# Biochemical Correlates of Respiratory Deficiency. I. The Isolation of a Respiratory Particle*

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A procedure is described for the preparation of a respiratory subparticle from yeast suitable for either small-scale or large-scale work and leading to a reproducible well-characterized fraction from commercial sources as well as from laboratory strains of both wild and respiratory-deficient (*petite colonie*) *Saccharomyces cerevisiae*. About 400 mg (protein) of this fraction is obtained per kg wet weight of commercial yeast, capable of oxidizing approximately 3.5 μmoles of DPNH, 1 μmole of succinate, and 0.1–0.2 μmole of D- or L-lactate per mg at 38° with oxygen as acceptor. The particle also catalyzes the appropriate partial reactions, including the primary dehydrogenase, cytochrome-reductase, and cytochrome c–oxidase reactions, with high efficiency. It contains cytochrome (a + $a_3$), cytochrome (c + $c_1$), cytochrome b, and total flavin in roughly equimolar proportions. Electron transport between substrate and cytochrome oxidase takes place virtually exclusively with particle-bound (internal) cytochrome c in separate sequences for succinate and DPNH, and solely with added soluble (external) cytochrome c with both D- and L-lactate. Extracts and particles of *petite* mutants are devoid of cytochrome (a + $a_3$) and cytochrome b, are incapable of catalyzing the reduction of cytochrome c by DPNH or succinate and the oxidation of reduced cytochrome c by oxygen, but exhibit a pattern of primary dehydrogenases similar to those of the wild type.

A number of different investigations currently in progress in our various laboratories all require the ready availability, in relatively large amounts, of the complex of respiratory enzymes, originally associated with the mitochondria, of *Saccharomyces cerevisiae* in as "native" and undegraded a form as possible. Although several procedures have been published (Utter *et al.*, 1958; Vitols *et al.*, 1961; Nygaard, 1961; Somlo, 1962; Mackler *et al.*, 1962) which describe the isolation and properties of such respiratory particles, we have found all of them to be unsatisfactory from the point of view of one or more of the criteria enumerated below.

A satisfactory procedure must conform to the following: (a) It must be rapid and the manipulations involved must be simple so that it may readily be adapted to both large- and small-scale operation. (b) It must be versatile: The same procedure (perhaps with minor modifications) must be applicable to different strains, grown under a variety of conditions; it should give comparable results with fresh, stored, or frozen cells. (c) It must be gentle: It must avoid as much as possible extremes of *p*H, temperature, harsh chemicals, enzymes, and detergents, all of which may affect the primary, secondary, and tertiary structure of the various constituent enzymic proteins and thus "modify" their properties. (d) It must be reproducible: The particulate enzyme complex so isolated must be easily characterizable by readily available techniques and replicate isolations, either from the same batch of cells or from one grown under similar conditions, must yield comparable results. (e) As a corollary to (d), differences between particles isolated by the same method but from different cell populations can then be regarded as significant.

The present publication describes the isolation and properties of such a complex of respiratory enzymes, or electron-transport particle, by a method which we believe conforms to all the above criteria. The differences and similarities between the particles isolated from wild type, respiratory-sufficient, and mutant, respiratory-deficient (cytoplasmic *petites*) (Slonimski, 1953) will be described and discussed.

## EXPERIMENTAL PROCEDURE

The polarographic and spectrophotometric assays for DPNH oxidase, succinate oxidase, cytochrome c oxidase, DPNH → cytochrome c reductase, and succinate → cytochrome c reductase were performed with prewarmed solutions in thermostatically controlled water baths or cell compartments kept at 29.0° or 38° (as indicated) in final volumes of 1.0 ml in the manner described previously (Mackler *et al.*, 1962). For D- and L-lactate dehydrogenase with cytochrome c as acceptor the conditions were those described by Somlo (1962). For the polarographic estimation of D-lactate oxidation by oxygen the amount of buffer used was lowered to 0.05 ml of the 0.2 M potassium phosphate and the cytochrome c was increased to 0.1 ml of a 1% solution; for L-lactate the buffer was that described (Mackler *et al.*, 1962), but the higher cytochrome c level was employed. Substrate concentrations of D- and L-lactate were 0.05 ml of a 0.20 M solution. For 2,6-dichlorophenol-indophenol-reductase activities 0.10 ml of a 0.01% solution of

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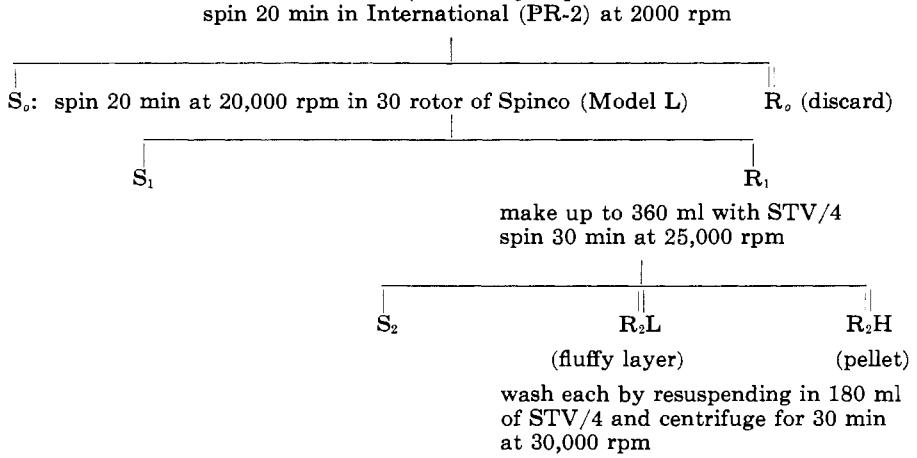
SCHEME I

PURIFICATION SCHEME FOR YEAST RESPIRATORY PARTICLES




* Time adjusted to liberate ≪20% of total protein. All operations at 2 ± 2°; resuspension of homogenates were by hand using Potter-Elvehjem homogenizers with Teflon pestles. In the separation of the three final fractions, $S_2$ was removed by decantation; $R_2L$ then constitutes a fluffy coherent layer which can easily be separated from the sedimented $R_2H$.

the dye was substituted for cytochrome c in the spectrophotometric-assay systems. When phenazine methosulfate was employed it was added in amounts of 0.02 ml of a 1% solution. Spectra were obtained with either the Cary Model 15 or a sensitive wavelength-scanning spectrophotometer (Estabrook, 1956). Routine assays were performed with a Beckman Model DU or Zeiss Model PMQ spectrophotometer. Extinction coefficients and wavelengths used were: for DPNH, 340 mμ ($\epsilon_{mM}$ = 6.30); for 2,6-dichlorophenol indophenol, 600 mμ ($\epsilon_{mM}$ = 19.1); for cytochrome c, 550 mμ ($\Delta\epsilon_{mM\ red-ox}$ = 19.1); for reduced cytochrome a absorbance at 630 subtracted from that at 605 mμ ($\Delta\epsilon_{mM}$ = 16.5); for reduced cytochrome $a_3$ absorbance at 454 subtracted from that at 444 mμ ($\Delta\epsilon_{mM}$ = 180); for reduced cytochrome b absorbance at 575 subtracted from that at 563 mμ ($\Delta\epsilon_{mM}$ = 20); cytochrome ($c + c_1$) at 550–553 corrected for absorbance at 540 mμ ($\Delta\epsilon_{mM}$ = 19.1). Methods for the estimation of protein, flavin, nonheme iron, and copper were those described by Mackler *et al.* (1962).

