10. The implications of these observations are discussed.

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Lactic Dehydrogenase and Cytochrome b_2 of Baker's Yeast

PURIFICATION AND CRYSTALLIZATION

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Meyerhof (1919) first showed that yeast could oxidize lactic acid in the absence of any added coenzyme. Bernheim (1928) later extracted acetone-dried baker's yeast with a phosphate buffer and, after dialysis, obtained a turbid solution which reduced methylene blue in the presence of lactate, glycolate and α-hydroxybutyrate. No added coenzyme was required. Reduction of both methylene blue and heart-muscle cytochrome *c* by a preparation from plasmolysed Delft yeast in the presence of lactate was shown by Ogston & Green (1935). Boyland & Boyland (1934) and Adler & Michaelis (1935) both showed that there was no activation of the yeast enzyme by added diphosphopyridine nucleotide or triphosphopyridine nucleotide.

Dixon and co-workers (Dixon & Zerfas, 1939; Bach, Dixon & Zerfas, 1942) extensively purified the enzyme from autolysates of air-dried baker's yeast. Added methylene blue and heart-muscle cytochrome *c*, and yeast cytochrome *c* present in the extract, were reduced with lactate as substrate. The rate of enzymic reduction of methylene blue was proportional to the concentration of a new protein haemochromogen which these workers called cytochrome b_2 (Bach, Dixon & Keilin, 1942). Bach, Dixon & Zerfas (1946) therefore concluded that 'cytochrome b_2 forms an essential part of the enzyme system, either as the dehydrogenase itself or as an essential intermediate carrier between lactate and methylene blue'.

This paper describes the purification and crystallization of yeast lactic dehydrogenase. This work, together with unpublished studies of the enzymic, chemical and physical properties of the enzyme, was carried out to determine the relationship between the dehydrogenase activity and cytochrome b_2. The results were described at the meetings of the Australian and New Zealand Association for the Advancement of Science in Canberra, 1953, and in Melbourne, 1955, and preliminary accounts have been published (Appleby & Morton, 1954; Morton, 1955*a*).

MATERIALS

DL-*Lactate*. A.R.-grade DL-lactic acid (British Drug Houses Ltd.) was diluted to 25% (w/v), the lactone hydrolysed by refluxing for 4 hr. and then adjusted to the required pH with 8·2N-NaOH and diluted to 2M-concentration.

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Organic solvents. Technical-grade solvents (Colonial Sugar Refining Co. Ltd.) were purified by distillation at atmospheric pressure, in each case collecting the fraction boiling at the temperature indicated: ethanol [undenatured, 95% (v/v) after refluxing over KOH], 76°; acetone (over K_2CO_3), 56°; butan-1-ol, 115–118°.

Cytochrome c. This was prepared from either horse heart or ox heart according to Keilin & Hartree (1952), except that the $(NH_4)_2SO_4$ was finally removed by exhaustive dialysis against 0·15% NaCl. A copious brown precipitate so obtained (see Henderson & Rawlinson, 1956) was removed by centrifuging.

Calcium phosphate gel. This was prepared as described by Keilin & Hartree (1938) and contained about 15 mg. dry wt./ml.

pH. All substrates and other materials were adjusted to the required pH with either N-HCl or N-NaOH as necessary. A glass electrode was used at room temperature (approx. 22°).

METHODS

Estimation of enzymic activity

The unit of activity is defined as the amount of enzyme which, with excess of DL-sodium lactate, catalyses reduction of 1 μmole of potassium ferricyanide (or of another specified hydrogen acceptor) in 1 hr. under defined conditions of pH, temperature and buffer composition. The specific activity is expressed as units/mg. of protein.

Reduction of cytochrome c. The initial rate of enzymic reduction at 20° was estimated spectrophotometrically at 549·5 mμ (see below). Cuvettes of 1 cm. light path were used in the constant-temperature holder of Dixon (1954). Assuming a molar extinction coefficient (ϵ) of $27{\cdot}7 \times 10^3$ for reduced heart-muscle cytochrome *c* at 550 mμ (Margoliash, 1954), $\epsilon_{(\text{reduced}-\text{oxidized})}$ was found to be $19{\cdot}8 \times 10^3$. After studies of the pH optimum and the finding of a protective effect of ethylenediaminetetra-acetate (EDTA), activities were estimated with a reaction mixture (3 ml.) containing 0·06 M-sodium phosphate buffer, pH 7·4, 0·05 M-DL-sodium lactate, mM-EDTA, pH 7·4, 0·05 mM-oxidized cytochrome *c*, and enzyme. Water replaced cytochrome *c* omitted from the reference cuvette and sodium lactate omitted from the control. During initial studies of enzyme purification, the following reaction mixture (3 ml.) was used: 0·066 M-sodium phosphate buffer, pH 7·0, 0·33 M-DL-sodium lactate, 0·02–0·03 mM-oxidized cytochrome *c* and enzyme. This reaction mixture is suboptimum in respect of cytochrome *c* concentration.

