

Refractive Indices of Amino Acids, Proteins, 2050 and Related Substances

The molar refractions of the amino acids were determined by measurements on their aqueous solutions and the expanded Lorenz-Lorentz equation. The refractive indices of a number of proteins were calculated from their amino acid compositions and the values for the refraction of the amino acid residues. These calculated results are in good agreement with those experimentally determined, demonstrating that refractive index is a unique characteristic of a protein. A comparison of the refractive index of heat denatured β -lactoglobulin with the native protein demonstrated that changes in structure produced a small change in refractive index, not associated with a change in volume.

Although the refractive index of a solution can be simply and precisely measured, it has been little used in characterizing proteins. The refraction of proteins is, however, frequently involved in measurements on protein solutions by such methods as light scattering, sedimentation, and electrophoresis. Previous investigations, summarized by Doty and Geiduschek (11), indicate the importance of composition, density, charge, and environmental factors in determining the refractive indices of proteins. They note that the values reported for the refractive indices of proteins are close to 1.60 and are nearly constant.

Adair and Robinson (1) indicate that the refractive index of a protein or an amino acid is approximately determined by its elementary composition; however, the structure of a molecule is also of importance. The values reported for amino acids are scattered and fragmentary (1, 10), and prior to our preliminary communication (25) no systematic investigation had accounted quantitatively for the relationship

Critical Tables and from Tilton and Taylor (32). The wavelength of light is given in millimicrons ($m\mu$).

Calculations. Refractive Indices and Molar Refractions of Solutions of Amino Acids and Proteins. The mean refractive indices of the amino acids and proteins were calculated by means of the following expanded Lorenz-Lorentz equation as given by Doty and Geiduschek (11):

$$\frac{n^2 - 1}{n^2 + 2} = c\bar{v} \frac{(n_p^2 - 1)}{(n_p^2 + 2)} + (1 - c\bar{v}) \frac{(n_0^2 - 1)}{(n_0^2 + 2)} \quad (1)$$

where n_p , n_0 , and n indicate the refractive indices of the water-free protein or amino acid, the solvent and the solution respectively, \bar{v} is the specific volume of the protein or amino acid and concentration, c , is expressed in grams per cubic centimeter. Molar refraction, R , is calculated by Equation 2.

$$[R] = \frac{n_p^2 - 1}{n_p^2 + 2} \times \frac{M}{\rho} \quad (2)$$

where n_p is the mean refractive index of the amino acid or protein, M , is the molecular weight of the amino acid (for proteins, 100 grams has been used instead of the molecular weight of the protein), and ρ is the density of the amino acid or protein, $1/\bar{v}$.

Refractive Index of Protein from Amino Acid Composition. The method used for calculating the refractive index of a protein from its amino acid composition is essentially the same as that described by Cohn and Edsall (9, Chap. 16) for calculating the specific volume of a protein from its amino acid composition.

The weight per cent of each amino acid found by analysis is converted into the weight per cent of its residue by multiplying by the ratio of the molecular weight of the residue (amino acid minus 1 mole of water) divided by the molecular weight of the amino acid. Then the weight per cent of each amino acid residue is multiplied by the value of the refraction of 1 gram of residue, as given in Table I, to give the total refractive volume in 100 grams of the protein due to a given amino acid residue. The total refractive volume of the amino acid residues in 100 grams of protein is obtained by adding the refractive volumes of the individual amino acid residues. Since an amino acid analysis is seldom perfect, a correction is made by multiplying the refraction per 100 grams of protein by percentage recovery, which is obtained by adding the weight percentages of amino acid residues and dividing by 100. The mean refractive index of the protein, n_p , is then calculated by solving for n_p in Equation 2, where $[R]$ is the value obtained for the refractive volume of 100 grams of protein and M is equal to 100. Amide nitrogen is arbitrarily calculated as being combined with the glutamic acid residues; any excess is assigned to aspartic acid residues.

In this paper, the results of a systematic study of the refractive indices of the amino acids, and some peptides and proteins, are described. The value for the refractive index of a protein calculated from the refractive increments of its amino acid residues and solution volume agrees with the experimental value and is a characteristic of the protein. The change in the refractive index of a protein as a result of denaturation has also been investigated.

