Tamm, I. (1956). *J. Bact.* **72**, 43.
Taylor, E. S. & Gale, E. F. (1945). *Biochem. J.* **39**, 52.
Waring, W. S. & Werkman, C. H. (1942). *Arch. Biochem.* **1**, 303.
Watkins, T. I. & Woolfe, G. (1952). *Nature, Lond.*, **169**, 506.
Webster, G. C. & Johnson, M. P. (1955). *J. biol. Chem.* **217**, 641.
Woolley, D. W. (1944). *J. biol. Chem.* **152**, 225.
Woolley, D. W. (1950). *Proc. Soc. exp. Biol., N.Y.*, **75**, 745.
Woolley, D. W. (1951). *J. exp. Med.* **93**, 13.
Woolley, D. W. (1952*a*). *J. Amer. chem. Soc.* **74**, 5450.
Woolley, D. W. (1952*b*). *A Study of Antimetabolites.* New York: John Wiley and Sons.
Woolley, D. W. & Pringle, A. (1952). *J. biol. Chem.* **194**, 729.
Woolley, D. W. & Schaffner, G. (1954). *Cancer Res.* **14**, 802.

The Solubility and Fractionation of β_1-Lactoglobulin

By M. P. TOMBS
Department of Biochemistry, University of Oxford

(*Received* 6 *May* 1957)

β-Lactoglobulin is an easily accessible crystalline protein and has been used in many experiments, often with the assumption that it is homogeneous. Since Li (1946) demonstrated electrophoretic heterogeneity, it has been firmly established that it is a mixture, and attempts at fractionation have been made (Polis, Schmukler, Custer & McMeekin, 1950; Preaux, Hulsmans & Lontie, 1954; Smithies, 1954). More recently it has been found that it is a member of the rapidly increasing number of proteins known to be under direct genetic control. Aschaffenburg & Drewry (1955) showed that some cows produced two lactoglobulins, β_1- and β_2-, some only β_1- and some only β_2-. β_1-Lactoglobulin was the faster in paper electrophoresis at pH 8·6. This made all work carried out on mixed milk preparations (the great majority) of uncertain validity. Ogston & Tombs (1957) studied the separate proteins, obtained from appropriate cows, and found that the titration curves and ultraviolet spectra were different, though the molecular weights were very similar. Green, North & Aschaffenburg (1956) had reached the latter conclusion from X-ray studies. However, the electrophoretic boundaries obtained with both separate proteins at pH 4·66 were complex, the feature which initially led to doubts on the homogeneity of mixed preparations, and leads equally to doubts of the homogeneity of the individual proteins.

Among previous workers only Ogston & Tilley (1955) made efforts to ensure that milk from single cows was used for their preparations, and they were able to distinguish 'normal' and 'abnormal' types of lactoglobulin. The 'normal' type (identified as β_1-lactoglobulin by Ogston & Tombs, 1957) gave a boundary pattern indicating two components on U-tube electrophoresis at pH 4·66, while the proportion of slow component varied with protein concentration down to a limit of 20 % at high concentrations. To account for this they supposed that this material consists of two proteins: one, about 80 % of the whole, polymerizes with increasing concentration; the other, about 20 % of the whole, migrates slowly and does not polymerize. The heterogeneity of β_1-lactoglobulin has now been investigated further by means of solubility tests.

EXPERIMENTAL

Typing of cows and preparation of β_1-lactoglobulin. This was by the method of Aschaffenburg & Drewry (1957), slightly modified as described by Ogston & Tombs (1957), and the samples of β_1-lactoglobulin, two in all, were the same as used by them.

Concentration. Concentrations (g./100 ml. of solution) were found with a refractometer (Cecil & Ogston, 1951). The specific refractive increment used was $1·8 \times 10^{-3}$ in all cases.

U-tube electrophoresis. A Hilger Tiselius apparatus was used as described by Ogston & Tilley (1955). The 3·5 ml. cell was used with a bath temperature of 1°. The current was always 10 mA at about 400 V. Boundaries were divided to estimate 'fast' and 'slow' components according to the method of Ogston & Tilley (1955).

Ultraviolet absorption. This was determined in a Hilger Uvispek spectrophotometer with matched 1 cm. cells of 3 ml. capacity.

pH *measurements.* These were made at room temperature with a Cambridge Instrument Co. glass electrode and valve voltmeter calibrated against potassium hydrogen phthalate, pH 4·0.

