Miura, K. (1964), *J. Biochem. (Tokyo) 55*, 452.
Neu, H. C., and Heppel, L. A. (1964), *J. Biol. Chem. 239*, 2927.
Plesner, P. (1955), *Acta Chem. Scand. 9*, 197.
Ralph, R. K., Young, R. J., and Khorana, H. G. (1963), *J. Am. Chem. Soc. 84*, 1490.
Singer, M. F., and Cantoni, G. L. (1960), *Biochim. Biophys. Acta 39*, 182.
Singh, H., and Lane, B. G. (1964a), *Can. J. Biochem. 42*, 87.
Singh, H., and Lane, B. G. (1964b), *Can. J. Biochem. 42*, 1011.
Smith, J. D., and Dunn, D. B. (1959a), *Biochim. Biophys. Acta 31*, 573.
Smith, J. D., and Dunn, D. B. (1959b), *Biochem. J. 72*, 294.
Zillig, W., Schachtschabel, D., and Krone, W. (1960), *Z. Physiol. Chem. 318*, 100.

Biochemical Correlates of Respiratory Deficiency. IV. Composition and Properties of Respiratory Particles from Mutant Yeasts*

Bruce Mackler†, Howard C. Douglas, Susan Will, Donald C. Hawthorne, and Henry R. Mahler‡

ABSTRACT: Respiratory deficient mutant yeasts (strains p_8, p_{13}, p_{12}, and a cytoplasmic mutant) have been studied in comparison with wild-type yeast. The mutant yeasts differ from the wild type in that they contain at most only traces of cytochromes *a* and a_3 and relatively low amounts of cytochromes *b*, *c*, and c_1 (with the exception of mutant p_{12} which contains normal amounts of cytochromes *c*, c_1, and *b*). Respiratory particles prepared from the mutant yeasts contain no cytochrome *a* or a_3 and very low amounts of cytochromes *b*, *c*, and c_1 (again with the exception of the electron-transport particles from mutant p_{12} which contain appreciable amounts of cytochromes *c* and c_1 but no cytochrome *b*). In addition, the respiratory particles from the mutant yeasts contain highly elevated levels of non-heme iron normal concentrations of copper, and low values of flavin and coenzyme Q as compared to the electron-transport particles prepared from wild-type yeast. Respiratory particles prepared from wild-type yeast harvested in the logarithmic phase of growth resemble the mutant respiratory particles in that the content of non-heme iron is high and levels of flavin and coenzyme Q are low compared to levels found in ETP from wild-type yeast in the stationary phase. The ETP's from the mutant yeasts show only traces of activity as DPNH and succinic oxidases and cytochrome *c* reductases, but are able to function as indophenol reductases with DPNH and succinate as substrates.

Previously, we have reported the isolation of electron-transport particles[1] (ETP) from wild-type *Saccharomyces cerevisiae* and from a cytoplasmic mutant strain (Mahler *et al.*, 1964a) and established that, although the latter is respiratory deficient (cannot grow aerobically on nonfermentable substrates), the primary dehydrogenases for D-lactate, L-lactate, succinate, and diphosphopyridine nucleotide (DPNH) are all present (Mahler *et al.*, 1964a). Furthermore, the ETP prepared from the cytoplasmic mutant, although lacking oxidase activity for these substrates, binds or reacts with an antiserum prepared against ETP from wild-type or respiratory-competent cells (Mahler *et al.*, 1964b), suggesting that at least some of the protein components of the two ETP's are similar. In the present communication, the composition and enzymatic properties of ETP prepared from several respiratory-deficient mutant strains and from wild-type *S. cerevisiae* will be reported. The mutants examined included both cytoplasmic (Ps) and segregational (or genic) ($P \times s^+/s^-$) types (Sherman, 1963; Sherman and Slonimski, 1964). The mutant strains were grown in media containing several different carbohydrates, *i,e.*, sucrose and galactose, to rule out possible differences due to glucose repression (Ephrussi *et al.*, 1956; Slonimski, 1956; Strittmatter, 1957; Polakis *et al.*, 1964; Tustanoff and Bartley, 1964a,b; Schatz, 1963).

