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The Use of Organic Solvents at Low Temperature for the Separation of Enzymes. Application to Aqueous Rabbit Muscle Extract

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Enzymes may be separated and purified by a number of methods, such as fractional adsorption and precipitation. The most widely used method has been that of salting out the proteins with neutral salts (cf. review of the literature by Cohn, Strong, Hughes, Mulford, Ashworth, Melin & Taylor, 1946). This method is safe, but the salting-out coefficient depends on rather unspecific characteristics, such as the shape and size of the protein molecule.

Organic solvents have not been applied so generally for the separation of enzymes, since they denature proteins at room temperature. During the war, Cohn, Leutscher, Oncley, Armstrong & Davis (1940) and Cohn *et al.* (1946) extensively developed the ethanol precipitation method of Mellanby (1908) and Hardy & Gardiner (1910). By working at low temperatures and low ionic strengths numerous protein fractions were isolated from blood plasma. Ethanol fractionation has various advantages over the salting-out method as pointed out by Edsall (1947). At low ionic strengths, the interaction between proteins and salts depends on specific properties of protein molecules, such as the distribution of electric charges. Small variations in dielectric constant and ionic strength will produce large and specific changes in the solubilities of proteins.

The present investigation was undertaken to study the separation of enzymes in tissue extracts from water-organic solvent mixtures. Enzyme tests provide a simpler and more specific measure of purity than the usual physical methods (such as solubility measurements and electrophoresis) which were applied by Cohn *et al.* (1946). Simple activity determinations will show where a protein is separating

out and how much denaturation (as measured by loss of biological activity) results from the fractionation.

Aqueous extract of rabbit muscle was chosen as source of enzymes. No systematic protein fractionation of rabbit-muscle extract has been reported, although much is known about its enzyme systems and a number of studies have appeared recently, e.g. salting-out curves (Distèche, 1948), electrophoretic patterns (Jacob, 1947, 1948; Dubuisson, 1950), and ultracentrifugal studies (Amberson, Erdös, Chinn & Ludes, 1949). Most of the glycolysis enzymes are part of the myogen complex with albumin-like properties and a very high solubility in water-salt mixtures. For these enzymes separation by organic solvents, with a lowered dielectric constant, should be useful.

METHODS

Preparation of rabbit-muscle extract

Minced rabbit muscle was extracted at 0° with an equal volume of distilled water for 20 min. with gentle stirring. After centrifuging for 15 min., the supernatant was dialysed first against cold running water for 3 hr., and then against 20 times its volume of distilled water at 4° for 6 hr. The conductivity then equalled that of about 0.007 M-KCl. The pH was adjusted to the required value with 0.05 M-acetate buffer (pH 4), or NaHCO₃. This extract will be referred to as 'original extract' in the text.

Fractionation with organic solvents, at 0° and below

The insoluble precipitate formed on dialysis (ppt. D, containing some myosin, globulin X and denatured proteins) was removed by centrifugation. Fractionation of the supernatant was carried out in a metal vessel to permit rapid heat

exchange. The organic solvent was added through a capillary jet and permitted to flow down the cold wall of the container slowly while the solution was kept rapidly stirred avoiding froth (Fig. 1). As soon as enough organic solvent had been added to keep the extract from freezing the temperature of the bath was lowered from 0° to -3° . When the solution contained 20 or 40% (v/v) of organic solvent, the temperature was reduced to -5° and -7° , respectively. Since long standing in the presence of organic solvents tends to denature enzymes even at low temperatures, the precipitate was centrifuged down, after 20 min. equilibration, in a cold

Fig. 1. Apparatus used for fractionations with organic solvents. A, cold bath; B, aluminium vessel; C, capillary tubing; D, graduated cylinder, containing the organic solvent; E, three-way tap; F, stirrer.

centrifuge set at the same temperature as the cold bath. The precipitates were taken up in a small volume of dilute buffer and dialysed overnight at 4° against 0.033M-phosphate buffer (pH 7.5). The fractions were then 'freeze-dried' and weighed. They could be kept for indefinite periods. After several fractionations it was found advantageous to compensate for dilution as well as removal of ions by the protein precipitate by adding 3M-sodium acetate (at pH 6.5) to give a final concentration of 0.015M, when the organic solvent level reached 40% (v/v). This was done in the case of the *n*-propanol fractionation.

