the best solvent for the reaction. Compounds 1–6 and 13–15 in Table II and compounds in Table III were all found subject to further reaction, *i.e.*, thiazoline formation and/or rearrangement (see text). The method designed to limit this secondary reaction in the sensitive isothiuronium compounds is given below for the synthesis of compound 1 (Table II).

S,2-Aminoethylisothiuronium·Br·HBr.—Thiourea (alcohol-soluble, recrystallized, 76.1 g., 1.0 mole) was added to 400 ml. of hot isopropyl alcohol (reagent ACS containing 0.5% water) in a 1-liter flask fitted with an efficient condenser, and was refluxed gently for 5 minutes to effect almost complete solution. Recrystallized 2-bromoethylamine·HBr (205 g., 1.0 mole) was added, and the refluxing continued. All was in solution within a few minutes and after about 10 minutes, crystallization of the product began, accompanied by vigorous boiling of the solvent. The reaction mixture was heated for an additional 20 minutes; the precipitated AET was filtered, washed with isopropyl alcohol and then ethyl acetate, and dried *in vacuo;* yield 230 g., *i.e.*, 82% of theoretical; m.p. 193–194° (ref. 34; m.p. 193–194°). A pure product requiring no recrystallization was obtained by this method if the starting materials were of high quality. The dry compound was stable under normal conditions of temperature and humidity (*i.e.*, a shelf life of at least a year in tightly closed bottles). AET is hygroscopic, however, under continued exposure to high humidity; it absorbs water, cakes together, and is converted in significant amounts to 2-aminothiazoline—a transformation that can be detected by a drop in melting point by as much as 30°.

Isothiuronium compounds also have been prepared by refluxing the reactants for many hours in ethanol,[34] propanol,[35] butanol[36] and toluene.[37] In our experiments, isopropyl alcohol, acetonitrile, absolute ethanol, and mixtures of absolute ethanol and ethyl acetate were the best solvents in terms of speed of reaction, yield and purity of products obtained.

2-Aminothiazoline·HBr.—AET·Br·HBr (28.1 g., 0.1 mole) was dissolved in 100 ml. of water, refluxed one-half hour and evaporated *in vacuo* to dryness. The residue was recrystallized twice from an isopropyl alcohol–ethyl acetate mixture; yield 15.5 g., 85%, m.p. 175–176°. A mixed melting point with an authentic sample of 2-aminothiazoline·HBr was unchanged. This compound was also obtained if AET was prepared in solvents containing an appreciable amount of water and refluxed for a long time; *e.g.*, 16 hours reflux in ethanol gave a 70% yield of the thiazoline.

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OAK RIDGE, TENN.

[CONTRIBUTION FROM THE KERCKHOFF LABORATORY OF BIOLOGY AND THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY OF THE CALIFORNIA INSTITUTE OF TECHNOLOGY]

Electrophoresis and Ultracentrifuge Studies of Milk Proteins. I. β_1- and β_2-Lactoglobulin[1,2]

BY HENRY KLOSTERGAARD AND R. A. PASTERNAK

RECEIVED MARCH 11, 1957

β_1- and β_2-lactoglobulin have been isolated from milk of individual cows. The properties of these two distinct protein species have been studied by electrophoresis and ultracentrifuge methods. The electrophoretic patterns indicate that at *p*H 4.8 both β_1- and β_2-lactoglobulin are inhomogeneous. Under similar conditions in the ultracentrifuge only β_1-lactoglobulin showed evidence of inhomogeneity.

Introduction

The whey protein, β-lactoglobulin, has been studied extensively since its isolation by Palmer[3] in 1934. It was considered to be a homogeneous protein, largely because of its easy crystallizability. The homogeneity of β-lactoglobulin was, however, made doubtful by the discovery of Li[4] that the protein showed composite electrophoretic boundaries near its isoelectric point.

Since then, many observations have indicated that the usual β-lactoglobulin preparations are composed of two species. Polis, *et al.*,[5] succeeded by repeated fractional crystallizations in getting in small yield a fraction which appeared to be electrophoretically homogeneous. This fraction was assumed to consist of one protein species only; it was called β_1-lactoglobulin. Polis, *et al.*, were unsuccessful in isolating the second species in pure form. Fractions were obtained which according to the electrophoretic pattern were not homogeneous but only enriched in the second component, β_2, and it was assumed that some residual β_1-lactoglobulin was still present.

