excluded volume of a linear polysaccharide is therefore expected to be larger. That the excluded volume decreases with increased dextran concentration is expected because of the increased overlapping of the neighbouring molecules.

There may be other explanations for the effect of dextran on the solubility of proteins. Formation of a complex between the protein and the polysaccharide, which had a lower solubility than its constituents, could account for some of the results that have been observed. A charge interaction is unlikely because the dextran is essentially uncharged, and, further, the effect persists even at very high concentrations of electrolytes and is independent of the electrolyte concentration and pH over a wide range. Other types of chemical interaction are equally improbable, for the effect seems to vary only with the dextran concentration while being independent of the albumin concentration even at very low concentrations.

In addition to the importance of this phenomenon in biological systems, it may have practical application in the fractionation of high-molecular-weight compounds.

SUMMARY

1. In media of high ionic strength the presence of dextran significantly decreases the solubility of albumin, γ -globulin, fibrinogen and α -crystallin.

2. The relative decrease in solubility is a function of the size of the protein and the concentration of dextran.

3. The effect on albumin is independent of the degree of polymerization of dextran (mol.wt. greater than 150000), the pH, the absolute salt concentration or the absolute protein concentration.

4. The results are discussed in terms of a large effective volume of dextran.

5. The importance of the phenomenon for natural polysaccharide-protein systems is emphasized.

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The Preparation of Crystalline Forms of Ferricytochrome b_2 and Ferrocyclochrome b_2

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Cytochrome b_2 is the L-lactate-cytochrome c oxidoreductase (EC 1.1.2.3) of baker's yeast, first partly purified and described by Bach, Dixon & Zervas (1946). A method for purification and crystallization was developed by Appleby & Morton

(1954), who showed that the crystalline enzyme was a flavohaemoprotein containing a DNA component (Appleby & Morton, 1954, 1959a, b, 1960; Appleby, Morton & Simmonds, 1960). Non-crystalline preparations of the enzyme, apparently

free of the DNA component, have been obtained by extension of the method of Bach *et al.* (1946) (see Boeri, Cutolo, Luzzati & Tosi, 1955; Appleby & Morton, 1959*a*; Boeri & Rippa, 1961; Hasegawa & Ogura, 1961). Appleby & Morton (1959*b*, 1960) showed that this labile enzyme is readily modified by loss of either or both of the flavin and DNA components, and that loss of the latter prevents crystallization by their procedure. The enzyme containing the DNA component is now known as Type I-cytochrome b_2 , and the enzyme without this component as Type II-cytochrome b_2 (Morton, 1961).

The present paper describes modified procedures for the preparation of crystalline Type I-ferrocytochrome b_2 , and new procedures for obtaining crystalline Type I-ferricytochrome b_2 , crystalline Type II-ferrocytochrome b_2 and crystalline Type II-ferricytochrome b_2 . Preliminary accounts of some of this work have been published (Morton, 1961; Morton & Shepley, 1961).

MATERIALS AND METHODS

Baker's yeast. Freshly compressed yeast was supplied by the Effront Yeast Co. Ltd., Melbourne. Air-dried yeast was prepared at room temperature and ground to a fine powder in ball mills at 2°.

Chemicals. Lactic acid (L.R. or A.R. grade) was obtained from British Drug Houses Ltd. or from May and Baker Ltd.; it contained an excess of the L-enantiomorph (Krebs, 1961). All concentrations given below refer to total lactate. Unsatisfactory results were obtained with some commercial preparations in that the enzyme was inactivated. Acetone and butan-1-ol were obtained from Colonial Sugar Refinery Chemicals Ltd., and were distilled before use. GSH was from Sigma Chemical Co. Ltd., U.S.A.

Anaerobic conditions. Solutions used in preparation and storage of enzyme were degassed and kept under oxygen-free nitrogen (see Appleby & Morton, 1959*a*; Armstrong, Coates & Morton, 1963).

General preparative procedures. Unless otherwise stated, centrifuging and other procedures were carried out at 0–2°.