Solubility tests

Variable-solvent solubility curves. The standard solvent system was similar to that used by Smithies (1954). The solutions were made from A.R. reagents and glass-distilled water. The acetate buffer, pH 4·95, contained 1·6 M-sodium acetate–1·06 M-acetic acid. Ammonium sulphate solutions in the range 2·53–3·2 M were made up by diluting a 3·2 M-standard solution (422·8 g./l.). Wet protein crystals (which had been dialysed against water) were separated by centrifuging and dissolved in 1·6 M-sodium chloride. The solution was then dialysed against 1·6 M-sodium chloride for at least 24 hr. Protein concentrations of about 1 % (w/v) were suitable for all these experiments. Acetate buffer, pH 4·95,

was then mixed with an equal volume of the dialysed protein solution and 1 ml. samples were pipetted into 5 ml. centrifuge tubes which had been cleaned with chromic acid. In many early tests 0·5 ml. of protein solution followed by 0·5 ml. of buffer solution was run into each tube. A slight increase in reproducibility was apparent when they were pre-mixed. Ammonium sulphate solution (3 ml.) was then added to give a final concentration of ammonium sulphate in the range 1·9–2·4 M. The tubes were sealed with clean rubber bungs lightly greased with silicone stopcock grease, and inverted twice to mix.

Care was taken throughout to pipette as accurately as possible, and it was found markedly advantageous to fill each pipette and empty it at least once before pipetting the ammonium sulphate solutions. Not more than twenty-four tubes could be handled at a time without loss of accuracy. The final buffer concentration was 0·2 M-acetate, which was sufficient to prevent significant change in pH caused by varying the ammonium sulphate concentration over the range used.

After being mixed the tubes were allowed to stand either at 25° in a water bath or at room temperature (15–17°). Equilibration times varied, but 24 hr. was the usual period. The curve after 6 hr. was indistinguishable from that after 24 hr.

After equilibration the tubes were centrifuged in batches of 6 in a fixed-head centrifuge at about 20 000 *g* for 20 min.: the clear supernatant was decanted straight into the Uvispek cell. Absorption at 278 mμ, the wavelength of maximum absorption of the main component of β_1-lactoglobulin under these conditions, was used as a direct measure of protein concentration.

Constant-solvent solubility curves. It was required to make up solutions containing varying amounts of protein, in a constant volume of solvent of constant composition. The solvent was chosen to correspond, with respect to the concentrations of ammonium sulphate, sodium chloride and buffer, with the point at 2·12 M-ammonium sulphate on the variable-solvent curve.

The weight of 0·5 ml. of protein solution was found: the weight of 0·5 ml. of diffusate differed negligibly from this. Samples (0·5 ml.) were then made up by weighing amounts of protein solution equal to or less than this weight, and making up to the required final weight (that of 0·5 ml. of solution), with diffusate. To each tube was then added 0·5 ml. of buffer and 3 ml. of ammonium sulphate solution. The final concentrations in each tube were then 0·2 M-sodium chloride, 0·2 M-acetate and 2·12 M-ammonium sulphate, while the weights of protein solution added gave a measure of the total amount of protein in the system. The procedures for equilibration and determination of the protein in solution were the same as in the variable-solvent test. This gave the result in Fig. 4.

The downward slope of the right-hand limb of the plot was unexpected. It was thought that this might be due to change of pH caused by the varying amount of protein. Accordingly, in all subsequent experiments the protein was dissolved in, and dialysed against, a solution containing 0·8 M-NaCl and acetate buffer, pH 4·95 (0·8 M-sodium acetate, 0·53 M-acetic acid). The tubes were made up as before, except that the protein–buffer mixture was made up by weighing to 1 ml., followed by 3 ml. of ammonium sulphate solution, giving final concentrations as before. This procedure eliminated the downward slope (Fig. 5).

In a few instances a calibrated 1 ml. pipette with a drawn-out tip was used to measure volumes directly, and gave satisfactory results. At a later stage the use of Agla micrometer syringes gave results comparable in every way with those obtained by weighing. The solvent system used was very sensitive to evaporation, and for long periods of equilibration it was necessary to layer liquid paraffin or a liquid silicone (M.S. 200/1, density 0·9 g./ml., Hopkins and Williams Ltd.) in the tubes before evaporation was completely prevented. Liquid paraffin was inferior to silicone because precipitate tended to stick in the interface during centrifuging. Some of the tubes equilibrated for long periods developed haze because of this effect. The absorption at 350 mμ was taken as a correction for haze; corrected readings were then identical with those of identical tubes in which haze was absent.