* From the Departments of Pediatrics, Microbiology, and Genetics, University of Washington, Seattle, and the Department of Chemistry, Indiana University, Bloomington. *Received June 4, 1965; revised July 19, 1965.* Supported in part by a grant (H-5457) from the National Heart Institute and a grant from fund 171, University of Washington, and in part by a grant (PHS GM 12228) from the National Institute of General Medical Sciences.

† Research Career Development Awardee of the National Institutes of Health.

‡ Recipient of a Research Career Award (No. PHS GM 05060) from the National Institutes of Health.

[1] Abbreviations used in this work: ETP, electron-transport particle; DPNH, diphosphopyridine nucleotide, reduced form.

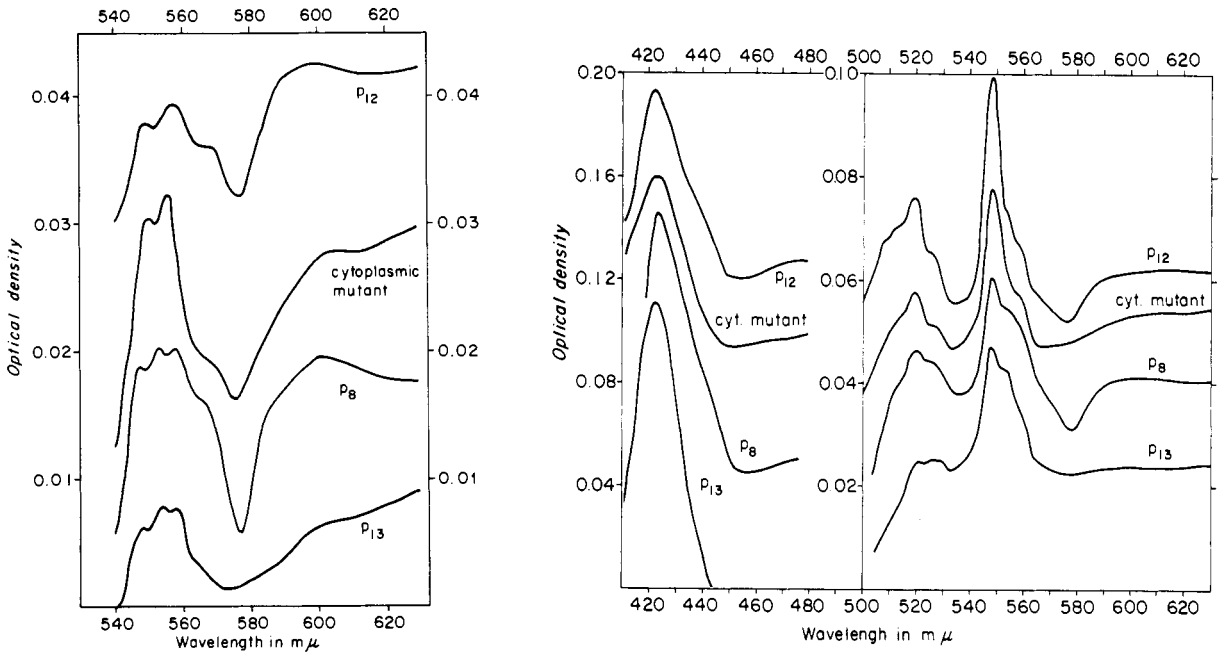

FIGURE 1: Low-temperature spectra of mutant yeasts. (a) Difference spectra (hydrosulfite reduced minus oxidized) were recorded at the temperature of liquid nitrogen as described by Estabrook and Mackler (1957a). (b) Difference spectra were recorded as in (a) with the difference that 0.01 ml of 3% H_2O_2 was added to the oxidized samples before freezing.