Enzymic activities. These were determined spectrophotometrically from the rate of reduction at 25° of either 1 mM-ferricyanide at 420 $m\mu$ or 0.07 mM-2,6-dichlorophenol-indophenol at 600 $m\mu$ in 0.03 M-tetrasodium pyrophosphate–0.3 M-DL-sodium lactate–0.01 mM-EDTA brought to pH 8.0 with HCl, or of 0.04 mM-heart-muscle cytochrome *c* at 550 $m\mu$ in similar buffer brought to pH 7.0 with HCl. Extinction values used were as follows: for reduction of ferricyanide, $\Delta\epsilon_{420\text{ }m\mu} = 1.04 \times 10^3$; for reduction of 2,6-dichlorophenol-indophenol, $\Delta\epsilon_{600\text{ }m\mu} = 21 \times 10^3$; for reduction of ferricytochrome *c*, $\Delta\epsilon_{550\text{ }m\mu} = 19.8 \times 10^3$. Other details are given by Appleby & Morton (1959*a*, *b*). Activities are expressed as μ moles of acceptor reduced/hr./mg. of protein at 25°. Protein concentrations were determined from extinctions of solutions of the ferrocytochrome at 423 $m\mu$, by using values of 232×10^3 for $\epsilon_{423\text{ }m\mu}$ (haem basis), and of 82000 g./mole of haem.

EXPERIMENTAL AND RESULTS

Type I-ferrocytochrome b_2

Extraction from air-dried yeast and crystallization at low ionic strength. The following modification of the method of Appleby & Morton (1959*a*) is suitable for large-scale preparation of the enzyme. About 1 kg. of powdered yeast was stirred with 2 l. of butan-1-ol and 5 l. of 0.1 M-sodium lactate–0.1 mM-EDTA–1 mM-magnesium sulphate, pH 6.8, either for 1–2 hr. at room temp. (18–22°) or for 14–16 hr. at about 2°. The material was centrifuged at 2000g for 30 min. and the aqueous layer was collected by suction. Approx. 1 ml. of M-magnesium sulphate/l. was added and the extract was then adjusted to pH 6.3–6.4 with N-acetic acid, cooled to –2° and brought to about 16% (v/v) with acetone. After centrifuging at 2000g for 10 min. at –5°, the clear brown supernatant was brought to 32–35% (v/v) with acetone. After 30–60 min. at –5°, the supernatant was decanted or the red precipitate was otherwise collected by centrifuging at 2000g for 10 min. at –5°. The red precipitate was extracted for about 1 hr. at –2 to 0° with about 1 l. of 0.15 M-sodium lactate–0.1 mM-EDTA–1 mM-magnesium sulphate, pH 6.8, containing 25% (v/v) of acetone. The clear red supernatant obtained by centrifuging at about 6000g for 20 min. at –2 to 0° was cooled to –5° and acetone was added until a viscous red precipitate was formed. The supernatant was decanted and the precipitate was dissolved in about 100 ml. of 0.1 M-sodium lactate–0.1 mM-EDTA–0.04 M-sodium pyrophosphate, pH 6.8, and centrifuged at 25000g for 30 min. The solution was dialysed against 0.05 M-sodium lactate–0.1 mM-EDTA–1 mM-magnesium sulphate, pH 6.8, and after about 20 hr. crystals of Type I-ferrocytochrome b_2 were collected by centrifuging at 6000g for 30 min. The crystals were dissolved in 0.1 M-sodium lactate–0.04 M-sodium pyrophosphate–0.1 mM-EDTA–1 mM-magnesium sulphate, the solution was centrifuged at 25000g for 30 min. at 0° and recrystallization was effected by dialysis as before; recrystallization was complete within 4 hr. Rapid recrystallization of the enzyme was also readily effected by pouring a solution of the enzyme (approx. 120 μ M-haem, in 0.3 M-sodium lactate–0.05 M-tetrasodium pyrophosphate–0.1 mM-EDTA) into water at 0° and stirring. Perfect crystals of Type I-ferrocytochrome b_2 were formed immediately. If extraction of 3 kg. of dried yeast is carried out overnight at 2° with mechanical stirring, the other procedures may readily be accomplished in 7–8 hr. on the following day.

The purification at each step was substantially the same as reported for the analogous steps by Appleby & Morton (1959*a*), and the crystalline Type I-ferrocytochrome b_2 (Plate 1*a*) had similar specific activities with ferricyanide and with horse-heart cytochrome *c* to those reported by Appleby & Morton (1959*a*). However, the yield of crystals (10–20 mg./kg. of yeast powder) was somewhat higher, owing apparently to a greater recovery between steps 2(*c*) and 3(*a*) of Table 1 of Appleby & Morton (1959*a*). In recrystallized ferrocytochrome so obtained, the $E_{264\text{ }m\mu}/E_{423\text{ }m\mu}$ ratio is about 0.86, and remains constant with further recrystallization.

