Crystallization of Goat β-Lactoglobulin

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Bain & Deutsch (1948) obtained lactoglobulin from goat milk by carrying out two ethanol fractionations of the whey proteins. The fraction they referred to as 'goat lactoglobulin' gave a single peak on electrophoresis at pH 8·0 in barbiturate buffer, but three boundaries separated at pH 7·0 and at least two at pH 4·2–6·0 after 2 hr. at 4·5 v/cm., $I = 0{\cdot}1$. The two main components had their isoelectric points at pH 5·2 and 5·9 respectively in sodium acetate buffer, $I = 0{\cdot}1$. 'Goat lactoglobulin' thus behaved in much the same way as crystalline bovine β-lactoglobulin, which also gives a single peak on electrophoresis at pH 8 but forms two or more boundaries at pH's 4·8 and 6·2 (Li, 1946). According to Polis, Schmukler, Custer & McMeekin (1950), crystalline bovine β-lactoglobulin shows multiple peaks at pH values acid to its isoelectric point but below pH 3·0 becomes once more a single peak.

In the present study goat lactoglobulin was crystallized from ammonium sulphate, and its electrophoretic behaviour was investigated at pH values ranging from pH 4·1 to 8·0. The three- and four-times recrystallized material appeared to be homogeneous on electrophoresis at pH 4·1 and 5·6–8·6, forming single symmetrical boundaries in runs lasting up to seven hours at about 5 v/cm. However, fairly concentrated solutions (above 1 %), analysed at pH 4·6 and 5·1 in sodium acetate buffer, $I = 0{\cdot}1$, produced markedly asymmetrical boundaries in the descending limb. The isoelectric point, sedimentation constant and molecular weight of the crystalline goat β-lactoglobulin were determined and some of its properties compared with those of crystalline bovine β-lactoglobulin.

METHODS

Goat milk was partially defatted by centrifuging at about 1500 *g*. The casein was precipitated by the dropwise addition of a mixture of N-HCl and N acetic acid (1:2, v/v) until the pH reached 4·5 and the casein precipitate was centrifuged off.

Preliminary fractionation of whey with $(NH_4)_2SO_4$

The pH of the whey was adjusted to 5·8 with 0·1 N-NaOH and solid $(NH_4)_2SO_4$ was added slowly with constant stirring to a saturation of 0·58 (380 g./l.). After standing for at least 1 hr. the precipitate was filtered off on a pad of Celite 545 (Johns Manville Co., London), previously washed with a solution of $(NH_4)_2SO_4$ (0·58 saturated). The precipitate was discarded. Solid $(NH_4)_2SO_4$ was then added to the clear supernatant to 0·9 saturation (requiring 230 g./l.) and the mixture left to stand several hours or overnight at room temperature. The mixture was filtered on a pad of Celite 545 and the filtrate discarded. The fraction insoluble in 0·58–0·9 saturated $(NH_4)_2SO_4$ was washed off the Celite with a minimum volume of 5% $(NH_4)_2SO_4$, dialysed for several hours against running tap water and then 48 hr. against four changes of distilled water. The dialysed material was freeze-dried *in vacuo* and contained β-lactoglobulin.

Crystallization of goat β-lactoglobulin

$(NH_4)_2SO_4$ (A.R.) was used and all steps were carried out at room temperature except for high-speed centrifuging at 4·5°. The above freeze-dried protein fraction (4–6 g.) was dissolved in 100 ml. water and the pH of the solution adjusted to pH 6·5. An equal volume of saturated $(NH_4)_2SO_4$ was added slowly with stirring and after 20 min. the small amount of precipitate was removed by filtration through Celite 545, previously washed with the same $(NH_4)_2SO_4$ mixture. The precipitate was discarded. Solid $(NH_4)_2SO_4$ (65 g./l. of filtrate) was then added slowly. After 1 hr. the precipitate (insoluble in 0·58–0·67 saturated $(NH_4)_2SO_4$) was spun down at 4·5° in a refrigerated high-speed centrifuge at about 15000 *g*. A further 65 g. $(NH_4)_2SO_4$/l. of supernatant precipitated the protein insoluble at 0·67–0·76-saturated $(NH_4)_2SO_4$ and the precipitate was spun down in the high-speed centrifuge. A third fraction insoluble at 0·76–0·86 saturation was precipitated by the addition of a further 70 g. $(NH_4)_2SO_4$/l.

