A Specific Property Solubility Test for Protein Purity, and its Application to the Preparation of Pure Liver Esterase

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The equilibrium between a solid protein and its solution was first comprehensively considered by Sorensen & Hoyrup (1917). They concluded from their investigations that egg albumin formed a true solution in water and that the equilibrium between this solution and solid egg albumin was governed by the phase rule.

This work was confirmed and extended by Northrop & Kunitz (1930) who obtained proteins of sufficient purity to have constant solubilities in solvents of constant composition, the solubility being independent of the amount of solid phase present. They showed, moreover, that, in the case of a protein which had a solubility independent of the amount of solid phase present, a consideration of that part of the solubility curve between the appearance of opalescence and the establishment of constant solubility might demonstrate the presence of an unsuspected impurity. They also demonstrated that some proteins did not conform to the 'Multiple Component System' theory of Sorensen (1930).

In the above-mentioned methods, the change in the amount of protein in solution, in a solvent of constant composition at constant temperature, is measured as a function of the total protein present. The alternative method of experimental analysis where the total protein, temperature and pH were constant and the salt concentration varied, was also considered by Sorensen & Hoyrup (1917). They plotted precipitation curves relating the amount of egg albumin in solution, in equilibrium with the crystalline solid, at constant temperature and pH, to the concentration of the precipitating ammonium sulphate. They then pointed out that the amount of albumin in solution was completely determined in the phase rule sense and that, at a given salt concentration, it would always be the same within the limits of experimental error. It is clear, therefore, that if ammonium sulphate is added to a solution of pure protein, the concentration of the protein in solution will remain merely the content, i.e. indeterminate, until the appearance of the solid phase, after which it becomes the solubility. Hence, the appearance of the solid phase will show itself in the precipitation curve as a break in continuity. Further, in the case of a complex protein solution, the appearance of each new solid phase will be indicated by a similar break. The realization of this has led to much interesting work at the hands of many workers, especially Butler, Blatt & Southgate (1935), Roche, Dorier & Samuel (1936 *a*, *b*) and Jameson & Roberts (1937).

Precipitation curves of this type could obviously be used as a test of purity, since with a pure protein only one inflexion in the graph should occur. Kuhn & Desnuelle (1937) have used data obtained in this way as evidence of purity in the case of Warburg's yellow enzyme. They also demonstrated that when ammonium sulphate was added to the solution, the yellow colour and protein began to come out of solution at the same salt concentration, and that the decrease in yellow colour and the decrease in protein content were parallel. They did not develop this method further, nor did they apply it to the analysis of impure solutions, an extension which might have yielded information useful in the purification of the yellow enzyme.

The value of a purity test depends not only on its sensitivity and accuracy, but also on the amount of information it yields about the concentrations and characteristics of the impurities present which would be of value in purifying the protein required. A consideration of one of the basic difficulties involved in the purification of a protein by salting out will make this point clearer. Suppose a solution contains five proteins

$$A \quad B \quad | \quad C \quad | \quad D \quad E$$

in order of increasing solubility, and suppose that C is the protein to be purified. The usual procedure is to add enough salt to precipitate A and B which are then filtered off. More salt is now added to precipitate C which is collected, leaving D and E behind in solution. Apart from the contamination of the precipitated C with mother liquor containing D and E, considerable impurities are almost always present, due to the overlapping of the precipitating ranges of proteins with closely related solubilities, in this case, B and D.