Plate 1. Crystals of cytochrome b_2 . The procedures used are described in the text. (a)–(d), Type I-ferrocytochrome b_2 ; (e) Type I-ferricytochrome b_2 ; (f)–(h), Type II-ferrocytochrome b_2 . The photographs are shown at the following magnifications: (a), $\times 800$; (b), $\times 320$; (c), $\times 260$; (d), $\times 160$; (e), $\times 536$; (f), $\times 536$; (g), $\times 160$; (h), $\times 88$.

Effect of Mg^{2+} ions on crystallization. Several preparations of Type I-ferrocytochrome b_2 failed to crystallize when the dialysis medium contained 5–10 mM-EDTA. However, crystallization from similar solutions occurred when magnesium sulphate was added in excess of the concentration of EDTA. Subsequently, therefore, magnesium sulphate was added at several stages of the preparation procedure and always during crystallization, since EDTA is required to protect the enzyme from inhibition by heavy-metal salts (see Appleby & Morton, 1959*a, b*).

Extraction from acetone-dried yeast powder and crystallization at low ionic strength. To determine whether possible autolysis during air-drying of yeast caused modification of the enzyme, it was extracted and crystallized from an acetone-dried powder under conditions that restricted autolysis to a minimum. Cakes of compressed yeast were stored at -20° for 48 hr. and the frozen yeast was crumbled in a mortar at -20° . The crumbled yeast was blended with about 3 vol. of butan-1-ol at -20° , the dispersion was stirred with about 10 vol. of acetone at -20° and the yeast was collected by filtration at -20° . The yeast was redispersed in about 10 vol. of acetone at -20° , washed on the filter paper with diethyl ether at -20° and dried in a stream of warm air. This powder (500 g.) was extracted with 600 ml. of butan-1-ol and 3 l. of 0.1 M-sodium lactate–0.1 mM-EDTA, pH 7.0, for 1–2 hr. at $0-2^\circ$. The material was centrifuged at 5000g for 30 min. at 2° , and the supernatant was adjusted to pH 6.4 with N-acetic acid and made 0.1 mM with respect to magnesium sulphate. The proteins were fractionated with acetone at -2° to 5° as described above. Crystals of Type I-ferrocytochrome b_2 were obtained in good yield after dialysis of the final red solution against 0.05 M-sodium lactate–0.1 mM-EDTA–1 mM-magnesium sulphate, pH 6.8. The spectral and enzymic properties of the recrystallized enzyme were similar to those of the enzyme obtained from air-dried yeast.

Crystallization by acetone treatment. A solution of once-crystallized Type I-ferrocytochrome b_2 containing about 120 μ M-haem in 0.3 M-sodium lactate–0.04 M-tetrasodium pyrophosphate–0.1 mM-EDTA, pH 6.8, at -2° was dialysed (with mechanical agitation) at -2° to -5° against a solution consisting of 100 ml. of 0.1 M-sodium lactate–0.1 mM-EDTA–5 mM-triethanolamine, pH 7.5, and 40 ml. of acetone. Small but perfect crystals (tetragonal shallow bipyramids; Plate 1*b*) appeared within 15 min. The spectral and enzymic properties were similar to those of crystals formed at low ionic strength.

Crystals were also obtained by the slow addition of acetone at -5° directly to another portion of the same solution of enzyme at -5° . A fine amorphous precipitate appeared initially, but dissolved when the solution was warmed. It reappeared on subsequent cooling. After this procedure had been repeated several times, fairly large pink crystals were obtained (Plate 1*c*). When acetone was added to a solution of the solvent, without protein, formation of long prismatic salt crystals was induced. Whereas these dissolved immediately in 0.05 M-sodium lactate, pH 6.8, at -1° , the enzyme crystals were relatively insoluble. The enzyme crystallized in more than one form, possibly owing to co-crystallization of enzyme and inorganic salt (lactate or pyrophosphate). The spectral properties of a solution of

these crystals were similar to those of crystals of Type I-ferrocytochrome b_2 obtained at low ionic strength.