The sources of the chemicals used (all of the highest purity obtainable) were as follows: DPNH horse-heart cytochrome c (Type III) and ADP from Sigma Chemical Co.; D- and L-lactate isomers from California Corp. for Biochemical Research; Seconal from Eli Lilly and Co.; antimycin A from the Wisconsin Alumni Research Foundation. Glass beads were either from Prismo-France (diameter 50–150 μ) or Superbrite size 071 (Minnesota Mining and Mfg., Co.)., pretreated and washed as described by Somlo (1962). The commercial yeasts were obtained from the following sources: Springer Brand; Fleischmann's from Standard Brands, Inc.; and Red Star from Red Star Yeast Co., Milwaukee, Wis. Yeast strain 59 R (grande) and 59 RA (cytoplasmic *petite* obtained from 59 R by the acriflavine technique) are standard strains from P.P.S.'s collection. Growth conditions and medium S were those described by Somlo (1962).

*Breakage of Cells and Isolation of Cell-free Extract*

*Small-Scale Isolation.*—Baker's yeast (20–100 g, wet wt) was washed twice with distilled water by centrifugation and suspended in STV[1] to give a final volume (in ml) equal to the amount of yeast used initially. An equal volume of glass beads was then added and the suspension, kept at 0° by immersion in an ice bath, was homogenized in the small-scale stainless-steel cup of a Lourdes Multi-Mixer for 6 minutes at half speed (setting of 50), an equal volume of STV/2 (STV diluted with an equal volume of distilled water) was then added, and the *p*H was adjusted to 7 with KOH. The subsequent operations were the same as those shown on the flow sheet (Scheme I) except that 0.1 of each volume indicated was used for the resuspension of particulate fractions.

*Large-Scale Isolation.*—Baker's yeast (750 g to 1 kg, wet wt) was washed as above and resuspended in STV to a final volume of 1 liter, mixed with 1 liter of beads, and homogenized for 5–7 minutes at 0° (ice bath) in a stainless-steel beaker by means of a large overhead blender equipped with a circular glass blade and operating at top speed. All subsequent operations are described on the flow sheet (Scheme I).

## RESULTS

The results obtained with wild type will be presented first and then compared with those characteristic of respiratory-deficient mutants. For convenience, however, the results for both types are frequently included in the same table.

*Isolation of Particles*

*Breakage and Partition between "Soluble" and "Particulate" Fractions.*—The time of blending with glass beads is adjusted in such a manner as to liberate into $S_0$, the initial homogenate (freed of whole cells, debris, and perhaps nuclei), approximately 15 g of protein/kg (wet wt) of starting yeast cake. Under these conditions 20–30% of the total cells are broken, and the large particle fraction $R_1$ will contain approximately 1.5 g of protein/kg (wet wt) of starting material; i.e., the particles contain about 10% of the protein of the homogenate. These are conditions which have been found to be optimal with a number of different

[1] Abbreviations used in this work: STV, a solution 1 M in sucrose, 0.02 M in Tris, and 0.001 M in EDTA, at *p*H 8.0; from Blair *et al.* (1963): ETP, electron-transport particles; $ETP^H$, electron-transport particle prepared from "heavy" mitochondria; EP, elementary (respiratory) particle.

TABLE I

DISTRIBUTION OF VARIOUS OXIDATIVE ENZYMES BETWEEN "PARTICULATE" ($R_1$) AND "SOLUBLE" ($S_1$) FRACTIONS[a]

| Prep. | Activity | $S_1$ Sp. Act. × 100 | $S_1$ Total Act. | $R_1$ Sp. Act. × 100 | $R_1$ Total Act. | Sp. Act. $R_1$ / Sp. Act. $S_1$ | Total Act. $R_1$ / Total Act. ($R_1$ + $S_1$) |
|---|---|---|---|---|---|---|---|
| *A. Wild-Type Yeast* | | | | | | | |
| No. 5 (Springer) | DPNH → $O_2$* | 13 | 1235 | 150 | 2150 | 12 | 0.64 |
| | Succ → $O_2$* | ≤2.0 | <150 | 64 | 918 | >32 | >0.86 |
| | D-Lact → $O_2$* | ≤4.4 | <330 | 23 | 330 | >5.2 | >0.50 |
| | Cyt c → $O_2$ | 9.5 | 95 | 57 | 820 | 79 | 0.90 |
| | DPNH → cyt c | 1.3 | 125 | 60 | 860 | 69 | 0.87 |
| | D-Lact → cyt c | 1.0 | 100 | 39 | 560 | 37 | 0.85 |
| | L-Lact → cyt c | 13 | 1225 | 39 | 560 | 2.9 | 0.31 |
| No. 13 (Springer) | DPNH → $O_2$ | 2.6 | 258 | 58 | 1010 | 22 | 0.75 |
| | Cyt c → $O_2$ | 5.4 | 530 | 87 | 1510 | 15 | 0.74 |
| | DPNH → cyt c | 1.7 | 163 | 27 | 470 | 16 | 0.73 |
| | Succ → cyt c | 0.63 | 62 | 6.4 | 112 | 12 | 0.65 |
| | D-Lact → cyt c | 0.53 | 52 | 5.3 | 92 | 10 | 0.64 |
| | L-Lact → cyt c | 9.8 | 910 | 3.9 | 68 | 0.4 | 0.064 |
| | DPNH → indoph | 3.9 | 388 | 30 | 520 | 7.7 | 0.57 |
| | Succ → indoph | <0.2 | <10 | 2.5 | 43 | >15 | >0.85 |
| No. 15 (59 R) | DPNH → $O_2$ | 0.38 | 44 | 38 | 430 | 100 | 0.91 |
| | Cyt c → $O_2$ | 2.0 | 24 | 19 | 212 | 8.5 | 0.90 |
| | DPNH → cyt c | 0.65 | 67 | 18 | 207 | 38 | 0.75 |
| | Succ → cyt c | <0.05 | <0.5 | 3.1 | 36 | >62 | 0.98 |
| | D-Lact → cyt c | 0.44 | 46 | 3.3 | 39 | 7.5 | 0.46 |
| | L-Lact → cyt c | 1.0 | 115 | 1.3 | 15 | 1.3 | 0.11 |
| | DPNH → indoph | 11.3 | 1300 | 27 | 320 | 2.4 | 0.20 |
| | Succ → indoph | <0.05 | <0.5 | 1.9 | 22 | >38 | 0.98 |
| *B. Cytoplasmic "Petites"* | | | | | | | |
| 1P (59 RA) | DPNH → cyt c | 0.016 | 1.7 | 1.7 | 22.6 | 105 | 0.98 |
| | D-Lact → cyt c | 0.15 | 15.4 | 3.8 | 50.5 | 25 | 0.75 |
| | L-Lact → cyt c | 1.2 | 123 | 3.0 | 40.0 | 2.5 | 0.25 |
| | DPNH → indoph | 0.24 | 255 | 18 | 240 | 7.5 | 0.49 |
| | Succ → indoph | 0.038 | 38 | 1.3 | 17 | 3.5 | 0.31 |
| 2P (59 RA) | DPNH → cyt c | 0.010 | 1.5 | 2.1 | 31 | 210 | 0.95 |
| | D-Lact → cyt c | 0.20 | 33 | 4 | 58.5 | 15 | 0.64 |
| | L-Lact → cyt c | 1.6 | 205 | 3.8 | 50.7 | 2.5 | 0.19 |
| | DPNH → indoph | 0.5 | 630 | 13 | 185 | 2.6 | 0.22 |
| | Succ → indoph | 0.080 | 10 | 0.62 | 9 | 7.8 | 0.48 |