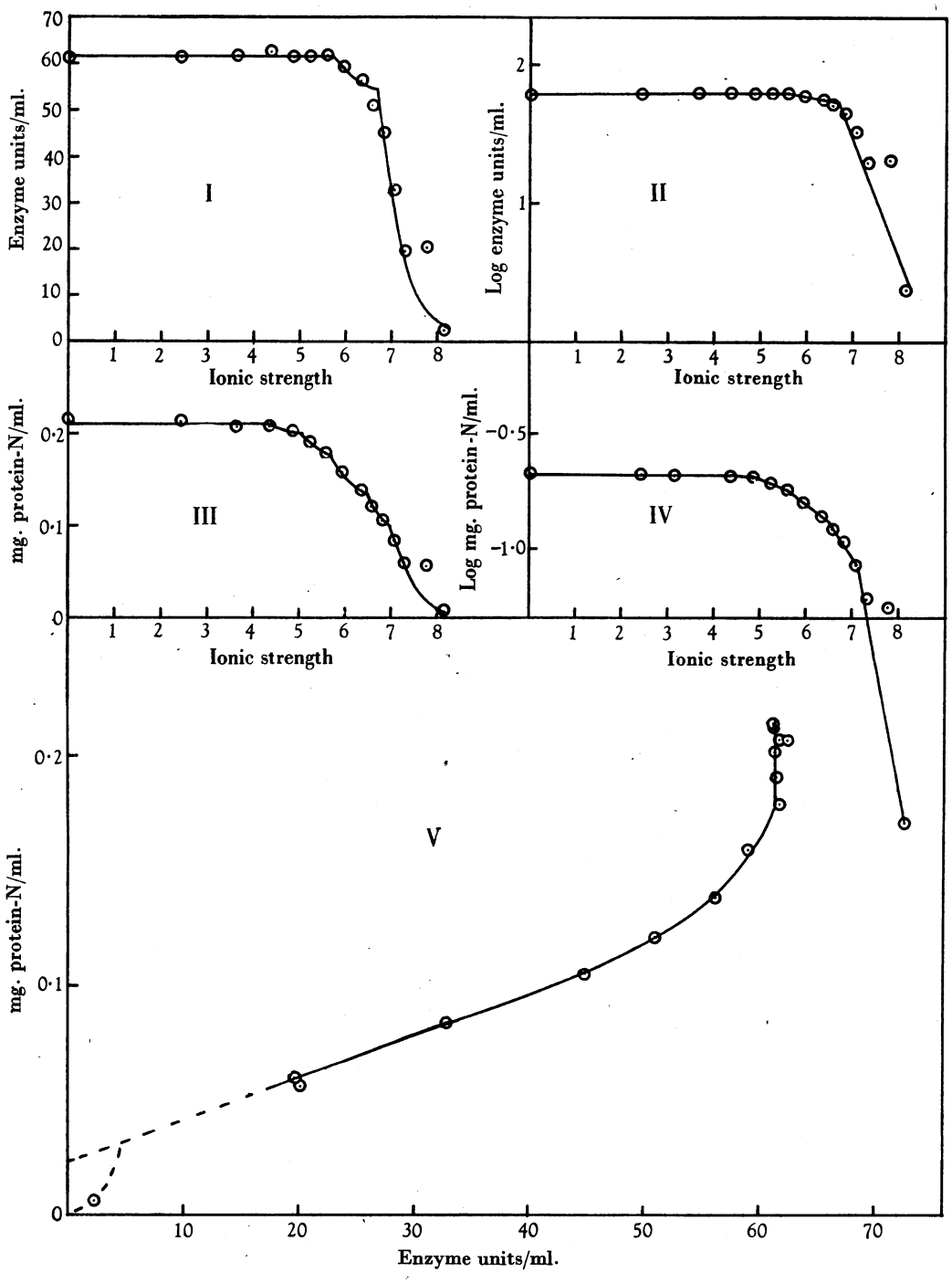

Fig. 1. V, Specific property solubility test of a partially purified solution of liver esterase showing the presence of two overlapping impurities. I, II, III, and IV, precipitation curves of esterases and proteins in the same solution.

The solubility test described in this paper presents a method of measuring the degree of this overlap, and so exposes the problem of the elimination of impurities to quantitative experimental investigation. Moreover, a consideration of the theory underlying the test enables the problem to be analyzed mathematically, thereby generalizing its applicability.

A preliminary account of part of this work has already been published (Falconer & Taylor, 1945).

EXPERIMENTAL

The methods and enzyme extract used were those described previously (Falconer & Taylor, 1946), except that protein-N/ml. of the solubility test filtrates was also determined and corrected for the diluting effect of the added ammonium sulphate in the same way as the esterase figures were corrected for this factor.

The solution used for Fig. 3 was prepared from that used for Fig. 1 by fractionation between the ionic strengths of 6·57 and 7·67 at pH 6, followed by dialysis. Ammonium sulphate was used as the precipitating reagent. All the solubility tests were carried out at pH 6·0.

Precipitation curves of both the esterases and the total protein nitrogen were plotted as a function of the ionic strength, and in order to find the relation between the precipitation of the impurities and the precipitation of the enzyme, the protein left in