Reduction of potassium ferricyanide. This was measured spectrophotometrically at 420 mμ and at 20° by a similar procedure to that used for studies with cytochrome *c*. At 420 mμ, ϵ for potassium ferricyanide was found to be $1{\cdot}04 \times 10^3$, whereas potassium ferrocyanide had negligible absorption. The reaction mixture (3 ml.) contained 0·03 M sodium pyrophosphate–HCl buffer, pH 8·0, 0·33 M-DL-sodium lactate, 0·83 mM-potassium ferricyanide, mM-EDTA, pH 8·0, and enzyme. In the initial studies of enzyme purification the reaction mixture (3 ml.) contained the following: 0·03 M-sodium pyrophosphate–acetic acid buffer, pH 8·5, 0·33 M-DL-sodium lactate, 0·5 mM-potassium ferricyanide and enzyme.

Reduction of methylene blue. Activity was determined anaerobically with Thunberg tubes as described by Bach *et al.* (1946). The stock assay solution contained 40 ml. of 0·1 M-sodium acetate buffer, pH 5·2, 10 ml. of 0·9 M-DL-sodium lactate and 10 ml. of 0·5 mM-methylene blue. Enzyme (0·1–0·5 ml.), diluted to give a reduction time of 50–70 sec., was tipped from the hollow stopper into 3 ml. of this stock solution and the time for 90% reduction of dye at 20° was estimated visually. For the control, water replaced sodium lactate. With crystalline enzyme the optimum pH for dye reduction was found to be pH 6·8 and the activity was then determined with a reaction mixture (3 ml.) containing 0·05 M-sodium acetate–0·05 M-sodium phosphate–HCl buffer (pH 6·8), approx. 0·3 M-DL-sodium lactate, mM-EDTA, pH 6·8, 0·08 mM-methylene blue and enzyme.

Spectrophotometry and spectroscopy. A Beckman spectrophotometer, model DU, Serial no. 61753, was used throughout these studies. The wavelength scale was calibrated against the emission spectrum of a mercury-discharge lamp. Between 226·2 and 656·3 mμ the scale was within the tolerances claimed by the manufacturers (Beckman Bulletin no. 89), the greatest errors being at 576·96 mμ (instrument reading, 576·4 mμ) and at 491·6 mμ (instrument reading 492·0 mμ). At 546·07 mμ the reading was 546·2 mμ, but the α-absorption bands of reduced heart-muscle cytochrome *c* (550·0 mμ; Margoliash, 1954) and of reduced pyridine protohaemochromogen (557·0 mμ; Lemberg & Legge, 1949) appeared at 549·5 and 556·5mμ respectively. In this region of the spectrum therefore the instrument may have given readings approx. 0·5 mμ low. In view of the uncertainty, however, wavelengths are given as directly determined.

The extinctions of two solutions of acid potassium dichromate at the wavelengths of the minima and maxima (234·4 and 257·6 mμ; and 313·2 and 349·7 mμ) were found to be within 0·5% of the values given by Gridgeman (1951).

When only small volumes of solutions were available, plastic inserts (Martin & Morton, 1956) were used with cuvettes of 1 cm. light path to reduce the volume to 1·2 ml. All measurements other than under aerobic conditions were carried out with Thunberg-type assemblies sealed to quartz cuvettes (1 cm. light path).

Visual observations of absorption bands were made with a Sorby-Browning type microspectroscope and a Hartridge reversion spectroscope. They were calibrated with a solution of reduced heart-muscle cytochrome *c* (α-band, 550·0 mμ; Margoliash, 1954).

Dialysis. This was carried out in cellulose tubing, the contents of the tubing and the diffusate being frequently stirred.

Other determinations

Dry weight. Enzyme solutions were dialysed against water and then dried to constant weight *in vacuo* over P_2O_5 at room temperature. Otherwise, the protein was precipitated with 15% (w/v) trichloroacetic acid, and collected by centrifuging, and washed once with 5% (w/v) trichloroacetic acid and dried to constant weight *in vacuo* over P_2O_5 at room temperature.

Protein nitrogen. This was determined by the Kjeldahl method, the ammonium sulphate formed being estimated with Nessler reagent (see Morton, 1955*b*).

