ENZYMES OF *ASPERGILLUS ORYZAE*

IV. FRACTIONATION AND PREPARATION OF CRYSTALS RICH IN PROTEASE

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Summary

Several methods have been examined for the separation of proteases from other enzymes and metabolic products in the culture filtrates obtained by surface culture of *Aspergillus oryzae* on sucrose-tartrate liquid medium. Concentration, by low-temperature precipitation with ethanol followed by ethanol fractionation of the dialysed concentrate at pH 4-5 in acetate buffer, is shown to be superior to adsorption methods or precipitation with flavianic acid or ammonium sulphate. At pH 4-5 the difference between the solubility of the proteases in aqueous ethanol solutions and that of other enzymes present is greater than at higher pH, and the addition of ethanol to the concentrate precipitates catalase, the carbohydrases amylase and sucrase, and finally the major proportion of the proteases and esterase, in that order.

Replacement of 0.1M sodium acetate by sodium valerate, ammonium chloride, and methylamine hydrochloride at the same concentration, produces minor changes in ethanol solubility of the various enzymes at pH 4. In the presence of glycine all the enzymes become completely soluble in 75 per cent. ethanol and in the presence of resonating aromatic amines, such as pyridine and aniline, the enzymes are almost completely soluble at all ethanol concentrations. By reducing the concentration of aromatic amine, precipitation of individual enzymes is possible.

By choosing suitable ethanol concentrations two fractions are obtained from the enzyme concentrate, the first containing catalase, sucrase, and amylase, with a little protease, and the second containing traces of sucrase and amylase with the greater proportion of the proteases and esterase. The addition of 4M ammonium sulphate to a concentrated solution of the second fraction results in the slow formation of needle-shaped crystals containing two proteases, glycylglycine dipeptidase and esterase, together with 70 per cent. ammonium sulphate. Recrystallization is most easily accomplished by cooling a saturated aqueous solution of the crystals.

I. INTRODUCTION

The previous studies on the enzymes of *Aspergillus oryzae* (Crewther and Lennox 1953) provide information concerning the nature, specificity, and stability of the various enzymes present in culture filtrates of the mould. Attention has now been turned to the fractionation of these enzymes with the primary object of purifying one or more of the proteases.

In this paper, further details of the optimal conditions for the formation of crystals containing the mould proteases (Crewther and Lennox 1950) are pro-

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vided, together with methods of recrystallization. The effects of pH and various anions and cations on the fractionation of the preparation with ethanol and methods of precipitation with other reagents are also described.

II. Methods

The culture filtrates were prepared by surface culture of *A. oryzae* on sucrose-tartrate medium, and the estimation of enzyme activity, total N, and pH was made as described previously (Crewther and Lennox 1953).

Although the activities of many of the enzymes in the mould culture filtrate were high enough to be accurately measured, the total protein concentration, particularly in early batches, was too low for efficient fractionation with ethanol. Concentration of the culture filtrate was attempted firstly by adsorption. Of a number of adsorbents tested, only carbon black and calcium phosphate gel removed the viscometric protease from solution. Recovery of the enzyme by elution from the gel could be effected using buffer at pH 9.0 but it was not recoverable from the carbon black. The amount of calcium phosphate gel required was excessive and the method was discarded in favour of evaporation and precipitation methods.

Vacuum evaporation of 20-l batches of mould culture filtrate was carried out in a glass, climbing-film, cyclic evaporator having five heat-exchange tubes connected in parallel with a 5-l flask. A large-surface brass condenser was used, through which refrigerated water was circulated, and a low pressure was maintained by two water pumps and an oil pump protected by a dry-ice trap. After 4-5 hr the batch was reduced to 2 l, the temperature having risen from 25 to 35°C as concentration proceeded.

The concentrate obtained by evaporation was dialysed and stored at a low temperature. Proteolysis, with considerable loss of enzyme activity, was found to have occurred during the evaporation and, as more active culture filtrates became available due to modification of the method of cultivating the mould (Maxwell 1952), vacuum concentration was discarded in favour of concentration by ethanol precipitation.

The 20-l batch of culture filtrate was chilled to 2°C either by standing overnight at 2° or, more rapidly, by agitation in a stainless steel can in a freezing chamber at −25°C, and 40 l of ethanol at −25°C was added with agitation. During the addition of the first 10 l the temperature rose momentarily to 8°C but it was rapidly reduced to −2°C on further addition of ethanol. The flocculent white precipitate was allowed to settle for 1 hr in a cabinet at −25°C, most of the supernatant was decanted or removed by suction and discarded, and the precipitate was recovered from the remainder of the solution by centrifuging in an imperforate stainless steel basket. The precipitate was suspended in water and the suspension diluted to 500 ml.

The yield of viscometric protease in the concentrate obtained by this method has been found to depend largely on the extent of proteolysis during treatment. When the culture filtrate was held for 18 hr at 20°C prior to precipitation, the yield of protease in the concentrate decreased by more than 50 per cent.

The thick suspension of the precipitate was dialysed for 1 hr in 5-cm diameter cellophane dialysis tubing in a vessel through which water was flowing vigorously, the volume increasing to approximately 650 ml. Insoluble salts were then removed by centrifuging, and dialysis of the solution was continued for a further period of 2 hr in a rotating dialyser* (Lennox 1949), using 2.7-cm diameter dialysis tubing. Dialysis and other operations were carried out rapidly in order to minimize the formation by proteolysis of compounds which inhibit the final crystallization.

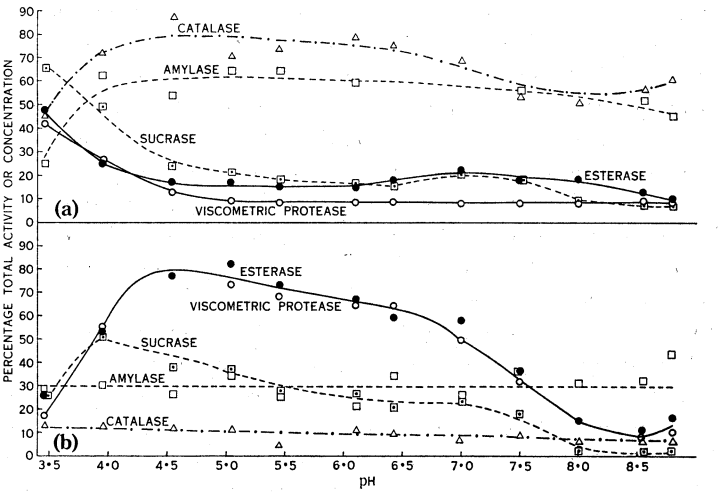

Fig. 1.—Activity of enzymes precipitated with ammonium sulphate at various pH values. (*a*) Precipitate by ammonium sulphate increment from 0 to 2.7M; (*b*) precipitate by ammonium sulphate increment from 2.7 to 3.6M.