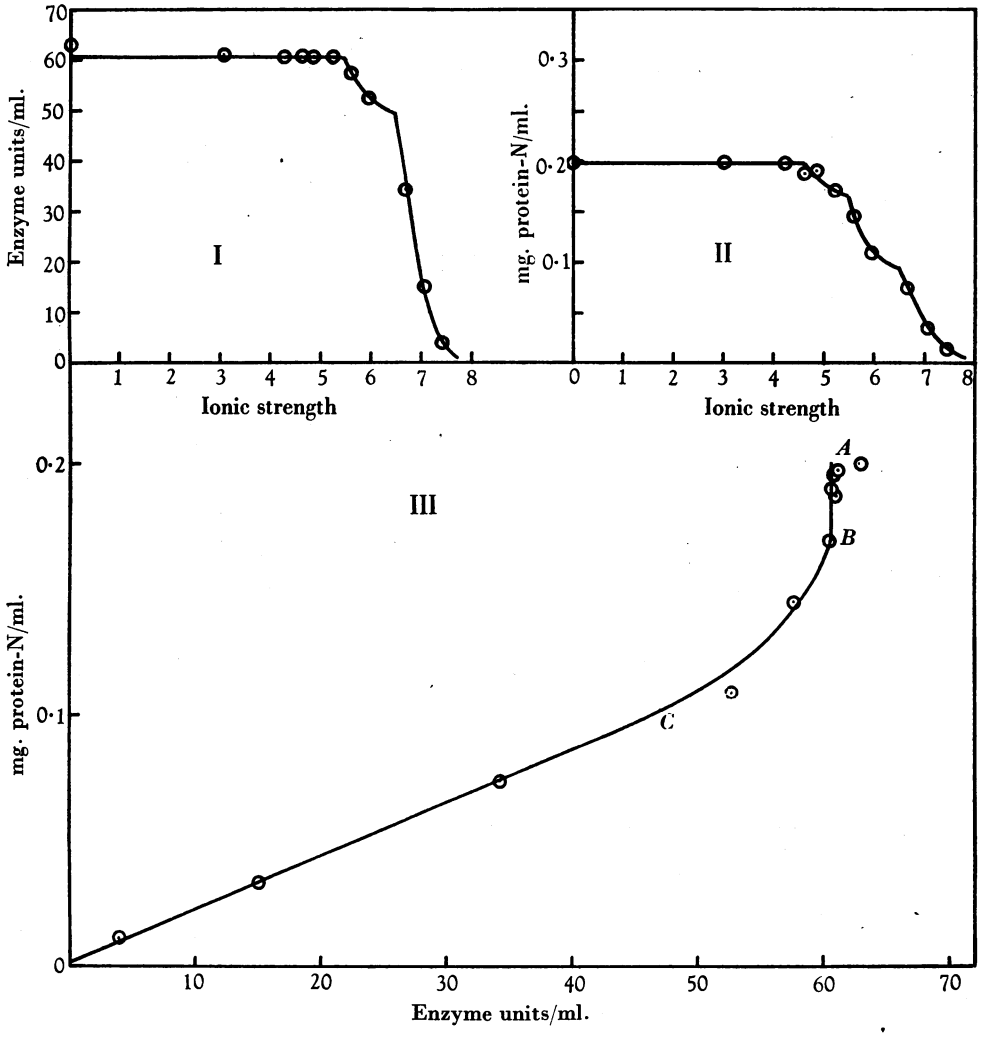

Fig. 2. Curve III. Specific property solubility test of a partially purified solution of liver esterase showing one less soluble overlapping impurity; also precipitation curves, I and II, of esterases and proteins in the same solution.

solution at each ionic strength was plotted against the enzyme in solution at the same point.

On examination of the protein and the enzyme precipitation curves in Figs. 1 and 2, it is difficult to decide exactly not only how many proteins there are, but also which parts of the protein precipitation curves correspond to the precipitation of enzyme protein. When, however, the enzyme concentration in purity (i.e. it runs along a straight line passing through the origin). The change in purity between points *B* and *C* is due to the overlapping of the precipitation ranges of the enzyme and the less soluble impurity, and the analysis of this overlap is important from the standpoint of its elimination.

Fig. 1, V shows the presence of two overlapping impurities, one more soluble and one less soluble than