Protein. This was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with heart-muscle cytochrome *c* (0·34% iron) as the reference standard. Later comparison with crystalline cytochrome b_2 (Appleby & Morton, 1954) showed that 0·76 mg. of cytochrome *c* and

1·00 mg. of cytochrome b_2 gave the same colour, and the earlier determinations were adjusted accordingly to obtain the amount of cytochrome b_2 protein. Enzyme solutions were diluted with water before determination of protein since the lactate and pyrophosphate buffers, in which enzyme was usually diluted, inhibited colour development.

EXPERIMENTAL AND RESULTS

Preliminary studies

Stability of the enzyme in yeast extract. Baker's yeast obtained locally was air-dried at room temperature and ground to a fine powder in a porcelain ball mill. A 20 % (w/v) suspension of the powder in 0·07 M-Na_2HPO_4 was stirred for 30 min. and then centrifuged at about 3000 *g* for 30 min. The cloudy supernatant had a lactic dehydrogenase activity (with potassium ferricyanide) of about 300 units/ml. and a specific activity of about 15 units/mg. Most of the enzymic activity was extracted, whereas extracts from fresh or freeze-dried baker's yeast in 0·07 M-Na_2HPO_4 had little activity, even after autolysis in 0·07 M-Na_2HPO_4 at 38° for 18 hr., or after freezing at −15° and slow thawing on several occasions. A suspension of fresh yeast (10 %, w/v) in 0·25 M-sucrose containing 0·01 M-sodium phosphate buffer, pH 7·4, was shaken with an equal volume of Ballotini no. 12 glass beads in a Mickle oscillator at 50 cyc./sec. for 30 min. in a cold room at 2°. Whereas the extract from a suspension of air-dried yeast similarly treated had high activity after centrifuging at 15 000 *g* for 35 min. at 2°, the supernatant from the fresh yeast had no activity.

Over the range pH 5·1–7·0 there was no loss of activity, 30 % loss at pH 4·7 and about 50 % loss at pH 9·1 when the extracts from air-dried yeast were maintained at these pH values for several minutes at 10°. There was no purification by these procedures. An extract was adjusted to pH 5·1, and centrifuged at 3000 *g* for 30 min. at room temperature. Less than 25 % of the initial activity remained in the supernatant, showing that most of the enzyme was associated with material insoluble in 0·07 M-sodium phosphate buffer at pH 5·1.

Dialysis of the extract against water at 0° for about 12 hr. under aerobic conditions caused about 10 % loss of activity. Much greater loss is found with purified enzyme.

Purification with calcium phosphate gel. The protection by lactate against heat-denaturation of the enzyme (Bach *et al.* 1946) was confirmed. Subsequently considerable purification was obtained as follows. A 20 % (w/v) yeast powder in 0·1 M-sodium lactate at pH 5·8 was heated to 53° for 7 min. and centrifuged at 2500 *g* for 1 hr. at about 20°. The cloudy extract (300 units/ml.; 28 units/mg.) was brought to 0·7 saturation with $(NH_4)_2SO_4$ at pH 7·4 and centrifuged at 20 000 *g* for 30 min. at about 20°. The supernatant contained much cytochrome *c* but no lactic dehydrogenase activity and was discarded. The precipitate was dissolved in 0·02 M-sodium lactate at pH 6·3, and dialysed against a large volume of the same solution and centrifuged at about 2500 *g* for 30 min. at about 20°. The precipitate was discarded. The supernatant (1250 units/ml.; 40 units/mg.) contained some cytochrome *c* and b_2. Calcium phosphate gel was added to 12 % (v/v), and after 20 min. the material was centrifuged at about 1500 *g* for 10 min. The precipitate contained much cytochrome *c* but no lactic dehydrogenase activity. Calcium phosphate gel was added to the supernatant to 25 % (v/v), and the precipitate collected as before. The yellow supernatant contained no cytochromes or lactic dehydrogenase activity and was discarded. The pink precipitate was washed by suspending and resedimenting, first in water and then three times in 0·1 M-sodium phosphate, pH 6·8, containing 0·05 M-sodium lactate. The washings, which contained no cytochromes, were discarded and the precipitate was treated as previously with a solution at pH 7·2 containing 0·1 M-sodium phosphate, 0·05 M-sodium lactate and 10 % (w/v) $(NH_4)_2SO_4$. The first pink supernatant (1820 units/ml.; 545 units/mg.) contained no detectable cytochrome *c* but showed strong absorption bands of reduced cytochrome b_2. A second eluate was less active. About 18 % of the activity of the yeast extract (40×10^4 units) was recovered in the eluates.

