AN ABSTRACT OF THE THESIS OF

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(Name) (Degree) (Major)

Date thesis is presented October 31, 1966

Title THE UREA DENATURATION OF BOVINE SERUM ALBUMIN, POLYGLYCYL BOVINE SERUM ALBUMIN AND POLY-L-PHENYLALANYL BOVINE SERUM ALBUMIN

Abstract approved Redacted for Privacy
(Major professor)

A comparative study was made on the effect of urea denaturation of bovine serum albumin (BSA), polyglycyl BSA and poly-L-phenylalanyl BSA. The denaturation process was followed by ultraviolet spectrophotometry and optical rotation.

A blue shift in the absorption maximum at 278 mμ was observed in urea denatured proteins. In 7M urea solution, the peak of the native BSA shifted from 278.4 mμ to 276.7 mμ. However, for urea denatured poly-L-phenylalanyl BSA, the extent of the shift was small, from 278.5 mμ to 278 mμ. Polyglycyl BSA had the same spectrum as native BSA, and the extent of the shift in 7M urea was about 1.5 mμ.

The difference spectra between native proteins and urea denatured proteins were investigated as the functions of molarities of urea from 1 M to 7 M. On exposure of native BSA, polyglycyl BSA

and poly-L-phenylalanyl BSA to urea concentrations above 2-3M at pH 7.6, an immediate blue shift in the absorption maximum at 278.5 mμ occurred. The extent of this shift was dependent on the urea concentration. A minimum in molar absorptivity difference ($\Delta\epsilon$) was also observed at 287 mμ. There was a strong dependency of $\Delta\epsilon_{287}$ on urea concentration for native BSA, polyglycyl BSA and poly-L-phenylalanyl BSA. No significant difference on the effect of urea concentration on $\Delta\epsilon_{287}$ was found between polyglycyl BSA and native BSA. However, poly-L-phenylalanyl BSA showed more stability toward urea denaturation.

The increased levorotation of the urea denatured proteins was dependent on the molarities of urea. At pH 7.6 and 7M urea, the specific rotation $[\alpha]_D$ of native BSA changed from -61° to -104°, polyglycyl BSA from -58° to -98°, and poly-L-phenylalanyl BSA from -57° to -76°. In pH 5.5, the effect of urea on $[\alpha]_D$ of polyglycyl BSA resembled native BSA. The $[\alpha]_D$ of polyglycyl BSA and native BSA at pH 5.5 changed from -60° to about -105° in 7M urea.

The stability of poly-L-phenylalanyl BSA toward urea denaturation was interpreted in terms of the protective effect of sodium dodecyl sulfate against urea denaturation of serum albumin.

THE UREA DENATURATION OF BOVINE SERUM ALBUMIN, POLYGLYCYL BOVINE SERUM ALBUMIN AND POLY-L-PHENYLALANYL BOVINE SERUM ALBUMIN

by

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A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1967

APPROVED:

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Date thesis is presented October 31, 1966

Typed by Donna Olson

ACKNOWLEDGEMENTS

I am deeply grateful to Professor Robert R. Becker whose innumerable suggestions were of invaluable aid to me during the course of my research. Above all, his understanding in human element encouraged me throughout the course of this work.

I would like to express my gratitude to Ward Harris for the supply of N-carboxy-glycine anhydride. I also wish to thank Jackson Lynn and Lily Wu for their help and inspiring discussions.

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I. INTRODUCTION

Chemical Modification of Proteins

The chemical modification of proteins has long been of interest to protein chemists as one of the approaches to the study of protein structure. Attempts have been made to modify proteins with chemical compounds which would attach to specific groups in the protein. By comparing the physical and biological properties of native and modified proteins, it is hoped to relate protein structure with its biological activities and functions.

Stahmann and Becker (1952) first succeeded in the preparation of polypeptidyl proteins using mild conditions (aqueous media, low temperature, and almost neutral pH) by reacting proteins with N-carboxyamino acid anhydrides. This method of preparation of modified proteins has several advantages:

(1) No foreign groups are introduced to the native protein;

(2) The reactions are specific for one group in the proteins; and

(3) The proteins remain native in this treatment.

The physical properties of polypeptidyl proteins have been subjected to extensive investigation. Results of electrophoresis,

sedimentation and counter current experiments showed that polypeptidyl proteins were reasonably homogeneous (Becker and Stahmann, 1953; Becker, 1962). Further study of electrophoresis on various polypeptidyl rabbit albumins and bovine albumins revealed changes in their mobilities (Tsuyuki, Van Kley and Stahmann, 1956; Van Kley and Stahmann, 1956; Van Kley and Stahmann, 1959). The sedimentaion and diffusion coefficients of polytyrosyl trypsin varied only slightly from those of unmodified trypsin (Glazer, Bar-Eli and Katchalski, 1962). The pH titration of polypeptidyl bovine and rabbit albumin has been systematically studied by Van Kley, Kornguth and Stahmann (1959). In all cases an increase in the number of α-amino groups was observed and a corresponding decrease in ϵ-amino groups indicating that some of the ϵ-amino groups were acylated.

The optical rotation and ultraviolet spectrum of bovine albumin modified by the addition of polypeptides of several amino acids were studied. Optical rotation showed that there was no significant change in the basic structure of the protein after the modification reaction. Ultraviolet absorptivities provided evidence for interactions when polypeptides of lysine were added to the protein; the absorption peaks due to phenylalanine residues were made more apparent in the polyphenylalanyl preparations (Van Kley and Stahmann, 1959).

Protein Denaturation[1]

Proteins as they are found in nature are called "native proteins". Any change in a protein which converts a protein to a state other than its native state is called denaturation. In general, denaturation reflects changes in the secondary and tertiary structures of proteins. There are various kinds of internal bonds that might contribute to the secondary and tertiary structure of a protein. These forces contributing to the overall conformation of a protein are described as following:

(1) Hydrogen bonds between peptide linkage

(2) Hydrophobic bonds between the nonpolar portions of the amino acid side chains

(3) Salt linkages of oppositely charged groups

(4) Hydrogen bonds other than those between peptide linkages, i. e., between phenol groups and carbonyl groups

(5) Covalent bonds such as the disulfide bridge between half cystine residues, or the phosphodiester links

[1] There is no precise definition of protein denaturation. Some workers prefer to define denaturation in terms of a change in a particular experimental property. In the experiments to be described, the term denaturation is used in terms of changes in the ultraviolet spectra and optical rotation.

(6) Dispersion forces

(7) Electron delocalization across the hydrogen bonds that join the peptide groups.

Among the above seven different types of intramolecular bonds, it is likely that hydrogen bonds and hydrophobic bonds are more important in determining the overall conformation of the protein molecule. Any severe change of environment (i. e., temperature, pressure, pH, presence of urea and guanidine, oxidation and reduction, enzymatic action) would disturb the equilibrium of the intramolecular forces in the protein. As a consequence, the conformation of the protein would be changed. For more information of protein denaturation, the reader is referred to the article by Kauzmann (1959).

Bovine Serum Albumin (BSA)[2]

Bovine serum albumin (BSA) has long received attention from biochemists since it is most abundant in a readily available protein system. By means of fractionation and crystallization techniques, BSA can be prepared easily in a sizable quantity. Purified by an ion-exchange procedure, BSA is essentially free of non-protein contaminants such as carbohydrates and heavy metal ions. However,

[2]Plasma albumin or serum albumin are used interchangeably by most workers in the protein field.

traces of fatty acids are still present in the BSA.

The amino acid composition of BSA is usually determined by ion-exchange chromatography pioneered by Stein and Moore (1949). The N-terminal and C-terminal groups of BSA are aspartic acid and alanine, respectively. The molecular weight is about 69, 000 with an uncertainty of $\pm$ 2000 because of some aggregation in the system. As for the structure of the protein, it is generally agreed that BSA has only one single polypeptide chain. This conclusion has been based on the following reasons (Phelps and Putnam, 1960):

(1) One N-terminal aspartic acid per mole of protein by both the fluorodinitrobenzene and the thiohydantoin methods.

(2) One cysteine residue per albumin molecule.

(3) No decrease in molecular weight after complete reduction of disulfide bonds followed by alkylation.

(4) One C-terminal alanine was detected by the hydrazinolysis method.

The characteristics of BSA in low pH have been subjected to much investigation. By the methods of optical rotation, intrinsic viscosity, sedimentation and light scattering it can be concluded that the BSA molecule, at low pH, undergoes expansion which is rapid and essentially reversible. This expansion is largely electrostatic in nature, and takes place after the formation of an

intermediate "expandable" form. The electrophoretic heterogeneity of this protein at pH 3.5 to 4.5 reveals that BSA exists in two forms, N and F, where the N form is the normal form existing above pH 5, and the F form is the "fast" form, which moved ahead of N in these experiments. These observations can be fitted quantitatively by assuming an equilibrium of the two forms:

$$N + 3H^{+} \rightleftharpoons F$$

The exact size and shape of BSA is not known. However, from the anomaly of the pH titration curve and electrophoretic data, it has been suggested that the protein consists of four similar subunits which can undergo isomerization independently of one another (Foster, 1960). Recent studies by Weber and Young (1964a, 1964b) have confirmed the proposal that bovine serum albumin consists of three to four fragments which are linked together by the continuity of the peptide chain.

Application of Ultraviolet Spectra in Proteins

Ultraviolet spectra of proteins have been widely used to give information about the composition and the conformation of proteins. As a first approximation, the ultraviolet spectrum of a protein is the sum of the spectra of the groups contained in it. This assumption of additivity is basic for useful analytical applications of

spectral measurements. One example is the estimation of the concentration of tyrosyl and tryptophyl residues in a protein from the determinations of absorbance and protein concentration, employing tryptophyl and tyrosyl residue extinction coefficients (Beaven and Holiday, 1952). Another example is the determination of protein concentration from a measurement of the absorbance in the peptide absorption band, employing a mean peptide extinction coefficient.

However, as more and more careful spectra were obtained and as better data for the amino acid composition of proteins became available, discrepancies in the intensity of the aromatic peaks of the order of 20% became increasingly common, and it was noted as well that the wavelengths of maximum absorption were often shifted from the values assigned to them in the amino acids and simple peptides. Furthermore, a pronounced splitting of the spectrum was observed in the case of helical peptide bond (Schellman and Schellman, 1964). This alteration of spectral properties affords the possibility of obtaining conformational information about proteins.

The earliest investigation of spectral shifts was by Crammer and Neuberger (1943) who noticed an abnormally high pK for several tyrosine residues of ovalbumin by performing spectrophotometric titrations and suggested that these residues were not free to titrate because of the formation of a hydrogen bond with carboxylate groups. In addition to hydrogen bond formation, a hydrophobic environment,

proximity of charges, and the perturbing effect of the polypeptide chain have been invoked as mechanisms for spectral shifts in proteins.

Extensive studies of ultraviolet spectra of polyglutamic acid and polylysine showed that peptide bond exhibited an absorption peak at 190 mμ. Upon further investigation, it was found that the peptide spectrum was conformation-dependent, and that the spectrum of helical polypeptides showed evidence of band splitting which could not be found in the conformation of random coil and β-form. Rosenheck and Doty (1961) have employed protein absorptivity values in this strong peptide absorption band (λ_{max}=190 mμ) to estimate the α-helical content of proteins. Their results for several proteins were in good to fair agreement in comparison with the estimations from optical rotatory dispersion studies (Yang and Doty, 1957). Detailed discussions can be found in the reviews of Scheraga (1961) and Wetlaufer (1962).