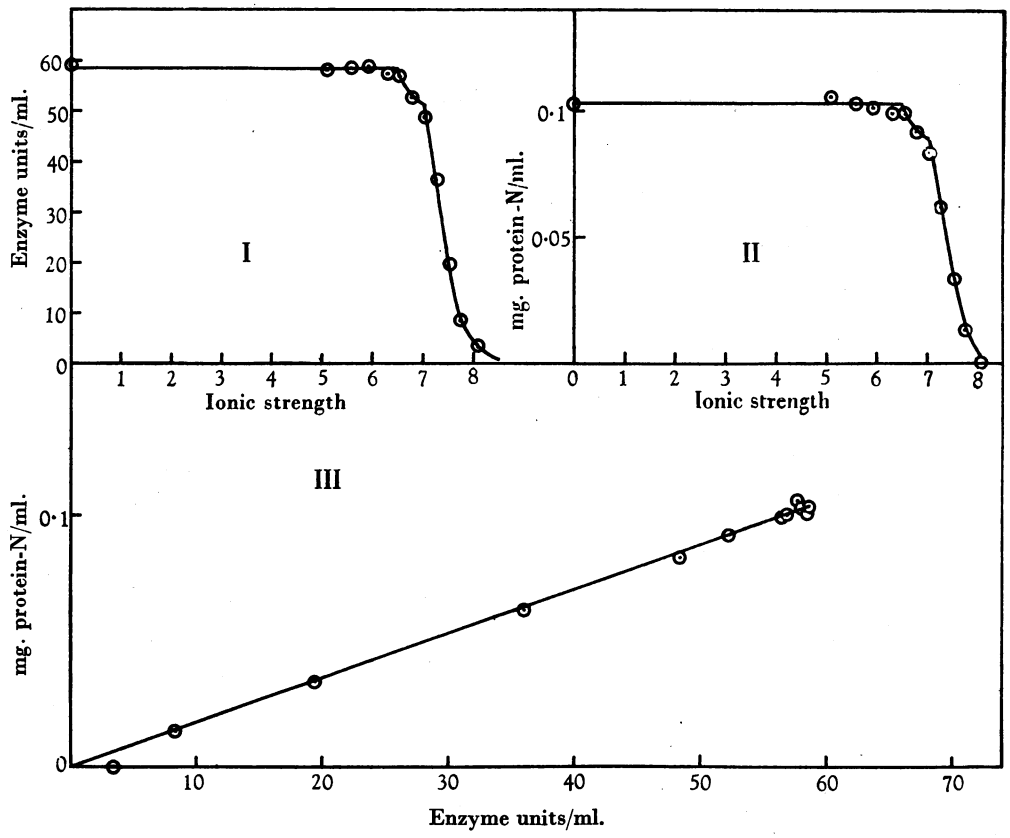

Fig. 3. Curve III. Specific property solubility test of pure liver esterase. Curves I and II, relationship of enzyme activities and protein concentration to ionic strength.

in solution is plotted against the total protein in solution, a clear picture of the relationship between the precipitation of the enzyme and the impurities appears.

For example, between the point *A* (Fig. 2, III) where the solution saturates with respect to non-enzyme protein, and the point *B* where it saturates with respect to the enzyme, there is precipitation of impurity without any change in enzyme concentration. This is also clear from a consideration of the separate enzyme and total protein precipitation curves. After *B*, the solution becomes progressively purer till the point *C* is reached, from which point to the origin the enzyme left in solution does not change the enzyme. Incidentally, the less soluble overlapping impurity in both Figs. 1 and 2 is the catalase referred to previously (Falconer & Taylor, 1946).

Fig. 3, III shows no inflexions or curves, and therefore indicates that the enzyme solution used to prepare it was pure. Fig. 3, II, however, suggests that there are two proteins present; that these correspond to the two enzymes is shown by Fig. 3, I. Fig. 3 therefore represents the purification of the liver esterases without their complete separation. The linearity of Fig. 3, III indicates that the activity of the two esterases in units/mg. of protein nitrogen is the same.

Graphical analysis

Fig. 4 represents the case of an enzyme with two overlapping impurities, one less and one more soluble than the enzyme itself.

All determinations made before any solid phase separates will be coincident at the point A and A–B is due to the precipitation of non-overlapping impurity. The solution saturates with respect to the enzyme at B, and the more soluble impurity at C. Extrapolation of BC to the vertical axis gives the point D, through which, if we draw DF parallel to the horizontal axis, we arrive at the situation as

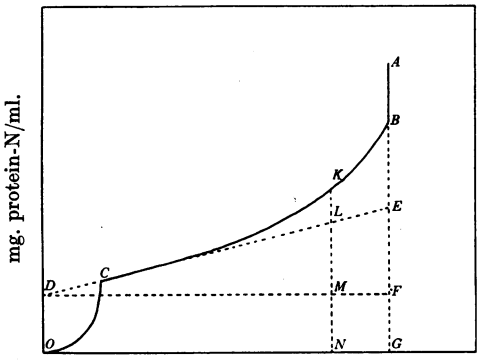

Fig. 4. To illustrate construction lines - - - -, used for graphical analysis of a hypothetical case, resembling Fig. 1, V, of an enzyme with two overlapping impurities one more and one less soluble than the enzyme itself.

it would occur if the more soluble protein impurity were absent, DF being the new abscissa. Further, if at the point D we draw the tangent DE to the curve BCD we get the graph that would occur if the enzyme were pure. The slope of this tangent, therefore, is the activity of the pure enzyme in units/mg. of protein nitrogen. The area BDE represents the overlap of the less-soluble impurity on to the precipitation range of the enzyme. The composition of the original solution can be obtained by extrapolating AB to the horizontal axis. Then AB represents less-soluble non-overlapping impurity, BE less-soluble overlapping impurity, EF the enzyme protein and FG the more-soluble overlapping impurity. The extent of the overlap of the less-soluble impurity into the precipitation range of the enzyme is given by the rate of approach of the curve BCD to the straight line ED. The faster BCD approaches ED the better it is from the point of view of separating the proteins. In order to analyze the composition of the solution at any point K on BCD, the vertical line KN is drawn, then KL represents the overlapping less-soluble protein, LM the enzyme, and MN the more-soluble protein impurity.

