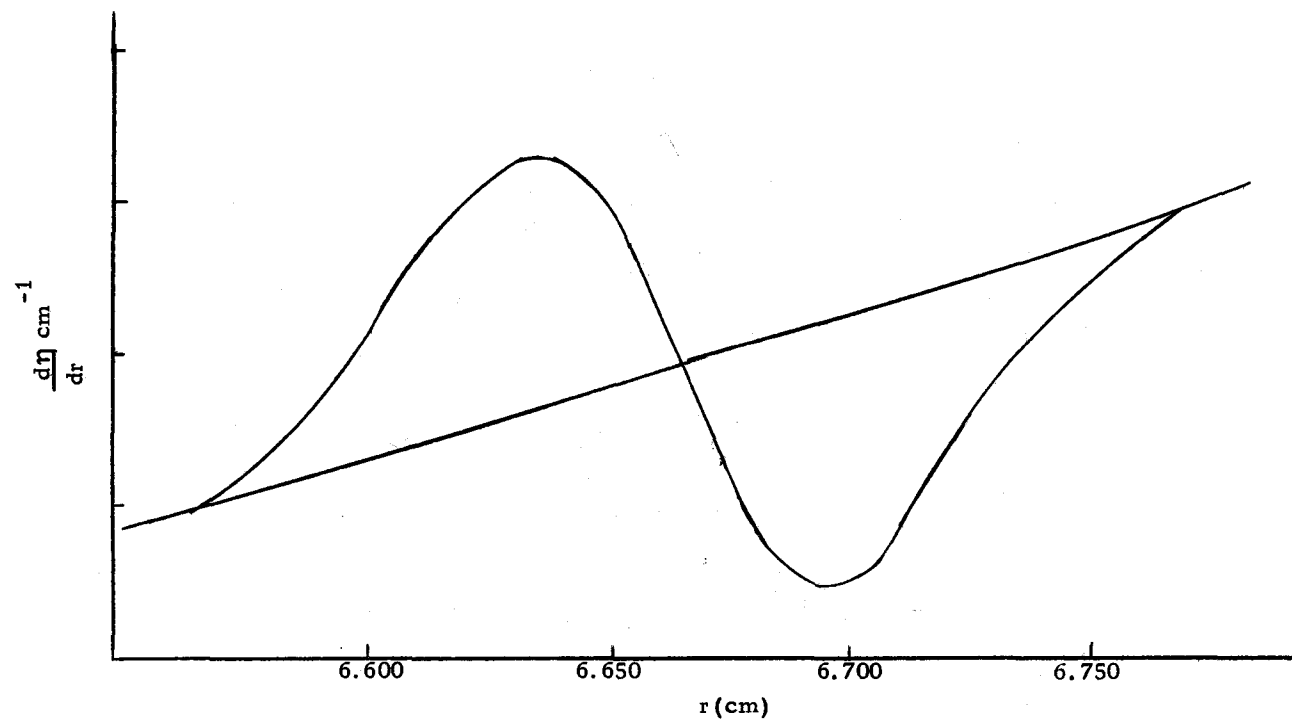


Figure 10. Schlieren image obtained from buoyant density determination of polyglycyl BSA in CsCl.