β_1-Lactoglobulin had a negative temperature coefficient of solubility in these solvents. In obtaining the points for Fig. 1 it was impracticable to centrifuge at all the different temperatures; instead precipitate was separated from supernatant by gently pushing a plug of cotton wool down the tube. Then 2 ml. samples were withdrawn and diluted to 3 ml. with water. This prevented precipitation on warming to room temperature. This procedure was not very efficient with the precipitates formed at low temperatures, and the results are not as accurate as in the experiments where centrifuging was possible. Experiments were carried out at room temperature instead of at 25° because centrifuging and fractionation were more conveniently performed there. The change in solubility over this range is small, but re-equilibration was taking place during centrifuging at room temperature after equilibration at 25°.

Fractionation of β_1-lactoglobulin

In the first attempt the solvent conditions fixed by the point at 2·19 M-ammonium sulphate in Fig. 3*A*, and the total protein concentration used for that curve, were reproduced, but in a volume of 800 ml. instead of 4 ml. Thus 200 ml. of a 0·6 % solution of protein in NaCl–buffer mixture was made up to 800 ml. with the appropriate ammonium sulphate solution to give a final concentration of 2·19 M-ammonium sulphate. With this volume about 1 g. of protein could be dealt with. Centrifuging was difficult on this scale and the precipitate was never firmly packed.

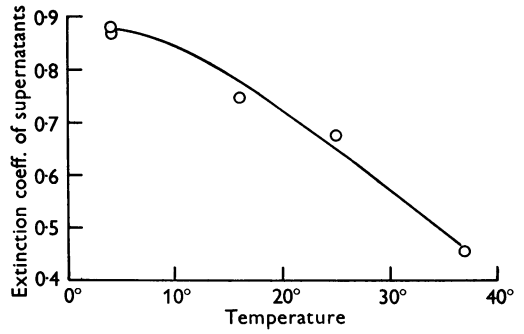

Fig. 1. Variation of solubility of β_1-lactoglobulin with temperature in 2·12 M-ammonium sulphate, 0·2 M-sodium chloride, 0·2 M-sodium acetate, with acetic acid to pH 4·95.

In the revised procedure the solvent conditions defined by the point at 2·15M-ammonium sulphate in the variable-solvent curve were preserved, but in a volume of 80 ml., and the protein concentration was increased so that about 2 g. was used. Precipitate was centrifuged out in a Spinco Model L preparative ultracentrifuge in the '30' head for 45 min. at 29 500 rev./min., giving about 50 000 *g*. Protein remaining in the supernatant was obtained by saturating it with ammonium sulphate and centrifuging out the precipitate as before. The sediments were well compacted and were collected with very little contamination from supernatants.

The precipitates were dissolved in the appropriate solvent for further tests, and dialysed against them. A small amount of insoluble material appeared and was removed by centrifuging after dialysis.

RESULTS AND DISCUSSION

Solubility methods, although they have been relatively little used, are among the most sensitive for investigating the homogeneity of proteins. The two methods are the variable-solvent method, where a constant amount of protein is precipitated to differing extents by different salt concentrations (e.g. Brown, 1952; Falconer & Taylor, 1946; Dervichian & Mossé, 1954; Roche, Derrien, Reynaud, Laurent & Roques, 1954) and the constant-solvent method, where protein is added to a fixed solvent system until saturated solubility is reached (Northrop & Kunitz, 1930).

Interpretation of solubility tests

In the variable-solvent test, every protein has a characteristic solubility curve, and if two or more proteins are present they give a combined curve with sharp breaks, each part of the curve following (Cohn, 1925) an equation of the form:

$$\ln S = \beta - KI, \qquad (1)$$

where S is the solubility, I the ionic strength and β and K are constants characteristic of the protein. The solubility of a substance, other things being equal, is determined by its solid state, and every break in the solubility curve represents the appearance of a new solid phase. This does not necessarily arise from a separate protein in solution. Smithies (1954) has described the effect of crystallization in introducing extra breaks into the solubility curve of mixtures of amorphous β_1- and β_2-lactoglobulins. However, the two different solid forms need not be obviously crystalline and apparently amorphous respectively; Ogston & Tombs (1956) have described the situation where two solid forms are produced from only one protein in solution, neither of them apparently crystalline. The break then represents a transition point. They showed that this kind of break is invariant, i.e. its position on the solubility curve does not change when total protein concentration is changed. On the other hand, a break representing the appearance of another solid phase from a distinct protein in solution is not invariant and will move up the solubility curve if the total protein concentration is increased, and down if it is decreased. No crystallization occurred in this work.

