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Studies on β-Lactoglobulins A, B, and C. I. Comparison of Chemical Properties*

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ABSTRACT: β-Lactoglobulins A, B, and C have been isolated, purified, and examined for amino acid composition. The previously described difference between β-A and β-B in content of aspartic acid, glycine, valine, and alanine is confirmed, and β-C is found to differ from β-B only in content of glutamine and histidine. β-Lactoglobulin C has 2 residues more of histidine and 2 residues fewer of glutamine than β-B per molecule of ~36,000 molecular weight. DEAE-cellulose column chromatography, using a phosphate buffer containing a NaCl gradient, has been extended to provide a separation of the three proteins. Photooxidation data and ultraviolet spectra are in agreement with the amino acid analyses.

An examination of the amino- and carboxyl-terminal sequences of the three variants yielded identical qualitative results; but a difference in the rate of release of amino acids from the carboxyl end of β-C as compared with β-A and β-B was observed. The conclusion is drawn that the amino acid substitutions among the β-lactoglobulins do not occur at or near the terminal portions of the peptide chain.

The genetically different bovine β-lactoglobulins A and B, discovered by Aschaffenburg and Drewry (1955, 1957a), have been chemically characterized by Gordon *et al.* (1961) and Piez *et al.* (1961). They found that the proteins differed in the content of four amino acids, β-A having 2 more aspartic acid and valine residues and 2 fewer glycine and alanine residues per mole than β-B. By studying peptides from chymotryptic digests of β-A and β-B, Kalan *et al.* (1962) showed that the differing amino acids are linked in pairs, aspartic acid being substituted for glycine and valine for alanine. The isolation and composition of the peptides containing the aspartic acid for glycine substitution were described but the valine for alanine substitution was not located. Townend *et al.* (1960a) have shown that each β-lactoglobulin molecule (~36,000 mw) consists of two identical chains of approximately 18,000 mw. Fraenkel-Conrat (1954, 1956) and Niu and Fraenkel-Conrat (1955) found, by chemical methods, 2 amino-terminal leucine residues and 2 carboxyl-terminal isoleucine residues per mole for mixed β-lactoglobulin. Neurath *et al.* (1954), using carboxypeptidase A, showed that histidine is the penultimate amino acid. Davie *et al.* (1959) investigated the kinetics of the release of isoleucine and histidine from a mixed preparation. Later work (Kalan and Greenberg, 1961) reported on the action of carboxypeptidases A and B on β-lactoglobulins A and B and their *S*-sulfo derivatives. These results revealed an identical C-terminal sequence, -Leu-His-Ileu, and identical rates of hydrolysis for both protein variants.

Bell (1962) reported the discovery of a third genetically determined β-lactoglobulin, β-C. This variant was shown to have a slightly slower mobility than β-B in starch-gel electrophoresis at *p*H 8.6. The immediate question arose as to the relationships in amino acid composition and structure of β-C to the previously analyzed A and B proteins. The opportunity to investigate the chemical properties of β-lactoglobulin C was

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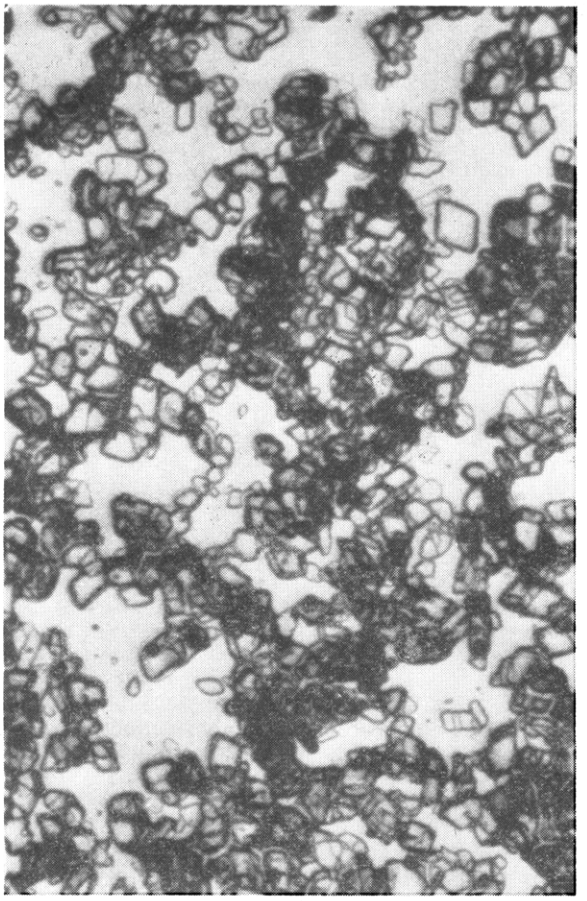