Enzyme estimations

For each test the enzyme fractions were diluted sufficiently for the rate of reaction to be proportional to the enzyme concentration. All phosphate determinations were carried out by the method of Fiske & Subbarow (1925) using a King photoelectric absorptiometer. As a measure of protein concentration, the absorption density at $278\text{ m}\mu$ was measured in the Beckman spectrophotometer; the purification achieved was calculated by comparing the activity (units of enzyme/ml./absorption density at $278\text{ m}\mu$) of a particular fraction with that of the original extract.

Adenosinetriphosphatase. The activity of Ca-activated adenosinetriphosphatase was tested in glycine buffer (pH 9.0) as described by Bailey (1949).

Myokinase. Increases in inorganic phosphate were determined after incubating at 28° (a) the enzyme fraction + myosin + 0.0017M-adenosinediphosphate (ADP) + 0.007M- Ca^{++} in glycine buffer pH 9.0 (total volume 1.5 ml.); (b) as control the same mixture as (a) without the enzyme fraction. After 20 min. the reaction was stopped with 1 ml. 10% trichloroacetic acid and the inorganic phosphate determined.

Phosphorylase. The method of Cori, Cori & Green (1943) was followed using glucose-1-phosphate as substrate. It was necessary to add adenylic acid since most of the phosphorylase was present as the phosphorylase b of Cori *et al.* (1943).

Lactic dehydrogenase. The enzyme was tested in 0.05M-KCN (neutralized), 0.067M-sodium lactate, coenzyme I (1 mg./ml. of reaction mixture, about 50% pure) and 0.05M-phosphate buffer (pH 7.4). The rate of reduction of coenzyme I was measured at 20° in the Beckman spectrophotometer by increase of absorption at $340\text{ m}\mu$.

Adenylic deaminase. The reaction mixture consisted of 0.05M-phosphate buffer pH 6.0, 0.002M-adenylic acid (neutralized to pH 6.0) and 0.5 mg. of enzyme/ml. Samples of 1 ml. were removed at zero time and after 5 and 10 min. of incubation at 38° . They were added to 0.2 ml. Nessler reagent to stop the reaction, then suitably diluted and the intensity of the colour complex formed with the liberated NH_3 measured in the Beckman spectrophotometer at $420\text{ m}\mu$ in glass cells.

Aldolase. The rate of breakdown of hexosediphosphate was determined according to the chemical method described by Taylor, Green & Cori (1948).

Enolase. The rate of formation of phospho-enolpyruvic acid from 2-phosphoglyceric acid was measured in the Beckman spectrophotometer by change of absorption at $240\text{ m}\mu$. (Warburg & Christian, 1941). 2-Phosphoglyceric acid was prepared by Kiessling's method from sodium β -phosphoglycerate, as described by Warburg & Christian (1941).

Creatinephosphokinase. The synthesis of phosphocreatine from adenosinetriphosphate (ATP) and creatine was determined as follows: the reaction mixture consisted of 0.05M-glycine buffer at pH 8.8, 0.05M-NaF (to inhibit at least partly any adenosinetriphosphatase and phosphatase present), 0.005M- Mg^{++} , 0.02M-creatine, 0.03M-cysteine (neutralized), 0.005M-ATP and the enzyme fraction. Samples (1 ml.) were removed at zero time and after 2 and 5 min. incubation at 30° , and added to 5 ml. trichloroacetic acid. Inorganic phosphate was determined after the hydrolysis of phosphocreatine in $\text{N-H}_2\text{SO}_4$ in the presence of molybdate for 45 min. at room temperature (Fiske & Subbarow, 1929). An increase in phosphate was due to the formation of phosphocreatine, since the values remained unchanged under the same conditions in the absence of creatine.

