With this established, the spectra of the mixtures of ester and sodium perchlorate were recorded again using an infrared microspectrometer previously described (Wood, 1950). The microspectra show somewhat less resolving power than the double beam spectra and the region showing the spectroscopic differences between the esters is given in

Fig. 2. Microspectra of *A*, synthetic acetyl- and propionylcholine perchlorates as mixtures with sodium perchlorate (19 and 16.5% ester respectively) and *B*, spleen ester sample. —, acetylcholine perchlorate mixture; - - - -, propionyl perchlorate mixture.

Fig. 2. The other regions of the spectrum where differences between the two esters also occurred are not included, because for various reasons the microspectra were considered unreliable (e.g. water vapour in the 6–7.5 μ . region). In comparing the two upper curves in Fig. 2 it will be noted that the acetyl ester has the band at 8.05 μ . lacking in the propionyl ester and the propionyl ester has strong absorption at 8.35 μ . where the acetyl ester has a region of maximum transmission. On the other side of the broad 9 μ . band due to perchlorate the acetyl ester has a broad multiple band near 10.5 μ . while the propionyl ester has a single sharp peak at 10.50 μ .

The curve *B* in Fig. 2 is the microspectrum of the sample of the biological extract thought to be propionylcholine perchlorate, and containing approximately 100 μg . of active material. This curve lacks the acetyl ester band at 8.04 μ ., but has the propionyl ester band at 8.35 μ . (as a shoulder). In addition, on the other side of the intense perchlorate band at 9 μ . the natural ester curve has a single peak at 10.50 μ . similar to that of the synthetic propionyl ester, and in contrast to the broad multiple band of the acetyl ester in the same region. With the exception of the small peak at 10.75 μ . in the natural extract the spectrum of the latter is identical with that of the synthetic propionylcholine perchlorate. The 10.75 μ . band is attributed to an unidentified impurity from the extraction procedure. On the basis of the infrared spectrum, the conclusion is that the extracted ester is indeed propionylcholine.

REFERENCE

Wood, D. L. (1950). *Rev. sci. Instrum.* **21**, 764.

The Application of Four Methods for Assessing Protein Homogeneity to Crystalline β -Lactoglobulin: an Anomaly in Phase Rule Solubility Tests

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In order to follow the purification of a protein a reliable and sensitive method for determining the number and amount of components present in different samples must be available. Ultracentrifugal, electrophoretic, immunological and solubility tests were carried out with crystalline β -lactoglobulin in order to find such a method for following

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further purification of this protein. The results of these tests are presented in the first part of this paper and the usefulness of each is considered.

In the second part of the paper the occurrence under certain conditions of an anomaly in the 'variable solvent' solubility test for protein homogeneity (Falconer & Taylor, 1946; Roche & Derrien, 1947; and Derrien, 1952) is described and discussed. This appears to be the first time that such an

anomaly in phase rule solubility tests has been demonstrated experimentally, although the theoretical possibility of a related phenomenon in 'constant solvent' solubility tests has been described (Northrop, Kunitz & Herriot, 1948).

EXPERIMENTAL METHODS

Protein. Two samples of crystalline β -lactoglobulin were used: LG I, a sample prepared by a modification of Cecil & Ogston's (1949) method (Smithies, 1953) and recrystallized 9 times from aqueous NaCl solutions by dialysis; and LG II, a sample prepared by uniting approximately equal quantities of once recrystallized lactoglobulin, prepared by the above method, with a 3 times recrystallized sample prepared by the method of Cecil & Ogston (1949) and recrystallizing the mixture once more. No difference between the two samples could be established in tests in which both were used. The crystals were stored at 4° under distilled water saturated with toluene.

Physical methods. In the ultracentrifugal test a Spinco ultracentrifuge was used, by the courtesy of H. F. Deutsch, Department of Physiological Chemistry, Wisconsin University. The electrophoretic studies were made with the apparatus and technique in use by R. A. Alberty, J. W. Williams and their co-workers at Wisconsin University (see, for example, Alberty, Anderson & Williams, 1948; and Baldwin, Laughton & Alberty, 1951).