Optical Rotation and Protein Conformation

Optical rotation and chain conformation in synthetic polypeptides and proteins have been studied extensively. Synthetic polypeptides are used as models for proteins because both classes of molecules share the same backbone of peptide bonds whose spatial disposition chiefly governs their rotatory power and at the same time

defines their secondary structures. Changes in the mutual orientation of peptide groups in the polymeric backbone are thus tantamount to the conformational changes, so that the special sensitivity of optical rotatory power to steric form renders it uniquely suited to the study of conformation and conformational changes of polypeptides and proteins.

For the study of helix-coil transition in synthetic polypeptides, optical rotation has been widely used. Nearly all transitions, whether accomplished by changes in solvent polarity, pH, or temperature, are centered about a distinct critical point and exhibit a relatively sharp sigmoid shape. In some cases of proteins, e.g. rabbit tropomyosin, ovalbumin and silk fibroin, the transitions brought about in nonaqueous solution by continuous variation of polar and nonpolar components in the solvent are sufficiently sharp and sigmoidal in shape. However, in other proteins, insulin, ribonuclease, and bovine serum albumin, the transition displayed in similar solvent mixtures is broad and is not sharpened appreciably by the breakage of restraining disulfide bonds. Broad transitions of this type could result either from relatively short helical segments or from helical regions with widely varying stabilities brought about by compositional heterogeneity.

Parallel to the helix-coil transition in nonaqueous solvent mixtures, globular proteins also show structural changes in aqueous

media when induced by pH, urea concentration, or temperature. Thus, protein denaturation can be followed also by changes in specific rotation which becomes more levorotary in the visible spectrum as the native structure is unfolded. As the disruption of a standard polypeptide helix brought about by the same agencies that denature proteins produces increased levorotation, these changes in specific rotation that accompanies the unfolding of a globular protein is compatible with its possession of helical regions in the native state (Urnes and Doty, 1961).

Thesis Statement

It was of interest to determine the effect of added peptide chains to the surface of a protein on the stability of the native structure. Glycyl side chains would be expected not to be involved in interactions with the peptide backbone of the molecule. On the other hand, a nonpolar side chain might interact sufficiently to provide stabilization toward denaturation.

Thus, the object of this research is to make a comparative study on the effect of urea denaturation of bovine serum albumin (BSA), polyglycyl BSA and poly-L-phenylalanyl BSA. The denaturation process will be followed by ultraviolet spectrophotometry and optical rotation.

II. MATERIAL

Protein and Amino Acids

Bovine serum albumin (BSA), from Pentex Incorporated, Kankakee, Illinois, Lot no. 11, Code No. BV0162, was used without purification.

L-phenylalanine, A grade, chromatographically homogeneous, was purchased from Calbiochem. $[\alpha]_D = -33.4^{\circ}$ (c = 2, water); percent nitrogen: 8.56%.

Glycine, ammonia-free, was purchased from Matheson Coleman & Bell.

Solvents

Ethyl acetate, was obtained from Fisher Scientific Company, as certified reagent. It was stored over anhydrous calcium chloride for at least two weeks and was distilled just before use. B. P. 77°C.

Petroleum ether, B&A reagent grade, B. P. 30° - 60° C, was stored over sodium ribbon for over a week and then distilled before use.

Ethyl ether, anhydrous, B&A reagent grade, was stored over calcium chloride and distilled just before use.

Tetrahydrofuran (THF), from Matheson Coleman & Bell, had

been refluxed with lithium aluminum hydride for two hours, and then distilled before use. The boiling point of the distillate was 65.5 - 66° C.

Dioxane, reagent grade, was obtained from Matheson Coleman & Bell. It was refluxed with potassium hydroxide for an hour and distilled from sodium.

Sodium methylate, was purchased from J. T. Baker Chemical Co., 2.16 ml of 25% solution in methanol and dilute to 100 ml. The normality was found to be 0.097N.

Thymol blue solution, was prepared by dissolving 1 gram of bromothymol blue in 100 ml of absolute methanol.

Organic Chemicals

Sodium phosphate (monobasic), reagent grade crystals, was from Matheson Coleman & Bell.

Sodium phosphate (dibasic), anhydrous, reagent grade.

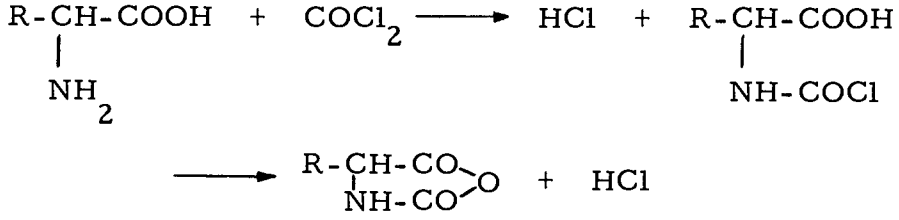
Urea, reagent grade, was obtained from Sigma Co. (pfs). Newly opened bottles were used without further purification.

III. EXPERIMENTAL

Preparation of N-Carboxy-α-Amino Acid Anhydrides (NCA)

General Remarks

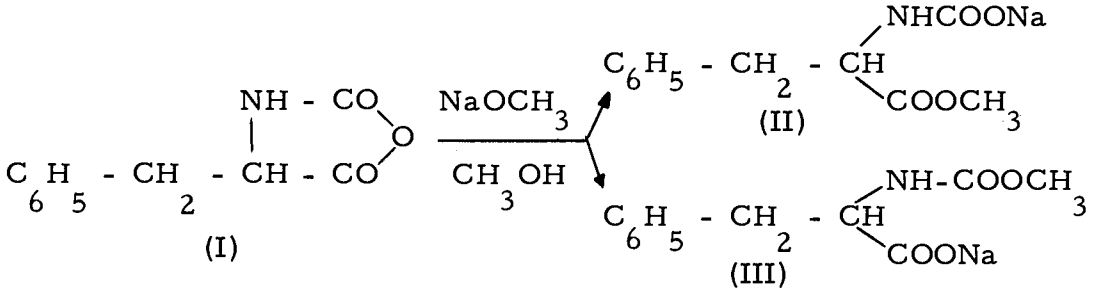
Whenever possible, the Fuchs-Farthing method for preparing N-carboxy-α-amino acid anhydride is preferred (Katchalski and Sela, 1958). An amino acid suspended in tetrahydrofuran is allowed to react with phosgene. The reaction proceeds according to the following equation:



However, for the synthesis of NCA's of trifunctional amino acids, it is necessary to protect the functional groups of the side chains prior to the reaction with the phosgene.

Since NCA's react readily with water, care should be taken to protect them from moisture. The crystallization should be performed in inert and dried solvents which are free of any traces of acid and base. Moreover, NCA's tend to polymerize at elevated temperature, even in the absence of initiator, and heating above 60° C should be avoided.

Two recrystallizations of NCA using ethyl acetate have proven to be satisfactory. The purity of the NCA's can be estimated by sharp melting points or by a titration method (Berger, Sela and Katchalski, 1953). In the latter method, NCA dissolved in dioxane is titrated with 0·1N sodium methoxide using thymol blue as the indicator. The color of the indicator changes sharply from yellow to deep blue upon addition of an equimolar quantity of the titrant. For example, N-carboxyphenylalanine anhydride (I) may react with sodium methylate and methanol to give either the sodium salt of N-carboxyphenylalanine methyl ester (II), or the sodium salt of N-carbomethoxy phenylalanine (III). The reaction goes as the following equation:



Preparation of L-Phenylalanine NCA

L-phenylalanine (4 gm, 0.024 moles), dried over P_2O_5 in vacuo at 100° C for three days was suspended in 100 ml of tetrahydrofuran. Phosgene was first passed through concentrated sulfuric acid to remove water and impurities, and then into the amino

acid suspension stirred with a teflon-coated magnetic stirrer for 3 hours at room temperature. The solution was clear and a straw-color. Air was passed through the solution for one hour to remove excess phosgene. The solvent was evaporated at room temperature until a yellowish oil resulted. On further evaporation yellowish white crystals formed and caked together.

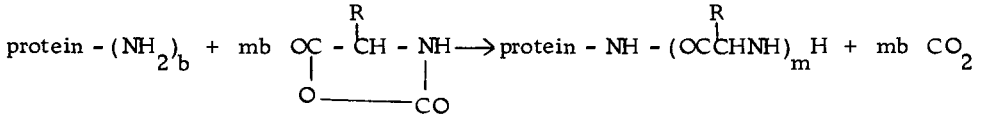
To the yellowish white crystal was added 8 ml ethyl ether followed by 12 ml ethyl acetate. The crystals gradually dissolved. After adding 40 ml petroleum ether, the solution became turbid. Upon cooling the solution in acetone-dry ice bath and scratching, white crystals were observed. Then another portion of 80 ml petroleum ether was added to the crystal-solvent mixture to cause further precipitation. The crystals were then filtered off and washed with petroleum ether. Recrystallization of the anhydride was found satisfactory.

The yield of L-phenylalanine NCA by this method was 89%. The melting point was 90 - 91° C [Literature, 91 - 92°C (Lynn, 1963); 95 - 96°C (Sela and Berger, 1955)]. The purity of the NCA was determined by titration (Berger, Sela and Katchalski, 1953), and was found to be 100%.

Modification of BSA with NCA's

General Remarks

The reaction of N-carboxyamino acid anhydrides (NCA) with proteins in aqueous buffer solution was reported by Stahmann and Becker (1952; Becker and Stahmann, 1953). The α - and ε - amino groups of the protein were found to initiate the polymerization leading to a polypeptidyl protein according to the following reaction:



The polymerization proceeds under mild conditions (aqueous media, low temperature, and almost neutral pH), in which most proteins remain native.

Modification of BSA with L-Phenylalanine NCA

0.5 gm of L-phenylalanine NCA was added to 100 ml 1.0 percent solution of BSA (molar ratio of NCA and protein 150:1) in 0.067M phosphate buffer (pH 7.6) at 4°C. The solution was stirred with a teflon coated magnetic stirrer for 24 hours in the cold room. The reaction mixture was then centrifuged at 22,000 x g to remove insoluble material. The supernatant was dialyzed in cold glass-distilled water for three days, and again was centrifuged for one hour at

22,000 x g. The supernatant was stored in the refrigerator. The concentration of poly-L-phenylalanyl BSA was determined by ultraviolet absorption at 280 mμ, using $A^{1\%}_{1cm} = 6.6$. The concentrations of different batches of poly-L-phenylalanyl BSA were in the range of 0.7 - 0.8 gm/100 ml in terms of native BSA.

Modification of BSA with Glycine NCA

The modification of BSA with glycine NCA was essentially the same as modification with L-phenylalanine NCA. The only difference was that 0.5 gm of glycine NCA was added to 50 ml of a 2.0 percent solution of BSA (molar ratio of NCA and protein 460:1) in 0.067M phosphate buffer (pH 7.3). The polyglycyl BSA was then lyophilized and stored in the refrigerator.