Electrophoresis. The original extract and 1.5% solutions of several fractions were dialysed overnight against 0.033M-phosphate buffer pH 7.5 (I, 0.09), and the electrophoresis carried out in a Tiselius apparatus at 4° , with a potential gradient of 7.5 V./cm.

RESULTS

Factors affecting the protein precipitation

The main factors affecting the solubility of proteins have to be carefully controlled during the experimental procedure. They are temperature, protein concentration, ionic strength, pH and dielectric constant. The variation in temperature was the same in all experiments (0 to -7°) and the protein concentration of the extracts was fairly constant (2.5–3% protein). The effect of varying the other three factors was studied to establish the best overall conditions for sharp separation with the least amount of denaturation.

Variation of ionic strength. Fig. 2 (a) shows how the proteins precipitate out when ethanol is added at pH 6.5. Since muscle contains many protein components, and there is some overlapping in the different fractions, a smooth precipitation curve is obtained. In one case the ionic strength of the extract was very low (after 9 hr. of dialysis) and the conductivity of the solution equalled that of 0.007 M-potassium chloride. The exact ionic strength cannot be given since the proportion of the different ions was not known, but it could not be more than 0.03.

Fig. 2a. Protein precipitation curves, using ethanol at pH 6.5. ○, ×, two separate fractionations, $I < 0.03$; ●, $I = 0.1$ at beginning of experiment. Ordinates represent the weight of the precipitated protein fractions, freeze-dried after dialysis. Each fraction contains a small amount of phosphate (accounting for less than 5% of the total weight). ⊗ = amount of freeze-dried protein present in the original extract (500 ml.); D, precipitate removed after dialysis, before adding ethanol.

When the ionic strength was lowered by further dialysis, any precipitate formed by ethanol was too fine to be centrifuged down. In the lower curve the ionic strength had been raised to 0.1 with phosphate buffer at the beginning of the experiment. As may be expected the proteins are more soluble, and a greater ethanol concentration is required to precipitate a given amount of protein. Under these conditions more denaturation results, and also the enzymes are found to be less sharply separated than at the lower ionic strength.

Variation in pH. A pH around 6.5 appears to be most favourable for protein precipitation. At both pH 5 and 8 the proteins are more soluble so that at a concentration of 50% (v/v) ethanol only half of the protein has come out of solution (Fig. 2b). Enzymic tests show that there are great losses by denaturation

and poor separation of the different components at pH 5 and 8.

Fig. 2b. Protein precipitation curves using ethanol at an ionic strength around 0.03 or less. ●, pH 8.0; ○, pH 6.5; ×, pH 5.0. ⊗, D, as in Fig. 2a. A, precipitate formed on acidifying to pH 5.0. The weights of A and D are included in the ethanol precipitation curves.

Fig. 3. Protein precipitation curves, using various organic solvents at pH 6.5 and $I < 0.03$. ■, methanol; ○, 97% ethanol; ×, n-propanol; ●, acetone (A.R.). ⊗, D, A, as in Figs. 2a, b.

Variation in dielectric constant. The dielectric constant was varied by using a number of different organic solvents (alcohols with one, two or three carbon atoms, and acetone) while the ionic strength and pH were kept constant (Fig. 3). Methanol has the highest dielectric constant of these and the proteins are most soluble in methanol-water mixtures. The other three curves are fairly similar; acetone

gives the steepest precipitation curve, but this flattens out before all proteins have come out of solution. Denaturation is not proportional to the chain length; *n*-propanol produces most denaturation, next comes methanol, and then ethanol. To avoid denaturation, it is best to keep the concentration of organic solvent as low as possible.

From these results it was evident that a pH of about 6.5 and a very low ionic strength (approx. 0.03) provided the most favourable conditions for fractionating the proteins, and these were adopted for the enzymic studies.