In the constant-solvent test, if two proteins are present, one will be precipitated first, but the solution is not saturated until precipitates of both are present. Solid protein will therefore appear before saturated solubility is reached and a plot of the type shown in Fig. 2 by *ABCD* will be obtained. Again, every break represents the appearance of a new solid phase, and the same considerations apply here as in the variable-solvent test. Since the solvent system is fixed, however, where a single protein gives two solids, one of them must be metastable and this will have the greater solubility. If so, *ABCD* will change to *ABF* on further equilibration.

Solubility curves of β_1-lactoglobulin

The variable-solvent solubility curve of β_1-lactoglobulin showed three breaks (Fig. 3). Ogston & Tombs (1956) have presented evidence showing that the break at 2·14M-ammonium sulphate (T_1) is a transition point. The break at 2·23M-ammonium sulphate (T_2) behaved in an exactly similar fashion. The other break at about 2·00M-ammonium sulphate was only easily detected at quite high protein concentrations, and must be due to a distinct minor component because it moved when protein concentration was varied. If its specific absorption is not very different from that of the rest of the material this minor component was present to the extent of about 10%.

The constant-solvent plot was made at an ammonium sulphate concentration above T_1. The initial experiment gave the results shown in Fig. 4. There was a clear break, but the saturated

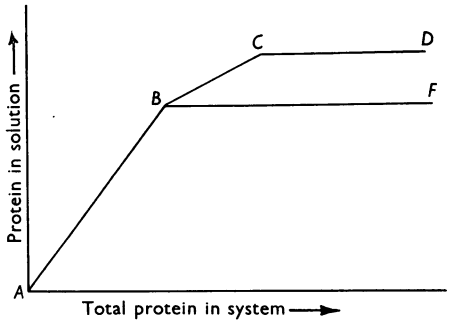

Fig. 2. Diagram of a constant-solvent solubility plot. *ABCD* is the result characteristic either of the presence of two distinct proteins or of a single protein having two different solid forms. In the latter system, *ABCD* should go to *ABF* on further equilibration.

solubility appeared to decrease as more protein was added. This may have been due to combination of the protein with buffer ion, since when the protein solution was pre-equilibrated with buffer the downward slope disappeared, though the break remained (Fig. 5).

It was difficult to establish that one of these solids was not metastable. On equilibration for several days the break remained, and experiments performed at 2·17 M-ammonium sulphate, below T_1, gave a very similar plot, including a break. In the variable-solvent experiments equilibration between the different solid forms was rapid, the curve after 6 hr. being indistinguishable from that after 24 hr., and it is unlikely that equilibration would be any slower in the constant-solvent experiments. The break remained for 8 days (Fig. 5). On equilibration for longer periods, up to 24 days, there was a slow rise in the ultraviolet absorption of the supernatants. A similar effect was noted by Northrop & Kunitz (1930) and was probably due to bacterial contamination. It was concluded that both the variable-solvent and the constant-solvent tests demonstrated heterogeneity.

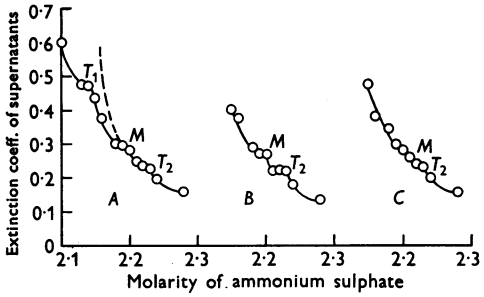

Fig. 3. Variable-solvent solubility curve of β_1-lactoglobulin. The solutions were 0·2 M-sodium chloride, 0·2 M-sodium acetate, with acetic acid to pH 4·95. Protein concentrations were in the ratio *A* 1·59, *B* 0·98, *C* 0·48. The equilibration time was 24 hr. at room temperature. T_1 and T_2 are transition points and do not vary with the concentration of protein. *M* moves as the concentration of protein is varied and therefore indicates a separate component.