Amino Acid Analysis and End Group Studies

Amino Acid Analysis

The method of amino acid analysis was essentially that described by Spackman, Stein and Moore (1958) for the quantitative determination of amino acid mixture where chromatographic columns of cross linked sulphonated polystyrene resins are used to separate the individual residues. Approximately 5 mg of protein was hydrolyzed. The hydrolysis was performed in sealed glass tubes from

which the air had been removed and replaced by nitrogen. In the tube was the dry protein in which 1 ml of 5.7 N HCl (constant boiling) was added. The tube was sealed under vacuum and placed in a bath of boiling toluene (110.6°C). The hydrolysis continued for 24 hours. HCl was then flash evaporated, and 5 ml of sample diluting buffer at pH 2.2 was added to the hydrolyzate. One ml of the hydrolyzate was added to the chromatographic column of the Spinco Automatic Amino Acid Analyzer Model 120B.

End Group Studies

These were performed using Sanger's method (Sanger, 1945). The modified protein and an equal weight of $NaHCO_3$ were dissolved in 10 times the weight of water. To this solution was added twice the volume of 5% (V/V) fluorodinitrobenzene (FDNB). The test tube was covered with aluminum foil since dinitrophenyl (DNP) derivatives are light sensitive. The solution was stirred with a teflon-coated magnetic stirrer for six hours. A yellow precipitate (insoluble DNP-protein) formed and was separated by centrifugation. The DNP-protein was washed three times with ether, and air dried overnight. About 5 mg was taken for acid hydrolysis at 110°C for 24 hours. The hydrolyzate was quantitatively analyzed on the Amino Acid Analyzer.

Spectrophotometric Studies of Native BSA and Poly-α-Amino Acid BSA's

Ultraviolet Spectra of Poly-L-Phenylalanine and Free L-Phenylanine

Since polyphenylalanine is practically insoluble in common solvents, it is impossible to obtain directly the ultraviolet spectrum of this polymer. In this experiment, it is assumed that the difference spectrum of poly-L-phenylalanyl BSA and native BSA should be equivalent to the spectrum of poly-L-phenylalanine. As for the spectrum of free L-phenylalanine, it was obtained as the difference spectrum of free L-phenylalanine + native BSA[3] and native BSA.

The ultraviolet difference spectra were obtained with a Cary model 11 recording spectrophotometer with matched 1.0 cm rectangular cells. The poly-L-phenylalanyl BSA, prepared as mentioned above, was diluted ten fold, and compared with native BSA which was dissolved first in distilled water. The final concentration of poly-L-phenylalanyl BSA was 0.079 gm/ 100 ml in terms of native BSA. Free L-phenylalanine was added to the native BSA solution and compared also with native BSA. The concentration of native BSA (0.079 gm/100 ml) in the reference cell was the same concentration as the BSA in poly-L-phenylalanyl BSA and free

[3]L-phenylalanine + native BSA means the mixture of L-phenylalanine and native BSA.

L-phenylalanine + BSA solutions. The free L-phenylalanine (0.019 gm/100 ml) added to the native BSA solution was the same concentration as the L-phenylalanine attached to the poly-L-phenylalanyl BSA. The pH of the solutions was adjusted with 0.1 N NaOH to pH 7. The molar absorptivities (ϵ) were calculated from the absorption spectra and are reported later.

Ultraviolet Spectra of Urea Denatured Native BSA and Poly-α-Amino Acid BSA's

The ultraviolet spectra of native BSA, polyglycyl BSA and poly-L-phenylalanyl BSA in the presence of urea were obtained using Cary model 11 recording spectrophotometer. The effect of urea on these proteins can be observed from:

1. the spectra of the proteins; and
2. the difference spectra of the proteins.

In both cases, the experimental procedures were essentially the same except that different cells were used.

The stock protein solutions were prepared by dissolving native BSA and polyglycyl BSA in 0.067 M phosphate buffer, pH 7.6 and ionic strength 0.16. In the case of poly-L-phenylalanyl BSA, the protein was dialyzed against the phosphate buffer solution. The concentration of the protein was determined by light absorption at 280 mμ with $A^{1\%}_{1\,cm} = 6.60$ (Glazer, McKenzie and Wake, 1963). The stock

urea buffer (10 M) solutions were always freshly prepared to avoid the complication of cyanate formation (Stark, Stein and Moore, 1960). Glass distilled water was used throughout the experiment.

For preparing the protein-urea solution, 0.5 ml of stock protein solution was pipetted into a 5 ml volumetric flask (in the case of poly-L-phenylalanyl BSA, 1 ml of the stock protein solution was used). Then, to this protein solution, the urea solution of required molarity was added. After gently stirring with a small glass rod with a flattened tip, the protein-urea solution was transferred to the cells. The measurements were taken within 10 to 20 minutes after the mixing. The experiment was performed at room temperature, $24^{o} \pm 1^{o}C$.

Spectra. The spectra of the proteins and urea treated proteins were obtained by using 1.0 cm rectangular cells. In all cases, 0.067M phosphate buffer was used as reference. The ultraviolet spectra of the proteins were recorded in the range 240-320 mμ.

Difference Spectra. All difference spectra measurements were carried out by employing pairs of matched cylindrical tandem double cells designed to subtract the solvent contribution to the difference spectrum directly in a single operation (Herskovits and Laskowski, 1962). As shown in Fig. 1, each cell has two compartments, one for the protein solution and one for the solvent blank,

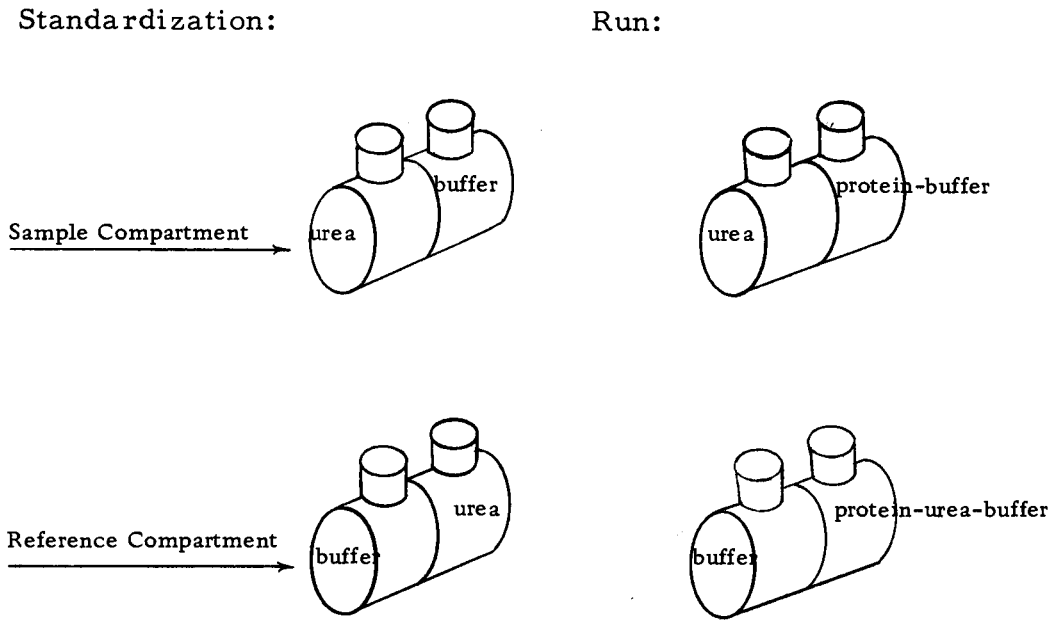


Figure 1. Double cells employed in difference spectra measurements. The path length for each of the two compartments is 1.0 cm.

with 1.0 cm separation between the silica windows.

In order to obtain a negative value of absorbance on the chart, the protein-urea-buffer solution was placed in the reference compartment of the instrument, and the protein-buffer solution was placed in the sample compartment. To compensate properly for the urea and solvent contribution to the difference spectrum, the required molar urea solution was placed in the front half of the double cell containing protein-buffer solution; buffer solution without protein was placed in the front half of the other cell, and the other half contained the protein-urea-buffer solution (Fig. 1). The two cells were positioned in the instrument with the front parts of the cells containing the blanks toward the source of radiation, so that the beam entered the solvent-containing sections of the two cells first.

Before each set of measurements, the spectrophotometer was compensated to give a satisfactorily straight base-line or "zero line". This was done with the cells in position, with both sample and protein compartments filled with urea and buffer solutions (Fig. 1). Then the front halves of the two cells were tightly stoppered, and the others were emptied, rinsed with distilled water, then with 95% ethanol, and dried with air.

Optical Rotation of Urea Denatured Native BSA and Poly-α-Amino Acid BSA's

A Rudolph photoelectric polarimeter model 80 equipped with a sodium light source and a 1-dm cell was used for all optical rotation measurements.

The protein solutions were prepared the same way as in the ultraviolet spectra experiments. As for pH 5.5 and ionic strength 0.15 solution, acetic acid-sodium acetate buffer was used.

For each measurement, a blank was taken so as to check the strain of the glass end plates. After mixing in the volumetric flask, the protein-urea solution was transferred to the polarimeter tube with a disposable pipette within 15-30 minutes. Care was taken to check for air bubbles. If there were air bubbles present, the tube was tilted and tapped gently until all air bubbles were removed.

After each measurement, the polarimeter tube was washed with distilled water, then rinsed with 95% ethanol and dried with air. All measurements were taken at room temperature, $24 \pm 1^{\circ}C$.

IV. RESULTS AND DISCUSSION

Preparation of L-Phenylalanine NCA

Among several methods for preparation of NCA (Leuchs method, Curtius method, Fuchs-Farthing method), Fuchs-Farthing method is the best, since it is the simplest method of synthesis and gives pure monomers in a high yield. NCA's prepared by the Leuchs method using PCl_5, may be contaminated with the chlorinating agent, with $POCl_3$, and carboalkoxyamino acid chlorides, while those formed by Fuchs-Farthing method may contain HCl and the N-carbonyl chloride of the corresponding amino acid. The removal of the final traces of the inorganic phosphorus derivatives from the anhydride is sometimes most troublesome.

Poly-α-Amino Acid BSA's

With the attachment of about 100 moles of L-phenylalanine per mole of protein, poly-L-phenylalanyl BSA was only slightly soluble in aqueous solution in comparison with native BSA. This property created a problem for storing poly-L-phenylalanyl BSA since the modified protein did not redissolve after lyophilization. Moreover, it tended to aggregate when kept in the freezer. Extreme cold, e.g., in the acetone-dry ice bath would cause precipitation immediately. Consequently the poly-L-phenylalanyl BSA solution was kept in the

refrigerator in solution after it was dialyzed. However, even prolonged storage was not advisable. The solutions became turbid after keeping in the refrigerator for more than two weeks. In these experiments, the poly-L-phenylalanyl BSA was always freshly prepared to assure no change in the protein.

In the case of polyglycyl BSA, it was lyophilized and kept in the refrigerator. No difficulty was observed in redissolving polyglycyl BSA.