Fractionation with organic solvents. Ethanol fractionation of extracts of yeast powder in sodium phosphate buffer at about −5° gave some purification but the fractions, dissolved in phosphate buffer, rapidly lost activity. When extracts in sodium lactate solution were similarly fractionated with either ethanol or acetone, and the precipitates were dissolved in lactate, substantial purification of the enzyme was achieved with good recovery of activity. By treatment of the dried yeast with butan-1-ol considerable amounts of lipid were extracted from the yeast, and the suspensions of the butanol-extracted yeast in 0·2 M-sodium lactate were readily clarified by centrifuging at about 3000 *g* for 30 min. The extracts so obtained had improved specific activity as compared with the yeast not treated with butanol. Moreover, the subsequent fractionation with acetone was considerably facilitated and gave products of very high specific activity.

Purification of lactic dehydrogenase by use of organic solvents

From the experiments described above, and after extensive further studies (Appleby, 1957), the following purification procedure was developed.

Dried yeast powder (2 kg.) was stirred for 1 hr. at room temp. (about 18°) in 7 l. of butan-1-ol. The smooth suspension was centrifuged at about 1500 *g* for 15 min. at room temp. in a centrifuge of capacity 3·6 l. The butanol-saturated yeast was thoroughly dispersed in 10 l. of a solution at pH 5·1 containing 0·1 M-sodium lactate and 0·02 mM-EDTA by vigorous mechanical stirring, forming a uniform suspension at pH 6·5. Then 1 l. of butan-1-ol was added to ensure saturation with butanol. The suspension was stirred mechanically for 2 hr. at room temperature and then centrifuged at approx. 2500 *g* for 30 min. as before, or alternatively filtered through a thin layer of Hyflo Supercel overlying filter paper (Whatman no. 1) on a large Büchner funnel. After centrifuging, the yellow butanol supernatant, which contained considerable amounts of phospholipid material, and the clear yellow–brown aqueous layer were separately collected. The emulsified material at the butanol–water interface and the compact viscous yeast precipitate were discarded.

The combined aqueous extracts (about 6 l.) were cooled to −2°. Acetone at −15° was added slowly with continuous stirring (see Askonas, 1951) to 18–20% (v/v). The material was maintained at −6° for about 10 min. The flocculent precipitate was allowed to settle out for about 1 hr., during which time the temperature of the material rose to about −4°. The clear red–brown supernatant was then decanted from the brown precipitate. The supernatant (at −6°) was brought to 27–30% (v/v) with acetone at −15°, and then held at −8° for 10 min. Stirring was stopped and a red precipitate was allowed to settle out for 30 min. The precipitate adhered to the stainless-steel container and the yellow supernatant, which contained a large amount of flavoprotein, was completely decanted and discarded.

The red precipitate was dispersed in about 300 ml. of a solution at pH 6·8 and at −4° containing 0·12 M-sodium lactate, 0·02 mM-EDTA and acetone (25%, v/v). The turbid suspension was maintained between −6° and −4° for about 30 min. and then was centrifuged at about 1600 *g* for 30 min. at −4°. The clear red supernatant was poured from the grey precipitate. For adequate extraction of the enzyme thorough dispersal of the precipitate is essential and may be facilitated with a Potter–Elvehjem-type homogenizer. If all of the enzyme is not extracted, a pink precipitate is obtained after centrifuging, in which case re-extraction is necessary.

The red supernatant was held at −4° and acetone (about 5–10 ml.) at −10° cautiously added until a slight turbidity appeared (at about 27%, v/v, of acetone). The grey precipitate was removed by centrifuging at about 1500 *g* at −4° for 15 min. and discarded. Upon further addition of acetone (usually 15–30 ml., to bring the concentration to about 30%, v/v, of acetone) to the red supernatant at −4°, there was a well-defined precipitation of red protein. At −4° this active precipitate has a characteristic appearance, resembling a red oil. It was collected by centrifuging at −4° as before, and the yellow supernatant was discarded.

The precipitate was dissolved at 0° in a solution at pH 6·8 containing 0·03 M-sodium pyrophosphate, 0·1 M-sodium lactate and 0·02 mM-EDTA. The viscous deep-red solution (40–80 ml.) so obtained was centrifuged at about 25 000 *g* for 15 min. at 0°, and the grey insoluble material discarded.

At this stage, the solution usually had a specific activity of about 600 units/mg. (see Table 1; stage 2*c*) and showed intense absorption bands of reduced cytochromes (b_2 and *c*). Separation of the two cytochromes and considerable further purification of the lactic dehydrogenase activity was achieved by crystallization of cytochrome b_2 as described below,

