252. *Cellular Constituents. The Chemistry of Xanthine Oxidase. Part II.* The Homogeneity of Crystalline Metalloflavoprotein Fractions.*

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The problem of homogeneity of crystalline molybdenoflavoprotein fractions [1] with high xanthine oxidase activity has been studied by sedimentation experiments, by electrophoresis, and by "constant protein" and "constant solvent" solubility analysis. All these tests, although in some instances not producing complete information, indicated that in our best preparations at least 90% of the material acted physicochemically as a single component. However, enzymic activity measurements, more fully discussed in Part III (following paper), made it clear that "active" and closely related "inactive" flavoproteins were present in this "component." From the sedimentation, diffusion, and density data, it has been calculated that the crystalline material has M about 290,000. Its isoelectric point is at pH 5·3—5·4.

Avis *et al.*[1] recently described a procedure for the preparation from cow's milk of a molybdenoflavoprotein with high xanthine oxidase activity and a low protein–flavin ratio, obtained for the first time in a crystalline form. It appears that xanthine oxidase, like

* Part I, *J.*, 1955, 1100.

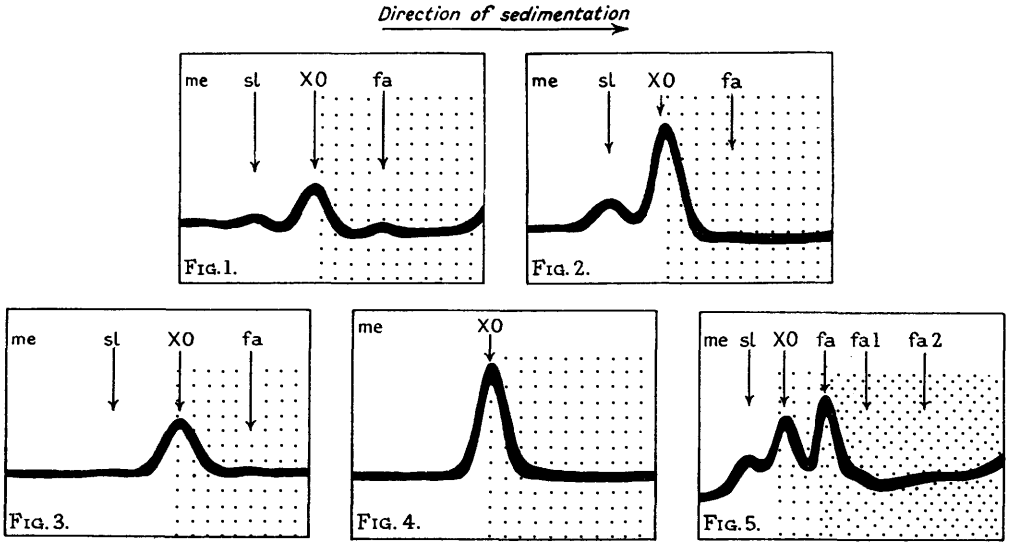
[1] Avis, Bergel, and Bray, *J.*, 1955, 1100.

other enzymic proteins, especially those with coenzyme- and metallo-prosthetic groups, decreases in activity during purification and on prolonged storage or mild chemical treatment, often without any obvious alteration of physicochemical properties. This may be due either to partial loss of its prosthetic groups, particularly of molybdenum, or to slight changes in its protein moiety.[2]

We now report below results of tests for homogeneity of some of our crystalline products in comparison with less pure fractions of our own and of other authors. The symbols M1—10 refer to numbers of different stages of the preparative procedure (see Avis *et al.*[1]), and LXO51—68 to those of the total batches, each from 20—40 l. of buttermilk. The xanthine oxidase activities of the various samples, expressed as " AFR " and " APR ", are given in Table 2, with other details of the specimens used.

