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The Heterogeneity of Bovine β -lactoglobulin

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Ogston & Tilley (1955) reviewed previous work on the heterogeneity of bovine β -lactoglobulins. A number of workers had concluded, on the evidence of electrophoresis, solubility and immunological measurements, that β -lactoglobulin is heterogeneous. Partial fractionation has been reported by Polis, Schmukler, Custer & McMeekin (1950), by Smithies (1954) and by Préaux, Hulsmans & Lontie (1954).

The meaning of much of this work has been made questionable by the discovery by Aschaffenburg & Drewry (1955) that one or both of two distinct β -lactoglobulins may occur in the milk of individual cows, their occurrence being determined genetically. It now appears likely that in all the work done with β -lactoglobulin prepared from pooled milk both β -lactoglobulins were present, and that the reported fractionations may have been of the two from each other. Only Ogston & Tilley (1955), of previous workers, are known to have worked on material prepared from the milk of single cows; they distinguished 'normal' and 'abnormal' types of β -lactoglobulin by the effect of the concentration of the protein on the electrophoretic pattern.

We describe here measurements made on samples of the two β -lactoglobulins, prepared from the milk of single cows, and on mixtures of the two; our object was to find how far previous observations on heterogeneity can be explained by the properties of these two proteins.

EXPERIMENTAL

Nomenclature. The nomenclature of Aschaffenburg & Drewry (1955) is used here: the protein migrating faster by paper electrophoresis at pH 8.6 is called β_1 -lactoglobulin, and the slower β_2 -lactoglobulin. Polis *et al.* (1950) used the terms β_1 - and β_2 -lactoglobulin for subfractions which appear to bear no simple relationship to the fractions as defined by Aschaffenburg & Drewry.

Typing of cows. Samples of milk from identified cows of the herd of the University of Oxford Department of Agriculture were typed by a slight modification of the method of Aschaffenburg (personal communication). Whole milk warmed to 40° was treated with anhydrous Na_2SO_4 (20 g./100 ml.); after being cooled to 25° it was filtered. The filtrate contained only the whey proteins; these were precipitated with $(\text{NH}_4)_2\text{SO}_4$ (20 g./100 ml. of filtrate) and filtered with the aid of Hyflo Supercel (Johns-Manville Co. Ltd., Artillery Row, London, S.W. 1). The mixture of precipitated proteins and Supercel, with a little water to make it fluid, was dialysed against water for 24 hr. The Supercel was then removed by filtration. The type or types of β -lactoglobulin in the filtrate were then found by paper electrophoresis at pH 8.6.

Preparation of lactoglobulins. Milk was obtained in 5 l. batches from individual cows. Each batch was received within 24 hr. of milking and was processed at once. β_1 - and β_2 -Lactoglobulins were prepared from milk identified as containing only β_1 - or β_2 -lactoglobulin by a slight modification of the method of Aschaffenburg & Drewry (1957); the final, concentrated, solution of lactoglobulin was dialysed first against acetate buffer, pH 5.2 (0.12*M*-sodium acetate; 0.04*M*-acetic acid) and then against three 2 l. batches of

glass-distilled water, saturated with toluene. Crystallization, or separation of oil, took place in 2–5 days. Preparations of β_2 -lactoglobulin were seeded with orthorhombic crystals from a previous preparation. The yields obtained at the first crystallization were 3–4 g./5 l. of milk.

Concentration. Concentrations (g./100 ml. of solution) were determined refractometrically (Cecil & Ogston, 1951); the value 1.80×10^{-3} was taken for the specific refractive increment in the buffers used (Ogston & Tilley, 1955).

Ultraviolet absorption. This was measured in 1 cm. matched cells in a Hilger Uvispek spectrophotometer.

U-tube electrophoresis. A Hilger Tiselius apparatus was used under approximately the same conditions as those used by Ogston & Tilley (1955). Measurements were made in Ogston & Tilley's acetate buffer (pH 4.66, I 0.1). The 3.5 ml. cell was most often used with a current of 10 ma at about 400 v and a bath temperature of 1°. The proportions of the fast and slow components in the descending boundaries were determined as described by Ogston & Tilley (1955).