Each of the three protein fractions thus obtained was treated as described below. A small amount of water was added slowly to each fraction until most of the protein had dissolved. The amorphous precpitates were centrifuged down at about 15000 *g* and the clear supernatant left to crystallize at room temperature in a desiccator over P_2O_5. With the right conditions crystals formed overnight. For this step high concentrations of protein (5–10%) are necessary.

Recrystallization. The crystal crops were collected by centrifuging and all samples recrystallized 3–4 times for the electrophoretic study. The crystals were washed with 0·62 saturated $(NH_4)_2SO_4$, dissolved in 0·5 saturated $(NH_4)_2SO_4$ and several drops of water if necessary. Solid $(NH_4)_2SO_4$ was added to cloudiness, and the samples were left to crystallize as before. Recrystallization of purified material may be carried out from more dilute solutions (about 1 % of protein) and if the protein is allowed to crystallize slowly over several days, perfectly shaped crystals up to 1–2 mm. in size may be obtained. Since the protein is more insoluble at high temperature it is important to avoid sudden increases in temperature after crystallization to prevent formation of

amorphous precipitates. The yield varied from 10 to 25% of the first $(NH_4)_2SO_4$ precipitate (0·58–0·9-saturated $(NH_4)_2SO_4$) depending on the scale of the experiment and the age and purity of the crude fraction.

Electrophoresis

Electrophoretic analyses were carried out in a Hilger Tiselius apparatus at +1° using acetate, phosphate or veronal buffers ($I=0·1$), at pH 4·1–8·0, with a potential gradient of about 5 v/cm. The 3–4 times recrystallized material was dissolved to yield solutions containing 0·5–1·3% of protein. The protein concentration could be estimated by measuring the refraction of the solution, or its ultraviolet absorption at 278 mμ. (the absorption maximum) in a Unicam spectrophotometer. In each case the descending boundary was utilized for the calculation of the electrophoretic mobility.

Ultracentrifuge experiments

Runs were carried out in a Spinco ultracentrifuge, analytical model E, at 187 100 or 259 700 *g*. The temperature of the free rotor couple was read at the start and finish of each run. Each point was corrected for temperature assuming an even change over the period of the run. The instrument has not been calibrated for actual cell temperature; hence the values were not corrected for the error arising from any difference between free couple and true cell temperature. Protein concentration was determined as described for the electrophoretic analyses. Solutions of protein (0·2–1%) were dialysed for 48 hr. against phosphate buffer pH 6·9, $I=0·2$, containing 0·1 M-NaCl, 0·0188 M-KH_2PO_4 and 0·0271 M-Na_2HPO_4.

S_{20} was calculated by the method described by Cecil & Ogston (1948); 0·751 being taken as the value for the partial specific volume of the protein. This is the value given by Pedersen (1936) for bovine β-lactoglobulin.

RESULTS

Crystalline form

β-Lactoglobulin from goat milk crystallizes from approximately 0·6–0·65 saturated ammonium sulphate solution in well-formed hexagonal bipyramids (Fig. 1). The actual ammonium sulphate concentration required depends on the concentration of protein present.

The solubility of the goat lactoglobulin crystals in ammonium sulphate is very similar to that of the needles obtained by Sørensen & Sørensen (1939) from cow's milk by similar methods. There are no electrophoretic data on the crystalline material obtained by Sørensen & Sørensen, and crystallization appears to have been very slow, taking at least 8–14 days, whereas in the present study the goat lactoglobulin forms crystals overnight in ammonium sulphate under the right conditions.

Crystalline bovine β-lactoglobulin is prepared usually by dialysing the protein at its isoelectric point (pH 5·2) free from salt (Palmer, 1934). Attempts at crystallization of the goat lactoglobulin by dialysing the preparation free from salt at its isoelectric point (pH 5·9) at 0° for 1–2 weeks have been unsuccessful so far, although as with bovine lactoglobulin (Palmer, 1934) an oily layer separated after dialysis against de-ionized water at pH 5·9.