Sedimentation.—Solutions of our molybdenoflavoprotein (LXO56, this being the batch described in Part I [1]), obtained at four successive stages of its preparation (M5, M6, M7, M8), have been examined in the ultracentrifuge. The sedimentation diagrams (Figs. 1—4)

FIGS. 1—5. *Sedimentation diagrams.*



clearly show the increase in the proportion of xanthine oxidase (peak " XO ") that was achieved at each step of the purification procedure. In the early stages, M5 and M6 (Figs. 1 and 2 respectively), considerable amounts of two major impurities (labelled " *sl* " and " *fa* ") were present, together with some polydisperse material of low molecular weight which produced an ill-defined peak between the meniscus (" *me* ") and the " *sl* "-peak. In the crystalline flavoprotein M7 (Fig. 3), the material of low molecular weight was absent and the greater portion of the " *sl* "- and the " *fa* "-component had disappeared. After recrystallisation leading to M8, the " *sl* "- and the " *fa* "-component were not detectable (Fig. 4).

From the changes in photographic density along the length of the sedimentation diagrams (indicated in the Figures by dotted areas), it could be deduced that the red-brown colour of the solution was associated, as would be expected, with the " XO "-peak. The " *sl* "-component was not coloured, but absorbed strongly in the ultraviolet region (250—265 mμ). Component " *fa* ", as shown in the diagram (Fig. 5) of one of the rejected fractions, S4 (see Avis *et al.*[1]), which contained an increased proportion of this substance,

[2] Cf. Corran *et al.*, *Biochem. J.*, 1938, **32**, 2231; 1939, **33**, 1694; Morell, *ibid.*, 1952, **51**, 657; Mahler and Green, *Science*, 1954, **120**, 7; Richert and Westerfeld, *J. Biol. Chem.*, 1954, **209**, 179; Mackler, Mahler, and Green, *ibid.*, 1954, **210**, 149; de Renzo, *Arch. Biochem. Biophys.*, 1954, **49**, 242; Mahler, Fairhurst, and Mackler, *J. Amer. Chem. Soc.*, 1955, **77**, 1514.

apparently possessed a colour similar to that of "XO". The two additional peaks in this diagram, "*fa* 1" and "*fa* 2", probably represent aggregates, resulting from incomplete dissolution. These results suggest that both "XO" and the impurity, "*fa*", contributed to the absorption of less purified fractions at 450 mμ ("flavin", see Avis *et al.*[1]).

The sedimentation coefficients ($s_{20,w}$) calculated for the three main components (see Experimental section) are given in Table 1.

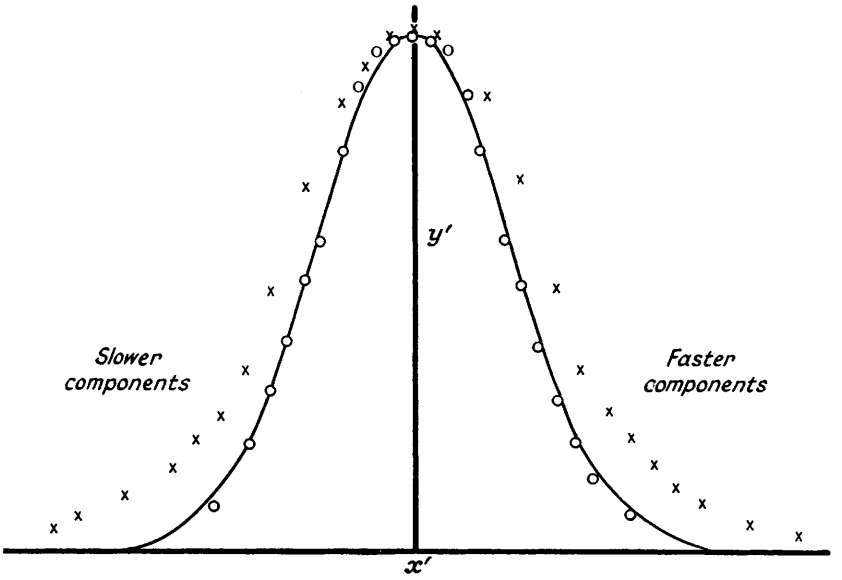
TABLE 1.

Stage	M5	M6	S4	M7	M8
$10^{13}s_{20,w}$: "*sl*"	6·9	6·2	6·95	*	†
"XO"	11·5	11·0	11·5	11·3	10·8
"*fa*"	15·7	*	16·5	*	†

* Amount too small for measurement of sedimentant constants. † Not detected.

