activity of the extract was such that 75 % of added ethyl butyrate was hydrolyzed in 3·5 hr. incubation time.

SUMMARY

1. The synthesis of an ester of glycerol with hippuric acid from glycerol chlorohydrin and sodium hippurate is described.

2. Cleavage effected by the action of a glycerol extract of pancreatin activated by sodium taurocholate amounted to 32·6 % after 270 min. The cleavage by the same extract in the absence of sodium taurocholate was 27·2 %.

3. Cleavage of hippurylglyceride was not effected by liver esterase.

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Yeast Lactic Dehydrogenase and Cytochrome b_2

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The dehydrogenases fall into three distinct groups, according to the nature of the intermediate hydrogen acceptors through which they react with O_2 (cf. Green & Brosteaux, 1936):

Group I, dehydrogenases which depend on the presence of one or other of the nicotinic amide nucleotides (coenzymes I and II). Through the latter substances, which act as intermediate acceptors or hydrogen carriers, they react with certain flavoproteins and so with cytochrome *c* and O_2. They cannot, however, react directly with flavoproteins, cytochrome or O_2, or with artificial acceptors like methylene blue.

Group II, flavoproteins, which do not require the addition of coenzymes, the prosthetic flavin group itself acting as intermediate acceptor. In some cases (aerobic dehydrogenases) this group can react directly with O_2; in other cases (diaphorases) the mediation of the cytochrome system is necessary.

Group III, the 'cytochrome-reducing dehydrogenases', so-called because (at any rate in the unpurified state) they readily reduce cytochrome *c*, though this may not be a direct reaction. These enzymes have a number of properties in common which distinguish them from those of the other groups. They do not depend on soluble coenzymes and readily reduce methylene blue in their absence.

Several dehydrogenases of group I have been isolated in the pure state as crystalline proteins. They do not contain any coloured prosthetic group. Several enzymes of group II have been obtained in a state approximating to purity. None of the enzymes of group III, on the other hand, has previously been isolated or purified to any great extent, and consequently the nature of the active groups of these enzymes is unknown. The reason for this is that almost all the enzymes of this class are attached to the insoluble part of the cell-structure and until they can be detached a high degree of purification is hardly possible.

Among the chief enzymes of group III are the succinic dehydrogenase, the lactic dehydrogenase of yeast (that of animal tissues is a quite distinct enzyme belonging to group I), the insoluble glycerophosphate dehydrogenase of animal tissues (the soluble glycerophosphate enzyme is again a different enzyme of group I) and probably choline dehydrogenase. The yeast lactic enzyme is of special interest because it is freely soluble and therefore its purification and isolation may be attempted with some hope of success. We undertook this with the object of obtaining information on the chemical nature of the enzyme and especially of its prosthetic group, if any, and also of studying the manner in which the purified enzyme reacts with cytochrome and other hydrogen acceptors.

Lactic dehydrogenase was first extracted from yeast by Bernheim (1928), who treated Delft baker's yeast with acetone and extracted the dry residue with alkaline phosphate solutions. Several later workers have failed to obtain active solutions in this way; the reason for this we have found to be that the acetone yeast is usually acid enough to neutralize the alkaline phosphate. If the solution is kept alkaline, Bernheim's results can be repeated.

* Preliminary notes on certain parts of this work have been published in *Nature* by Dixon & Zerfas (1939), Bach, Dixon & Keilin (1942) and Bach, Dixon & Zerfas (1942).

Even under the best conditions, however, only a small part of the enzyme is extracted by this method, and the activity of the solutions is not great. Much better results are given by autolysis under certain conditions, and after purification and concentration we have obtained solutions some thousands of times as active as those described previously. The final degree of purification, calculated on a dry weight basis with respect to the original Lebedew juice, was some 100 times; the purification in relation to the original yeast cannot be definitely stated, as it is difficult to determine the proportion of the total enzyme extracted, but it was probably over 1000-fold.

At this stage the enzyme was still not pure, but unfortunately the work had to be terminated, owing to war conditions, and the impossibility of obtaining further supplies of Delft yeast. Various English yeasts were tried, but they behaved differently, and although the enzyme could be prepared from some of them, the method had to be altered considerably and the difficulties then became so great that work on a sufficiently large scale was impracticable. Moreover, a higher degree of purification than this will probably be very difficult to achieve, on account of the inherent instability of the enzyme which increases in direct proportion with the purity of the preparation. The purest fractions lost their activity completely on standing for 20 min. at room temperature in neutral solution, although the Lebedew juice could be kept for many days without loss of activity. This makes further stages of purification extremely difficult. The work is therefore being reported in its present stage.

MEASUREMENT OF ENZYME ACTIVITY

The pH curve of the enzyme shows an optimum at about pH 5·2 (Fig. 1), and as instability of the enzyme seems to be least in this region, this pH was chosen for the standard activity tests. The curve is not symmetrical, owing to destruction of the enzyme below pH 4·5.

The Thunberg technique as modified by Green & Dixon (1934) offers a rapid and accurate quantitative test of the activity of the enzyme. No coenzyme or carrier is needed and the test is very simple. A stock test solution is made up by mixing 50 ml. M/2000 methylene blue, 50 ml. 10% sodium lactate and 200 ml. acetate buffer, pH 5·2. Into each Thunberg tube 3 ml. of this solution is pipetted and the enzyme solution (usually 0·1 or 0·5 ml.) is placed in the hollow stopper. After the tube is evacuated, filled with O_2-free N_2 and re-evacuated, it is placed in the water-bath at 37°, and the contents mixed after allowing about 2 min. to reach the temperature of the bath. The reduction time is measured with a stop-watch from the instant of mixing. Duplicate results agreed to within 3%, and the reciprocal of the reduction time was found to be exactly proportional to the amount of enzyme present within wide limits. 0·5 ml. of our most active solutions gave a reduction time of about 1 sec., but for best results the amount of enzyme taken should be adjusted to give a reduction time between 10 sec. and 1 min. Except in the initial stages of purification, no reduction occurred in the absence of lactate. With the yeast enzyme, unlike the muscle enzyme, it is not necessary to add any fixative for the pyruvic acid formed (e.g. HCN), as the reaction velocity is unaffected thereby.