III. Experimental

(*a*) *Precipitation of Enzymes with Ammonium Sulphate and Flavianic Acid*

Enzyme concentrate obtained by evaporation was mixed with 3 volumes of 3.5M ammonium sulphate containing suitable amounts of H_2SO_4 or NH_4OH and equilibrated for 18 hr at 18°C. The precipitates, when dissolved in 2 vol. of water, were found to have the activities shown in Figure 1(*a*). Addition to the supernatants of 4 vol. of 4M $(NH_4)_2SO_4$ having the same pH values produced a further series of precipitates which, when dissolved in 2 vol. water, yielded solutions with the activities shown in Figure 1(*b*). At the lower salt concentration catalase and amylase precipitated over a wide range of pH, whereas sucrase, esterase, and the viscometric protease precipitated maximally at pH 3.5, the minimum pH possible without considerable inactivation. With increase in salt concentration the sucrase, esterase, and protease precipitated at higher pH than previously and over a wide range of pH; the solutions at pH

* Melbourne tap water contains *c.* 50 p.p.m. total solids, most of which is organic in nature.

3.5, having been partly depleted of these enzymes, did not produce very active precipitates.

Reduction of the pH of an enzyme concentrate to 4.0 by addition of flavianic acid caused precipitation of fine granules. When these were dissolved in 0.005N NaOH to give a pH of 7.0 and diluted to the original volume, a solution was obtained with proteolytic activity equal to that of the original. By shaking the active enzyme flavianate solution with "Amberlite" IR4 the yellow flavianate was completely removed without affecting the proteolytic activity. However, on adding greater amounts of flavianic acid and thereby reducing the pH to 3.5, a flaky precipitate formed which was devoid of activity.

The addition of 1.0M acetate buffer at pH 4.0 to mixed enzyme concentrate to reduce the pH value to 4.5 and fractionation by adding 10 equal increments of 1 per cent. flavianic acid did not result in clear-cut separation of the enzymes. Esterase and catalase were most active in fraction 5, gravimetric protease and amylase in fraction 6, viscometric protease and phosphatase in fraction 7, and sucrase in fraction 8. The total nitrogen content, determined after removal of the flavianic acid on "Amberlite" IR4, was maximal in fraction 6.

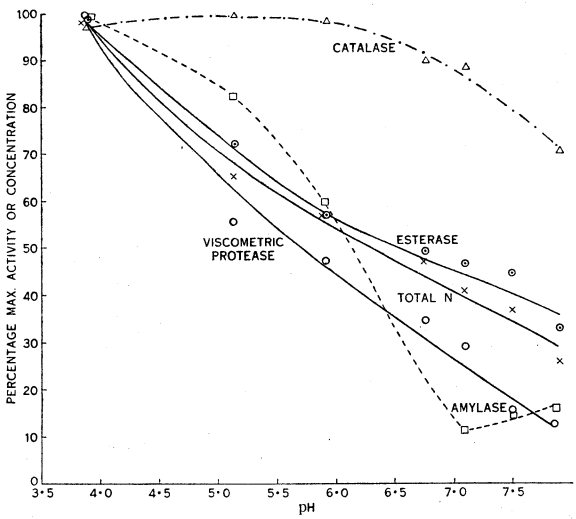

Fig. 2.—Activity of ethanol precipitates formed in presence of 0.05M phosphate buffers. Ethanol increment 0-56 per cent.

(*b*) *Effect of pH on Precipitation with Ethanol*

Figures 2-4 show the effects of pH on the ethanol precipitation of the various enzymes present in the concentrated filtrate using 0.05M phosphate, HCl in the absence of buffer, or 0.05M acetate to adjust the pH. An ethanol increment from 0 to 56 per cent. was tested for each buffer, and a further increment from 56 to 69 per cent. for concentrate adjusted with hydrochloric acid and acetate.

(*c*) *Precipitation with Ethanol in the Presence of Organic Ions*

The data presented in the preceding section suggest that the various enzymes in the concentrates exhibit greatest differences in their solubility in ethanol at low pH, and a pH of 4.0 has therefore been used to test the effects of different buffering ions at 0.1M concentration.

Mould culture filtrate, which had been concentrated under vacuum, was dialysed against running tap water, precipitated with two volumes of ethanol at 2°C, filtered, and the precipitate dissolved in a solution of the electrolyte being tested to give the original volume of filtrate at pH 4.0. Aliquots of this solution were then precipitated with various amounts of ethanol at 2°C and held at that temperature for 15 hr. Each precipitate was centrifuged off, dissolved in 10 vol. of water, and the solution used for the estimation of protease by both the gravimetric and viscometric methods. Catalase, esterase, sucrase, amylase, and total nitrogen were also estimated.

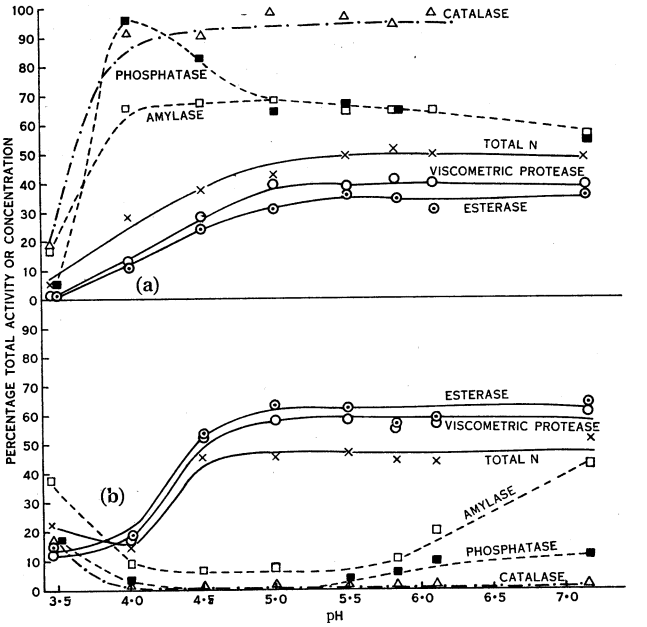

Fig. 3.—Activity of enzymes precipitated by ethanol after adjusting the pH with hydrochloric acid. (*a*) Precipitate by ethanol increment from 0 to 56 per cent.; (*b*) precipitate by ethanol increment from 56 to 69 per cent.