Paper electrophoresis. This was measured in an apparatus similar to that described by Flynn & De Mayo (1951). Solutions were applied with a paint brush in a band 2 cm. wide, either at the apex or (for longer runs) half way between the apex and the cathode buffer, to strips of Whatman no. 4 chromatography paper, 4 cm. $\times$ 35 cm. The current was usually 10 ma/4 cm. width of paper at 500 v, applied for 10–15 hr. The strips were stained with bromophenol blue [1% (w/v) in 90% (v/v) ethanol containing 10 g. of HgCl_2 /100 ml.] for 10 min. and then washed with 2% (v/v) acetic acid and tap water until the background was clear. Most runs were done in veronal buffer, pH 8.6, I 0.05 (10.3 g. of sodium diethylbarbiturate and 1.84 g. of diethylbarbituric acid/l.); some were done in acetate buffer (pH 4.6, I 0.05) or in acetate-HCl buffer, pH 3, I 0.05.

Sedimentation. This was observed in a Svedberg oil-turbine ultracentrifuge at 900 rev./sec. at approximately 26°. The optical system of Baldwin (1953) was used. Solutions were approximately 1% in buffer, pH 5.14 (Cecil & Ogston, 1948). The sedimentation coefficients were measured by the method of Cecil & Ogston (1948). The areas of the boundary schlieren curves were measured by trapezoidal integration from a base line determined in a separate run (Baldwin, 1957).

pH measurements. These were made at room temperature (18°) with a Cambridge glass electrode and valve voltmeter calibrated against potassium hydrogen phthalate, pH 4.00. AnalR reagents were used for all solutions.

Titration curves. Samples (2 ml.) of protein dissolved in M-KCl were titrated with 0.103N-HCl or 0.111N-NaOH (CO_2 -free) by using a microburette made from a Conway tube. The pH was found as described. After each titration the pH meter was restandardized against phthalate (pH 4.00) and if any drift had occurred the results were discarded. The titration vessel could be sealed or rotated but not both. For acid titration mixing was by rotation, for alkali titration the vessel was sealed and mixing was achieved by bubbling CO_2 -free nitrogen. Frothing was suppressed by a drop of octanol. The reference electrode was Ag/AgCl in saturated KCl, with a 0.1M- NH_4NO_3 bridge. The pH readings over the whole range were steady for 10 min. The initial pH of the solutions varied between 5.2 and 5.3.

RESULTS

Of the six cows typed, one produced only β_1 -, one produced only β_2 - and four produced both β_1 - and β_2 -lactoglobulin.

Attempted separation of β_1 - and β_2 -lactoglobulins by recrystallization. Mixtures were made up in 0.1M-NaCl containing (i) 4.6 g. of total protein/100 ml. (11% β_1 , 89% β_2); (ii) 3.8 g. of total protein/100 ml. (27% β_1 , 73% β_2); (iii) 2.9 g. of total protein/100 ml. (52% β_1 , 48% β_2). They were recrystallized by dialysis against glass-distilled water for 24 hr. Crystallization was not quite complete under these conditions. Crystals were then spun out and redissolved in 0.1M-NaCl. Each was recrystallized four times. Solutions of the crystals, and the supernatant solutions, were examined by paper electrophoresis at pH 8.6 to determine the relative proportions of β_1 - and β_2 -lactoglobulins. For this purpose, scanning of the stained strips with a densitometer proved unsatisfactory, because of incomplete resolution of the β -lactoglobulins on the paper. It was found, however, that the proportions in an unknown sample could be estimated with fair accuracy by visual comparison of the stained strips with those obtained by electrophoresis of known mixtures.

The supernatants from the first recrystallizations of (i) and (ii) contained almost exclusively β_2 -lactoglobulin. The second recrystallization of the crystals from (i) again gave a supernatant containing almost exclusively β_2 -lactoglobulin: the second recrystallization of the crystals from (ii) gave a supernatant containing both β_1 - and β_2 -, but an excess of β_2 -lactoglobulin as compared with the crystals. Recrystallization of (iii) and subsequent recrystallizations of (i) and (ii) gave no further changes of composition. It appears, therefore, that recrystallization under those conditions can reduce the proportion of β_2 -lactoglobulin to about 50%, if it is initially higher, but will not reduce it further.

Sedimentation. The boundary schlieren curves were symmetrical; plots of (area/height)² against time were strictly linear, the slopes being: β_1 , 4.10×10^{-5} ; β_2 , 3.84×10^{-5} . Baldwin (1957) has shown that heterogeneity of sedimentation rate causes curvature of this plot; in the absence of curvature the slope is determined largely by the diffusion coefficient. Both lactoglobulins had the same sedimentation coefficient 2.77×10^{-13} sec. at 1 g./100 ml.