Experimental

ETP was prepared as described by Mahler *et al.* (1964a); the particles here referred to as ETP correspond to the fraction called R_2L_w in that earlier paper. The spectrophotometric and polarographic assays for DPNH oxidase, succinoxidase, DPNH cytochrome *c* reductase, succinate cytochrome *c* reductase, DPNH dehydrogenase, and succinic dehydrogenase were performed at 38° as described previously (Mahler *et al.*, 1964a). Dehydrogenase activity was measured using 2,6-dichlorophenolindophenol (indophenol) as electron acceptor. Methods for the estimation of the concentrations of cytochromes, protein, flavin, iron, copper, lipid, and coenzyme Q (ubiquinone) were modifications of those described previously by Mackler *et al.* (1962).

The sources of the chemicals were the same as described by Mahler *et al.* (1964a). Commercial yeast (Fleischmann's) was obtained from Standard Brands, Inc. The respiratory-deficient cytoplasmic mutant was isolated from cultures of the commercial yeast treated with acriflavine as described by Slonimski (1953). The respiratory deficient nuclear mutants p_{13}, p_8, and p_{12} were strains from the collection maintained by Dr. Hawthorne. Wild-type and mutant yeasts were grown in 10 l. of media of pH 6 containing 10 g of KH_2PO_4, 5 g of $MgSO_4 \cdot 7H_2O$, 10 g of $(NH_4)_2SO_4$, 20 g of Difco yeast extract, and 6.04 g of anhydrous sodium acetate, in addition to a carbohydrate, at 28° with vigorous aeration. The carbohydrate source differed in the various experiments and consisted of either 5 or 1% sucrose, or 1% galactose. In experiments in which the yeast was grown in the presence of galactose, 10 ml of Tween 80, 100 mg of ergosterol, and 100 g of yeast extract were added to the media.

At the time of harvest the cultures were examined microscopically for bacterial contamination. Reversions in the nuclear mutants to the respiratory-sufficient condition were measured by plating on lactate agar. In all cases values of less than 1 in 10^6 cells were observed. The fraction of cells in the nuclear mutants containing the cytoplasmic respiratory factor was taken as the fraction of cells that would complement with a cytoplasmic mutant to form respiratory-sufficient diploids. In p_8 and p_{13} this was approximately 50 and 90%, respectively, and in p_{12} it was zero. The wild-type yeast was harvested either at the middle of the log phase of growth or 4–5 hr after having reached the stationary phase. The mutant yeasts were harvested in the stationary phase after reaching cell populations similar to those of the stationary wild-type cultures at harvest time.

Results

Composition of the Intact Mutant Yeasts. The cytochrome composition of the respiratory-deficient mutant yeasts grown aerobically in 1% sucrose and harvested during the stationary phase was determined qualitatively from difference spectra (hydrosulfite reduced minus oxidized) of suspensions of the various yeasts recorded at the temperature of liquid nitrogen (Estabrook and Mackler, 1957a,b), as shown in Figure 1. All mutants contained cytochrome *c* (absorption maxi-

TABLE I: Cytochrome Content of Various Yeasts Grown in 1% Sucrose and Harvested during the Stationary Phase.

Yeast	Cytochrome Concn. (mμmole/mg protein)		
	$c + c_1$	b	$a + a_3$
Wild Type	0.40	0.20	0.15
Cytoplasmic mutant	0.15	0.06	0
p_{13}	0.10	0.04	0
p_8	0.09	0.05	0
p_{12}	0.49	0.25	0