Amino Acid Analysis and End Group Studies

The results of amino acid analysis and end group studies of polypeptidyl BSA's are shown in Table I. After dinitrophenylation of the derivatives, analysis showed for each amino group acylated, there was an N-terminal dinitrophenyl-L-phenylalanine or dinitrophenylglycine. The moles of DNP-L-phenylalanine (glycine)[4] were calculated by subtracting the total moles of L-phenylalanine (glycine) in DNP-polypeptidyl-BSA from moles of L-phenylalanine (glycine) in polypeptidyl BSA. The moles of lysine in DNP-polypeptidyl-BSA should be equal to the number of amino groups acylated per mole of protein. It must be pointed out the the polypeptidyl BSA's are heterogeneous with respect to the extent of modification.

[4] DNP-L-phenylalanine (glycine) means DNP-L-phenylalanine or DNP-glycine.

Table I. Results of Amino Acid Analysis and End Group Studies of Polypeptidyl BSA's

	poly-L-phenylalanyl BSA		polyglycyl BSA
	I	II	
1. Moles of amino acid added per mole of BSA[a]	100	86	135
2. Moles of added amino acid remaining per mole of DNP-polypeptidyl-BSA[a]	67	53	111
3. Moles of ϵ-DNP-lysine per mole of DNP-polypeptidyl-BSA[b]	24	29	38
4. Moles of lysine per mole of DNP-polypeptidyl-BSA[a]	35	35	21
5. Amino groups acylated per mole of BSA[c]	33	33	24
6. Average chain length[d]	3	2.6	5.6

[a]Determined by the method of Spackman, Stein and Moore (1958).

[b]Determined by Sanger's method (1945).

[c]Calculated by subtracting 2 from 1.

[d]Calculated by dividing 1 by 5.

As shown in Table I, the results agree quite well with each other. The average chain length of the polyglycyl chains was about twice that of poly-L-phenylalanine. This is reasonable, as poly-L-phenylalanine is insoluble in aqueous solution. With longer chain lengths of poly-L-phenylalanine, poly-L-phenylalanyl BSA would precipitate out. On the other hand, polyglycyl derivatives are quite soluble in aqueous solution.

Spectroscopic Studies of Native BSA and Poly-α-Amino Acid BSA's

Ultraviolet Spectra of Poly-L-Phenylalanine and Free L-Phenylalanine

The absorption curves of poly-L-phenylalanine BSA and free L-phenylalanine + BSA using native BSA as reference are shown in Fig. 2. Although it is assumed that these spectra are equivalent to poly-L-phenylalanine and free L-phenylalanine, it must be emphasized that the polypeptidyl BSA is heterogeneous with respect to the extent of modification. The chain lengths of 3 and 2.6 (Table I) are the average chain lengths of the added L-phenylalanine peptides.

The fine structure of L-phenylalanine agrees quite well with that reported (Beaven, 1952), although there is approximately 1.2-1.5 mμ long wavelength shift in poly-L-phenylalanine. It is a well known fact that for aromatic amino acids, peptide combination involving the carboxyl and α-amino groups usually results in some loss

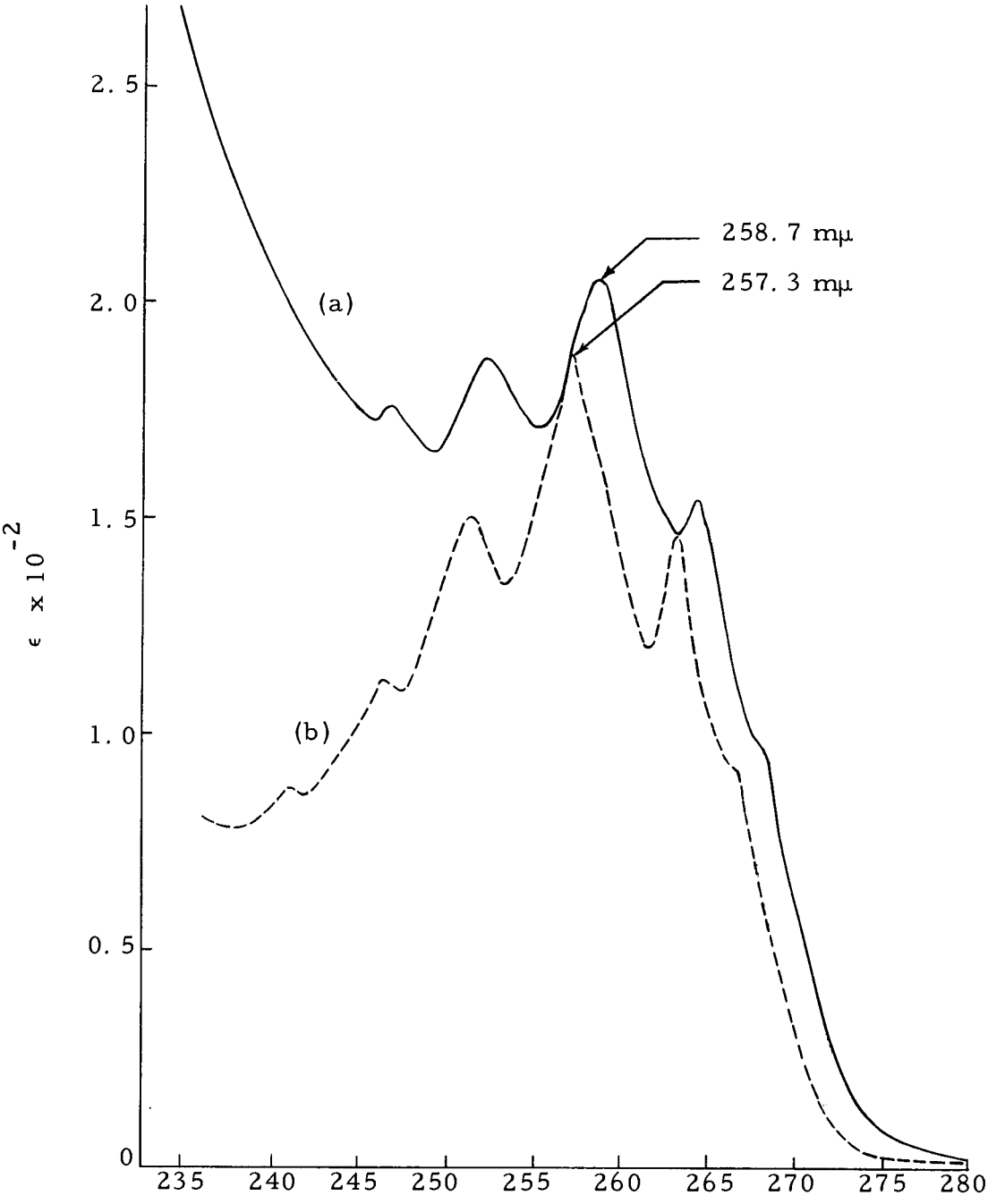


Figure 2. Ultraviolet spectra (molar absorptivity, ϵ, versus wavelength in mμ) of poly-L-phenylalanyl BSA and free L-phenylalanine + native BSA at pH 7 using native BSA as reference in both cases.

(a) Poly-L-phenylalanyl BSA (0.079 gm/100 ml in terms of native BSA).

(b) Free L-phenylalanine (0.019 gm/100 ml) + native BSA (0.079 gm/100 ml).

of resolution of the vibrational fine structures, and a shift of the absorption bands to longer wavelengths. Spectral studies of simple di- and tripeptides by several workers (Fruton and Lavin, 1939; Barkdoll and Ross, 1944) have shown both long wavelength shifts and loss of fine structure resolution when the peptides are compared to the amino acids. Barkdoll (1944) has shown 1.5 mμ long wavelength shift in a tripeptide of tyrosine in comparison with monomer L-tyrosine. The result with poly-L-phenylalanine is comparable to this poly-L-tyrosine shift.

Ultraviolet Spectra of Urea Denatured Native BSA and Poly-α-Amino Acid BSA's

Spectra. The ultraviolet spectra of native BSA and denatured BSA in 7M urea are shown in Fig. 3. The spectra of poly-L-phenylalanyl BSA and denatured poly-L-phenylalanyl BSA in 7M urea are shown in Fig. 4. There is an observable blue (short wavelength) shift in the absorption maximum at 278.4 mμ of urea denatured native BSA. The extent of this shift was dependent on the urea concentration. In 7M urea solution, the peak of the native BSA shifts from 278.4 mμ to 276.7 mμ. However, as for urea treated poly-L-phenylalanyl BSA,the extent of the shift is small, from 278.5 mμ to 278 mμ. The extent of the shift in both proteins increases a little after 24 hours. The polyglycyl BSA has the same spectrum as native BSA, and the extent of the shift in 7M urea is

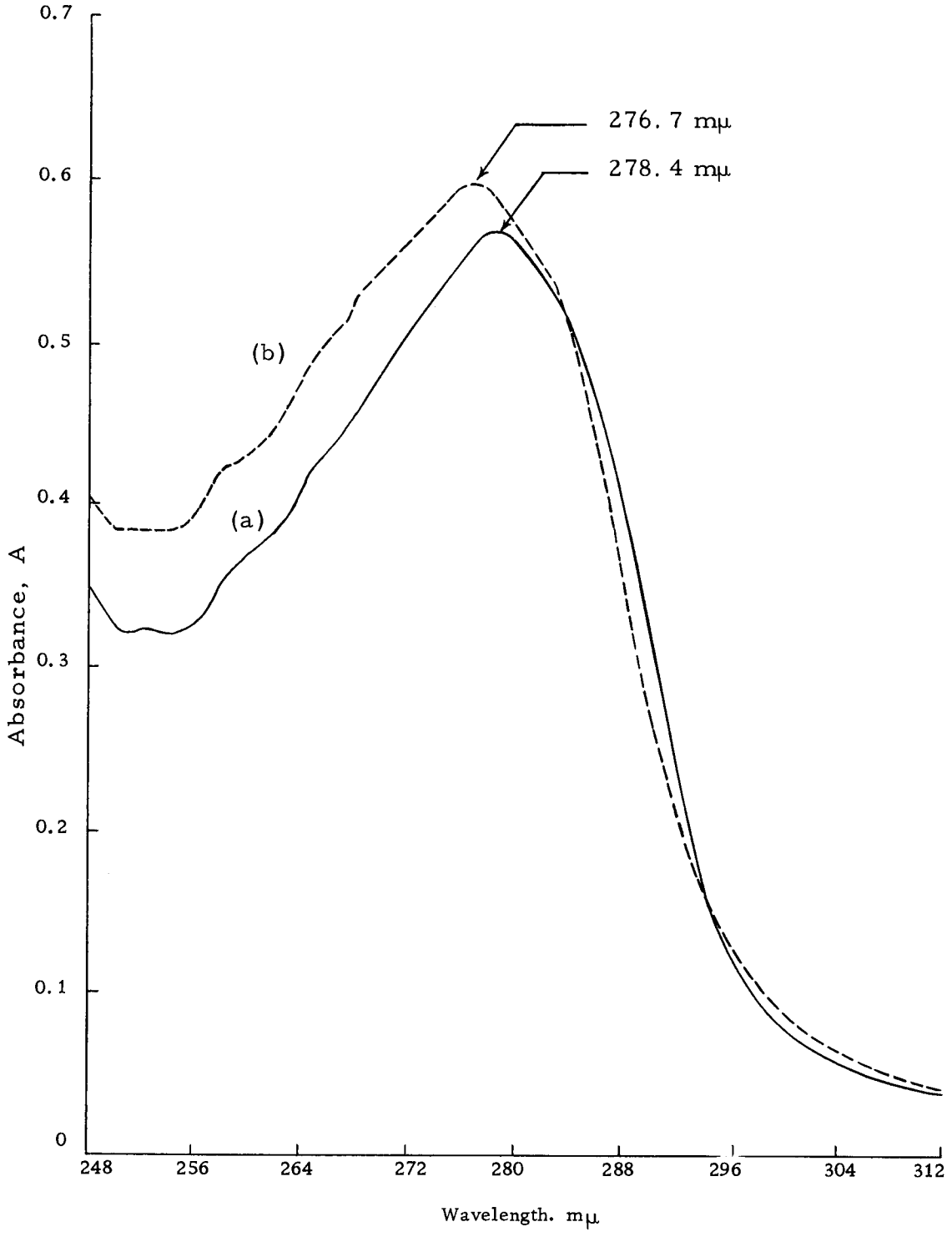


Figure 3. Ultraviolet spectra of native BSA and denatured BSA in 7M urea. Both proteins in 0.067M phosphate buffer, pH 7.6, and ionic strength 0.16.
(a) Native BSA
(b) Denatured BSA in 7M urea

