ON THE MULTIPLICITY OF LACTIC DEHYDROGENASES IN YEAST†

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It was recently observed (Lindenmayer and Smith, 1957; Slonimski and Tysarowski, 1958; Boeri et al., 1958; Singer and Kearney, 1958) that anaerobic yeast, although devoid of the hemoflavoprotein cytochrome b_2 (b_2), is capable of rapid oxidation of lactate. Labeyrie et al. (1959) later showed that the enzyme from anaerobic cells is specific for the D(-) form of lactate whereas b_2 is known to be specific for L(+) lactate (Boeri et al., 1955). It appeared from this work that during the transition from anaerobiosis to aerobiosis (Slonimski, 1953) there was a change in the type of lactic dehydrogenase (LD) present. Slonimski and Tysarowski (1958) suggested that the dehydrogenase of anaerobic cells might be a precursor of b_2. Following the recognition of the differences in the optical properties and kinetic constants of the two LD's, this hypothesis was abandoned by its proponents (Labeyrie et al., 1959), but has recently been revived by Nygaard (1960) and by Kattermann and Slonimski (1960).

Two avenues appeared to be open toward the resolution of the important question as to whether the D(-) LD is a precursor of b_2. One involved the isolation of the former and a comparison of its properties with those of b_2. The properties of the partially purified enzyme (reversibility, dependence of its activity on a divalent metal, effect of inhibitors, its ability to act on a wide range of D-α-hydroxy acids (Boeri et al., 1960)), are so completely different from those of the b_2 that a precursor—product relationship appears extremely unlikely.

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The other approach, the results of which are reported here, exploited the fact that "petite" cells are unable to synthesize b_2 (Slonimski, 1953). Thus, with "petites" one could determine whether the loss of D(-) LD during O_2-adaptation of anaerobic cells was necessarily related to the synthesis of b_2 or was a consequence of the conditions of adaptation.

TABLE I

D-α-OH ACID DEHYDROGENASE AND CYTOCHROME B_2 ACTIVITIES OF ANAEROBICALLY GROWN YEAST AFTER VARIOUS CONDITIONS OF ADAPTATION*

Exp.	Conditions	Q_{O_2}	μMoles lactate oxidized/min.			
			D(-)lactate—$K_3Fe(CN)_6$		L(+)lactate—cyt. c	
			per ml.	per mg.**	per ml.	per mg.**
1	"Grandes"		32	0.62		
	Same, adapted 6 hrs. in O_2		0	0		
	Same, adapted 6 hrs. in N_2		0	0		
2	"Petites"		17.8	0.35		
	Same, adapted 6 hrs. in O_2		0.18	0.005		
	Same, adapted 6 hrs. in N_2		0	0		
3	"Grandes"	< 10	12.6	0.23	0	0
	Same, adapted 4 hrs. in air	22	13.9		0.37	
	Same, adapted 22.5 hrs. in air	53	11.3	0.2	3.81	0.071

Yeasts were grown anaerobically in 10% glucose, 1% yeast extract, 1% peptone. Adaptations were performed with thoroughly washed cells with constant bubbling with the gas indicated at 30° in the following media: Exp. 1, 0.066 M KH_2PO_4—10% glucose; Exp. 2, 0.1 M acetate, pH 4.0—10% glucose—0.01% DL-lactate; Exp. 3, 0.066 M KH_2PO_4—7 mM glucose—54 mM DL-lactate. *Adaptation means incubation in stationary conditions. ** Activity per mg. biuret protein.