The existence of two species of β-lactoglobulin also was suggested by the observation of Block and Zweig[6] that two bands appeared in paper electrophoresis of the protein in barbitol buffer at *p*H 8.6.

Recently β-lactoglobulin was reinvestigated extensively by Ogston and Tilley.[7] They concluded that β-lactoglobulin was composed of two very similar species, one of which dimerized reversibly.[8]

Aschaffenburg and Drewry[9] recently discovered by the use of paper electrophoresis as a test procedure that the whey of individual cows differed in its protein composition; some samples showed the two β-lactoglobulin bands, others only one or the other. These authors isolated the two proteins in crystalline form.

In the present study, two cows were selected such that the milk of one contained β_1-lactoglobulin alone and the milk of the other β_2-lactoglobulin alone. From these two sources the two β-lactoglobulins were isolated in crystalline form. Each

(1) Contribution No. 2156 from the Gates and Crellin Laboratories of Chemistry.
(2) Supported by the Carnation Company and by a grant from the National Science Foundation.
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was investigated for homogeneity by ultracentrifuge and electrophoretic techniques over ranges of temperature, *p*H and concentration.

Experimental

Paper Electrophoresis.—Twenty-four samples of raw milk[10] from individual cows were screened to find sources of milk containing one of the lactoglobulins but not the other. The samples for the screening test were prepared by removing the casein and part of the whey proteins by half saturation with $(NH_4)_2SO_4$. The lactoglobulins were precipitated by increasing the degree of saturation of the whey with $(NH_4)_2SO_4$ to 80%, and the precipitate was dialyzed against distilled water. The protein concentration of the solution was about 1%. A Spinco paper electrophoresis unit was used with barbitol buffer, $I = 0.1$, *p*H 8.6; 10 γ of the protein solution was applied to the paper strips. Electrophoresis for 16 hours at a potential gradient of about 7 v. cm.$^{-1}$ resulted in a fair resolution of the lactoglobulins.

Isolation and Purification of Lactoglobulins.—For both β_1- and β_2-lactoglobulin, an isolation procedure essentially identical with that used for β-lactoglobulin by Palmer[3] was originally tried. With β_2 no difficulties were encountered and good crystals were obtained; they appeared under the microscope to be identical in shape with β-lactoglobulin crystals from pooled milk. Preparations of β_1-lactoglobulin, however, could not be induced to crystallize nor to precipitate as an oil from the whey at a *p*H near its isoelectric point when dialyzed against distilled water. The protein was finally brought to crystallization in the form of hexagonal prisms; the method employed will be reported elsewhere.

The two proteins were recrystallized repeatedly and stored as wet crystals at about 5°.

Preparation of Solutions.—The solutions for each electrophoresis or ultracentrifuge run were prepared freshly from the wet crystals and equilibrated by dialysis in the cold for at least 20 hours against a large volume of buffer. On prolonged standing β_2-lactoglobulin solutions became turbid, indicative of instability of that protein. The *p*H of the solutions were determined at room temperature with a glass electrode; the approximate protein concentrations were measured with a differential refractometer.

Boundary Electrophoresis Experiments.—A micro-cell of the Perkin–Elmer unit was used with a current of 9 ma., the potential gradient depending on the resistance of the solutions. Usually the runs lasted until the boundaries had swept through the entire cell. At least two photographs were taken, one halfway through and one at the end of the run. Mobilities were computed from the displacement of the maxima without correcting for overlap of peaks.

Ultracentrifuge Experiments.—The Spinco ultracentrifuge Model E was used at 59780 r.p.m. nominal speed. For runs at room temperature advantage was taken of the automatic temperature control. In the runs at lower temperature a drift of about 1° per hour occurred, for which a correction was introduced into the calculations. Photographs were taken at intervals of 16 minutes. They were evaluated with a micro-comparator, the centers of the peaks were measured at three different heights with the double purpose of increasing the accuracy and checking the symmetry of the boundaries. Except for β_1-lactoglobulin at 8°, all the boundaries were symmetrical within the accuracy of the measurements. The sedimentation constants were calculated in intervals; no systematic variation of the constant for an individual run was detected.

In the case of β_1-lactoglobulin at 8°, the sedimentation constant of the fast component was calculated from the displacement of the maximum; that of the slow component was estimated only roughly from the final photograph.