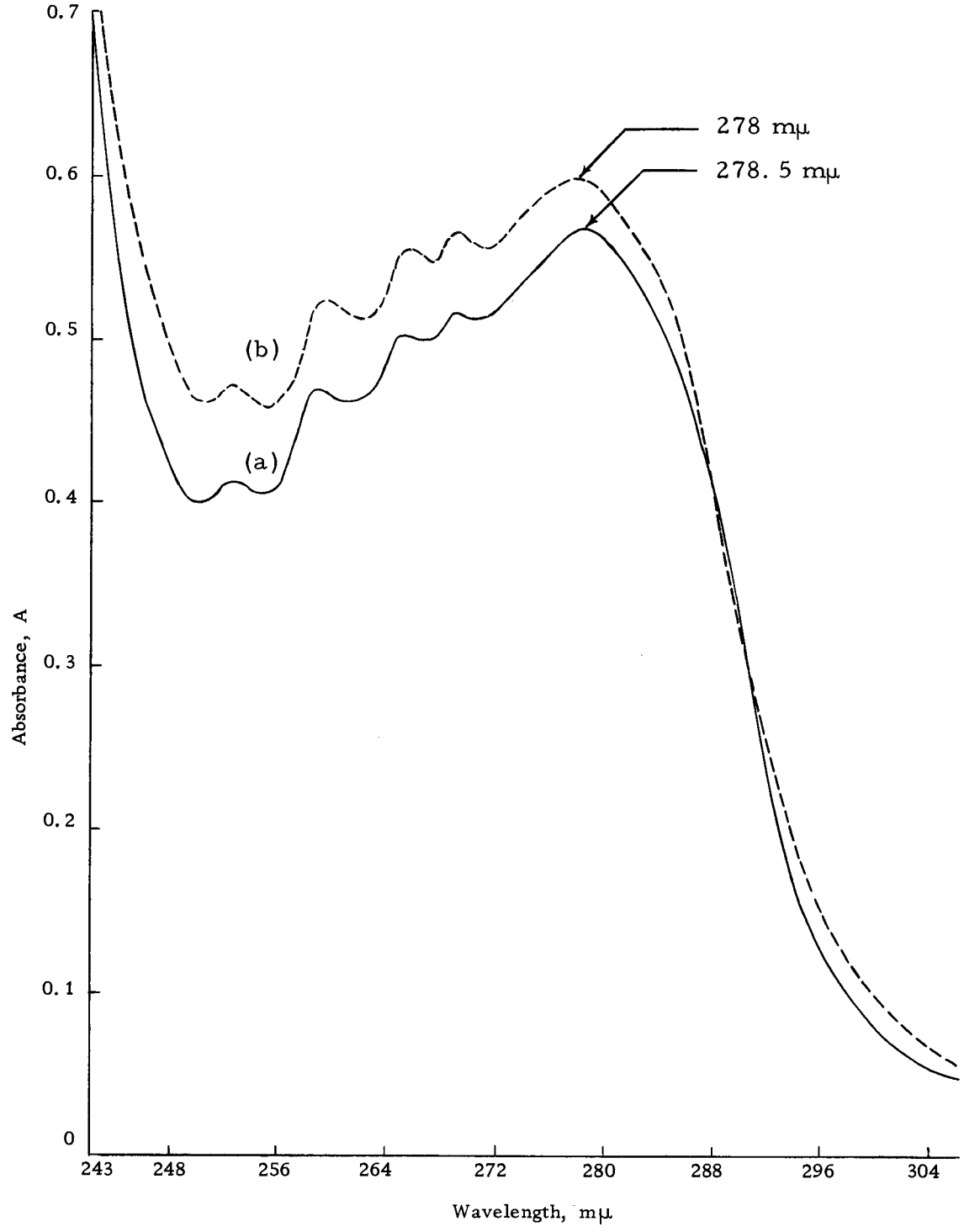


Figure 4. Ultraviolet spectra of poly-L-phenylalanyl BSA and denatured poly-L-phenylalanyl BSA in 7M urea. Both proteins in 0. 067M phosphate buffer, pH 7. 6, and ionic strength 0. 16.
(a) Poly-L-phenylalanyl BSA
(b) Denatured poly-L-phenylalanyl BSA in 7M urea

about 1.5 mμ. The results for the proteins studied are shown in Table II.

Table II. Blue (Short Wavelength) Shifts at Maximum Peaks of Urea-Denatured Native BSA and Polypeptidyl BSA's

Molarity of urea	O M	7 M
Native BSA	278.4 mμ	276.7 mμ
Polyglycyl BSA	278.1	276.5
Poly-L-phenylalanyl BSA	278.5	278

Difference Spectra. The difference spectra of proteins can be obtained by two methods, subtraction of separately determined spectra and direct measurement. Measurement by the subtraction method has a larger error than the direct method (Wetlaufer, 1962). In general, if stray light and fluorescence errors can be excluded, direct measurement of a difference spectrum is highly preferable.

A typical difference spectrum is shown in Fig. 5. On exposure of native BSA, polyglycyl BSA and poly-L-phenylalanyl BSA to urea concentrations above 2-3M at pH 7.6, an immediate blue shift in the absorption maximum at 278.5 mμ occurred. The extent of this shift was dependent on the urea concentration. A minimum in $\Delta\epsilon$ was also observed at 287 mμ. The 287 mμ minimum is due to differences in the environment of the tyrosine residues (Donovan, Laskowski and Scheraga, 1958; Yanari and Bovey, 1960). A strong

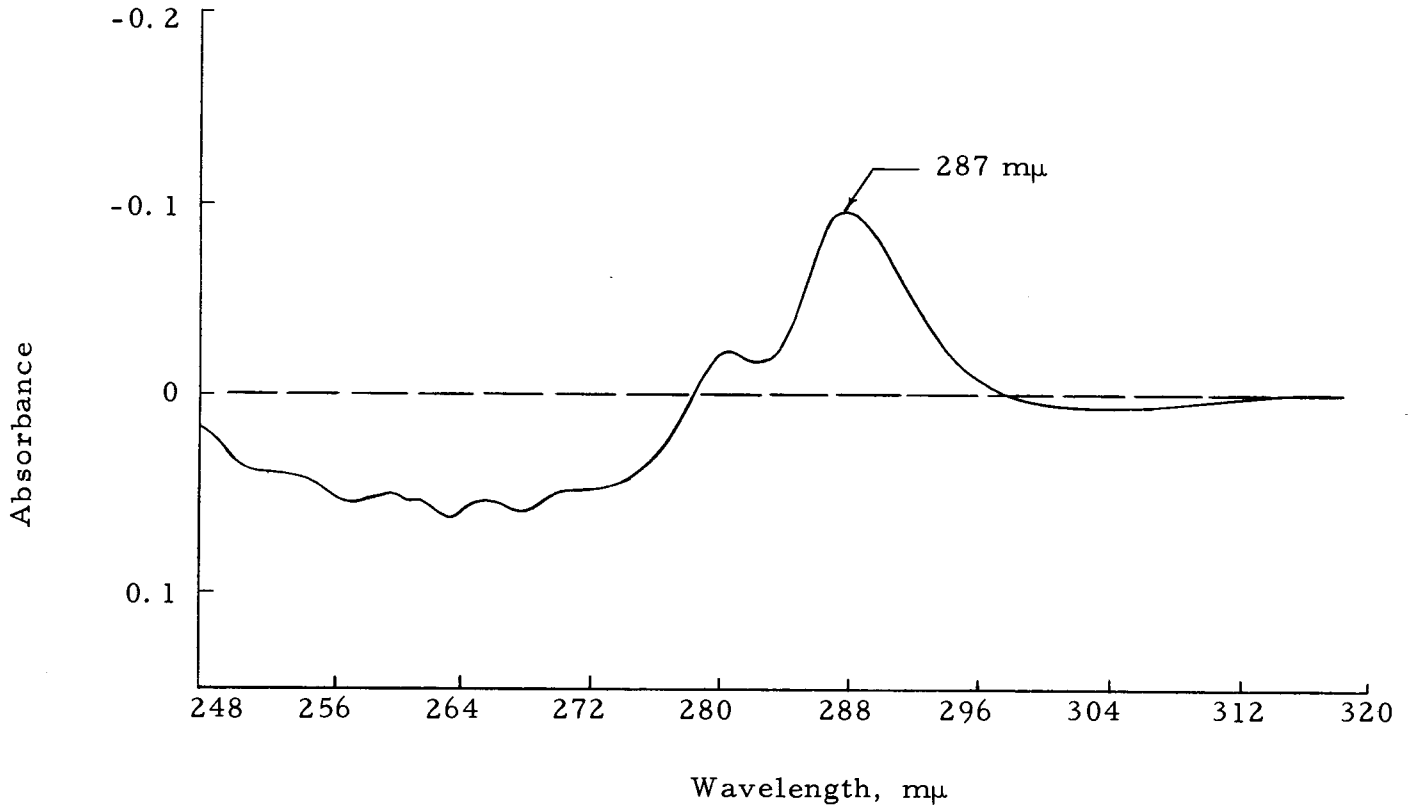


Figure 5. Difference spectrum of native BSA and denatured BSA in 7M urea. Protein concentration: 0.16 gm/100 ml (pH 7.6, 0.067M phosphate buffer, and ionic strength 0.16).

dependency of $\Delta\epsilon$ on urea concentration is shown in Fig. 6 for native BSA; Fig. 7 for polyglycyl BSA and Fig. 8 for poly-L-phenylalanyl BSA. There were further changes in $\Delta\epsilon$ of native BSA and polyglycyl BSA over 24 hours. This change may be due to the sulfhydryl-disulfide exchange reactions in the BSA. In urea denatured BSA, Hospelhorn, Cross and Jensen (1954) observed two phases of reaction, an initial rapid increase in viscosity followed by a slower rise. The slow reaction was particularly sensitive to sulfhydryl reagents. These phenomena have been interpreted by these workers as due to a type of chain reaction in which there is interchange of -SH and -S-S groups.

The ultraviolet absorption maxima of proteins undergo shifts to shorter wavelengths when the secondary and tertiary structures of the molecules are disrupted. The interpretations of this effect that have been advanced so far involve changes in hydrogen bonding, vicinal charges, ion-dipole bonding, dipole-dipole interactions, and hydrophobic bonding.