FIGURE 1: Photomicrograph of crystals of β-lactoglobulin C.

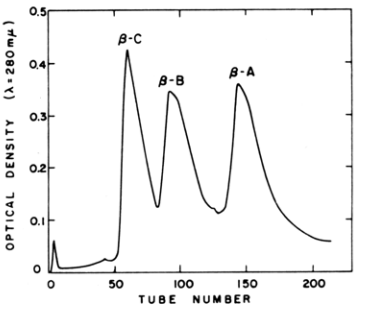

FIGURE 2: DEAE-cellulose column chromatography of β-lactoglobulins A, B, and C. Each tube contained 15–17 ml and flow rate was four tubes per hour. For elution gradient, see text.

provided by the location of a cow typed homozygous for this protein.

Concurrent with the study of the C protein, it was decided to reexamine β-A and β-B prepared from milks of single homozygous cows. A preliminary report of the results of this study has appeared (Kalan *et al.*, 1964) showing the finding of a single amino acid substitution between β-lactoglobulins C and B. This report presents in detail the results of the investigations of the chemical properties of β-lactoglobulins A, B, and C, including studies on the amino- and carboxyl-terminal end groups of the three variants.

Experimental

β-Lactoglobulins A and B were prepared according to the method of Aschaffenburg and Drewry (1957b) from milks of individual, genetically typed cows. The proteins were recrystallized four times, lyophilized, and stored at a constant humidity of 50%. The preparation of β-lactoglobulin C, also from a single typed cow, required slight modification of the above-mentioned isolation procedure. In the case of β-C, as contrasted with β-A and β-B, a considerable portion of the protein came down in the "*p*H 2.0 lactalbumin" fraction instead of remaining in solution. This necessitated careful reworking of this precipitate to achieve a separation of the β-lactoglobulin and α-lactalbumin. The precipitate was dissolved with dilute NH_3 solution, and the *p*H was adjusted to 3.5 with dilute HCl. At this point, the β-lactoglobulin did remain in solution and the α-lactalbumin precipitated. This filtrate was then treated as "F2" (Aschaffenburg and Drewry, 1957b) and the purification and crystallization were carried out in the same manner as with β-A and β-B. However, because of its great solubility, β-C must be in a reduced volume of very high concentration to effect the first crystallization. Recrystallization proceeded without difficulty and was repeated four times, followed by lyophilization. The crystals (Figure 1) in a slurry slowly dissolved at room temperature, and when allowed to dry out on a slide melted into a glassy film.

All three proteins were examined for purity by polyacrylamide-gel electrophoresis at *p*H 8.6 where a single band was obtained for each preparation. DEAE-cellulose column chromatography, as discussed later, resulted in a single peak for each protein and rechromatography of the material revealed elution behavior identical to the original.

The *S*-sulfo-β-lactoglobulins A, B, and C were prepared from the native materials by a modification (Weil and Seibles, 1959) of the method of Swan (1957). The sulfur content of the derivatives was 2.29, 2.30, and 2.49%, respectively, for *S*-β-A, *S*-β-B, and *S*-β-C.

Separation by Column Chromatography. The DEAE-cellulose column chromatography procedure of Piez *et al.* (1961) was modified to provide a separation of β-C as well as β-B and β-A. A 1.8- × 35-cm DEAE-cellulose column and increasing NaCl gradient in a constant phosphate buffer (0.05 M, *p*H 5.8) at room temperature were employed. Figure 2 shows the separation of a mixture of 200 mg of each protein using the following elution gradient: seven chambers, each of 500 ml of 0.01, 0.03, 0.05, 0.07, 0.09, 0.11, and 0.14 M NaCl in phosphate buffer. Rechromatography of individual peaks was carried out in the same manner.