A second crystalline form was at times obtained from the mother liquor after the hexagonal bipyramids had crystallized out. These crystals were highly birefringent and cubic in shape, but they were obtained in amounts too small for extensive investigation. They had the same sedimentation constant as the other type of crystalline protein ($S_{20}=2·87$ in phosphate–NaCl buffer pH 6·9, $I=0·2$, at a concentration of 0·52% protein). The electrophoretic mobility determined in three runs at different pH values (0·7–0·8% protein) was slightly higher (around 10%) than that of the usual type of crystalline form of goat β-lactoglobulin (see Fig. 2 for mobility of the hexagonal crystals at different pH values). The crystals shaped like cubes had the following electrophoretic mobilities (in cm.²/v/sec.) at an ionic strength of 0·1: $+3·8\times10^{-5}$ and $+2·1\times10^{-5}$ in sodium acetate buffer at pH 4·55 and 5·1 respectively, and $-2·4\times10^{-5}$ in phosphate buffer at pH 7·0. It is, therefore, not quite clear whether these are a different form of β-lactoglobulin crystals or crystals of a different protein. All other results in this study refer to the goat β-lactoglobulin crystallized as shown in Fig. 1.

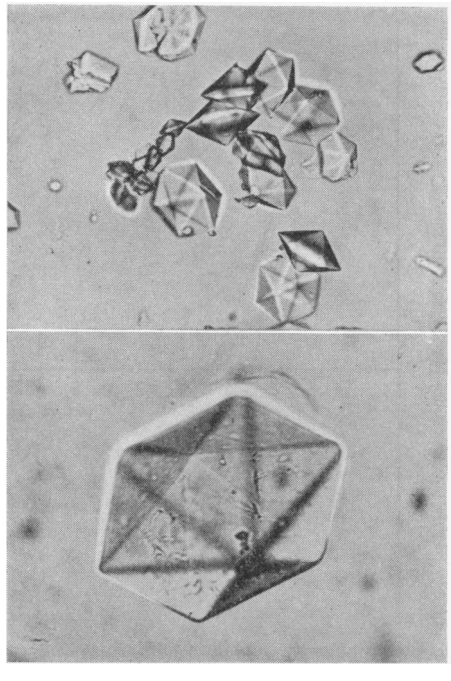

Fig. 1. Goat β-lactoglobulin crystallized from $(NH_4)_2SO_4$. Magnification, large crystal × 270; small crystals × 156.

Electrophoretic behaviour

Isoelectric point. In Fig. 2, mobility of the crystalline goat β-lactoglobulin is plotted against the pH of the electrophoretic runs. It may be seen that in sodium acetate, $I = 0{\cdot}1$, the isoionic point of the protein lies at pH 5·9. Goat lactoglobulin thus has a higher isoelectric point than bovine lactoglobulin (pH 5·2 in the same buffer, cf. Longsworth & Jacobsen, 1949). The value obtained agrees with that quoted by Bain & Deutsch (1948) for one of the components of their goat lactoglobulin. It may also be noted on Fig. 2 that increasing the ionic strength of the sodium acetate buffer to 0·2 at pH 4·6 reduces the mobility of the protein from 3·4 to $2{\cdot}3 \times 10^{-5}$. The same effect was noted by Longsworth & Jacobsen (1949) with bovine β-lactoglobulin and with serum albumin, suggesting binding of the salt by the protein.

Homogeneity of crystalline goat β-lactoglobulin. Fig. 3 shows some of the electrophoretic patterns obtained by examining 3- to 4-times recrystallized lactoglobulin at different pH values. The material appears to be homogeneous, forming a perfectly symmetrical boundary at pH 5·6–8·0 and at pH 4·15 using 0·7–1·5% solution of protein (see Fig. 3, *a*, *b*, *c*, *g*). If electrophoretic analysis is carried out at pH 5·1 and about 4·6 in acetate buffer, $I = 0{\cdot}1$, using solutions of protein at concentrations higher than 1%, the descending boundary is markedly asymmetric in runs lasting for 4–6 hr. (Fig. 3, *d*, *e*).

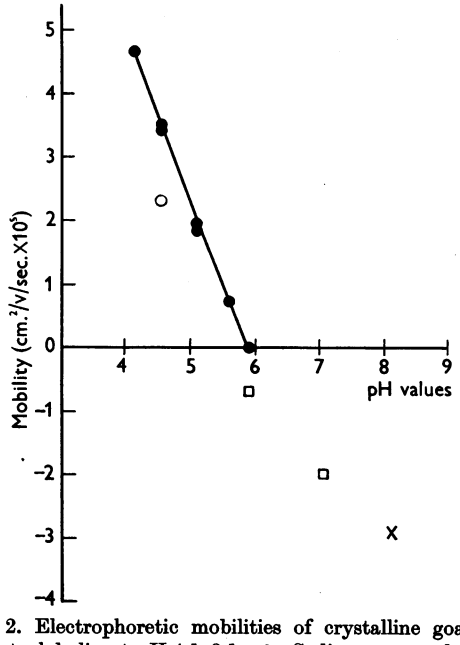

Fig. 2. Electrophoretic mobilities of crystalline goat β-lactoglobulin at pH 4·1–8·1. ●, Sodium acetate buffer, $I = 0{\cdot}1$; ○, sodium acetate buffer, $I = 0{\cdot}2$; □, sodium phosphate buffer, $I = 0{\cdot}1$; ×, veronal buffer, $I = 0{\cdot}1$.