From Figs. 3 and 4, it can be deduced that 95% or more of our crystallisates in solution sedimented to form a single peak, "XO". However, on closer inspection of the sediment-

FIG. 6. *Normalised sedimentation diagram* (42,000 r.p.m.).



× *After* 17 *min.* ○ *After* 123 *min.*
The curve is calculated from the diffusion coefficient.

ation results, it was found that the corrected areas under the single peaks diminished as sedimentation proceeded, indicating the presence of polydisperse "impurities".[3] For stage M7, the decrease in area after 55 minutes' centrifugation amounted to 15%, only a third of which could be accounted for by the small quantities of "*sl*" and "*fa*" still present in this material. This preparation contained, therefore, in addition, at least 10% of polydisperse "impurities". Recrystallisation, leading to M8, reduced the total "impurities", since centrifugation for the same time as for M7 produced only a 5% decrease in area. After more prolonged centrifuged (2 hr.) it was calculated that M8 contained 10% of total impurities.

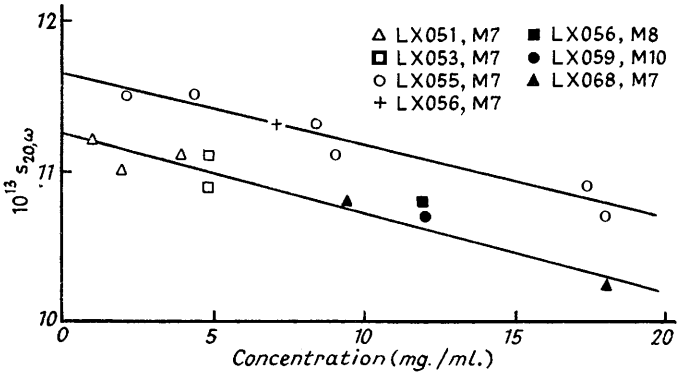
The presence of these small amounts of extraneous material can be discerned still more clearly when "normalised" sedimentation curves are considered.[4] When this method is applied to fraction M8, Fig. 6 discloses that at the beginning of the sedimentation process the spread of the peak was greater than could be accounted for by calculation from

[3] Svedberg, Pedersen, Lamm, and Kraemer, "The Ultracentrifuge," Oxford Univ. Press, 1940.
[4] Davison, James, Shooler, and Butler, *Biochim. Biophys. Acta*, 1954, **15**, 415.

our diffusion values for the " XO "-component (see p. 1218). But after 2 hours' sedimentation the theoretical curve and that calculated from experiment agreed well, which meant that the polydisperse " impurities " had disappeared from under the main peak. This confirmed our estimate of the amount of impurity in M8 arrived at by the area method given above. It also showed that some of the " impurities " sedimented faster and some slower than the main component.

The sedimentation coefficients for the " XO " peak have been measured on seven crystalline preparations from different batches at varying concentrations of the solution (Fig. 7; M7 of LXO51, 53, 55, 56, 68, M8 of LXO56, and M10 of LXO59). Extrapolation of these results to zero concentration gives an average $s_{20,w} = 11{\cdot}4 \pm 0{\cdot}3 \times 10^{-13}$ sec. It appears that the sedimentation coefficients of different batches may vary slightly, but significantly (two separate lines as seen in Fig. 7), perhaps as the result of the presence of different amounts of polydisperse " impurities " or because of their varying xanthine oxidase activities (cf. following paper, also Table 2 and the remark on the loss of activity through storage, p. 1217). It may be noted that one of our specimens, derived from washings of LXO60, M7, which had almost completely lost its xanthine oxidase but not its DPNH-dehydrogenase activity during prolonged storage (see Table 2 and following paper),

FIG. 7. *Effect of concentration on sedimentation coefficients.*



gave a sedimentation coefficient s for the main peak " XO " of $11{\cdot}2 \times 10^{-13}$ sec. at a concentration of 3 mg./ml., which falls inside the lower values noted in Fig. 7.

Our findings agree with those given by Philpot [5] who based them on a preparation by Corran *et al.*[2] (1939). He found three components, which correspond to our " *sl* ", " *fa* ", and " XO ".