The changes which occur in precipitation of the enzymes when acetate is replaced by valerate are shown in Figure 5. The order of appearance of the enzymes in the precipitates is similar but the range of ethanol concentrations over which the enzymes precipitate is greater with valerate than with acetate. However, the slope of the curves relating ethanol concentrations to the amount of each enzyme precipitated is less with valerate than with acetate; there is therefore little to choose between the anions from the point of view of separation of the proteases from other enzymes.

When using glycine in place of acetate as the buffer (Fig. 6) the sequence of appearance of the enzymes and the ethanol concentrations required for precipitation of each enzyme were not markedly changed. It was found, however, that in 75 per cent. ethanol the enzymes became completely soluble or remained in the liquid phase as a slight opalescence which could not be centrifuged out.

Other amino acids used as buffers were lysine, δ-aminovaleric acid, α-amino-*n*-valeric acid, arginine, and cysteine hydrochloride. Their characteristics compared with acetate as buffer are summarized in Table 1. Unlike glycine, no appreciable tendency to increased solubility at high concentrations of ethanol was observed with other amino acids.

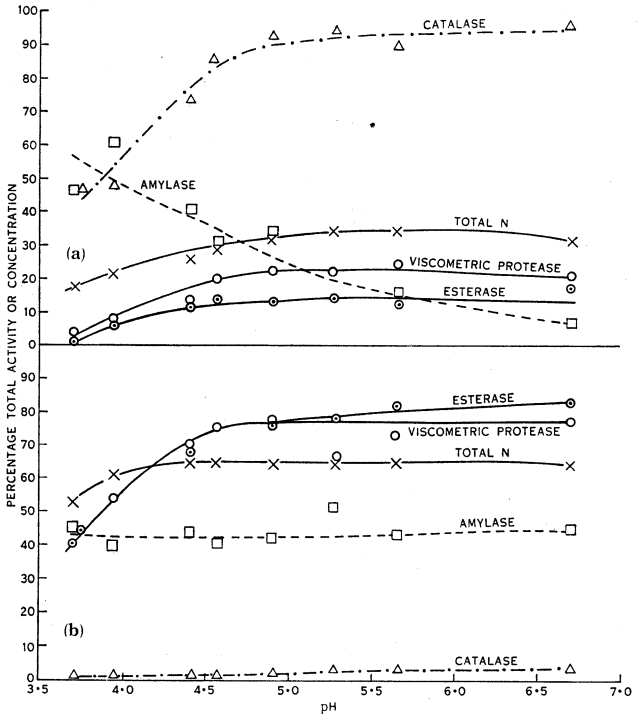

Fig. 4.—Activity of enzymes precipitated by ethanol in presence of 0.05M acetate buffers. (*a*) Precipitate by ethanol increment from 0 to 56 per cent.; (*b*) precipitate by ethanol increment from 56 to 69 per cent.

A number of simple amines have also been used under the same conditions as the anions and amino acids. As a basis for comparison, ammonium chloride was first tested and provided the results shown in Figure 7. Comparing it with acetate the most noteworthy difference is to be found in the tendency for catalase, sucrase, and amylase to precipitate at higher ethanol concentrations. A second difference is to be found in the delayed appearance of esterase in the precipitates. The results for methylamine (Fig. 7), like those for di- and trimethylamine, were similar to the curves obtained with ammonium chloride.

Tetramethyl ammonium bromide provided curves which were almost identical with those given by ammonium chloride.

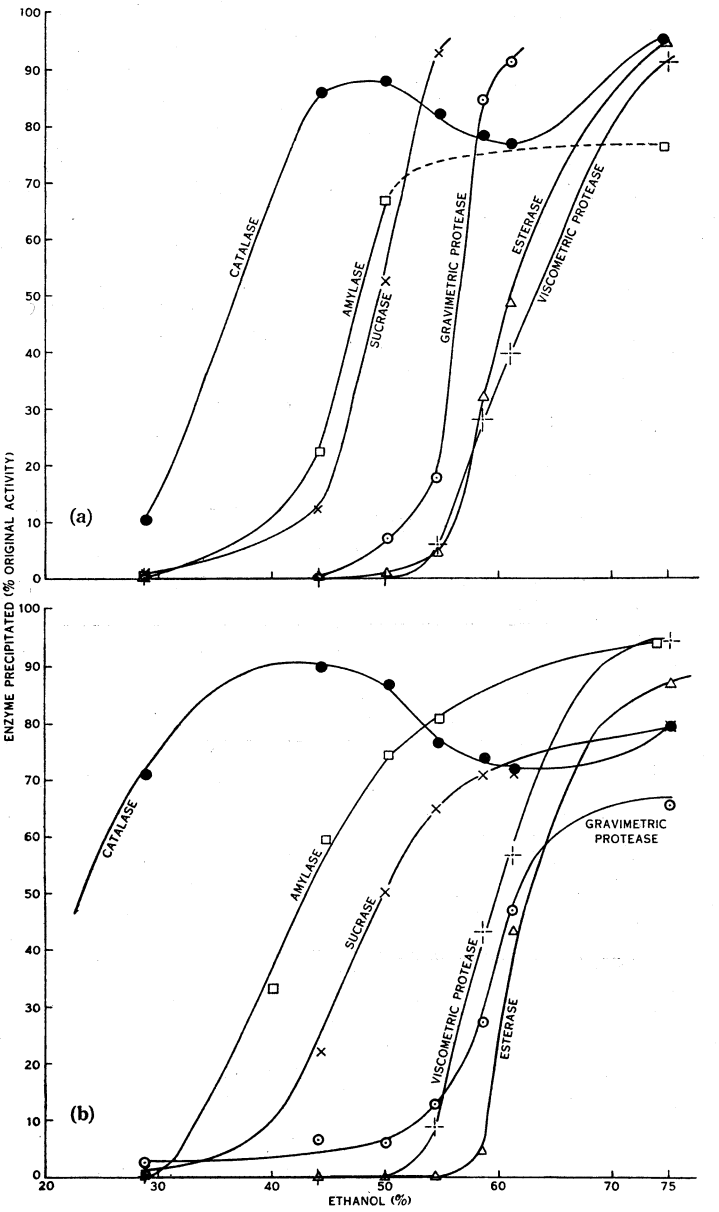

Fig. 5.—Ethanol precipitation curves in presence of 0.1M acetate (*a*) and valerate (*b*) buffers at pH 4.0.