Amino Acid Composition. Samples of each protein

TABLE I: Amino Acid Composition of β-Lactoglobulins.

Amino Acid	Residues per Mole of Protein								
	A		B		C		Nearest Even Integer		
	Average	95% Confidence Limits[a]	Average	95% Confidence Limits	Average	95% Confidence Limits	A	B	C
Aspartic acid	31.68	0.196	30.44	0.196	30.05	0.216	32	30	30
Threonine	16.2		15.9		15.6		16	16	16
Serine	13.6		13.5		13.6		14	14	14
Glutamic acid	49.11	0.314	50.56	0.294	48.16	0.216	50	50	48
Proline	17.47	0.118	17.24	0.133	17.40	0.110	16[b]	16	16
Glycine	6.19	0.037	8.23	0.049	8.20	0.063	6	8	8
Alanine	27.64	0.151	29.79	0.176	29.78	0.186	28	30	30
Half-cystine[c]	6.78	0.114	6.73	0.145	6.77	0.127	6	6	6
Valine	20.04	0.125	18.19	0.092	18.21	0.088	20	18	18
Methionine	7.99	0.049	7.98	0.049	7.95	0.049	8	8	8
Isoleucine	19.49	0.118	19.57	0.104	19.67	0.046	20	20	20
Leucine	42.44	0.216	43.02	0.240	43.12	0.250	43[d]	43	43
Tyrosine	8.03	0.053	8.03	0.051	8.00	0.055	8	8	8
Phenylalanine	8.05	0.047	7.97	0.049	8.06	0.049	8	8	8
Lysine	29.88	0.176	29.59	0.172	29.51	0.127	30	30	30
Histidine	3.98	0.037	3.95	0.067	5.97	0.043	4	4	6
Arginine	6.19	0.037	6.12	0.039	6.14	0.039	6	6	6
Tryptophan	3.9–4.6		3.9–4.6		3.9–4.5		4	4	4
Cysteine[e]							2	2	
Sulfur (%)	1.50		1.46		1.40				

[a] If the experiment were repeated, the average would be expected to be within the computed average ± the 95% confidence limits, 95% of the time. [b] Value set at lower even integer; see text. [c] Uncorrected for destruction during hydrolysis. [d] For discussion of uneven integer see text. [e] Value taken from Cole *et al.* (1958).

were hydrolyzed in 6 N HCl in sealed evacuated tubes at 110° for 24, 72, and 96 hours. The analyses were carried out using the automatic recording apparatus described by Piez and Morris (1960). All determinations were run in triplicate.

Amino-Terminal End Groups. The Edman degradation as applied for paper strips by Fraenkel-Conrat *et al.* (1955) and Schroeder *et al.* (1961) was employed for the qualitative determination of the N-terminal amino acids of the β-lactoglobulins. Chromatography with Sjøquist Solvent A (Fraenkel-Conrat *et al.*, 1955) and visualization with the starch-iodide detection method proved satisfactory for the identification of the PTH-amino acids released.[1]

The N-terminal group was verified by using the FDNB method of Sanger. The procedures of reaction with FDNB, hydrolysis, and identification of the DNP-amino acid were essentially those outlined by Levy (Fraenkel-Conrat *et al.*, 1955).

Carboxyl-Terminal Amino Acids. For the determination of the C-terminal amino acids of the native proteins (mw about 36,000) carboxypeptidase A was employed. Carboxypeptidase A was obtained from Worthington Biochemical Corp.[2] as a water suspension of three-times-recrystallized material. It was treated with DFP prior to use. The reactions were carried out at *p*H 8.0 in an unbuffered system at 37° with a weight ratio of enzyme to protein of 1:100. Aliquots of the digestion mixture were withdrawn at specific intervals ranging from 15 minutes to 72 hours. These were precipitated with 0.5 ml of 30% trichloroacetic acid and centrifuged in the cold, and the supernatants were decanted and frozen until analyzed. The amino acids released were identified and quantitated with the aforementioned automatic recording chromatographic apparatus using samples corresponding to 0.3 μmole of protein for analysis. The *S*-sulfo-β-lactoglobulins (mw about 38,000) were hydrolyzed with carboxypeptidase A in exactly the same manner as were the native materials.