mum at 549 mμ), cytochrome c_1 (absorption maximum or shoulder at 553–554 mμ), and a type b cytochrome (absorption maximum at 556–558 mμ). The small absorption maximum at 600 mμ seen in all spectra in Figure 1a may represent trace amounts of cytochromes $a + a_3$. When a small amount of hydrogen peroxide was added to the oxidized sample to ensure complete oxidation of all cytochromes, and the spectra (reduced minus oxidized) were again recorded at liquid nitrogen temperatures, much larger amounts of cytochrome c were found as shown in Figure 1b, suggesting that although most of cytochromes b and c_1 were present in an oxidized form a major portion of the cytochrome c in the mutant yeast cells was present in the reduced form. The concentrations of the various cytochromes were determined from difference spectra (hydrosulfite reduced minus hydrogen peroxide oxidized) of suspensions of the yeasts recorded at room temperature as previously described by Estabrook and Mackler (1957b), and the values are shown in Table I. As shown in the table the wild type yeast contained more of all cytochrome types than did the mutant yeasts, but the p_{12} mutant although lacking cytochromes $a + a_3$ differed from the other mutants in having normal amounts of cytochromes c, c_1, and b. When the mutant yeasts were grown aerobically in media containing 1% galactose (instead of sucrose) as described in the section on Methods, their cytochrome content was the same as described above for growth in sucrose, *i.e.*, even in the latter medium the cells were completely derepressed, at least with respect to their complement of respiratory carriers.

Properties of Isolated Respiratory Particles (ETP). The rates of various enzymatic activities are shown in Table II for ETP isolated from commercial yeast and wild-type and mutant yeasts grown in our laboratory as described in the section on Methods. As shown in the table the activities of the ETP prepared from commercial yeast and aerobically grown wild-type yeast harvested in the stationary growth phase were essentially the same. However, all ETP's from the mutant yeasts showed only traces of oxidase or cytochrome c reductase activities, but did possess some indophenol reductase activity with both DPNH and succinate as substrates, in accord with results previously published in studies of the cytoplasmic mutant (Mahler *et al.*, 1964a). When ETP was prepared from wild-type yeast harvested in logarithmic phase of growth instead of the stationary phase, the pattern of activities closely resembled those of the mutant yeasts.

Difference spectra (hydrosulfite reduced minus oxidized) of preparations of ETP from the mutant yeasts recorded at both room temperature and liquid nitrogen temperature showed that the ETP's contained only traces of cytochromes c and b with the exception of the ETP prepared from mutant p_{12} which contained

TABLE II: Activities of ETP from Wild-Type and Mutant Yeasts.[a]

Yeast ETP Preparation	Activity (μmole Substrate/mg/min)					
	DPNH→O_2	Succinate→ O_2	Cytochrome c Reduction		Indophenol Reduction	
			DPNH	Succinate	DPNH	Succinate
Commercial yeast	3.4	1.3	0.57	0.30	0.59	0.09
Wild-type yeast sucrose 1% stationary phase	2.0	0.40	0.43	0.15	0.45	0.15
Wild-type yeast 1% sucrose log phase (a)	0.10	<0.01	0.05	0.01	0.17	0.01
Cytoplasmic mutant 1% sucrose	0.02	<0.01	0.01	<0.01	0.12	0.01
Cytoplasmic mutant 5% sucrose (b)	0	0	0.01	<0.01	0.07	0.01
p_8 mutant 1% sucrose	0.02	<0.01	0.01	<0.01	0.10	0.01
p_{13} mutant 1% sucrose	0.02	<0.01	0.03	<0.01	0.13	0.01
p_{12} mutant 1% sucrose	0.01	0	0.05	<0.01	0.13	0.01

[a] All values represent the averages of data from 3 or more preparations, with the exception of a and b.