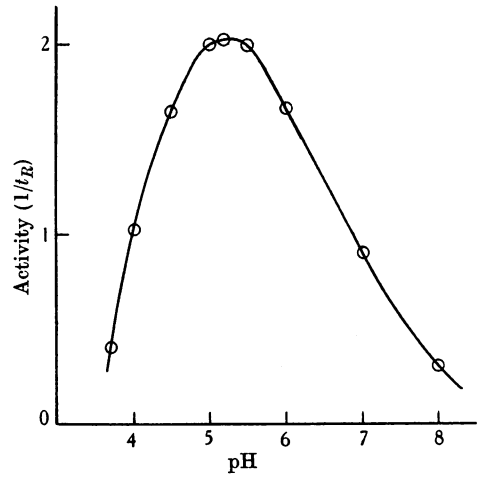


Fig. 1. pH-activity curve of enzyme.

Measurements may also be made manometrically by O_2 uptake, using methylene blue as carrier. Identical rates are obtained, as is to be expected, but the method is much less convenient.

Activity units. The reciprocal of the reduction time is a measure of the activity of a solution, i.e. of the enzyme concentration. In this paper t_R denotes the reduction time in sec. corresponding to 1 ml. of the enzyme solution. The purity is measured by the Q_{MB}, which denotes the amount of methylene blue (in μl. H_2) reduced per hr. and per mg. of protein, 1 mol. of the dye being taken as equivalent to 1 mol. of H_2.* Since the amount of methylene blue taken is equivalent to 5·6 μl. H_2, $Q_{MB} = \frac{60 \times 5{\cdot}6}{t_R \times w}$, where t_R is expressed in min. and w mg. of protein are taken. The protein was determined by precipitating with trichloroacetic acid, washing free from ammonium salts with dilute trichloroacetic acid and estimating N by Kjeldahl.

* In expressing methylene blue in μl. one may consider either the amount of H_2 required to reduce it or the amount of O_2 required to reoxidize it. As 1 mol. of the dye is equivalent to 1 mol. of H_2 or $\frac{1}{2}$ mol. of O_2 the results will differ by a factor of 2. The term Q_{MB} was first introduced by Dixon & Keilin (1936), who defined it with respect to O_2. It was later used by Corran, Dewan, Gordon & Green (1939*a*) as defined on the H_2 basis. This produced some confusion, especially as both papers referred to the same enzyme. In the present paper we shall use Q_{MB} as defined by Corran *et al.*, as we are dealing with an anaerobic dehydrogenase.

We define the *unit of enzyme* as the amount present in 1 mg. of a preparation with a Q value of 1. We suggest this as a rational and consistent unit which might with advantage be used for many other enzymes also. If generally adopted it would avoid the present multiplicity of arbitrary enzyme units bearing no relation to one another.

PURIFICATION OF THE ENZYME

Much preliminary work was done to determine the most promising procedures for purification, and a number of special properties of the enzyme were discovered of which good use could be made.

We were not successful in showing the presence of the enzyme in several brewer's yeasts which were tested, but large amounts could be extracted by

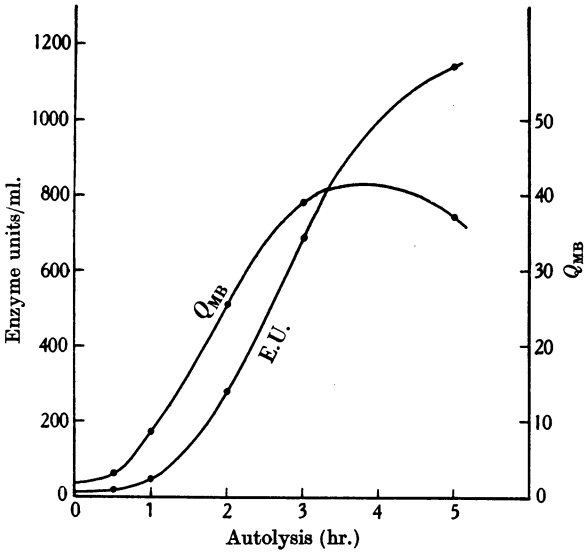


Fig. 2. Course of extraction of enzyme during autolysis of Delft yeast.

autolysis of dried Delft baker's yeast. The enzyme began to pass out of the cells at a very early stage of the autolysis and the Q_{MB} of the extracted material rose rapidly to a maximum (see Fig. 2), after which it fell, no doubt owing to the extraction of other proteins. At the time of maximum Q_{MB} the rate of extraction of enzyme also began to decrease; thus by choosing the time suitably a starting material of high specific activity and a good yield of enzyme could both be obtained. The resulting Lebedew juice could be kept for a few days in the cold without loss of activity. Much cytochrome *c* is also extracted, but this is strongly adsorbed by alumina, which does not adsorb the enzyme from the Lebedew juice.

The enzyme is comparatively easily destroyed by heating, but it was found that in the presence of its substrate it could be heated to a very much higher temperature without loss of activity. This made it possible to coagulate and remove a large amount of other protein while retaining practically the whole of the enzyme. The effect of lactate was no doubt specific to this enzyme, but analogous effects probably occur with other enzymes and could probably be made use of with advantage in their preparation.

The enzyme is well adsorbed by calcium phosphate gel at pH 5–6, but no trace can be eluted with slightly alkaline phosphate solutions. This led Bernheim to believe that the enzyme was completely destroyed by the adsorption, but we have found that this is not the case. By repeatedly eluting the gel with phosphate until no further protein is extracted, large quantities of material are removed, but the enzyme remains firmly fixed to the adsorbent. It can, however, be removed by eluting with phosphate solution containing ammonium sulphate. This procedure is particularly useful in the purification.

The solution, concentrated and examined spectroscopically, showed the cytochrome *c* absorption band, but on adding lactate a new band at 5565 A. appeared. Thereafter the strength of this band in the presence of lactate ran parallel with the enzyme activity, and by spectroscopic observations the subsequent fractionations with ammonium sulphate could be carried out rapidly. The cytochrome *c* could be removed by ammonium sulphate fractionations in acid solution.

The method of purification finally worked out was based upon these properties. A number of other procedures, such as fractionation of the Lebedew juice with acetone, with lead acetate, or with ammonium sulphate seemed promising at first, but were later abandoned because they gave very unstable enzyme solutions.