A different crystalline form of the flavohaemoprotein was obtained as follows. Approx. 1 ml. of a solution of 5 mg. of Type I-ferrocytochrome b_2 in 0.5 M-sodium lactate, pH 6.8, was dialysed at -2° to -5° against 2 l. of 0.3 M-sodium lactate–0.1 M-KCN–0.03 M-sodium pyrophosphate–0.1 mM-EDTA, pH 7.4, for 18 hr. It was then dialysed against 2 l. of 0.05 M-sodium lactate–0.1 M-KCN–0.01 M-magnesium sulphate–0.01 M-sodium pyrophosphate, pH 7.5, for 3 hr. at 0° , and afterwards at -3° against a solution consisting of 100 ml. of 0.05 M-sodium lactate–0.1 M-KCN–0.1 mM-magnesium sulphate, pH 7.5, and 42 ml. of acetone. During the final dialysis, the protein solution was frozen and thawed twice. After about 2 hr., large pink crystals appeared. These consisted of octagonal prisms with pyramids at both ends (Plate 1*d*). Before the final dialysis, the specific activities of the enzyme solution, determined with ferricyanide (5100 units/mg.) and with 2,6-dichlorophenol-indophenol (1400 units/mg.), compared well with the activities of the initial enzyme solution (6100 ferricyanide units/mg. and 2200 2,6-dichlorophenol-indophenol units/mg. respectively). Activities of the crystalline preparation were not determined. The absorption spectrum of a solution of the crystals was similar to that of Type I-ferrocytochrome b_2 obtained by crystallization at low ionic strength.

Type I-ferricytochrome b_2

Although cytochrome b_2 is most stable when reduced and in the presence of lactate and EDTA (Bach *et al.* 1946; Appleby & Morton, 1954, 1959*a, b*), a stable active preparation of the ferricytochrome could be obtained as follows. About 20 mg. of crystals of Type I-ferrocytochrome b_2 , obtained by dialysis to low ionic strength (cf. Plate 1*a*), were washed free of lactate with 50 ml. of 0.05 M-sodium acetate–0.005 M-sodium phosphate–0.01 M-magnesium sulphate, pH 6.8, at 0° , and then dissolved in 5 ml. of 0.3 M-sodium acetate–0.04 M-sodium pyrophosphate–0.1 M-magnesium sulphate, pH 6.8. The enzyme was then oxidized by the addition of 0.25 ml. of 0.1 M-potassium ferricyanide. After the absence of ferrocytochrome b_2 had been confirmed by spectroscopic observation, approx. 1 mg. of GSH was added to the solution; this does not reduce ferricytochrome b_2 (see below) but protects the enzyme. The enzyme was dialysed against 1 l. of 0.05 M-sodium acetate–0.1 mM-EDTA–1 mM-magnesium sulphate–2 mM-triethanolamine, pH 6.8, at $0-2^\circ$. Tan-coloured crystals (Plate 1*e*) were rapidly formed. These dissolved readily in 0.3 M-sodium acetate–0.05 M-sodium pyrophosphate–0.1 mM-EDTA, pH 7. Sodium acetate may be replaced by KCl in these solutions. Crystals of the ferricytochrome have also been obtained by precipitation with acetone essentially as described for the ferrocytochrome. The crystals were similar to those of Plate 1*e*).

Crystals of the Type I-ferricytochrome b_2 obtained at low ionic strength differ from crystals of the ferrocytochrome (Plate 1*a*) in that the crystals of the ferricytochrome appear by phase-contrast to be thicker at the edge than in the centre. Solutions of the crystals are burgundy-red when concentrated and brown when diluted. All of the haem of the ferricytochrome was reduced immediately on the

addition of sodium lactate (final concn. 0.08 M). The position and extinction of the ultraviolet-absorption band indicated that the ferriocytochrome retained all of the DNA component. The enzymic activities of the crystalline preparation at 25° were 7100 ferri-cyanide units/mg. and 1950 2,6-dichlorophenol-indophenol units/mg. These compare well with those of Type I-ferrocytochrome b_2 .