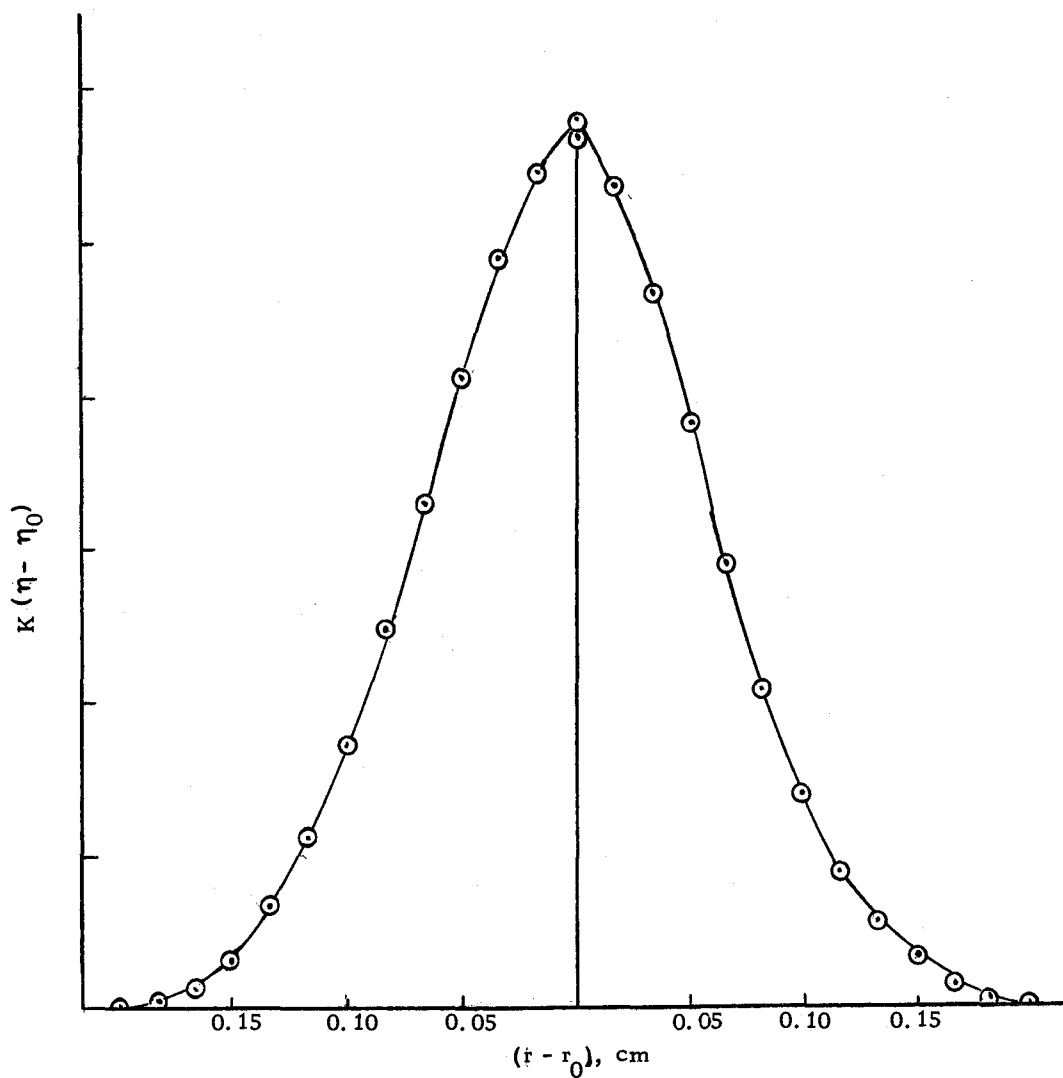


Figure 11. Concentration distribution of polyglycyl BSA in CsCl gradient at sedimentation equilibrium.