Method of purification from Delft yeast. The fresh yeast cake was first uniformly broken up by pressing through an ordinary potato-masher, and was then air-dried at room temperature in batches of 1 cwt.* For each autolysis 3 kg. of dried yeast (which retained its activity for many weeks) were dispersed in 6 l. warm tap water and kept at 37° for 4½ hr. (cf. Fig. 2). 4·5 l. of cold tap water were then added, and the mixture stirred and allowed to stand 15 min. at room temperature, after which it was centrifuged for 20 min. at 3000 r.p.m. The supernatant Lebedew juice was sucked off (6250 ml., t_R 14 sec., Q_{MB} 40). The Q_{MB} at this stage varied from

* We wish to express our thanks to the Marmite Food Extract Co. Ltd. and especially to their Chief Chemist, Mr A. E. Andrews, for testing a number of different methods of drying to determine the most suitable conditions, and for carrying out the drying of the main bulk of the yeast for us.

30 to 60 in different batches. The opaque juice was coloured reddish brown, owing to the presence of cytochrome *c*, flavoproteins and yellow pigments other than flavin compounds.

To 6250 ml. Lebedew juice about 1 l. of alumina C_γ suspension (15 mg. dry weight/ml.) was added in portions, until about 15 % of the enzyme had been adsorbed, the pH being kept at 5–6. The alumina, which was centrifuged off and discarded, was coloured deep red by transmitted light; the solution (7 l., t_R 17 sec.) was pure yellow and showed no absorption band of cytochrome *c*.

To 7 l. of solution, 140 ml. 50 % sodium lactate were added, and the mixture was heated rapidly (in 500 ml. portions) to 53° and held at this temperature for 7 min. A bulky precipitate of coagulated protein was centrifuged off. (Without lactate the enzyme was destroyed by heating to 40°, and the amount of protein coagulated at this temperature was not large.)

(t_R 1·25 sec.). Some loss of activity occurred during this process, which required about 5 hr. Instability of the enzyme began to be noticeable at this point and thereafter increased roughly in proportion with the Q_{MB}.

The solution, which was now coloured pink, mainly owing to the concentration of small amounts of cytochrome *c* which had escaped the initial alumina adsorption, was next fractionated with ammonium sulphate as follows. The solution (A) was placed in a centrifuge tube, 25·24 g. solid ammonium sulphate (A.R.) stirred in, the precipitate (B) centrifuged down, the supernatant fluid poured off into another centrifuge tube, 5·8 g. ammonium sulphate added, and so on, as shown in Table 1. The various precipitates B–G were dissolved in water and made up to the volumes shown in col. 3. The final supernatant fluid, which was discarded, still contained much protein. Fraction E was the purest and also gave the most active solution (t_R 0·6 sec.).

Table 1. 130 *ml. of solution A, fractionation at* pH 7·2

Amm. sulph. added (g.)	Fraction	Ppt. made up to (ml.)	t_R (sec.)	Units/ml.	Total units	Q_{MB}	
25·24	B	7·5	6·1	3,300	24,800	—	discarded
+5·8	C	5	2·7	7,500	37,500	—	discarded
+5·2	D	20	1·5	13,500	269,000	1730	
+6·3	E	15	0·6	33,500	504,000	1750	mixed (=H)
+7·0	F	22	0·9	22,500	493,000	1330	
+9·6	G	3·5	3·0	6,700	23,500	—	discarded

Supernatant solution inactive.

To the resulting solution (7 l., t_R 19 sec.) was added 3·5 l. Ca phosphate gel suspension (40 mg. dry wt./ml.). After centrifuging, the supernatant solution contained only a small amount of unadsorbed enzyme (t_R 390 sec.), but much protein. The Ca phosphate was eluted five times in succession, each time with 2·5–3 l. M/15-phosphate buffer, pH 7·2. The eluates were quite inactive, but contained much protein and practically all the yellow colour. The enzyme was then eluted from the Ca phosphate by three successive extractions with pH 7·2 phosphate buffer containing 10 % ammonium sulphate, 400, 1200 and 1200 ml. respectively being added. The t_R of the three eluates were 9, 8 and 24 sec. respectively. The three eluates, which were water-clear, were mixed (2800 ml.; t_R 16 sec.) and kept cold overnight without loss of activity.

The enzyme solution was then concentrated on a battery of Bechhold ultrafilters fitted with an automatic feed and with brushes lightly sweeping the surface in order to increase the rate of filtration. '3 %' Schleicher collodian ultrafilter membranes were used and 100 ml. of solution could be filtered in an hour by each ultrafilter. In this way 2800 ml. (t_R 16 sec.) were concentrated to 130 ml.

In another experiment the t_R was 0·3 sec., this being the most active solution obtained in this work.

Fractions D, E and F were mixed, giving a clear red solution (H) showing a strong cytochrome *c* spectrum (Fig. 3*a*). On the addition of a small amount of lactate, however, a new band at 5565 A. instantly appeared, the spectrum then being as

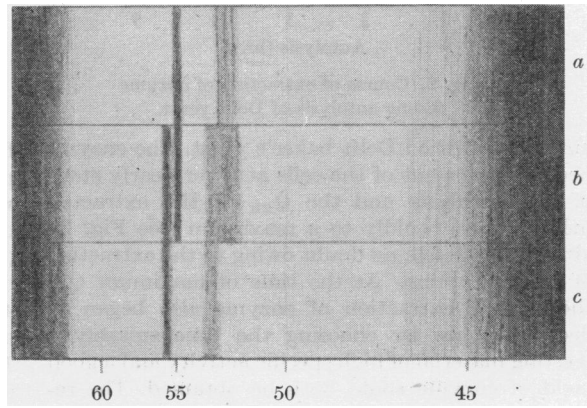


Fig. 3. Absorption spectra of purified enzyme fractions. (*a*) fraction H; (*b*) fraction H + lactate; (*c*) fraction M + lactate.

shown in Fig. 3*b*. As the band at 5565 A. in presence of lactate invariably accompanies the enzyme through all the fractionations, it can be used as a quick test for controlling the ammonium sulphate precipitations.

Preparation from Manchester yeast. When it became impossible to obtain Delft yeast, owing to war conditions, we attempted to use English baker's yeast, but found that we were unable to prepare the enzyme by the above method, on account of the much greater difficulty of extracting the enzyme from the cells. An entirely new method had to be worked out, and although this was done successfully, the new method is too laborious to be practicable for general use. As some essential data were obtained with such preparations, however, and as they yielded an important piece of evidence as to the nature of the system, the method is given here.