At pH values below the isoelectric point of the protein there is a striking difference between the patterns of the ascending and descending boundaries. The ascending boundary stays extremely sharp throughout the whole run with no evidence of any asymmetry or of a second component. This same sharpening effect has been described by Longsworth & Jacobsen (1949) for electrophoresis of bovine β-lactoglobulin and serum albumin. When the ionic strength of the acetate buffer was raised to 0·2, a 1·2% solution of protein produced a symmetrical peak even after a 7 hr. run (Fig. 3, *f*).

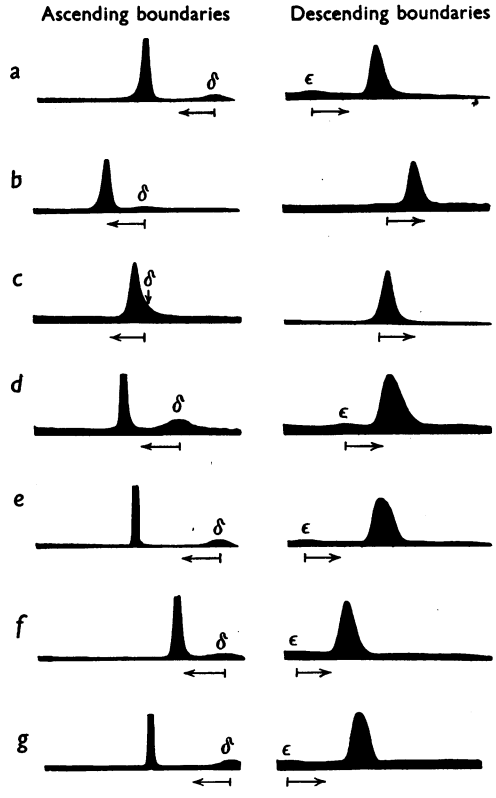

Fig. 3. Electrophoretic patterns of crystalline goat β-lactoglobulin. The arrows below the patterns indicate the position of the boundary at start of run and direction of movement. Potential gradient was about 5 v/cm. $I = 0{\cdot}1$, unless otherwise indicated.

	Buffer	pH	Protein concentration (%)	Duration of run (hr.)
a	Sodium veronal	8·1	1	4·5
b	Sodium phosphate	7·05	1	4·0
c	Sodium acetate	5·9	1·3	6·5
d	Sodium acetate	5·1	1·2	5·25
e	Sodium acetate	4·55	1·3	4·25
f	Sodium acetate ($I = 0{\cdot}2$)	4·65	1·2	7·0
g	Sodium acetate	4·15	1	3·5

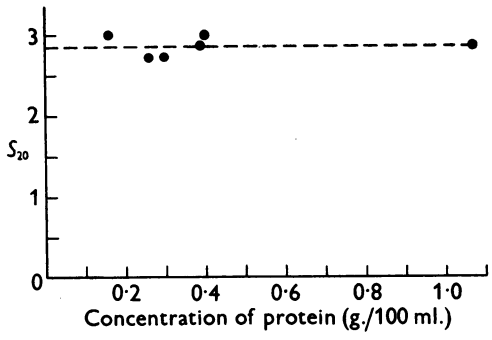

Fig. 4. Sedimentation constant of crystalline goat β-lactoglobulin at different protein concentrations. Average $S_{20}=2{\cdot}85$.

Sedimentation constant

Fig. 4 shows several values of S_{20} obtained by using different concentrations of crystalline goat β-lactoglobulin. Ultracentrifuging was carried out at pH 6·9 in phosphate–sodium chloride buffer, $I=0{\cdot}2$. It is not possible to extrapolate these few values to infinite dilution. They suggest that S_{20} is independent of the protein concentration. More points would be required to establish whether the apparent dip in S_{20} between 0·2 and 0·4 % protein is significant or whether the spread in the points is due purely to experimental error. The six values were, therefore, averaged, giving a mean value of 2·85 for S_{20} at pH 6·9.

