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Reduced Nicotinamide Adenine Dinucleotide Dehydrogenase, Piericidin Sensitivity, and Site 1 Phosphorylation in Different Growth Phases of *Candida utilis**

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SUMMARY

Reports in the literature indicate that during the exponential phase of growth of *Candida utilis* NADH oxidation is insensitive to rotenone, that rotenone sensitivity is acquired during the transition to the late stationary phase and is again lost on catabolite repression. The acquisition and loss of rotenone sensitivity appears to be accompanied by similar changes in Site 1 phosphorylation but does not appear to be reflected in the rate of oxidation of NADH (by mitochondria) or of NAD-linked substrates (by mitochondria or whole cells). In the present paper evidence is presented that these fluctuations in sensitivity to inhibitors of NADH oxidation reflect the presence of different types of inner membrane-bound NADH dehydrogenases in different phases of growth. Thus inner membrane preparations from exponential phase cells contain an NADH dehydrogenase which reacts equally well with ferricyanide and juglone as electron acceptor, appears to be very labile, and lacks EPR signals corresponding to iron-sulfur Centers 1 and 2, whereas a new species, probably an iron-sulfur protein, with resonances at $g_{\parallel}$ = 2.01, and $g_{\perp}$ = 1.92 in the reduced state, is present. This species is not significantly reduced by NADH. In corresponding preparations from late stationary phase cells NADH-ferricyanide activity is high, juglone reductase activity is low, and the enzyme is stable and exhibits the EPR signals of iron-sulfur Centers 1 and 2, whereas the EPR signals of iron-sulfur Centers 3 + 4 change very little on transition from exponential to stationary phase cells. There is also a decrease in cytochrome concentration. Most prominent among these is a *b*-type cytochrome (g = 2.54; 2.23; 1.87) which decreases 2- to 3-fold. The EPR detectable species with $g_{\parallel}$ = 2.01 and $g_{\perp}$ = 1.92 in the reduced state is no longer detected. On catabolite repression of late stationary phase cells there is an 80 to 90% decline in NADH-ferricyanide activity, of iron-sulfur Centers 1 and 2, a 50 to 60% decrease of Centers 3 + 4, and an increase in a *b* cytochrome, but the specific activity in NADH-juglone reductase and NADH oxidase assays increases, the enzyme becomes once again labile, and the EPR detectable species with $g_{\parallel}$ = 2.01 and $g_{\perp}$ = 1.92 appears on reduction with dithionite. All these changes are prevented by cycloheximide. The data suggest that sensitivity to piericidin A and coupling to energy conservation Site 1 are properties of the type of NADH dehydrogenase present in late stationary phase cells but not in exponential phase or of catabolite-repressed cells.

Katz *et al.* (1, 2) reported that the oxidation of NAD-linked substrates by cells of *Candida utilis* is insensitive to rotenone and piericidin during the exponential phase of growth but becomes rotenone- and piericidin-sensitive in the stationary phase and becomes again insensitive to these inhibitors on the addition of ethanol as a carbon source to stationary phase cells. The same reports indicate that the appearance and disappearance of Site 1 phosphorylation parallels rotenone sensitivity. No attempt was made in the study quoted to follow NADH dehydrogenase activity in the different growth states. Thus it is not known whether the appearance and decay of rotenone sensitivity and of energy coupling site 1 reflect changes in the concentration of the inner membrane-bound NADH dehydrogenase or whether, in fact, the same type of NADH dehydrogenase is present in these different phases of growth.

Correlation of rotenone- and piericidin-sensitivity with the type of respiratory chain-linked NADH dehydrogenase present

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appeared important for several reasons. First, since, by analogy with the mammalian enzyme (3, 4), the dehydrogenase is likely to be the binding site of these inhibitors, it would be difficult to see how the same enzyme could be sensitive to the inhibitors in some growth phases but not in others. Second, there are conflicting reports in the literature (5, 6) as to whether phosphorylation at Site 1 may be dissociated from piericidin sensitivity by aeration of cells, grown in iron-limited media, in the presence of cycloheximide. Since both of these properties appear during the transition from the exponential to the stationary phase of growth (1), this system may provide an opportunity to see whether a kinetic separation of the two properties occurs, if, indeed, the same type of NADH dehydrogenase is present throughout growth. Third, if the type of NADH dehydrogenase present is constant, comparison of the iron-sulfur composition of the enzyme in the exponential phase (where Site 1 is absent) and in the stationary phase (Site 1 present) could provide additional support for the conclusion drawn from previous findings (7, 8), namely that iron-sulfur Center 2 is intimately involved in, if not itself, the locus of coupling Site 1.

As briefly reported (9) and documented in detail in this paper, data on the kinetic properties, stability, and EPR signals of the enzyme suggest that the type of NADH dehydrogenase present in piericidin-sensitive cells is different from that present in cells insensitive to this inhibitor.

EXPERIMENTAL PROCEDURE

Unless otherwise mentioned, *Candida utilis* was grown in batch cultures in the medium of Galzy and Slonimski (10) at 30°, except that the concentration of $FeCl_3$ was reduced to 10 μM, $MnSO_4$ and mesoinositol were omitted, $CuSO_4$ was increased to 0.5 μM, and 1.5% (v/v) ethanol served as the carbon source. Growth was conducted in a New Brunswick model 614 fermentor in a 10 to 12.5 liter volume with 12 to 24 liters of air flow per min, constant stirring, and with the pH maintained automatically at 5.0 by addition of 6 N KOH. The complex medium described by Biggs *et al.* (11) was used in growth on glucose, except that the pH was automatically maintained at 5.0. Mitochondria were prepared as per the method of Light and Garland (12) but 2 mg per ml of bovine serum albumin was present during osmotic breakage. ETP_c[1] was prepared by shaking 10 g of well washed yeast cells, suspended with 10 ml of 0.5 M sucrose-20 mM Tris-2 mM EDTA, pH 7.4, with 50 g of 0.2 mm Ballotini beads in 75-ml Pyrex bottles for 30 s at 0 to 4° in a Braun shaker at 4000 rpm. The particles were isolated by differential centrifugation, essentially as in previous work (small scale method) (11).