Table 2. *Solution H, fractionation at* pH 4·8

53 ml. solution H + 26 ml. M/5-acetate (pH 4·8) + 53 ml. water

Amm. sulph. added (g.)	Fraction	Ppt. made up to (ml.)	t_R (sec.)	Units/ml.	Total units	E_{5500}	E_{5565}	
32·0	J	8	3·4	5,900	47,500	—	0·4	discarded
+4·8	K	7	1·4	14,400	101,000	0	0·75	discarded
+3·9	L	8	0·65	31,000	248,000	0	1·32	(see Fig. 4)
+3·7	M	8	0·75	26,900	215,000	0	1·18	mixed (=Q)
+6·4	N	8·5	1·1	18,300	156,000	trace	0·97	
+7·3	P	4	8·0	2,500	10,100	0·4	0·1	discarded

Supernatant solution inactive.

The enzyme was then further purified, and the cytochrome *c* completely eliminated, by fractional precipitation by ammonium sulphate in acid solution, as follows: 53 ml. solution H (t_R 0·9 sec., Q_{MB} 1550, 1,180,000 units) was mixed with 26 ml. M/5-acetate buffer (pH 4·8) and diluted with 53 ml. water to facilitate separation of the precipitates. It was then separated into six fractions (J–P) with ammonium sulphate, as shown in Table 2. The first fraction gave a slightly opalescent, pale and not very active solution; the next fractions gave perfectly clear, deep-red and very active solutions, quite free from all traces of cytochrome *c*, but showing on reduction a very strong 5565 band, as shown in Fig. 3*c*; the last fraction was much less active, and had a weak 5565 band, but a stronger cytochrome *c* band at 5500 A.; the final supernatant fluid showed a strong spectrum of pure cytochrome *c* and was quite inactive. The extinctions ($E = \log_{10} I_0/I$) of these solutions at 5500 and 5565 A. were measured in a Hilger-Nutting spectrophotometer with a 2 cm. cell, and are shown in Table 2 (cf. also Fig. 5). The absorption spectrum curve of the most active solution (fraction L) was determined in the same instrument, and is shown in Fig. 4. Fractions L, M and N were mixed, giving solution Q (24 ml., t_R 0·8 sec., 600,000 units). The instability of the enzyme was now so marked that further attempts at purification resulted in the loss of most of the activity. The Q_{MB} of solution Q was unfortunately not exactly determined in this particular preparation, but in other similar preparations it was about 2500. The progress of the purification is shown in Table 3. The highest Q_{MB} obtained by us was nearly 3000, after adsorbing the enzyme first on calcium phosphate and then on alumina, followed by ammonium sulphate fractionation, but the yield was much smaller than by the method given above.

Table 3. *Course of purification from Delft yeast*

Stage	Volume (ml.)	t_R (sec.)	Q_{MB}	Total units
Lebedew juice	6250	14	40	9,000,000
After alumina	7000	17	—	8,300,000
After heating	7000	19	—	7,430,000
Eluate from Ca phosphate	2800	16	—	3,530,000
Ultrafilter concentrate	130	1·25	—	2,100,000
$(NH_4)_2SO_4$ fraction H	57	0·9	1550	1,280,000
Acid $(NH_4)_2SO_4$ fraction Q	24	0·8	*c.* 2500	600,000

20 kg. fresh Manchester yeast were plasmolyzed by adding solid NaCl. The liquefied mass (17 l.) was ground in a high-speed metal ball-mill in batches of 130 ml. for 20 min. each, in order to break the cells. It was then incubated at 37° for 3 hr. with mechanical stirring, during which time toluene was slowly added up to a final concentration of 7%. 9 l. of M/15-phosphate buffer (pH 7·2) were then added and the whole was centrifuged, giving 12 l. of Lebedew juice (t_R 105 sec.). The enzyme from 11 l. of juice was then adsorbed on 550 g. of kaolin, which was centrifuged off and eluted with 1·8 l. M/15-phosphate buffer (pH 7·2) containing 20% of ammonium sulphate (t_R of eluate 45 sec.). On standing, some activity was lost. 600 g. solid ammonium sulphate were added, the precipitate filtered off after standing overnight in the refrigerator, dissolved in water and dialyzed for 3 hr. (1100 ml., t_R 35 sec.). 165 ml. calcium phosphate gel were then added to adsorb the enzyme washed with 600 ml. phosphate buffer (pH 7·2) and eluted with 700 ml. of the same buffer containing 20% of ammonium sulphate

(t_R 28 sec.). On standing, half the activity was lost (t_R 55 sec.). The solution was saturated with ammonium sulphate and placed in the refrigerator. The precipitate which by fractional precipitation with ammonium sulphate, first at pH 7·2, then at pH 4·8. The final fraction (T), corresponding to fraction L of the previous preparation, had