DISCUSSION

Comparison with bovine β-lactoglobulin

The goat β-lactoglobulin appears to be a molecule similar in size to that of bovine β-lactoglobulin. D_{20} and S_{20} are of the same order in the two proteins. A number of values for S_{20} of bovine lactoglobulin have been reported in the literature. Thus Pedersen (1936) obtained mean values of 3·12 at pH 5·2–5·7. Cecil & Ogston (1949) observed an S_{20} of 2·83 at infinite dilution in sodium acetate buffer, pH 5·14, and Miller & Golden (1952) a value of 2·91 at pH 5·0 using a Spinco ultracentrifuge. The authors found the usual decrease in S_{20} with increasing protein concentration.

The sedimentation constant of crystalline goat β-lactoglobulin was found to equal 2·85 (mean value, 0·2–1 % protein) at pH 6·9 in phosphate buffer, $I=0{\cdot}2$. S_{20} appeared independent of the protein concentration but is very similar to the values reported for the bovine β-lactoglobulin.

The diffusion coefficients were determined on solutions containing 0·26 and 0·38 % protein (see Addendum). It may be assumed that D_{20} shows no appreciable variation at this range of concentration (cf. Cecil & Ogston, 1949); therefore the two values of D_{20} obtained were averaged. Thus D_{20} of 7·48 and S_{20} of 2·85 gave a molecular weight of 37100 for goat β-lactoglobulin. This lies within the range of values (35000–39000) reported by various authors for the molecular weight of bovine lactoglobulin (cf. Cecil & Ogston, 1949; Pedersen, 1936; Polis *et al.* 1950; Bull & Currie, 1946).

Bovine β-lactoglobulin has been the subject of extensive study. For a long time it was believed to be a good example of a homogeneous protein. Li (1946), however, showed that although the crystalline bovine β-lactoglobulin appeared to be homogeneous at pH 7 and 8·6, electrophoresis at pH 4·8 and 6·2 showed a heterogeneous pattern with two to three components separating. This heterogeneity has been investigated by McMeekin, Polis, Della-Monica & Custer (1948) and Polis *et al.* (1950). Electrophoresis of 4-times recrystallized bovine β-lactoglobulin in sodium acetate buffer pH 4·8 showed 40 % of a slow- and 60 % of a fast-moving component. Although the ascending and descending boundaries were not mirror images, both showed two components. Polis *et al.* (1950) isolated the slower moving component (β_1) in crystalline form in small yield (1 g. from 50 g. crystalline β-lactoglobulin). This component had the same molecular weight as β-lactoglobulin and the suggestion of Lundgren & Ward (1949) that the heterogeneity is due to the presence of immune globulin is thus ruled out.

That the different components, if they are different proteins, interact strongly has been shown by Polis *et al.* (1950). Substitution of sodium chloride for sodium acetate at a constant ionic strength of 0·1 changes the proportion of the two components in electrophoretic patterns of bovine β-lactoglobulin.

Heterogeneous electrophoretic patterns have also been noted with other proteins previously presumed to be pure, when electrophoresis is carried out in sodium acetate buffer at pH values below the isoelectric point. Li & Pedersen (1953) found aggregation of growth hormone molecules at pH 4·0 in sodium acetate buffer with a preparation homogeneous at pH 2·32 and 9·93. In crystalline human and horse serum albumins, also, more than one component may be identified by electrophoresis at pH 4·0 in sodium acetate buffer, $I=0{\cdot}02$ (Luetscher, 1939).

The present study showed that crystalline goat lactoglobulin formed symmetrical boundaries at pH 4·1 and pH 5·6–8·0. Electrophoresis continued for more than 4 hr. at pH 4·6 and 5·1 in sodium acetate, $I=0{\cdot}1$, at a protein concentration over 1 %, however, produced asymmetric boundaries in the descending limb. Under the conditions of electrophoresis used it has not been possible to get separation of more than one component. It is not quite

clear whether the asymmetry of the descending boundary is due to the presence of more than one protein component in the crystal or merely to some reversible change in the molecule below the isoelectric point of the protein. It is not likely that the asymmetry is due to changes in the size of the molecule. Ultracentrifuging of a 0·5 % solution for 2 hr. at 187 100 *g* in sodium acetate (pH 4·6, $I = 0·2$) showed no sign of heterogeneity. Any striking change in the size of the molecule by dissociation or aggregation at pH 4·6 should show up by this method.

