Note added in Proof. While this paper was in the press, we noted the work of Werner & Odin (1952) on the reaction of diphenylamine with crystalline sialic acid. The absorption curve which they report for the coloured complex produced is very similar to that shown above in Fig. 4, and it appears likely that our method of estimating urinary mucoprotein depends mainly upon its sialic acid content.

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Studies on the Heterogeneity of Crystallized β-Lactoglobulin

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In spite of evidence to the contrary, it seems to have been assumed that β-lactoglobulin of cow's milk is sufficiently homogeneous to make its amino acid analysis (Brand & Kassell, 1942; Brand, Saidel, Goldwater, Kassell & Ryan, 1945; Stein & Moore, 1949) and its physico-chemical properties (Cohn & Edsall, 1943) worth measuring. This view may have been encouraged by the ease with which clean crystalline preparations can be made.

Evidence for its heterogeneity has come primarily from electrophoretic studies. Pedersen (1936) first reported that it migrated as a single component over a wide range of pH; these measurements were made at 20° and at low concentration of protein. All subsequent work has been at 0·5–2° and at higher concentration of protein (Li, 1946, 1951; Polis, Schmukler, Custer & McMeekin, 1950; McMeekin, Polis, DellaMonica & Custer, 1948; Alberty, Anderson & Williams, 1948; Bain & Deutsch, 1948; Deutsch, 1950; Jacobsen, 1949; Smithies, 1954). Direct comparison of the results of these workers is difficult because of the variety of conditions of pH and ionic strength used; however, all agree that β-lactoglobulin migrates as two or sometimes three components and that two main components can be distinguished in acetate and acetate–chloride buffers of pH 4·2–4·8. Polis *et al.* (1950) showed that the ratio of acetate to chloride appears to affect both the mobilities and the proportions of the two components at pH 4·8. Some authors have also commented on a marked difference between the ascending and descending boundaries, especially at pH 4·8 and lower. Lundgren & Ward (1949) have suggested that the heterogeneity might be due to contamination with other whey proteins, but Polis *et al.* (1950) rejected

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this suggestion; the electrophoretic properties of known whey proteins do not support it.

Solubility measurements at constant ionic strength have been made by Sørensen & Palmer (1938) and Grönwall (1942). These showed little variation of solubility with the amount of protein, but at least twice the amount of protein needed to saturate the solution was used. Polis *et al.* (1950) state that the solubility varies with small excess amounts of protein. Smithies (1954) has used the 'variable solvent' test to show the presence of two main components, and possibly one minor component. Other solubility measurements (see Jacobsen, 1949) have shown differences between different samples under identical conditions, and Jacobsen has shown that such samples may differ in chemical composition.

McMeekin *et al.* (1948) claimed to have separated fractions with differing electrophoretic behaviour. Polis *et al.* (1950) reported the isolation in 1 % yield of a fraction which migrated as a single component at pH 4·8, with a mobility equal to that of the slower component seen in ordinary crystalline samples at this pH. It seems doubtful whether the determinations were accurate enough to support the claim that the N content and molecular weight of this fraction were significantly different from those of the parent material (cf. Halwer, Nutting & Brice, 1951). The difference of molecular weights of two samples claimed by Christensen (1952) is certainly not statistically significant (Smithies, 1953).

Deutsch (1950) and Smithies (1954) have obtained immunological evidence of heterogeneity.

There is thus ample evidence that crystalline β-lactoglobulin is not a single homogeneous protein. There is little to indicate the nature of its heterogeneity, apart from Polis *et al.*'s (1950) isolation of a fraction in very small yield. It must be remembered that methods of preparation have varied slightly, Palmer (1934) using alkali to dissolve crystals before recrystallization, while most later workers (see Bull & Currie, 1946) have used neutral salt solutions. Most preparations have been made from pooled milk, which may have obscured a natural variation of the proportions of the components.

METHODS

Preparation of β-lactoglobulin. Milk was collected at the time of milking from the T.T. Guernsey herd of S. E. Howse and Sons, Botley, Oxford. One- or two-gallon batches were used, the whole of each batch, or in a few cases nearly the whole, coming from a single cow in mid-lactation. The milk was brought uncooled straight to the laboratory and was twice separated on a de Laval separator, using the 39·5 mm. gravity ring; 200–300 ml. left in the separator bowl was rejected. The skimmed milk was worked up by the method described by Smithies (1953), with the following modifications: (i) preliminary dialysis of the lactoglobulin solution was against acetate buffer, pH 5·2, $I = 0·2$; (ii) subsequent dialysis was against de-gassed, glass-distilled water, saturated with toluene; (iii) after 2–3 days of (ii), but before precipitation began, the solution was centrifuged at 12 000 rev./min. to remove a small amount of a grey amorphous sediment.