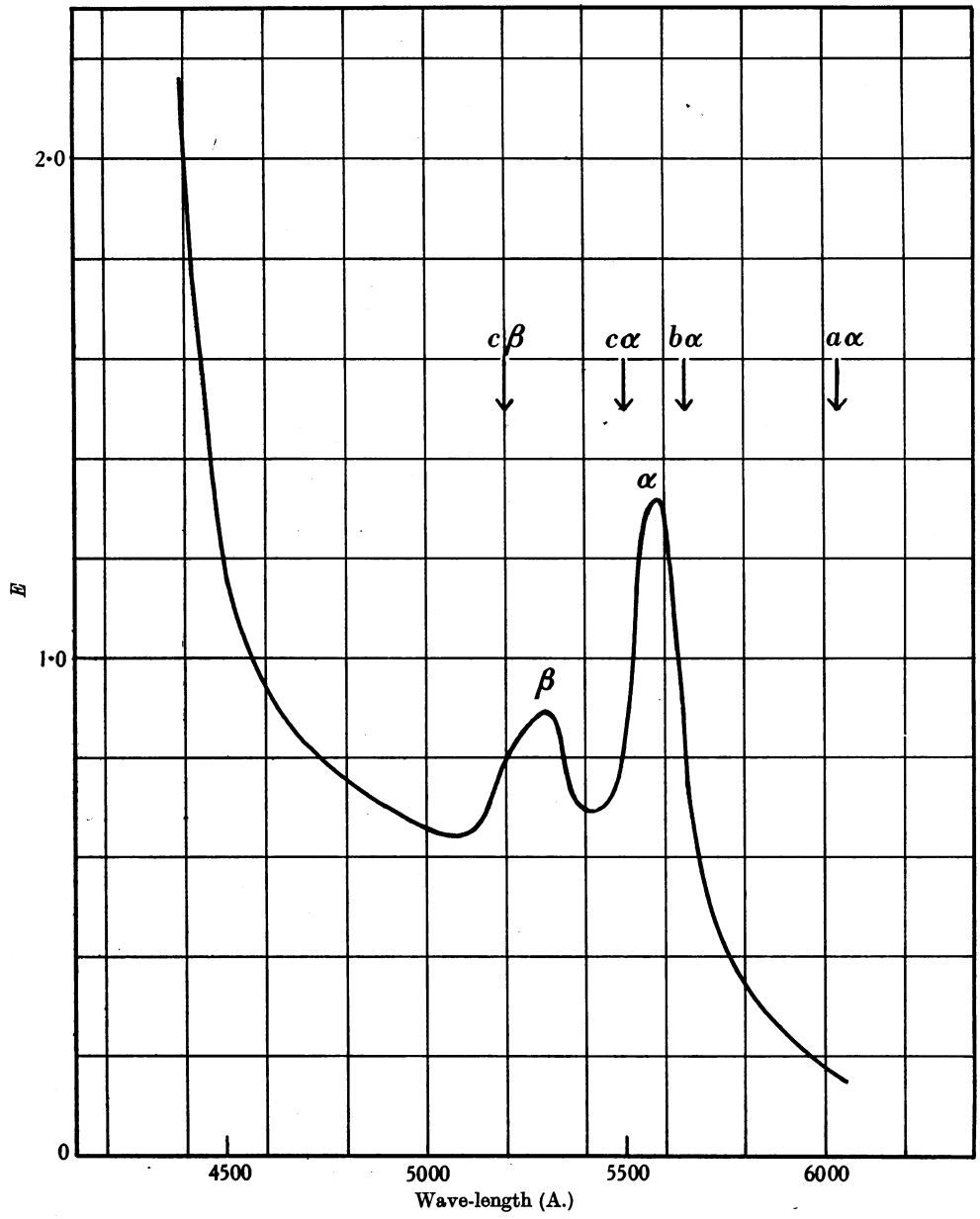


Fig. 4. Absorption spectrum curve of cytochrome b_2.

formed overnight was taken up in 10 ml. of water (t_R 1·8 sec.). On adding lactate to this solution the absorption band at 5565 A. was clearly seen as in the enzyme preparation from Delft yeast. From this point the purification followed the procedure of the method already described, about the same Q_{MB}, representing a purification of more than 200 times with respect to the Lebedew juice. It was used for spectrophotometric determinations needed for calculations given later. The general course of the purification by this method is shown in Table 4.

Table 4. *Course of purification from Manchester yeast*

Stage	Volume (ml.)	t_R (sec.)	Q_{MB}	Total units
Lebedew juice	12,000	105	10·5	2,300,000
Eluate from kaolin	1,800	45	55	800,000
Pptd. and dialyzed	1,100	35	250	630,000
Eluate from Ca phosphate	700	28	1100	500,000
Eluate from Ca phosphate (after standing)	700	55	556	257,000
$(NH_4)_2SO_4$ ppt.	10	1·8	—	110,000
Acid $(NH_4)_2SO_4$ fraction T	4	1·7	2280	47,500

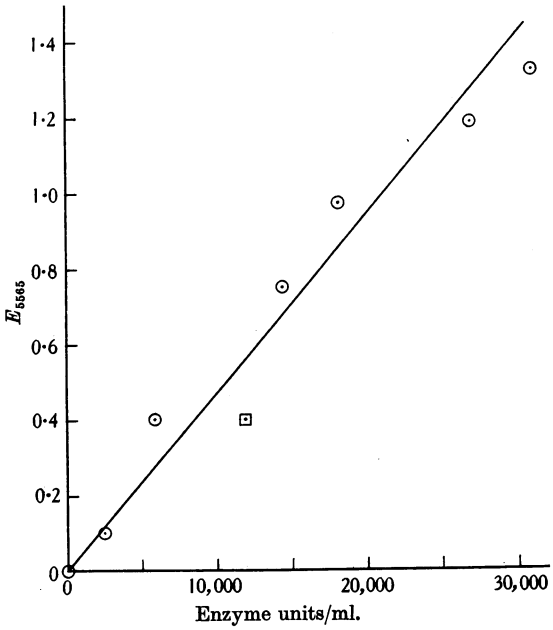


Fig. 5. Relation between enzyme activity and intensity of cytochrome b_2 absorption band in different fractions. The circles represent points given by fractions J–P from Delft yeast, the square was given by fraction T from Manchester yeast.

CYTOCHROME b_2

The coloured substance which accompanies the enzyme and is responsible for the absorption spectrum shown in Fig. 3*c* and Fig. 4 is evidently a haemochromogen protein. Further examination has shown that it is definitely a cytochrome, though not identical with any of those previously known. As it is most closely allied to cytochrome *b*, we have proposed the name cytochrome b_2, in order to distinguish it from other components and to avoid any implication as to its identity with the enzyme. (The name cytochrome b_1 has been given to a modified cytochrome present in certain bacteria; Keilin, 1934.)

The new cytochrome differs from those previously described, with the exception of cytochrome *c*, in being extremely soluble in water. The α band of its spectrum, as will be seen in Fig. 4, lies almost midway between those of cytochromes *b* and *c*. The Hartridge reversion spectroscope gives its position as 5563 A. It is not visible in the absorption spectrum of yeast as its concentration there is far too low.

The absorption spectrum, which is that of the reduced form of cytochrome b_2, appears instantly on the addition of either lactate or hyposulphite ($Na_2S_2O_4$). The reduced form is slowly autoxidizable and in either case the bands gradually disappear on shaking with air, provided that only a small amount of reducer has been added. When the enzyme activity of the solution has been lost after keeping, the bands do not appear on adding lactate, but they appear, apparently unchanged, on reduction by hyposulphite.