U-tube electrophoresis. β_1 - and β_2 -Lactoglobulins, and mixtures of them, were investigated. All showed boundaries of the general character of those observed by Ogston & Tilley (1955); the descending boundaries were broad, showing partial resolution into two or more components, and the ascending boundaries had very sharp advancing fronts and

appeared to be self-sharpening. Typical tracings are shown in Fig. 1. The boundaries obtained with β_1 -lactoglobulin closely resembled those observed by Ogston & Tilley with their 'normal' samples; they were resolvable into fast and slow components in proportions depending upon concentration (Fig. 2) in a manner again closely resembling the 'normal' samples of Ogston & Tilley. The boundaries obtained with β_2 -lactoglobulin were markedly different in shape, showing larger proportions of slow component (Figs. 1, 2), and the possibility that three components might be resolved by sufficiently prolonged electrophoresis. Mixtures gave boundaries intermediate in character between those of β_1 - and β_2 -lactoglobulins; the variations of the proportions of fast and slow components with concentration resembled those shown by Ogston & Tilley's 'abnormal' samples.

Other methods of electrophoresis. Paper electrophoresis never gave more than one band for either β_1 - or β_2 -lactoglobulin under a variety of conditions. At pH 3.0 and 4.6 at room temperature the mixture

gave only one band, with considerable trailing. Smithies (1955*a*) noted that lactoglobulin gave two bands in the starch gel described by him. Use of his method (Smithies, 1955*b*) showed that β_1 -lactoglobulin gave only one band, with a considerable amount of trail. β_2 -Lactoglobulin likewise gave only one, and the mixture gave two.

Titration curves. The acid or alkali reacting with the protein was determined by subtracting from the titre of the protein solution at each pH the corresponding blank titre, corrected for difference of total volume; since the volumes of titrant solutions were small the effect of dilution on the ionic strength was neglected.

The curves (Fig. 3*a*) suggest that β_1 -lactoglobulin has over 50 acid-binding groups, while β_2 -lactoglobulin has about 40, calculated on the basis of a molecular weight of 35 000 (Cecil & Ogston, 1949). A high absolute accuracy is not claimed for these curves.

Ultraviolet absorption. The absorption curves of β_1 - and β_2 -lactoglobulins in 0.1*N*-NaOH are shown in Fig. 4. Significant differences appear in the region of 280 μ . Analysis of these curves for the contributions of tyrosine and tryptophan by the method of Goodwin & Morton (1946) gave for β_1 -lactoglobulin: tyrosine 5.5 g. mol., tryptophan 3.2 g. mol.; and for β_2 -lactoglobulin: tyrosine 6.2 g. mol., tryptophan 3.5 g. mol./35 000 g. of protein.

Fig. 1. Tracings of typical electrophoretic boundaries. A, β_1 -Lactoglobulin (1.18 g./100 ml.) after 93 min.; B, β_2 -lactoglobulin (3.64 g./100 ml.) after 88 min.; C, mixture of β_1 - and β_2 -lactoglobulins, from a cow which produced both in approximately equal amounts (1.64 g. of total protein/100 ml.) after 103 min. For conditions of electrophoresis, see text. The starting positions are represented by vertical lines O in A.

Fig. 2. Variations in the proportion of slow component in electrophoresis with concentration of protein, in acetate buffer (pH 4.66, *I* 0.1). ●, β_1 -Lactoglobulin; ●, β_2 -lactoglobulin; ○, mixture of β_1 - and β_2 -lactoglobulins, from a cow producing both in approximately equal amounts.

Fig. 3a. Titration curves of β -lactoglobulins with acid (see text). Each titration started at pH 5.2. $\bullet$, β_1 -Lactoglobulin; $\bullet$, β_2 -lactoglobulin; $\circ$, mixture of β_1 - and β_2 -lactoglobulins, 40:60 by weight; $\circ$, mixture of β_1 - and β_2 -lactoglobulins, 22:78 by weight.

Fig. 3b. Titration curves of β -lactoglobulins with alkali (see text). Each titration started at pH 5.2. $\bullet$, β_1 -Lactoglobulin; $\circ$, β_2 -lactoglobulin.

Fig. 4. Ultraviolet-absorption curves of β_1 -lactoglobulin (broken line) and β_2 -lactoglobulin (full line). The measurements were made at 0.07 g./100 ml. in 0.1 M-NaOH; the extinctions plotted have been calculated for 1.0 g./100 ml.