All samples gave on dialysis an oil of high protein content, but different samples differed in their subsequent behaviour. If the oil did not crystallize spontaneously within a few days, it was seeded with crystals from a previous batch. If this failed, the oil and supernatant were suspended in $(NH_4)_2SO_4$ (27·8 g./100 ml.), and the precipitate was again dialysed against pH 5·2 buffer and water. Recrystallization was performed by dissolving crystals in 1·0 or 0·1 M-NaCl, or in pH 5·2 buffer, and dialysing against water. Electrophoresis of the supernatant from the first recrystallization gave a complex boundary pattern, showing that considerable amounts of other whey proteins had accompanied the first crystallization. Most samples were therefore recrystallized 2 or 3 times before use. The final products were either freeze-dried or kept as crystals in water under toluene at 1°. In all, thirteen separate preparations were made. Difficulty in crystallization was always experienced with samples which did not have the normal electrophoretic composition.

Solutions were usually made by dissolving wet crystals in buffer and dialysing against a large volume of buffer at 1°. For solubility measurements (q.v.) freeze-dried protein was dissolved directly in the buffer or salt solution.

Estimation of concentration. Concentration was estimated with the refractometer of Cecil & Ogston (1951), using the procedure of Smithies (1953). A value of $1·82 \times 10^{-3}$ at 546 mμ. was taken as the specific refractive increment against water, on the basis of concentration g./100 ml. of solution (Pedersen, 1936; Halwer *et al.* 1951; Johnston, 1945); this corresponds to $1·80 \times 10^{-3}$ in the buffers used.

pH *of buffers.* This was determined at 18° with a Cambridge glass electrode and valve voltmeter, calibrated against KH phthalate pH 4·00 (British Standards Institution (1950), 1647). AnalaR reagents and glass-distilled water were used.

Electrophoresis. Standard Hilger equipment was used. The biprism and mask were removed from the camera and the cylindrical lens was moved into the conjugate position nearer the camera lens, giving approximately a 4-times greater schlieren magnification without appreciable loss of optical performance. A 10 ml. Hilger U-tube was used in most of the work. The usual current at 1° was 10 mA. The normal bath temperature was 0·5° maintained by thermostatic control of the refrigerator. When required, the bath was warmed by means of a 3 kw immersion heater; the refrigerator was cut off during the heating period (45 min. from 0·5 to 17°) but full stirring was maintained. This rise of temperature could be produced without any sign of disturbance of the boundaries in the U-tube. When the desired temperature had been reached (1–1·5° below room temperature), the bath was again controlled by the refrigerator, with stirrers and filter-pump running at maximum speed. The bath temperature then became constant within ±0·1° after the first half hour. Cooling again to 0·5° required 4–5 hr. Current was not passed while the temperature was being changed.

A preparative 'flow-through' cell (Kekwick, Lyttleton, Brewer & Dreblow, 1951) was used for electrophoretic fractionation at 1°.

Sedimentation in the ultracentrifuge. Sedimentation at *ca.* 25° was observed in a Svedberg oil-turbine ultracentri-

fuge at 900 rev./sec. by the method of Cecil & Ogston (1948), using the optical system described by Baldwin (1953). Four measurements were performed for us by Dr J. M. Creeth (Courtauld Institute of Biochemistry, Middlesex Hospital) on a Spinco model E analytical ultracentrifuge, two of which were at *ca.* 5°.

Solubility. This was measured in dilute salt or buffer, by equilibrating freeze-dried solid with solvent. The problem of achieving mixing of solid and solvent, while avoiding denaturation, was solved, after preliminary experiments, as follows: freeze-dried protein was weighed into 5 ml. Pyrex test tubes, each containing a 4 mm. Pyrex bead, tube and bead having been first cleaned in conc. HNO_3, rinsed in dil. NaOH, thoroughly washed with dist. water and oven-dried. Solvent (4–4·5 ml.) was weighed into each tube. Each tube was then cautiously evacuated in a desiccator, to dislodge and remove air bubbles and to wet the solid. Care was taken not to boil the solvent; the loss of weight was never greater than 0·25% of solvent. Each tube was then nearly filled with paraffin (B.P.) and a carefully cleaned rubber bung, having a 1 mm. hole, was pushed firmly in. Displaced paraffin was removed and the hole was finally sealed with Chatterton cement. In this way, all air bubbles were excluded.

The tubes were tapped, to mix the contents, and were placed in Terry clips on the periphery of a 6 in. diam. wheel (axis horizontal) in a water bath at $20 \pm 0{\cdot}1°$; the wheel was rotated at 120 rev./min. After 24–72 hr., the tubes were removed; solid was centrifuged down; the supernatant solutions were removed and their concentrations measured refractometrically against pure solvent; 24 hr. was sufficient for equilibration.

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

EXPERIMENTS AND RESULTS

Electrophoresis at 1° *and* pH 4·66 (0·1 M Na *acetate*, 0·088 M *acetic acid*)

This proved to be the major and central part of this work. An acetate buffer pH 4·66, $I = 0{\cdot}1$ was chosen, because under similar conditions β-lactoglobulin had been repeatedly found by previous workers to show two components in the descending limb. The original intention was to use this method to follow fractionation. However, it was noticed early in the work that the proportions of the two components varied markedly with the concentration of protein; this phenomenon seems to have escaped notice, though Polis *et al.* (1950) found that the concentration of NaCl affects the proportions of components. Fig. 1 shows records of descending and ascending boundaries at various concentrations.