Materials and Methods

Amino Acids and Peptides. The amino acids and peptides used were high grade commercial products, further purified by recrystallization from alcohol-water mixtures, except when they were chromatographically pure. Glycolamide was prepared by passing dry ammonia into cold, freshly distilled ethyl glycolate. Pure lactamide was obtained from E. H. Harris of this laboratory.

Proteins. Crystalline lysozyme, bovine serum albumin, ribonuclease, and pepsin were obtained from the Armour Laboratories and crystalline β -lactoglobulin was prepared from skimmed milk. α -Lactalbumin was obtained from W. G. Gordon of this laboratory. Crystalline ovalbumin and human serum albumin were obtained from Nutritional Biochemical Corp. and purified pigskin gelatin from the Eastman Kodak Co.

Refractive Index Determination. The refractive indices of amino acids were determined by means of a dipping refractometer, using a sodium light, and also with the Brice-Hawler (4) differential refractometer. Concentrations of solutions were based on the dry weight of an aliquot at 110° C. and also on moisture determinations. The values obtained were in essential agreement. No difference was found between the DL-amino acids and the corresponding optically active amino acids; consequently, most of the measurements were made on solutions varying in concentration from 1 to 10%, depending on the solubility of the amino acid. No difference in refractive increment was found due to variations in concentration, except in the case of glycine solutions, where the difference could be correlated with variations in specific volume and were essentially eliminated by applying the appropriate volume for a given concentration of glycine. Cystine, tyrosine, and aspartic acid are not sufficiently soluble in water for accurate measurement of refractive indices. The molar refraction of cystine was calculated by using the molar atomic refractions (in cc.) given by Fajans (12): C 2.418, H 1.10, O 1.525, O⁻² 2.211, and N 2.322, and S 8.11 given by Cohen (8). The molar refractions of tyrosine and aspartic acid were estimated from the molar refractions of glycyl tyrosine and glycyl aspartate, respectively, by subtracting the refraction due to the glycyl residue. Protein solutions of 1 and 2% concentrations were used for making refractive index measurements. The values for water as a function of wavelength of light and temperature were taken from the International

4. McMEEKIN ET AL. *Refractive Indices of Amino Acids*

Table I. Refractive Indices, Molar Refractions of Amino Acids,
25°C

Amino Acid	Specific Volume ^a , Cc.	Refractive Index
Glycine	0.58	1.685
Alanine	0.68	1.606
α -Aminobutyric acid	0.74	1.587
Valine	0.79	1.571
α -Aminovaleric acid ^b	0.79	1.577
Leucine	0.83	1.565
Isoleucine	0.83	1.568
α -Aminocaproic acid	0.83	1.565
Serine	0.58	1.676
Threonine	0.66	1.618
Hydroxyproline	0.64	1.618
Proline	0.70	1.596
Methionine	0.71	1.646
Cystine	0.59	-
Phenylalanine	0.74	1.682
Tyrosine	0.68	-
Tryptophan	0.71	1.754
Histidine	0.64	1.700
Arginine	0.67	1.664
Lysine	0.74	1.615
Aspartic acid	0.56	-
Glutamic acid	0.63	1.655
Asparagine	0.59	1.691
Glutamine	0.64	1.670

^a (9).

^b Calculated from data of Craig and Schmidt (10).

^c 2 moles of water subtracted (7.46 cc.).

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and Calculated Refractions of Amino Acid Residues
($\lambda = 589 \text{ m}\mu$)

Amino Acid Observed	Residue (Ref. of Amino Acid - 3.73)	Refraction per G. Residue (<u>Mol. Ref. Res.</u>) (Mol. Wt. Res.), Cc.
16.54 ± 0.1	12.81	0.225
20.88 ± 0.15	17.15	0.242
25.67 ± 0.15	21.94	0.258
30.46 ± 0.13	26.73	0.270
30.72 ± 0.15	26.99	0.272
35.32 ± 0.15	31.59	0.279
35.60 ± 0.20	31.87	0.282
35.17 ± 0.15	31.44	0.278
22.89 ± 0.10	19.16	0.220
27.55 ± 0.10	23.82	0.236
29.57 ± 0.10	25.84	0.229
27.47 ± 0.10	23.74	0.245
38.18 ± 0.05	34.45	0.263
56.04	48.58 ^c	0.238
45.94 ± 0.15	42.21	0.287
48.07	44.34	0.272
58.97 ± 0.30	55.24	0.297
38.35 ± 0.15	34.62	0.253
43.20 ± 0.10	39.47	0.253
37.83 ± 0.2	34.10	0.266
28.54	24.81	0.216
33.80 ± 0.15	30.07	0.233
29.82 ± 0.20	26.09	0.229
34.10 ± 0.20	30.37	0.237