Miscellaneous. Photooxidation was performed on all three β-lactoglobulins by the method of Weil *et al.* (1951). Approximately 15 mg of each protein was dissolved in 1.5 ml of phosphate buffer (0.067 M, *p*H 7.0), and 0.5 ml of solution containing 0.1 mg of methylene blue was added after equilibration at 10°. Ultraviolet

[1] Abbreviations used in this work: PTH, phenylthiohydantoin; FDNB, 1-fluoro-2,4-dinitrobenzene.

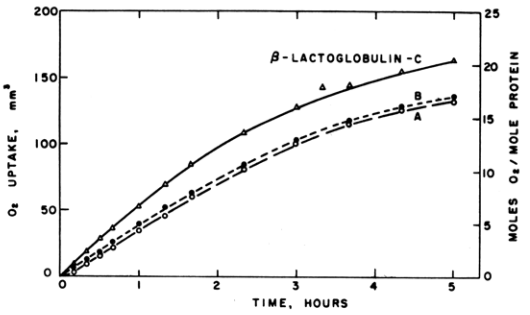

FIGURE 3: Photooxidation of β-lactoglobulins A, B, and C. See text for experimental conditions.

spectra of the proteins were examined in 0.15 M KCl solution at *p*H 5.2 in the Cary recording spectrophotometer Model 14.[2]

Results

A summary of the amino acid composition data is presented in Table I. For the majority of the amino acids the values represent the data of nine determinations—triplicates of 24-, 72-, and 96-hour hydrolysates. The residues per mole found in the table are grand averages which were obtained in the following manner. For each separate analysis, calculations of the molar ratios were made based on ten different amino acids (aspartic acid, glycine, alanine, methionine, leucine, tyrosine, phenylalanine, lysine, histidine, and arginine). Thus, ten values for each of the nine determinations or ninety values were then considered for each amino acid. From these were eliminated the nine values where the amino acid in question was considered as the base and assigned a whole number. The results reported for the ten amino acids mentioned are thus based on the average of eighty-one values. For half-cystine and proline ninety values were averaged. Glutamic acid, valine, and isoleucine were calculated by considering only the 72- and 96-hour values. Threonine and serine were obtained from the data, using linear regression analysis by the method of least squares. No attempt was made to oxidize the cysteine, probably present in the hydrolysates, to cystine. Therefore, since cysteine emerges under the proline peak in this analytical system, the proline values are predictably high. The cystine values are uncorrected for destruction during hydrolysis.

The last three columns of Table I clearly demonstrate the differences in amino acid composition among the three β-lactoglobulins. β-Lactoglobulin A has 2 more each of aspartic acid and valine residues and 2 fewer each of glycine and alanine residues than β-B. This confirms the previous work of Piez *et al.* (1961) and Gordon *et al.* (1961) on samples prepared from pooled milks. β-Lactoglobulin C is identical to β-B with respect to the above-mentioned four amino acids, but differs from it in one other pair. β-Lactoglobulin C has 2 more histidine residues and 2 fewer glutamic acid (or glutamine) residues than the β-B variant.