DISCUSSION

Measurements made on separate β_1 - and β_2 -lactoglobulins show that they are closely similar in some respects, and distinctly different in others. Thus their crystallographic dimensions differ very little from each other (Green, North & Aschaffenburg, 1956) and they readily form mixed crystals under conditions which include those ordinarily used for the preparation of β -lactoglobulin. Both appear homogeneous in the ultracentrifuge at 26°, their sedimentation rates being identical, and it appears from the similarity of the rate of spreading of their sedimentation boundaries that their diffusion coefficients must be closely similar. Their molecular weights are therefore probably identical, as the crystallographic data of Green *et al.* (1956) suggest. On the other hand, they have different solubilities, different titration curves and different electrophoretic behaviour, and their absorptions in the ultraviolet are different. These observations are consistent with the two proteins differing somewhat in their amino acid composition.

In spite of their differences of solubility, separation is very difficult. Our experiments show that, after the removal of excess of β_2 -lactoglobulin, fractional crystallization at pH 5.2 produces no further separation; examination by paper electrophoresis of the sample, nine times crystallized, on which Smithies (1954) made most of his measurements showed that it contained a substantial proportion of β_2 -lactoglobulin. It is therefore possible that the partial fractionations reported by Polis *et al.* (1950) and by Smithies (1954) by salting-out, and by Ogston & Tilley (1955) by electrophoresis, consisted of the fractionation of β_1 - and β_2 -lactoglobulins from samples which contained both;

unfortunately, it cannot be ascertained whether the sample used by Ogston & Tilley in this experiment was a 'normal' one. The differences of solubility and of other properties reported by Jacobsen (1949) and by Grönwall (1942) could well have arisen from the presence of different proportions of β_1 - and β_2 -lactoglobulins in the samples studied. All these observations could therefore be consistent with the observed heterogeneity of β -lactoglobulin being due to the presence of β_1 - and β_2 -components.

The fact remains that both β_1 - and β_2 -lactoglobulins show complex boundaries in U-tube electrophoresis, the property which first (Li, 1946) raised doubts about its homogeneity. Comparison of our present results (Figs. 1, 2) with those of Ogston & Tilley (1955) make it clear that their 'abnormal' samples must have contained varying proportions of β_1 - and β_2 -lactoglobulins; the occurrence of such samples, and probably the electrophoretic fractionation reported by Ogston & Tilley, may therefore be explained by the existence of these two components. It is also clear, however, from the identity of the boundary patterns and the identical variations of the proportions of 'fast' and 'slow' electrophoretic components that Ogston & Tilley's 'normal' samples consisted of pure β_1 -lactoglobulin.

Now Ogston & Tilley argued that, although the presence of two electrophoretic boundaries is due in part to association of 'slow' into 'fast' material, a behaviour reproduced in sedimentation at low temperature, confirmed by Townend & Timasheff (1956), there must be two chemically distinct components even in the 'normal' samples. Otherwise, the proportion of 'fast' (associated) material should approach 100% as the concentration of protein is raised, instead of reaching a limit of about 70–80%. This conclusion is unaffected by the work of Gilbert (1955), who has shown that two boundaries will be observed in an associating system, even when the association-dissociation equilibrium is rapidly established: although his theory predicts that a slow boundary will be present at all concentrations, its size should reach a limiting value at a certain critical concentration (at which the fast boundary first appears), so that the proportion of 'fast' boundary should approach 100% at high concentration. Since it appears likely that Ogston & Tilley's 'normal' samples contained only β_1 -lactoglobulin, it is possible that β_1 -lactoglobulin is itself heterogeneous. The evidence of the constant-solvent solubility curve found by Ogston & Tilley for a normal sample supports this possibility.

The material isolated by Polis *et al.* (1950) in small yield by salt fractionation does not appear to have had electrophoretic properties corresponding with those of either β_1 - or β_2 -lactoglobulin.

We conclude that, although much of the work on the heterogeneity of β -lactoglobulins can be ex-

plained by the presence of β_1 - and β_2 -components in varying proportions in the samples studied, there remains evidence of heterogeneity which cannot be so explained; and that it is possible that both β_1 - and β_2 -lactoglobulins are mixtures of closely related proteins.

SUMMARY

1. Samples of β_1 - and β_2 -lactoglobulins, and mixed samples, have been prepared from the milk of individual typed cows.

2. β_1 - and β_2 -Lactoglobulins appear to be identical in sedimentation behaviour and similar in molecular weight, but to differ in solubility, ultra-violet absorption, titration curves and electrophoretic properties. Both give complex electrophoretic boundaries.

3. The two proteins are very hard to separate by crystallization: a 9-times crystallized sample contained a substantial proportion of β_2 -lactoglobulin.

4. The bearing of the properties of these proteins on previous observations on the heterogeneity of β -lactoglobulin is discussed. It is concluded that their existence and properties can explain most of, but not all, these observations, and that it remains possible that β_1 - and β_2 -lactoglobulins are themselves heterogeneous.

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