had already shown that solubility and titration measurement gave comparable results.

The results for glycylglycine agreed well with those of Monk (1951*c*). They were all measured at pH more acid than 8·0. At more alkaline pH values Dobbie, Kermack & Lees (1951) have shown that with cupric ions the peptide nitrogen also takes part in chelation. Evidence of this further chelation was obtained with mercury in this study. The titration curve obtained was symmetrical and yielded satisfactory results for log K_1 and log K_2.

The marked decrease in stabilities from glycine to glycylglycine is of interest, as according to Albert (1950) the metal bridges the carboxyl and amino groups of glycine. In the case of glycylglycine such ring formation is unlikely in view of the size of such a ring. As, however, there is evidence for the peptide losing a second proton per molecule (Dobbie *et al.* 1951) it is more probable that the metal chelates first with the terminal amino groups and then with the peptide nitrogen. Further work on metal peptide complexes is in progress.

The effect of lengthening the chain length on stability constants was small, but produced a still detectable decrease in stability from glycine to norvaline (Table 1). Norleucine, however, gave an unexpected increase in stability. The effect of isomerism in the chain was also studied. From Table 1 it will be seen that the isomers of norleucine gave different stability constants but the isomers of norvaline gave identical values.

SUMMARY

1. The stability constants of some complexes of α-amino-acids with metals of Group II of the periodic classification were measured by a potentiometric method.

2. The predominant part played by the metal in determining the stability constant was confirmed.

3. Metals of Group II*a* formed complexes with stability constants too low to be measured. Group II*b* metal complexes yielded measurable constants in the order of magnitude Hg > Be > Zn > Cd. The results of a number of investigators using a range of metals with glycine, α-alanine and glycylglycine were compared.

4. The effect of substituting the hydrogen atoms of the amino group of glycine on stability constants was studied. The only appreciable structural effect observed was the decrease in stability produced by peptide formation. Increase of chain length resulted in decreased stabilities.

I wish to thank Dr N. H. Martin for his advice and encouragement during the course of this work.

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The Crystallization of Fumarase

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Fumarase, the enzyme catalysing the reversible hydration of fumaric acid to L-malic acid, was discovered in 1911 by Batelli & Stern. Since then it has been shown to occur in a large number of plant and animal tissues, and in lower organisms.

In 1941 Laki & Laki claimed to have isolated crystalline fumarase from pig-heart muscle, but Scott (1948), Scott & Powell (1948) and Racker (1950) have shown this preparation to contain considerable quantities of aconitase and lactic dehydrogenase. Scott (1948) has also obtained amorphous fractions with over three times the specific activity of the preparation of Laki & Laki. In this communication is reported the isolation from pig-heart muscle of crystalline fumarase, homogeneous by sedimentation and electrophoretic criteria, which has a specific activity almost ten times that reported by Laki & Laki. A preliminary account of this work has been published (Massey, 1951).

METHODS

Enzyme activity determinations. Fumarase activity was determined by the spectrophotometric method of Racker (1950). The reaction was measured in the presence of 0·017 M-sodium fumarate and 0·033 M-phosphate buffer, pH 7·3, using a Beckman model DU spectrophotometer. One unit of activity was chosen arbitrarily as the amount of enzyme required to reduce the optical density of fumaric acid at 3000 A. by 0·01/min. at 20° and pH 7·3. The initial rate of reaction was calculated by plotting a graph of optical density against time of reaction.

Protein concentration. This was measured by Kjeldahl nitrogen estimations on dialysed extracts. In all calculations the protein concentration was assumed to be 6·25 times the nitrogen content. Approximate concentrations were also obtained by optical density determinations at 2770A. The optical density of 1 ml. of protein solution in a final volume of 3 ml., determined through a quartz cell of 1 cm. thickness was referred to as density/ml. (*d*/ml.).

Sedimentation and diffusion. The sedimentation constant was determined using a Svedberg oil-turbine ultracentrifuge and the diffusion constant using a Gouy diffusiometer (see Addendum).

Electrophoresis. Electrophoretic mobilities were determined with a Perkin Elmer electrophoresis apparatus at 1°. The current passed was 8 ma.