It is clear, therefore, that the determination of the concentration, pH, temperature and precipitating reagent which make the overlap a minimum, constitutes a general method for the purification of any protein with a specific accurately measurable property such as the activity in the case of an enzyme.

Mathematical analysis

Since generality is more easily achieved in theory than in practice, it will be sufficient for the purposes of analysis to consider two cases, that of the pure enzyme and that of the enzyme with one less-soluble overlapping impurity.

Calculation of precipitation equations for pure enzyme. In this case, the protein solubility P as a function of the enzyme solubility S_E (in enzyme units) is given by the equation

$$P=\frac{1}{K_E}\times S_E, \tag{1}$$

where K_E is the activity of the pure enzyme in units/mg. protein nitrogen.

Further, accepting Cohn's equation (1925) and letting β_E and k_E be the intercept and slope constants respectively of the enzyme and I the ionic strength, we have

$$\log S_E=\beta_E-k_EI, \tag{2}$$

therefore

$$S_E=\exp(\beta_E-k_EI). \tag{3}$$

Substituting in equation (1) we get:

$$P=\frac{1}{K_E}\exp(\beta_E-k_EI). \tag{4}$$

It is clear, therefore, that equations (1) and (4) are the equations to the line ED, Fig. 4.

Calculation of equations for the case of the enzyme with one less-soluble overlapping impurity. Fig. 2, III, is such a case, and we will attempt to calculate the equation to the curve between the point where the solution saturates with respect to the enzyme and the origin, also the equations for the overlap and the overlap ratio, and finally the activity of the pure enzyme.

(1) *Calculation of equation to curve.* In the case of one less-soluble overlapping impurity, we have, where the subscripts i and E refer to the impurity and enzyme respectively,

$$\log S_i=\beta_i-k_iI \tag{5}$$

and

$$\log S_E=\beta_E-k_EI, \tag{6}$$

therefore

$$S_i=\exp(\beta_i-k_iI) \tag{7}$$

and

$$S_E=\exp(\beta_E-k_EI). \tag{8}$$

Now since the protein concentration P at any point on the curve must be equal to the sum of the concentrations of the impurity and the enzyme protein, we have

$$P=\exp(\beta_i-k_iI)+\frac{1}{K_E}\exp(\beta_E-k_EI), \tag{9}$$

which is therefore the equation to the curve in question.

(2) *Calculation of the overlap and overlap ratio.* The amount of impurity left in solution when the ionic strength is such as just to saturate the solution with respect to the enzyme is the overlap and is given by equation (7), where I has the value specified. The value of the overlap itself is not as important as the ratio of the overlap to the amount of enzyme present. Here we have

$$\text{Overlap ratio}=\frac{\exp(\beta_i-k_iI)}{\frac{1}{K_E}\exp(\beta_E-k_EI)}, \tag{10}$$

where I has the value just mentioned.

(3) *Calculation of the activity of the pure enzyme.* The activity A in enzyme units/mg. protein nitrogen at any point on the curve is obviously given by

$$A = \frac{\exp(\beta_E - I_E k)}{\frac{1}{K_E}\exp(\beta_E - k_E I) + \exp(\beta_i - k_i I)}, \qquad (11)$$

from which we can easily calculate K_E. When the amount of impurity relative to the enzyme tends to zero A becomes K_E, i.e. the tangent to the curve at the origin.

If now our experimental data permit us to determine the slope and intercept constants used in the above equations, we can determine the activity of the pure enzyme and the overlap ratio, both useful in purification.

(4) *Calculation of terms limiting extent to which proteins can be separated.* Fig. 5 represents two hypothetical cases RSQ and RTQ of overlap by a less-soluble impurity, the precipitation of the enzyme protein overlapped being represented by VQ, while in each case the actual amount of the overlap, represented by RV, is the same; the impurity in the case of RTQ could be removed much more easily, since the curve approaches VQ more rapidly. It is therefore important to calculate the terms determining the rate of approach of any curve of the same type as RTQ to the straight line VQ, since this rate limits our ability to separate the two proteins, and to purify the enzyme. This calculation can be done by considering the slope dP/dE of the tangent TX to the curve at any point T. Since the variation of total protein P relative to the fall in enzyme solubility is equal to the sum of the rates of change of the impurity and the enzyme protein relative to the ionic strength divided by the rate of change of the enzyme solubility relative to the ionic strength, we have

$$\frac{dP}{dE} = \frac{\left(\frac{\delta S_i}{\delta I}\right)_{\text{Enz.}} + \left(\delta\frac{1}{K_E}\frac{S_E}{\delta I}\right)_{\text{Imp.}}}{\left(\frac{\delta S_E}{\delta I}\right)_{\text{Imp.}}} \qquad (12)$$