Crystallization of cytochrome b_2

During all subsequent stages, as far as possible the enzyme solutions were maintained under anaerobic conditions by displacement of air with oxygen-free nitrogen, since it was found that prolonged dialysis under aerobic conditions caused considerable loss of activity. The enzyme solution obtained as above was dialysed anaerobically at 0° against 4 l. of a solution at pH 6·8 containing 0·05 M-sodium lactate and 0·01 mM-EDTA. The dialysis sac was frequently inverted to ensure proper mixing of the viscous contents. This is facilitated by inclusion of a glass marble in the sac. The dialysate was renewed about every 8 hr. After dialysis for 18–24 hr. crystals were observed in the dialysis sac. Dialysis was continued for a further 12–18 hr., after which the crystals were collected by centrifuging the dialysed material at about 2500 *g* for 15 min. at 0°. The red supernatant (containing cytochrome *c*) was removed and the coral-pink pellet of crystals of cytochrome b_2 was washed by resuspending in 0·05 M-sodium lactate–0·01 mM-EDTA, pH 6·8, at 0° and resedimenting as before. The crystals were then dissolved at 0° in the minimum amount (5–10 ml.) of 0·5 M-sodium lactate–0·1 mM-EDTA, pH 6·8. The solution was centrifuged at 25 000 *g* for 30 min. at 0°, and the water-clear coral-pink supernatant was stored anaerobically at −15°.

Recrystallization was carried out by redialysis of the enzyme solution for 4–12 hr. as already described. The crystals were collected and washed and then dissolved at 0° in 0·5 M-sodium lactate–0·1 mM-EDTA, pH 6·8. The supernatant obtained after centrifuging at 25 000 *g* for 30 min. at 0° was stored anaerobically at −15°. Under these conditions, activity is retained for several months.

A summary of the course of purification in a typical preparation as obtained in 1953 is given in Table 1. The overall purification of the initial extract was about 1300 times with a recovery of about 10% of the activity as crystalline enzyme with a specific activity (determined under sub-optimum conditions) of about 5820 units/mg. of protein.

It is preferable to proceed as described as rapidly as possible, without interruption to the process at any stage. Crystals are normally obtained between 24 and 30 hr. after commencement of a preparation. Over 50 batches of crystals have been prepared since this procedure was developed in 1953.

Table 1. *Summary of the purification and crystallization of lactic dehydrogenase (cytochrome* b_2)

Activities were determined with potassium ferricyanide at pH 8·5* as described under Methods. Protein was estimated from protein N × 6·25. Details of the purification procedures are given in the text.

Stage	Purification procedure	Volume† (ml.)	Protein (mg./ml.)	Lactic dehydrogenase activity: Units/ml.	Units/mg.	Total units ($\times 10^{-3}$)	% of original extract
1 (*a*)	Lactate extract of butanol-treated yeast	3150	14·9	690‡	46	2180	100
(*b*)	Ppt. with 20% (v/v) acetone at −6°: (discarded)	140	55	6 180‡	112	860	40
(*c*)	Ppt. with 30% (v/v) acetone at −6°: Resuspended in 300 ml. of 0·12M-sodium lactate (pH 6·8)–25% (v/v) acetone at −4°, and centrifuged	330	§	§	—	—	—
2 (*a*)	Ppt. from stage 1*c*: (discarded)	32	18·4	9 450	514	302	14
(*b*)	Ppt. from the supernatant from stage 1*c* brought to 27% (v/v) acetone: (discarded)	9	21	4 220	201	38	2
(*c*)	Ppt. from supernatant stage 2*b* brought to 33% acetone	40	40	24 000	600	960	44
3 (*a*)	Crystals by dialysis of ppt. from stage 2*c*	11·5	3·4	19 800	5820	228	10
(*b*)	Mother liquor from stage 3*a*: (discarded)	75	13·1	1 390	106	104	5

* These conditions of assay were suboptimum, as discussed in the text.
† Other than for the initial extract, these represent volumes of material dissolved in 0·2M-sodium lactate.
‡ These activities are not corrected for non-enzymic reduction of potassium ferricyanide, and may be about 40% higher than the true enzymic activities.
§ Not determined.

Fig. 1 shows a photomicrograph of the crystals, as obtained after rapid recrystallization. As viewed with a light microscope, the crystals appear as four-sided plates (when flat) and as narrow needles or rectangles (when edge-on). Examination under polarized monochromatic light (589·6 mμ) showed an axis of asymmetry and it is probable that the crystals belong to a tetragonal system, being formed as shallow bi-pyramids.

Spectroscopic observations during purification

During the purification of the enzyme with calcium phosphate gel as already described, lactate solutions of enzyme preparations of specific activities of about 250 units/mg. and higher all showed absorption bands at 556 and 527 mμ. These are the positions of the α- and β-bands respectively of reduced cytochrome b_2 as described by Bach *et al.* (1946). The most active preparation obtained by this method contained 545 units/mg. and in sodium lactate showed maxima at 556, 527 and 423 mμ, with extinction values of 0·146, 0·100 and 0·788. Comparison with crystalline cytochrome b_2 (Appleby & Morton, 1954, and in preparation) shows that this preparation was substantially free of other haem pigments, such as cytochrome *c*.