TABLE III: Composition of ETP from Grande and Mutant Yeasts.[a]

Yeast ETP Preparation	Fe[b] (non-heme)	Cu[b]	Total Flavin[b]	Acid[b] Heat-Labile Flavin	Cytochromes[b] $c + c_1$	Cytochromes[b] b	Cytochromes[b] $a + a_3$	CoQ[b]	Total Lipid (% dry wt)
Commercial yeast	3.1	1.5	0.72	0.40	0.45	0.48	0.47	2.2	37
Wild-type yeast sucrose 1% stationary phase	2.6	1.0	0.95	0.36	0.61	0.77	0.78	1.6	28
Wild-type yeast 1% sucrose log phase (a)	17.5	2.2	0.57	..	0.30	0.32	0.14	0.21	13
Cytoplasmic mutant 1% sucrose	16.7	1.5	0.48	0.13	0.08	0	0	0.37	23
Cytoplasmic mutant 5% sucrose (b)	3.2	1.4	0.19	0.09	0.01	0	0	0.51	18
p_8 mutant 1% sucrose	12.1	1.7	0.44	0.07	0.06	0.05	0	0.20	18
p_{13} mutant 1% sucrose	9.5	1.3	0.32	0.11	0.06	0.05	0	0.21	26
p_{12} mutant 1% sucrose	13.5	3.2	0.27	0.17	0.26	0	0	0.40	27

[a] All values represent the averages of data from three or more preparations, with the exception of a and b. [b] mμ-mole/mg of protein.

relatively high amounts of cytochromes *c* and c_1. The ETP preparations differed from the intact yeasts in that the cytochromes were now present in a fully oxidized state, perhaps due to removal of endogenous substrate during the course of the preparation of the particle.

The composition of the ETP's derived from the various yeasts with respect to various respiratory carriers is shown in Table III. ETP's from commercial and wild-type yeast harvested in the stationary phase are similar; however, the composition of ETP's from the mutant yeasts differ from that of the wild type in several ways. Cytochromes *a* and a_3 were absent from all mutant ETP's and cytochromes *b*, *c*, and c_1 were present in only very small amounts, with the exception of the ETP from p_{12} which contained appreciable cytochrome *c* and c_1. Copper was present in normal amounts in all ETP's, but coenzyme Q and flavin although present in the mutant ETP's were found in reduced amounts. Of particular interest were the greatly elevated concentrations of non-heme iron found in the ETP's from all mutant yeasts. The ETP prepared from the wild-type yeast harvested in the logarithmic growth phase resembled the mutant ETP's in having an elevated level of non-heme iron and relatively low levels of flavin and coenzyme Q. However, when the cytoplasmic mutant was grown in a medium containing 5% sucrose (a level which caused marked repression of the oxidative systems of the wild type), ETP's prepared from the mutant contained low levels of non-heme iron similar to those of the ETP's from commercial and stationary wild type yeasts.

Discussion

The mutant yeasts reported above and the ETP's prepared from them differ in several ways from wild-type yeasts grown under similar conditions and their derived ETP's. The most striking difference between the mutant and wild-type yeasts is the absence of cytochromes *a* and a_3 in all the mutants. In this respect and in the lowered amount of cytochrome *b* observable in the mutant cells tne results of quantitative spectrophotometry at room temperatures neatly confirm and complement those obtained by Sherman and Slonimski (1964) by means of spectroscopy at liquid nitrogen temperatures. The one major discrepancy between the two sets of results, namely, the presence of significant amounts of cytochrome *b* in cells of our cytoplasmic mutant (P_S^-) as compared to its complete absence in an analogous mutant examined by Sherman and Slonimski (*cf.* spectrum II of their Figure 3), may be due to actual differences either in the strains utilized or, more likely, in culture conditions and the extent of residual glucose repression.