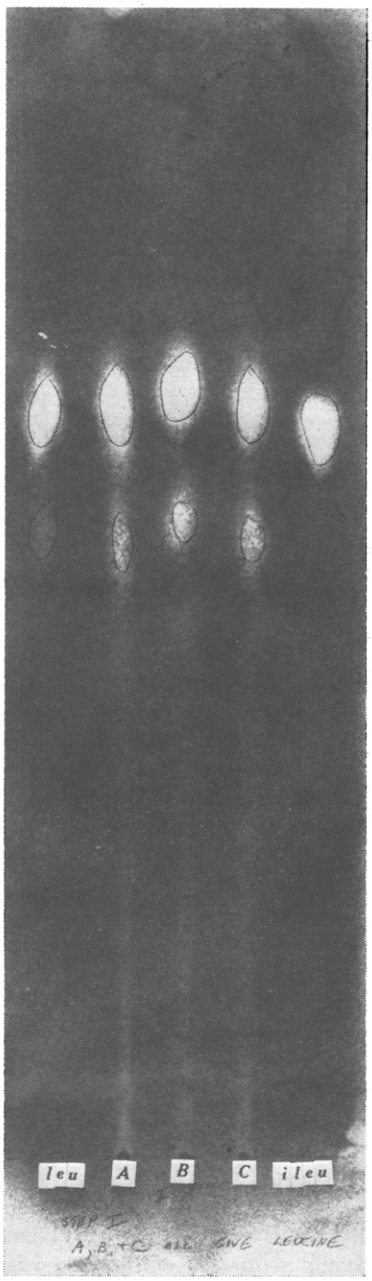

FIGURE 4: Chromatographic identification of the N-terminal PTH–amino acid released by the Edman procedure. From left to right: PTH-leucine, unknowns from β-lactoglobulins A, B, and C, and PTH-isoleucine.

The photooxidation of the proteins, as shown in Figure 3, presents results which can be correlated with the amino acid composition data. The increased rate of oxygen uptake for the C protein and the greater

[2] It is not implied that the U.S. Department of Agriculture recommends the above-mentioned company or its product to the possible exclusion of others in the same business.

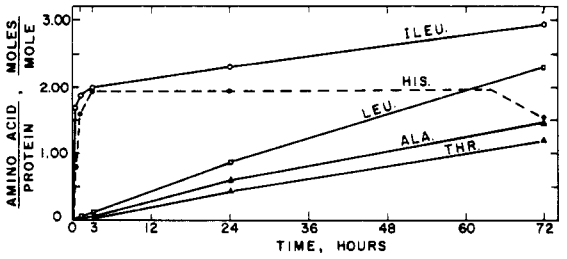

FIGURE 5: Hydrolysis of β-lactoglobulin A by carboxypeptidase A at *p*H 8.0, 37°, with enzyme-substrate ratio 1:100.

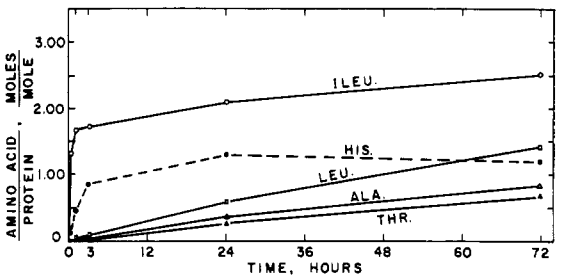

FIGURE 6: Hydrolysis of β-lactoglobulin C by carboxypeptidase A. Conditions same as in Figure 5.

extent of photooxidation are presumably due to its greater histidine content. The similarity of β-A and β-B is attributable to equal content of photooxidizable amino acids (methionine, histidine, tyrosine, and tryptophan).

The ultraviolet spectra of the β-lactoglobulins are included in the work of Townend *et al.* (1964). They are identical, and this confirms the equal content of tyrosine, cystine, and tryptophan. By using the ultraviolet absorptivities of Beaven and Holiday (1952) and Wetlaufer (1962) for these three amino acids, and the known content of cystine and tyrosine, it is possible to calculate the tryptophan. This was carried out for a range of specific extinction coefficients between 9.3 and 9.7 and for molecular weights between 35,500 and 37,700. The values for tryptophan in Table I, 3.9–4.6, are those which were obtained in this manner. The calculation using the widely accepted values, $\epsilon = 9.6$ and molecular weight of 36,500, produced a result of 4.1 tryptophan residues per mole. This agrees with the value of Brand *et al.* (1945) of 3.5 residues per 36,500 as determined by ultraviolet absorption of the tryptophan mercury complex, and satisfies the even-whole-number requirement of the two-identical-chain structure of the β-lactoglobulins.