The red enzymically-active precipitates obtained with organic solvents all showed the absorption bands of reduced cytochrome b_2 and cytochrome *c*. Much of the cytochrome *c* extracted from the yeast by the sodium lactate was removed with the bulky precipitate obtained with 20% (v/v) acetone (Table 1, stage 1*b*). The cytochrome *c* which remained in association with the cytochrome b_2 at stage 2*c* (Table 1) stayed in the mother liquor when the cytochrome b_2 crystallized. Recrystallization removed all detectable traces of cytochrome *c* from the cytochrome b_2.

As pointed out by Bach *et al.* (1946), intensity of the cytochrome b_2 absorption bands is useful as an indication of activity during purification of the lactic dehydrogenase.

Activities with potassium ferricyanide, methylene blue and cytochrome c

The activity with each hydrogen acceptor was measured during the preliminary studies and at several stages of the purification with organic solvents. The relative activities with potassium ferricyanide, methylene blue and heart-muscle cytochrome *c* remained substantially the same throughout and, as shown in Table 2, was very similar for an extract of yeast powder in 0·15M-NaCl and for the crystalline protein.

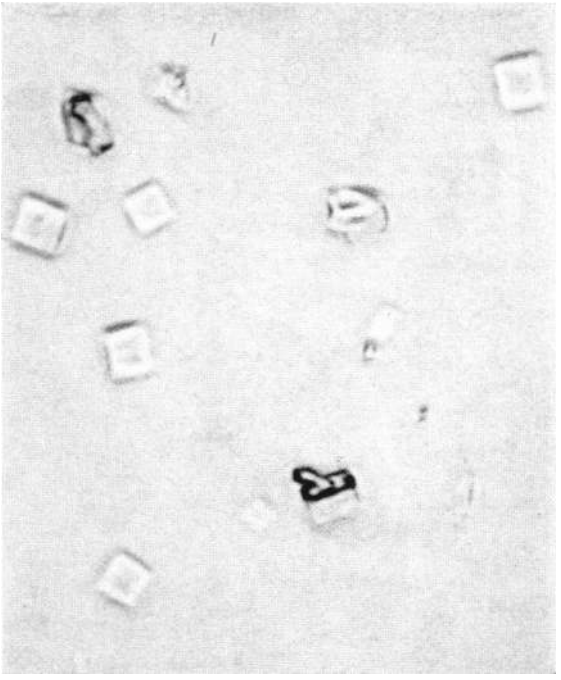

Fig. 1. Crystals of yeast lactic dehydrogenase (cytochrome b_2). Photograph with direct illumination. Recrystallized material. As shown, ×750.

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Table 2. *Activities of a yeast extract and of crystalline lactic dehydrogenase with different hydrogen acceptors*

A suspension (20%, w/v) of dried yeast was extracted with 0·15 M-NaCl at 38° for 1 hr. and centrifuged at 100 000 *g* for 1 hr. at about 5°. The activities of the clear supernatant (2·6 mg. of protein N/ml.) were determined at room temperature (18–20°) as described under Methods except that the sodium diethyl barbiturate–sodium acetate–HCl buffer of Michaelis (1931) was used with each acceptor. Activities of the crystalline enzyme were determined as described under Methods, in each case the assay system containing EDTA being used. Unless otherwise indicated, the pH values are optimum for each system used.

Hydrogen acceptor		Lactic dehydrogenase activities: Yeast extract				Crystalline enzyme		
	Concn. (mM)	pH	Units/ml.	Units/mg.	Relative activity	pH	Units/mg.	Relative activity
Potassium ferricyanide	0·5	8·4	580	36	100	8·0	7750	100
Heart-muscle cytochrome *c*	0·02	6·0	107	6·6	18	7·0*	1730	22
	0·05	—	—	—	—	7·4	5870	76
Methylene blue	0·08	5·2	48	2·9	8 (16)†	5·2*	1030	13 (26)†
		—	—	—	—	6·8	2025	26 (52)†

* These pH values are suboptimum.
† Figures in parentheses represent the relative activity on the basis of the transfer of one hydrogen atom/mole.

Table 3. *Specific activity of yeast lactic dehydrogenase after recrystallization*

The enzyme was recrystallized on each occasion by dissolving the crystalline material in 0·3 M-sodium lactate, pH 6·8, and dialysing anaerobically against 0·05 M-sodium lactate, pH 6·8. Activities were determined with potassium ferricyanide with buffer at pH 8·0 as described under Methods. (EDTA was omitted from the dialysis fluid and the assay system.) Protein was estimated by the method of Lowry *et al.* (1951) as described under Methods.