Results and Discussion

There is some evidence from the Aschaffenburg, *et al.*, experiments that the two species of β-lactoglobulin produced simultaneously by some cows are the same as those produced separately by other cows. In screening milk from a number of cows for the present study it was observed that the β-lactoglobulin fraction in about 50% of the cases yielded two spots (A fast and B slow) in paper electrophoresis. Using a standardized procedure all A spots could be superimposed, as could the B spots. In the other 50% of the cases only one spot was obtained. It would coincide closely to either an A or B spot.

(10) All milk was supplied by the courtesy of the Carnation Milk Farms, Carnation, Wash.

In this investigation the procedure of Polis, *et al.*,[5] was used to isolate a fraction rich in β_1-lactoglobulin. This protein was subjected to paper electrophoresis along with mixtures of it with the β-lactoglobulins isolated from milks which in paper electrophoresis showed a single component only. The results of this experiment showed that β_1-lactoglobulin is the slower moving component in barbitol buffer at *p*H 8.6; the nomenclature of the present paper is based on this finding. Aschaffenburg, *et al.*,[9] have used the opposite nomenclature, but the reasons for their choice are not clear. Additional data on electrophoretic mobilities and isoelectric points favoring our choice are given below. The present work falls naturally into two sections, ultracentrifuge study and electrophoretic study, and will be reported in that order.

Ultracentrifuge Study.—In order to minimize charge effects, acetate buffer at *p*H 4.8 was used, this *p*H differing only slightly from the isoelectric points of the proteins; for evaluating the effect of temperature the experiments were carried out both at room temperature and at approximately 8°. Tracings of the ultracentrifuge patterns are shown in Fig. 1 and the data are listed in Table I.

TABLE I

SEDIMENTATION CONSTANTS OF β-, β_1- AND β_2-LACTOGLOBULIN IN ACETATE BUFFER, $I = 0.1$, *p*H 4.8

	Concn., %	Temp., °C.	$S_{20} \times 10^{13}$
β	2.5	25	2.80
β_1	1.50	29.0	2.88
β_1	1.50	8.0 to 9.3	3 and 4.6
β_2	1.65	28.4	2.68
β_2	1.65	7.0 to 8.2	2.65

The ultracentrifuge patterns (Fig. 1) indicate that β_2-lactoglobulin is homogeneous over the temperature range of the experiment. On the other hand, β_1-lactoglobulin at 8° is clearly inhomogeneous; at 29° β_1 shows no double boundary, yet, compared to β_2 of similar concentration, the boundary is broader and the calculated sedimentation constant is higher. An inhomogeneity in one of the lactoglobulins was anticipated by Ogston and Tilley.[7] The pattern of β_1-lactoglobulin at 8° suggests that this protein undergoes reversible aggregation. If the two boundaries in this pattern arose from two species with no interdependence, a better resolution would have been expected in view of the indicated difference in sedimentation constants.

The data in Table I indicate that in ultracentrifuge experiments (at 8°) with pooled milk the β_2-species and the slow component of β_1, having about the same sedimentation constants, would move together as one boundary, and that the fast component of β_1 would move ahead as a single boundary by itself. This conclusion is in agreement

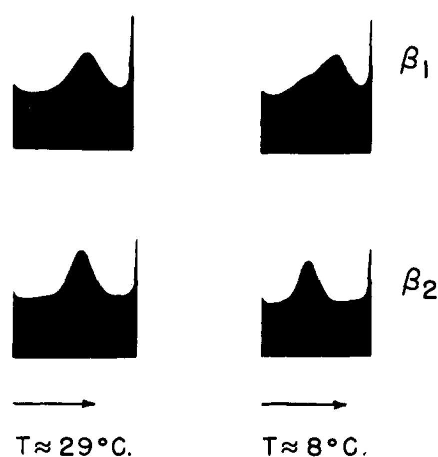

Fig. 1.—Ultracentrifuge patterns of β_1- and β_2-lactoglobulin, about 2 hours after start of run. Diaphragm angle 60° for β_2 at 8°, 55° for the other patterns. Nominal speed 59780 r.p.m.; concentration of β_1, 1.50%; of β_2, 1.65%.

with the observation of Ogston and Tilley who found approximately 2.8 and 4.57 for the sedimentation constants of the slow and fast moving components, respectively.

Electrophoretic Study.—Both lactoglobulins show inhomogeneity in boundary electrophoresis. The degree of inhomogeneity varies with protein concentration and *p*H; moreover β_1 and β_2 behave differently.