Prof. Keilin very kindly made an examination of fraction L and made the following observations. The preparation was free from cytochrome *c*. On denaturation with dilute NaOH and addition of pyridine and hyposulphite the haematin of cytochrome b_2 was converted into the corresponding pyridine haemochromogen. The spectrum of this was identical with that given by pyridine protohaemochromogen, showing that cytochrome b_2 has protohaematin as its prosthetic group, like cytochrome *b* and unlike cytochromes *a* and *c*. It resembles cytochrome *b* also in being slightly autoxidizable. No change in the spectrum was produced by saturating with carbon monoxide in neutral solution, showing that, like cytochromes *a*, *b* and *c* but unlike other haemochromogens, cytochrome b_2 does not combine with CO under these conditions. When the solution was frozen in liquid air the absorption bands became much sharper and the α band was then seen to be double; this has also been seen with some other haemochromogens. When a strong solution of oxidized cytochrome *c* was added to the preparation, followed by lactate, the cytochrome *c* became reduced as well as the cytochrome b_2.

By converting the cytochrome b_2 into pyridine haemochromogen, as described above, and comparing the solution with standard pyridine haemochromogen solutions by the spectroscopic method described by Keilin (1933, 1941), its concentration may be accurately determined. In this way Prof. Keilin kindly determined the concentration of cytochrome b_2 haematin in solution T, the final fraction from the Manchester yeast preparation. This was found to be equivalent to 0·0040 mg. haemin/ml. The absorption curve of this solution, after reduction with hyposulphite, had been determined in the spectrophotometer and E_{5565} estimated as 0·4, using

a 2 cm. cell. The absorption coefficient β (where $\beta=\frac{1}{cd}\ln\frac{I_0}{I}$, and c is the concentration and d the thickness of the cell) corresponding to the α band is therefore given by

$$\beta_{5565}=\frac{2\cdot3\times1000\times652\times0\cdot4}{2\times0\cdot0040}=0\cdot75\times10^8\ \frac{\text{cm.}^2}{\text{g. atom Fe}}.$$

This is of the same order as that of the α band of cytochrome *c*.

FURTHER PROPERTIES OF THE ENZYME

Instability. The greatest difficulty in this work was caused by the increasing instability of the enzyme as purification proceeds, and until some means of preventing it is found, it is unlikely that the purification will be carried much further. Many unsuccessful attempts to stabilize the enzyme were made by the recognized methods. The use of glass-distilled water throughout, alterations of pH, and the addition of glycine, gum arabic, lactose, various proteins, cyanide, cysteine or glutathione, were all ineffective. The possibility that the destruction might be due to another enzyme (e.g. a proteinase) was considered and many attempts were made to separate it from the lactic enzyme but without success. Indeed, if this were the cause, the properties of the two enzymes would have to be remarkably similar to account for the fact that the more the lactic enzyme is purified, the more rapidly is it destroyed.

Two substances had a marked stabilizing effect, namely lactate, the substrate of the enzyme, as already mentioned, and ammonium sulphate in high concentration. By adding both together, preparations of moderate purity may be made almost completely stable. Thus rather impure preparations (Q_{MB} up to 1000) are fairly stable; moderately purified fractions (Q_{MB} 1000–2000) lose most of their activity in a few hours, but can be very largely stabilized by ammonium sulphate in presence of lactate; and the purest fractions (Q_{MB} 2000–3000) become inactive in about 20 min. without ammonium sulphate, and even when kept saturated with ammonium sulphate at 0° in neutral solution lose 90% of their activity in a few hours. This makes it extremely difficult to carry the purification further.

For the same reason it was not possible to investigate the homogeneity of the final fractions in the Tiselius apparatus, since this requires a preliminary dialysis for some hours against dilute buffer solution, during which the enzyme would be completely destroyed.

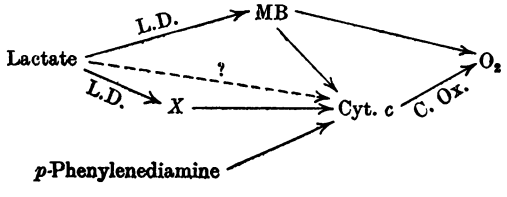
Coenzyme. It seems certain that the lactic dehydrogenase of yeast, unlike that of muscle, does not involve any dissociable coenzyme. The method of purification is such that any such coenzymes would be very completely removed, e.g. by the exhaustive washing of the enzyme adsorbed on calcium phosphate. The purified preparations are, in fact, quite free from all traces of the well-known coenzymes, and we have been unable to detect them even in small traces by delicate enzyme tests, both before and after denaturing the solutions by heat or acid. Actually they are found to be removed in the early stages of the purification. The addition of the usual coenzymes does not increase the activity of the enzyme. It is true that activity is lost by dialysis, but the non-dialyzed solutions also lose activity at the same time, so that the loss is not due to dialysis. As already mentioned, fractions of only moderate purity are completely stabilized by ammonium sulphate, and such solutions may be dialyzed for many days against ammonium sulphate solutions without loss of activity. Furthermore, we have attempted in many ways to resolve our enzyme preparations into two fractions having a greater activity when mixed than they have separately, and we have been unable to do so.

Flavoprotein. The final fractions obtained by the method of purification described above are not altogether free from flavoprotein. We think, however, that it is unlikely that flavoprotein plays any part in the system. There is no parallelism between the amount present and the activity, and by a modified method of preparation we were able to obtain highly active solutions in which no traces of flavoprotein could be detected. By repeatedly adsorbing the enzyme on alumina, followed by ammonium sulphate fractionation, active solutions could be obtained (though with a much smaller yield and containing much more cytochrome *c*) which (*a*) showed no decrease of absorption at 4500A. on addition of hyposulphite, (*b*) after denaturation gave no increase of activity with the flavin-freed *d*-amino-acid oxidase, and (*c*) showed no trace of fluorescence in ultra-violet light, either before or after denaturation by heat or acid. Test (*a*) is not so sensitive as the other two. Test (*b*), though sensitive, detects only flavoproteins containing flavin dinucleotide. Test (*c*) is highly sensitive, and although not all flavoproteins fluoresce, they all give a strong fluorescence when the prosthetic group has been split off by denaturation. It is clear that if the methylene blue reduction by the system takes place through the mediation of a flavoprotein, it must have a catalytic activity far higher than that of any of the flavoproteins known at present. Whether the reduction of cytochrome *c* by the system involves a flavoprotein cannot as yet be definitely stated.