A pure strain was obtained by single colony isolation from baker's yeast (Distillerie Italiane), and "petite" mutants were isolated by the acriflavin technique (Slonimski, 1953). Cells were grown at 27° in Slonimski's Medium No. 3, supplemented with 1% peptone (Slonimski, 1953), or in 1% peptone—1% yeast extract—2 to 10% glucose, with or without added DL-lactate, for 40 to 48 hours. The degree of aerobiosis of cells was controlled by the Q_{O_2} on glucose and by the cyto-

chromes present. Samples of D(-) and L(+) lactate which contained in excess of 0.3% of the antipode were purified by treatment with crystalline yeast L(+) lactic dehydrogenase or D-α-hydroxy acid dehydrogenase (Boeri et al., 1960), respectively. Since ferricyanide served as the oxidant for this purpose, all traces of ferri- and ferrocyanide were removed before use. Dehydrogenase activities were assayed spectrophotometrically at 30°, pH 8, in 0.05 M Tris buffer with ferricyanide or cyt. c as electron acceptor, with 5 mM L(+) or 10 mM D(-) lactate as substrate with fresh autolysates of thoroughly washed, air-dried yeast (3 hours autolysis at 37°; 1 gm. dried yeast in 5 ml. H_2O). The protein content of the autolysates was 40 to 55 mg. per ml. (biuret). The activities were essentially the same in autolysates as in cells broken with Ballotini beads.

Exp. 1, Table I, shows that the D(-) lactate-ferricyanide (D-α-hydroxy acid dehydrogenase , D-α-HAD) activity of anaerobically grown normal cells disappears in the absence of growth on incubation under anaerobic as well as aerobic conditions of "adaptation". This decline is particularly rapid if the "adaptation" is carried out in the presence of high glucose. Exp. 2 demonstrates the same for "petite" cells, while Exp. 3 shows that under certain conditions extensive synthesis of b_2 can occur without a concomitant decrease in D-α-HAD. Generally, however, D-α-HAD is readily lost from both "grandes" and "petites" after active cell division ceases, whether or not b_2 is being synthesized.

During this study it was also found that both anaerobically and aerobically grown cells contain a number of enzymes for the oxidation of both isomers of lactate. Table II presents representative data from a large number of experiments illustrating this point. These data, suitable kinetic experiments, studies with inhibitors, and fractionation have permitted distinguishing the following enzymes concerned with lactate oxidation in yeast.

(1) The highest activity is observed with D(-) lactate and $K_3Fe(CN)_6$ in anaerobic cells. The enzyme responsible for the rapid oxidation of D(-) lactate with ferricyanide as acceptor is the general D-α-HAD (Boeri et al., 1960) which has now been partially purified from both normal and "petite" yeasts. As first shown by Slonimski and Tysarowski (1958), it does not react with cyt. c. The

TABLE II

THE OXIDATION OF D(-) AND L(+) LACTATE IN YEAST AUTOLYSATES

Growth Conditions	Substrate D or L lactate	μMoles lactate oxidized/min./ml. autolysate			
		with $K_3Fe(CN)_6$		with Cyt. c	
Normal yeast, grown in 10% glucose — 1% y.e. —1% peptone, solid medium (aerobic)	D(-)	5.3	(0.13)	0.14	(0.0033)
	L(+)	0.45	(0.011)	0.16	(0.0038)
Normal yeast, Slonimski Medium No. 3, solid medium (aerobic)	D(-)	3.1 - 3.7	(0.072 - 0.14)	0.27	(0.010)
	L(+)	0.41 - 0.66	(0.010 - 0.015)	0.92	(0.034)
"Petite", same medium as above (aerobic)	D(-)	1.8 - 6.3	(0.045 - 0.11)	0.066 - 0.18	(0.0013 - 0.0034)
	L(+)	0.2 - 2.1	(0.004 - 0.039)	0.066 - 1.98	(0.0013 - 0.036)
Normal yeast, Slonimski Medium No. 3, anaerobic	D(-)	12.3 - 27.0	(0.21 - 0.49)	0.088	(0.0015)
	L(+)	0 - 0.36	(0 -0.064)	0.053	(0.0009)
Normal yeast, grown in 10% glucose — 1% y.e. —1% peptone, anaerobic	D(-)	13.0 - 33.0	(0.24 - 0.81)	0 - 0.082	(0 - 0.0015)
	L(+)	0 - 0.38	(0 - 0.007)	0	
"Petite", same medium as above, anaerobic	D(-)	14.2 - 29.0	(0.26 - 0.65)	0	
	L(+)	0 - 0.81	(0 - 0.017)	0	
"Petite", Slonimski Medium No. 3, ± DL-lactate, anaerobic	D(-)	6.6 - 20.4	(0.12 - 0.41)	0 - 0.077	(0 - 0.0013)
	L(+)	0 - 0.59	(0 - 0.01)	0	