No. of crystallizations	Volume (ml.)	Protein (mg./ml.)	10^{-4} × Lactic dehydrogenase activity: Units/ml.	Units/mg.	Total units	Recovery (%)
1*	51	6·5	4·41	0·68	225	100
2†	45	5·2	4·20	0·81	189	84
3†	28	7·9	5·45	0·69	153	68
4	—	7·25	4·72	0·65	—	—
5	—	10·5	5·30	0·50	—	—

* Initial material was pooled first crystals from a number of preparations.
† Approx. 1390 units/ml. (total 14×10^4 units) and 1080 units/ml. (total $9·3 \times 10^4$ units) were found in the mother liquors at the second and third crystallizations respectively.

The activity with potassium ferricyanide of well-washed crystals is substantially the same after recrystallization several times (Table 3). The crystalline enzyme is somewhat unstable. Maximum activity was determined by diluting 50 μl. of a suspension of crystalline material (in 0·05 M-sodium lactate containing 0·01 mM-EDTA, pH 6·8) into 5 ml. of de-aerated 0·5 M-sodium lactate containing 0·1 mM-EDTA, pH 6·8, and rapidly determining activity at 20° at pH 8·0 as described under Methods. A similar dilution of the original enzyme sample was made in water, and protein was estimated in this diluted material by the method of Lowry *et al.* (1951), as described in the Methods section. A specific activity of 7750 units/mg. of protein was obtained. The specific activities of different batches of enzyme (crystallized once) ranged from 5820 to 7750 units/mg. of protein. The variation partly reflects the experimental difficulties in handling the highly active enzyme preparations. A batch of twice-crystallized enzyme gave an activity of 6620 units/mg. dry wt., as determined directly, and 6550 units/mg. of protein as estimated from protein N × 6·25 (see under Methods).

Instability of the enzyme is particularly apparent after repeated crystallization (see Table 3). The crystalline enzyme is rapidly inactivated by oxygen. A solution of oxidized enzyme was prepared by dissolving the reduced crystalline material in 0·5 M-NaCl containing 0·1 mM-EDTA, pH 6·8, and exposing to air for a few minutes. The pink solution changed to brown, indicating oxidation of the cytochrome. Oxidized solutions so obtained rapidly lost activity, even if retained at −15°. This loss was greater in the absence of EDTA.

DISCUSSION

With purification procedures based on those of Bach *et al.* (1946) it was confirmed that the lactic dehydrogenase activity and cytochrome b_2 concentration of yeast extracts were closely related

(Bach *et al.* 1942, 1946). Boeri, Cutolo, Luzzati & Tosi (1955) have reported similar findings. This relationship could have arisen from a failure to separate two distinct proteins. Therefore an entirely different purification procedure was developed. This led to the crystallization of cytochrome b_2 (Appleby & Morton, 1954).

The intracellular localization of lactic dehydrogenase in yeast cells is not known. Attempts to extract the enzyme from fresh, frozen and thawed or from freeze-dried yeast were unsuccessful. Nossal, Keech & Morton (1956) found that much of the lactic dehydrogenase activity of baker's yeast was sedimented between 5000 and 25 000 *g* with a particulate fraction from disrupted cells. This may indicate that the enzyme is associated with insoluble lipoprotein material, from which it is released by air-drying.

In principle, the purification method involves: (1) modification of cellular association of the enzyme by air-drying of yeast; (2) extraction of lipid material with butan-1-ol; (3) fractionation of a lactate extract of the yeast with acetone at low temperature; (4) crystallization at low ionic strength. The treatment of yeast and lactate extracts with butan-1-ol (see Morton, 1950, 1955*c*) is not essential to bring the enzyme into aqueous solution but it considerably facilitates subsequent purification. The acetone fractionation of the lactate extract is carried out at quite high ionic strength, whereas low ionic strength is usually found to be essential to prevent denaturation of enzymes during fractionation with organic solvents (see Askonas, 1951). The extraction of the enzyme from a precipitate obtained with acetone (27–30 %, v/v) into 25 % (v/v) acetone–lactate (Table 1, stage 2*a*) undoubtedly is a highly selective purification procedure. The solubility of the enzyme in 25 % (v/v) acetone probably depends on the higher concentration (0·12 M) of sodium lactate in the extracting solvent, since the sodium lactate concentration at the prior precipitation with acetone is about 0·07 M, owing to dilution of the lactate extract of the yeast with acetone.

The protection of the enzyme by its substrate during the fractionation appears to be essential. Chelation of inhibitory metals (such as copper) by EDTA, which partially protects against the harmful effects of oxygen, is also valuable during purification. Boeri *et al.* (1955) have also used EDTA in purification and assay procedures for this enzyme.