Descending boundaries. These always showed a partial resolution of a fast component and a slow component, except at the lowest concentration, where only the slow component was present. As the concentration was increased, fast component appeared in increasing proportion up to a limiting value reached at 2–3 g. total protein/100 ml. (normal samples). At higher concentrations still, the slow component boundary tended to appear sharper, and at very high concentration the fast component boundary tended to become skew. Since the two component boundaries were never fully resolved, the allocation of areas in the schlieren diagrams could be only approximate. In the earliest photographs, the boundary areas were divided by a vertical line from the minimum between the two peaks; in the latest photographs, where resolution was most complete, the trailing limb of the slow component boundary was reflected about its maximum, and the remaining area ascribed to

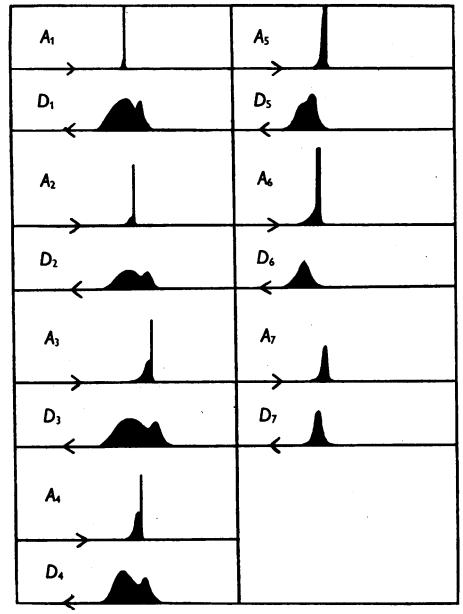

Fig. 1. Tracings of the electrophoretic boundaries of β-lactoglobulin LG-15 at 1° in 0·1 M Na acetate, 0·088 M acetic acid, pH 4·66. Ascending (*A*) and descending (*D*) boundaries are shown at the following concentrations: (1) 9·7; (2) 6·4; (3) 5·1; (4) 3·7; (5) 0·88; (6) 0·45; (7) 0·22 g./100 ml. The directions of migration are shown by arrows. The δ and ε boundaries are not included.

The following are the times (t) and the schlieren edge angles (θ):

Photograph	t (min.)	θ°
1*A*	61	85
1*D*	180	60
2*A*	192	70
2*D*	193	70
3*A*	183	70
3*D*	184	70
4*A*	189	60
4*D*	190	60
5*A*	240	30
5*D*	241	30
6*A*	243	30
6*D*	244	30
7*A*	185	30
7*D*	187	20

the fast component; in intermediate photographs, the trailing limb of the slow component boundary was reflected about the position of its maximum, estimated from its mobility, which was obtained from the positions of the maxima in the latest photographs. In every case the ε boundary was subtracted before performing the resolution. These methods are illustrated in Table 1 and Fig. 2. The values obtained were constant within 2–3% throughout a run and showed no trend. Fig. 3 gives examples, from a number of experiments, of the variation of the estimated amounts of the two components with concentration. In a typical experiment (0·93 g. protein/100 ml.) the ratio of the mobilities of the two maxima was estimated to be 2·73:1·7, which agrees closely with the ratio found by Jacobsen (1949) in a similar experiment.

Table 1. *An example of the resolution of the descending electrophoretic boundary into fast and slow components*

Sample LG-19; 0·93 g./100 ml.; 1°; 0·1 M Na acetate, 0·088 M acetic acid, pH 4·66. Analysis performed by the method illustrated in Fig. 2. Areas are given in arbitrary units. The ε-boundary has been subtracted.

Time hr.	min.	Fast component Area	Fast component % of total	Slow component Area	Slow component % of total	Combined area
3	0	84	59	58	41	142
4	0	82	61	52	39	134
7	22	77	62	48	38	125
8	35	83	59	57	41	140
9	40	82	60	55	40	137

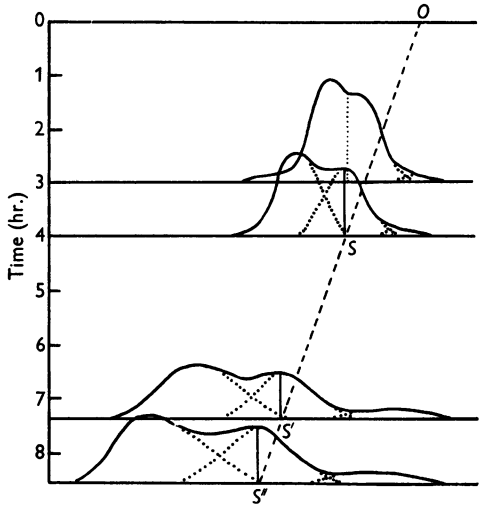

Fig. 2. Tracings of the descending boundary, at various times, in the electrophoresis of β-lactoglobulin in 0·1 M Na acetate, 0·088 M acetic acid, pH 4·66 at 1°, concentration 0·93 g./100 ml. The results of the analysis are given in Table 1; the method is described in the text. *O* represents the position of the sharp boundary at zero time; the line *OSS'S''* shows the movement of the slow component. In the record at 3 hr., the areas were divided by the dotted line; in later records, the slow boundary was drawn symmetrically about the full vertical lines representing the positions of its maximum.