The activities are minimal values, since endogenous blank was subtracted. The range indicated is that found in different lots of yeast grown under the conditions given. The values in parentheses denote μmoles/min./mg. biuret protein.

dehydrogenase is a metal-flavoprotein; the metal may be reversibly removed with complete loss of activity (Boeri et al., 1960; Curdel et al., 1959). It is competitively inhibited by analogs of the substrate, e.g., oxalate, it is inactivated by EDTA and cyanide, and its action on D(-) lactate is readily reversible (Boeri et al., 1960).

(2) There is in both normal and "petite" cells a D(-) lactate-cytochrome c reductase, the activity of which is much lower than that of the D-α-HAD and is generally higher in aerobic than in anaerobic cells. Its activity increases on O_2-adaptation of anaerobically grown normal or "petite" yeast, but its rate of development is generally slower than that of b_2. It may be readily distinguished from D-α-HAD by the different effect of growth conditions on the two enzymes (Table II) and by the facts that the D(-) lactate-cyt. c reductase is not inhibited by concentrations of L-malate and of oxalate which inhibit the former enzyme completely and that it does not act on D-malate, an excellent substrate of D-α-HAD. The existence of this enzyme has been independently recognized by Nygaard who has suggested that it is an intermediate in the transformation of D-α-HAD to b_2 during O_2 adaptation (Nygaard, 1960). This suggestion is unlikely in view of the extensive differences in specificity for substrates and substrate competitors between the two enzymes.

(3) Among the enzymes for the oxidation of L(+) lactate, b_2 is best known. It reacts with ferricyanide and cyt. c at about equal rates (Boeri and Tosi, 1956); it is formed aerobically and is absent in "petite" cells (Slonimski, 1953); and it is insensitive to cyanide.

(4) The fact that "petite" cells, devoid of cytochrome b_2, nevertheless oxidize L(+) lactate fairly readily (Table II) suggests the existence of one or more additional LD's. The existence of an enzyme capable of oxidizing L(+) lactate with ferricyanide as acceptor but not cyt. c is suggested by the results from anaerobic cells (Table II). This activity cannot be ascribed to the operation of a racemase, since it is HCN-insensitive; it is not inhibited by 10^{-4} M p-chloromercuribenzoate, and cannot act on L-malate, whereas the D-α-HAD is inactivated by HCN and mercurials and acts well on malate. The occasional pres-

ence of this enzyme in normal, anaerobically grown cells, devoid of b_2 (Table II) might account for their ability to catalyze the L(+) lactate-ferricyanide reaction and its possible presence in normal aerobic cells might account for the higher rate of L(+) lactate oxidation via ferricyanide than via cyt. c.

(5) While anaerobic "petites" are usually devoid of L(+) lactic-cyt. reductase activity, aerobic cells possess an appreciable activity (Table II), and the activity also develops on O_2-adaptation of anaerobic cells. This raises the question of the possible existence of an enzyme capable of oxidizing L(+) lactate with cyt. c as acceptor, but differing from b_2 and from the L(+) lactic-ferricyanide enzyme just discussed.

Lastly, it is emphasized that the activities listed in Table II are minimal values, since the conditions of assay were not necessarily optimal for each enzyme and since they were calculated by subtracting the endogenous oxidation, much of which now appears to be due to the presence of considerable amounts of lactate itself in autolysates. Thus, in two anaerobically grown preparations, one normal, one "petite", the autolysates contained per gm. of dried yeast 17.5 and 8.5 μmoles of lactate, respectively, by chemical and enzymatic analysis; most of the lactate was present in the D(-) form. When grown in the presence of DL-lactate, the washed cells contained considerably more lactate, virtually all in the D(-) form, and the endogenous reduction of ferricyanide was completely inhibited by traces of oxalate. This suggests that all or most of the blank is D-α-hydroxy acid oxidation.

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