Molecular Weight.—The molecular weight has been calculated by using the equation $M = \boldsymbol{R}Ts/(1 - \bar{v}\rho)D$ (Svedberg *et al.*[3]). Combining the extrapolated value for $s_{20,w}$ (see above) with the diffusion constant, $D = (3{\cdot}6 \pm 0{\cdot}2) \times 10^{-7}$ cm.^{2} sec.$^{-1}$, and the partial specific volume, $\bar{v} = 0{\cdot}74 \pm 0{\cdot}02$ c.c./g. (see p. 1218), one obtains $M = 290{,}000$, and after allowance for the extreme limits of error in s, D, and $\bar{v}$ the maximum possible error is $\pm 40{,}000$. This value of M agrees with that deduced by Philpot [5a] from a measured value of s and assumed values for D and $\bar{v}$, *viz.*, $M = 220{,}000$—$320{,}000$. The molecular weight, obtained from chemical analysis of the enzyme and agreeing with the above, is discussed in the following paper.

The axial ratio of the molecule calculated from the diffusion coefficient [3] is 7 : 1 (on the assumption that there is no hydration).

Electrophoresis.—The only electrophoretic work on purified milk xanthine oxidase reported to date is that of Ball [6] who used Theorell's technique; [7] he found that the isoelectric point of his enzyme preparation was at pH about 6·2. No details of experimental conditions were given.

[5] Philpot, *Biochem. J.*, (*a*) 1939, **33**, 1707; (*b*) 1938, **32**, 2240.
[6] Ball, *J. Biol. Chem.*, 1939, **128**, 51.
[7] Theorell, *Biochem. Z.*, 1934, **275**, 1.

We have examined specimens of our crystalline fractions in an apparatus of the Tiselius type. For LXO60, M7 in preliminary experiments no migration was observed after 6 hr. at pH 5·3. When LXO62, M7 was examined at pH 4·50, 5·28, 5·46, 6·32, and 8·15 some precipitation was noted at the lowest pH, and at the highest, gelation occurred. From the mobilities it was concluded that the isoelectric point was between pH 5·28 and 5·46 (acetate buffer, $I = 0{\cdot}2$). Since in some of these experiments, particularly in that at pH 6·32, the single peak observed was slightly skew, indicating the presence of some impurities, a further test was done with a purer specimen LXO59, M10 which migrated as a single symmetrical peak after 5 hr. at pH 6·6. As the apparatus available to us did not warrant a more detailed analysis of the boundary curves (cf. Alberty [8]), these electrophoretic results were not adequate to elucidate the finer aspects of homogeneity of our preparations.

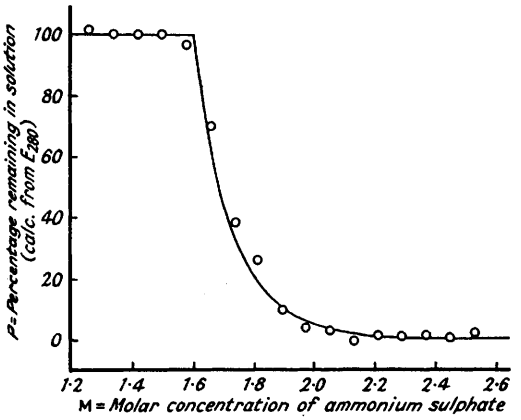


FIG. 8. *Solubility of xanthine oxidase as a function of salt concentration ("constant protein" method).*

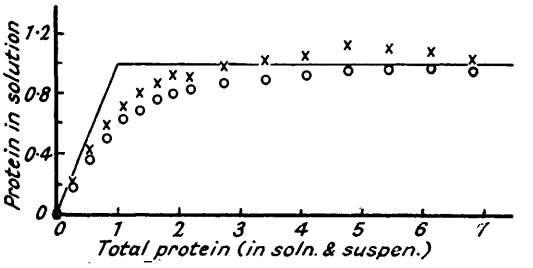


FIG. 9. *Effect of total protein concentration on the solubility of xanthine oxidase ("constant solvent" method).*

Protein concns. in arbitrary units:

○ *from* E_{280};
× *from enzyme assays.*

Solubility Analyses.—Two tests for homogeneity of proteins are available, the "constant solvent" test [9] and the "constant protein" test.[10] In the "constant protein" method sample LXO59, M10 gave an exponential curve (Fig. 8) showing no breaks, in agreement with the theory for a single protein component. However, for this sample in the "constant solvent" solubility test some deviation from the ideal behaviour was found (Fig. 9) when the dissolved protein was estimated by means of xanthine oxidase activity or ultraviolet absorption at 280 mμ. Since fairly rapid loss of activity occurred under our conditions, which allowed the measurements to be carried out only at one time of equilibration between solids and solutions, no certainty existed that final equilibrium had been reached and that the results were therefore significant.