EXPERIMENTAL

Method of isolation

Fresh pigs' hearts were cleaned of fat and connective tissue, minced, and washed with large quantities of tap water until the washings were almost colourless. The washed mince was squeezed in cheesecloth, and homogenized with three times its weight of cold 0·01 M-Na_2HPO_4 for 3 min. The homogenate was spun for 30 min. at 1800 *g* in an International Serum Centrifuge, and the supernatant poured from the residue. The pH of the supernatant was adjusted to 5·2 with 1 M-acetate buffer, and the inactive precipitate centrifuged off. The clear, slightly reddish supernatant contained fumarase in almost the same quantities as the supernatant before adjusting the pH to 5·2, but the protein concentration was reduced to 10–14% of the initial value. The fumarase was then adsorbed quantitatively on $Ca_3(PO_4)_2$ gel. The amount necessary varies from preparation to preparation, and must be determined by pilot lots for maximum yield of enzyme. It is generally in the range of 4–7 ml. of gel (containing 30 mg. dry wt./ml.) for 100 ml. of solution. The $Ca_3(PO_4)_2$ was centrifuged and the supernatant discarded. The enzyme was next eluted from the gel with cold 0·1 M-phosphate buffer, pH 7·3, containing 50 g. $(NH_4)_2SO_4$/l. The fumarase can be eluted almost quantitatively from the gel—three to four elutions are sufficient to remove the bulk of the enzyme. The combined eluates were then fractionated between 45 and 60% saturation with $(NH_4)_2SO_4$, the 45–60% fraction containing the enzyme. This fraction was dissolved in distilled water, and dialysed overnight against distilled water. Considerable purification was then achieved by the addition of small amounts of $Ca_3(PO_4)_2$ to this concentrated protein solution. Sufficient $Ca_3(PO_4)_2$ was added to combine with impurities, but not sufficient to adsorb fumarase. The quantities vary from one preparation to another, and are best determined by the addition of gradually increasing amounts of gel, followed each time by centrifugation and activity and protein determinations. This was followed by another $(NH_4)_2SO_4$ fractionation, the precipitate formed on the addition of $(NH_4)_2SO_4$ to 60% saturation was centrifuged at high speed and dissolved in the minimum amount of 15% saturated $(NH_4)_2SO_4$ solution. Saturated $(NH_4)_2SO_4$ was added drop by drop until a faint turbidity was formed. The solutions of $(NH_4)_2SO_4$, which were of A.R. grade, had not been neutralized. The pH of the mixture before crystallization was between 6·1 and 6·4. After two days in the refrigerator, crystals formed in large amounts (Fig. 1). These are relatively insoluble in water; they can be washed quickly and centrifuged without much loss through solution.

Recrystallization. Fumarase crystals were centrifuged, washed with cold distilled water, centrifuged again, and dissolved in 0·01 M-phosphate buffer, pH 7·3. From this, the enzyme was precipitated by 60% saturation with $(NH_4)_2SO_4$, dissolved in 15% saturated $(NH_4)_2SO_4$, and saturated $(NH_4)_2SO_4$ added to produce turbidity as in the original crystallization. Crystallization can be greatly accelerated by seeding from a previous preparation. After two recrystallizations the preparation reaches a constant specific activity, which is from 2000 to 3000 times that of the original extract. With care, the overall yield can be 40–50%. The activity and purity data for a representative preparation are shown in Table 1.

Recrystallization from phosphate buffer, pH 6·3. Fumarase can also be obtained in crystalline form by dialysis against 0·1 M-phosphate buffer, pH 6·3. Fumarase crystals from $(NH_4)_2SO_4$ were dissolved in 0·01 M-phosphate buffer, pH 7·3, and the enzyme precipitated in amorphous form by 60% saturation with $(NH_4)_2SO_4$. The precipitate was dissolved in 0·1 M-phosphate, pH 7·3, and the pH gradually lowered by dialysis against 0·1 M-phosphate, pH 6·3.

The enzyme crystallized in the form of thin rectangular plates (Fig. 2). These crystals are almost insoluble in water and dilute phosphate solutions, but can be dissolved in dilute NaOH solution. If this is done at 0° and the pH adjusted to neutrality as soon as the crystals are dissolved, there is no loss in activity. The solution so obtained has the same specific activity as solutions of fumarase obtained from $(NH_4)_2SO_4$. All the other results reported in this paper however have been obtained with the crystals from $(NH_4)_2SO_4$.