[a] All activities were measured spectrophotometrically at 29° except those marked with an asterisk, which were determined polarographically at 38°; for details see Experimental Procedure. Sp. Act. = specific activity, μmoles of substrate oxidized/min per mg; total act. = total activity = μmoles/min calculated for 1 kg of yeast. $S_1$ and $R_1$ are the supernatant and the residue after centrifuging for 20 minutes in the Spinco Model L ultracentrifuge using the No. 30 rotor at a speed of 20,000 rpm and the medium described under Experimental Procedure. Preparations of No. 5, 13, 1P, and 2P were on a large scale (750–1000 g of yeast, wet wt); preparation No. 15 was a small-scale preparation. Nos. 5 and 13 used two different lots of yeast obtained commercially (Springer) and stored at 0°. Preparation 15 used a genetically homogeneous wild-type strain (59 R) harvested at the end of the exponential phase. Preparations 1P and 2P were of a cytoplasmic *petite colonie* mutant (59 RA) isolated from 59 R by acriflavine treatment and otherwise isogenic with it; growth and culture conditions are indicated under Experimental Procedure.

yeast strains under a variety of growth conditions: Shorter blending times give insufficient breakage of cells without any improvement in the retention of key respiratory enzymes on the particles ($R_1$) or enhancement of their specific activity; more prolonged blending (which does, of course, result in more complete liberation of proteins into the homogenate) leads to a decrease in specific activity in particulate indicator enzymes and/or their gradual liberation into the supernatant fraction $S_1$. As indicated under Experimental Procedure, the procedure is applicable to both large- (1 kg) and small-scale (50 g) isolations.

In Table I are shown the distributions of a number of respiratory activities between the $R_1$ and $S_1$ fractions. Two criteria have been employed: the ratio between the two specific activities and the proportion of the total activity residing in $R_1$. For an enzyme or activity to qualify as "particulate" the former is required to be $\gg 1$ and the latter $\geq 50\%$. The two preparations derived from commercial yeast represent the limits observed for that type of preparation; the wild-type yeast 59 R was grown aerobically on glucose and harvested at the end of the exponential phase. As might have been anticipated from our own and the results of others (Slonimski, 1953; Utter *et al.*, 1958; Hebb *et al.*, 1959; Chance, 1959; Vitols *et al.*, 1961; Mackler *et al.*, 1962) or from analogy to mammalian systems, the cytochrome c–linked components of the respiratory chain (DPNH → cytochrome c reductase, succinate → cytochrome c reductase, cytochrome c oxidase, and the complete DPNH- and succinoxidase) are indeed particulate, and the relatively small proportion of these activities found in the $S_1$ fraction is almost certainly due to disruption of the particles to yield populations no longer sedimentable at the actual speeds employed. Conversely, the disruption procedure used has served, as had been hoped, to retain the bulk of these activities in the particles. The results obtained suggest that these conditions are analogous to 10 seconds of dis-

TABLE II
FRACTIONATION OF PROTEINS IN VARICUS PREPARATIONS

| Preparation No. | Remarks | Total Protein (mg) in $S_o$ | $S_1$ | $R_1$ | $S_2$ | $R_2H$ | $S_3$ | $R_2L_w$ | DPNH Ox of $R_2L_w$ ($\mu$moles oxidized/mg protein) |
|---|---|---|---|---|---|---|---|---|---|
| 5 | *a, b* | | 9500 | 1435 | 210 | 525[c] | | 640[c] | 3.1 |
| 5a | *a, b* | | 10000 | | | 220 | | 350 | 2.9 |
| 6 | *a, b* | | 10000 | | | | | | 4.2 |
| 7 | *a, b* | | 15000 | | | 320 | | 400 | 3.4[d] |
| 8 | *a, b* | | 10700 | | | 264 | | 432 | 4.4[d] |
| 9 | *a, b* | | | | | 240 | | 455 | 3.9 |
| 9a | *a, b, e* | | | | | 630 | | 220 | 3.9 |
| 10 | *a, b* | | | | | | | 420 | 3.4 |
| 12 | *a, b* | | | | | | | 450 | 5.2 |
| 13 | *a, b* | 12700 | 9800 | 1740 | 700 | 485[c] | 140 | 220 | 4.7[d] |
| 14 | *b, f* | 17400 | 17000 | 1600 | 720 | 300[c] | | 336 | 3.5[d] |
| 15 | *d, g* | 13000 | 10350 | 1150 | 300 | 338[c] | | 78 | 1.5[d] |
| 1P | *a, h* | 18100 | 10300 | 1330 | 390 | 427[c] | 100 | 138 | $\ll$0.05[d] |
| 2P | *a, h* | 18100 | 12600 | 1450 | 480 | 710[c] | 154 | 105 | $\ll$0.05[d] |