Conclusions.—The physicochemical data obtained from sedimentation experiments and from "constant protein" solubility analysis show that, at this stage of our work on crystalline molybdenoflavoprotein fractions from milk, we are dealing with preparations in which at least 90% of the material is behaving as a single component. While the observation of single peaks in the electrophoretic tests at several pH values supports this

[8] Alberty in "The Proteins," ed. Neurath and Bailey, Acad. Press, New York, 1953, Vol. IA, p. 461.
[9] See Herriot, *Chem. Rev.*, 1942, **30**, 413.
[10] See Cohn, *Physiol. Rev.*, 1925, **5**, 349.

conclusion, one is forced by the variation in xanthine oxidase activity to assume the presence in the " component " of at least two closely related molecular species.

Experimental

Flavoprotein Solutions.—The fractions used for the physicochemical measurements were derived from batches, prepared according to the procedures described by Avis *et al.*,[1] the batches being designated LXO51 to LXO68. The different stages of the purification were given the symbols M1—M10 and S1—S7 as explained for most of them in the previous communication,[1] where in fact LXO56 was the batch in question. No mention was made previously of M9 and M10; the former was obtained by recrystallisation of M8, and the latter by the same process from M9, as reported in detail for M8 by Avis *et al.*[1] The ratios " AFR ", " APR ", and " PFR " for the different batches are given in Table 2, and refer to the assay with xanthine as substrate

TABLE 2. *Properties of various batches.*

Batch	Stage	" AFR " [a]	" APR " [a]	" PFR " [a]	Batch	Stage	" AFR " [a]	" APR " [a]	" PFR " :
LXO51	M6 [b]	62	7·3	8·5	LXO56	M8 [c]	71	14·2	5·0
LXO51	M7 [b]	53	10·4	5·1	LXO59	M5	79	7·9	9·9
LXO53	M7 [b]	32	6·1	5·3	LXO59	M10	43	8·8	5·0
LXO55	M7	69	12·4	5·6	LXO60	M7	70	14·0	5·0
LXO56	M5 [c]	70	7·4	9·4	LXO60	*ex* M7	0·3	0·04	6·8
LXO56	M6 [c]	88	11·1	8·0	LXO62	M7	28	5·2	5·4
LXO56	M7 [c]	79	15·2	5·2	LXO68	M7	18	3·1	5·7

[a] For definition of symbols see ref. 1. [b] Values given previously (*Nature*, 1954, **173**, 1230) [a] LXO51 was batch B; LXO53, batch A. [c] Values given in ref. 1.

and oxygen as electron acceptor, carried out immediately after preparing the solution. In some instances there were considerable delays, accompanied by variable reductions of activity (cf. following paper), before the ultracentrifuge and electrophoresis experiments were carried out. In all cases, the samples of the preparations for the experiments described in this and the following paper were stored as aqueous solutions with total protein concentrations of about 1—2%, in the presence either of phosphate buffer (pH 6; 0·1—1·0M) or of sodium chloride (0·2M; pH near to 6), at 0—5°. Aliquot parts of these solutions were removed for use as required. For measurements of s, D, and $\bar{v}$, the solutions were dialysed thoroughly against 0·2M-sodium chloride at 0—5° before use. Before the electrophoretic experiments dialysis against the appropriate buffer was carried out.