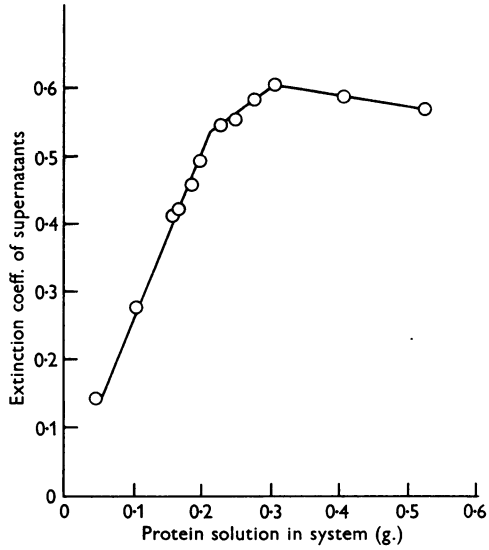

Fig. 4. Constant-solvent solubility curve of β_1-lactoglobulin. The solvent was 2·12 M-ammonium sulphate, 0·2 M-sodium chloride, 0·2 M-sodium acetate, with acetic acid to pH 4·95. Equilibration time was 24 hr. at room temperature.

Salt fractionation

The variable-solvent solubility curve lies at the basis of the experimental approach to salt fractionation. It is clear that the concentration not only of salt, but also that of protein, must be carefully controlled. The variable-solvent curve gives information on both these points; unfortunately it is adapted to use little protein, whereas in fractionation it is desirable to use larger amounts. Simply scaling up the appropriate conditions derived from the variable-solvent curve leads to inconveniently large volumes which are difficult to centrifuge. Nevertheless, at some sacrifice of efficiency conditions may be found where protein concentration is practically immaterial. The steeper the solubility curve the less a break will move along the salt axis of the plot with change in protein concentration. If the curve T_2M in Fig. 3*A* is extrapolated back it becomes nearly vertical at about 2·15 M-ammonium

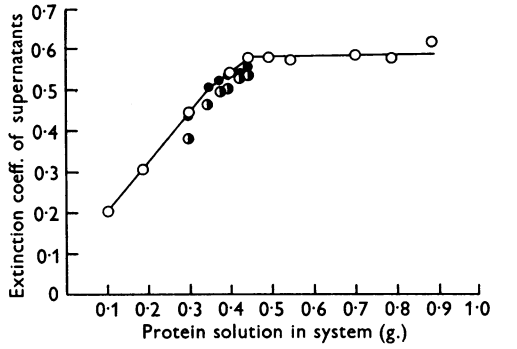

Fig. 5. Constant-solvent solubility plot of β_1-lactoglobulin, under the same conditions as for Fig. 4, showing the persistence of the break with longer periods of equilibration, and the absence of slope of the right-hand limb of the plot. The protein solution was first dialysed to pH 4·95, before the tubes were made up (see text). ○, Equilibrated for 24 hr. In a duplicate experiment, each solution was made up freshly and then equilibrated for 24 hr. (◑) and for 8 days (●).

sulphate. Fractionation at any protein concentration at this point will give as the precipitate nearly pure major component, leaving in solution major component enriched with minor component.

β_1-Lactoglobulin was fractionated by both these methods, with equivalent results. The major component was first spun out, the supernatant saturated with ammonium sulphate, and major component enriched with minor component precipitated. Major component was tested for homogeneity by both solubility methods. The variable-solvent curve (Fig. 6) at a protein concentration comparable with that of Fig. 3*A* showed very little or no minor component, though both transition points remained. The constant-solvent curve likewise demonstrated that most of the minor component was removed. Precipitate, in extremely small amount, first appeared at the saturation point (Fig. 7).

U-*tube electrophoresis*

Fractions were examined by U-tube electrophoresis in acetate buffer, pH 4·66 (0·1 M-sodium acetate–0·088 M-acetic acid). Fig. 8 shows typical boundaries obtained with this and unfractionated material. Pure major component still shows two peaks, though the trailing is of a slightly different shape from that of unfractionated protein. The proportion of slow component at high concentration was about 10 % instead of about 20 % in the unfractionated material. On the other hand, the fraction enriched with minor component contains more slow component than unfractionated β_1-lactoglobulin. The minor component evidently migrates as part of the slow boundary in the mixture.