The oxidation of pyruvate plus malate (12.5 mM each) and of ethanol (20 mM) in mitochondria was measured polarographically at 30° in a 2-ml volume in 0.65 M D-sorbitol-0.1 mM EDTA-20 mM 2-(*N*-morpholino)ethanesulfonate-10 mM P_i buffer, pH 6.5, containing 1 mg of bovine serum albumin per ml. The piericidin sensitivity of this oxidation was measured in State 3 (0.5 mM ADP), in the presence of 0.3 to 0.5 nmole of inhibitor per mg of protein. P/O ratios were measured with 20 mM ethanol or 12.5 mM each of pyruvate plus malate in the medium above by the procedure of Estabrook (13).

NADH-$Fe(CN)_6^{-3}$ assays in ETP_c particles were conducted at 25° under conditions described elsewhere (11, 14) and activities were expressed for V_{max} with respect to $Fe(CN)_6^{-3}$. NADH oxidase and NADH-juglone reductase activities were measured polarographically at 30° in 0.1 M P_i-50 μM EDTA, pH 7.4, in the presence of 0.75 mM NADH. In the juglone reductase assay the concentration of the electron acceptor was 100 μM and 5 nmoles of antimycin A per mg of protein were present. Juglone was purchased from the Sigma Chemical Co. Other materials were as in previous work (11, 15).

Oxidized samples for EPR and optical reflectance spectroscopy were prepared by aerating 1 ml of ETP_c in conical centrifuge tubes (15 mm inside diameter) for 10 s at 0° with a Vortex mixer. Aliquots of 0.4 ml were immediately withdrawn with a syringe into quartz EPR tubes and frozen in liquid nitrogen. NADH- and dithionite-reduced samples were incubated for 60 s at 0° with 2.5 μmoles of NADH plus 200 nmoles of antimycin A or 1 mg of solid dithionite per ml of ETP_c, respectively, prior to withdrawing and freezing the aliquots.

Low temperature optical reflectance spectroscopy was carried out at 95° K essentially as described by Palmer and Beinert (16). The reflectance spectra allowed an assessment of the oxidation state of the samples independent of EPR spectroscopy. Thus samples in which the desired oxidation state was not realized could be eliminated from consideration.

The instrumentation and conditions of EPR spectroscopy were essentially as given in previous papers (17–19). Unless specifically stated otherwise, first derivative spectra were obtained at 3 mwatts microwave power, modulation amplitude and frequency of 8 G and 100 kHz, respectively, a temperature of 13.3 ± 0.15° K, a scan rate of 200 or 400 G per min, and a time constant of 0.5 s. As in previous reports (18, 19), the values given on a g factor scale are the actually measured values of prominent peaks or shoulders in the spectra but are not necessarily true g factors of the materials studied. In many instances, however, the values given will be very close to the actual g factors. Since a detailed analysis of the EPR spectra of the individual iron-sulfur centers of the NADH dehydrogenase of *C. utilis*, requiring isolation and characterization work under a number of conditions, is not available to date, we have based the evaluation of the EPR spectra on our knowledge of the properties of the iron-sulfur centers of the analogous enzyme from beef heart mitochondria (18, 19). The relative concentrations of the centers were estimated from peaks as indicated: Center 1 ($g = 1.94$); Center 2 ($g = 2.05$); Centers 3 + 4 ($g = 1.86$). For Center 1 the distance between the extrema at $g = 1.94$ and $g = 1.95$ was measured, while for the other centers the peak height with respect to the low or high field base-lines, respectively, was determined. These peaks were chosen because they are least interfered with by other signals. Resolution of Centers 3 and 4 (*cf.* Ref. 18) can only be achieved by reductive titration under favorable conditions of signal to noise. In view of the signal to noise ratios obtained in the present experiments, it did not seem practical to attempt this. The ratio of the peaks at $g = 1.94$ (Center 1) and $g = 1.92$ (Centers 1 + 2) observed after reduction with NADH in the presence of antimycin is different from that in beef heart ETP as can be seen from a comparison of *Spectrum 2* of Fig. 6 of this paper with data in Refs. 18 and 19, whereas after reduction with dithionite the ratio approaches that seen in beef heart (Fig. 6, *Spectrum 3*). This may be due to somewhat different shapes of the signals from the two sources or different concentrations of the centers. As evident from the records of Figs. 3 and 6, the ratio of the peak heights at $g = 1.94$ and 1.92 after maximal reduction with NADH in the presence of antimycin is practically constant at the various times of sampling. This indicates that the ratio of the concentrations of Centers 1 and 2 does not vary significantly. In the presentation of the data in Table I we have therefore plotted the changes of Centers 1 + 2, as measured from the excursion between the peak at $g = 1.95$ (maximum) and the peak at $g = 1.92$ (minimum). That these peaks originate from signals due to Centers 1 + 2 and not Center 1 alone is evident from the presence of the line at $g = 2.05$ in spectra where sufficient resolution was achieved (*cf.* Figs. 3 and 6).

RESULTS

Piericidin Sensitivity in Mitochondria and Submitochondrial Particles—It has been reported (20) that *Candida utilis* contains three NADH dehydrogenases. One, thought to be located in the outer membrane, catalyzes the antimycin A- and piericidin-insensitive oxidation of NADH. The other two are thought to be located in the inner membrane, one oriented toward the inner surface of the cristae, the other toward the outer surface. Of these two enzymes only the one oriented inward is thought to be inhibited by piericidin and rotenone and coupled to energy conservation Site 1 (20). Among these enzymes only the piericidin-

[1] The abbreviations used are: ETP_c, inner membrane preparation from *Candida utilis*, obtained by shaking with glass beads; ETP, inner membrane preparation, usually isolated from heart mitochondria.