Data from experiments in acetate buffer at *p*H 4.85 at various protein concentrations are given in Table II and Figs. 2 and 3. In these experiments β_1-lactoglobulin (Fig. 2) shows only one peak, but the peak is certainly unsymmetrical at higher concentration. More detailed information on molecular structure of proteins would be required in order to make a reliable prediction of the effect of dimerization on electrophoretic mobility. Therefore it is not possible to state that the unsymmetrical peak obtained in electrophoresis of β_1 is at all related to the aggregation indicated by the ultracentrifuge pattern.

TABLE II

ELECTROPHORETIC MOBILITIES OF β_1- AND β_2-LACTOGLOBULIN IN ACETATE BUFFER, I = 0.1, *p*H 4.85

	Protein concn., %	$\mu \times 10^5$ Descending	Ascending
β_1	2.4	1.3	2.9
	1.0	1.7	2.5
	0.4	1.9	2.1
	1.0[a]	1.8[a]	
β_2	1.25	1.0 and 3.4	3.1
	0.6	1.2 and 3.0	2.5
	0.25	1.5 and 2.2	1.9
β[a]	1.0[a]	3.0[a]	

[a] Data from Polis, *et al.*[5]; *p*H 4.8.

In the case of β_2-lactoglobulin (Fig. 3) the electrophoretic heterogeneity is indicated by two peaks which differ markedly in their mobilities. Evidently in the solutions used in these experiments β_2-lactoglobulin was present mainly in two molecular forms, the relative quantities of which depend on the total protein concentration. Unlike

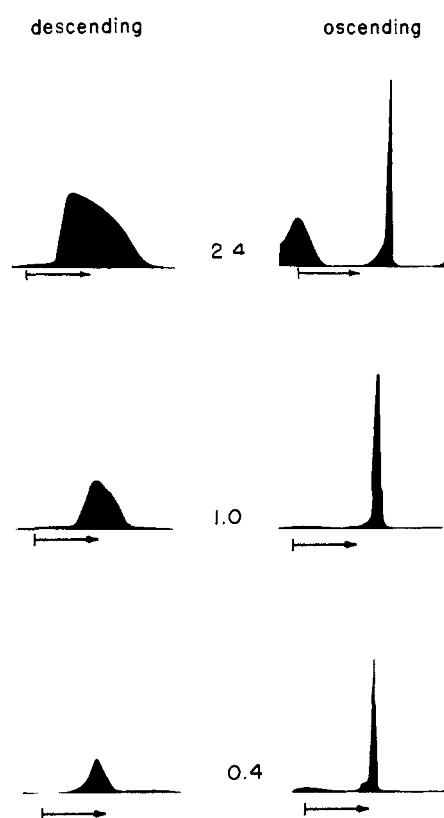

Fig. 2.—Electrophoresis patterns of β_1-lactoglobulin in acetate buffer I = 0.1, *p*H 4.85. Photographs are taken at approximately the same time after start of run. The numbers signify protein concentration in per cent.

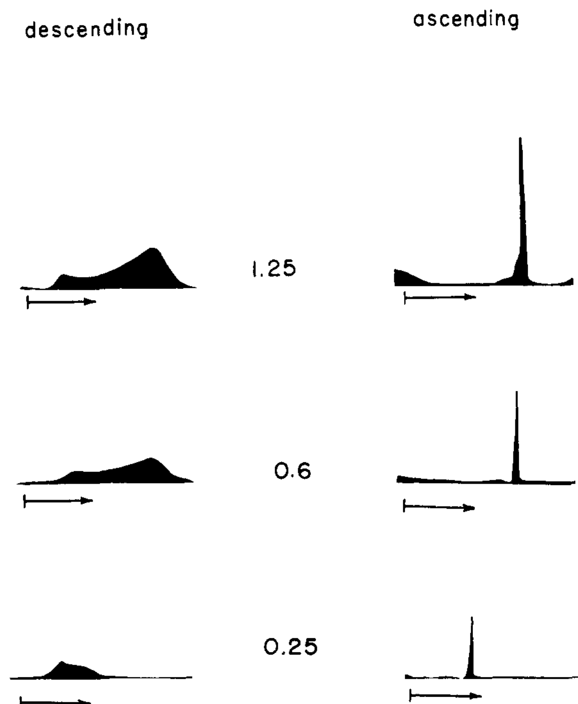

Fig. 3.—Electrophoresis patterns of β_2-lactoglobulin analogous to Fig. 2.

the case of β_1, the ultracentrifuge experiment with β_2-lactoglobulin gives no evidence of molecular

aggregation and therefore the complex electrophoretic pattern must be due to some other phenomena. Apparently the absolute amount of the slow component present in the electrophoretic cell remained approximately constant over the range of concentration examined. Table II also contains some data taken from Polis, *et al.*[5] At 1% protein concentration they found a mobility of 1.8×10^{-5} for β_1 at *p*H 4.8 compared to 1.7×10^{-5} at *p*H 4.85 in the present study. For the *fast* moving component they indicate a mobility of 3.0×10^{-5}; the fast component of β_2 in the present study has a mobility of approximately 3.0×10^{-5} at about 0.5% concentration.