The unusual crystallization procedure reflects the relative insolubility of the material at low ionic strength and at acid pH values. In 0·25 M-sodium lactate, the enzyme is slightly soluble (4 mg./ml.) at pH 6·8 but quite insoluble at pH 5·0. Some poorly formed crystals have been obtained by dialysis at lower pH values than 6·8. However, such preparations usually contain much amorphous material and have relatively low activity. Prolonged dialysis of the crystalline enzyme also may cause formation of amorphous material and a corresponding fall in the specific activity of the enzyme. There is about a tenfold increase of specific activity on crystallization (Table 1) and this remains relatively constant during several recrystallizations (Table 3).

When tested at the appropriate pH optima, the relative rates of reduction of potassium ferricyanide, heart-muscle cytochrome *c* and methylene blue remained relatively constant during purification of the lactic dehydrogenase activity (Table 2, and Appleby & Morton, 1954). In the earlier work of Bach *et al.* (1946) it was found that the relative rate of reduction of cytochrome *c* as compared with that of methylene blue was considerably less in more purified as compared with crude preparations of the enzyme. These findings were probably due to the use of the same pH value for estimating the activity with cytochrome *c* and with methylene blue. The optimum pH values for reduction of these hydrogen acceptors differ considerably and change somewhat during purification (Table 2, and Appleby & Morton, 1954). Moreover, the activity with heart-muscle cytochrome *c* is markedly dependent on the concentration of cytochrome *c* (Table 2). The activity of the crystalline enzyme with 0·05 mM-cytochrome *c* is 5870 units/mg. of protein (Table 2). At saturation with cytochrome *c* and at the optimum pH with this acceptor therefore the activity would be expected to be similar to that obtained at the optimum pH and at saturation with potassium ferricyanide (7750 units/mg. of protein, Table 2). The activity previously reported by Appleby & Morton (1954) (1800 units/mg. of protein) was obtained at low concentration (0·02 mM) of cytochrome *c*, and in the absence of EDTA. The enzyme purified from yeast by Boeri *et al.* (1955) by adsorption and salting-out procedures (see Bach *et al.* 1946; Dixon, 1955) also reacts with the three hydrogen acceptors. However, from the results obtained by Boeri *et al.* (1955) and by Boeri & Tosi (1956), it appears that the best activities obtained by these workers were 3650, 3660 and 580 units/mg. of protein, as compared with 7750, 5870 and 2025 units/mg. of protein for the crystalline cytochrome b_2, as estimated with potassium ferricyanide, heart-muscle cytochrome *c* and methylene blue respectively (Table 2, and Appleby & Morton, 1954).

No attempts were made to obtain crystals by salting-out procedures. The enzyme is stable to ammonium sulphate precipitation, but treatment at high ionic strength partially dissociates the deoxyribopolynucleotide (see Morton, 1955*a*, 1958; Appleby, 1957) which is integrally associated with

the crystalline enzyme. Yamashita *et al.* (1957) have obtained from yeast an enzymically inactive haemoprotein with an absorption spectrum resembling that of cytochrome b_2. This haemoprotein was obtained as extremely slender needle-like crystals by salting out with ammonium sulphate. The properties of the material of Yamashita *et al.* (1957) and of the enzyme preparation of Boeri *et al.* (1955) will be compared with the properties of the crystalline cytochrome b_2 of Appleby & Morton (1954, and this paper) in subsequent papers in this series. Morton (1958) has recently reviewed the properties of cytochrome b_2.

SUMMARY

1. Lactic dehydrogenase was partially purified from extracts of baker's yeast by selective heat-denaturation of proteins, salting-out and adsorption and elution from calcium phosphate gel. A close relationship between the enzymic activity and the concentration of cytochrome b_2 (Bach *et al.* 1946) was observed.

2. The cytochrome b_2 and lactic dehydrogenase activity were purified together by treatment of air-dried yeast with butan-1-ol, fractionation of butanol-saturated extracts of yeast in sodium lactate with acetone at low temperature and crystallization by anaerobic dialysis at 0° against 0·05 M-sodium lactate–0·01 mM-ethylenediaminetetra-acetate, at pH 6·8.

3. The coral-pink crystals of the flavohaemoprotein (Appleby & Morton, 1954) probably belong to a tetragonal system. The lactic dehydrogenase activities of the crystals, expressed as μmoles of hydrogen acceptor reduced/hr./mg. of protein at 20° were as follows: with 0·5 mM-potassium ferricyanide, 7750; with 0·05 mM-heart-muscle cytochrome *c*, 5870; with 0·08 mM-methylene blue, 2025. These activities are substantially greater than those described for yeast lactic dehydrogenase as purified by other workers.

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