Ascending boundaries. Only at the lowest concentration (*ca.* 0·2 g. protein/100 ml.) did these have nearly normal form; they then appeared as rather sharp and somewhat skew single peaks, migrating about 12% faster than the corresponding descending (purely slow component) boundaries. This difference of mobility may be explained by the dilution of an ascending boundary (Longsworth, 1947). At higher concentration, the faster part of the ascending boundary had a very sharp leading edge, behind which was a narrow region from which light was deflected outside the optical system. Behind this region there appeared, at intermediate concentration, to be a partial resolution of a slower moving boundary but this resolution was absent again at the highest concentrations. Up to 4 g./100 ml. the leading part of the ascending boundary moved slower than the descending fast component boundary, but above this concentration it again moved faster. As far as could be estimated, the slower part of the ascending boundary when present always migrated considerably faster than the descending slow component boundary; the area of this slower part increased with time.

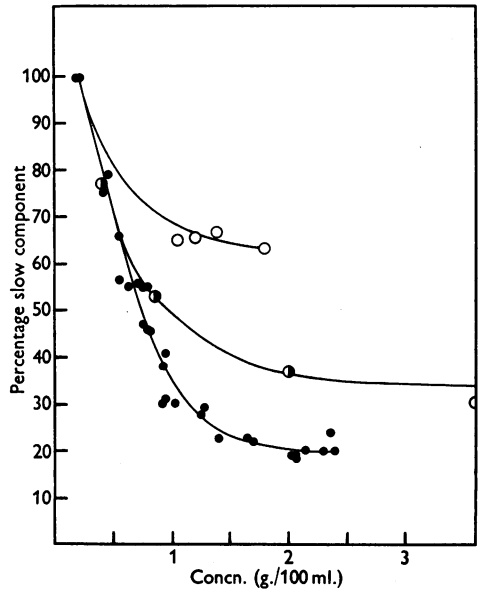

Fig. 3. Proportions of the slow component at varying concentration (1°, in 0·1 M Na acetate, 0·088 M acetic acid, pH 4·66). ●, Points obtained with nine normal samples; ◑, LG-14 and ○, LG-15, showing abnormally high proportions of slow component (cf. Table 2).

Table 2. *Proportions of fast and slow electrophoretic components in various preparations of β-lactoglobulin*

All measurements at 1°; 0·1 M Na acetate, 0·088 M acetic acid, pH 4·66. Normal values under the same conditions are given in brackets beside the abnormal values.

Sample	Concn. (g./100 ml.)	Fast component (%)	Slow component (%)
Mean of 'normal samples'	1·00	66	34
LG-15	0·85	47 (55)	53 (45)
LG-14	1·05	35 (67)	65 (33)
LG-17	1·15	36 (69)	64 (31)
LG-9	1·38	37 (75)	63 (25)
Electrophoretic fractions of normal samples:			
Descending limb fractions	0·75	37 (47)	63 (53)
	1·21	57 (70)	43 (30)
Ascending limb fractions	1·50	72 (75)	28 (25)

Variation between samples. Of the thirteen preparations made, nine showed closely similar behaviour with regard to the apparent proportions of components in the descending boundary; four others, however, had relatively larger proportions of slow component (Fig. 3; Table 2). Of these, sample LG-14 was never crystallized, in spite of repeated attempts. That crystallization, in itself, does not affect the ratio of components, was shown by tests on the normal sample LG-16 before and after recrystallization. A mixed solution containing equal concentrations of LG-16 and of LG-14 (which contained an abnormally high proportion of slow component) was also studied, at a total concentration of 1 g./100 ml. The descending boundary showed 56 % of slow component, compared with 54 % expected from simple mixing.

Electrophoresis at other values of pH *and* 1°

Various single, or small series of, experiments were done.

(*a*) pH 3·98 (0·1 M sodium acetate; 0·43 M acetic acid). No resolution was obtained at 0·98 g./100 ml. in the descending boundary, though this was much more diffuse than the ascending boundary. Re-adjustment by dialysis to pH 4·66 gave the normal pattern for that pH.

(*b*) pH 4·68–5·03. The results are shown in Table 3. There seemed to be a steady increase of slow component at around 1 g./100 ml., as the isoelectric point (5·22) was approached.

(*c*) Higher pH. A number of experiments was done in the range 5·22–7·29. The results nearly always indicated heterogeneity, in agreement with Li (1946, 1951). It was, however, found that either high field strength (20 mA current) or a long experiment (9 hr. at 10 mA) were required before resolution was apparent.

(*d*) Effect of $CaCl_2$ at pH 4·66. A sample of β-lactoglobulin in 0·05 M Na acetate, 0·048 M acetic acid; 0·017 M-$CaCl_2$, $I = 0·1$, pH 4·64 was compared with the same sample, at the same concentration in the usual pH 4·66 buffer. The results were indistinguishable.