Pig-heart protein A. From the mother liquor of the crystallization of fumarase, another crystalline protein can be obtained on the addition of a little more $(NH_4)_2SO_4$. Here crystallization proceeds best at 20–25°. The crystals, unlike those of fumarase, are very soluble in water, and appear as long thin needles without sharply defined edges. Attempts to identify the crystals have not yet been successful.

Properties of fumarase

Like other crystalline enzymes, fumarase is protein in nature; it gives a strong biuret reaction, is denatured by heating, and by extremes of acidity and alkalinity. It is reasonably stable over the pH range 5–9. The crystals are faintly birefringent in polarized light, and do not contain any ammonium sulphate. They are slightly soluble in distilled water, but readily soluble in 0·01 M-phosphate

Table 1. *Details of a representative preparation of fumarase*

Treatment	Activity (units/ml.)	Protein concn. (*d*/ml.)*	Purity (units/*d*)	Volume (ml.)	Total units	Times purified	Yield (%)
Pigs' hearts homogenized with 0·01 M-Na_2HPO_4. Supernatant	9	6·53	1·37	4800	43000	—	—
Adjusted to pH 5·2. Supernatant	8·5	0·45	19	4750	40050	14	94
Added 185 ml. $Ca_3(PO_4)_2$ gel (30 mg./ml.). Supernatant	0	—	—	—	—	—	—
Eluted four times with 0·1 M-phosphate, pH 7·3, containing 50 g. $(NH_4)_2SO_4$/l.	66	1·35	49	590	39000	36	91
Fractionated between 45–60% saturation with $(NH_4)_2SO_4$	1050	5·85	180	30	31500	132	73
Dialysed overnight against distilled water	750	3·90	192	42	31500	140	73
Added $Ca_3(PO_4)_2$ gel. Supernatant from							
0·5 ml.	750	3·70	203	42	31500	148	73
1·0 ml.	750	3·15	238	42	31500	174	73
2·5 ml.	700	2·45	286	43	30000	209	70
5·0 ml.	679	1·85	362	45	30000	265	70
10·0 ml.	510	0·85	600	50	25500	438	59
Added $(NH_4)_2SO_4$ to 60% saturation, precipitate dissolved in 15% sat. $(NH_4)_2SO_4$. Crystallized	—	—	—	—	—	—	—
Crystals dissolved in 0·01 M-phosphate buffer, pH 7·3	1200	0·48	2500	15·1	18100	1830	42
1st Recrystallization	1800	0·62	2900	9·5	17100	2110	40
2nd Recrystallization	1560	0·54	2900	10·5	16400	2110	38

* For definition of *d*/ml. see Methods.

buffer, pH 7·3. At pH 7·3 and 20°, in the presence of 0·033 M-phosphate, 1 g. of crystals is capable of converting 0·55 mole (i.e. 64 g.) of fumaric acid to L-malic acid/min. On the basis of a molecular weight of about 200000, as indicated by sedimentation and diffusion, this represents a turnover number of over 100000 moles substrate/min./mole fumarase, under the above conditions.

At pH 6·7 and 39° in the presence of 0·1 M-phosphate, the conditions which were used by Laki & Laki (1941), the activity was 1·30 moles of fumaric acid converted to L-malic acid/min./g. enzyme. The activity reported by Laki & Laki (1941) was 0·14 mole/min./g. enzyme.

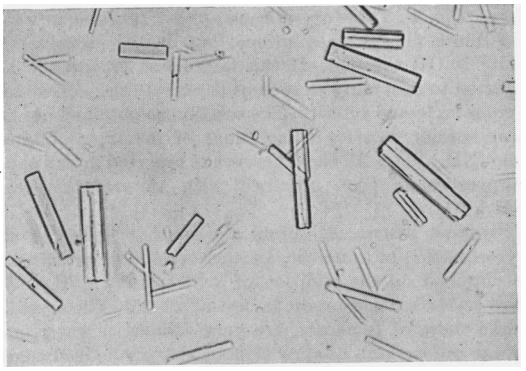


Fig. 1. Fumarase crystals from ammonium sulphate. ×600.