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TABLE I

EPR signals during transition of Candida cells from exponential to stationary phase and during catabolite repression

In Experiments 1 and 2 *Candida* cells were grown on ethanol and in Experiments 3 and 4 (duplicate experiments on the same batch of cells) glucose was the carbon source during growth (*cf.* "Experimental Procedure"). The concentration of ethanol during repression was 1% (v/v) in Experiments 1 and 2, and 3% (v/v) in Experiments 3 and 4. The signal heights are given in millimeters and represent ETP_c samples reduced by NADH, calculated for 50 mg per ml of protein concentration in each case. The values for Centers 1 + 2 were from measurement between g = 1.95 and 1.92, those for Center 2 from g = 2.05, and those for Centers 3 + 4 from g = 1.86. The g = 2.02 component in the last column represents a high potential iron-sulfur protein (see text).

	Signal height									
	Centers 1 + 2				Center 2		Centers 3 + 4		g = 2.02	
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 3	Expt. 4	Expt. 3	Expt. 4	Expt. 3	Expt. 4
Hours of growth										
29	35.5									
37	30.5									
41.5	30.5									
45.5	33.5									
49	109.5									
55.5	151.5									
59.5	159									
Hours of repression										
0	110.5	129.5	(≧98.5)	(≧98.5)	(≧14)	(≧14)	(≧9.9)	(≧9.9)	84.5	84.5
0.5	104	71								
1.0	41.7	44	82.5	83.5	11	10	6.5	5.3	94	91.5
2.0	27.5	9	81.5	80	10	9	5.8	5.5	119.5	112.5
3.0	17.5		61.5	47.5	9.5	6.3	7.2	4.2	133.5	102
4.0	6									
4.5			30	27	3.3	3.6	3.3	3.6	142	118
5.0	27.6									
5.0 + CHI[a]	132.5									
6.0			17	17	2.2	1.8	4.3	3.5	134	117

[a] CHI, cycloheximide.

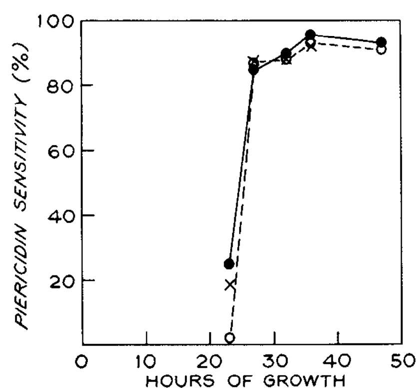

FIG. 1. Correspondence of piericidin sensitivities of the oxidation of ethanol and of malate + pyruvate in mitochondria and of the oxidation of NADH in ETP_c at various stages of growth. ●, NADH oxidation in ETP_c; ×, respiration of mitochondria on ethanol; ○, respiration of mitochondria on pyruvate + malate. Mitochondrial oxidations were measured in State 3; piericidin A concentration was 300 pmoles per mg of protein in all experiments.

sensitive one has been purified and partially characterized (11, 21). An additional complication is the observation (20) that in mitochondria from exponential phase cells grown at low rate of aeration (0.75 liter per min per liter of medium) the oxidation of malate plus pyruvate and of ethanol is insensitive to piericidin, but in submitochondrial particles from such cells the oxidation of NADH is extensively inhibited by piericidin. It was, in fact, concluded from this observation that the piericidin-sensitive enzyme is present but bypassed, *i.e.* inoperative, in exponential phase cells.

In view of this complex situation, it was important to define which NADH dehydrogenase was being measured in the ETP_c particles in the experiments to be described. The outer membrane enzyme could be ruled out, since NADH oxidation was fully antimycin-sensitive under all conditions. In late stationary phase cells grown on ethanol, the mitochondrial oxidation of NAD-linked substrates and the oxidation of NADH in ETP_c were always over 90% inhibited by piericidin (Fig. 1). Therefore, the NADH dehydrogenase present in ETP_c from such cells has the properties ascribed (20) to the inward-oriented inner membrane enzyme. Available data do not permit conclusions as regards the "sidedness" of the inner membrane enzyme in ETP_c from exponential phase cells, but, as shown below, the dehydrogenase is clearly different from that present in stationary phase cells. It is important to emphasize that in none of our experiments was any indication obtained for a latent, inoperative NADH dehydrogenase, as has been postulated by Ohnishi (20). Thus, as shown in Fig. 1, at all stages of growth, including the exponential phase, the oxidation of ethanol or of pyruvate plus malate in the mitochondria was inhibited by piericidin to the same extent as the oxidation of NADH in ETP_c. It should be noted in this context that the cells used in this study were always grown at high rates of aeration (*cf.* "Experimental Procedure"), that increasing the air flow to 3 liters per min per liter of medium did not affect the results, and that the generation time was 2 to 2.5 hours, as in Ohnishi's (20) "high aeration"

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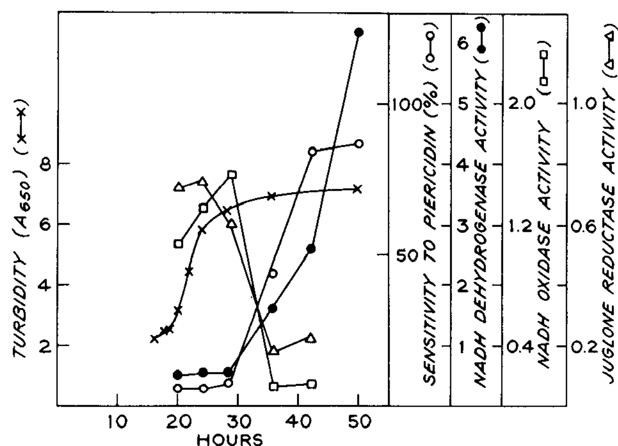