Entirely different results were obtained when the aromatic amines were tested. With 0.1M aniline or pyridine at pH 4.0 no precipitate was obtained at any of the ethanol concentrations tested. In the presence of *p*-phenylene-

diamine and 28 per cent. ethanol, a slight precipitate was obtained which contained about 30 per cent. of the total catalase but no other enzyme. At higher concentrations of ethanol no precipitate was obtained. As shown in Figure 8,

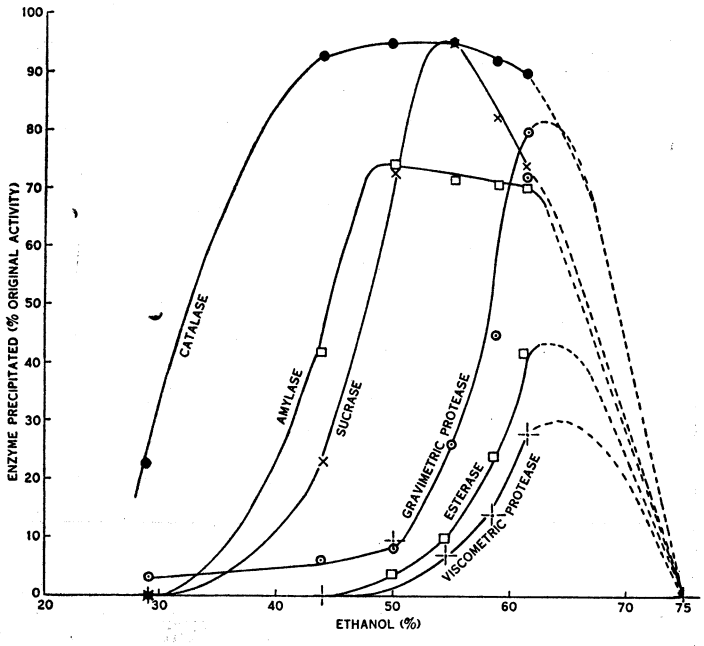

Fig. 6.—Ethanol precipitation curves in presence of 0.1M glycine at pH 4.0.

TABLE 1

EFFECT OF PRESENCE OF 0.1M AMINO ACIDS DURING PRECIPITATION OF MOULD ENZYMES WITH ETHANOL

The results are compared with those obtained in 0·1M acetate (Fig. 5)

Amino Acid	Sequence of Appearance of Enzymes
Glycine	As with acetate*
Lysine	Sucrase and amylase precipitation delayed†
δ-Aminovaleric acid ..	As with lysine†
Valine	As with acetate
Arginine	Amylase partly precipitated at low ethanol concentration. Sucrase precipitation delayed
Cysteine	As with acetate but all enzymes first precipitated at higher ethanol concentrations

*Enzymes become soluble at high ethanol concentrations.

†Two peaks of maximum precipitations of sucrase.

p-aminophenol was slightly less effective in repressing precipitation, though catalase was the only enzyme precipitated in any quantity.

Benzylamine, on the other hand, behaved in much the same way as methylamine (Fig. 8) and although piperidine repressed precipitation at low ethanol

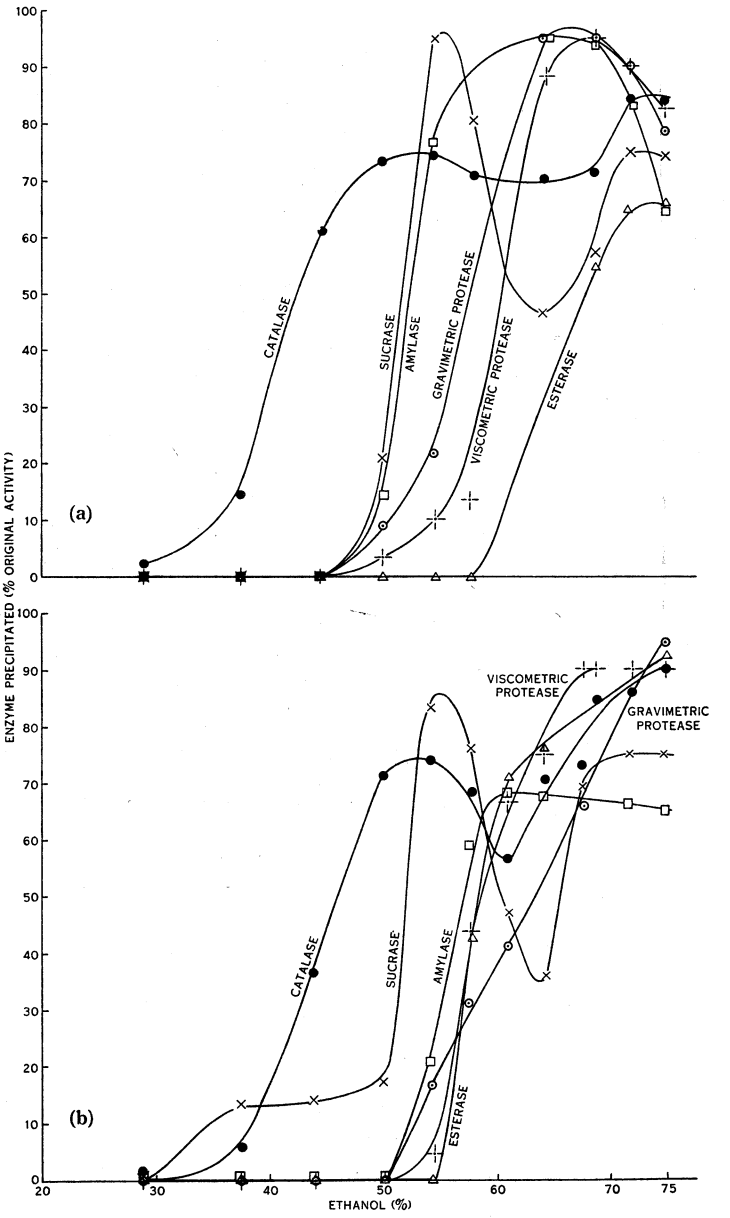

Fig. 7.—Ethanol precipitation curves in presence of 0.1M ammonium chloride (*a*) and methylamine hydrochloride (*b*) at pH 4.0.

concentrations it allowed considerable precipitation at higher ethanol concentrations. The divergence in behaviour in the presence of piperidine from that observed in the presence of a typical aliphatic amine may be due to the

presence of small amounts of pyridine. The most noteworthy effect of benzylamine was the suppression of precipitation of amylase, or alternatively its inactivation.