Fig. 4. Electrophoretic pattern (anodic) of aqueous extract of rabbit muscle dialysed against phosphate buffer pH 7.5, $I=0.09$. The arrows below the picture indicate the position of the boundary at zero time and the direction of movement. The pictures were taken after 1 and 2 hr. (7.5 V/cm.). Left, ascending boundary; right, descending boundary.

Fig. 4 shows the electrophoretic pattern of the original extract. At least eight components are visible, but muscle extract is known to contain many more different enzymes.

Separation and purification of enzymes

Seven enzymes were tested and Fig. 5 shows how they separated out with ethanol. The percentage of enzyme activity recovered in a particular fraction was plotted against the ethanol concentration (as percentage v/v) at which this fraction had precipitated out. The method appeared to be better for some enzymes than for others. Aldolase, for example, precipitated over a wide range of ethanol concentration with hardly any purification. On the other hand, 86 % of the phosphorylase was recovered between 10 and 20 % (v/v) ethanol with a 12-fold purification in one step. Adenosinetriphosphatase and adenylic deaminase were removed from the preparation at very low ethanol concentrations, i.e. 15 % (v/v). This is useful because adenosinetriphosphatase is usually difficult to get rid of and is apt to interfere with enzymic tests using the adenylic system. Enolase and creatinephosphokinase were

Fig. 5. Separation of enzymes by an ethanol fractionation, pH 6.5, $I<0.03$. Ordinates, percentage of the original amount of enzyme activity appearing in each fraction. Abscissae, ethanol concentration (% v/v). Numbers above histograms indicate the purification achieved compared with the original extract (enzyme activity being taken as units/ml./adsorption density at 278 m μ). Figures in brackets represent the total percentage recovery of the enzyme in all the fractions. D, precipitate formed on dialysis before ethanol addition. S, supernatant after centrifuging down the last precipitate.

Fig. 6. (a) Separation of enzymes by a fractionation with *n*-propanol, pH 6.5, $I<0.03$. (b) Separation of enzymes by a methanol fractionation, pH 6.5, $I<0.03$. Ordinates, percentage of the original amount of enzyme activity appearing in each fraction. Abscissae, alcohol concentration (% v/v). Numbers above histograms, figures in brackets and D as in Fig. 5.

the most soluble of the enzymes tested in ethanol-water mixtures. They came out of solution with the last protein fraction which could be precipitated.

Methanol (Fig. 6*b*) produces hardly any separation or purification, and is the least useful of the solvents tried. Fig. 6*a* gives the results from a fractionation with *n*-propanol. This solvent leads to a good separation in some cases, but the recovery of enzyme activity is too low for general use. However, propanol may be very useful for some enzymes which are particularly stable to alcohol, such as creatine-phosphokinase. Of this enzyme 55% was recovered in one *n*-propanol fraction with a sixfold purification. This sample was free of adenosinetriphosphatase, adenylic deaminase and myokinase.

Fig. 7. Separation of enzymes by an acetone fractionation, pH 6.5, $I < 0.03$. Ordinates, percentage of the original amount of enzyme activity appearing in each fraction. Abscissae, acetone concentration (% v/v). Numbers above histograms, figures in brackets and *D* as in Fig. 5.

Acetone (Fig. 7) seems to be superior to the other solvents for enzyme separation. Since the precipitation curve is steep it is essential to take very small steps (at times a 2 vol. % increase is sufficient). An almost complete recovery and fairly sharp separation is obtained with most of the enzymes tested, and with the precautions mentioned the products are completely soluble in water. With both acetone and ethanol the results are easily reproducible as long as the temperature, ionic strength, pH and dielectric constant are carefully controlled.