The leucine value of 43 residues should be 44 to agree with the even-number concept. Piez *et al.* (1961) did find 44 residues and later data in this investigation confirmed their findings.[3]

Amino End. Chromatographic identification of the N-terminal amino acid as released by the Edman procedure is shown in Figure 4. Leucine is revealed as the N-terminal residue for all three β-lactoglobulins. It is clearly differentiated from isoleucine, although it has the same mobility, by the appearance of a double spot which is characteristic of leucine in the solvent system employed (Fraenkel-Conrat *et al.*, 1955). The second step of the degradation produced a trace of leucine from the first step and a strong spot of the next amino acid, threonine. The results were identical for A, B, and C. Further sequence could not be elucidated as the chromatograms became too complex for accurate interpretation.

[3] Presented in the next paper of this series.

The application of the FDNB procedure resulted in a spot at the position of DNP-leucine and/or DNP-isoleucine which are not resolvable under the chromatographic conditions utilized (Fraenkel-Conrat *et al.*, 1955). Basic hydrolysis and further chromatography of the unknown and standard DNP-amino acids were performed to distinguish between the two. The results confirmed the identity of the N-terminal amino acid as leucine, and agree with the previous determinations made by Fraenkel-Conrat (1954, 1956) and Niu and Fraenkel-Conrat (1955), which were carried out on mixed β-lactoglobulins. All three variants, therefore, begin with the same amino-terminal sequence, Leu-Thr-.

Carboxyl End. The results of the hydrolysis of the native β-lactoglobulins A, B, and C with carboxypeptidase A are shown in Figures 5 and 6. As β-A and β-B yielded identical results, only the figure for β-A is shown. In all three cases isoleucine was liberated first, with histidine next. This is in agreement with results of previous experiments on β-A and β-B (Kalan and Greenberg, 1961) and with the work of Davie *et al.* (1959) on mixed β-lactoglobulins. In contrast to these earlier findings of only isoleucine and histidine released from the native proteins by carboxypeptidase A, now a spectrum of amino acids was released in addition. These results are similar to those found when the combination of carboxypeptidases A and B was employed for hydrolysis (Kalan and Greenberg, 1961). This fact, combined with the finding of small amounts of lysine at 24 and 72 hours, leads to the conclusion that the enzyme in the present experiments was contaminated to a slight degree with carboxypeptidase B.

Leucine was the next amino acid released, followed by alanine, for all three β-lactoglobulins. Threonine and a second isoleucine occur in the carboxyl-terminal portion of the chain although their exact order cannot be ascertained from this experiment. The qualitative order of amino acids liberated from the three proteins is the same but, as can be observed from a comparison of the figures, the rate of release from β-C is greatly reduced. Histidine and leucine never reach a full two equivalents as they do with β-A and β-B. The apparent decrease of the histidine values after 24 hours is a phenomenon not previously observed and for which no explanation is proposed.

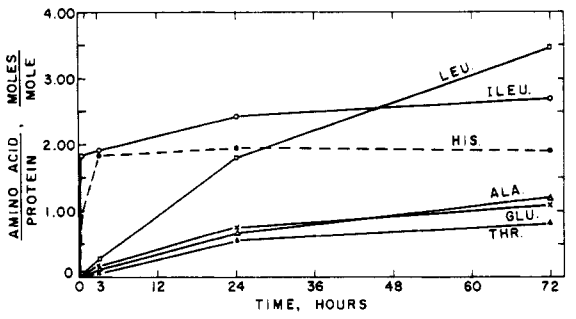

FIGURE 7: Hydrolysis of *S*-sulfo-β-lactoglobulin A by carboxypeptidase A. Conditions same as in Figure 5.

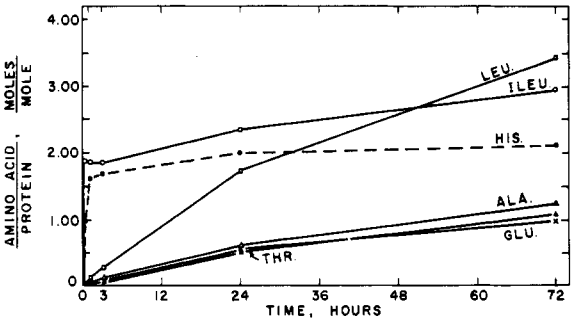

FIGURE 8: Hydrolysis of *S*-sulfo-β-lactoglobulin C by carboxypeptidase A. Conditions same as in Figure 5.