At low concentrations of the aromatic amines (aniline and pyridine) it is possible to obtain considerable amounts of precipitate, particularly at low ethanol concentrations. Different enzymes show differing tendencies to remain in solution at high ethanol concentrations under these conditions and it is possible to obtain partial separation of the enzymes in this way. The preparation of solutions containing the gravimetric protease in the absence of viscometric

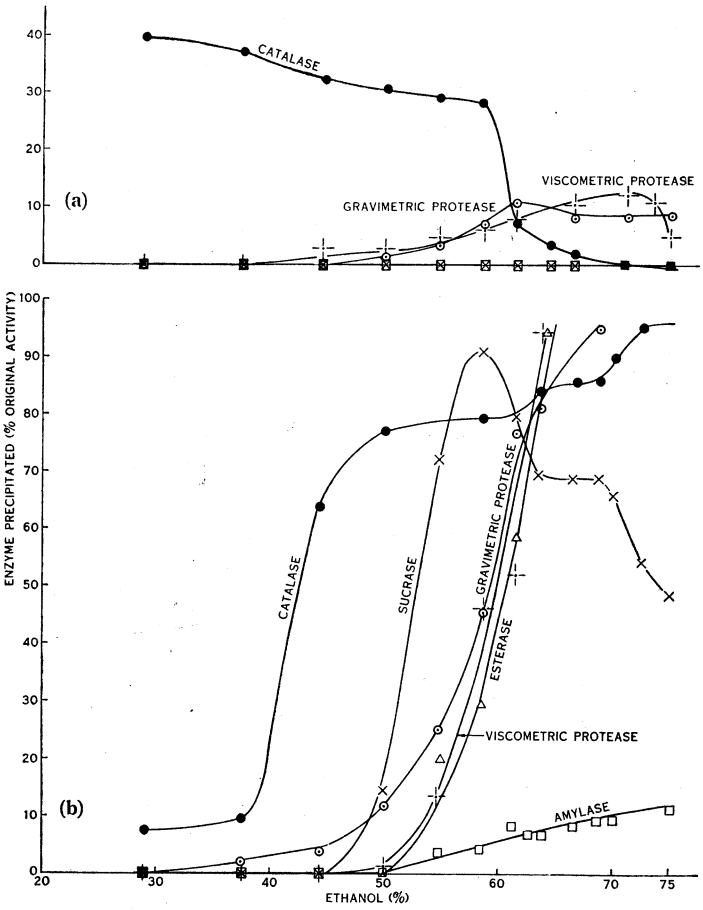

Fig. 8.—Ethanol precipitation curves in presence of 0.1M *p*-aminophenol (*a*) and benzylamine (*b*) at pH 4.0.

activity, using this method, has already been described (Crewther and Lennox 1953). However, a more general application of the method to the fractionation of culture filtrates has proved difficult owing to considerable variation between batches of filtrate. For this reason acetate buffer was used for the subsequent experiments.

Low-temperature fractionation of an enzyme concentrate in the presence of 0.05M acetate, to give a pH of 4.7, yielded solutions having the activities shown in Table 2. Estimation of the total solids, protein N, and reducing substances (after acid hydrolysis) indicated the presence of carbohydrate in each fraction, amounting to approximately 15 per cent. of the total solids. Estimation of the total N indicated that the remaining 85 per cent. was almost entirely protein. Jermyn (1952) has identified the hexoses in a hydrolysate of the mould polysaccharide as mannose and galactose. The results in Table 2 indicate that catalase, the proteases, and possibly esterase are the major constituents of the protein in the concentrate.

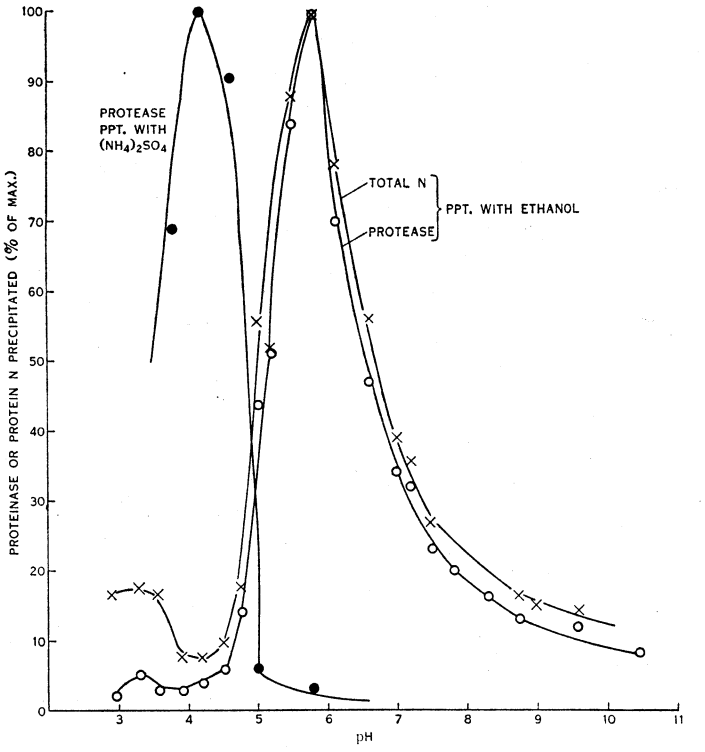

Fig. 9.—Effect of pH on viscometric protease and protein precipitation from a protease-rich fraction.

(d) Subfractionation and pH-Solubility Relationships of Protease-rich Fractions

A fraction obtained from the enzyme concentrate by precipitation with ethanol in the presence of acetate buffer at pH 4.0 over the range of ethanol concentrations 58-64 per cent., was re-precipitated with 2 vol. of ethanol, dissolved in water, and precipitated with 2.8M $(NH_4)_2SO_4$ at different pH values. Figure 9 shows that viscometric protease was precipitated maximally at a higher pH value than with crude enzyme solutions (Fig. 1) and the pH range was greatly restricted. When 67 per cent. ethanol was used as precipitant for a similar experiment (Fig. 9) the pH of maximum precipitation was again higher

than that for crude enzyme (Fig. 4) and the pH range of precipitation was decreased.

Ethanol subfractionation of fractions 5 and 6, obtained by fractionation of mixed enzyme concentrate at pH 4.0, yielded the results shown in Figure 10.

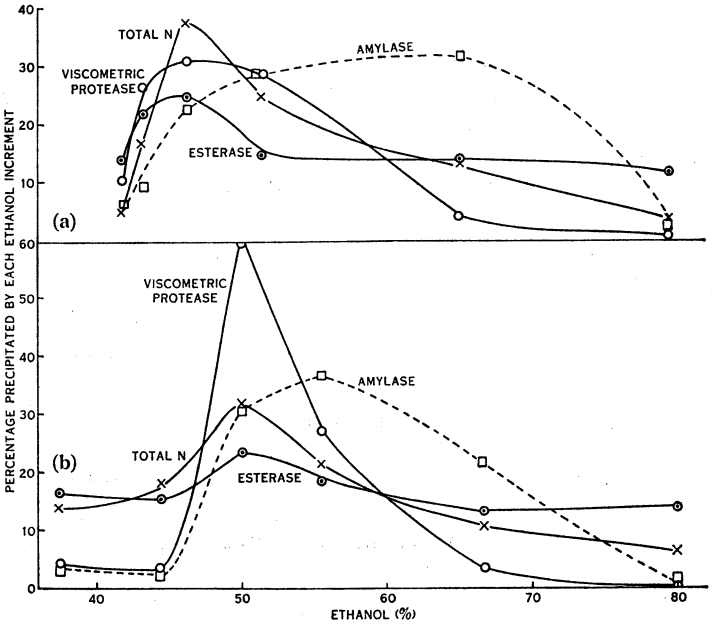

Fig. 10.—Subfractionation of protease-rich fractions with ethanol. (*a*) Subfractionation of fraction 5 (51-57 per cent. ethanol); (*b*) subfractionation of fraction 6 (57-61 per cent. ethanol).