$$= \frac{\delta S_i}{\delta S_E} + \frac{1}{K_E}. \qquad (13)$$

Now, since $1/K_E$ is the slope of VQ and since dP/dE is the slope of TX, the rate of approach of the curve RTQ to the straight line VQ is determined by the rate of removal of the impurity relative to the rate of precipitation of the enzyme, a rather obvious conclusion. Moreover, by differentiating equations (5) and (6), relative to I and dividing we get that

$$\frac{\delta S_i}{\delta S_E} = \frac{k_i \times \exp(\beta_i - k_i I)}{k_E \times \exp(\beta_E - k_E I)}; \qquad (14)$$

this difference therefore between RTQ and VQ can be quantitatively expressed in terms of the slope and intercept constants of the proteins in question. Substituting in equation (13) we get

$$\frac{dP}{dE} = \frac{k_i \times \exp(\beta_i - k_i I)}{k_E \times \exp(\beta_E - k_E I)} - \frac{1}{K_E}. \qquad (15)$$

Therefore in the limit when $\frac{k_i \times \exp(\beta_i - k_i I)}{k_E \times \exp(\beta_E - k_E I)}$ tends to zero we have

$$\frac{dP}{dE} = \frac{1}{K_E}. \qquad (16)$$

That is, the tangent to the curve RTQ at the origin is VQ and is equal to the activity of the pure enzyme, a conclusion which confirms the use of the tangent in the graphical analysis. The tendency of equation (14) to zero will be favoured by a high k and a low β constant for the impurity relative to the corresponding constants for the enzyme, and since the slope constants are independent of pH and temperature (Cohn, 1941), the pH and temperature which make $\beta_E - \beta_i$ a maximum will be the optimum for the purification of the enzyme.

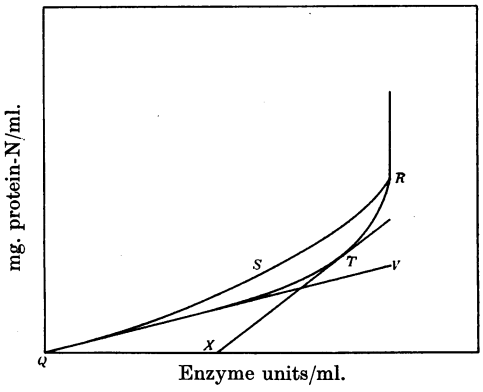

Fig. 5. Diagram to illustrate two possible cases of overlap RSQ and RTQ by a less-soluble impurity, the actual amount of the overlapping impurity being the same in each case, but the extent of the overlap into the precipitation range of the enzyme being much greater in the case of RSQ. This diagram is to illustrate the calculation of the terms limiting the extent to which two proteins can be separated.

QUANTITATIVE ANALYSIS OF EXPERIMENTAL DATA

This can be carried out in two ways. First, we can attempt to estimate the intercept and slope constants β and k respectively of each protein from the precipitation curves, and from these calculate the data we want by substituting them in the appropriate equations. Secondly, we can carry out the relatively much easier graphical analysis as described earlier.

The algebraic approach has the disadvantage that its validity depends on how accurately the proteins obey the equations we use to relate solubility to ionic strength (in this case Cohn's equation). Moreover, apart altogether from the intrinsic accuracy of the function we use, either solution in or adsorption on to an already formed protein precipitate would upset the analysis. This method has, however, the great advantage that it permits easy extrapolation of the experimental data to other concentrations.

The graphical analysis, on the other hand, is independent of the relationship between the solubility of any of the proteins and the ionic strength, nor does the formation of solid solutions or adsorption

interfere with it provided the enzyme is not involved. The method cannot, however, be used to predict what would happen if we concentrate or dilute the original solution. So, although much more exact, the graphical lacks the generality of the mathematical method. These two methods can therefore be regarded as complementary.

Since the object of the analysis is the deduction of a method of purification, we require an estimate of the activity of the pure enzyme and also data giving the extent to which the precipitation ranges of any impurities overlap that of the enzyme to be purified.

The results of the graphical analysis of Fig. 1, V, are shown as circles in Fig. 6.

because if the precipitation of the catalase had been logarithmic, the curve could at best only have approached the pure enzyme line asymptotically. The actual precipitation curve of the catalase (circles, Fig. 6, III) shows that the curve becomes much steeper after the point at which the solution saturates with respect to esterase I. It appears that the catalase is forming a solid solution in the esterase precipitate or being adsorbed on to it. The further possibility that it combines with it in solution cannot be excluded.