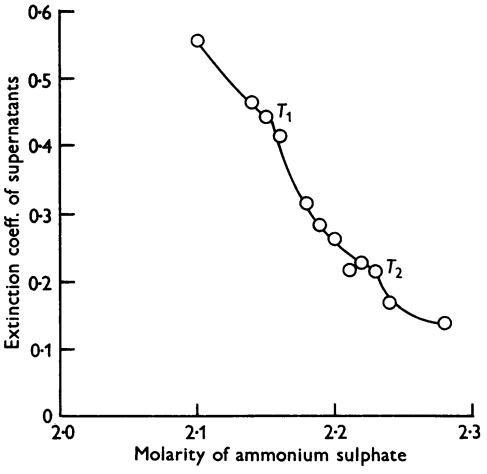

Fig. 6. Variable-solvent solubility curve of purified major component, under the same conditions as for Fig. 3, showing the absence of the break *M*. The protein concentration was 1·25 on the scale used for Fig. 3.

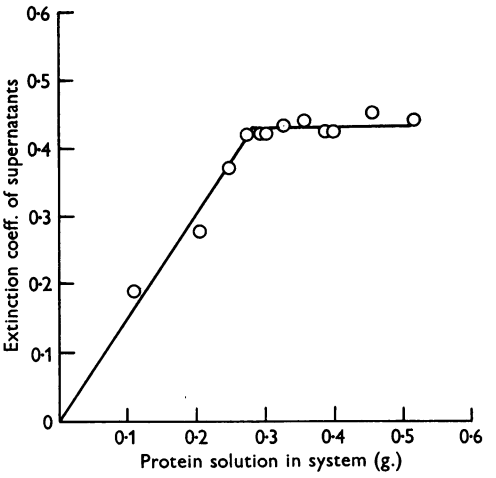

Fig. 7. Constant-solvent solubility plot of purified major component under the same conditions as for Fig. 4, showing no break indicative of heterogeneity.

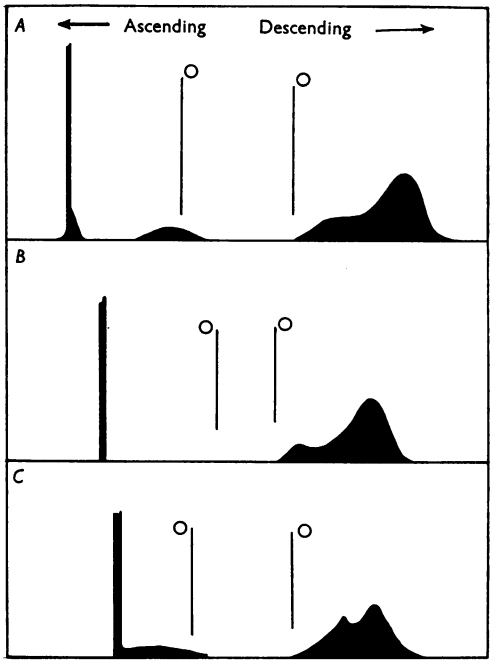

Fig. 8. Tracings of typical electrophoretic boundaries. *A*, β_1-Lactoglobulin, 1·18 g./100 ml. after 90 min.; *B*, purified major component, 1·58 g./100 ml. after 80 min. *C*, major component enriched with minor component, 0·92 g./100 ml. after 60 min. Origins are indicated by the vertical lines O.

Polymerization of β_1-lactoglobulin

The percentage of slow component in the major fraction and unfractionated material varied with protein concentration (Fig. 9). Gilbert (1955) has given a theoretical treatment of polymerization during electrophoresis or ultracentrifuging. He concluded that if the polymer consists of more than two monomer units, and if equilibration is instantaneous, then a partly resolved boundary indicating two components will be formed. The apparent percentage of slow component derived from boundary analysis will vary from 100 % at a concentration when fast component is just formed to zero, approached asymptotically, as total protein concentration is increased.

The theoretical curve may be calculated from the relation:

$$\text{Percentage slow component} = \frac{1}{2}\left\{\frac{\text{Total concn. at which slow component forms 50 \% of total}}{\text{Total concn.}}\right\} \times 100.$$

Fig. 9 shows this curve for unfractionated and fractionated material. That for the unfractionated material levels off at about 15 % more slow component than the curve indicates.