FIG. 2. Events accompanying the transformation from exponential to stationary phase. *Candida utilis* was grown in 1.5% (v/v) ethanol in a fermentor at 30°. Cells were harvested at the times shown and washed twice with water, and ETP_c and mitochondria were prepared as described under "Experimental Procedure." NADH oxidase is expressed as microatoms of O_2 per min per mg of protein at 30°; NADH dehydrogenase is expressed as micromoles of NADH oxidized per min per mg of protein in ETP_c at 25° at V_{max} with respect to $Fe(CN)_6^{-3}$; sensitivity to piericidin A (0.5 nmole per mg of protein) is expressed as per cent inhibition of NADH oxidase; and turbidity is given as absorbance at 650 nm in 1-cm light path. The pH was maintained during growth by automatic addition of 6 N KOH (pH-stat) at 5.0 until 25 hours, after which no further acid development occurred but pH rose to between 5.0 and 6.2.

experiments, while the observations on the latent NADH dehydrogenase were made with restricted air supply during growth, resulting in a long generation time of 5 hours (20).

Characteristics of NADH Oxidation in Different Phases of Growth—The experiment of Fig. 2 confirms the report (1) that, during the exponential phase of growth of *C. utilis* in ethanol, NADH oxidase activity is high and piericidin sensitivity nearly absent but in the late stationary phase sensitivity to piericidin appears, with concomitant decline of NADH oxidase activity. These observations are at variance with the report (20) that differences in piericidin sensitivity during growth are observed only when growth is slow because of limited O_2 supply, since the experiment was conducted at high rate of aeration. Fig. 2 also shows that the rise in the sensitivity of NADH oxidation during the transition from the exponential to the stationary phase is accompanied by a manifold increase in NADH-$Fe(CN)_6^{-3}$ activity, while the NADH-juglone reductase activity declines during this period. Although not shown in the figure, the respiratory control was comparable in mitochondria from exponential and late stationary phase cells (~2.5) but the P/O ratio was approximately 50% higher in the latter than in the former, suggesting the acquisition of coupling Site 1.

Fig. 3 reproduces EPR spectra of ETP_c samples from typical exponential and late stationary phase cells, in the oxidized state and after reduction with NADH and dithionite, respectively, in the presence of antimycin A.

The resonances in the region of g = 2.2 are center lines of low spin ferric heme protein spectra. Corresponding high field resonances are therefore expected to occur at values of g < 2. These resonances obviously interfere with an evaluation of the iron-sulfur resonances which occur in the same region. The evaluation is further complicated by the possibility that the respective heme compounds may not be present at the same concentration and may not be reduced to the same extent in the

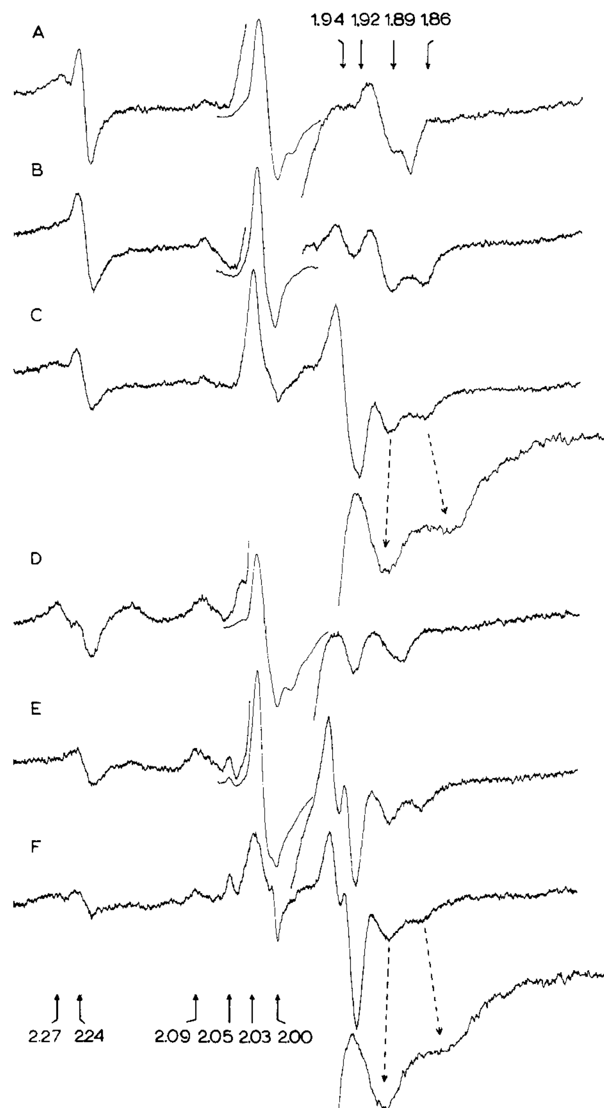

FIG. 3. EPR spectra of ETP_c particles obtained from cells sampled at different times during growth. The samples were either oxidized by aeration (*A* and *D*) or reduced with NADH (*B* and *E*) or dithionite (*C* and *F*), both in the presence of antimycin A. *Spectra A* to *C* were derived from cells harvested at 20 hours and *D* to *F* from cells harvested at 38 hours. The conditions of EPR spectroscopy were as given under "Experimental Procedure." Under *Spectra C* and *F*, samples reduced with dithionite, are shown expanded spectra of the high field portion (*cf. dashed arrows*) from which information on changes of Centers 3 + 4 has been obtained. The vertical scale is expanded approximately 3-fold and the horizontal scale 2-fold. The expanded spectra shown are the averages of four spectra accumulated by the Nicolet 1020 signal averager. All spectra of this figure are quantitatively comparable without corrections.

samples obtained under a variety of conditions. We have, however, been able to obtain what we consider to be satisfactory answers on the behavior of Centers 3 + 4 in two situations, namely, in the transition from exponential to stationary phase on reduction with dithionite (Fig. 3, *Spectra C* and *F*), when the heme compounds are partly reduced, as seen from a comparison of the lines at g = 2.2, and in catabolite repression experiments of cells grown on glucose to be described below, since in such cells the interfering heme compounds are present at considerably lower concentration (*cf.* Figs. 6 and 7).