Sedimentation Experiments.—These were performed with a Spinco analytical ultracentrifuge, running at speeds of 42,000 or 50,000 r.p.m. Photographs of the boundary formed between solution and solvent during sedimentation were taken with the Philpot–Svensson optical system.[11] Visible light was used, except in one experiment with ultraviolet light. The concentrations of the xanthine oxidase solutions were determined from the absorption at 450 mμ, the absorption coefficient $E^{1\%}_{1\,cm.}$ (450 mμ) being 2·3 (cf. following paper). For a few solutions, absorption data were not available, and then the concentrations were estimated by comparing the areas under the peaks of the sedimentation diagrams with the corresponding area for a solution of known concentration. These areas were obtained from enlargements of the boundary photograph drawn on graph paper, and were corrected for the dilution effect resulting from the use of a sector-shaped cell.[3]

The sedimentation coefficients were calculated from the rate of movement of the maximum of the peak in the usual manner and were then corrected for the adiabatic cooling of the rotor during acceleration, the variation of the viscosity of the solvent with temperature, and the difference between the viscosity of the solvent and of water, to give the sedimentation coefficient $s_{20,w}$ in water at 20° (see Table 1 and Fig. 7). Previous experiments have shown that s can in general be determined with an accuracy of ±2% with this apparatus.

Two methods of determining the degree of purity of the crystalline preparations were used: (*a*) The area under the peak of the sedimentation diagram, after different times of sedimentation, was measured as described above. (*b*) [4] The peaks were drawn on graph paper (see above); the y axis was taken as a line drawn vertically through the maximum of the peak, and the x axis as the base of the peak. The peaks were then normalised by means of the transformations $x' = xt^{-\frac{1}{2}}$ and $y' = yt^{\frac{1}{2}} \exp 2w^2st$. With LXO56, M8 (Fig. 6) the theoretical curve was calculated from $D = 3{\cdot}5 \times 10^{-7}$ cm.^{2} sec.$^{-1}$.

[11] Philpot, *Nature*, 1938, **141**, 283; Svensson, *Kolloid Z.*, 1939, **87**, 181.

Diffusion.—For three of the experiments (at 20°), with LXO68, M7, the Hilger electrophoresis apparatus fitted with a tungsten lamp and a red filter (Wratten 29) was used. Photographs were taken at intervals up to 3—5 days on Kodak IRER plates with the Philpot–Svensson optical system. The boundaries, between the solvent and the enzyme solution, formed by sliding the two halves of the electrophoresis cell together, were slowly moved into the centre of the cell and carefully sharpened. One experiment with LXO56, M7 (at the same temperature) was performed with the Perkin-Elmer electrophoresis apparatus and the Longsworth scanning technique. The diffusion coefficients were calculated by the height–area method.[12]

For LXO68, M7 it was found that at concentrations of 1·75, 0·94, and 0·55% of the protein, $10^7\ D_{20,w} = 3{\cdot}74 \pm 0{\cdot}12$, $3{\cdot}57 \pm 0{\cdot}07$, and $3{\cdot}51 \pm 0{\cdot}14$ cm.^{2} sec.$^{-1}$ respectively. For a 0·5% solution of LXO56, M7, it was 3·5 cm.^{2} sec.$^{-1}$. Since the diffusion coefficient did not vary with concentration, within the experimental error, the mean value of $D_{20,w} = (3{\cdot}6 \pm 0{\cdot}2) \times 10^{-7}$ cm.^{2} sec.$^{-1}$ was used in the calculations of molecular weight.

Partial Specific Volume.—The densities of a sample of LXO68, M7 and of the 0·2M-sodium chloride against which it had been dialysed, were measured at 21°, with a 3 ml. pycnometer.[13] The concentration of the solution (2·05%) was determined from the value of E_{280} (following paper). The density of the solution was found to be 1·0111 and that of the solvent 1·0059. The value of the partial specific volume was therefore $\bar{v} = 0{\cdot}74 \pm 0{\cdot}02$ (the limits of error on this quantity allow for weighing errors and for possible errors in the $E^{1\%}_{1\text{cm.}}$ value given in the following paper). It is assumed [13] that $\bar{v}$ equals ϕ.

Electrophoresis.—Buffer solutions were made up to a constant ionic strength (I 0·2) according to the data given by Miller and Golder.[14] Solutions of xanthine oxidase at a concentration of about 0·8% were employed.