Table 3. *Proportions of fast and slow electrophoretic components in a normal sample of β-lactoglobulin, measured at different values of* pH *in acetate buffers at* 1°

The corresponding values at pH 4·66 are given in brackets.

pH	Concn. (g./100 ml.)	Fast component (%)	Slow component (%)
4·68	1·25	69 (70)	31 (30)
4·82	1·25	52 (70)	48 (30)
4·99	1·26	39 (70)	61 (30)
5·03	0·88	17 (58)	83 (42)
5·03	1·22	13 (70)	87 (30)

Electrophoresis at 8° *and at* 17° *at* pH 4·66

Experiments were made with a normal sample of β-lactoglobulin, 0·93 g./100 ml., at pH 4·66. Electrophoresis could be carried out at about 17° using a low current density (3 mA in the 10 ml. U-tube) without any sign of convection. No resolution was obtained and both ascending and descending boundaries were symmetrical, though the ascending was somewhat less diffuse. With the same current density at 8° marked resolution was obtained in the descending boundary, with approximately 70 % slow component; the proportion of slow component at 1° was only 32 %. On lowering the temperature from 17 to 8°, after electrophoresis at 17°, resolution was obtained, with the same proportion of slow component (70 %) as was obtained when the temperature was 8° throughout.

When resolution was first carried out at 1°, and the temperature then raised to 17°, the form of the descending boundary remained unchanged; but the two parts of the boundary now migrated at the same rate, no further resolution being produced.

The same equal rates were observed if, after resolution at 1°, the current was reversed at 17°. This rate was identical with that of the unresolved descending boundary observed when the temperature was 17° throughout.

Chemical fractionation

A preliminary fractionation of a once-recrystallized sample several times reprecipitated with $(NH_4)_2SO_4$ was performed by adding solid $(NH_4)_2SO_4$ to a solution in 0·1 M-NaCl (2·75 g. protein/100 ml.) at 20° to give 50 % saturation. The precipitate was removed by centrifuging at 12 000 rev./min.; the supernatant was then made 55 % saturated and the precipitate removed; and so, by increments of 5 % up to 70 % saturation. Each precipitate was dialysed against acetate buffer, pH 4·66, $I = 0·1$, and examined by electrophoresis at 1°. The first two fractions appeared to be of normal composition (see below) having two components migrating to the cathode. In the last two fractions, a component appeared which migrated to the anode. This anionic component (at pH 4·66) was absent from a sample which had been 4 times crystallized; its total amount was small, and this suggests that it was a contaminating whey protein. In subsequent experiments, several-times recrystallized material was used and the following procedures were examined as means of fractionation:

Ammonium sulphate. β-Lactoglobulin in 45 % saturated salt was placed in a dialysis sac in an open beaker which was filled with 45 % sat. salt. Slow evaporation was allowed to occur at room temperature until a part of the protein had precipitated.

Ethanol. β-Lactoglobulin in acetate buffer pH 4·66, $I = 0·1$, was precipitated with 15–25 % ethanol (v/v) at −5°. The precipitates were dissolved in 1 M-NaCl and dialysed against water. All crystallized within 24 hr.

Crystallization. When the oil, first obtained on dialysis, begins to crystallize, large crystals also appear almost at once in the supernatant. A sample, previously recrystallized 3 times, was dissolved in 0·1 M-NaCl and dialysed against water; as soon as crystals appeared, the supernatant was rapidly withdrawn into another tube; after 1 min. crystals had formed; on again decanting the supernatant into a third tube, a further crop was obtained. The uncrystallized part of the oil was also separated from its crystals, and allowed to crystallize in another tube.

Leaching. A large excess of crystals were stood for 24 hr. periods with successive portions of acetate buffer, pH 5·13, $I = 0·01$.

Urea. An attempt was made to bring about a differential denaturation as described by Jacobsen & Christensen (1948). A solution (10 ml.) containing 4 M urea, 0·1 M α-picoline, 0·015 M-HCl (pH 7·0) was mixed at 30° with 0·217 g. freeze-dried protein. After 5 min. half the solution was withdrawn and added to 10 vol. of a solution containing 0·8 M acetic acid, 0·4 M Na acetate, 0·5 M-$MgSO_4$ to precipitate denatured material; 79 % of the protein in this portion remained soluble. The remaining half of the solution was treated in the same way after 8 hr. at 30°; 46 % of the protein in this portion remained soluble.

All fractions obtained by these procedures were examined by electrophoresis, at pH 4·66, $I = 0·1$, 1°, but no differences from the parent material were found.

Electrophoretic fractionation

Before attempting to fractionate β-lactoglobulin in the preparative cell, a test was made with a mixture of bovine serum albumin (Armour Laboratories) and β-lactoglobulin in acetate buffer pH 4·99, $I = 0·1$, 2 g. of each/100 ml.; the products on subsequent electrophoretic analysis were as follows: cathode fraction, serum albumin 7 %, β-lactoglobulin 93 %; anode fraction, serum albumin 92 %, β-lactoglobulin 8 %. Two fractionation experiments were then performed, using a normal sample of β-lactoglobulin 2 g./100 ml. in pH 4·66 acetate buffer. Slow material was isolated from the descending boundary, and material was also isolated from the region of the ascending boundary. The isolated solutions were then analysed electrophoretically. The results are given in Table 2, together with the proportions of components at the same concentration expected if no fractionation had taken place. It is clear that slow material has been concentrated in the slow descending boundary, but that no corresponding concentration of fast material has occurred in the ascending boundary.