[a] Standard large-scale preparation (1 kg). [b] Commercial yeast (Springer); preparations 5–7, 8–10, 12–13 used three different batches of yeast. [c] Fraction not washed. [d] Determined spectrophotometrically at 29° and multiplied by a factor of 2.1 found empirically to relate the two assay systems for DPNH oxidase (see Experimental), i.e., the spectrophotometric one at 29° and the polarographic one at 38°. [e] Yeast stored frozen. [f] Small-scale preparation (50 g); all quantities calculated on the basis of 1 kg yeast. [g] Strain 59 R (wild type). [h] Strain 59 RA (cytoplasmic *petite*).

integration in the Nossall shaker (Somlo, 1962). (DPNH oxidase has been used as reference activity in most of the work reported below.) D-lactate → cytochrome c reductase has been reported to be associated with particles (Nygaard, 1961; Gregolin and Singer, 1961, 1963), which may, however, be either not identical with or more easily detached from those carrying the main components of the electron-transport system.[2] In the present studies, a maximum of 85% of the D-lactate activity has been found to be associated with the particles. Since in preparation 15 (59 R) this has dropped to less than 50% one might suspect that the localization (and/or ease of detachment) is to some extent under the control of the physiological environment. L-Lactate → cytochrome c activity as found by Somlo (1962) and ourselves is largely nonparticulate, although under optimal conditions as much as 30% of this activity may be associated with the $R_1$ fraction. The distribution observed for the two primary dehydrogenases for DPNH and succinate, respectively (here measured by their indophenol-reductase activities[3]), are of some interest: A significant fraction, which may actually constitute the majority of the DPNH dehydrogenase under certain conditions, is not associated with the particles, while in wild-type cells, at least, essentially all the succinate dehydrogenase is particulate.

*Fractionation and Isolation of Subparticles.*—The large particle fraction is further subfractionated by lowering of the sucrose concentration and adjustment of the *p*H to 8.0 as shown in Scheme I. Under these mild conditions two reproducible subfractions called $R_2H$ ($R_2H_w$ when washed) and $R_2L$ ($R_2L_w$ when washed) are obtained. In Table II we have summarized the distribution of protein between the various fractions and show the specific DPNH-oxidase activities obtained in the $R_2L_w$ fraction for twelve different preparations (differing strains, growth conditions, scale of preparation, etc.). It will be seen that approximately the same amount of protein is obtained in the $R_2L_w$ fraction (~400 mg for commercial yeast) and that the protein has associated with it a high DPNH-oxidase activity (~3–4.5 $\mu$moles oxidized/min per mg at 38° for commercial yeasts).

The purification obtained for various respiratory activities in going from the first extract ($S_o$) to the final $R_2L_w$ particle is shown in Table III. The overall purification is some 20-fold for both DPNH and succinoxidase.

*Properties of Isolated Particles*

*Enzymatic Activities.*—The rates of various enzymatic activities characteristic for the $R_2L_w$ particle are shown in Table IV. Preparations from commercial yeasts oxidize 3–5 $\mu$moles of DPNH or 1 $\mu$mole of succinate per minute per mg of protein at 38°. The partial activities (DPNH → indophenol or ferricyanide, succinate → indophenol or ferricyanide, DPNH → cytochrome c, succinate → cytochrome c reductases, and cytochrome c oxidase) are uniformly lower than the corresponding overall activities. D-Lactate → cytochrome c–reductase activity is generally of the same magnitude as that obtained with succinate. No phosphorylation of ADP to ATP accompanies the oxidation of DPNH or succinate. The oxidation of pyruvate, malate, $\alpha$-ketoglutarate, or $\beta$-hydroxybutyrate could not be detected. The preparations are stable up to 10 days when stored at 0° and lose no activity upon freezing, storage at −20°, and subsequent thawing after several weeks. The ability of externally added cytochrome c to stimulate oxidation with $O_2$ as acceptor (as measured either polarographically for DPNH, succin-, or D-lactate oxidase, or spectrophotometrically for DPNH oxidase) depends markedly on the nature of the substrate employed (Fig. 1). Succinoxidase is virtually not stimulated at all (<1.5-fold), DPNH oxidase is stimulated 1.5- to 2.4-fold (average 1.8-fold) at a saturating concentration of cytochrome c of $5 \times 10^{-6}$ M, while D- and L-lactate oxidation require $5 \times 10^{-5}$ and $1 \times 10^{-4}$ M cytochrome c, with <10% of the maximal rate in the absence of the cytochrome.

[2] Unpublished observations of J. Pennock, Y. Graver, and H. R. Mahler; similar results have also been obtained by Somlo (private communication).

[3] The values obtained under optimal conditions with ferricyanide as acceptor at the same temperature (29°) are approximately equal to that with indophenol for DPNH and 2–3 times the indophenol value for succinate.

TABLE III
PURIFICATION OF RESPIRATORY PARTICLES (NO. 13)[a]

| Activity | $S_o$ | | $R_1$ | | $R_2L_w$ | |
|---|---|---|---|---|---|---|
| | Total | Specific | Total | Specific | Total | Specific |
| DPNH → $O_2$ | 1270 | 0.10 | 1010 | 0.58 | 506 | 2.30 |
| Cyt → $O_2$ | 2040 | 0.16 | 1510 | 0.87 | 485 | 2.20 |
| DPNH → cyt c | 633 | 0.049 | 470 | 0.27 | 143 | 0.65 |
| Succ → cyt c | 175 | 0.014 | 112 | 0.064 | 24 | 0.11 |
| D-Lact → cyt c | 144 | 0.009 | 92 | 0.053 | 26 | 0.12 |
| L-Lact → cyt c | 998 | | 68 | 0.039 | 9 | 0.040 |
| Protein (g) | 12.7 | | 1.74 | | 0.22 | |

[a] All activities in μmoles substrate oxidized/min at 29°; specific = total/mg protein.

TABLE IV
COMPARISON OF VARIOUS ENZYMATIC ACTIVITIES IN $R_2L_w$[a]

| Activity | Preparations | | | | | |
|---|---|---|---|---|---|---|
| | 5 | 12 | 13 | 1F | 15 | 1P |
| DPNH → $O_2$ | 3100[b] | 2100, 5200[b] | 2300 | 3300[b,c] | 730 | <1 |
| Succinate → $O_2$ | 870[b] | | | 1300[b,c] | | |
| D-Lactate → $O_2$ | 200[b] | | | | | |
| L-Lactate → $O_2$ | 110[b] | | | | | |
| Cyt c → $O_2$ | | 2000 | 2200 | | | <0.5 |
| DPNH → cyt c | | 620 | 650 | | 300 | 10 |
| Succinate → cyt c | | | 110 | | 43 | 5 |
| D-Lact → cyt c | | | 120 | | 58 | 40 |
| L-Lact → cyt c | | | 40 | | 16 | 35 |
| DPNH → indophenol | | 770 | 720 | 1200[b,c] | 310 | 170 |
| Succinate → indophenol | | 74 | 68 | 650[b,c] | 40 | 17 |

[a] All in mμmoles substrate oxidized/min per mg protein at 29°. All preparations except 1F have been described in the legend to Table II; preparation 12 was an exact duplicate of 13 using the same lot of yeast, several days later; preparation 1F was one using an American yeast (Fleischmann's) carried out in Seattle. [b] Polarographic estimation. [c] At 38°.