Immunological methods. In the immunological work two samples (one completely free from visible haemolysis products) of a pooled serum were obtained from two rabbits, following seven and nine subcutaneous injections (spread over 3 weeks) each of 1 ml. of an approximately 1 g./100 ml. solution of LG I in 0.15 M-NaCl. The rabbits were bled from the ear 5 days after the last injection. The serum, after the addition of merthiolate to 1 part in 10000, was stored at -20° until required.

The precipitation curve for LG I against these antiserum samples was determined as follows. A series of tubes was set up each containing 0.2 ml. of the serum and 0.2 ml. of LG I solution in approximately isotonic pH 7.4 phosphate-NaCl buffer. The lactoglobulin solutions covered dilutions of from 1 part in 50 (w/v) to 1 in 3280000. After mixing and standing at room temperature for 2.5 hr. the relative amount of precipitate was determined for the contents of each tube by measuring the absorption of light of wavelength 346 m μ . in a Beckman spectrophotometer, using a suitably masked Perspex cell of 0.3 ml. capacity. In the region of maximum precipitation the supernatant solutions (separated by centrifuging after having stood for 24 hr. with the precipitates) were tested for excess antigen and antibody by means of ring tests with the undiluted serum and 1 in 50000 LG I respectively.

Solubility test methods. In the solubility work the 'variable solvent' solubility test was used, and the salt precipitation curves were determined as follows, with unimportant differences in the earlier tests. The protein sample to be tested was dissolved in 2.4 M-NaCl and the solution centrifuged at 20000 g to ensure its clarity. Equal volumes of the protein solution and centrifuged 2.4 M sodium acetate buffer of the required pH were mixed, and 0.2 ml. portions of the mixture transferred to dry glass test tubes (10 $\times$ 75 mm.) previously cleaned with alkali, hot chromic acid and distilled water. To each tube 1 ml. of (NH₄)₂SO₄ solution was

then added, the concentration of (NH₄)₂SO₄ being increased stepwise from tube to tube. The acetate concentration in the resulting solutions was 0.2 M so that they were adequately buffered. The tubes were sealed with Parafilm sheet (Marathon Corporation, Menasha, Wisconsin, U.S.A.), as corks give impurities absorbing in the ultraviolet; their contents were mixed by inversion. The tubes were transferred to a constant temperature bath at 25° until ready for the solubility determinations, when the contents were once again mixed by inversion and centrifuged for 20 min. at about 20 000 g in a Servall SS 1a centrifuge. (Filtration was avoided as all types of paper tried contained water-soluble substances absorbing in the ultraviolet). From 50 to 67 % of the supernatant of each tube was removed without disturbing the solid. In doing this the tube was held at approximately the same angle and position as during centrifuging and the supernatant was withdrawn by means of an acid cleaned dry Pasteur pipette with the tip of the pipette almost touching the lowest part of the tube opposite the pellet. The optical densities of the supernatant solutions were determined at 278 m μ . in a Beckman spectrophotometer fitted with a mask limiting the light beam to a size of 2 $\times$ 6 mm. so that a modified quartz cell of solution size 3 $\times$ 10 $\times$ 15 mm. could be used. The initial protein concentration was adjusted so that the maximum optical densities of these final solutions were between 0.40 and 0.45. This corresponds to a concentration of about 0.05 g. lactoglobulin/100 ml., so a test of twenty-four tubes could be made with less than 20 mg. of protein.

The precipitation curves were found to be sensitive to small changes in the system, for example in the buffer composition, consequently in comparing any two curves only one component at a time (protein, buffer or (NH₄)₂SO₄ solutions) was varied. The procedure described facilitates this. The initial protein concentration was also kept the same in any two tests to be compared, as it was found that the precipitation zone of a protein is influenced by the total amount of protein present (cf. Falconer & Taylor, 1947). When checking a result all solutions were freshly made up to minimize accidental effects.