Sedimentation

Sedimentation at 25°. This was observed between 0·22 and 6·32 g./100 ml. in pH 4·66 acetate buffer $I = 0·1$. The sedimentation coefficients (Table 4), corrected to water at 20°, agreed well with those of Cecil & Ogston (1948) measured in 0·1 M-NaCl, 0·1 M Na acetate, 0·04 M acetic acid, of pH 5·01 on the scale used in this paper. The boundary appeared in all cases to be symmetrical. In two cases (2·37

Table 4. *Sedimentation coefficients measured in the Svedberg ultracentrifuge at* ca. 25° *in* 0·1 M Na *acetate,* 0·088 M *acetic acid,* pH 4·66

Concn. (g./100 ml.)	$10^{13} \times S_{20,w}$
0·22	2·81
0·45	2·78
0·86	2·80
2·37	2·73
6·23	2·54

and 0·88 g./100 ml.), symmetry was tested by comparing the areas of the boundary curves of each side of the maximum; these agreed within the error of measurement (S.D. ± 4 %).

Sedimentation at 5°. Two solutions in pH 4·66 acetate buffer, 2·48 and 0·66 g./100 ml., were run in the Spinco ultracentrifuge at the Courtauld Institute, Middlesex Hospital, by Dr J. M. Creeth. Each solution was run at near 22° and at near 5°. At the higher temperature the boundary appeared symmetrical and the sedimentation coefficients agreed closely with the oil-turbine values. At the lower temperature, there was an obvious partial resolution of the higher concentration; at the lower concentration, the boundary showed a marked broadening and asymmetry consistent with partial resolution of a faster component (Fig. 4). At both concentrations,

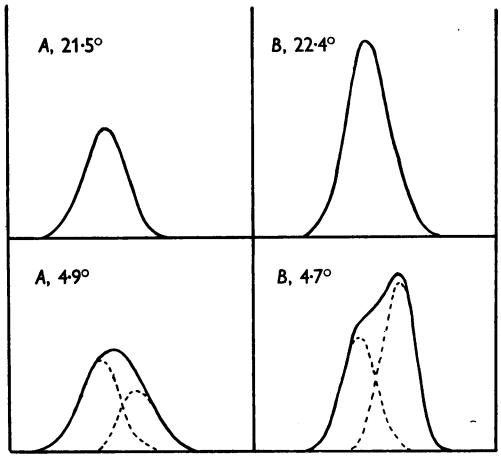

Fig. 4. Tracings of sedimentation boundaries observed in the Spinco ultracentrifuge, in 0·1 M Na acetate, 0·088 M acetic acid. Direction of sedimentation, left to right. Each pair of tracings, *A* and *B*, are taken at approximately corresponding points in the two runs concerned. In each case, the upper tracing is at the higher, the lower tracing at the lower temperature. Concentration in *A* 0·66, in *B* 2·48 g./100 ml. Resolution of the boundaries has been performed as described in the text; the proportions of components are given in Table 5.

the lower sedimentation coefficient, estimated directly from the record, was near to 3×10^{-13}. The areas due to the two components were approximately estimated. Because of the rather wide bar used in this optical system, the shapes of the upper and lower edges differ markedly: the centre of the trace, in the ordinate direction, was taken as measuring the gradient of refractive index. It was assumed that the slower component had the same S_{20} as was obtained with the same solution at 22°, and the positions of the centre of its boundary were calculated from this. The boundary curve of the slower component was drawn in symmetrically about these positions, and the remaining area ascribed to the fast component. As only a few photographs in each run was suitable for analysis in this way, the sedimentation coefficient of the faster component was estimated as follows: $\log x$ (x = distance from the centre of rotation) was plotted against time t (from an arbitrary zero) for the slow component, and the straight line obtained was extrapolated back to the meniscus. Through this point ($\log X, t_X$), a straight line was drawn through the $\log x$, t values for the faster component; the slope of this line is $2{\cdot}303\ \omega^2 S$ (ω = angular velocity). The results are given in Table 5.

Solubility

Preliminary measurements in 0·01 M-NaCl gave high solubility values compared with those obtained by other authors. Since the protein had not been electrodialysed, it was suspected that this might be due to its not being strictly isoelectric: Grönwall (1942) showed that the solubility varies rapidly with pH. Subsequent measurements were therefore made in 0·01 M Na acetate, 0·003 M acetic acid, $I = 0{\cdot}01$, pH (glass electrode) = 5·25 at 20°. A complete solubility curve was measured for a normal lactoglobulin (LG-18A); saturation was reached at 0·4 g./100 ml.; the curve (Fig. 5) was carried up to 2·2 g./100 ml. representing 3·5-fold excess. A few measurements were also made on LG-17, which had an abnormally high proportion of slow material (see Table 2); values were measured from 24 to

Table 5. *Sedimentation coefficients, and proportions of components, measured in the Spinco ultracentrifuge, in* 0·1 M Na *acetate,* 0·088 M *acetic acid,* pH 4·66

The temperature range during the run is given in brackets.