Reaction with cytochrome c. In their preliminary note, Dixon & Zerfas (1939) stated that the yeast lactic enzyme loses its power of reducing cytochrome *c* when it is purified, whereas it was stated above

that the purest fractions could be seen spectroscopically to reduce cytochrome *c* rapidly. The explanation of this apparent discrepancy is that although the enzyme can apparently react with cytochrome *c* to some extent, the rate of this reaction was too small to be effective in catalyzing any appreciable O_2 uptake when the partially purified enzyme solutions of Dixon & Zerfas were used. When, however, the enzyme is highly concentrated, as in the extremely active solutions now obtained (which are far more concentrated than any available in 1939), the reaction rate is sufficient to show a rapid reduction of cytochrome *c* under the spectroscope. We cannot say at present whether the completely pure enzyme would be able to react with cytochrome *c*, or whether the reaction is due to some additional factor still present in the final solutions.

Evidence confirming the view of Dixon & Zerfas, that the rapid reduction of cytochrome *c* by crude preparations of the enzyme depends on the presence of an additional factor which is removed in the purification and which is not required for the reduction of methylene blue, has been obtained, using a purified preparation from Manchester yeast. This was prepared as described above, but was not taken to the final stages of purification. The rates of reduction of cytochrome *c* and methylene blue by the system were conveniently compared by O_2 uptake measurements after the addition of either cytochrome *c* plus excess of cytochrome oxidase or of methylene blue, since the reduced cytochrome and the reduced methylene blue are both reoxidized with uptake of O_2. The reactions taking place are shown in the diagram, the arrows showing the direction of hydrogen transfer.



On carrying out the experiment at pH 5·2, the optimum pH of the lactic dehydrogenase, no O_2 uptake was obtained in either case. With methylene blue, this was due to the failure of the reduced dye to react with O_2 at the acid pH, as shown by the fact that the solution was completely decolorized. The addition of cytochrome oxidase, however, catalyzed the reoxidation of the dye and a rapid O_2 uptake was then obtained. The absence of O_2 uptake with the cytochrome system without methylene blue, however, did not necessarily show that the cytochrome was not reduced by the lactic dehydrogenase system, as the cytochrome-oxidase system was found to be practically inactive at this pH, giving no O_2 uptake with *p*-phenylenediamine. It is not clear why the oxidase should be able to oxidize leucomethylene blue but not *p*-phenylenediamine at this pH.

At pH 7·6 these difficulties were avoided, and the lactic dehydrogenase still retained sufficient activity at this pH for the O_2 uptake measurements, while the cytochrome oxidase gave a rapid O_2 uptake with *p*-phenylenediamine. Fig. 6 shows results obtained under these conditions, and it will be seen

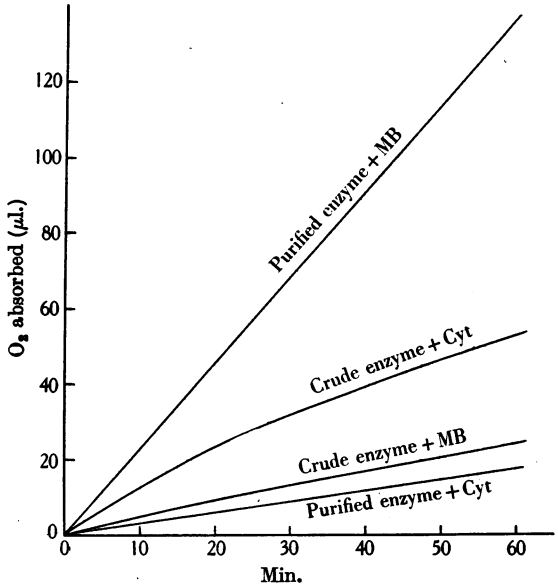


Fig. 6. Reaction of crude and purified enzyme with cytochrome *c* and with methylene blue.

that with the purified enzyme the reaction with cytochrome is extremely slow compared with the reaction with methylene blue. Similar results have been obtained with other purified preparations. With cruder preparations, on the other hand, it will be seen that the rates are reversed and the reaction with cytochrome is much more rapid than the reaction with methylene blue. This is evidence for the presence in the unpurified preparations of a factor (*X*) catalyzing the reaction of the lactic system with cytochrome, but not required for the reaction with methylene blue. The experiments of Fig. 6 were done at the same time and with the same cytochrome oxidase preparation.

DISCUSSION

This work was undertaken mainly with the object of throwing some light on the chemical nature of dehydrogenases of this group, which are not flavoproteins and do not depend on coenzyme I or II.

The most interesting question which arises is, therefore, whether the yeast lactic dehydrogenase is identical with the cytochrome b_2 which invariably accompanies it or not. If it were identical, this would be a new kind of function for a haematin-protein, but there is no inherent improbability in such an assumption and cases of reactions between methylene blue and haemochromogens are already known.

Evidence for identity is provided by the close similarity in the properties of the enzyme and cytochrome b_2, which causes the pigment to accompany the enzyme activity through all the fractionations involved in the purification, so that the absorption band can be used as a quick test for locating the enzyme during fractionation. Moreover, there is a quantitative parallelism between the strength of the absorption band and the enzyme activity in different solutions, as shown in Fig. 5. We have noticed in many preparations that, as the enzyme is concentrated, the first appearance of the band occurs as the t_R approaches 5 sec. The preparation from Manchester yeast yielded strong confirmatory evidence, for in spite of the fact that the method of preparation was almost entirely changed from that previously used, the same band appeared when about the same activity was reached. Moreover, the strength of the band in the final fraction T was approximately that predicted from the activity: E_{5565} was found to be 0·4 for this solution, using a 2 cm. cell, and this gives the point shown by a square in Fig. 5. It will be seen that this falls fairly close to the line previously obtained. If cytochrome b_2 has no connexion with the lactic dehydrogenase system this would indeed be a most remarkable coincidence.

It is true that the same absorption band can be seen when the solution is reduced with hyposulphite after the enzyme has become inactivated on standing, but this is not necessarily an argument against identity, since the lactate-activating group in the enzyme might become inactivated without affecting the haematin group. A somewhat similar case is that of xanthine oxidase, in which, according to Corran *et al.* (1939*a*), the xanthine-activating part of the enzyme may become inactivated while the flavin group remains intact.