The precision of the test was later found to be improved and the labour of setting up greatly reduced by the use of automatic pipettes, but the results here presented were obtained with simple calibrated glass pipettes.

RESULTS AND DISCUSSION

(1) Homogeneity tests

Fig. 1 shows a tracing of a photograph of the boundary formed after 2.5 hr. of centrifuging at 59780 rev./min. of an approximately 1 g./100 ml. solution of LG I in 0.2 M sodium acetate buffer, pH 5.1, at an average temperature of 24.1°.

Fig. 2 illustrates the electrophoretic patterns obtained with various solutions of LG II at pH values close to the isoelectric point of the protein.

Fig. 3 shows the results obtained by the author in two determinations of the precipitin curve for LG I against two samples of its rabbit antiserum. The relative amount of precipitate formed is plotted against the final LG I concentration present. The broken line and half filled points show some com-

parable results obtained by Deutsch (1950) with a preparation of crystalline lactoglobulin made by the method of Cecil & Ogston (1949). The results of tests for excess antigen and antibody are also shown. The dotted line and open points show Deutsch's

results with material prepared by the method of Palmer (1934).

Fig. 4 shows the 'variable solvent' solubility (salt precipitation) curve obtained at pH 4.8 with LGII. It is the combined results of two determina-

Fig. 1. Sedimentation diagram of an approximately 1 g./100 ml. solution of LGI in 0.2M sodium acetate buffer, pH 5.1. Time 2.5 hr. Speed 59 780 rev./min. Temperature 24.1°.

Fig. 2. Electrophoretic patterns of LGII in various solutions. The initial boundary positions are indicated by vertical lines and the directions of migration by arrows. (a) Approximately 1% (w/v) protein; 0.05M-NaCl, pH 5.60 $\pm$ 0.05, or 0.05M Na acetate, pH 5.50 $\pm$ 0.05. (b) 0.7 or 1.3% protein; 0.1M-NaCl, pH 5.50 $\pm$ 0.05. (c) 1.3% protein, 0.09M-NaCl + 0.01M Na acetate, pH 5.15 $\pm$ 0.05.

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Fig. 3. Precipitation curves for various crystalline lactoglobulin samples against their corresponding antisera. The author's results with LGI are shown by a solid line and filled points. Data obtained by Deutsch (1950) are shown by a broken line and half filled points (for lactoglobulin prepared by the method of Cecil & Ogston, 1949), and by a dotted line and open points (for a sample prepared by the method of Palmer, 1934). The results of tests for excess of antibody and antigen on the supernatants for the LGI and Cecil & Ogston lactoglobulin (C and O) experiments are shown above the curves. The relative amount of precipitate is calculated as $100 \times \text{increase in optical density at } 346 \mu\text{ over blank} \div \text{maximum increase}$. The final LGI concn. present is expressed as $\log_2$ LGI in g./100 ml. The vertical axis scale for the Deutsch data is $100 \times \text{mg. N in precipitate} \div \text{mg. N in precipitate at first maximum}$.

Fig. 4. The 'variable solvent' solubility (salt precipitation) curve for LGII at pH 4.8. The original solutions contained approximately 0.05 g./100 ml. of protein and were 0.2M in sodium acetate, with sufficient acetic acid present to give the required pH. The curve gives the combined results of two experiments with equilibrium times of 2 days.

tions with equilibrium times of 2 days. The vertical axis represents the percentage of the original protein remaining in solution. This figure is obtained from the optical densities of the supernatant solutions at 278 $m\mu$. (corrected for the blank with no protein and expressed as the percentage of the optical density for the tubes in which no precipitation has occurred) on the assumption that the extinction coefficients of each of the protein components is the same. Since Tilley (private communication) finds no significant change in the ultraviolet absorption of lactoglobulin on fractionation, this assumption is probably justified. The horizontal axis shows the corresponding ammonium sulphate concentration in the supernatant solutions.