The hydrolysis of the *S*-sulfoproteins with the carboxypeptidase preparation (Figures 7 and 8) revealed essentially the same order of release of amino acids as the native material. However, leucine was liberated at a much more rapid rate, and glutamic acid, which previously appeared as a trace, now appeared to be of the same magnitude as threonine. The overall rates of hydrolysis were the same for the three sulfoproteins. *S*-Sulfo-B, again identical to *S*-sulfo-A, is not shown. The marked decrease in rate evident for native C was not seen with the derivatives.

Discussion

From the analysis it is evident that β-lactoglobulin C is a "mutant" or genetic variant of β-lactoglobulin B, having 2 residues of histidine more and 2 residues of glutamic acid (or glutamine) fewer than the B protein. Bell and McKenzie (1964) recently published similar findings. According to the genetic codes described by both Jukes (1963) and Eck (1963), either a single amino acid mutation involving histidine/glutamine or one involving histidine/glutamic acid is feasible. Several other factors seem to favor the choice of glutamine as more likely. On polyacrylamide-gel electrophoresis β-C has only a slightly slower mobility than β-B. The difference in mobility between β-A and β-B, which is attributed to the charge of the carboxyl groups of the 2 additional aspartic acid residues in A, is much greater than the difference between β-B and β-C. If the substitution in β-C involved 2 fewer glutamic acid residues, the difference in mobility might be expected to be as great as the β-A–β-B difference, or even greater, considering the contribution of the charge of the 2 extra histidine residues. This suggests that the substitution involves glutamine and the mobility difference is owing mainly to the presence of the 2 additional histidine residues in β-C. From these observations it is assumed in the subsequent discussion that the genetic difference between β-lactoglobulins B and C involves a glutamine/histidine substitution.

Similar results for β-lactoglobulins A, B, and C have been obtained upon examination of the N-terminal and C-terminal sequences. Therefore the amino acid substitutions among the variants do not occur at either the amino-terminal position or in the region close to the carboxyl end of the molecules. The only observable variation among the proteins in this regard was the markedly slower rate of hydrolysis of native β-C with carboxypeptidase. The difference in glutamine/histidine content between β-B and β-C has probably caused a modification in the tertiary structure which makes the latter protein more resistant to the action of the enzyme. Upon denaturation or when disulfide bonds are broken by derivative formation as with the *S*-sulfo compounds, all three proteins are hydrolyzed at the same rate, which is somewhat more rapid than that of the native materials.

It is interesting to note that in almost all cases isoleucine and histidine are released to a full 2 equivalents in 3 hours while the sequential amino acids are liberated at a much slower rate. This behavior made possible the isolation and crystallization of derivatives lacking the last 2 amino acids, as described in the next paper of this series.

The requirement of the presence of carboxypeptidase B to liberate amino acids beyond the penultimate and terminal locations without the finding of a basic residue in the third position has been discussed in connection with earlier work on β-A and β-B (Kalan and Greenberg, 1961). This was ascribed to changes in the tertiary structure caused by the action of the combination of enzymes, an explanation still considered valid.

Kalan *et al.* (1962), using high-voltage paper electrophoresis, located the peptides containing one of the substitutions (glycine/aspartic acid) between β-lactoglobulins A and B from proteolytic digests of these proteins. The chemical characterization of β-C as a genetic variant of β-B containing a histidine/glutamine substitution provides a base for further investigations to locate this substitution within the primary structure.

The photooxidation experiment becomes of interest as a tool with which to study some other comparative structural properties of the β-lactoglobulins. Weil and Buchert (1951) made a comprehensive examination of the disappearance of the susceptible amino acids during photooxidation of mixed β-lactoglobulins. The specific destruction of histidine, tryptophan, and

perhaps other amino acids in this manner might be used in connection with further study of the tetramerization mechanism as described by Townend *et al.* (1960b), Townend and Timasheff (1960), and Timasheff and Townend (1961), and of the failure of the β-lactoglobulin variants to hybridize (Townend *et al.*, 1961).

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