Our EPR experiments, as documented in Fig. 3, allow us to draw the following conclusions. Iron-sulfur Centers 1 and 2

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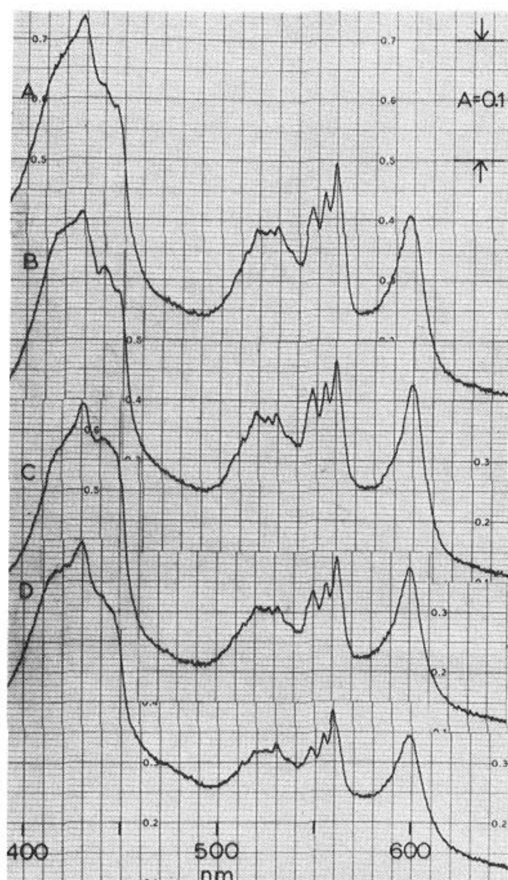

FIG. 4. Optical reflectance spectra of ETP_c particles from cells in the logarithmic or stationary phase of growth, reduced with NADH or dithionite, both in the presence of antimycin A. *Spectra A* and *B*, log phase ETP_c, reduced with dithionite and NADH, respectively; *C* and *D*, stationary phase ETP_c, reduced with dithionite and NADH, respectively. The protein concentrations were 37.5 mg per ml for *A* and *B* and 32 mg per ml for *C* and *D*. *Samples A, B, C,* and *D* are those whose EPR spectra are shown in Fig. 3, *C, B, F,* and *D*, respectively.

of NADH dehydrogenase are practically undetectable in exponential phase cells, as opposed to cells harvested in the stationary growth phase (Fig. 3, *Spectra B* and *C versus E* and *F*), whereas little change is observed in the intensity of the signals of Centers 3 and 4 (see enlarged *Spectra C* and *F*). Since we do not distinguish between these centers in the present work, we are unable to say whether both are present and whether or not one of these centers may, in fact, undergo significant changes. In exponential phase cells, however, there is present an unidentified species with resonances observed under reducing conditions at $g_{\parallel} = 2.01$ and $g_{\perp} = 1.92$ (Fig. 3, *Spectrum C*). According to these g values and other EPR characteristics, such as temperature and power sensitivity, these resonances are presumably those of an iron-sulfur center. We are not certain as to what functional unit the new species belongs. It is significant that it is at most 25% reduced by NADH in the presence of antimycin A (Fig. 3, *Spectrum B*). That this iron-sulfur center is not identical with either Center 1 or Center 2 is apparent from the absence of (Fig. 3, *Spectrum C*) the low field resonance ($g = 2.05$) characteristic of Center 2 and the absence of the peak at $g = 1.94$ (g_y), characteristic of Center 1. The new species has a temperature sensitivity similar to that of Center 1 rather than Center 2. Other resonances which are seen in Fig. 3 are the center line of the low spin heme of cytochrome *c* oxidase at $g = 2.26$ (peak resonance at $g = 2.27$) (in Fig. 3, *Spectra A* and *D*), the center of a *b*-type cytochrome at $g = 2.23$ (peak at $g = 2.24$), the corresponding high field line at $g = 1.87$, and possibly resonances of other low spin heme compounds between $g = 2.1$ and 1.87. Among the heme resonances, those of the *b*-type cytochrome ($g_z = 2.54$, $g_y = 2.23$, and $g_x = 1.87$) are the most prominent features and it is significant that this cytochrome appears to be present at much higher concentration in log phase cells than in cells grown to the stationary phase (Fig. 3, *Spectra A* and *B versus D* and *E*). The behavior of the cytochromes has also been followed by reflectance spectroscopy, where their reduced forms are readily detected (Fig. 4). It is also apparent from Fig. 4 that the cytochromes are not completely reduced by NADH in the presence of antimycin and that all cytochromes appear to be present in exponential phase cells at a significantly higher concentration than in stationary phase cells. The *b* cytochrome, which is so prominent in the EPR spectra, has an α-band located at 560 nm at 95° K. It is interesting to note that, despite the strong absorption indicating the presence of the reduced *b* cytochrome in Fig. 4, *Spectrum B*, for instance, there is nevertheless a strong signal at $g = 2.23$, indicating the simultaneous presence of sizeable amounts of the oxidized form (Fig. 3, *Spectrum B*). The significance of this cytochrome or of the changes in cytochrome concentrations which are, apparently, part of the metabolic adaptation taking place under the experimental conditions, is presently unknown. In addition to the resonances from heme and iron-sulfur proteins there is, except in samples reduced with dithionite, a strong signal in the center at $g = 2.02$. This signal is due to a protein with characteristics of a high-potential iron-sulfur protein.[2] There is little change in the concentration of this component during the transition between the growth phases of *C. utilis*.