4. McMEEKIN ET AL. Refractive Indices of Amino Acids

Results

Amino Acids. The values for the refractive indices and molar refractions of the amino acids calculated from the refractive indices by Lorenz-Lorentz Equations 1 and 2 are recorded in Table I. Values for molar refractions of the aliphatic amino acids are in good agreement with values calculated from atomic refraction factors. However, the molar refractions of tryptophan, tyrosine, phenylalanine, and histidine are larger than those calculated from atomic refraction factors and larger than might be expected from their comparative specific volumes.

The refractivity per gram of amino acid residue (Table I) is obtained from the molar refraction of the amino acid by subtracting the value of 3.73 due to the loss of a molecule of water in forming the residue from the amino acid. This value is the sum of its atomic refraction factors, $2H = 2.2$ and $O = 1.53$. It is in essential agreement with the value deduced from the molar refractions of glycine and its peptides. The molar refraction of glycine is 16.54, of diglycine 29.89, of triglycine 41.33 and of glycyl leucine 48.04 (Table III). Thus, by difference, the loss of a mole of water in making diglycine decreases molar refraction by 3.19 and the loss of 2 moles of water in making triglycine decreases refraction by 4.15 per mole of water. A value of 3.8 cc. is obtained for water in the formation of glycyl leucine, giving an average value of 3.72 for water from the three peptides.

Specific Volumes and Refractive Indices of Proteins. The specific volumes and refractive indices, as well as the refractive indices calculated from the amino acid compositions of a number of proteins, are recorded in Table II. The refractive indices calculated by using the amino acid composition of the protein are in good agreement with the values as determined in the case of most proteins, except in the cases of α -casein, gelatin, and α -lactalbumin, where the measurements were made away from the isoelectric point. This difference could be due to a charge effect, as was found by Perlmann and Longsworth (26), or to inaccurate specific volumes, since the blank correction used in specific volume calculations from density determinations is of questionable applicability on protein solutions containing alkali. The close agreement between the determined value for the refractive index of ovalbumin was unexpected, since ovalbumin contains carbohydrate and no estimate is made of the refraction due to carbohydrate. The calculated refractive index is, however, based on the assumption that ovalbumin is composed entirely of amino acid residues, which indicates that the refraction of the carbohydrate does not greatly differ from that of the average amino acid.

The importance of specific volume or density in determining refractive index is apparent in Equations 1 and 2, which are used in calculating refractive index and molar refraction. This inverse relationship between specific volume and refractive index is illustrated in Table II (cf. columns 2 and 4). The necessity of obtaining an accurate value for the specific volume of a protein in order to obtain agreement between its refractive index calculated from the amino acid composition and the determined value can be illustrated in the case of ribonu-

ADVANCES IN CHEMISTRY SERIES

Protein	Solvent, pH	Refrac- tion/100 G.	Specific Volume, Cc.	Refractive Index	
				Detd.	Calcd. from amino acid comp.
α -Casein	NaOH, 7.0	25.50	0.728	1.618	1.607 ^a
Gelatin	Water, 5.0	24.27	0.682	1.630	1.618 ^b
α -Lactalbumin	NaOH, 7.0	25.64	0.735	1.615	1.601 ^c
β -Lactoglobulin	0.1M NaCl, 5.2	25.44	0.751	1.594	1.590 ^d
Lysozyme	Water, 6.0	25.34	0.718	1.624	1.620 ^b
Ovalbumin	Water, 5.0	25.35	0.745	1.596	1.593 ^b
Pepsin	Water, 5.0	24.91	0.725	1.603	1.605 ^e
Ribonuclease	Water, 4.8	24.66	0.693	1.630	1.630 ^f
Bovine serum albumin	Water, 5.0	25.32	0.734	1.606	1.599 ^g
Horse serum albumin	Water, 5.0	25.12	0.734	1.600	
Human serum albumin	Water, 5.0	25.28	0.736	1.603	1.602 ^b

^a(14); ^b(33); ^c(15); ^d(13); ^e(3); ^f(20); ^g(30).