A number of potential reducing reagents were added to small portions of a solution of Type I-ferriocytochrome b_2 containing about 120 μ M-haem in 0.05 M-sodium pyrophosphate, pH 7.0. The solutions were in air and observations were made with a microspectroscope at room temp. Sodium lactate, sodium dithionite, sodium thioglycollate and 2,3-dimercaptopropanol caused rapid and complete reduction of the haematin. Sodium sulphite reduced the haematin more slowly. However, GSH and cysteine hydrochloride, in concentrations up to 10 mg./ml. of enzyme, caused no reduction. Ascorbic acid in concentrations of less than about 2 mg./ml. had no effect, but at high concentrations (about 20 mg./ml.) there was approx. 30 % reduction of the haematin as determined spectrophotometrically. It was observed that GSH (about 10 μ g./ml. of solution containing 50 μ M-cytochrome b_2 haematin) considerably decreased the loss of activity that occurs when solutions of the enzyme are stored aerobically (cf. Appleby & Morton, 1959*a*, *b*). At pH 6.4–6.8, a solution containing about 44 μ M-cytochrome b_2 haematin and about 10 μ g. of GSH/ml. in 0.05 M-sodium pyrophosphate–0.1 mM-EDTA lost about 10 % of its activity when stored anaerobically at 0° for about 32 hr., and about 20 % of its activity when stored aerobically at 0°. About 70 % of activity was lost by a similar solution stored aerobically at 0° in the absence of GSH for the same period.

Type II-cytochrome b_2

Crystallization of Type II-ferrocytochrome b_2 by precipitation with salt. Appleby & Morton (1959*a*, *b*, 1960) showed that the DNA component of Type I-ferrocytochrome b_2 may be removed (with retention of enzymic activity) by treatment with deoxy-ribonuclease, or by dialysis against 70 % saturated ammonium sulphate.

A solution of once-crystallized Type I-ferrocytochrome b_2 containing approx. 105 μ M-haem was dialysed with continuous agitation, successively and for 6–12 hr. on each occasion, against solutions of 20%, 30% and 40% saturated ammonium sulphate in 0.1 M-sodium lactate–0.1 M-tetrasodium pyrophosphate–0.1 mM-EDTA, pH 8.0, at 2°. After about 6 hr. of dialysis against the 40% saturated ammonium sulphate solution, a crystalline precipitate formed. It consisted of small somewhat irregular crystals of Type II-ferrocytochrome b_2 (Plate 1*f*).

Crystallization of Type II-ferrocytochrome b_2 at low ionic strength. A solution of three-times crystallized Type I-ferrocytochrome b_2 containing about 100 μ M-haem in 0.3 M-sodium lactate–0.05 M-sodium pyrophosphate–0.1 mM-EDTA, pH 6.8, was dialysed successively at pH 8.0 against 50% and 70% saturated solutions of ammonium sulphate in 0.15 M-sodium lactate–0.1 mM-EDTA at 0–2°. Each dialysis was for 24 hr., and any precipitate formed after the first treatment was collected by centrifuging, redissolved and re-treated. The pink amorphous precipitate was collected by centrifuging at 2°, washed with 70% ammonium sulphate in 0.15 M-sodium lactate–0.1 mM-EDTA, pH 8.0, and then dissolved in a minimum volume of 0.3 M-sodium lactate–0.1 mM-EDTA–1 mM-magnesium sulphate, pH 7.0. The solution was centrifuged at 25000*g* for 1 hr. at 2°, and the supernatant was dialysed against 0.05 M-sodium lactate–0.1 mM-EDTA–1 mM-magnesium sulphate, pH 6.8, at 0–2°. After 2–5 days, large red crystals appeared (Plate 1*g*) which adhered to the walls of the cellulose dialysis sac. It is convenient to use for dialysis a thistle funnel secured with a cellulose membrane, and containing a glass slide; large crystals grow on the slide, which may be removed without damage to the fragile crystals.

Crystals of Type II-cytochrome b_2 have been obtained over the pH range 6–7.5, and with solutions containing up to 0.2 M-lactate instead of 0.05 M-lactate. On similar dialysis to low ionic strength, recrystallization is usually complete within 24 hr., but the crystals may be smaller than those shown in Plate 1(*g*). Recrystallization has also been effected by the slow addition of acetone under conditions similar to those used for the crystallization of Type I-cytochrome b_2 by acetone precipitation. Another form of Type II-cytochrome b_2 commonly obtained by dialysis at low ionic strength is shown in Plate 1(*h*).