Purity of lactoglobulin

Inspection of the ultracentrifugal result presented in Fig. 1 shows no evidence for the existence of more than one component in LGI. Confirmation of this result is provided by Baldwin (personal communication), who was unable to find evidence for more than one component from detailed mathematical analysis of the boundary shape in the ultracentrifuge at 28° of solutions of recrystallized β -lactoglobulin also prepared by the modified Cecil & Ogston method, although he points out that even with this analysis two components present in equal amounts could only be detected reliably in this case if their sedimentation coefficients differed by > 10%.

Since in the ultracentrifuge at these temperatures crystalline β -lactoglobulin appears already homogeneous, within the present resolving power of the method, use of the ultracentrifuge to follow further purification would be pointless.

The electrophoretic studies indicate the presence of at least two components in LGII. These lactoglobulin solutions, however, showed marked anomalies in electrophoresis, as can be seen from the illustrative results presented in Fig. 2. The ascending and descending patterns differ widely, and small changes in the system bring about large changes in the apparent component analysis. Thus an unequivocal analysis of the component composition of various lactoglobulin samples by electrophoresis would clearly require extensive investigations for each sample at a series of protein concentrations and in various buffers. It would thus be impractical to use the method to follow further purification of lactoglobulin. This conclusion is supported by more extensive electrophoretic studies made by Tilley (private communication) with the same protein.

Of the various immunological methods available for investigating protein homogeneity the determination of the precipitin curve for the protein against its rabbit antiserum was chosen for this study because of its quantitative nature. An immunologically pure protein should give a symmetrical

precipitin curve with a single maximum when the amount of precipitate formed is plotted against the logarithm of the amount of protein antigen added to a constant amount of its rabbit antiserum. An equivalence zone should be observed over which no excess of either antibody or antigen is detectable in the supernatant solution after removal of the precipitate.

Of the precipitation curves plotted in Fig. 3, that due to Deutsch with the Palmer lactoglobulin shows a clear indication of the existence in this preparation of more than one component, in that more than one maximum is present. The author's results with LGI and Deutsch's with the Cecil & Ogston lactoglobulin are closely similar and, as only one maximum is clearly seen, offer little evidence for the presence of more than one component in these preparations. Consequently, this immunological test was also regarded as unsatisfactory for following further purification of lactoglobulin.

It should be pointed out however that, while Deutsch in his immunological test on the Cecil & Ogston lactoglobulin obtained no equivalence zone, in the present work with LGI an equivalence zone was observed (see Fig. 3). The significance of this observation as a criterion of the greater immunological homogeneity of LGI is, however, rendered uncertain for two reasons: (a) there is a small rise in the precipitin curve for LGI, which should not occur with an immunologically pure protein, at very high LGI concentrations (250 times that at the maximum) in the one experiment carried beyond 0.25 g. LGI/100 ml.; and (b) that as the demonstration of an equivalence zone is the result of negative tests for antigen and antibody excess it may only reflect insufficiently sensitive tests for their presence.

Previous workers (see, for example, McMeekin, Polis, Dellamonica & Custer, 1948, who give earlier references) have found evidence for the inhomogeneity of crystalline β -lactoglobulin from solubility studies, using the 'constant solvent' method. Their results do not, however, permit a quantitative estimation of the components present. For the solubility studies here described the 'variable solvent' solubility test was therefore chosen as it can more readily provide this quantitative data.

The 'variable solvent' solubility curve for LGII plotted in Fig. 4 was obtained at pH 4.8. Similar curves were also obtained with LGII at pH's 4.5 and 5.1, and with LGI at pH's 4.8 and 5.1. This type of curve, in which two breaks are clearly visible, could regularly be obtained with lactoglobulin at any of these pH values with equilibrium times ranging from 1 to 9 days. Examination of the precipitates obtained showed them to consist in part of completely amorphous (gelatinous) material, and in part of solid, the structure of which, even when best defined, could not be fully resolved by an

oil immersion objective, but which had the appearance of short branched strings of beads. The gelatinous precipitate appeared first in the course of the precipitation and may have corresponded to the material represented by the initial break in the curves.