lease, where a number of different values for its specific volume have been reported. Rothen's (28) value of 0.709 on the ribonuclease prepared by Kunitz is in good agreement with that calculated from its amino acid composition. Using ribonuclease obtained from the Armour Laboratory, Buzzell and Tanford (6) found 0.728 for its specific volume, while Harrington and Schellman (17) reported 0.692 to 0.696. Since the reported variations in the specific volume of ribonuclease are large, it was desirable to determine the specific volume on the sample used. The value of 0.695 found is in excellent agreement with the value of Harrington and Schellman (17). Its nitrogen content, found to be 16.6%, is also in agreement with 16.8% found by Harrington and Schellman. The value for the specific volume of ribonuclease of 0.693 was used in calculating its refractive index from the amino acid composition. In the case of pepsin, no value for its specific volume could be located. The specific volume of a 2% solution was found to be 0.725 cc., also in agreement with the 0.725 calculated from its amino acid composition. The remainder of the values for specific volumes given in Table II were taken from our previous compilation (24).

Refractive Indices of Peptides. To determine the effect of peptide formation on refractive index, the refractive indices of several peptides were determined (Table III). The average molar refraction of water produced in peptide formation can be estimated, empirically, by subtracting the observed molar refraction of the peptide from the sum of molar refractions of its constituent amino acids.

Tyrosine and aspartic acid are not sufficiently soluble for accurate measurements of the refractive indices of their solutions. Consequently, their molar refractions have been estimated from their more soluble glycyl peptides. From the results given in Table III, the molar refractions of tyrosine (48.07) and aspartic acid (28.54) are obtained by

4. **McMEEKIN ET AL.** *Refractive Indices of Amino Acids*

Substance	Specific Volume, Cc.	Refractive Index	Molar Refraction	
			Obsd.	Calcd. with Atomic factors
Glycine	0.581 ^a	1.685	16.54	16.38
Diglycine	0.584 ^a	1.702	29.89	29.09
Triglycine	0.600 ^a	1.649	41.33	41.38
Glycylleucine	0.741	1.606	48.04	47.52
Glycyltyrosine	0.664	1.694	60.73	54.10
Glycylaspartate	0.557	1.706	41.2	39.82

^a (9).

subtracting the calculated molar refraction of the glycyl residue [12.66 (molar refraction of glycine minus the refraction of 1 mole of water)] from the refraction of the peptide. The molar refractions calculated for the peptides in Table III are in good agreement with the observed except in the case of the tyrosine peptide and to a lesser extent the aspartic acid peptide; consequently, it is felt that the values for the molar refraction of tyrosine and aspartic acid deduced from experimental measurements on their peptides are more accurate than the values calculated from atomic factors. Since our preliminary publication (25), the molar refractivity of glycyl aspartate has been redetermined on a highly purified sample. The value of 41.2 cc. for its molar refraction is about 2% lower than that previously reported. This has reduced the value for the molar refractivity of the aspartic acid residue from 26.06 to 24.81. Since aspartic acid is present in considerable amounts in proteins, the lowering of the value for its refractivity has slightly reduced the calculated values for the refractive indices of proteins given in Table II, as compared to the previously published values (25).

Comparison of Molar Refractions of Amino Acids with Their Uncharged Isomers. Numerous comparisons have been made between the properties of amino acids and the properties of their nonzwitterion isomers (9). By comparing the solution densities of glycine and its isomer, glycolamide, and alanine with its isomer, lactamide, it has been found that the amino acid occupies about 13 cc. per mole less volume than its uncharged isomer. Consequently, it is also of interest to compare the solution refractive indices of these amino acids with their isomers. Table IV shows that the refractive indices of the amino acids are considerably greater than those of their uncharged isomers; however, the molar refractions are the same within the experimental variation. This is because the larger specific volume of the uncharged isomer compensate for its lower refractive index in calculating the molar refraction by Equation 2. It can be concluded that electrostriction of water by an amino acid in solution increases its refractive index but has no effect on its molar refraction and that the molar refractions do not change significantly with temperature.