Individual progress curves and EPR spectra on the changes of signals just described have been shown in a previous publication (9). Table I presents in tabular form the actual values of the components measured, in a number of experiments, including those on catabolite repression (see below). These components are: Centers 1 + 2 from the total excursion between $g = 1.92$ and $g = 1.94$, Center 2, when feasible, from the line at $g = 2.05$; Centers 3 + 4 from the line at $g = 1.86$, and the species with a signal at $g = 2.02$.

As shown in Table I, during the transition from the exponential to the stationary phase, the sharp rise in NADH-$Fe(CN)_6^{-3}$ activity (Fig. 2) is accompanied by an increase in the EPR signals evoked by NADH in ETP_c at $g = 1.94$ and 1.92, which represent iron-sulfur Centers 1 + 2 of the dehydrogenase.

The fact that the appearance of sensitivity to piericidin during the transition to the stationary phase is accompanied by a large increase in NADH-ferricyanide activity and in the EPR signals of the reduced iron-sulfur centers associated with this enzyme but a decline in the juglone reductase activity suggests that a different type of NADH dehydrogenase may be synthesized during this transition (rather than more of the same type of enzyme as occurs in the exponential phase). This interpretation is supported by the following observations.

In assays of NADH-$Fe(CN)_6^{-3}$ activity in ETP_c from exponential phase cells double reciprocal plots are biphasic, while in particles from late stationary phase cells such plots show no break (Fig. 5). Biphasic double reciprocal plots in this assay have been seen in ETP from heart mitochondria (22) and were interpreted to suggest the occurrence of two reaction sites for $Fe(CN)_6^{-3}$ in the respiratory chain, one with the dehydrogenase,

[2] F. J. Ruzicka and H. Beinert, manuscript in preparation.

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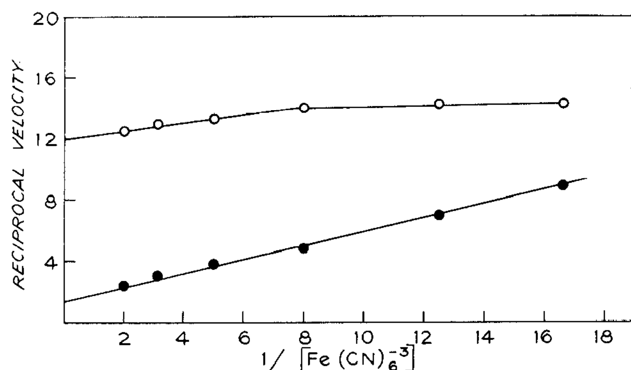

FIG. 5. Behavior of NADH dehydrogenase in ETP_c from exponential and stationary phase cells in the NADH-$Fe(CN)_6^{-3}$ assay. *Abscissa*, reciprocal concentration of $Fe(CN)_6^{-3}$, expressed in milliliters of 10^{-2} M dye per 3 ml of reaction volume; *ordinate*, absorbance change at 420 nm per min. Protein concentration was 0.23 mg per 3 ml in the exponential phase preparation (○) and 0.51 mg per 3 ml in the stationary phase preparation (●).

TABLE II

Effect of washing of submitochondrial particles on NADH dehydrogenase activity

The ETP_c samples were those used in the experiments of Fig. 2. Of each particle preparation 110 mg of protein were suspended in 15 ml of 1 mM 2-(*N*-morpholino)ethanesulfonate buffer-1 mM EDTA, pH 6.5, and allowed to stand for 5 min at 0°. The suspension then was centrifuged for 30 min at 144,000 × *g* and the pellet was resuspended to 3 ml in the same buffer. Two milliliters of this suspension were diluted in 10 ml of the same buffer, incubated for 5 min at 0°, and centrifuged as above. The pellet was resuspended to 2 ml in the same medium; 1 ml of this was diluted with 5 ml of 2-(*N*-morpholino)ethanesulfonate-EDTA and centrifuged again. The final pellet was suspended in 1 ml of buffer. Dehydrogenase assays are V_{max} values at 25°, oxidase assays polarographic measurements in 0.1 M P_i, pH 7.4, at 30°.

Treatment	Specific activity			
	Log phase cells		Stationary phase cells	
	NADH-$Fe(CN)_6^{-3}$ assay	NADH oxidase assay	NADH-$Fe(CN)_6^{-3}$ assay	NADH oxidase assay
	μmoles NADH × min⁻¹ × mg⁻¹			
None	1.4	0.8	5.6	0.27
After first wash	0.5	0.5	6.9	0.28
After second wash	0.3	0.2	7.3	0.22
After third wash	0.3	0.2	6.9	0.25

one in the cytochrome c-c_1 region, because antimycin A, Amytal, and rotenone abolished the break in the curve. This interpretation cannot explain the data for *C. utilis* particles, since inclusion of antimycin A in the assay, using particles from exponential phase cells, had no effect. More likely, this kinetic behavior is a property of the type of NADH dehydrogenase present in the exponential phase of growth. Further, successive washing of the exponential phase particles with low osmolarity buffer leads to extensive loss of NADH dehydrogenase, while the same treatment results in no loss of activity from stationary phase particles (Table II). The loss shown in Table II represents instability of the enzyme, rather than ease of extraction, since the supernatant solutions obtained on centrifugation of the exponential phase particles showed no detectable NADH-$Fe(CN)_6^{-3}$ activity.

NADH Dehydrogenase Activity and Piericidin Sensitivity during Catabolite Repression—It has been reported that the addition of ethanol to substrate-limited, late stationary phase cells produces a decline in the sensitivity of respiration to rotenone and piericidin (1). As documented elsewhere (9), this decline is accompanied by a major fall in NADH-$Fe(CN)_6^{-3}$ activity, while the specific activity of NADH oxidase and of NADH-juglone reductase rises during this period. The presence of cycloheximide prevented all these changes (9).