In view of the way in which these curves could repeatedly be obtained with either lactoglobulin sample at three different pH's and with considerably different equilibrium times it is concluded that the two breaks represent two 'true' components present in lactoglobulin as prepared by the Cecil & Ogston method or its modification.

The precipitation curves follow the form described by Cohn (1925), viz.

$$\text{Solubility} = e^{(\beta - \alpha \cdot c)}$$

(where α and β are constants, and c is the salt concentration). The smooth curves in Fig. 4 were drawn to fit this relationship.

Application of variable solvent solubility test

In view of the relative simplicity and reliability of this 'variable solvent' solubility test it was regarded as the most suitable method of the four tested for following attempts at fractionating lactoglobulin. Two applications of the method may be quoted. The first of these was to find out whether the repeated recrystallizations during the preparation of LGI had affected its component analysis. A sample of crystalline lactoglobulin was therefore prepared by the modified Cecil & Ogston method but was not recrystallized. The solubility curve at pH 5.1 of this material was compared with that of LGI, the 9 times recrystallized sample. No difference between the two samples could be demonstrated in this solubility test, despite the fact that LGI had been stored for nearly a year and the once-crystallized material for less than 2 weeks. Some data presented later in this paper throw light on the ineffectiveness of these repeated recrystallizations in modifying the component analysis of the protein.

The second application of the method was to follow an attempt at fractionating β -lactoglobulin by using the change with temperature of the solubility of the protein in dilute salt solutions. Several saturated solutions of LGI in 0.05M-NaCl were prepared at 25°, and centrifuged to ensure their clarity. An equal volume of 0.05M-NaCl was added to each and the mixture cooled to 0°, when gelation occurred in a few minutes. After standing at 0° for varying times from 2 to 5 hr., the gels (usually about 16% of the total material) were separated as precipitates in a cooled centrifuge. In all but one out of the five gelation experiments of this type which were made, no significant difference between the precipitate and supernatant could be demonstrated by the solubility test. To illustrate these

experiments, the solubility curves determined at pH 5.1 with the products of a typical gelation (IV), and of the one (II) in which appreciable fractionation was obtained, are presented in Fig. 5. The atypical result could not be repeated but serves to show that fractionation can be followed by this test when it occurs.

It will be noted that the curves shown in Fig. 5 suggest the presence of three components in β -lactoglobulin. The one present in a small amount was not observed in the earlier solubility experiments quoted (see, for example, Fig. 4) because at that time random errors, probably in the pipetting, obscured its presence. Insufficient experiments were performed however to exclude the possibility that this component was a small amount of denatured material.

These solubility tests suggest the presence of two (possibly three) components in crystalline β -lactoglobulin prepared by the modified Cecil & Ogston method. The observed proportion of the components is approximately 1:5:20, assuming that each has the same extinction coefficient (see above). These proportions will, however, only represent those present in the original solution provided that the precipitation of any one of the components does not cause the

Fig. 5. The solubility curves at pH 5.1 for the supernatant solutions and precipitates from two gelation experiments with LGI. The upper two curves are the combined results of three determinations of the solubility curves for the products of gelation experiment no. II, with equilibrium times of 5-8 days; the lower two are the results of a single determination of the curves for the products of gelation IV, with an equilibrium time of 3 days. The vertical axis is numbered only for supernatant IV to avoid confusion.

co-precipitation of any of the others. This condition is probably fulfilled by the present system at pH 5.1, as equivalent results can be obtained at two other pH values.

(2) *An anomaly in the 'variable solvent' solubility test for protein homogeneity*

Fig. 6 shows the results obtained with LGII at pH 5.4 in the 'variable solvent' solubility test in a single determination of the solubility curve with an equilibrium time of 2 days. Fig. 7 shows two repetitions of this experiment with LGI. In the first of these, five sets of tubes were set up and the solubilities were measured at daily intervals from 1 to 5 days. In the second repetition, two sets of tubes were used and the solubilities were measured after 2 and 5 days.