DISCUSSION

The above results, obtained from one stage of fractionation of crude muscle extract, have shown that the use of pure acetone with the strict temperature precautions described, at about pH 6.5 and an ionic strength of approximately 0.03, provides the most favourable conditions for enzyme separations. The advantages of the use of organic solvents appear to be that generally the separation is sharper than with ammonium sulphate, where the enzymes tend to spread over wide salt concentrations. Since any lipid material goes into solution, the products from an acetone fractionation tend to be much cleaner than those from an ammonium sulphate fractionation and they are soluble in water. A number of enzymes may be prepared from the same extract with almost 100% recoveries. The above conditions may probably also be applied to other tissue extracts and they may be recommended especially for the first step in fractionations, with possible changes in pH and ionic strength for sub-fractionations, or followed by ammonium sulphate precipitation and adsorption on calcium phosphate gel.

It is not possible to say why some enzymes show more tendency to spread over a number of fractions than others. The spreading may be due to protein interactions and the adsorption of proteins on one another. Too little is known about these factors to control them at this stage. Adenylic deaminase, for example, precipitates with adenosinetriphosphatase, with the amount of purification running roughly parallel in the different fractions, although it is not certain whether the two are identical proteins. Hermann & Josepovits (1949) have not been able to separate them, but according to Ferdman & Nachiropenko (1946) some of the adenylic deaminase stays in the supernatant if myosin is reprecipitated.

Both the nature and concentration of electrolytes influence the fractionation profoundly. The presence of some electrolytes is necessary to protect the proteins from denaturation and to help form complexes favouring precipitation. On the other hand, an increase in ionic strength from 0.03 to 0.1 decreases the sharpness of separation and the recovery of enzyme activity considerably. The nature of the ions added has to be carefully considered. Phosphate tends to keep the proteins in solution longer; this may be due to a specific interaction of phosphate with the muscle proteins. A trace of calcium is at times useful to decrease the solubility of some proteins, or to flocculate very fine precipitates. Negative ions may be of importance in forming complexes with the proteins; a small amount of acetate, for example, has in some cases increased the recovery of an enzyme. Not enough work has been done to

determine whether this effect is general or is due to a specific protection.

Several conductivity measurements (Table 1) have shown that the conductivity of the muscle extract after precipitating some proteins with ethanol is lower than that of a pure calcium chloride solution to which an equivalent amount of ethanol had been

Table 1. *Effect of protein precipitation by means of ethanol on the conductivity of solutions*

	Ethanol concn. (% v/v)	Specific conductance (expressed as equivalent molarity of KCl)
Aqueous muscle extract (dialysed 20 hr.)	0	0.002
Dialysed muscle extract containing 0.0036 M-CaCl ₂	0	0.0085
Dialysed muscle extract containing 0.0036 M-CaCl ₂	20	0.0038
Dialysed muscle extract containing 0.0036 M-CaCl ₂	40	0.0019
0.0045 M-CaCl ₂ solution	0	0.00805
0.0045 M-CaCl ₂ solution	20	0.0051
0.0045 M-CaCl ₂ solution	40	0.0033

added. Thus dilution with organic solvents as well as the formation of protein precipitates will deplete the solution of salts. The addition of sodium acetate at pH 6.5 to give a final concentration of 0.015 M when the organic solvent level reached 40 % (v/v) was found useful in the *n*-propanol fractionation and it is recommended that some salt should be added with the organic solvent to avoid depletion.

It was found that the enzymes precipitated out of solution in a definite order, i.e. each enzyme tended to come out of solution when a constant percentage of the total protein had precipitated, regardless of the alcohol concentration at which this level of precipitation may be reached. This order was constant under the conditions of pH and ionic strength tested in the present study or after the addition of lysine (3 g./100 ml.).

A number of the fractions obtained were analysed electrophoretically at pH 7.5 in phosphate buffer (*I*, 0.09). Except for myoalbumin, the most rapid component, it was found that the faster moving components were precipitated by lower alcohol concentrations than the slower components. There seemed to be a rough parallelism between mobilities and the concentrations of alcohol needed for precipitation. These findings are opposite to the results on blood plasma (Cohn *et al.* 1946), where the proteins with the lowest mobilities were the most in-

soluble in alcohol-water mixtures and were precipitated out first.