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Temp., °C.	Substance	Specific Volume ^a , Cc.	Refractive Index	Molar Refraction, Cc.
5	Glycine		1.691	16.85
	Glycolamide		1.516	17.00
	Alanine		1.615	21.13
	Lactamide		1.496	21.47
25	Glycine	0.58	1.685	16.58
	Glycolamide	0.75	1.506	16.73
	Alanine	0.68	1.606	20.88
	Lactamide	0.83	1.490	20.99
40	Glycine		1.676	16.38
	Glycolamide		1.506	16.73
	Alanine		1.612	21.06
	Lactamide		1.489	21.21

^a Specific volumes obtained at 25° also used to calculate refractive index values at 5° and 40°.

Effect of Ionization on the Refractive Index and Molar Refraction of Amino Acids and Proteins. Since the electrostriction produced by an amino acid does not affect its molar refraction, the ionization of an amino acid might be expected to produce no significant change in molar refraction. Table V indicates that this is the case, provided the large change in the volume of the amino acid as a result of ionization, found by Kauzmann, Bodanszky, and Rasper (23), is used in calculating molar refraction. The refractive index of an equivalent concentration of hy-

Table V. Effect of Ionization on the Refractive Index and Molar Refraction of Alanine and Ovalbumin
25° ($\lambda = 589 \text{ m}\mu$)

Substance	Refractive Index	Specific Volume, Cc.	Molar Refraction, Cc.
Alanine	1.615	0.682	21.1
Alanine ⁺ Cl ⁻	1.539	0.767	21.4
Alanine ⁻ Na ⁺	1.473	0.924	22.0
Ovalbumin			
pH 4.6	1.600	0.745	11,470
pH 3.8	1.598	0.747	11,475
pH 3.2	1.593	0.749	11,425
pH 2.5	1.592	0.753	11,466
pH 2.0	1.588	0.757	11,466

4. **McMEEKIN ET AL.** *Refractive Indices of Amino Acids*

drochloric acid or sodium hydroxide was used instead of water in calculating molar refraction of the salts of alanine. Similarly, the data on the refractive index and molar refraction of ovalbumin in Table V indicate that acidification reduces the refractive index of ovalbumin; but if its volume changes, reported by Kauzmann, are taken into consideration, the molar refraction of ovalbumin does not change on acidification. This lack of change of molar refraction with charge may appear to be contrary to the findings of Perlmann and Longworth (26) on the effect of charge on the refractive increment of proteins. However, this is not necessarily true, since they only calculated refractive increments. Their results do not take into consideration volume changes which take place with change in charge, reported by Kauzmann (22).

Specific Refractive Increments of Proteins. The specific refractive increments, $(n - n_0)c$, of a number of proteins have been determined by Armstrong et al. (2), Perlmann and Longworth (26), Halwer, Nutting, and Brice (16), and Charlwood (7). These are of practical value in determining the concentration of protein solutions by means of the refractive index of the solution and have an accuracy of about $\pm 0.5\%$, as stated by Halwer, Nutting, and Brice (16). Consequently, values in Table VI are given to only three significant figures. In general, the

Table VI. Specific Refractive Increments of
Certain Proteins at 25°
 $(n - n_0)c$, g/ml

Protein	Wavelength		
	589 $m\mu$	546 $m\mu$	436 $m\mu$
Gelatin	0.184	0.186	0.191
α -Lactalbumin	0.188	0.189	0.195
β -Lactoglobulin	0.180	0.181	0.187
Lysozyme	0.184	-	-
Ovalbumin	0.178	0.181	0.185
Pepsin	0.177	0.182	0.188
Ribonuclease	0.185	0.186	0.192
Bovine serum albumin	0.183	0.188	0.193
Horse serum albumin	0.177	0.185	0.191
Human serum albumin	0.180	0.186	0.188

values listed in Table VI agree with those reported by Halwer, Nutting, and Brice (16) and Charlwood (7) for the same proteins.

Refractive Index of Proteins at Different Wavelengths. The refractive indices of amandin and heamocyanin were found by Putzeys and Brosteaux (27) to vary with the inverse square of the wavelength. A similar relation for the specific refractive increments of a number of proteins was reported by Perlmann and Longworth (26). The refractive indices of several proteins are plotted as a function of the inverse square of wavelength for three wavelengths ($m\mu$) in Figure 1. The slopes are essentially the same for the proteins listed and differ