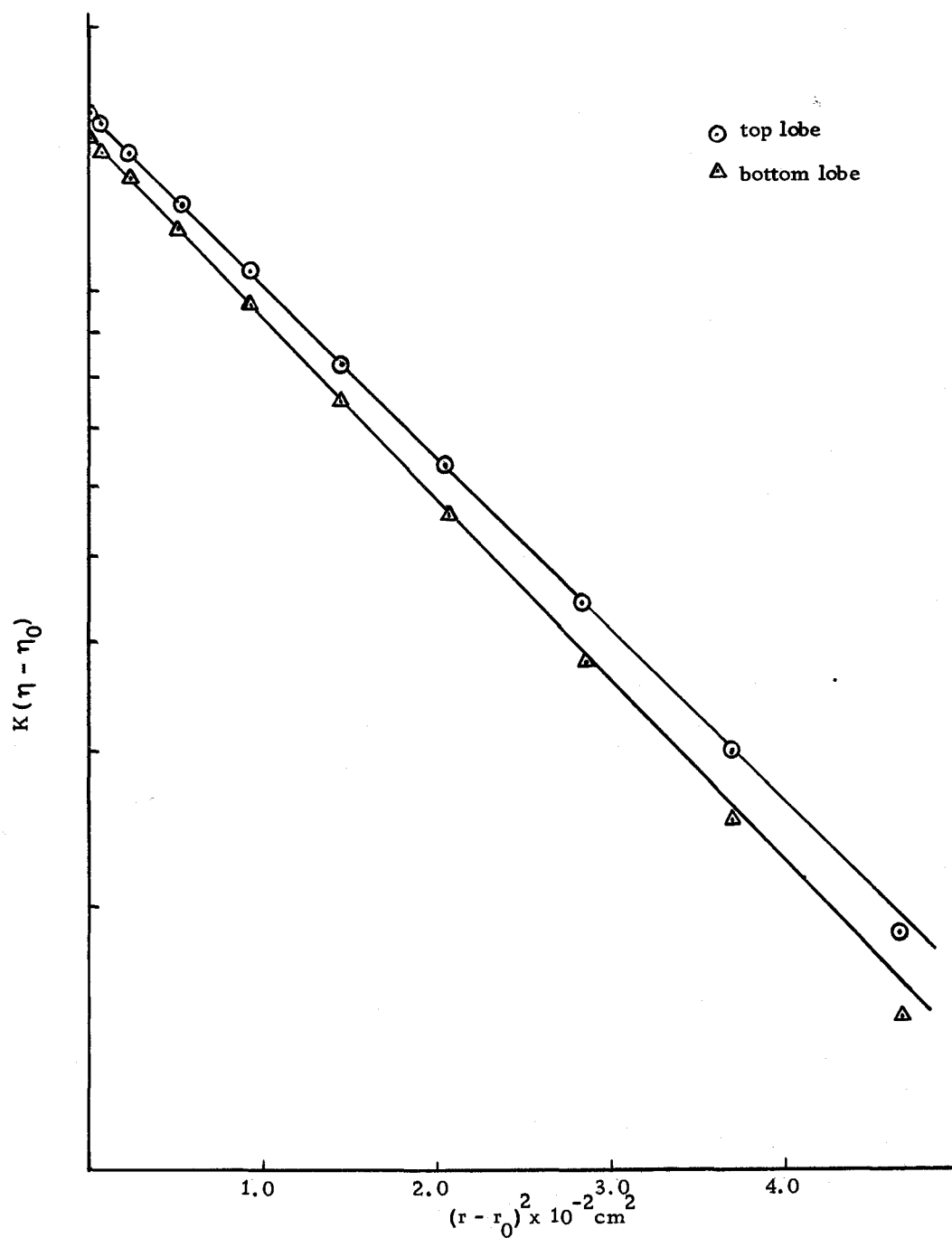


Figure 12. Logarithmic plot of concentration distribution of polyglycyl BSA in CsCl gradient at sedimentation equilibrium.

presented in Figure 12. The deviation from the initial slope at the extreme end of the plot is typical and may be explained on the grounds that the gradient is nonlinear over relatively long distances in the centrifuge cell.

Table 3 shows the buoyant densities that have been determined for polyglycyl BSA in three salt solutions and for native BSA in cesium chloride. For comparison the data determined by Ifft and Vinograd for bovine mercaptalbumin in the same salt solutions has been included. The standard deviation,  $\sigma$ , given in Table 3 was the average of four values determined from a single, double cell run. The hydration,  $\Gamma$ , was estimated using the relation between the buoyant density,  $\rho_0$ , the hydration, and the partial specific volume of the protein expressed as

$$\rho_0 = \frac{1 + \Gamma}{\bar{V}_3 + \Gamma \bar{V}_1}$$

where  $\bar{V}_1$  is the partial specific volume of water which is taken as one ml/gm. This estimation does not include the effect of the anions that are bound to the protein.

The buoyant density of the polyglycyl BSA in each of the salts was greater than the buoyant density of BMA in the corresponding salt solution. This difference was to be expected as the added glycine residues have a lower partial specific volume than the average partial specific volume of the native protein. As was stated earlier,

the buoyant density is affected by two factors, the anhydrous composition and the hydration. It is seen that the composition of the derivative will tend to increase the density. The data given in the last column of Table 3, which does not contain corrections for the bound anions, indicated that the hydration of the derivative is less than that of the native protein.