Fig. 8 shows some of the results obtained with LGI at pH 5.7. Determinations of the solubilities were made 1, 2, 3, 6, 9, 13, 20 and 30 days after setting up, with several duplicates, but for clarity of presentation only the results after 1, 3, 13 and 30 days are plotted.

In part (1) of the Results it was established that lactoglobulin shows two main components in the 'variable solvent' solubility test (see Fig. 4) at pH values of 4.5, 4.8 and 5.1, and that the type of curve obtained was unaffected by the time allowed for equilibrium to be established.

At pH 5.4, however, with both LGI and LGII, a discontinuity suggesting the presence of a considerable amount of an additional 'component' is seen (the regions of open points in Figs. 6 and 7), and in Fig. 7 it can be seen that in this region the two repetitions of the experiment diverge considerably. This is in marked contrast to the other regions of the curve where good agreement between the two repetitions was found. Furthermore, the precipitates in the tubes indicated by the open squares in Fig. 6 were noticeably different in character (appearing granular) from those in the other regions of the curve; and examination under the microscope of precipitates from duplicate tubes showed them to contain clusters of parallel needle-like crystals. This observation was regarded as evidence that the additional break in the curves at pH 5.4 is due to the presence of a different (crystalline) form of the precipitate, stable at this pH only over a narrow range of salt concentrations, and does not represent a 'true' additional component. The variability of the results in this region supports this conclusion. It can be explained as due to differences in the proportion of the total material in the crystalline form, which proportion is probably very sensitive to small differences in the system such as may be inadvertently made in repeating an experiment.

Yet more confirmation of the correctness of these ideas is found in the results obtained at pH 5.7, at

which pH the crystalline form is the stable solid phase at all salt concentrations, though at short equilibrium times the amorphous type of curve can be seen. The results of an extensive experiment at pH 5.7 with LGI are shown in Fig. 8. Observations of the form of the precipitate were made on every tube and the approach to final equilibrium carefully followed.

Fig. 6. The solubility curve for LGII at pH 5.4. A single determination with an equilibrium time of 2 days.

Fig. 7. Solubility curves for LGI at pH 5.4. Δ are the results of an experiment consisting of five determinations with equilibrium times of 1-5 days; $\square$ are the results of a repetition of the experiment with two determinations with equilibrium times of 2 and 5 days. $\blacksquare$ the combined results of both experiments where no difference between them was apparent.

Fig. 8. Solubility curves for LGI at pH 5.7 determined at varying intervals from 1 to 30 days after setting up (i.e. with equilibrium times of 1-30 days).

The curve obtained 1 day after setting up is in general form like the normal curve showing two components, except in the region indicated by the open points where the two points *A* and *C* are seen to lie considerably below the expected curve. The precipitates from those two tubes were found to be almost completely crystalline, the crystals being needle-like and arranged in sheaves. In no other tubes were crystals present to any considerable extent. Thus despite the difficulty of observing under the microscope the proportions of crystalline and amorphous material, there was no doubt that the precipitate from tube *B* was largely of the character described in part (1) of the Results for the pH 4.8 precipitates and contained only a few poorly defined crystals. Consequently, point *B* lies close to the curve (dotted line) expected for precipitates of the bead-like form.

After 3 days the precipitates were all largely of the sheaf-like crystal form, except in tube *D* (the point for which lies above the general curve for 3 days). This tube contained a good deal of amorphous and bead-like material, such crystals as were present being poorly defined.