TABLE 2

FRACTIONATION OF MOULD CULTURE CONCENTRATE WITH ETHANOL IN pH 4.7 ACETATE BUFFER

Enzyme activities expressed as percentage of total precipitable activity

Fraction (% ethanol v/v)	Catalase	Sucrase	Amylase	Phosphatase	Gravimetric Protease	Viscometric Protease	Esterase	Total N
0-50	97	32	18	9	2	Nil	4	19
50-56	3	40	22	23	18	2	4	8
56-64	Nil	25	49	53	32	22	17	23
64-69	Nil	3	9	9	35	41	46	33
69-74	Nil	Nil	2	6	13	35	29	17

The pH values of the fractions were not altered from their initial values of 4.6 and 4.8 respectively. Owing to the high concentration of viscometric protease in these fractions it precipitated at low ethanol concentrations, particularly in fraction 5.

Although maximum esterase activity was detected in the richest protease subfraction, almost as much was also detected in the later subfractions.

(*e*) *Formation of Protease-rich Crystals by Addition of Ammonium Sulphate to Ethanol Fractions*

Each of the fractions obtained by ethanol fractionation of the concentrate was dissolved in a minimum volume of water and treated with 4M ammonium sulphate until a faint cloud appeared. This was filtered off and the solutions were held at 2°C for several days. The fractions with the greatest protease content were then found to contain needle crystals either free or in rosettes, whereas those in which catalase or the carbohydrases preponderated produced granular material.

On suspending the crystalline precipitate in water, pronounced double refraction of flow was observed and some fractions, which appeared to be granular, also showed this phenomenon. In such instances the granules probably break up into minute needle crystals too small to be distinguished under the microscope.

TABLE 3

SUMMARY OF ACTIVITIES AND NITROGEN CONTENTS OF ENZYME SOLUTIONS AT VARIOUS STAGES IN A TYPICAL PURIFICATION PRIOR TO CRYSTALLIZATION FROM FRACTION 2

Solution	Volume (ml)	Viscometric Protease (arbitrary units/ml.)	Total Viscometric Protease (arbitrary units)	Non-dialysable N (mg/ml)	Viscometric Protease (arbitrary units/mg N)
Culture filtrate	20,000	25·0	500,000	0·21	119·0
Ethanol ppt. dissolved in water	500	268·5	134,250	2·50	107·2
After 1st dialysis (1 hr)	680	225·0	153,000	2·10	107·1
After 2nd dialysis (2 hr)	960	155·0	149,000	1·25	124·0
Fraction 1	200	131·0	26,200	1·35	97·1
Fraction 2	200	388·5	77,700	3·00	129·5

Further experiments showed that fractionation with ethanol into more than two fractions before crystallization was unnecessary and a better yield of crystals was obtained as follows: The concentrate from 20 l of culture filtrate (500 ml) was dialysed, mixed with 200 ml of 1.0M acetate buffer at pH 4.0, diluted to 2 l, and cooled to 2°C. An equal volume of ethanol* at -20 to -25°C was then added with agitation and the mixture held at about -10°C for 30 min. The precipitate was removed in an imperforate basket centrifuge in the cold room at -5°C and the supernatant precipitated with further ethanol (2 l). The second precipitate was recovered in the centrifuge and dissolved in a minimum

* The optimal ethanol concentrations for fractionation of different batches have been found to vary considerably. The best criterion for determining the limit of ethanol concentration for the first fraction is the appearance of a white precipitate in the rather brownish precipitate which is first formed.

volume of water (usually 200 ml) to yield a solution at pH 4.6 which, on addition of ammonium sulphate as described above, produced a heavy deposit of crystals. Yields of up to 10 g of crystalline material have been obtained from 20 l of culture filtrate. A summary of the volumes of solution, viscometric protease activities, and non-dialysable N contents up to the stage of crystallization is reported in Table 3.

The crystals have been found to possess many of the characteristics of other protein crystals produced by salt precipitation. They are formed from the enzyme solution slowly over periods of several weeks, and likewise they dissolve slowly on suspension in water. They have a much lower solubility than the amorphous material obtained by rapid precipitation with ethanol under similar conditions. Cooling the enzyme-ammonium sulphate solution is not necessary for the production of crystals, though there is an increase in the solubility of the crystals with increasing temperature.

Crystallization was found to take place over the pH range 4.0-5.2 with greatest yields of crystalline material and best crystal form at pH 4.6-4.7 (Fig. 11). In general, inadequate dialysis or crystallization under suboptimal conditions resulted in granules which sometimes had dark centres and developed into rosettes of crystals after prolonged standing at 2°C.

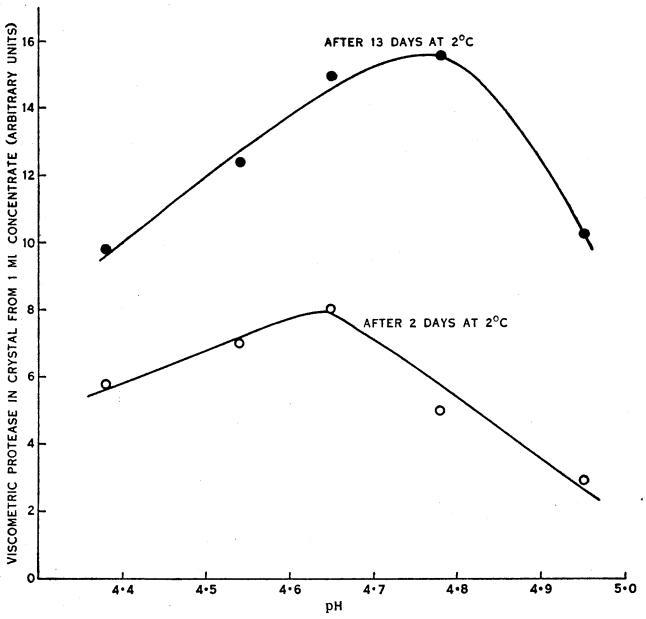

Fig. 11.—Activity of crystals obtained by precipitation with ammonium sulphate at various pH values.