NADH-$Fe(CN)_6^{-3}$ assays in ETP_c obtained from cells which had been repressed 3 to 5 hours showed the same biphasicity as preparations from exponential phase cells. Since mitochondria from cells repressed with ethanol showed little or no respiratory control, the loss of coupling Site 1 could not be followed. It is clear from Table I, however, that the EPR signals attributed to iron-sulfur Centers 1 and 2 also decline after the addition of ethanol and that cycloheximide prevents this decline. The changes in the EPR signals of Centers 1 and 2, and in two experiments also of 3 + 4, in the NADH-reduced state at various times after the addition of ethanol, are summarized in Table I. When samples were reduced with dithionite, it also became apparent that the signal with lines at g = 2.01 and 1.92, observed in exponential phase cells on dithionite reduction, was present in ETP_c from stationary phase cells repressed with ethanol. As already noted, the signal is not that of Center 1 or 2 and may represent an unknown species. Concomitant with these changes there was an approximately 2-fold increase in a *b*-type cytochrome (*cf.* Fig. 4). With cells grown on glucose this cytochrome was present at a much lower level than in cells grown on ethanol.

Figs. 6 and 7 show typical EPR spectra of samples from experiments with cells grown to the late stationary phase on glucose. In ETP_c from cells grown on the complex glucose medium of Biggs *et al.* (11) the specific activity of NADH dehydrogenase is considerably higher than in cells grown on ethanol. The EPR signals of NADH dehydrogenase were therefore expected to be more distinct.

As mentioned above, the low level of *b*-type cytochromes in cells grown on glucose was very helpful in determining the behavior of Centers 3 + 4. Fig. 6 shows the EPR spectra of samples withdrawn immediately before ethanol addition and 6 hours thereafter, oxidized, and reduced with NADH or dithionite, both in the presence of antimycin A. The features discussed above, *viz.* extensive disappearance of Centers 1 and 2, partial disappearance of Centers 3 + 4, and appearance of the new species with $g_\parallel = 2.01$; $g_\perp = 1.92$ can be seen in Fig. 6 at good signal to noise ratio. In addition, the species responsible for the strong and highly temperature sensitive signal at g = 2.02 seen in the oxidized and NADH-reduced samples (*cf.* Fig. 3) clearly increases with catabolite repression.

It is interesting to compare Fig. 3, showing the events on transition between growth phases with the similarly organized Fig. 6, which depicts events brought about by catabolite repression. In many respects Fig. 6 is the inverse of Fig. 3, *i.e.* the events in going from the log to the stationary growth phase are the reverse of those seen in going from the stationary phase to the catabolite-repressed state. This applies to the appearance and disappearance of iron-sulfur Centers 1 and 2 and the opposite behavior of a new (presumably iron-sulfur) species and changes in the concentrations of cytochromes. Most prominent among these is a *b*-type cytochrome, which is readily detected by EPR spectroscopy. Fig. 7 shows expanded and enlarged scans of the high field region of the iron-sulfur protein spectra, to demonstrate unambiguously the behavior of the line at g = 1.86, which we have taken as a measure of the presence of Centers 3 + 4. This line is clearly absent in the oxidized state (*top*

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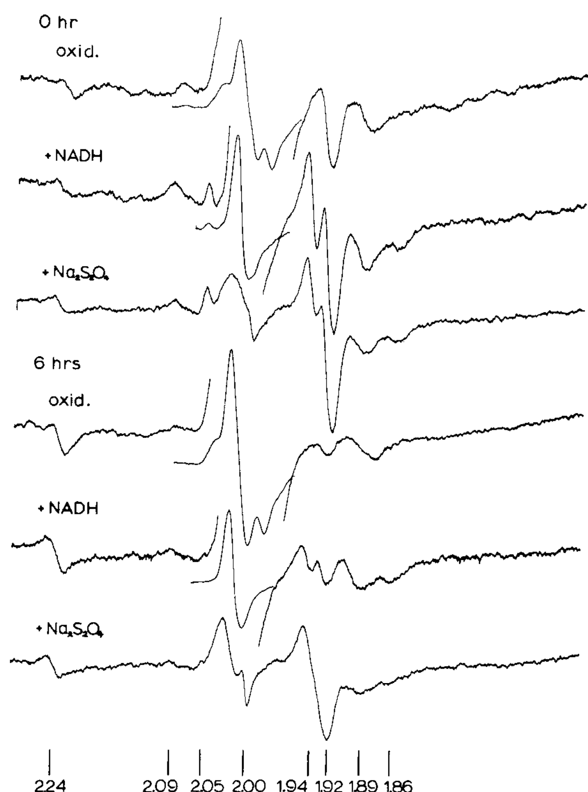

FIG. 6. EPR spectra of ETP_c particles from cells grown on glucose and then exposed to ethanol. The cells were grown to the late stationary phase (24 hours in this medium) with 16 liters per min forced air flow. The cell suspension (10 mg dry weight of cell per ml) was then diluted with 3 volumes of growth medium, less glucose, and 10 liters of the diluted suspension were aerated at 24 liters per min at 30° in the presence of 3% (v/v) ethanol. The spectra shown are from samples obtained immediately before addition of ethanol (0 hours) and 6 hours after addition. The conditions are as indicated. Antimycin A had been added to all samples reduced with NADH or $Na_2S_2O_4$. The conditions of EPR spectroscopy were as given under "Experimental Procedure." For quantitative comparison corrections arising from differences in protein concentrations and instrument settings require multiplication of the signal heights with the following coefficients (from top to bottom): 1.0, 1.0, 1.25, 0.93, 0.75, and 0.93. For the *insets* (g ~ 2) these coefficients are: 1.0, 0.79, 0.94, 1.51, for the first, second, fourth, and fifth spectra, respectively.