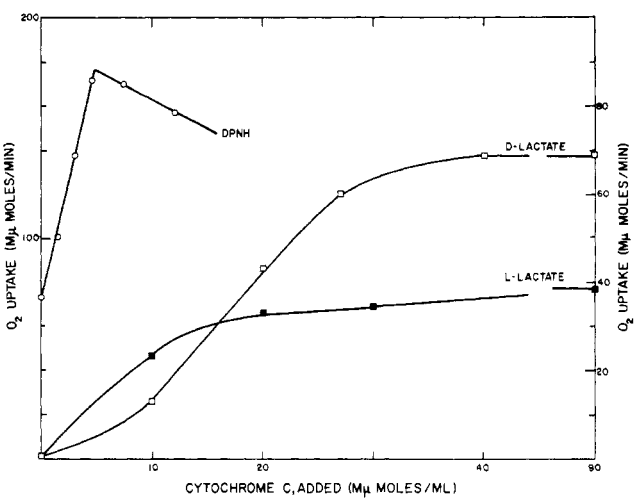



FIG. 1.—Stimulation of various oxidase activities of yeast respiratory particles by external cytochrome c. The polarographic assay procedures described under Experimental Procedure were employed. For DPNH oxidase the system included 0.4 ml of 0.2 M phosphate buffer of *p*H 7.5, the amount of cytochrome c indicated, 1.5 ml of $H_2O$, and 0.01 ml of $R_2L_w$ of preparation 1F (97 μg protein). The reaction was started by the addition of 0.1 ml of 1% DPNH and was measured at 38°. For the D- and L-lactate oxidases the system contained 0.1 ml of the same buffer, the amount of cytochrome c indicated, 0.1 ml of $R_1$ (4 mg total), and 1.5 ml of $H_2O$. The reaction was started by the addition of 0.1 ml of 0.2 M D- or L-lactate and was measured at 30°.

*Action of Respiratory Inhibitors.*—The succinic and DPNH-oxidase activities of $R_2L_w$ are inhibited 90–100% by cyanide (0.001 M) or azide (0.01 M). Similar inhibitions are also obtained for the D-lactate, L-lactate, and reduced cytochrome c–oxidase activities; but the same inhibitors have no effect on any of the other activities recorded in Table IV. Antimycin A (1 μg/ml) also completely inhibits the DPNH- and succinic-oxidase as well as DPNH- and succinic–cytochrome c–reductase activities, but is without effect on the D- and L-lactate oxidases and on the cytochrome c–oxidase, DPNH → ferricyanide or indophenol, and succinate → ferricyanide or indophenol–reductase activities. Both Seconal ($2 \times 10^{-3}$ M) and rotenone ($1 \times 10^{-6}$ M) fail to inhibit any of the activities described.

*Spectra.*—The difference spectrum (dithionite reduced–oxidized) of a typical $R_2L_w$ preparation recorded at room temperature is shown in Figure 2. Absorption maxima at 605, 562, 552, and 444 mμ (not shown) demonstrate the presence of cytochromes a, b, and (c + $c_1$) in the preparation. Either DPNH (as shown) or succinate is capable of effecting the rapid reduction of all cytochrome components, while D- or L-lactate, even with the less pure $R_1$ preparation, is completely incapable of reducing any of the cytochromes of the respiratory chain, even in the Soret region where, of course, the extinction coefficients and thus the sensitivity are much greater.

*Composition.*—The cytochrome content of four different preparations of $R_2L_w$ from commercial yeasts is given in Table V. For one preparation from an American yeast (Fleischmann) we also show the composition with regard to total flavin (released by acid after trypsin digestion), nonheme iron, and copper (see Table IX). The ratios for the various components are roughly: cytochrome (a + $a_3$)–b–(c + $c_1$)–flavin–Fe–Cu = $\geq 1 : \geq 1 : 1 : 1 : 8 : \geq 3$.

*Centrifugation in a Sucrose Gradient.*—Some idea concerning possible heterogeneity of particle preparations may be obtained by allowing the latter to sediment through a linear gradient of some inert solute

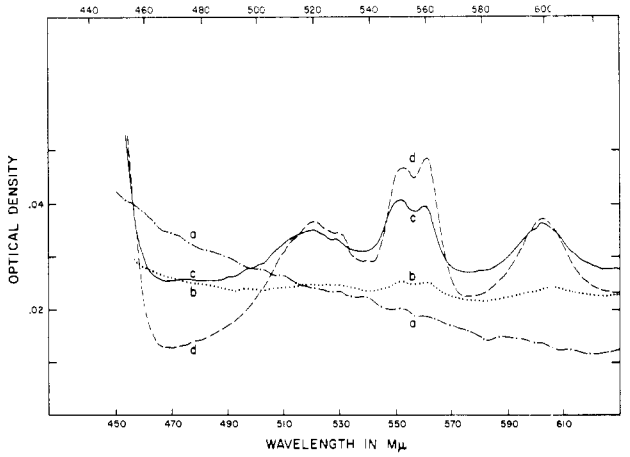



FIG. 2.—Absorption spectrum of $R_2L_w$. Spectra were obtained on 3.0 ml of $R_2L_w$ (2.8 mg protein/ml) in STV/4 in a 3-ml cuvet with a 10-mm light path. The spectra shown are difference spectra (reduced-oxidized) and were obtained against a control containing the same enzyme solution. Curve (a) is the base line before addition of any reductant, curve (b) is that observed immediately after addition of DPNH (steady state), curve (c) is the spectrum after complete reduction by DPNH, and curve (d) is the spectrum obtained after addition of dithionite.