Solubility changes continued still further until true equilibrium was reached between 20 and 30 days, when the solid phase was found to consist entirely of crystals, now flat diamond-shaped—some arranged in clusters obviously derived from the earlier sheaf-like bundles of needles. At this stage the character of the precipitation curve (filled squares) is quite different from that observed at equilibrium at, for example, pH 4.8. The curve is sigmoid and shows no marked breaks. The precipitation zone is much narrower (> 66% of the protein precipitated with < 2.5% change in the salt concentration). This type of curve, in a 'variable solvent' solubility test, may be analogous to the rounded curves obtained in the 'constant solvent' test when mixed crystals of variable composition are present.

The decreases in solubility with time at this pH are thus due to the change from a metastable solid to the stable crystalline form. It is concluded that if the range of salt concentrations over which the alternative form of the solid is stable, is narrow (as the results suggest is the case at pH 5.4), this decreased solubility can lead to the appearance of an additional break in the solubility curves, which suggests erroneously the existence of an additional component in the protein under study. This anomaly should be suspected when precipitation curves are obtained in which any region is found to be un-reproducible. It may be recognized by performing the test at several pH values with different lengths of time for equilibrium, and by observing the appearance of the precipitates.

A suggestion which follows from the results

presented here is that crystallization should be avoided in the further purification of proteins which have already been crystallized once (cf. Pirie, 1940). The steep precipitation curves with no clear steps, obtained under conditions of crystallization, indicate the danger that all the protein components present in solution, if sufficiently similar, may be included in the highly stable protein crystals if these are once formed. This is probably the explanation of the absence of any change in the component composition of lactoglobulin (noted in part (1) of this paper) following repeated recrystallizations. However, under conditions leading to amorphous precipitates the components come out of solution less abruptly, and to some extent separately, so that further purification by salt fractionation might be accomplished. It should also be noted how small is the change in pH which makes the difference between lactoglobulin being precipitated from these very dilute solutions as an amorphous solid or as crystals.

SUMMARY

1. A method for following attempts at further purifying crystalline β -lactoglobulin was sought.

2. The ultracentrifugal and immunological (precipitation curve determination) methods tried were unsatisfactory as they showed no evidence for inhomogeneity in the sample tested.

3. Electrophoretic analysis of lactoglobulin was subject to such marked anomalies as to be impractical.

4. 'Variable solvent' solubility tests were found most satisfactory, and indicated the presence of two main components (ratio approximately 1:4) in lactoglobulin at pH's 4.5, 4.8 and 5.1.

5. Repeated recrystallizations do not affect this ratio.

6. At pH 5.4 an additional discontinuity occurs in the solubility curves for lactoglobulin associated with a different form of the solid phase (previously amorphous, now crystalline). It is concluded that it does not represent a 'true' additional component in the protein.

7. At pH 5.7 the solubility behaviour of lactoglobulin is markedly time dependent. The results are shown to be due to progressive crystallization, and provide confirmation for the conclusion that the additional break in the curves at pH 5.4 is due to the taking on by the solid phase of a different (crystalline) form stable, at this pH, only over a limited range of salt concentrations.

8. The ways of detecting this anomalous behaviour (when more breaks occur in the solubility curve than there are components in solution) are discussed.

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REFERENCES

- Alberty, R. A. Anderson, E. A. & Williams, J. W. (1948). *J. phys. Chem.* **52**, 217.
 Baldwin, R. L., Laughton, P. M. & Alberty, R. A. (1951). *J. phys. Chem.* **55**, 111.
 Cecil, R. & Ogston, A. G. (1949). *Biochem. J.* **44**, 33.
 Cohn, E. J. (1925). *Physiol. Rev.* **5**, 349.
 Derrien, Y. (1952). *Biochim. biophys. Acta*, **8**, 631.
 Deutsch, H. F. (1950). *J. Immunol.* **185**, 377.
 Falconer, J. S. & Taylor, D. B. (1946). *Biochem. J.* **40**, 835.
 Falconer, J. S. & Taylor, D. B. (1947). *Proc. XIth Int. Congr. Chem., Lond.*, **2**, 413.
 McMeekin, T. L., Polis, B. D., Dellamonica, E. S. & Custer, J. H. (1948). *J. Amer. chem. Soc.* **70**, 881.
 Northrop, J. H., Kunitz, M. & Herriot, R. M. (1948). *Crystalline Enzymes*, 2nd ed. p. 293. New York: Columbia University Press.
 Palmer, A. H. (1934). *J. biol. Chem.* **104**, 359.
 Pirie, N. W. (1940). *Biol. Rev.* **15**, 377.
 Roche, J. & Derrien, Y. (1947). *Proc. XIth Int. Congr. Chem., Lond.*, **2**, 463.
 Smithies, O. (1953). *Biochem. J.* **55**, 57.