The extent of this interaction can be estimated from the mathematical analysis. Table 1 shows the precipitation constants used for these calculations,

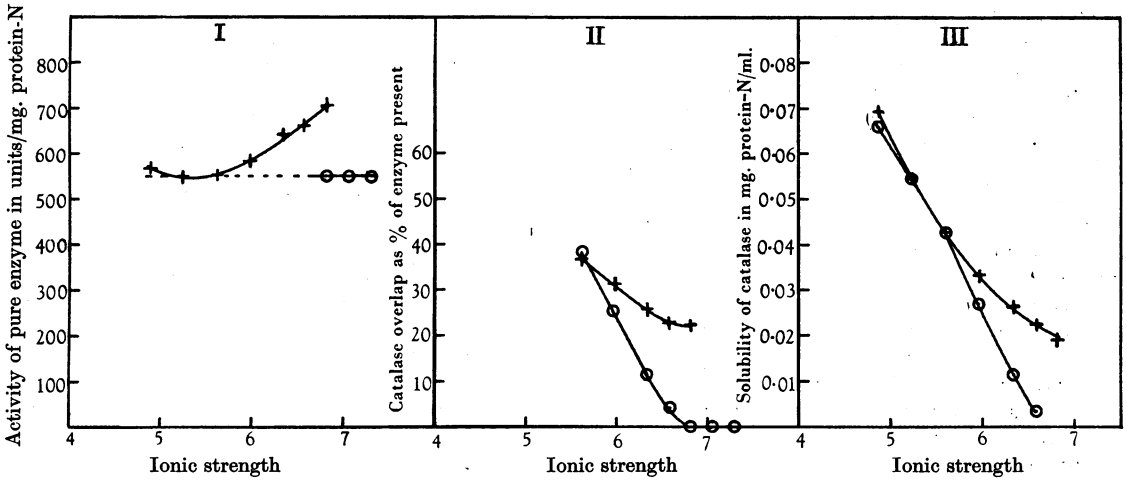

Fig. 6. Analysis of Fig. 1, V, showing discrepancy between actual and calculated values of the activity of the pure enzyme, the percentage overlap of the less soluble impurity, and the solubility of the catalase. ⊙—⊙ Actual values from graphical analysis. ×—× Values calculated from constants given in Table 1 using equations (11), (10) and (5).

The activity of the pure enzyme by this method works out at 550 units/mg. protein-N. Fig. 3, III, confirms this figure. The extent to which the precipitation range of the catalase overlaps that of the enzymes is shown in Fig. 6, II, as a function of the ionic strength. When the ionic strength is 6·81 this overlap is zero, an unexpected but useful result,

Table 1. *Results of graphical analysis of Fig.* 1, *including the precipitation constants used in the calculations for Fig.* 6

Proteins present	β	k	% of total esterase	% of total protein
Most-insoluble impurity	—	—	—	4·6
Less-soluble overlapping impurity (catalase)	0·254	0·29	—	32·3
Liver esterase I	3·94	0·50	19	9·7
Liver esterase II	7·42	0·85	81	42·2
Most-soluble impurity	—	—	—	11·1

and it also includes the approximate composition from graphical analysis of the solution analysed.

The solution of pure enzyme used for Fig. 3, III, was obtained by removal, by fractionation, of that part of curve, Fig. 1, V, showing no overlap, i.e. between 45 and 4 enzyme units/ml.

The precipitation constants for catalase are expressed as mg. protein nitrogen/ml., and for the enzyme in esterase units/ml. Using these values and equations (5), (10) and (11) the values shown by the crosses in Fig. 6 were calculated. The divergence between the calculated and the measured values in Fig. 6, III, shows that the discrepancy occurs at the ionic strength at which the esterase I begins to come out of solution, lending further support to the assumption already made that the catalase and the esterase are interacting; this also explains the discrepancy in Fig. 6, I and II.

The accuracy of the constants in Table 1 is limited by the fact that they were obtained by analysis of limited portions of the graphs in Fig. 1;

precise evaluation will have to await the separation of the enzymes. Some idea of the fit obtainable with these constants can be obtained from Fig. 1, I, where the continuous lines were calculated from the constants for the two enzymes given in Table 1.

DISCUSSION

Types of solubility test and a suggested terminology. There are three types of solubility test in use in protein chemistry at present. First we have the test (Sorensen & Hoŷrup, 1917; Northrop & Kunitz, 1930) in which an analysis is made of the solubility of increasing quantities of a mixture of proteins in a solvent of constant composition at constant pH and temperature. We suggest that this test be called the 'Constant solvent solubility test'.

Secondly, the precipitating effect of a salt on a solution of several proteins at constant pH and temperature has been used to provide much useful information (Derrien, 1944; Roche, Derrien & Moutte, 1941). We will refer to this as the 'Variable solvent solubility test'.

Finally we suggest that the test described in this paper should be called the 'Specific property solubility test'.

The relation of the three tests to one another. If it were possible, and in some instances it seems to be, to make a complete and accurate quantitative analysis of the results of a variable solvent solubility test for each protein in all solvents within the range of salt concentrations used, we would, as a result, be able to calculate and construct the constant solvent solubility test corresponding to any ionic strength in the range studied. Theoretically at least, therefore, the constant solvent solubility test is a special case of the variable solvent solubility test. The constant solvent solubility test is in fact a cross-section of the variable solvent solubility test at a given ionic strength.