By centrifuging 1-ml portions of the crystal suspension and diluting both the deposit and supernatant to 10 ml for measurement of enzyme activity, most of the viscometric protease in the fraction was shown to be precipitated in the crystals.

(*f*) *Composition of the Enzyme Crystals and Recrystallization*

Recrystallization was complicated by the presence of a considerable proportion of ammonium sulphate in the crystals. Addition of 4M ammonium sulphate at pH 4.7 to concentrated solutions of the crystals yielded non-crystalline precipitates. Dialysis of a solution of crystals and freeze-drying of the protein solution made possible the testing of different protein concentrations under the usual conditions of crystallization. However, although hexagonal plates were obtained from the more concentrated protein solutions, no needle forms appeared even after seeding with needle crystals.

TABLE 4

COMPOSITION OF CRYSTALS AND SUPERNATANT LIQUID DURING THE PROGRESS OF RECRYSTALLIZATION

Enzyme Preparation	Enzyme Activity (arbitrary units/mg total N)				Ammonia N (mg/mg total N)
	Viscometric Protease	Gravimetric Protease	Esterase	Glycyl-glycine Dipeptidase	
1st Crop crystals	15·1	5310	0·34	0·157	0·69
1st Supernatant	12·9	5770	0·26	0·178	0·72
Ratio of activity: 1st Supernatant / 1st Crystals	0·86	1·09	0·77	1·13	(1·04)
2nd Crop crystals	13·1	3470	0·53	0·124	0·71
2nd Supernatant	18·0	3140	0·57	0·089	0·77
Ratio of activity: 2nd Supernatant / 2nd Crystals	1·37	0·91	1·08	0·72	(1·08)
3rd Crop crystals	11·3	1740	0·51	0·057	0·78
3rd Supernatant	12·0	1340	0·43	0·028	0·80
Ratio of activity: 3rd Supernatant / 3rd Crystals	1·06	0·77	0·84	0·50	(1·03)

A satisfactory yield of recrystallized material in the form of needle crystals was finally accomplished by dissolving enzyme crystals in a minimum volume of distilled water at 30°C (about 0.2 g/ml) leaving a few crystals undissolved. On cooling to 2°C and holding at that temperature for a few days, a heavy precipitate formed consisting of large rosettes of long needle crystals; crystallization continued for several weeks. By holding the solution for 18 hr at 30°C prior to cooling, the traces of amylase and sucrase, which are always present after the first crystallization, were almost completely eliminated.

The crystalline preparations have a number of enzymic activities of which the gravimetric and viscometric proteases, esterase, and glycylglycine dipeptidase appear to be the most important. During the recrystallization process it is

possible to recover the crystals at intervals as they are formed and examine their composition. The data for Table 4 were obtained in this way. The crystalline preparation used for this particular recrystallization contained a much higher proportion of the gravimetric protease in relation to the esterase and the viscometric protease than most others. On recrystallization the first crystals formed had more viscometric protease and esterase activity per mg N than the supernatant, but less gravimetric protease and dipeptidase activity. In the second and third crops of crystals, however, the trend was reversed.

A similar experiment was conducted with a crystal preparation having a ratio of activity of the gravimetric to the viscometric protease of 110 : 1 as compared with the ratio of 450 : 1 in the preparation to which Table 4 refers. In this instance the ratio of activities of the gravimetric to the viscometric proteases increased during recrystallization to 135. Crystals prepared under suboptimum conditions contained a low percentage of protein. As shown in Table 5, with repeated recrystallization the enzymes were enriched at the expense of the ammonia N. This approached a ratio to the total N of 0.7, the figure attained also in the other recrystallizations.

TABLE 5

REPEATED CRYSTALLIZATION OF A MIXED CRYSTAL PREPARATION

Enzyme Preparation	Enzyme Activity (arbitrary units/mg total N)			Ammonia N (mg/mg total N)
	Viscometric Protease	Gravimetric Protease	Esterase	
Original crystals	11·5	700	0·32	0·86
Once recrystallized	12·2	770	0·33	0·84
Twice recrystallized	34·2	4770	0·82	0·70

Recrystallizations have been successfully carried out on crystal preparations after holding in the dry state for 2 years. Both protease activities and the esterase activity of the crystals are unchanged at room temperature but the glycylglycine dipeptidase loses activity and finally disappears.

Solubility experiments were complicated by the presence of ammonium sulphate in the crystals. Varying amounts of a crystal preparation were shaken in a bath for periods of 1.5 and 3 hr at 18°C with 2.5M ammonium sulphate, which had previously been found to cause partial solution of the crystals. The relationship between the viscometric protease activities of the supernatant solutions and the amount of crystalline material used is shown in Figure 12. The appearance of a peak of maximum solubility indicates the effect of ammonium sulphate dissolved from the crystals on the amount of protein remaining in solution.

IV. Discussion

The pH-solubility curves of various proteins in ethanol-water mixtures and in concentrated salt solutions have been shown to have sharp solubility minima (Green 1931; Surgenor *et al.* 1949), and the method devised by Distèche (1948) for obtaining crystalline preparations from mixtures of proteins depends for its success on each component having low solubility over a rather narrow band of pH values. Thus the pH-solubility curves obtained with dialysed mould enzyme concentrate (Figs. 1-4) were atypical, whereas on repeated fractionation and precipitation with ethanol typical narrow pH ranges of low solubility were obtained (Figs. 9 and 10). These results suggest that the dialysed concentrate contains a component or components which interfere with the solubility characteristics of the proteins in ethanol and salt solutions, this component being removed during fractionation of the proteins with ethanol. Recent work by Gillespie and Woods (1953) has demonstrated that interference with ethanol fractionation by the considerable amount of non-dialysable ionic material in the dialysed concentrate is responsible for these results.

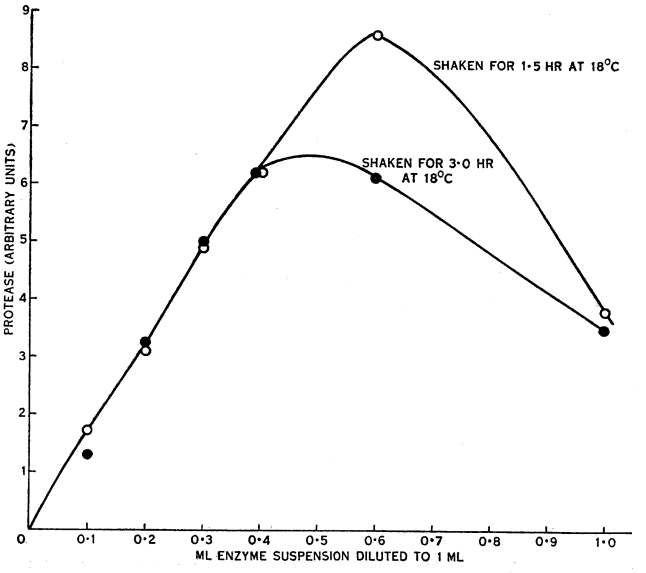

Fig. 12.—Solubility of viscometric protease in 2.5M ammonium sulphate.