Concn. (g./100 ml.)	Mean cell temperature (°)	$10^{13} \times S_{20,w}$ Fast component	$10^{13} \times S_{20,w}$ Slow component	Percentage of total Fast component	Percentage of total Slow component
0·66	21·5 (21·4–21·6)	—	2·76	0	100
0·66	4·92 (3·22–6·62)	3·92	2·76*	39	61
2·48	22·4 (22·3–22·5)	—	2·79	0	100
2·48	4·68 (3·37–6·0)	4·57	2·79*	61	39

* Value assumed equal to that in the corresponding run at the higher temperature; see text.

72 hr. and showed that equilibrium was attained in 24 hr. The marked difference of solubility of the two samples agrees with the findings of previous workers, particularly with Polis *et al.* (1950), who found their isolated slow material more than twice as soluble (in water) as the parent material.

Ultraviolet absorption

The absorption spectra of a β-lactoglobulin sample of normal composition, and one with an abnormally large content of slow component (LG-14: see Table 2) were compared both at pH 4·66 and in 0·1 M-NaOH, between 250 and 350 mμ. Only very slight differences in the shapes of the absorption curves were observed, indicating (Beavan, Holiday & Jope, 1950) that the tyrosine/tryptophan ratio was closely similar in both samples. LG-14 appeared to have a specific extinction coefficient about 15% less than that of LG-19, but this difference might have been due to errors of the refractometric measurement of the rather low concentrations used (*ca.* 0·05 g./100 ml.).

DISCUSSION

Our experiments confirm those already quoted in showing that crystalline β-lactoglobulin consists of more than a single component. The presence of more than one electrophoretic component has been confirmed. The proportions of these components are different in some samples from those usually found. The proportions of components can be changed by electrophoretic fractionation. The constant-solvent solubility curve gives clear evidence of heterogeneity, when measured under conditions of small solid excess.

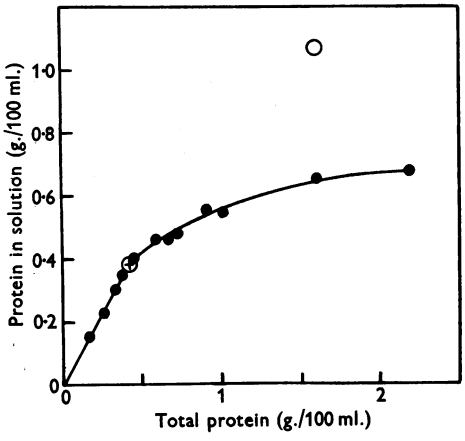

Fig. 5. Solubility curve for a normal sample of β-lactoglobulin in 0·01 M Na acetate, 0·003 M acetic acid, pH 5·25 at 20°. The point at which saturation is first reached is shown ⊕. ○ is a value obtained with a sample containing an abnormally high proportion of slow component (LG-17; cf. Table 2).

Smithies's (1954) solubility measurements are the only ones so far to give quantitative information about this heterogeneity. They indicate the presence, in crystalline samples, of two major components in proportions 1:4, with the possibility of a third minor component. These proportions agree strikingly, at first sight, with our finding that the slow and fast components in electrophoresis occur in proportions of 1:4, when they are observed in the descending limb at 1°, pH 4·66 and at protein concentration 2 g./100 ml. and above. However, this apparent correspondence requires careful examination. First, Svensson (1946) and Hoch-Ligeti & Hoch (1948) have shown that, through purely physical causes operating across migrating boundaries, the areas in the boundary diagrams may not correctly represent the amounts of migrating components; calculation, by their methods and using quantities proper to the present case, has shown that the error at 2 g./100 ml. will not exceed a few per cent of the quantities estimated. Secondly, the apparent proportions of electrophoretic components are strongly dependent on the concentration of protein, the temperature and on the ionic composition and pH. Under the conditions which we have investigated most fully, a dilution of the protein down to 0·2 g./100 ml. caused a progressive disappearance of the fast component, raising the temperature to 8° caused a partial, and to 17° a complete, disappearance of the fast component; in each case a corresponding increase of the amount of the slow component took place. These changes were fully reversible.

It does not seem that this behaviour can be explained by any form of boundary anomaly. Therefore one must conclude that the fast component is actually formed from slowly migrating material. Since the amount of fast material appears to reach a limiting value of about 80% of the whole, the original material must contain 20% of a substance which cannot, and 80% of a substance which can form the fast component. At sufficiently low concentration or sufficiently high temperature, these two substances migrate identically, at the slow rate. Under constant conditions of solvent and temperature, the proportion of the fast component depends solely on the concentration of protein, increasing to a limit as this increases; this indicates that the fast component is formed by an association process. This is most probably a protein–protein association, such as

$$nA \rightleftarrows (A)_n.$$

If A constitutes 80% of the material, then this will be the limiting proportion of fast component.

The few sedimentation measurements made show the presence of faster sedimenting material at 5°, the proportion of this corresponding approximately with that of the faster electrophoretic component under the same conditions. The rate at which this sediments is consistent with its being a dimer, the value of the sedimentation coefficient expected for a dimer, without change of the frictional ratio, being $4{\cdot}45 \times 10^{-13}$ (compare Table 5). Dimerization does not, in itself, explain the change of electrophoretic mobility, as it does that of sedimentation coefficient. There is, however, no reason why dimerization should not affect ionizing groups in such a way as to cause an increase of mobility; or perhaps affect the binding of other ions by the protein so as to change its mobility.