Type II-ferricytochrome b_2

Crystallization at low ionic strength was induced after treatment of a solution of Type II-ferrocytochrome b_2 essentially as described above for the preparation and crystallization of Type I-ferriocytochrome b_2 .

Enzymically-inactive crystalline flavohaemoprotein derivatives of cytochrome b_2

A solution of twice-crystallized Type I-cytochrome b_2 containing approx. 120 μ M-haem was dialysed for 48 hr. at 2° against a solution of 0.05 M-sodium lactate–1 mM-magnesium sulphate–0.5 M-cupric sulphate–0.5 mM-ferrous sulphate, pH 6.8. The crystals so obtained were regular in shape and appeared similar to those of Type I-cytochrome b_2 that had been obtained by dialysis to low ionic strength (see Plate 1*a*). However, the solution obtained by dissolving the washed crystals in 0.5 M-sodium lactate had negligible lactate-dehydrogenase activity with ferricyanide. Under anaerobic

conditions, the solutions showed a strong flavin fluorescence, but the haem was reduced. On exposure of the solution to air, the fluorescence increased and the solution became orange; subsequently, the haem group became oxidized. When the concentrations of cupric sulphate and ferrous sulphate were doubled, poor crystals of partly oxidized Type I-cytochrome b_2 were obtained. A small yield of enzymically inactive crystals was also obtained by acetone precipitation of protein that had previously been exposed to pH 4.0 for 45 min. at 0° and subsequently to pH 8.0 with triethanolamine. The crystals were brown, and were partly oxidized, but contained both flavin and haem groups.

DISCUSSION

One purpose of the present studies was to prepare cytochrome b_2 as single crystals suitable for X-ray crystallography, since the crystals obtained by Appleby & Morton (1954, 1959*a*) were unsuitable. The results show that cytochrome b_2 may be obtained as crystals, both in the reduced and oxidized states, and with and without the specific DNA component of the enzyme. Crystallization by salting-out procedures has not been extensively investigated, and possibly larger and more perfect crystals than those shown in Plate 1 (*f*) could be obtained by precipitation with ammonium sulphate under suitable conditions.

The changes in the procedure of Appleby & Morton (1954, 1959*a*) for the isolation of crystalline Type I-ferrocycytochrome b_2 were developed to facilitate large-scale preparation of the enzyme. The advantages of adding magnesium sulphate (see above) are understandable since crystalline Type I-ferrocycytochrome b_2 was found by Appleby & Morton (1959*b*) to contain approx. 1 g. atom of magnesium/mole of haem. Unspecific associations of the magnesium with the DNA component of the enzyme was suggested by Appleby & Morton (1959*b*), but this hypothesis is unlikely since there are 15 nucleotide residues/haem group (Appleby & Morton, 1960). The magnesium atom may be involved in the binding of riboflavin phosphate via co-ordination with the phosphate oxygen atoms and tyrosine or other residues of a polypeptide chain of the enzyme.

The crystalline form of the enzyme is more influenced by the presence or absence of the DNA component than by the relative state of oxidation or reduction. Within the range pH 6–7, there was apparently little influence of pH on the crystalline form of either Type I- or Type II-cycytochrome b_2 . Type I-cycytochrome b_2 , both when reduced and when oxidized, forms small tetragonal crystals at low ionic strength and when precipitated with acetone from its dilute solutions in 0.1 M-sodium lactate.

The octagonal prisms with pyramids at both ends (Plate 1*d*) are an unusual form of Type I-ferrocycytochrome b_2 and may be suitable for single-crystal X-ray studies.

Under comparable conditions, crystals of Type II-ferrocycytochrome b_2 form more slowly than those of Type I-ferrocycytochrome b_2 . Having once been crystallized, Type II-cycytochrome b_2 may be readily recrystallized. When obtained by dialysis to low ionic strength, the crystals of Type II-cycytochrome b_2 always belong to the hexagonal system and are always large; individual crystals up to 0.5 mm. across are frequently obtained, and these may be suitable for single-crystal X-ray studies.