Some difficulty arises in interpreting the different effects of aromatic and aliphatic amines. The most reasonable explanation for the increase in solubility of the proteins in ethanol-water mixtures in the presence of aniline, *p*-aminophenol, *p*-phenylenediamine, or pyridine appears to be combination of protein with the compound through its amino group, either by hydrogen bond formation or by ionic attraction, with a consequent masking of the polar groups of the protein. If the effect were produced by the ionic form of the compound, no great difference in ethanol precipitation of the enzymes would be expected

in the presence of either aromatic or aliphatic amines since both types are largely ionized under the experimental conditions. Phenyl ammonium ions and benzyl ammonium ions should then have analogous effects.

It is pointed out by Pauling (1945) that an ionized amino group will donate a proton to form a hydrogen bond more readily than an un-ionized amino group. For this reason the un-ionized form of aniline, which resonates with forms having a positive charge on the nitrogen and a negative charge on the aromatic ring, would form hydrogen bonds more readily than an un-ionized aliphatic amine in which the nitrogen is practically uncharged. The overall positive charge of the ionized form of the amines may prevent complex formation with the proteins, most of which are positively charged under the experimental conditions. The smaller solubilizing effects of substituted anilines can be explained in terms of the greater polarity of the aromatic ring in these compounds. The solubilizing effect of glycine on proteins in ethanol-water mixtures has been observed previously and employed in protein fractionation (Cohn *et al.* 1950).

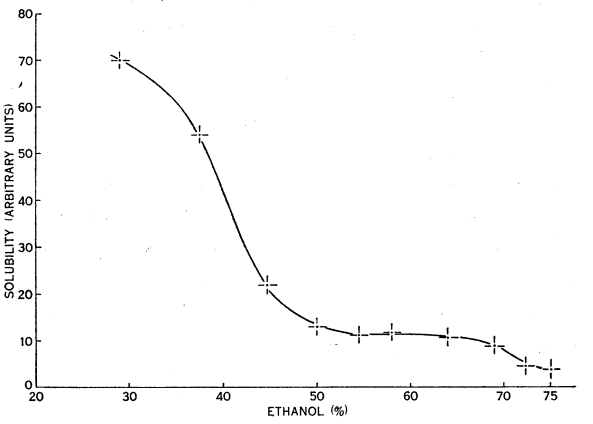

Fig. 13.—Ethanol solubility of catalase in presence of of 0.1M ammonium chloride.

The fractionation results have provided further evidence for the view already proposed (Crewther and Lennox 1953) that the gravimetric and viscometric proteases are separate enzymes. There is a marked tendency for the gravimetric protease to commence precipitation at a lower ethanol concentration than the viscometric protease. The distribution of the two enzymes in crystalline preparations and supernatant solutions is also markedly different.

There is also evidence that catalase consists of two enzymes, as shown by the typical two-component solubility curve obtained by converting the precipitation curve for catalase, in the presence of ammonium chloride, to a curve relating the solubility of catalase in the ethanol-water mixtures to the ethanol content (Fig. 13). The dual peaks obtained when precipitating sucrase in the presence of aliphatic amines do not correspond with the curves expected for a two-component system and although more than one enzyme is involved (Gillespie, Jermyn, and Woods 1952) it seems probable that one component redissolved at high ethanol concentrations.

Gillespie, Jermyn, and Woods (1952) have shown that the difficulties encountered in obtaining relatively pure preparations of the mould proteases are attributable to the complexity of the various enzyme systems present in the culture filtrates. However, paper electrophoresis has shown that fractionation under the conditions described in this paper for preparing crystalline material does, in fact, separate a number of major components of the system (Gillespie and Woods 1953).

On recrystallization from water the composition of the crystals is found to be similar in most enzyme activities and in ammonium ion to that of the solution remaining (Table 4), though as successive batches of crystals are removed from a concentrated liquor there is found to be a decrease in the activity of the gravimetric protease and the glycylglycine dipeptidase per mg total N which probably indicates a progressive inactivation and breakdown of these enzymes. The slight increase in the ammonia N content per mg total N may be due to protein decomposition. In general, however, the constancy of the ammonia content of the crystals is remarkable and there seems little doubt that ammonium sulphate forms part of the crystal structure. It seems probable that the enzymes form a series of mixed cystals having lower solubilities than the component proteins or ammonium salts and that several eutectic mixtures are possible. It is not unlikely that many well-known salt-precipitated enzyme crystals are also mixed crystals of the salts and proteins.

Astrup and Alkjaersig (1952) have recently published a classification of proteolytic enzymes in terms of their inhibition by various specific inhibitors and surface active agents. They showed that the crystalline proteases of *A. oryzae*, described in the present paper, were atypical in that they were activated by both cationic and anionic detergents. The presence of at least two, and possibly three, proteases in the crystalline material may explain this apparent anomaly.

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VI. References

Astrup, T., and Alkjaersig, N. (1952).—*Nature* **169**: 314.
Cohn, E. J., et al. (1950).—*J. Amer. Chem. Soc.* **72**: 465.
Crewther, W. G., and Lennox, F. G. (1950).—*Nature* **165**: 680.
Crewther, W. G., and Lennox, F. G. (1953).—*Aust. J. Biol. Sci.* **6**: 410.
Distèche, A. (1948).—*Biochim. Biophys. Acta* **2**: 265.
Gillespie, J. M., Jermyn, M. A., and Woods, E. F. (1952).—*Nature* **169**: 487.
Gillespie, J. M., and Woods, E. F. (1953).—*Aust. J. Biol. Sci.* **6**: 447.
Green, H. A. (1931).—*J. Biol. Chem.* **93**: 517.
Jermyn, M. A. (1952).—*Aust. J. Sci. Res.* B **5**: 409.
Lennox, F. G. (1949).—*Aust. J. Sci.* **12**: 110.
Maxwell, Margaret E. (1952).—*Aust. J. Sci. Res.* B **5**: 42.
Pauling, L. (1945).—"The Nature of the Chemical Bond." (Cornell Univ. Press: New York.)
Surgenor, D. M., Strong, L. E., Taylor, H. L., Gordon, R. S., and Gibson, D. M. (1949).—*J. Amer. Chem. Soc.* **71**: 1223.