The curve for the separated major component follows the theoretical curve more closely, particularly in tending to zero as concentration is increased, to a much greater extent than unfractionated. Nevertheless, it shows signs of levelling off at about 10 % slow component. No doubt the simple fractionation procedure used left the major component still contaminated to some extent with minor. The discrepancy between the theoretical curve and that of unfractionated material suggests the presence of about 15 % of a slowly migrating minor component. This agrees with the estimate of minor component obtained from the solubility studies. The curve given by the major component suggests that it still contains about 5 % of minor component after fractionation. However, Gilbert's relationship is based on the hypothesis of infinitely rapid establishment of equilibration in relation to the rate of resolution in electrophoresis. A finite rate of equilibration would lead to disagreement between the theoretical and experimental curves. Moreover, the requirement that the fast component be at least a trimer of the slow may be relaxed. Ogston & Tilley (1955) tentatively suggested that the faster migrating component of 'normal' β-lactoglobulin was a dimer. If equilibration times were infinitely slow this situation would equally lead to an electrophoretic pattern with two peaks, representing monomer and dimer respectively. With more rapid equilibration, to obtain two peaks requires the existence of a trimer rather than a dimer; it is not possible at present to calculate the critical rate of equilibration at which trimer becomes necessary.

The possibility that the faster migrating component in electrophoresis may be a dimer of the slow component, and not of a higher order of polymerization, cannot therefore be ruled out on the basis of present evidence. It must be concluded that although the purified major component of β_1-lactoglobulin shows a complex boundary on electrophoresis, this is due to polymerization in solution and not to the presence of other distinct proteins. If Gilbert's hypothesis is accepted, the extent of polymerization must be at least three and representable by: monomer$\rightleftharpoons$dimer$\rightleftharpoons$ polymer. Such a system is a single component in the phase-rule sense, and would be expected to show only a single component in phase-rule tests.

The possibility that heterogeneity observed on electrophoresis or in the ultracentrifuge may not always indicate distinct proteins (though it does of course show the presence of different molecular species) means that much care is necessary in interpreting reports on the 'micro-heterogeneity' of proteins.

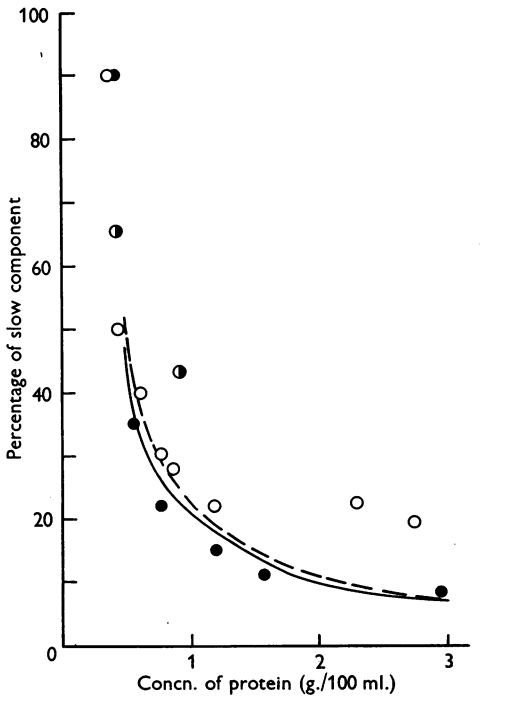

Fig. 9. Variation of percentage of slow component with protein concentration. ○, Unfractionated β_1-lactoglobulin; ●, purified major component; ◑, major component enriched with minor. The theoretical curve for major component is shown by a full line, for unfractionated material by a broken line.

SUMMARY

1. β_1-Lactoglobulin has been examined for heterogeneity by the variable-solvent and constant-solvent solubility tests.

2. Both tests indicate that β_1-lactoglobulin contains two proteins, the minor accounting for about 10 % of the total.

3. The theoretical basis of solubility tests and their application to salt fractionation are discussed.

4. Fractionation of β_1-lactoglobulin has produced the major component in a more nearly pure state.

5. The behaviour of the major component on U-tube electrophoresis is discussed.

I am indebted to Dr A. G. Ogston, F.R.S., who initiated this work and under whose guidance it was performed. The work was supported by a grant from the Agricultural Research Council.