Although mutant *cells* contain cytochromes *b*, *c*, and c_1 in appreciable amounts, the corresponding respiratory particles frequently are almost completely lacking in the same components (Table I compared to Table III). These observations suggest that one of the consequences of the mutations to respiratory deficiency may be an inability to bind or integrate cytochromes into the mitochondrial membranes, leading to detachment of the carriers in the course of isolation of respiratory particles. This appears to be especially true of cytochrome *b*, since in mutant p_{12}, an organism apparently capable of synthesizing all cytochromes except *a* and a_3 in normal amounts, an appreciable amount of cytochrome *c* plus c_1 is retained in the respiratory particle. The interpretation of these observations is rendered more difficult, however, by the fact that it is this very strain which, among all the genic mutants described here, is the only one which also

lacks the cytoplasmic factor required for respiratory sufficiency, *i.e.*, it is of the genotype $p_{12}s^{-}$. Conversely then one is struck by the fact that, contrary to earlier expectations, loss of this factor does *not* invariably result in a complete inability to produce cytochromes *b* and c_1.

ETP's derived from the mutant yeasts contain flavin (both total and acid extractable) and coenzyme Q in amounts considerably lower than those found in the corresponding particles isolated from wild-type cells in the stationary phase, but resembling those observed in particles obtained from the same cells but in the logarithmic phase. Similar observations for coenzyme Q and other lipids in whole cells have already been reported by us previously. There appears to be a good correlation between the amounts of these components found in the particles and their ability to function as indophenol (or other acceptor) reductases.

The significance of the highly elevated concentration of non-heme iron found in respiratory particles isolated from the mutant yeasts is not immediately obvious at this time. Since, however, the particles obtained from wild-type yeasts harvested in the logarithmic phase of growth also contain similar high levels of this component, and in this respect as well as in their flavin, coenzyme Q, and dehydrogenase content resemble the particles from mutant yeast, it is possible that all these properties characterize the state of an electron-transport system just prior to the elaboration by the yeast cell of fully functional respiratory particles. The relevance of this hypothesis to the problem of mitochondriogenesis and the possible association of the various carriers and other components described with various mitochondrial precursors (Schatz, 1963; Schatz *et al.*, 1964; Linnane, 1964) are currently under investigation.

Acknowledgments

The authors wish to thank Standard Brands, Inc., for generous supplies of Fleischmann's yeast, and Mrs. Helen Talbott for her valuable technical assistance.

References

Ephrussi, B., Slonimski, P. P., Yotsuayanagi, Y., and Tavlitzki, (1956), *Compt. Rend. Trav. Lab. Carlsberg, Ser. Physiol. 26*, 87.

Estabrook, R. W., and Mackler, B. (1957a), *J. Biol. Chem. 224*, 637.

Estabrook, R. W., and Mackler, B. (1957b), *J. Biol. Chem. 229*, 1091.

Mackler, B., Collipp, P. J., Duncan, H. M., Rao, N. A., and Huennekens, F. M. (1962), *J. Biol. Chem. 237*, 2968.

Mahler, H. R., Mackler, B., Grandchamp, S., and Slonimski, P. P. (1964a), *Biochemistry 3*, 668.

Mahler, H. R., Mackler, B., Slonimski, P. P., and Grandchamp, S. (1964b), *Biochemistry 3*, 677.

Polakis, E. S., Bartley, W., and Meek, G. A. (1964), *Biochem. J. 90*, 369.

Schatz, G. (1963), *Biochem. Biophys. Res. Commun. 12*, 448.

Schatz, G., Tuppy, H., and Klima, J. (1964), *Z. Naturforsch. 18b*, 145.

Sherman, F. (1963), *Genetics 48*, 375.

Sherman, F., and Slonimski, P. P. (1964), *Biochim. Biophys. Acta 90*, 1.

Slonimski, P. P. (1953), La Formation des Enzymes Respiratories chez la Levure, Paris, Masson.

Slonimski, P. P. (1956), *Proc. 3rd Intern. Congr. Biochem., Brussels*, 242.

Strittmatter, C. F. (1957), *J. Gen. Mircobiol. 16*, 169.

Tustanoff, E. R., and Bartley, W. (1964a), *Biochem. J. 91*, 595.

Tustanoff, E. R., and Bartley, W. (1964b), *Can. J. Biochem. 42*, 651.