In 1943, Crammer and Neuberger suggested that tyrosyl groups of the protein are involved as hydrogen bond donors with carboxylate, citing salicylic acid as an example of a compound showing such bonding (Crammer, 1943). The titration of ovalbumin shows that there are as many abnormally strong carboxyl groups as there are abnormally weak phenolic groups (Harrington, 1955), which can be

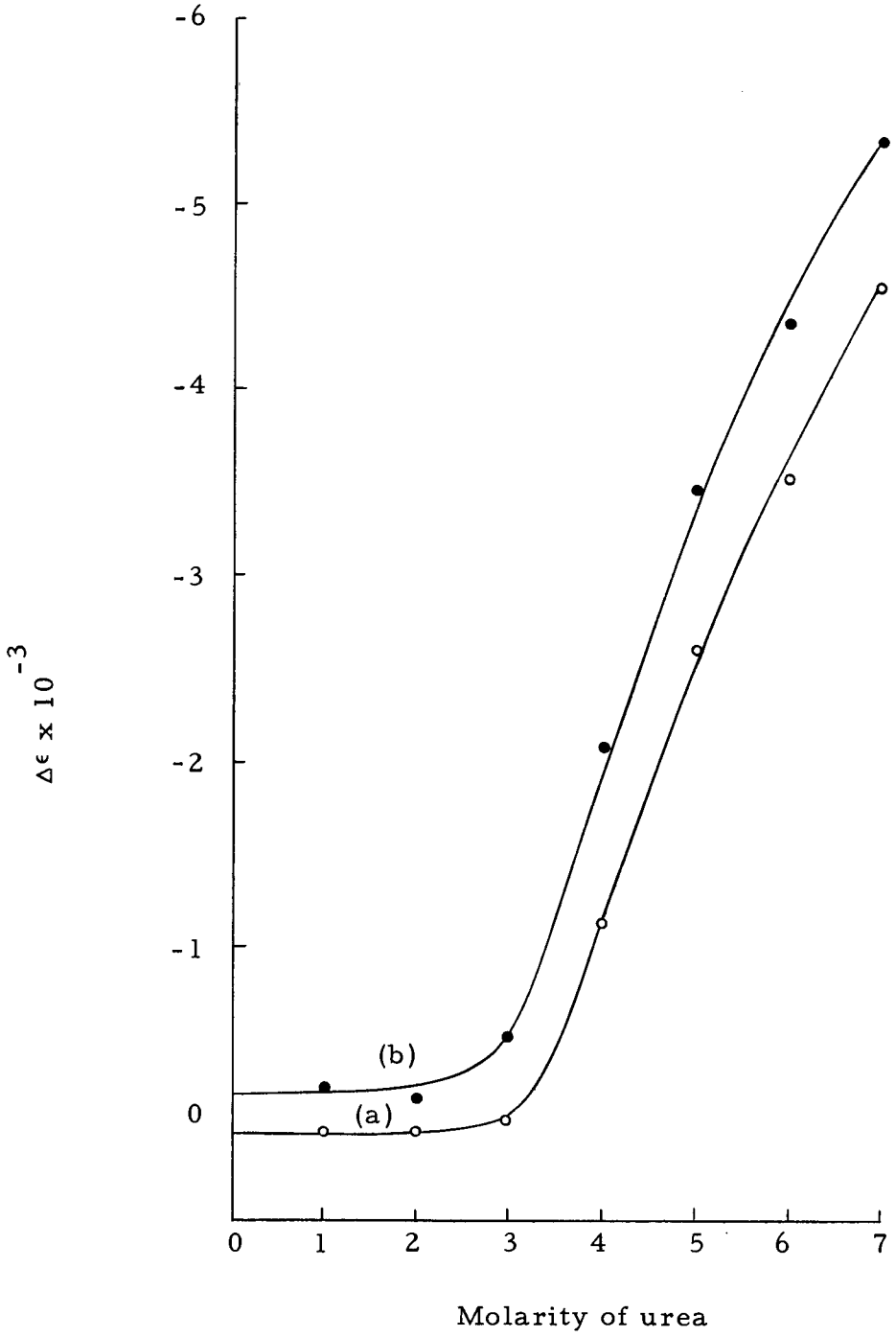


Figure 6. Effect of urea concentration on molar absorptivity difference at 287 mμ, $\Delta\epsilon_{287}$, for native BSA at 24 ± 1°C. Protein concentration: 0.16 gm/100 ml (pH 7.6, ~ 0.067M phosphate buffer, and ionic strength 0.16). Measured (a) within 10-15 mins., and (b) after 24 hours.

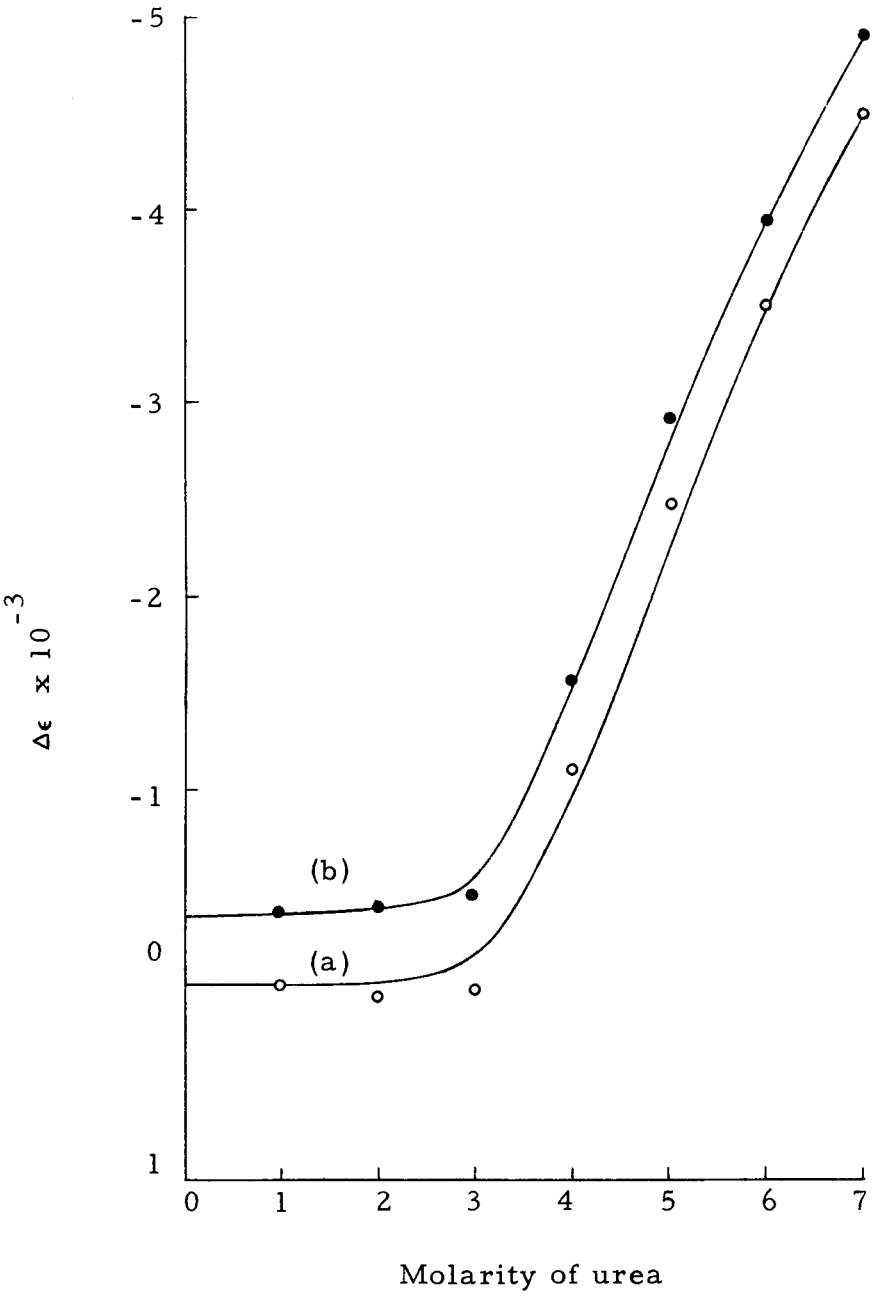


Figure 7. Effect of urea concentration on molar absorptivity difference at 287 mμ, $\Delta\epsilon_{287}$ for polyglycyl BSA at 24 ± 1°C. Protein concentration: 0.173 gm/100 ml in terms of native BSA (pH 7.6, 0.067M phosphate buffer, and ionic strength 0.16). Measured (a) within 10 minutes, and (b) after 24 hours.

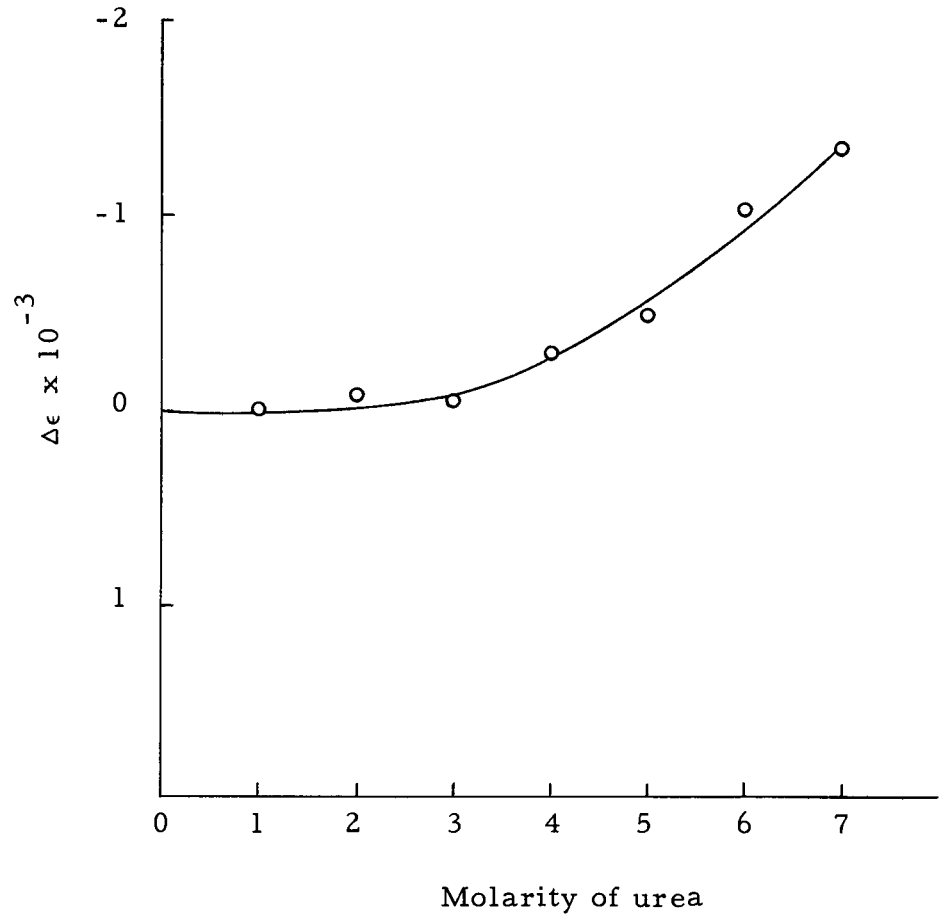


Figure 8. Effect of urea concentration on molar absorptivity difference at 287 mμ, $\Delta\epsilon_{287}$, for poly-L-phenylalanyl BSA at 24± 1° C. Protein concentration: 0.155 gm/100 ml in terms of native BSA (pH 7.6, 0.067M phosphate buffer, and ionic strength 0.16). Measured within 10 minutes.

taken as supporting evidence of this hypothesis. Furthermore, Laskowski et al. (1956), Scheraga (1957) and their co-workers have obtained spectrophotometric data which are regarded as evidence for the existence of hydrogen bonds or ion dipole bonds to tyrosyl groups in a number of proteins. The pH dependence of the insulin difference spectrum suggested a pK of 3 for the groups involved in the spectral transition. This lower-than-normal carboxyl pK is consistent with hydrogen bonded carboxylate groups. Gordon and Jencks (1963) studied the effectivenss of various denaturing agents for proteins, and found no support for the involvement of hydrogen bonding in protein denaturation.