The general character of the electrophoretic boundaries is in agreement with the hypothesis of reversible, concentration dependent, association. In the descending limb, ahead of the faster boundary the faster (associated) material is migrating in a constant, equilibrium, concentration of its dissociated form; its amount therefore remains constant with time. Within the region of the faster boundary, the concentration of the faster, and to a less extent that of the slower, material is changing. This variation does not appear to disturb the equilibrium sufficiently to make the area of the faster boundary change noticeably with time, but it explains why complete resolution of the two boundaries is not obtained, even on prolonged migration. In the ascending limb, the associated material is migrating out into buffer; this will cause it to dissociate and to revert at once to the slowly migrating monomer, and this explains the sharp advancing front in the ascending limb, the intermediate value of its mobility and the failure of resolution.

The effect of higher temperature could be interpreted in one or both of two ways, applicable equally to electrophoresis and to sedimentation: (i) The association constant may be strongly temperature dependent, so that little association occurs at near 20° at a concentration at which association is nearly complete at 1°; (ii) the association and dissociation processes may become rapid compared with the rate of resolution. The fact that at 7° resolution is obtained by electrophoresis and sedimentation, but with a smaller proportion of fast component, favours the first of these.

Osmotic pressure measurements provide the only evidence which is possibly inconsistent with the occurrence of association. Measurements at 25° (Johnston & Ogston, 1946; Christensen, 1952; Smithies, 1953) and at 0° (Gutfreund, 1945) have agreed in giving values for the molecular weight between 37 000 and 40 000. While these values are higher than the accepted value near 35 000 obtained by various methods (Cecil & Ogston, 1949; Heller & Klevens, 1946; Halwer *et al.* 1951; McMeekin & Warner, 1942; Senti & Warner, 1948; Riley, 1951; Bull & Currie, 1946), there is no marked difference between those at 25° and those at 0° such as would be expected if association were occurring at the lower temperature. However, the measurements at 0° were made near the isoelectric point, and in NaCl solution, both of which conditions minimize association.

SUMMARY

1. Measurements have been made of the electrophoretic migration of β-lactoglobulin under various conditions of concentration, pH and temperature. Evidence is obtained of the presence of two very similar chemical species, in proportions which may vary from one sample to another, and which can be separated only with great difficulty.

2. One of these species associates reversibly to form a complex which migrates and sediments more rapidly than the simple material. Sedimentation suggests that this is a dimer. This association is strongly dependent on temperature and pH.

3. Solubility measurements confirm the heterogeneity of β-lactoglobulin.

4. The form of migration boundaries is discussed in the light of this conclusion.

5. Previous data on the osmotic pressure of β-lactoglobulin are discussed.

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Sulphydryl Groups in Haemoglobins

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Recently Green, Ingram & Perutz (1954) crystallized derivatives of horse haemoglobin in which two sulphydryl groups were combined either with *p*-mercuribenzoate groups or with silver ions. The crystals obtained from these mercaptide compounds proved to be isomorphous with free oxyhaemoglobin, but they showed significant changes in the X-ray diffraction pattern from which the phases of the diffracted rays could be deduced. Further development of this method required a more detailed knowledge of the number and the reactivity of the SH groups in various haemoglobins than was available in the literature; for this purpose the present study was undertaken.

The presence of sulphydryl groups in horse globin was first demonstrated by Anson & Mirsky (1931). The same authors later titrated horse haemoglobin with potassium ferricyanide and found no reactive SH groups at pH 6·8, whereas raising the pH to 9·5 led to the oxidation of 1·3 SH groups per four iron atoms. The maximum number of reactive groups was found in denatured horse globin in guanidine hydrochloride solution and amounted to two SH for four iron atoms (Mirsky & Anson, 1936). Greenstein (1939), using the porphyrindin titration, concluded that there are three sulphydryl groups per four iron atoms in horse globin. No further work on horse haemoglobin seems to have been reported. According to Hughes (1950) a molecule of human haemoglobin at pH 7·5 reacts with 2 molecules of methylmercury iodide. Ingbar & Kass (1951) titrated human haemoglobin amperometrically with silver nitrate in ammoniacal 0·85% sodium chloride. They found two SH groups per molecule which increased to 4–5 with the addition of 20% methanol. By a similar method Benesch (1950) found four thiol groups in dog haemoglobin.

The proteins studied in the present experiments included the adult haemoglobins of horse, man, ox and sheep. The number of available SH groups was determined by measuring the number of silver atoms per molecule bound as Ag-S-protein. For this the amperometric titration method with silver nitrate was used (Benesch & Benesch, 1948; Ingbar & Kass, 1951). Silver nitrate is added to the protein in weakly ammoniacal solution until unbound silver ammine ions are present; the end point is detected by the appearance of the diffusion current of these ions to a rotating platinum electrode. The binding of mercuric chloride by the haemoglobins was studied with a similar method (Kolthoff, Stricks & Morren, 1954).

The reactivity of the SH groups was investigated by allowing them to react with known molecular proportions of such mercury derivatives as mercuric chloride, sodium *p*-chloromercuribenzoate or