It seems clear, therefore, that cytochrome b_2 forms an essential part of the catalyst causing the reduction of methylene blue by lactate. In this connexion it is very interesting to note that in heart muscle cytochrome *b* is intimately associated with the succinic dehydrogenase, a dehydrogenase of the same class as the yeast lactic enzyme (Keilin & Hartree, 1939). It is not impossible, therefore, that the enzymes of this group are cytochromes of the *b* type.

The available evidence, however, is insufficient to show whether cytochrome b_2 is identical with the lactic dehydrogenase itself, or whether it forms an intermediate carrier acting between the dehydrogenase and methylene blue. The proportionality between the rate of reduction of the dye and the amount of cytochrome b_2 present could be accounted for on either view. If the pigment were identical with the enzyme a proportionality would obviously be expected; if it were a carrier this would also be the case, provided that the dehydrogenase itself is always present in excess, so that the carrier is the limiting factor, and provided that the methylene blue reacts only through the carrier. It is not easy to see, however, why the dehydrogenase should still be present in excess after the whole fractionation procedure, resulting in a high degree of purification and a great increase in activity.

An argument for the carrier hypothesis might at first sight seem to be provided by an observation by Haas, Horecker & Hogness. They observed (1940) 'a small band at 557 mμ' in impure preparations of their cytochrome *c* reductase from yeast. Later (1942), after the appearance of our preliminary note on this work, they assumed that the substance responsible for this absorption band was identical with cytochrome b_2.* They found that the substance was reduced on adding hexosemonophosphate, hexosemonophosphate dehydrogenase and coenzyme II to the reductase preparation. If we assume that this observation refers to cytochrome b_2, it would seem to suggest that it can act as a carrier in the hexosemonophosphate system as well as in the lactate system. In reality, however, the two reactions are quite different. In the former case the reaction consists in the oxidation of reduced coenzyme II, while in the latter case this coenzyme is not a component of the system and the reaction consists in the linking of lactate with methylene blue. It does not seem impossible that the impure reductase preparation may have contained lactic dehydrogenase and also enzymes capable of forming traces of lactate from the hexosephosphate, in which case cytochrome b_2 would certainly be reduced. The traces of lactate required to produce the 5565 band in our preparations are extremely small, and a momentary contact with the human skin is quite sufficient to cause its almost instantaneous appearance.

* It is doubtful whether the evidence is adequate for such an identification. Their published spectrophotometric curve (1942) shows the band only as a small rise on a curve indicating a considerable general absorption. Its intensity is less than one-fiftieth of the α band shown in Fig. 4, and there is no trace whatever of any β band. Under these circumstances it is not easy to determine the position of the band with precision, but even if it were coincident with the α band of cytochrome b_2 it would not necessarily prove identity. For example, the α bands of cytochromes *a* and a_3 coincide, although they are not identical. It is not stated whether the preparation contained lactic dehydrogenase.

It is clear that it must still remain an open question whether cytochrome b_2 is identical with the yeast lactic dehydrogenase or whether it forms an essential intermediate carrier in the system.

In either case it is possible to calculate the catalytic activity of pure cytochrome b_2 and to draw certain conclusions. If we consider fraction T, which contained 0·0040 mg. cytochrome b_2 haemin/ml., as already stated, we can calculate the Turnover Number (T.N., i.e. the number of times it must be reduced and oxidized per minute in order to account for its observed catalytic activity) as follows. Bearing in mind that 1 mol. of haemin requires 1 equivalent of H for reduction, while 1 mol. of methylene blue requires 2 equivalents, we see that 1 ml. of M/1000-methylene blue is equivalent to 1·30 mg. of haemin (M.W. 652). Thus 0·5 ml. M/2000 methylene blue (the amount taken in the t_R test) is equivalent to 0·325 mg. haemin. This was reduced by 1 ml. of solution T in 1·7 sec. or 0·028 min., so that the amount per min. would be equivalent to 11·6 mg. haemin. The turnover number is then given by $\text{T.N.} = \frac{11{\cdot}6}{0{\cdot}0040} = 2900$. This is an entirely reasonable value, and may be compared with the T.N. of pure cytochrome *c*, which is 1400 in the succinic system *in vitro* and 3800 in the living yeast cell (Keilin & Hartree, 1940). The T.N. of diaphorase varies from 1000 to 8000, according to the dilution (Corran, Green & Straub, 1939*b*).

As 1 ml. of solution T contained 11,900 enzyme units, 1 mg. of haemin would correspond to 3,000,000 units within the limit of error.

The Q_{MB} of the pure catalyst depends on the molecular weight assumed. If the M.W. per haematin group is 68,000, as in catalase, the Q_{MB} when pure will be $\frac{3{,}000{,}000 \times 652}{68{,}000} = 29{,}000$. The purity of our best fractions ($Q_{MB} = 3000$) would then be a little over 10%. If, on the other hand, the M.W. per haemin group is 17,000, as in cytochrome *c* and haemoglobin, the Q_{MB} when pure would be $\frac{3{,}000{,}000 \times 652}{17{,}000} = 115{,}000$ and the purity of our best fractions would be only 2·5%. For comparison with these figures we may mention that pure crystalline lactic dehydrogenase from heart muscle, a dehydrogenase of fairly average activity, appears from the results of Straub (1940) to have a Q_{MB} of 60,000, assuming that the O_2 taken up was converted into H_2O_2, as it probably would be under Straub's conditions. Other dehydrogenases are known with up to twice this activity, as well as some with lower activities. Thus calculations based on the assumption that cytochrome b_2 is responsible for the catalytic activity of the yeast lactic dehydrogenase lead to quite reasonable figures.

It can be calculated from these results that the Lebedew juice obtained from 1 kg. yeast (fresh weight) contains on the average only 5–10 mg. of the pure protein.

SUMMARY

1. The lactic dehydrogenase of yeast (which belongs to the 'cytochrome-reducing' group) has been extracted and purified; solutions some thousands of times as active as those previously known have been obtained. The enzyme is still far from pure, but increasing instability makes further purification difficult.

2. The enzyme does not depend on soluble coenzymes, nor apparently on flavin groups, for its activity.

3. A new cytochrome ('cytochrome b_2'), freely soluble in water, has been found in the concentrated enzyme fractions. The nature of its prosthetic group, its absorption spectrum and other properties have been determined.

4. Evidence is presented showing 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.

5. There is evidence that an additional factor is necessary to enable the system to react with cytochrome *c*.

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