Phosphorylation of Carbohydrates in Retinal Extracts

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Warburg, Posener & Negelein (1924) first observed that the isolated rat retina together with tumour cells, the grey matter of brain and embryonic tissue possessed comparatively high glycolytic activity. Subsequently, numerous investigations were carried out on the metabolic activity of the isolated retina (e.g. Dickens & Greville, 1933; Laser, 1935). Kerly & Bourne (1939, 1940) and Süllman & Vos (1939), independently, were the first to study glycolysis in retinal extracts. Such extracts were found to convert added glucose, fructose, hexose diphosphate and glycogen into lactic acid; the latter workers also observed the formation of lactic acid from mannose and glucose 1-phosphate. Studies of the effects of dialysis and of activators and inhibitors supported the view that the pathway of lactic acid formation involved the phosphorylating glycolysis which operates in muscle extracts. However, no intermediary phosphate esters were isolated, nor were any of the individual enzymes studied. Kerly (1948) has reported preliminary experiments on a hexokinase in retinal extracts, but again the products of the reaction were not isolated nor identified. Lenti (1939) also studied lactic acid formation in retinal extracts and concluded that phosphorylation was not involved; he failed in all attempts to isolate phosphorylated intermediates (Lenti, 1943).

The present paper provides direct evidence for the occurrence in retinal extracts of the three glycolytic enzymes, hexokinase, phosphohexose isomerase and 6-phosphofructokinase. Some of the characteristics of the hexokinase have been examined and compared with those of yeast hexokinase; certain intermediate phosphoric esters have also been isolated and identified.

METHODS

Materials

AnalaR grade sugars were used without further purification. D-Glucosamine hydrochloride was purified by two recrystallizations from ethanol-water and had a purity of 98–99% on the basis of its nitrogen content. Glucose 1-phosphate (G 1-P) was prepared by the method of Hanes (1940) and was recrystallized as the di-potassium salt. We are indebted to Dr J. Ashby for samples of synthetic D-glucose 6-phosphate (G 6-P) and D-fructose 1:6-diphosphate (Fru 1:6-P) and to Dr C. W. Parr for D-fructose 6-phosphate (Fru 6-P). Adenosine triphosphate (ATP) was made from rabbit muscle by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948), and inosine triphosphate (ITP) was made from this by the method of Kleinzeller (1942). All the phosphate esters except Fru 1:6-P were prepared as the barium salts and were converted to the soluble sodium salts before use; Fru 1:6-P was prepared and used as the lithium salt, experiments having shown that lithium ions at the concentration employed did not inhibit the phosphorylating enzymes. All solutions were adjusted to pH 7.5 before use.

Preparation of extracts

(a) *From retina.* Ox eyes, in batches of one dozen, were fetched on ice from the slaughter house. The retinas were dissected out as soon as possible on arrival in the laboratory and were immediately suspended in 10 ml. ice-cold glass-distilled water. The suspension was ground in a chilled mortar with 2 g. acid-washed sand for 1 min. and was then centrifuged in thoroughly chilled tubes for 5 min. at 1500–2000 g. The top, slightly turbid, pink layer was decanted off (or removed with a pasteur pipette) and the residue (a small dark brown layer together with cell debris and sand) was re-extracted with a further 10 ml. glass-distilled water, again centrifuged and the top layer added to the first extract. The combined extracts were dialysed in tightly filled cellophan sacs against running ice-cold glass-distilled