Wetlaufer (1956) showed that the spectrum of phenol in strong urea and sodium acetate is perturbed in a similar fashion to that of tyrosyl groups in insulin. Similar effects of urea and acetate on the spectrum of N-acetyltyrosine ethyl ester have been attributed to secondary bonding to tyrosyl phenolic groups. However, Wetlaufer, Edsall and Hollingworth (1958) have shown a very similar difference spectrum of O-methyltyrosine to that of tyrosine in strong urea and sodium acetate aqueous solutions. Since the hydrogen of O-methyltyrosine is replaced by a methyl group, it is clear that it cannot participate in a hydrogen-bonding mechanism as donor. Therefore, Wetlaufer, Edsall and Hollingworth (1958) postulated that the tyrosyl absorption of proteins may be affected by neighboring ions and dipoles. This conclusion is supported by Donovan, Laskowski

and Scheraga (1958) who by studying the spectral changes of phenylalanyl and tryptophyl residues have postulated that modification in the absorption of these residues in proteins may be produced by neighboring charges.

The acid transformation of serum albumin has been studied further with the solvent-perturbation technique by Leonard and Foster (1961), and by Herskovits and Laskowski (1962). The results of these studies support the idea that the tyrosyl groups of serum albumin do become progressively more exposed to solvent, both in the N-F transformation and in the molecular expansion at slightly lower pH. However, this hypothesis of tyrosyl-carboxylate bonding does not explain the changes on the tyrosyl absorption of pepsin (Yanari and Bovey, 1958; Williams and Foster, 1959). Bigelow and Geschwind (1960) examined the effect of media of high refractive index on the spectra of the model compounds tryptophan, tyrosine, phenylalanine, indole, phenol and O-methyltyrosine. In nearly all cases there was a red (long wavelength) shift. The red shift was explained in terms of increase in refractive index of the solvent. Under non-denaturing conditions, salts and urea sometimes produce red shifts for normal tyrosine and tryptophan residues in proteins; except LiBr, NaBr and NaCl which produce blue shifts for normal tyrosine residues. Under denaturing concentrations of salts and urea, blue shifts are invariably produced. While this blue

shift for abnormal tyrosyl residues may be due to changes in hydrogen bonding of tyrosine hydroxyl groups, this explanation cannot be valid in the case of shifts for tryptophan residues.

A number of workers (Williams and Foster, 1959; Edelhoch, 1958) have suggested that some of the aromatic residues in proteins are involved in hydrophobic bonding, and are in an environment of high refractive index and blue shifts may be expected (Yanari and Bovey, 1960). Abu-Hamdiyyah (1965) suggested that nonpolar solutes, especially hydrocarbons and hydrocarbon moieties, dissolve interstitially in water and in aqueous urea solutions. Physicochemical properties of aqueous urea solutions indicate that urea does not influence the structure of water but actively participates in the formation of clusters. Weakening of hydrophobic bonding upon addition of urea is due to the increased ease of cluster formation. Bovine serum albumin consists of nonpolar aliphatic and aromatic side chains which could provide hydrocarbon-like environment for aromatic side chain, i. e., leucyl, isoleucyl, valyl, phenylalanyl and possible alanyl side chains. In the addition of urea to BSA solution, urea tends to weaken the hydrophobic bonds and causes the spectral change.

From the observation in Fig. 6, 7, and 8, it is observed that there is no significant difference between the denaturation of polyglycyl BSA and native BSA. It is a well known fact that

polyglycyl proteins showed no significant change in biological and chemical properties in comparison with the native proteins (Becker and Stahmann, 1953). However, in polypeptidyl proteins modified with nonpolar amino acids, there were changes in both enzymatic and physical properties compared with the native protein (Becker, 1962; Stracher and Becker, 1959). These properties of polypeptidyl proteins can be related to the hydrophobic character of the added amino acids. The stability of poly-L-phenylalanyl BSA toward urea denaturation may be related to its hydrophobic nature.

Optical Rotation of Urea Denatured Native BSA and Poly-α-Amino Acid BSA's

The increased levorotation of the urea denatured proteins at pH 7.6 are shown in Fig. 9 and Fig. 10, at pH 5.5. In 7M urea the specific rotation $[\alpha]_D$ of native BSA changed from -61^o to -104^o as compared with the reported -60^o to -100-110^o (Glazer, McKenzie and Wake, 1963).

At pH 7.6, there is a decrease of net positive charge of the modified proteins when compared to the native protein. This decreased net positive charge is caused by substitution of α-amino groups with pK_a of 7.6 for ε-amino groups with pK_a of 10.4. To minimize this slight difference in net charge, an experiment was also performed at pH 5.5 where almost all the α-amino and ε-amino groups are charged. There is little difference in $[\alpha]_D$ between

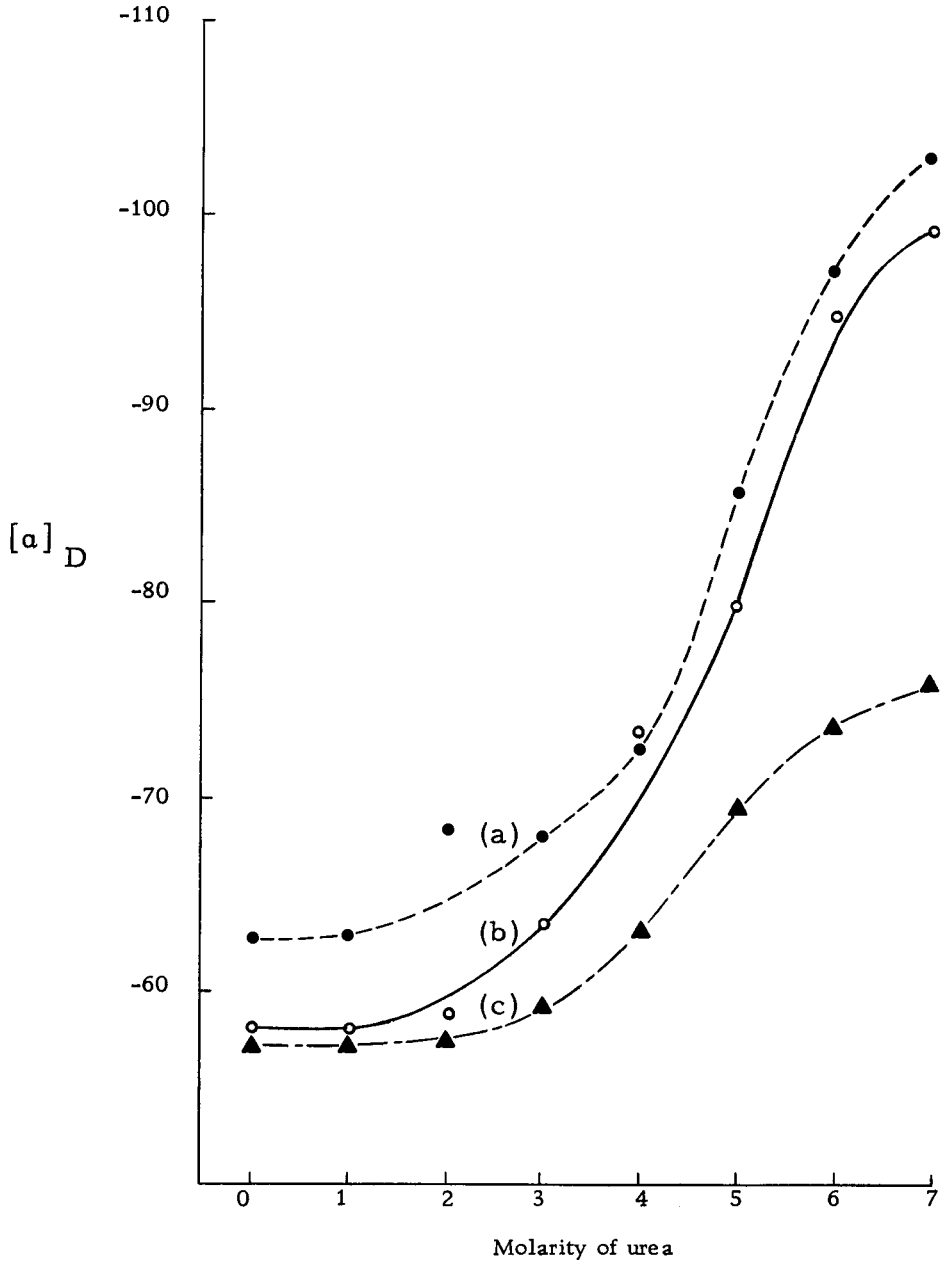


Figure 9. Effect of urea concentration on specific rotation $[a]_D$ of native BSA, polyglycyl BSA and poly-L-phenylalanyl BSA at pH 7.6. All in 0.067M phosphate buffer and ionic strength 0.16. Measured within 10-15 minutes at 24± 1°C.