The difference between the specific property solubility test and the variable solvent solubility test is one of function rather than of type, since in both the composition of the solvent is varied. In the variable solvent solubility test, the relationship between the precipitation of the proteins in solution and the salt strength is analyzed, whereas in the specific property solubility test it is the relationship between the precipitation of one particular protein and the precipitation of the others that is the subject of inquiry.

The relationships of these three solubility tests to one another and the differences between them result in each test having advantages and disadvantages of considerable practical importance. We can compare and contrast these three tests in three different ways, as tests of protein purity, as sources of information for further purification, and finally from the standpoint of the practical difficulties involved.

Comparison of the three tests as criteria of protein purity. The constancy of solubility of increasing quantities of protein in a solvent of constant composition is a very sensitive test of its purity, and is limited only by the amount of solid phase available and the accuracy of the determination of protein in solution. While larger initial quantities of protein increase the delicacy of the variable solvent test, and since the sensitivity of the specific property test depends on the accuracy of the activity measurements, they are probably not so sensitive as their constant solvent relative.

It has been pointed out by various authors (e.g. Shedlovsky, 1943) that it is possible in the case of a constant solvent solubility test for a solid solution of an impurity, present in just the right ratio to the pure substance, to imitate the behaviour of a pure substance. The chance of this type of compensation occurring is minimized by repeating the test under different conditions. In the case of a variable solvent solubility test, it is possible, after the solution has saturated with respect to the first protein, for a second protein to dissolve in the precipitate of the first to such an extent as to prevent the occurrence of a second inflexion. Moreover, it seems quite possible for this to occur at two different pH values. The position of the specific property test with respect to this point is however unique since the type of solid solution that would give a straight line passing through the origin, when the activities of the protein left in solution are plotted against the amounts, would be of a most complex and unusual nature. The solubility of the impurity in the protein under investigation would have to be independent of the salt strength of the solvent, for the specific property curve to be straight. Moreover, the amount of impurity present would need to tend to zero in the same way, with increasing salt strength, as the main protein did, in order that the graph should pass through the origin. Whilst the impurity might be present in the right amount for the curve to go through the origin it is extremely unlikely that its solubility in the main protein would be independent of the salt strength.

Comparison of the tests as sources of methods for further purification. The use of the constant solvent solubility test for this purpose has been developed by Herriott, Desreux & Northrop (1940) and by Northrop (1941). While the methods derived from this test are interesting, they are limited to the manipulation of a solvent of constant composition. This is a serious limitation since salt fractionation depends on the use of solvents of different composition. It is here that the specific property test is most useful since it enables the overlap of the precipitation ranges of the protein under investigation

and its impurities to be analyzed and related to the ionic strength of the precipitating salt.

Comparison of technical difficulties involved in performing each test. In performing a constant solvent solubility test the composition of the solvent must be chosen with great care. For a satisfactory result can be attained only if the most soluble of all the proteins in the mixture saturates the solvent while at the same time the solvent must permit an analyzable quantity of the least soluble protein to dissolve. Now in order to determine the true composition of a protein solution it is necessary to proceed from the supersaturated side so that the alteration in composition caused by solvent washing of the solid protein mixture can be avoided. Working from the supersaturated side in this case is usually more difficult and less accurate.

In the case of the variable solvent and specific property solubility tests these difficulties do not occur since all that has to be done is to add increasing quantities of salt until all the protein present has been precipitated.

SUMMARY AND CONCLUSIONS

1. A new type of phase rule solubility test for protein purity is described, and quantitative graphical and mathematical methods are provided for its analysis.

2. A method by which the test described could be extended to form the basis of a general method of purification of any protein with a specific accurately measurable quality is indicated.

3. The relationship of the new test to the two previously described solubility tests is indicated and the relative merits of the three tests discussed.

4. The new test has been applied to the analysis of a liver extract relative to its content of liver esterase.

5. The preparation of pure pig liver esterase is described.

It is a pleasure to express our indebtedness to both Mr G. S. Adair, F.R.S. and Dr D. W. G. Style for their invaluable advice on many points.

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Observations on the Nicotinic Acid Requirements of Pigs

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While a large number of methods or their modifications has been described for the chemical estimation of nicotinic acid, the specificity of the rather complicated procedure has only seldom been confirmed by biological test. The work of Harris & Raymond (1939) in this country and of Waisman, Mickelsen, McKibbin & Elvehjem (1940) in America may be quoted as the exception rather than the rule.* Both groups of workers used dogs, and it is obvious that in this country assays of this type are difficult to carry out.

* The same remark may also be applied to the numerous methods for the microbiological assay of nicotinic acid (cf. Snell & Wright, 1941).