Table 3. Buoyant densities, net hydration, and standard deviation of native and polyglycyl BSA

Protein	Salt	$\rho_0$ gm/ml	$\Gamma$ (gm H <sub>2</sub> O/gm Protein)	$\sigma$
Native BSA	CsCl	1.290	0.182	0.110 <sub>1</sub>
Polyglycyl BSA	CsCl	1.317	0.155	0.087 <sub>6</sub>
Polyglycyl BSA	RbBr	1.353	0.064	0.088 <sub>4</sub>
Polyglycyl BSA	KBr	1.350	0.073	0.128 <sub>2</sub>
BMA <sup>a</sup>	CsCl	1.282	0.200	0.106
BMA <sup>a</sup>	RbBr	1.310	0.115	0.103
BMA <sup>a</sup>	KBr	1.302	0.138	0.143

<sup>a</sup>from Ifft and Vinograd (33)

The value of the buoyant density of native BSA in cesium chloride of 1.290 gm/ml was higher than that obtained by Ifft and Vinograd for BMA of 1.282 gm/ml. This discrepancy may be due to a slightly different conformation of the two molecules or more likely due to the microheterogeneity of the BSA as compared to the repeated purification steps that are necessary in the preparation of the mercaptalbumin. The mercaptalbumin has exactly one sulfhydryl residue per mole of

protein while BSA has 0.75 sulfhydryl per mole.

The evaluation of the number of anions bound to the protein in the salt solution was necessary for the determination of the hydration of the protein-salt complex. The method used was that of Scatchard and Black (51) in which the shift of the pH was measured between an isoionic protein solution and the identical concentration of protein in the buoyant salt solution. The average increase in negative charge of the protein is related to the number of anions bound by the relation

$$v = \frac{2.303 \Delta \text{pH}}{2 w}$$

where the value of the electrostatic free energy term,  $w$ , was determined from the equation

$$w = 0.1190 \frac{[1 + 0.581 (I)^{1/2}]^2}{[1 + 8.125 (I)^{1/2}]^2}$$

in which  $I = \sum m z^2$ , is the double ionic strength. The value was calculated using the method and assumptions given by the authors. Further in the discussion of this study, it is shown that the most probable spatial arrangement of the added peptides is with them folding back along the protein matrix. For this reason the radius of\* the native protein was used to compute the number of anions bound to the polyglycyl derivative.

Table 4 shows the anion-binding values for the derivative in the three salt solutions used in the buoyant density determinations,

and the native protein in cesium chloride.

Table 4. Anion binding of native and polyglycyl BSA

Protein	Salt	$\Delta\text{pH}$	W	Number of Anions
Native BSA	CsCl	0.730	0.0145	57
Polyglycyl BSA	CsCl	0.763	0.0142	61
Polyglycyl BSA	RbBr	0.853	0.0139	71
Polyglycyl BSA	KBr	0.900	0.0131	79

Using the data obtained as indicated above, it is possible to determine a quantity defined by Vinograd as the net hydration of the protein-salt complex. It is mathematically defined as

$$\rho_0 = \frac{1 + Z_{xy} + \Gamma^*}{\bar{V}_3 + \bar{Z}_{xy} \bar{V}_{xy} + \Gamma^* \bar{V}_1}$$

where  $Z_{xy}$  and  $\bar{V}_{xy}$  are the weight fraction and the partial specific volume of the salt that is bound to the protein.  $Z_{xy}$  was determined with the relation  $Z_{xy} = \frac{\bar{V}_{xy} M}{M \text{ protein}}$ . The partial volume of cesium chloride was taken from a recent paper by Ifft (34) and that of rubidium bromide and potassium bromide from a paper by Muskerjee (45). The value of  $\Gamma^*$ , the hydration of the salt-protein complex, was the actual hydration that existed in the buoyant solution.

Another quantity that compensates for the high salt in the

buoyant solution is the derived buoyant density which is defined by the relation

$$\rho_0^* = \frac{1 + \Gamma^*}{\bar{V}_3 + \Gamma^* \bar{V}_1}$$

and equals that density which would be found if no anions were bound to the protein.

The values tabulated in Table 5 are those determined in this study using the expression shown. The solvated molecular weight was determined from the previously given equation. With the knowledge of the hydration of the protein-salt complex it was possible to compute the anhydrous molecular weight of the protein and thus provide a check on the accuracy of the experimental methods.

$$M_a = \frac{M_s}{1 + Z_{xy} + \Gamma^*}$$

The last column in the table shows that reasonable agreement of the anhydrous molecular weight calculated and the value of 86,100 gm/mole is found. All the calculated values of the anhydrous molecular weight are slightly high which may indicate a systematic error, but it is felt that the precision with which the standard deviation could be determined and the known heterogeneity of the polyglycyl preparation were responsible for this.