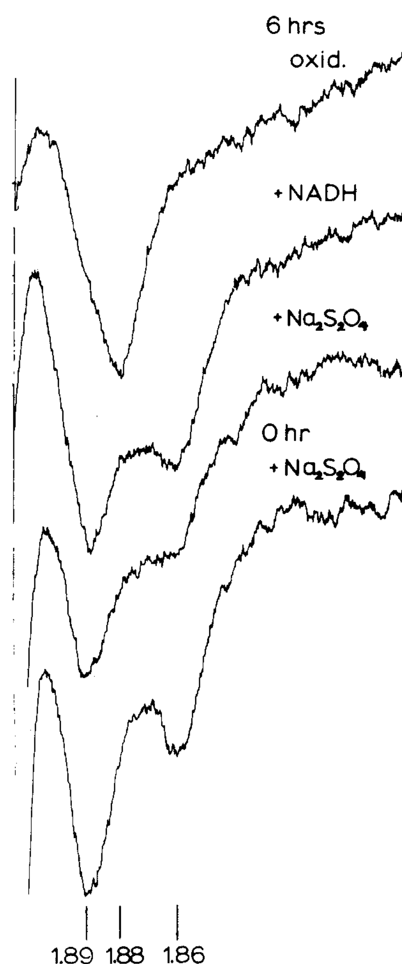

FIG. 7. High field part of EPR spectra of samples from the experiment of Fig. 6, showing the changes occurring in iron-sulfur Centers 3 + 4. The conditions of the experiments are as in Fig. 6. The conditions of EPR spectroscopy were the following: eight spectra were accumulated in the Nicolet 1020 signal averager at high amplifier gain but otherwise the conditions were as given under "Experimental Procedure." For quantitative comparison the signal heights require multiplication by the following coefficients (from top to bottom): 1.0, 1.14, 1.0, 1.51.

curve), ensuring that there are no heme absorptions superimposed which would interfere with an evaluation of the resonances due to Centers 3 + 4. As seen from the remaining spectra, the line at g = 1.86 is decreased during catabolite repression, but no more than 50 to 60% from its original value (*cf. bottom curve*). This contrasts to the decrease in Centers 1 and 2 (Fig. 6) which is close to 90%.

The changes in catalytic activities in the transition from the exponential to the stationary phase, although not documented, were similar to those illustrated in Fig. 2 for ethanol-grown cells, but were even more pronounced with glucose as the carbon source during growth. The decline of NADH-$Fe(CN)_6^{-3}$ activity and of sensitivity to piericidin and the increase in NADH oxidase activity on the addition of ethanol to such glucose-grown cells were also comparable to the events observed on the addition of ethanol to cells grown on ethanol.

DISCUSSION

The data presented in this paper demonstrate that the acquisition of piericidin sensitivity and of energy coupling at Site 1 during the transition of exponential phase cells of *C. utilis* to stationary phase cells is accompanied by a large increase in NADH dehydrogenase activity, as measured by the ferricyanide assay, and of EPR signals from substrate reducible iron-sulfur centers of the enzyme. The qualitative differences noted between the "internal" NADH dehydrogenases of exponential and stationary phase cells further suggest that a different type of enzyme is present in these two phases of growth. The dehydrogenase present in inner membrane preparations from stationary phase cells resembles closely the mammalian enzyme in catalytic properties (11), including, as shown in this paper, high reactivity with ferricyanide but low juglone reductase activity, in stability, and in the presence of the EPR signals of Centers 1, 2, and 3 + 4. Little is known about the type of NADH dehydrogenase present in exponential phase cells, except that, in contrast to the stationary phase enzyme, it appears to react with juglone and ferricyanide at similar rates, that it seems to lack iron-sulfur Centers 1 and 2, and that it appears to be more labile than the stationary phase enzyme under certain conditions. Further, it is not coupled to phosphorylation Site 1 and is insensitive to piericidin and rotenone. On the addition of oxidizable substrates, such as ethanol, the stationary phase enzyme disap-

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pears and a dehydrogenase resembling that present in exponential phase cells appears.

Disappearance of the stationary phase enzyme has also been observed on adding 2% (w/v) glucose in lieu of ethanol. As in the catabolite repression of succinate dehydrogenase induced by the addition of glucose to aerobic cells of *Saccharomyces cerevisiae* (23), the process appears to involve active destruction of the enzyme present prior to addition of the catabolite. One or more of the enzymes involved in this event are synthesized extramitochondrially, as judged by the fact that in *S. cerevisiae*, as well as in *C. utilis*, catabolite repression is blocked by cycloheximide.

The experiments presented also bear on arguments in the literature (5, 6) as to whether Site 1 of energy conservation may be dissociated from sensitivity to piericidin in *C. utilis*. The technique used to explore this question was aeration of cells grown in a chemostat on iron- or sulfur-limited media. Aeration of such cells, with or without addition of the limiting component, was said to induce only coupling Site 1 (5) or both this site and sensitivity to piericidin (6). Prior to the observations reported in the present paper, it was difficult to see how the mere process of aeration would lead to the acquisition of either Site 1 or of piericidin sensitivity. Since in the experiments referred to (5, 6) the cells were almost certainly in the exponential phase (particularly in studies where O_2 supply was limited (6)), it seems likely that aeration in the absence of substrate transformed the cells to the stationary phase type, in which NADH dehydrogenase is endowed with both coupling Site 1 and sensitivity to piericidin. While the authors do not wish to negate the possibility that coupling Site 1 and sensitivity to piericidin might be dissociated under appropriate conditions, they wish to call attention to the likelihood that in this type of experiment, superimposed on the effect of iron or sulfur limitation, a transition from exponential phase cells to the stationary phase type may occur, with consequent differences in the NADH dehydrogenase present.

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