Isoelectric Points.—In Table III are listed the electrophoretic mobilities of the two proteins in acetate buffer at *p*H's near their isoelectric points. From these data the isoelectric points were calculated for both the ascending and descending sides; the averages of these values are reported in the table as the isoelectric points of the β_1- and β_2-lactoglobulins.

TABLE III

ELECTROPHORETIC MOBILITIES OF β_1- AND β_2-LACTOGLOBULIN NEAR THE ISOELECTRIC POINT IN ACETATE BUFFER $I = 0.1$

Concentration of β_1, 0.5%; of β_2, 1.1%

	*p*H	$\mu \times 10^5$ Desc.	Asc.	
β_1	5.20	+0.1[a]	+0.4[a]	
	5.32	− .6	− .8	*p*I = 5.23
β_2	5.13	− .4	.0	
	5.23	− .9	− .5	*p*I = 5.09

[a] Differences primarily due to boundary asymmetry.

The difference in isoelectric point may not be impressive, but it is real. It is therefore of interest to compare these isoelectric points with the values of Polis, *et al.*,[5] who found *p*I = 5.3 for β_1-lactoglobulin and *p*I = 5.1 for pooled β-lactoglobulin.

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[CONTRIBUTION FROM THE KERCKHOFF LABORATORY OF BIOLOGY AND THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY OF THE CALIFORNIA INSTITUTE OF TECHNOLOGY]

Electrophoresis and Ultracentrifuge Studies of Milk Proteins. II. α-Lactalbumin[1,2]

BY HENRY KLOSTERGAARD AND R. A. PASTERNAK

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α-Lactalbumin shows various degrees of heterogeneity in boundary electrophoresis at *p*H's outside its isoelectric range. The degree of heterogeneity depends also on the protein concentration and the buffer ions. In the ultracentrifuge α-lactalbumin is homogeneous at room temperature and at 7°. The isoelectric point has been found to be *p*I = 5.1, which lies outside the *p*H range of minimum solubility.

Introduction

The name lactalbumin has been applied to various protein preparations derived from milk even before criteria were adopted for the practical definition of the terms albumin and globulin in protein chemistry. A crystalline material which he called lactalbumin was observed and reported first by Wichmann.[3] He removed the casein and crude globulins by precipitation with magnesium sulfate and acid and obtained crystals from the remaining albumin fractions by addition of ammonium sulfate and acid. Sjögren and Svedberg[4] obtained crystals in a similar way. In both cases, the term lactalbumin was applied to the crystals, but it is now clear that the crystals were not pure and that they consisted primarily of the protein now called β-lactoglobulin.

Pedersen[5] in his extensive ultracentrifuge studies found several components in bovine whey; he called the slowest moving component α, the next β, etc. His β is found in the term β-lactoglobulin, which it represents. The α-component was isolated by Kekwick in 1935 (unpublished results quoted in reference 5). Its sedimentation constant was determined[6] and reported to be $S_{20} = 2 \times 10^{-13}$. The term α-lactalbumin was first used by Svedberg.[6] Sørensen and Sørensen[7] gave a procedure for isolating a "crystalline insoluble substance" from bovine whey, and showed that it was not β-lactoglobulin. This procedure was the basis of the method used by Gordon and Semmett,[8] who suggested the name α-lactalbumin for such preparations. Further variations of this method have been described by Gordon, *et al.*,[9,10] and by Zweig and Block.[11]

The present authors first became interested in α-lactalbumin as an attractive protein for X-ray diffraction investigations; it has a low molecular weight (about 16,000) and readily forms large crystals which can be obtained in abundance. At first, the work reported in the present paper was undertaken as an investigation of a possible heterogeneity of the protein, an indication of which was

(1) Contribution No. 2155 from the Gates and Crellin Laboratories of Chemistry.

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