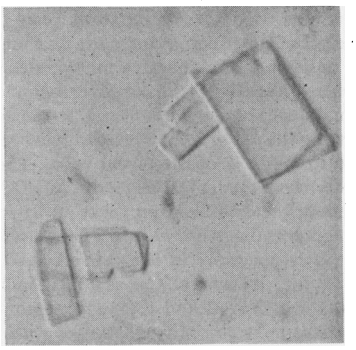
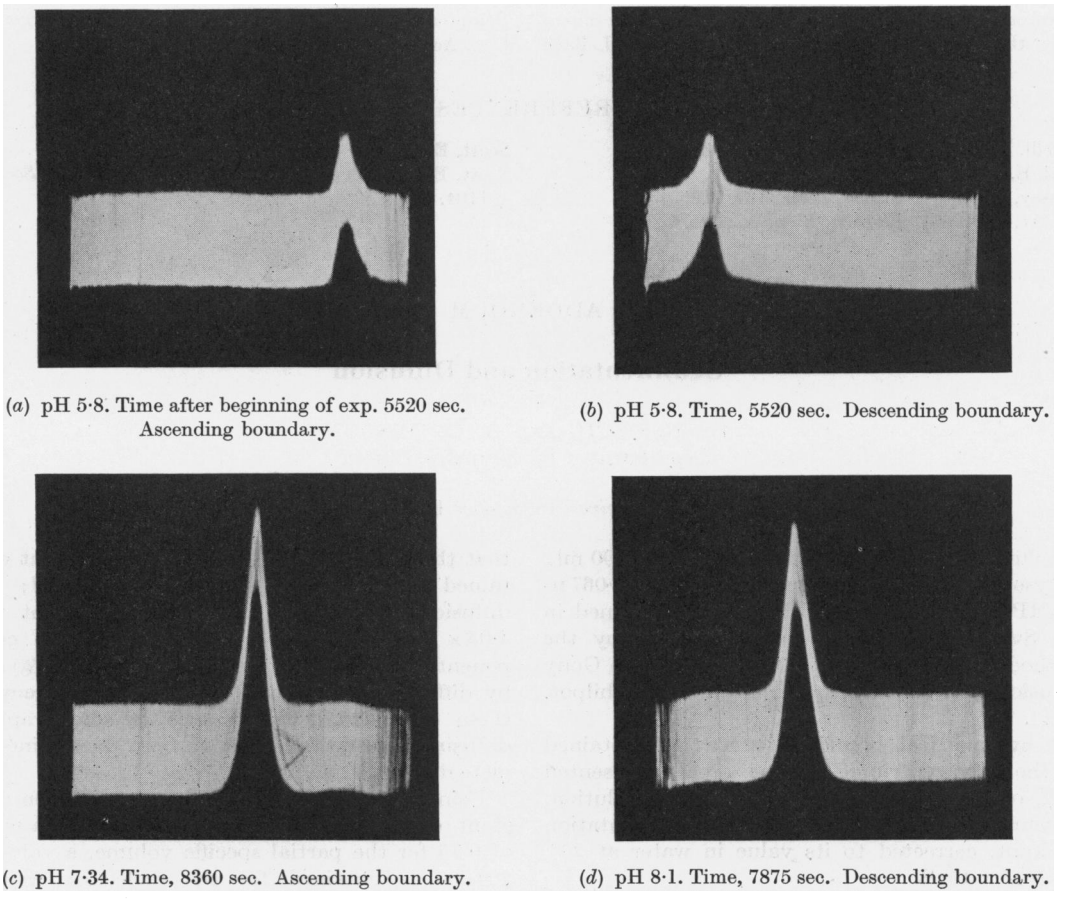


Fig. 2. Fumarase crystals from 0·1 M-phosphate, pH 6·3. ×950.

As indicated by previous studies with partially purified preparations, crystalline fumarase has a very high specificity, attacking only fumaric and L-malic acids. Maleic, D-malic, *trans*- and *cis*-aconitic, tartaric, aspartic and crotonic acids are completely unattacked by even large concentrations of fumarase.