(a) Native BSA (0.164 gm/100 ml)

(b) Polyglycyl BSA (0.152 gm/100 ml in terms of native BSA)

(c) Poly-L-phenylalanyl BSA (0.157 gm/100 ml in terms of native BSA)

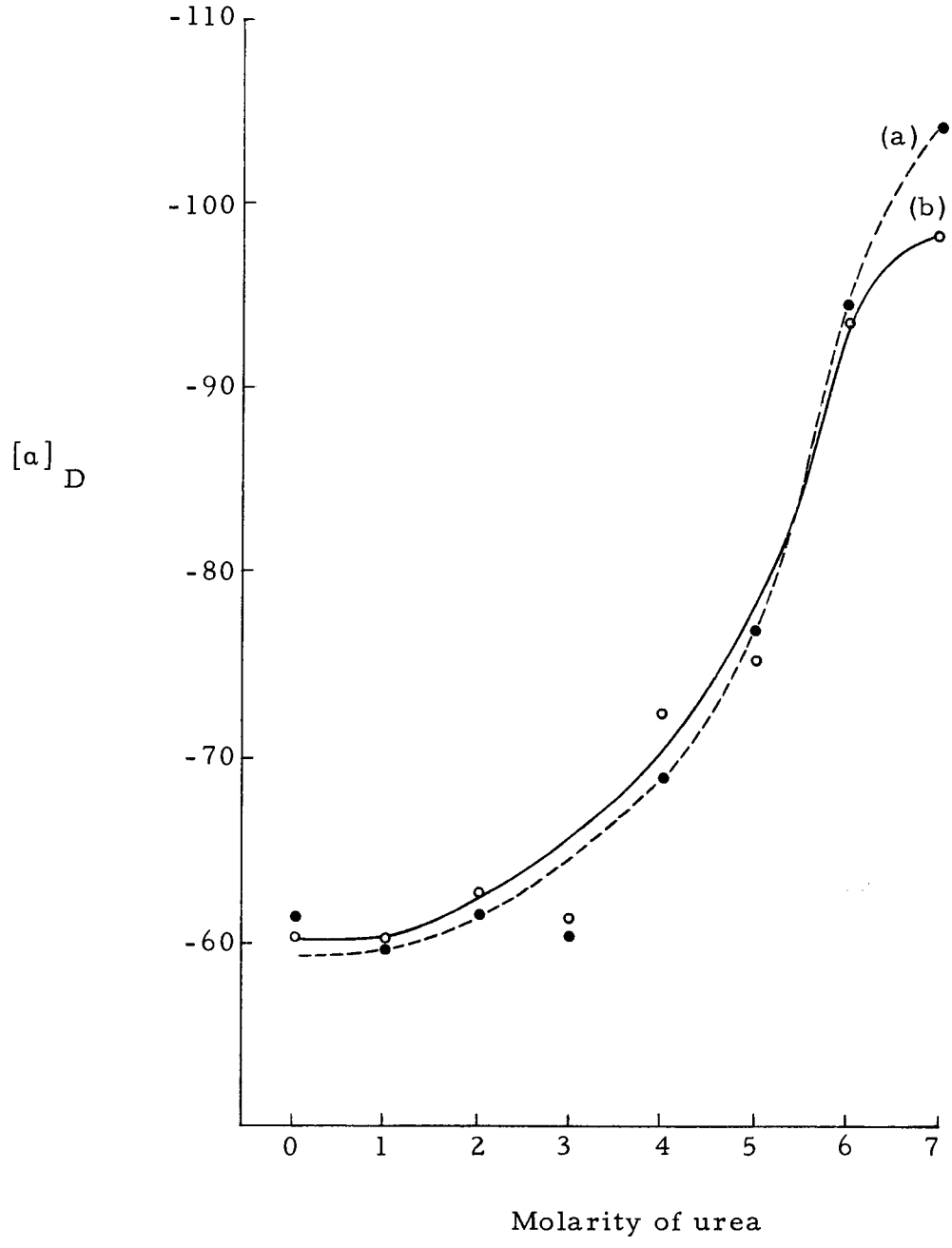


Figure 10. Effect of urea concentration on specific rotation $[\alpha]_D$ for native BSA and polyglycyl BSA at pH 5.5. All in acetic acid-sodium acetate buffer and ionic strength 0.15. Measured within 30 minutes at 24± 1°C.
(a) Native BSA (0.162 gm/100 ml)
(b) Polyglycyl BSA (0.157 gm/100 ml in terms of native BSA)

polyglycyl BSA and BSA as influenced by urea (Fig. 10). Until further investigation, the slight difference may be considered within the experimental error (Van Kley and Stahmann, 1956).

The sigmoid curves for the increases in levorotation of the proteins with increasing urea concentration has also been described by Kauzmann and Simpson (1953), and Gordon and Jencks (1963). There is not much difference in change of $[\alpha]_D$ of polglycyl BSA and native BSA. However, marked stability of poly-L-phenylalanyl BSA toward urea denaturation was observed.

This result of less increased levorotation of poly-L-phenylalanyl BSA adds to the evidences of the hydrophobic property of the modified protein. The introduced non-polar aromatic groups of L-phenylalanine peptides may form intramolecular or intermolecular bonds. The intramolecular hydrophobic bonds of poly-L-phenylalanyl BSA render the protein less susceptible to urea denaturation. The intermolecular hydrophobic bonds can be illustrated by the aggregation phenomena.

It is a well known fact that native proteins are levorotatory and on denaturation their levorotation increases (Kauzmann and Simpson, 1953). Attempts have been made to interpret configuration of the protein. However, the physical principles underlying optical rotation are complex, and it is not possible to interpret changes in it in precise structural terms (Kauzmann, 1959). In general, it can be

said that optical rotation arises through the interactions of the chromophoric electrons ("vicinal action") in different groups in asymmetric molecules. Any structural change which alters the positions of the groups in an asymmetric molecule produces a marked change in the optical rotation.

In the case of urea denaturation of BSA, the rapid increased levorotation of the protein has been interpreted as related to the "unfolding" of the protein (Kauzmann and Simpson, 1953). This is supported by sedimentation and diffusion measurements (Glazer, McKenzie and Wake, 1963). Yang and Foster (1964), on the other hand, who have studied the streaming birefringence of serum albumin under equivalent conditions, have concluded, because of the absence of the birefringence, that the molecule is not unfolded but expanded.

Another interpretation of this increase in levorotation is based on the fact that when synthetic L-polypeptides in their helical form are transformed into the random coil form, they become more levorotatory (Yang and Doty, 1957). This observation leads one to conclude that during denaturation the protein molecule becomes extensively disorganized and consequently causes the change of rotation. Moreover, Markus and Karush (1957) have shown that the splitting of the cystine disulfide groups of serum albumin either by reduction or by oxidation results in a decrease in levorotation of the protein; they have interpreted this to mean that disulfide groups in

serum albumin introduce constraints which reduce the amount of helical structure possible for the polypeptide chain. However, Turner and co-workers (Turner, Bottle and Haurowitz, 1958) have noted that cystine and cystine-like compounds have unusually high levorotation, which are markedly reduced when the disulfide link is ruptured, and that the order of magnitude of the increased levorotation in serum albumin and other proteins is about the same as one would expect from the change of rotation contributed by their cystine content. Therefore, until we have independent evidence to know exactly about the relationship between protein structure and optical rotation, it is not certain how the protein structure changed during denaturation.

Nature of Stabilization

It has been found from this experiment that poly-L-phenylalanine BSA was more stable toward urea denaturation in comparison with native BSA and polyglycyl BSA when optical rotation and ultraviolet spectrum were used as parameters for the evaluation of the extent of denaturation. Although the nature of this stabilization of poly-L-phenylalanine toward urea denaturation is not exactly known, this property can be compared with the protective character of certain anionic detergents toward protein denaturation.

It is a well known fact that certain organic anions, for

example, sodium dodecyl sulfate, in appropriate concentrations prevented the rise in levorotation of human serum albumin caused by 6M urea (Markus and Karush, 1957). Markus, Love and Wissler (1963) studied the mechanism of this protection of human serum albumin against urea denaturation as a function of the amount of sodium dodecyl sulfate bound, the concentration of urea, and the state of ionization of the cationic side chains of the serum albumin. They concluded that the protection is in part based on electrostatic interaction between the positively charged ϵ -ammonium groups of some lysine residues and the negatively charged sulfate moiety of the bound detergent ions. Binding of the detergent by both electrostatic and nonpolar forces suggests that the protection might be the result of a cross-linking function.

In the cases of polypeptidyl BSA's, the poly-L-phenylalanine of polyglycine attaches to the ϵ -lysine groups of the BSA by covalent bonds. The nonpolar residues (the benzene group) of poly-L-phenylalanine can be bound to the nonpolar side chains of the native BSA. The cross-link formed may be similar to those of adding sodium dodecyl sulfate to BSA, where the nonpolar groups of the detergent and the protein are cross-linked. Consequently, poly-L-phenylalanine acts more or less as a protective agent toward urea denaturation. To illustrate the similarity and difference of these three cases, a highly simplified diagram for the comparison of the binding of sodium dodecyl sulfate, poly-L-phenylalanine and polyglycine to the protein is shown in Fig. 11.

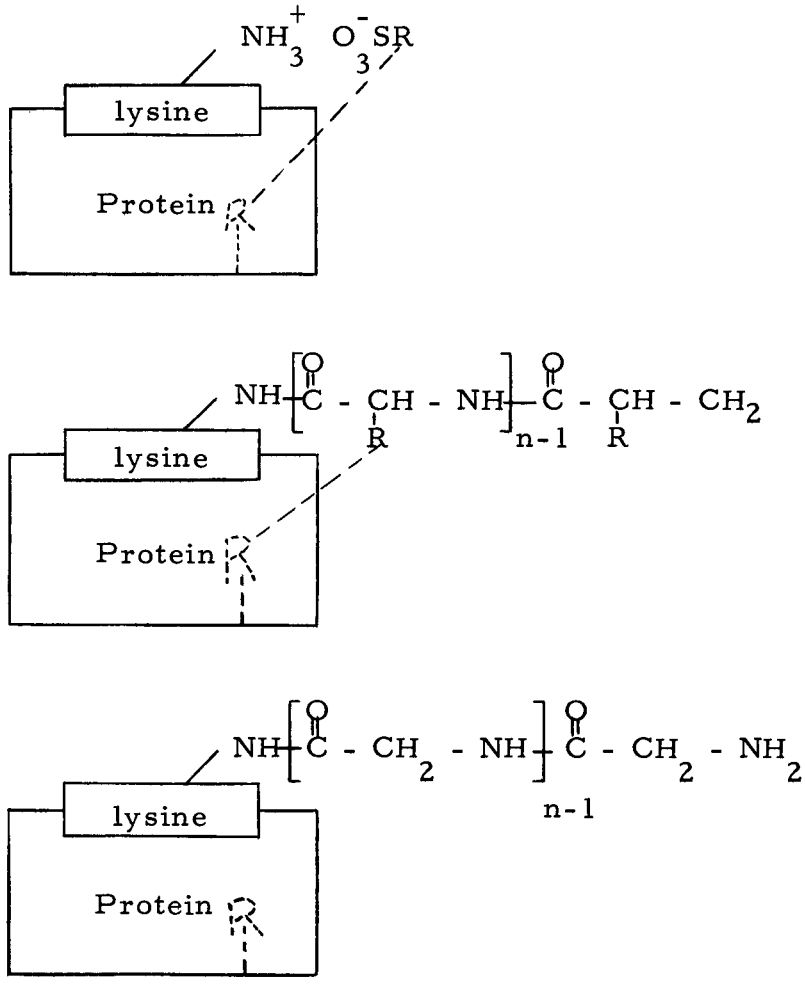


Figure 11. A highly simplified diagram for the comparison of the bindings of sodium dodecyl sulfate, poly-L-phenylalanine, and polyglycine to the protein. R represents non-polar group.

CONCLUSIONS

The effect of urea denaturation on native BSA, polyglycyl BSA and poly-L-phenylalanyl BSA has been investigated by ultraviolet spectrophotometry and optical rotation. Polyglycyl BSA was chosen as a control in this study because of the fact that polyglycyl proteins have shown similar properties as native proteins.

It was found that there was no significant difference in urea denaturation of polyglycyl BSA and native BSA. However, poly-L-phenylalanyl BSA was more stable toward urea denaturation in comparison with native BSA and poly-L-phenylalanyl BSA. This property of poly-L-phenylalanyl BSA can be related to the protective effect of sodium dodecyl sulfate against urea denaturation of serum albumin.

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