Electrophoresis was performed with a Perkin-Elmer apparatus fitted with the Longsworth optical measuring system,[15] working at 0° and a constant current of 15 milliamp. Since the solutions were deep red the photographs of the boundary were taken on the same type of red-sensitive photographic plates, as for the diffusion experiments (above). Conductivities were measured at 0° with a Leeds–Northrup bridge. LXO62, M7 migrated cathodically at pH 4·5 (electrophoretic mobility $\mu = 1{\cdot}7 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$) and with lower mobility in the same direction at pH 5·28. At pH 5·46 the migration was anodic ($\mu = 0{\cdot}09 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$), with increasing mobility at pH 6·32 ($\mu = 2{\cdot}9 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$).

"Constant Protein" Solubility Test (Effect of Salt Concentration).[10]—The sample LXO59, M10 was diluted with phosphate buffer of pH 6, and portions (2 ml.) of the diluted solution were placed in a series of centrifuge tubes, together with varying quantities (0—4 ml.) of 50% w/v aqueous ammonium sulphate, and 25% w/v ammonium sulphate to bring the total volume in all samples up to 6 ml. The tubes were closed with rubber caps, and mixed by inversion; after 20 hr. at about 3° with very occasional inversion to assist equilibration, the tubes were centrifuged (10 min. at 6000 ***g*** and +3°). 2 ml. of each of the clear supernatant liquids were removed and diluted with water (2 ml. portions), and the light absorption at 280 mμ measured in a 4 cm. cell with a plastic fitting designed to reduce the volume of liquid required. Small corrections for the low absorption due to ammonium sulphate were applied,[16] the corrections being calculated from measurements on the stock 50% and 25% ammonium sulphate solutions. The percentage of protein remaining in solution (Fig. 8) was calculated from these E_{280} values, the E values corresponding to 0 and 100% precipitation being determined empirically. The molarities of ammonium sulphate were calculated by ignoring volume changes on mixing of the solutions. In this experiment, the initial protein content was about 0·2 mg./ml., the final phosphate concentration was 0·3M, and the calculated pH in the final concentrated salt solutions was about 5. The curve through the experimental points in the Figure was calculated from Cohn's equation in the form: $\text{M} = A - B \log_{10} P$, where M is the molarity of ammonium sulphate, P is the percentage of protein remaining in solution, and A and B are constants, taken here as 2·2 and 0·3, respectively.

"Constant Solvent" Solubility Test (Effect of Protein Concentration).—The sample LXO59, M10, after some weeks' storage, was dialysed against 0·1M-phosphate buffer (pH 6) and centrifuged to remove small amounts of precipitated material. Samples of the solution (up to 0·5 ml.) were placed in a series of centrifuge tubes, and portions of the buffer used in the dialysis

[12] Alexander and Johnson, "Colloid Science," Oxford Univ. Press, 1949.
[13] Cf. Koenig, *Arch. Biochem.*, 1950, **25**, 241.
[14] Miller and Golder, *ibid.*, 1950, **29**, 420.
[15] Longsworth, *Ind. Eng. Chem. Anal.*, 1946, **18**, 219.
[16] Derrien, *Biochim. Biophys. Acta*, 1952, **9**, 49.

were added to bring the total volume in all tubes to 0·5 ml. Portions (5 ml.) of a solution containing ammonium sulphate and phosphate buffer were added to each tube, which was then closed and equilibrated as for the " constant protein " determinations. All operations, including making up of the mixtures, were carried out at about +3°. The samples were centrifuged, the supernatant liquids were diluted, and the absorption at 280 mμ was measured as before. Aliquot parts of the diluted solutions were also assayed for xanthine oxidase activity (cf. ref. 1); from these figures the " protein in solution " values (Fig. 9) were obtained. The absorption and activity figures for the total protein, in solution and in suspension, were obtained by removing samples of a few of the suspensions, before the centrifugation, and diluting and measuring these in the same way as for the supernatant liquors. Graphs were plotted of E_{280}, or of activity, against the volumes of the enzyme solution originally introduced into the tubes and, from these, the " total protein " values for all the tubes were deduced. In the case of the E_{280} measurements, the intercept of the graph on the E axis also served to evaluate the blank correction, which was due to absorption by the salt mixture and had to be subtracted from both the " total protein " and the " protein in solution " figures. The maximum solubility found in this experiment corresponded to about 0·2 mg. of protein per ml.; the buffer concentration and pH were as in the previous experiment; the ammonium sulphate concentration was 1·66M.

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