REFERENCES

Aschaffenburg, R. & Drewry, J. (1955). *Nature, Lond.*, **176**, 218.
Aschaffenburg, R. & Drewry, J. (1957). *Biochem. J.* **65**, 273.
Brown, G. L. (1952). *Biochem. J.* **51**, 570.
Cecil, R. & Ogston, A. G. (1951). *J. sci. Instrum.* **28**, 253.
Cohn, E. J. (1925). *Physiol. Rev.* **5**, 349.
Dervichian, D. G. & Mossé, T. (1954). *Bull. Soc. Chim. biol., Paris*, **35**, 1289.
Falconer, J. S. & Taylor, D. B. (1946). *Biochem. J.* **40**, 835.
Gilbert, G. A. (1955). *Disc. Faraday Soc.* no. 20, p. 68.
Green, D. W., North, A. C. T. & Aschaffenburg, R. (1956). *Biochim. biophys. Acta*, **21**, 583.
Li, C. H. (1946). *J. Amer. chem. Soc.* **68**, 2746.
Northrop, J. H. & Kunitz, M. (1930). *J. gen. Physiol.* **13**, 750.
Ogston, A. G. & Tilley, J. M. A. (1955). *Biochem. J.* **59**, 644.
Ogston, A. G. & Tombs, M. P. (1956). *Nature, Lond.*, **178**, 200.
Ogston, A. G. & Tombs, M. P. (1957). *Biochem. J.* **66**, 399.
Polis, B. D., Schmukler, H. W., Custer, J. H. & McMeekin, T. L. (1950). *J. Amer. chem. Soc.* **72**, 4965.
Preaux, G., Hulsmans, J. & Lontie, R. (1954). *Arch. int. Physiol.* **62**, 574.
Roche, J., Derrien, Y., Reynaud, T., Laurent, G. & Roques, M. (1954). *Bull. Soc. Chim. biol., Paris*, **36**, 51.
Smithies, O. (1954). *Biochem. J.* **58**, 31.

The Isolation of Inositol Monophosphate from Liver

By G. HÜBSCHER and J. N. HAWTHORNE
Department of Medical Biochemistry and Pharmacology, The Medical School, Birmingham 15

(*Received* 9 *May* 1957)

Phospholipids containing inositol (phosphoinositides) are known to occur in a great variety of animal tissues such as ox brain (Folch, 1949), ox heart (Faure & Morelec-Coulon, 1954), liver (Macpherson & Lucas, 1947; McKibbin, 1956; Hawthorne & Hawthorne, 1955) and egg yolk (Malangeau, 1955; Dils & Hawthorne, 1956). Several recent observations emphasize the high turnover rate of the phosphoric acid component in the phosphoinositides of brain and pancreas (Dawson, 1954; Hokin & Hokin, 1955*a*, *b*, 1956) as compared with the other phospholipids present. Also, certain drugs seem to influence the turnover of phospholipids at pharmacologically significant concentrations (Magee, Berry & Rossiter, 1956; Ansell & Dohmen, 1956, 1957).

It seems therefore that phosphoinositides may have biochemical functions of dynamic rather than structural importance. A study of the biosynthesis of phosphoinositides might contribute to a better understanding of their metabolic significance.

The evidence is presented in this paper for the occurrence of free inositol monophosphate in liver; this has already been reported as a preliminary communication (Hawthorne & Hübscher, 1956).

MATERIALS AND METHODS

Paper chromatography. The solvent system propan-2-ol–acetic acid–water (3:1:1, by vol.) was used for the separation of ethanolamine, serine and amino acids (descending 18 hr.), of choline (descending 6 hr.) and of inositol and glycerol (descending 11 hr.). For the separation of inositol and glycerol, *n*-butanol–ethanol–water (4:1·9:9, by vol.; ascending 16 hr.; Hough, 1950) was also used.

The chromatograms were either sprayed with the ninhydrin reagent for the detection of ethanolamine, serine and amino acids, or according to the method of Chargaff, Levine & Green (1948) for the detection of choline. Inositol, glycerol and sugars were detected by dipping the chromatograms according to the method of Trevelyan, Procter & Harrison (1950). The solutions for dipping the chromatograms were prepared as follows. Solution 1: 0·1 ml. of saturated aq. $AgNO_3$ soln. was added to 20 ml. of acetone. Water was added with constant stirring until all the precipitate had just redissolved. Solution 2: saturated aq. NaOH soln. was diluted with 50 vol. of ethanol. The papers were dipped in solution 1, allowed to dry, and then dipped in solution 2. Inositol, glycerol and sugars showed up as dark-brown spots on a yellow background.

The phosphoric acid esters were separated by paper chromatography in the above propan-2-ol–acetic acid solvent and also in propan-1-ol–aq. NH_3 soln.–water