SUMMARY

1. β-Lactoglobulin may be crystallized from goat milk by means of ammonium sulphate. The isoelectric point of the protein lies at pH 5·9 in sodium acetate buffer, $I = 0·1$.

2. The electrophoretic behaviour of the protein was studied at pH values ranging from 4·1 to 8·1. The protein appears homogeneous forming a symmetrical boundary at pH 4·15 and 5·6–8·1. An asymmetric boundary may be noted in the descending limb at about pH 4·6 and 5·1 in sodium acetate buffer, $I = 0·1$. This asymmetry disappears on raising the concentration of the sodium acetate to an ionic strength of 0·2 at pH 4·65.

3. The goat β-lactoglobulin was found to have a molecular weight of the same order as bovine β-lactoglobulin (i.e. 37 100). S_{20} was 2·85 and D_{20} $7·48 \times 10^{-7}$ (see Addendum).

I should like to express my thanks to Dr T. S. Work and Dr P. N. Campbell for their interest and encouragement and to Dr R. A. Kekwick and Mr E. Caspary for their helpfulness with the sedimentation and diffusion measurements. I am most grateful to Mrs A. Dovey for carrying out the electrophoretic runs, to Mr J. Orr for doing the runs in the Spinco ultracentrifuge and Mr Young for the photographs of the crystals.

REFERENCES

Bain, J. A. & Deutsch, H. F. (1948). *Arch. Biochem.* **16**, 221.
Bull, H. B. & Currie, B. T. (1946). *J. Amer. chem. Soc.* **68**, 742.
Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **43**, 592.
Cecil, R. & Ogston, A. G. (1949). *Biochem. J.* **44**, 33.
Li, C. H. (1946). *J. Amer. chem. Soc.* **68**, 2756.
Li, C. H. & Pedersen, K. O. (1953). *J. biol. Chem.* **201**, 595.
Longsworth, L. G. & Jacobsen, C. F. (1949). *J. phys. Chem.* **53**, 126.
Luetscher, J. A. (1939). *J. Amer. chem. Soc.* **61**, 2888.
Lundgren, H. P. & Ward, W. H. (1949). *Annu. Rev. Biochem.* **18**, 121.
McMeekin, T. L., Polis, B. D., DellaMonica, E. S. & Custer, J. H. (1948). *J. Amer. chem. Soc.* **70**, 881.
Miller, G. L. & Golden, R. H. (1952). *Arch. Biochem. Biophys.* **36**, 249.
Palmer, A. H. (1934). *J. biol. Chem.* **104**, 359.
Pedersen, K. O. (1936). *Biochem. J.* **30**, 961.
Polis, B. D., Schmukler, H. W., Custer, J. H. & McMeekin, T. L. (1950). *J. Amer. chem. Soc.* **72**, 4965.
Sørensen, M. & Sørensen, S. P. L. (1939). *C.R. Lab. Carlsberg*, **23**, 55.

ADDENDUM

Diffusion Coefficient of Crystalline Goat β-Lactoglobulin

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The diffusion measurements were carried out by the Gouy interferometric method (Kegeles & Gosting, 1947) in a modification of the apparatus of Gosting, Hanson, Kegeles & Morris (1949) with a parallel light beam through the cell at 25 ± 0·01°. Increased sensitivity was obtained by using the blue line, $\lambda = 4360$ Å, isolated from a high-pressure mercury arc by an interference filter. Boundaries were made by flowing through a capillary (Kahn & Polson, 1947).

Diffusion coefficients were measured in the same phosphate–sodium chloride buffer used for the sedimentation-runs (pH 6·9, $I = 0·2$) at two concentrations of protein. $D_{20,w}$ was found to equal 7·38 and $7·58 \times 10^{-7}$ cm.²/sec. using 0·38 and 0·26 % solutions of protein respectively. Only slight deviation from the ideal form, defined by the probability integral, was observed.

REFERENCES

Kahn, D. S. & Polson, A. (1947). *J. phys. Chem.* **51**, 815.
Kegeles, G. & Gosting, L. J. (1947). *J. Amer. chem. Soc.* **69**, 2516.
Gosting, L. J., Hanson, E. M., Kegeles, G. & Morris, M. S. (1949). *Rev. sci. Instrum.* **20**, 209.