Molecular weight. The molecular weight was determined by sedimentation and diffusion measurements of a 0·7% (w/v) solution of fumarase in 0·087M-phosphate buffer of pH 7·34. The results, which are given in the Addendum, show a molecular weight of 204000 under these conditions, assuming a partial specific volume of 0·75.



(*a*) pH 5·8. Time after beginning of exp. 5520 sec. Ascending boundary.

(*b*) pH 5·8. Time, 5520 sec. Descending boundary.

(*c*) pH 7·34. Time, 8360 sec. Ascending boundary.

(*d*) pH 8·1. Time, 7875 sec. Descending boundary.

Fig. 3. Electrophoresis schlieren diagrams.

Electrophoretic behaviour. Electrophoresis was performed at pH 8·1, 7·34 and 5·8, at a constant ionic strength of 0·21. At these pH values the material appeared to be perfectly homogeneous. The results are shown in Fig. 3 and Table 2. The slight bumps, away from the main peaks, in Fig. 3 (*c*) and (*d*) show the initial position of the boundary and are the usual boundary anomalies found with high protein concentrations.

Table 2. *Electrophoretic mobilities of fumarase*

pH	Mobility (cm.^{2} sec.$^{-1}$ V.$^{-1}$ × 10^{-5})
5·8	0·93
7·34	2·25
8·1	2·45

From the mobilities at these pH values, an extrapolated value for the isoelectric point of fumarase is between pH 5·0 and 5·4. Unfortunately, the electrophoretic behaviour of fumarase at pH values lower than 5·8 could not be studied, because of the low solubility in this pH region. However, the extrapolated value agrees well with the isoionic point of pH 5·0 as determined by exhaustive dialysis of a salt-free solution of fumarase.

SUMMARY

1. The isolation of crystalline fumarase and another, as yet unidentified, crystalline protein from pig heart is described.

2. Sedimentation and diffusion constants indicate that fumarase is a homogeneous protein of molecular weight about 200000.

3. Electrophoresis studies at three pH values indicate a homogeneous protein. The results give an isoelectric point for fumarase between pH values of 5·0 and 5·4.

4. The turnover number of fumarase is over 100000 at 20° and pH 7·3 in the presence of 0·033 M-phosphate.

The principle of the initial stages in the purification was based on results obtained in an advanced class experiment under the direction of Dr M. Dixon, F.R.S., Dr S. J. Bach and Dr E. C. Webb at the Biochemistry Department, Cambridge. I am indebted to Dr R. Cecil and Dr A. G. Ogston of the Biochemistry Department, Oxford, for the determination of sedimentation and diffusion constants, and to Dr B. Conway and Mr D. W. F. James of the Chester Beatty Research Institute, London, for electrophoretic mobility measurements. I wish to thank Dr M. Dixon for suggesting the problem and for his advice and help in this work. The work was carried out with a grant from the Commonwealth Scientific and Industrial Research Organization, Australia.

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ADDENDUM

Sedimentation and Diffusion

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A solution of fumarase containing 0·7 g./100 ml., dialysed against a buffer containing 0·067 M-Na_2HPO_4 and 0·017 M-KH_2PO_4, was examined in the Svedberg oil-turbine ultracentrifuge by the method of Cecil & Ogston (1948) and in the Gouy diffusiometer (Coulson, Cox, Ogston & Philpot, 1948).

A symmetrical boundary curve was obtained in the ultracentrifuge, whose area represented 96 % of the refractive increment of the solution, measured against diffusate. The sedimentation constant, corrected to its value in water at 20°, was $8{\cdot}51 \times 10^{-13}$.

The mean diffusion constant, corrected to its value in water at 20°, was $4{\cdot}45 \times 10^{-7}$. Analysis of the Gouy interference pattern (Ogston, 1949) showed that the material was not homogeneous, but contained 7 % of rapidly diffusing material; the diffusion constant of the main component was $4{\cdot}05 \times 10^{-7}$. The proportions of the main component estimated by the ultracentrifuge (96 %) and by diffusion (93 %) agree within the accuracy of these estimates. The presence of some rapidly diffusing material might have been due to incomplete dialysis.

Using the corrected value for the diffusion constant of the main component and assuming a value of 0·75 for the partial specific volume, a value of 204000 is obtained for the molecular weight. Although this is derived from quantities measured at a single concentration, it is not likely to differ much from the true value.

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