TABLE V

CYTOCHROME CONTENT OF VARIOUS PREPARATIONS OF $R_2L_w$

| Preparations | mμMoles of Component/mg Protein | | | |
|---|---|---|---|---|
| | a | $a_3$ | b | c + $c_1$ |
| $R_2L_w$ of No. 5 | 0.28 | 0.24 | 0.48 | 0.33 |
| $R_2L_w$ of No. 9[a] | 0.38 | 0.17 | 0.61 | 0.48 |
| $R_2L_w$ of No. 12 | 0.24 | 0.23 | 0.62 | 0.38[a] |
| $R_2L_w$ of No. F1[c] | 0.40[b] | | 0.45 | 0.40 |
| $R_2L_w$ of P1[c] | 0[d] | 0 | 0 | <0.085 |
| $R_2H$ of $P_1$[c] | 0 | 0 | 0 | <0.071 |

[a] Spectroscopic examination at the temperature of liquid N indicated approximately equal concentrations of cytochrome c and $c_1$. [b] Cytochrome (a + $a_3$). [c] For an explanation, see legends to Tables I and IV. [d] A zero (0) means not detectable.

such as sucrose (Kuff *et al.*, 1956; de Duve *et al.*, 1958). An experiment of this type is shown in Figure 3. It can be seen that the particles sediment in a reasonably symmetrical band and that the DPNH → $O_2$ and DPNH → cytochrome c activities appear to be associated with the same species of particle.

*Comparison of Different Strains*

As shown in Table IV, different samples of commercial baker's yeasts (presumably differing in strain but grown under analogous conditions) give $R_2L_w$ particles which are very similar in enzyme content and activities. There appears to be no significant difference between the yeast used routinely in France (Springer) and two available in the U.S. (Fleischmann's and Red Star). A wild-type yeast (59 R) grown on glucose in the laboratory and harvested at the end of the exponential phase did appear to differ significantly from the others in its particles especially with regard to cytochrome oxidase (and hence DPNH oxidase).

Turning now to a comparison between preparations from wild-type yeasts and a respiratory-deficient mutant (cytoplasmic *petite*) strain derived from 59 R we first examine the total activities estimated in the respective cell-free extracts (Table VI). We find that only those activities are grossly impaired

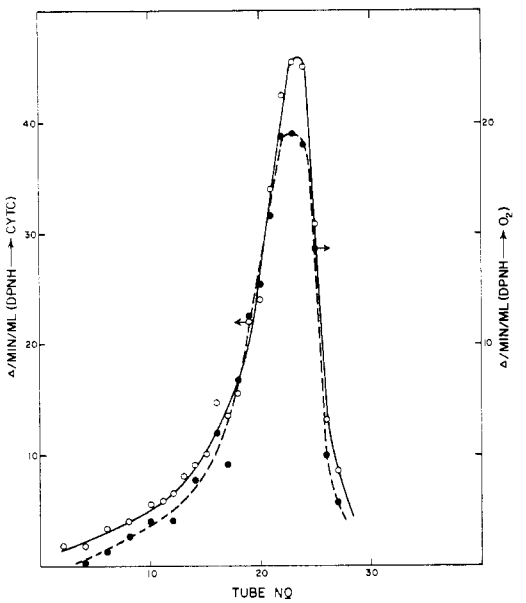



FIG. 3.—Centrifugation of $R_2L_w$ in a sucrose gradient. One ml of $R_2L_w$ No. 9a (Table II) concentrated to 18 mg protein/ml by centrifugation and resuspension in STV/4 were layered on top of 28 ml of a linear sucrose gradient (4–20% by weight). Centrifugation was at 22F for 45 minutes at 10,000 rpm (plus additional time required to stop rotor without braking) in the SW 25 rotor of the Spinco Model L ultracentrifuge. One-ml fractions were then collected after piercing the bottom of the tube, and aliquots of each were assayed for their DPNH–cytochrome c–reductase and DPNH-oxidase activities: ●, DPNH → cytochrome c reductase, $\Delta A_{550}$/min per ml; ○, DPNH oxidase, $\Delta A_{340}$/min per ml.

which require the participation of cytochromes of the respiratory chain proper, i.e, DPNH → cytochrome c and succinate → cytochrome c reductase, cytochrome c oxidase, and, consequently, the oxidase activities for both DPNH and succinate. It must be stressed here, however, that the cytochrome employed in these tests is always *mammalian* cytochrome c. Whether the same would hold true with yeast cytochrome c remains a moot point. On the other hand, all the *primary* dehydrogenases, i.e., those for DPNH, succinate, D- and L-lactate are present in the mutant with activities not differing greatly from those found in the wild type. As a corollary to these observations, it is clear that not even in this crude extract of the mutant is external cytochrome c capable of reacting *directly* with the primary particle-bound (flavoprotein) dehydrogenases for DPNH and succinate. We recognize of course that the acceptor used here for the primary succinate and DPNH dehydrogenases (i.e., 2,6-dichlorophenol indophenol) may be less than ideal for this purpose and that its reduction may require a step or steps beyond that of the dehydrogenase proper (Singer and Kearney, 1957; Minakami *et al.*, 1962). We have found, however, that in the particular case of yeast extracts and particles the DPNH → ferricyanide activity is always approximately equal to the DPNH → indophenol one, and while succinate → ferricyanide is sometimes somewhat higher than succinate → indophenol, the latter is not increased by the addition of catalytic amounts of phenazine methosulfate. Its high extinction coefficient and its great convenience, as well as its use for comparative purposes only, thus vitiate the disadvantages of 2,6-dichlorophenol iodophenol as an acceptor.

The distribution of the various respiratory enzymes of the mutant between the particulate and soluble

TABLE VI
COMPARISON OF ACTIVITIES IN TOTAL EXTRACTS[a]

| Activity | Preparations | | | | |
|---|---|---|---|---|---|
| | 13 | 15 | 1P | 13/1P | 15/1P |
| DPNH → $O_2$ | 1268 | 474 | <1 | ~1200 | ~400 |
| Cyt c → $O_2$ | 2040 | 234 | <1 | ~2000 | ~200 |
| DPNH → cyt c | 633 | 274 | 24 | 26 | 11 |
| Succinate → cyt c | 175 | 37 | <1 | >175 | >37 |
| D-Lact → cyt c | 144 | 85 | 66 | 2.2 | 1.3 |
| L-Lact → cyt c | 998 | 120 | 163 | 6.1 | 0.74 |
| DPNH → indoph | 908 | 1620 | 500 | 1.8 | 3.2 |
| Succinate → indoph | 50 | 23 | 55 | 0.91 | 0.42 |

[a] μMoles/min at 29°. All activities measured spectrophotometrically as described under Experimental Procedure. Total activities are calculated for 1 kg (wet wt) of yeast. Preparation 13 was a large-scale preparation using commercial yeast (Springer); preparation 15 was on a small scale (50 g) using yeast strain 59 R (wild type) grown to the end of the exponential phase; preparation 1P was on a large scale using yeast strain 59 RA (cytoplasmic *petite* isogenic with 59 R).