Crystals of the enzyme obtained by acetone precipitation at low ionic strength, and by salting-out with ammonium sulphate, have essentially the same lactate-dehydrogenase activities with a variety of acceptors; this supports the extensive physicochemical and other evidence for the homogeneity of cytochrome b_2 as prepared by Appleby & Morton's (1954, 1959*a*) procedure. Crystallization is used as a routine method in this Laboratory to separate enzymically active from modified or inactive protein. However, inactive flavohaemoprotein crystals may be obtained from solutions containing relatively high concentrations of heavy-metal ions. It may thus be possible to prepare crystals with a heavy metal attached and in a crystalline form suitable for X-ray studies by the principle of isomorphous replacement.

Crystallization of ferrocycytochrome b_2 and of ferri-cycytochrome b_2 as described above provides a convenient method for obtaining the enzyme in high concentration for chemical, spectrophotometric and kinetic studies; the crystalline Type I-ferri-cycytochrome, stabilized with EDTA and GSH, is particularly suitable. Hasegawa & Ogura (1961) estimated that the E'_0 of a preparation of cytochrome b_2 (which was probably free of the DNA component) was +0.12 v between pH 6 and 8 at 25°. Reduced cysteine (E'_0 -0.340 v at pH 7; Burton, 1961) and GSH (E'_0 -0.340 v at pH 7; Burton, 1961) do not reduce the haematin group of the Type I-ferri-cycytochrome, but GSH may stabilize the enzyme by maintaining in the reduced form the essential thiol groups ((Morton, Armstrong & Appleby, 1961; Armstrong *et al.* 1961, 1963), thereby preventing dissociation of the flavin prosthetic group. Sodium borohydride and some other reducing agents in high concentration appear to modify the haematin group of Type I-ferri-cycytochrome b_2 . The partial reduction of the haematin group of Type I-ferri-cycytochrome b_2 by ascorbic acid (E'_0 +0.166 v at pH 4; Burton, 1961) in high concentration is consistent with Hasegawa & Ogura's (1961) estimate of the E'_0 of this group (E'_0 +0.12 v) in their material.

Type I-cytochrome b_2 has associated with it, as an integral part of the enzyme, a specific DNA component. Although initially isolated from air-dried yeast, in which autolysis may possibly occur, Type I-cytochrome b_2 has now been isolated from a butanol-acetone-dried powder (Morton, 1955) under conditions that keep autolysis to a minimum. By a modification of Appleby & Morton's (1954, 1959a) procedure, Rippa (1961) isolated crystalline Type I-ferrocycytochrome b_2 from fresh baker's yeast which was disrupted by blending with glass beads. Air-drying of yeast is therefore not essential for obtaining crystalline enzyme containing the DNA component. The DNA component is present in constant proportion in Type I-cytochrome b_2 crystallized from a number of strains of yeast used in Australia and in other countries. It is therefore improbable that the DNA component is an artifact arising during purification and crystallization. Crystallization of Type II-cytochrome b_2 has facilitated studies of the specificity of the association of the DNA component with the enzyme (R. K. Morton & K. Shepley, unpublished work). The evidence suggests that Type I-cytochrome b_2 , which contains the DNA component, is the naturally occurring form of the enzyme.

SUMMARY

1. A modified procedure is described for obtaining crystalline Type I-ferrocycytochrome b_2 from air-dried baker's yeast. The addition of magnesium sulphate facilitates crystallization, and EDTA in high concentrations prevents it.

2. A procedure for obtaining crystalline Type I-ferrocycytochrome b_2 from a butan-1-ol-acetone-dried powder of yeast is described.

3. After isolation in crystalline form, Type I-ferrocycytochrome b_2 is readily recrystallized either at low ionic strength or by acetone precipitation. According to the conditions of acetone precipitation, a number of crystalline forms of Type I-ferrocycytochrome b_2 were obtained.

4. A procedure is described for obtaining crystalline Type I-ferrocycytochrome b_2 . The addition of GSH does not reduce the haematin group but the haematin group is partly reduced by a large excess of ascorbic acid.

5. After removal of the DNA component by slow fractionation of the protein with ammonium sulphate, Type II-ferrocycytochrome b_2 and Type II-

ferrocycytochrome b_2 may be isolated in crystalline form at low ionic strength. Crystals of Type II-ferrocycytochrome b_2 up to 0.5 mm. across may be obtained. The crystalline form differs considerably from that of Type I-ferrocycytochrome b_2 . Less perfect crystals of Type II-ferrocycytochrome b_2 are obtained by slow precipitation with ammonium sulphate or with acetone.

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