Rabbit-muscle extract consists of such a complex mixture of proteins that it was not possible to identify all the peaks on the electrophoretic picture (Fig. 4). The individual fractions were not sufficiently homogeneous, and only seven of the numerous enzymes were tested for. The problem is complicated by the fact that a protein will not necessarily move at the same rate when pure, as in the presence of a large number of other components. The difficulties in trying to establish the relationship between the classical muscle proteins and the peaks have been discussed by Jacob (1947). Aldolase, phosphorylase and triosephosphate dehydrogenase have been placed by mobility determinations on crystalline enzymes (Taylor *et al.* 1948; Green, 1945; Cori, Stein & Cori, 1948). From the present study it may be concluded that adenylic deaminase, adenosinetriphosphatase, phosphorylase and lactic dehydrogenase are among the smaller and faster moving components. Most of the other glycolysis enzymes (enolase, aldolase, triosephosphate dehydrogenase, creatinephosphokinase) form part of the large complex of proteins moving most slowly at pH 7.5 (components *l* and *m* of Jacob, 1947).

SUMMARY

1. A fractionation of the water-soluble proteins of rabbit muscle has been carried out using organic solvents at low temperatures and low ionic strengths. Enzyme estimations were used to follow the separation of the proteins.

2. The following conditions were, in general, found best for sharp separation of the enzymes with the least amount of denaturation: precipitation of the proteins with acetone at pH 6.5, at very low ionic strengths (around 0.03) and low temperatures (0° and below).

3. The results obtained are easily reproducible and a four- to five-fold purification in one stage is achieved on the average. The procedure compares favourably with other methods of enzyme purification.

4. Most of the glycolysis enzymes separated out free from adenylic deaminase and adenosinetriphosphatase. Creatinephosphokinase was obtained free from myokinase.

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The Preparation of Permanent Turbidimetric Standards for Use in the Determination of Small Amounts of Chloride

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Permanent turbidimetric standards are useful in the determination of small amounts of chloride. They are of particular value in the control of the purity of Analar and B.P. chemicals, since they avoid the repeated and costly preparation of fresh standards; and they may be used for other turbidimetric tests, e.g. the determination of proteins by precipitation with sulphosalicylic or trichloroacetic acid.

We feel that such standards may be of value to workers in biochemical and other fields, and the purpose of this paper is to describe the preparation of permanent standards of cloudy Perspex which we have found useful in the routine determination of chloride, and which have been employed by King (1950) for urinary and C.S.F. protein estimations.

The method of preparation of the standards involves, in the first place, the production of a clear interpolymers of 50% methyl methacrylate and 50% styrene. In the second stage of the process, this interpolymers is dissolved in methyl methacrylate monomer; this monomer is then polymerized under conditions which involve uniform precipitation of the styrene/methyl methacrylate interpolymers.

catalyst with stirring in a conical flask (600 ml.) at 70° under reflux, until the viscosity of the syrup is approximately 4 poises. This operation takes about three-quarters of an hour. The syrup is cooled and then evacuated free from air by means of a water pump. The cold syrup is poured into a cell formed by two glass plates (30.5 × 30.5 × 0.6 cm.) separated from one another by rubber tubing (6 mm. diam., 1 mm. wall) and bound by screw clips (Fig. 1). After filling and binding, the cell is maintained at 75° for about 6 hr. in an air oven. A steady current of air is circulated through the oven throughout this period. After cooling, the polymer sheet is detached from the glass plates, ground and bottled.

Fig. 1. Cell for forming interpolymers.

METHODS

Interpolymer. A polymethyl methacrylate-polystyrene interpolymers is prepared as follows: 200 g. of redistilled methyl methacrylate monomer are heated with 200 g. of redistilled styrene monomer and 1.6 g. benzoyl peroxide

Standards. Turbidimetric standards are prepared in the following way: a known weight of the ground polymer (see below) is dissolved in 50 ml. of redistilled methyl methacrylate monomer by heating under reflux with stirring for 1 hr. at 50° in the apparatus shown in Fig. 2. 1.1 ml. of a