TABLE VII
COMPARISON OF $R_1$ WITH $R_2L$ AND $R_2H$[a]

| | $R_1$ | | $R_2H$ | | $R_2L$ | |
|---|---|---|---|---|---|---|
| | G | P | G | P | G | P |
| DPNH → $O_2$ | 0.58 | <0.001 | 0.77 | <0.001 | 2.30 | <0.001 |
| Cyt c → $O_2$ | 0.87 | <0.0005 | 0.70 | <0.0005 | 2.20 | <0.0005 |
| DPNH → cyt c | 0.27 | 0.017 | 0.33 | 0.019 | 0.65 | 0.010 |
| Succ → cyt c | 0.064 | 0.003 | 0.037 | 0.002 | 0.11 | 0.005 |
| D-Lact → cyt c | 0.053 | 0.038 | 0.055 | 0.053 | 0.12 | 0.040 |
| L-Lact → cyt c | 0.039 | 0.030 | 0.024 | 0.062 | 0.040 | 0.035 |
| DPNH → indoph | 0.30 | 0.18 | 0.20 | 0.24 | 0.79 | 0.17 |
| Succ → indoph | 0.025 | 0.013 | 0.030 | 0.012 | 0.068 | 0.017 |

[a] G = preparation 13; P = preparation P1. All assays as described under Experimental Procedure. Specific activity = μmoles/min per mg protein at 29°.

fractions is also of interest. As shown in Table I, the low-residual DPNH → cytochrome c–reductase activity is unambiguously particulate, as is the D-lactate → cytochrome c reductase. In the mutant as in the wild type an appreciable proportion of the L-lactate dehydrogenase also appears to be associated with particulate matter. The distribution of the DPNH dehydrogenase is reminiscent of that observed with the parent wild-type strain grown under somewhat similar conditions, while there emerges a significant difference with respect to succinic dehydrogenase: For the first time a considerable proportion of this activity now makes its appearance in the $S_1$ fraction. Similar observations have also been reported by F. C. Charalampous (private communication). Whether this corresponds to a truly soluble protein or to an association with smaller particles, perhaps analogous to the mitochondrial precursor observed by Schatz (1963), is currently under investigation.

In the course of further fractionation of the $R_1$ particle an additional difference between the wild type and the mutant becomes apparent (Table VII). While all specific activities (except for L-lactate dehydrogenase) of the wild type increase in going from $R_1$ to $R_2L$, with constant or slightly lowered values characterizing the $R_2H$, the reverse is true for the mutant: Specific activities of $R_2L$ are either the same as, or lower than, the $R_1$ while the $R_2H$ activities if anything are higher. This is the more remarkable since both types of particles can be obtained from extracts of the mutant in a completely reproducible and routine manner by the use of the method developed for the wild type as described before, and the amount and distribution of protein in the various fractions is quite similar for the two strains. As a consequence of this phenomenon the activities of the $R_2L$ fraction from the mutant (Table IV) reflect in a somewhat exaggerated manner the results obtained with the crude extract: in any event it is clear that even in the particle the primary dehydrogenases are present in significant amounts.

One additional observation is of interest: Whole cells of 59 RA contain cytochrome (c + $c_1$) in amounts at least equal to those characteristic of the parent strain 59 R. Yet, as shown in Table V, the $R_2L_w$ particles obtained for 59 RA retain these cytochromes in much lower amounts (one-third to one-fourth). Thus the mutant is either incapable of incorporating cytochrome c (and/or $c_1$) into the mitochondrial matrix or else the cytochrome is more easily detached therefrom in the course of the mild purification procedures employed here.

## DISCUSSION

*Comparative Activity of the $R_2L_w$ Particle.*—Some idea concerning the activity of the preparation can be obtained by an estimate of the catalytic efficiencies (turnover numbers) of the various cytochromes implicated in the respiratory chain. Such a calculation, including the assumptions used in its execution, is shown in Table VIII. Of the various efficiencies listed the one for cytochrome $a_3$ is probably subject to the fewest ambiguities. In any event, it would appear that all three classes of cytochromes, (a + $a_3$), (c + $c_1$), and b are capable of transferring of the order of $10^4$ electron equivalents/heme per minute at 29°. The two hypothetical results calculated for cytochrome b are of some interest: If we assume that the reoxidation of cytochrome b by cytochrome c is rate limiting in both the DPNH–cytochrome c–reductase and the cytochrome c–stimulated DPNH-oxidase reactions, then we see that the former is considerably slower than the latter, i.e., the reduction

TABLE VIII

CALCULATION OF CATALYTIC EFFICIENCIES OF CYTOCHROMES IN $R_2L_w$[a]

| Activity | Method of Calculation | Numerical Values | Result (electron equivalents/ heme × min) |
|---|---|---|---|
| Cytochrome oxidase | Specific activity/ (cyt $a_3$) | $2 \times 1000/0.21$ | $1.0 \times 10^4$ |
| DPNH-cytochrome reductase | Specific activity/ (cyt b) | $2 \times 630/0.57$ | $0.22 \times 10^4$ |
| DPNH-oxidase (without external c) | Specific activity/ (cyt c + $c_1$) | $2 \times 1250/0.40$ | $0.62 \times 10^4$ |
| DPNH-oxidase (external c added) | Specific activity/ (cyt b) | $2 \times 2200/0.57$ | $0.77 \times 10^4$ |

[a] All calculations for activities at 29°; specific activities in μmoles/min per mg protein (average of two determinations, preparations 12 and 13 of Table VI); cytochrome concentrations in mμmoles per mg protein (average of the three determinations of Table V).

TABLE IX

COMPARISON OF YEAST $R_2L_w$ WITH MITOCHONDRIA AND RESPIRATORY SUBPARTICLES[a]

| | Yeast $R_2L_w$ | | Yeast[c] | | Heart Muscle[d] | |
|---|---|---|---|---|---|---|
| | Springer[b] | Fleisch-mann | Intact Cells[e] | Mito-chondria | ETP$^H$ | EP |
| Total cytochromes | 1.48 | 1.47 | 1.30 | 0.51 | 3.1 | 5.05 |
| Cytochrome (a + $a_3$) | 0.51 | 0.40 | 0.52 | 0.22 | 1.62 | 2.90 |
| Cytochrome (c + $c_1$) | 0.40 | 0.48 | 0.50 | 0.14 | 0.60 | 0.64 |
| Cytochrome b | 0.57 | 0.59 | 0.28 | 0.15 | 0.85 | 1.51 |
| Total flavin | | 0.64 | 0.15[f] | | 0.66 | 0.91 |
| Nonheme iron | | 3.9 | | | | 7.15 |
| Copper | | 1.5 | | | | 3.32 |
| $DPNH_2 \rightarrow O_2$ (38°) | 4.1 | 3.3 | | 0.45[g] | 4.2 | 8.4 |
| Succinate $\rightarrow O_2$ (38°) | | 1.3 | | | | 3.6 |