Table 5. Measured and calculated net hydration of protein-salt complex, derived buoyant densities, and solvated and anhydrous molecular weights

Protein	Salt	$Z_{xy}$	$M_s \times 10^{-3}$	$\Gamma^*$ gm H <sub>2</sub> O/gm protein	$\rho_0^*$ gm/ml	$M_{\text{anhydr.}}$ $\times 10^{-3}$	$\Gamma^*$ calc. gm H <sub>2</sub> O/gm protein
Native BSA	CsCl	0.111	104	0.49	1.218	67	--
Polyglycyl BSA	CsCl	0.119	135	0.42	1.241	88	0.42
Polyglycyl BSA	RbBr	0.143	138	0.37	1.262	91	0.38
Polyglycyl BSA	KBr	0.109	128	0.33	1.264	89	0.31
BMA <sup>a</sup>	CsCl	0.127	102	0.51	1.21	63	--
BMA <sup>a</sup>	RbBr	0.155	107	0.46	1.22	67	--
BMA <sup>a</sup>	KBr	0.113	103	0.37	1.24	69	--

<sup>a</sup>from Ifft and Vinograd (33)

As is seen in the third column of Table 5 the hydration of the protein-salt complex of the polyglycyl BSA was lower than that of the native protein in all of the salts investigated. In the paper by Ifft and Vinograd (33) a correlation between the hydration of the protein-salt complex and the activity of water in the buoyant solution was made. Figure 13 shows the curve they obtained by plotting the hydration of the protein salt complex against the activity of water. Included in the figure are the three points for the polyglycyl derivative measured in this study. The general relation appears to be valid even though the polyglycyl derivative was less hydrated. More experimental points would be needed if this relation was to be firmly established for the polyglycyl BSA.

The hydration measured in the buoyant density equilibrium and the hydration calculated from the diffusion coefficient were not identical. As was pointed out in the section on diffusion the system containing the protein in solution was considered to be a two component system containing only the protein and water. The dilute salt or buffer solution was ignored since it was distributed uniformly throughout the system. In addition, the protein was considered to be a rigid hydrodynamic particle. It is realized that the surface of the protein is irregular with various indentations and folds and that these may be occupied by solvent. The solvent will travel with the protein as if it were part of the particle. It is this associated solvent



that is considered the hydration of the protein in the diffusion calculation. Scheraga and Mandelkern (52) disagree with this concept that the protein may be considered a rigid particle with a definite hydration, and suggest a new function that is defined using only hydrodynamic measurements. The solution used in the buoyant density equilibrium measurements must be considered to include the salt and thus to be comprised of three components. A simplified diagram of the surface of a protein is shown in Figure 14. In this figure, the salt is shown as having a definite radius and thus a definite distance of closest approach to the protein surface. Water also has a definite radius which in the case of the salts used, is smaller than that of the salts. If the salt and water are replaced by a point source at their center of mass, two regions are created on the protein surface. It is seen that the water may approach closer to the protein than the salt. This creates a situation of preferential solvation of the type measured in the buoyant density experiments. This phenomenon is evident when Figure 13 of this study and Figure 4 of reference 28 is reexamined considering the size of the anion as compared to the size of the water. The relation between the activity of water and net hydration is valid but the anion size will also affect the system. Assuming that this was the type of hydration measured in the buoyant density experiments, a calculation of the change of the surface to volume ratio in the derivative should indicate the spatial arrangement of the added glycol chains.