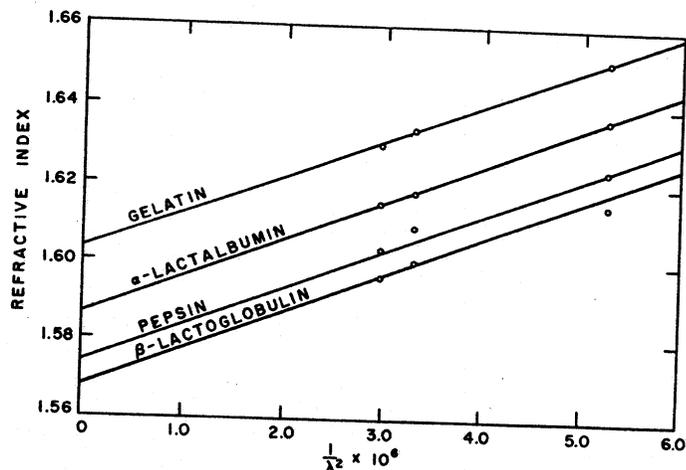


Figure 1. Variations in refractive index of proteins with wavelength in millimicrons, $m\mu$

slightly from those reported by Putzeys and Brosteaux (27). The equation relating refractive index of the protein to wavelength is

$$n_p = B + (9.6 \times 10^3)/\lambda^2$$

where intercept B for gelatin, α -lactalbumin, pepsin, and β -lactoglobulin is 1.603, 1.586, 1.574, and 1.568, respectively.

Effect of Heat Denaturation on Refractive Index. Kauzmann (21) has pointed out that it should be possible to observe small changes in the index of refraction of a protein as a result of denaturation. In fact, he reported small changes in the refractive index of ovalbumin solutions denatured by urea, but, he found that these changes due to denaturation could be accounted for by accompanying changes in volume. Stauff and Rasper (29) also found small changes in the refractive index of chymotrypsinogen solutions on heating.

The effect of heat denaturation on the index of refraction of β -lactoglobulin solutions has been investigated. A 1% solution of β -lactoglobulin dissolved in phosphate buffer of pH 7 and 0.1 ionic strength (a half of which was sodium chloride) was denatured by heating at 80° for 150 minutes. These are the conditions used by Briggs and Hull (5) in their electrophoretic studies of the denaturation of β -lactoglobulin. The changes in refractive index due to denaturation were measured with the Brice-Halwer differential refractometer. An increase in the refractive index of a 1% solution of β -lactoglobulin was produced by heat denaturation, which amounted to a small but significant difference of 0.000017 in the refractive index between the heated and unheated solutions for the three wavelengths—436, 546, and 589 $m\mu$.

Kauzmann (21) has stated that denaturation is accompanied by a contraction of several hundred cubic centimeters per 100,000 grams of

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4. McMEEKIN ET AL. *Refractive Indices of Amino Acids*

protein. However, in the literature only urea denaturation is given. In the case of heat denaturation, no change in volume was found by Haurowitz (18), while Heymann (19) has reported an increase. We found no change in the specific volume of β -lactoglobulin due to heat denaturation; native β -lactoglobulin in veronal buffer, pH 8.5, and 0.1 ionic strength, had a specific volume of 0.755 and the denatured a value of 0.753.

Discussion

The essential agreement between the refractive indices of proteins calculated from their amino acid compositions and the experimentally determined values given in Table II is good evidence that the refractive index of protein solutions is largely determined by atomic refractory factors. This is in agreement with Tanford's (31) views concerning the fundamental factors determining the refractive index of protein solutions—that the refractive increment is a measure of local polarizability due to electron deformation and is little affected by long-range forces concerned in large molecules. However, the values calculated for the refractive indices of proteins tend to be slightly lower than the determined values. Thus, of ten proteins in Table II, eight give lower calculated than determined values for the refractive indices. This indicates that the spatial arrangement of the amino acid residues in a protein has a small effect on the refractive index of the protein, which is also indicated by the change in the refractive index of β -lactoglobulin on heat denaturation.

The small but significant increase in the refractive increment of heat-denatured β -lactoglobulin solutions is of the same order as found by Stauff and Rasper (29) for changes in the refractive index of heated chymotrypsin solutions. Kauzmann believes that this small change in refractive index can be accounted for by changes in volume. However, no change in specific volume of β -lactoglobulin solution due to denaturation could be detected by a method sensitive to changes in volume of the order of 0.3%. Consequently, it is felt that the small change in the refractive index of the β -lactoglobulin solution produced by heat denaturation is due to changes in polarizability rather than in volume.

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