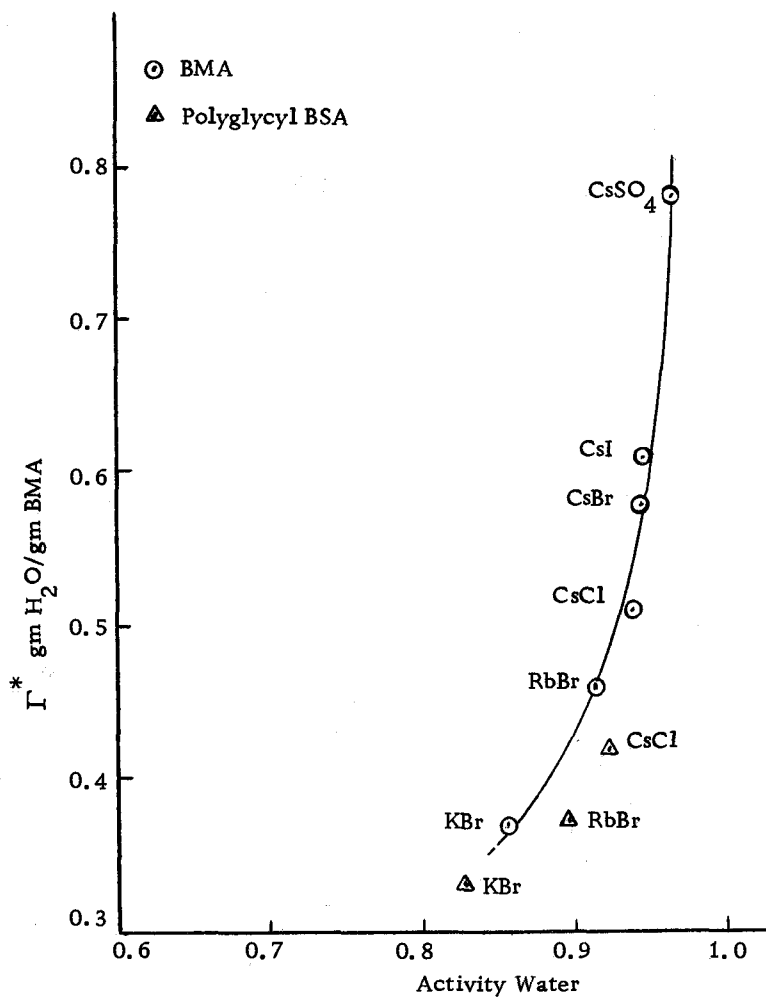


Figure 13. Net hydration of bovine mercaptalbumin\* and polyglycyl BSA.

\* from Ifft and Vinograd (33)

If the added glycyll chains were fully extended into the solvent, a large increase in surface area as compared to volume would occur. Representing the added chains by a cylinder, an admittedly crude analogy, the calculated change in surface to volume ratio was 1:1.5 for this model. This means there should be a proportional increase in hydration. If the glycyll chains were folded back along the protein surface, still considering them as cylinders, the surface area gained by adding the peptides is the difference between a hemicylinder and a plane surface. The surface to volume ratio in this model was 1:0.83, a remarkably different value from that given above. The hydration of the polyglycyll derivative in this case should be 0.83 that of the native protein. As is seen in Table 5 there is excellent correlation between the change in surface to volume ratio and the measured hydration indicating that the added glycine residues fold back along the protein surface.

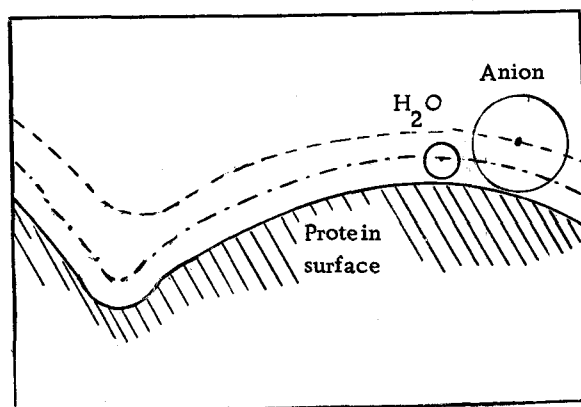


Figure 14. Differential solvation of protein surface

The fact that polyglycyl BSA was soluble is unusual considering the solubility of glycyll peptides in water. The reason for the insolubility of the glycine homopolymers does not reflect their non-polarity, but rather the converse, their high potential to hydrogen bond and form large aggregates. The ability of the carboxyl oxygen and the amide nitrogen of the peptide bond to form hydrogen bonds was one of the requirements given in the Pauling, Corey, Branson theory for the formation of the  $\alpha$ -helix. Polyglycine has no side chains attached to the  $\alpha$ -carbon which would sterically hinder the formation of a very closely packed structure. Such a structure has been described by Crick and Rich (13). This structure, polyglycine II, is only possible for polyglycine peptides since in this structure there is no room for side chains larger than a hydrogen atom. It is very similar to the structure of collagen which consists of multiple peptide helices, and therefore would exhibit similar solubility characteristics. It was shown earlier that the surface of BSA is large enough to minimize the probability of the added peptide chains overlapping. Therefore, they could not form the above structure, but they still retain the ability to hydrogen-bond. The added glycyll chains in the modified protein, having no steric restrictions, can form hydrogen bonds with sites on the surface of the protein thus giving the derivative additional stability.

BSA has been shown by Foster (14) and Markus (41) to have ten

strongly hydrophobic sites on the protein surface and approximately 80 weaker ones. These sites were measured by the binding of fatty acids or anionic detergents. If the added glycyl chains were covering part of the hydrophobic areas, the solubility and stability of the derivative would be increased. Stabilization would be due to the entropy gained by water when the hydrophobic regions are shielded by the glycyl chains.

An increase in hydrogen bonding as well as an apparent decrease in hydrophobic area would be expected if the added glycine chains do fold back along the protein surface. This would result in added stability of the derivative as discussed above.

## SUMMARY AND CONCLUSIONS

A heat stable derivative of bovine serum albumin containing 300 residues of glycine added to 30 sites on the surface of the protein has been prepared. This derivative may be heated to 100° C for prolonged periods of time without aggregation. By comparison, native BSA aggregates at 62° C under similar conditions. The spatial arrangement of the added glycine peptides was thought to impart the observed stabilization to the modified protein. The experimental evidence greatly favors an arrangement in which the added glycine chains are folded back along the protein surface rather than fully extended into the surrounding solvent.

Using trinitrobenzene sulfonic acid, it was determined that there were thirty reactive amino groups on the surface of BSA. It is most likely that these sites were reacted with the N-carboxy glycine anhydride.

The conformation characteristic of native BSA appears to be maintained in the derivative as indicated by various experimental measurements. The change in rotation associated with the acid expansion of BSA is paralleled by a similar but smaller change in the derivative. The specific rotation of the derivative is several degrees lower than would be expected if it had the identical molar rotation as the native protein. The ultraviolet difference spectra

observed by perturbing the native and derivative protein by pH and heat indicated that the main matrix of each protein was undergoing similar transitions. In all cases observed the derivative showed less change in optical density than did the native protein.

The hydrodynamic measurements also substantiate the hypothesis that the structure of the protein core of the derivative was retained. The derivative undergoes the same reversible acid expansion as the native protein. By approximations that may be made from the diffusion coefficient, it was shown that the degree of hydration of the derivative could have a large variation depending upon the spatial arrangement of the added peptides. If the added peptides were folded, the hydration would be less than that of the native and if they were extended, the hydration would be greater than the native protein. The hydration was evaluated by the determination of the buoyant density of polyglycyl BSA in a series of salt solutions. The results from these experiments indicated that the derivative was less solvated than the native protein in all salt solutions examined. An increase of the activity of water was accompanied by an increase in the hydration of the protein in the limited number of salt solutions that were studied. This confirms the relation between the activity of water and the protein hydration presented by Ifft and Vinograd (33). The decrease in hydration found in the derivative may be adequately explained by the change in the surface to volume ratio that would be expected if the

added peptides were folded back along the protein surface.

Considering the experimental evidence that has been presented it is felt that the added polyglycyl chains are folded back along the surface of the protein. When they are in this configuration they form hydrogen bonds with groups on the surface of the protein and would cover some of the hydrophobic regions on the surface of the protein. Both of these effects would cause the derivative to be more stable than the native protein.



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APPENDIX



## APPENDIX

## Sample calculation of Buoyant Density Equilibrium Data

measured distances

cell bottom	$r_b$	7.226	initial density $\rho_e = 1.3204$
equilibrium position	$r_0$	6.625	
cell top	$r_a$	6.037	Temperature $25.0^\circ \text{C}$

Isoconcentration distance  $r_e$ 

$$r_e = \left[ \frac{r_a^2 + r_b^2}{2} \right]^{1/2} = 6.658$$

Buoyant density gradient

$$\begin{aligned} \frac{d\rho}{dr}_{\text{buoy}} &= \left[ \frac{1}{\beta_0} + \frac{k - k_s}{1 - a} \rho_0^2 \right] \omega^2 r_0 \\ &= \left[ \frac{1}{1.495 \times 10^9} + \frac{(35.1 - 9.1) \times 10^{10}}{(1 - 0.21)} (1.317)^2 \right] 2.308 \times 10^8 \\ \frac{d\rho}{dr}_{\text{buoy}} &= 0.167 \end{aligned}$$

Effective density gradient

$$\frac{d\rho}{dr}_{\text{eff}} = (1 - a) \frac{d\rho}{dr}_{\text{buoy}} = 0.132$$

Buoyant density protein

$$\rho_0 = \rho_e + \Delta r \frac{d\rho}{dr}_{\text{eff}} = 1.3204 + (0.132) (6.625 - 6.658)$$

$$\rho_0 = 1.317$$

Standard Deviation of Concentration Distribution

K( $\eta$ )	K( $\eta_0$ )	K( $\eta - \eta_0$ )	$\Sigma K(\eta - \eta_0)$	( $r - r_0$ ) <sup>2</sup>
1.390	1.390	0.000	0.000	
1.417	1.410	2.007	0.007	0.0006
1.464	1.427	0.037	0.044	0.0023
1.526	1.442	0.084	0.128	0.0052
1.590	1.456	0.134	0.262	0.0092
1.055	1.469	0.186	0.448	0.0144
1.730	1.484	0.246	0.694	0.0207
1.797	1.498	0.299	0.993	0.0282
1.842	1.514	0.328	1.321	0.0369
1.865	1.529	0.336	1.657	0.0467
1.862	1.545	0.317	1.974	0.0576
1.784	1.660	0.224	2.198	0.0697
1.709	1.574	0.135	2.333	0.0829
1.590	1.590	0.000	( $r_0$ )	
1.458	1.605	0.147	2.292	
1.347	1.618	0.271	2.145	
1.290	1.636	0.346	1.874	
1.285	1.650	0.363	1.528	
1.338	1.666	0.325	1.165	
1.410	1.682	0.272	0.837	
1.500	1.698	0.198	0.565	
1.583	1.714	0.131	0.367	
1.633	1.731	0.098	0.236	
1.675	1.746	0.071	0.138	
1.711	1.760	0.049	0.067	
1.790	1.790	0.000	0.000	

$\eta$  = refractive index of protein solution

$\eta_0$  = refractive index of reference solution

by determination of the slope of a  $\ln K (\eta - \eta_0)$  versus  $(r - r_0)^2$  plot  
in figure  $\sigma^2$  is determined

$$\text{slope} = -\frac{1}{2\sigma^2} = -0.0156$$

$$\sigma^2 = 0.0078$$

### Solvated Molecular Weight

$$M_S = \frac{RT \rho_0}{\omega^2 \frac{d\rho}{dr}_{\text{eff}} r_0 \sigma^2}$$

$$= \frac{(8.314 \times 10^7) (298) (1.317)}{(3.451 \times 10^7) (0.132) (6.625) (0.0078)} = 135.5 \times 10^3$$

### Hydration Protein

$$\rho_0 = \frac{1 + \Gamma}{\bar{V}_s + \Gamma \bar{V}_1} = 1.317 = \frac{1 + \Gamma}{0.722 + \Gamma \bar{V}}$$

$$\Gamma = 0.155$$

### Weight Fraction of Salt Bound to Protein

number of Anions bound  $V = 61$

$$Z_{xy} = \frac{V M_{xy}}{M_{\text{protein}}} = \frac{(61) (168.4)}{86,100} = 0.119$$

$$\bar{V}_{\text{CsCl}} \rho = 1.317 = 0.263$$

Hydration-Protein-Salt Complex

$$\rho_0 = \frac{1 + Z_{xy} + \Gamma^*}{V_3 + Z_{xy} \bar{V}_{xy} + \Gamma^* \bar{V}_1} = \frac{1 + 0.119 + \Gamma^*}{0.722 + (0.119)(0.263) + \Gamma^*}$$

$$\Gamma^* = 0.419$$

Anhydrous Molecular Weight

$$M_{\text{anhy.}} = \frac{M_S}{1 + Z_{xy} + \Gamma^*} = \frac{135.5 \times 10^3}{1 + 0.419 + 0.419} = 88 \times 10^3$$