[a] All values in mμmoles (content), or μmoles of substrate oxidized/min (activity), per mg protein. [b] Average of three determinations, on three different lots (prep. No. 5, 9a, and 12, Table II). [c] From the data of Chance and Hess (1959); strain not given. [d] From the data of Blair *et al.* (1963). [e] Values per 100 mg cells. [f] Given as "flavoprotein" reducible by substrate. [g] Temperature not specified.

of externally added cytochrome c *cannot* be an obligatory step in the overall DPNH-oxidase reaction. This is true even if we use only the difference between the unstimulated and the stimulated oxidase, i.e., $2 \times (2200-1250)/0.57$ or $0.33 \times 10^4$; and our conclusion is unaffected by the assumption that the oxidation of cytochrome $c_1$ rather than that of cytochrome b is the actual rate-limiting step, as long as the same assumption is used for both cases. It must also be remembered that the concentration of cytochrome c actually used in both the DPNH → cyt c and cyt c → $O_2$ reaction is more than ten times that required for and employed in the cytochrome-stimulated DPNH oxidase. From this we conclude that the function of the externally added cytochrome c in the latter reaction is simply to substitute for and bind to the particle in place of the cytochrome c lost from the chain in the course of the isolation of the particle, and that it *cannot* be that of a soluble component, continuously turning over by virtue of its reduction by DPNH and subsequent carriers, and reoxidation by cytochrome oxidase.

Another useful comparison is one between the particles described here and intact yeast cells, mitochondria, and other electron-transport particles prepared therefrom on the one hand, and the respiratory subparticles obtained from heart muscle on the other (Table IX). We observe that a most significant change in composition occurs in the relative proportion of cytochromes in going from the intact yeast cell to mitochondria. Whereas in the former the ratio of (a + $a_3$)–(c + $c_1$)–(b) ≅ 2:2:1; in the latter it is 1:1:1. There is no further significant change in going from mitochondria to any of the subparticles described; the ratio remains 1:1:1. The loss of cytochrome c in the course of isolation of a respiratory particle is not surprising (one might even estimate that approximately 40% of this component had become solubilized on the basis of the extent of stimulation of DPNH oxidase already described). But that the content of mitochondria with regard to (a + $a_3$) should be lower relative to cytochrome b is surprising and so far remains without an explanation. It is of interest to note in this context that with heart muscle particles the ratio of (a + $a_3$)–b remains at 2:1 (cytochrome c + $c_1$ does not provide a useful basis since the bulk of the cytochrome c, at least, was probably lost during the extensive purification). The relative flavin content of the derivative particles is apparently of the same order of magnitude.

If we now turn our attention to a comparison of activities we find that (assuming Chance and Hess' experiments (1959) to have been carried out at a temperature of 25–26°) the DPNH-oxidase activity of their mitochondrial preparation is quite comparable to that reported here for our $R_1$ particle (an activity of 0.45 at 25° would correspond to one of 1.2 at 38°). The ETP of Mackler *et al.* (1962), although containing all cytochromes and total flavin at a level approximately twice that of $R_2L_w$, exhibits a specific activity certainly no greater than that reported here. In other words its specific activity on a cytochrome or flavin basis is approximately 0.5 that of the $R_2L_w$. Perhaps the high *p*H and the relatively long work-up period employed in its isolation has led to a decreased activity; perhaps also this is related to its quite low content of nonheme iron. Another important difference is that Mackler's yeast ETP is stimulated some 1.5-fold in its succinoxidase and some 3.0-fold

in its DPNH oxidase by the addition of external cytochrome c. In comparing $R_2L_w$ with electron-transport particles from heart we find that, except for its significantly lowered cytochrome (a + $a_3$) content, $R_2L_w$ is comparable in every other respect to ETP[H] and shows approximately 0.3–0.5 of the enzymatic activity, cytochrome b, total flavin, nonheme iron, and copper content of EP, the supposed ultimate subunit of electron transport. It is comforting to know that particles so similar in composition, activity, and function can be obtained from cells so disparate in origin as beef cardiac muscle and aerobic *S. cerevisiae*—yet with one main common feature: an extremely efficient and rapid aerobic metabolism.

It must be emphasized, however, that the particles just described, unlike EP, are made up of sizable fragments of the starting mitochondria, and that if a yeast EP should be shown to exist the $R_2L_w$ must be composed of a considerable number of these subunits.

*Electron Transport to and from the $R_2L_w$ Particle.*—As has already been discussed, we now believe that the enhanced rate of oxidation of DPNH by oxygen in the presence of external cytochrome c is brought about by virtue of the latter's becoming linked to unoccupied combining sites on the particle. In this reaction the added cytochrome c then functions as "internal" cytochrome, and has therefore become indistinguishable from the cytochrome still retained on the particle and required for the unstimulated DPNH-oxidase reaction (as measured with no cytochrome c added). If this premise is correct, then the reduction of external cytochrome c by DPNH (through the intermediation of the primary dehydrogenase and one or more carriers of the electron-transport sequence, including the antimycin-sensitive site) and its reoxidation (by cytochrome oxidase plus oxygen) catalyzed by the particle are interesting, and possibly significant model reactions, but cannot be considered *by themselves* to duplicate particulate electron transport in the immediate vicinity of cytochrome c. Furthermore the virtual absence of *any* stimulation of succinoxidase by external cytochrome c provides strong support to the contention of Mackler *et al.* (1962) that in these yeast particles there appear to be present distinct and separate electron-transport chains for succinate and DPNH.

What then of the oxidation of D- and L-lactate catalyzed by the preparation? It would appear that in complete contrast to DPNH and succinate the only portion of the particulate electron-transport chain required for the oxidation of these substrates is that contributed by cytochrome oxidase. In other words, electron transport is in the sequence substrate → dehydrogenase → cytochrome c (external) → [cytochrome oxidase] → $O_2$ (all the components not in brackets are considered to be soluble and freely diffusible). This postulate is based on the following characteristics of the oxidation of D- and L-lactate by the particles already described: (a) its antimycin insensitivity, (b) its absolute requirement for cytochrome c at substrate levels, (c) the inability of the substrates by themselves of reducing any of the cytochromes of the particles including cytochrome (a + $a_3$), and (d) the retention of the ability to reduce cytochrome c with either enantiomer of lactate in a mutant incapable of catalyzing the reduction of this acceptor when either succinate or DPNH is used as substrate. As applied to D-lactate these results confirm and extend the conclusions reached by Gregolin and Singer (1961).

The presence of the primary dehydrogenases for succinate, D-lactate, and L-lactate in certain *petite* mutants has already been reported by Linnane and Still (1956), F. C. Charalampous (private communication), and Gregolin and Ghiretti-Magaldi (1961), respectively.

## Added in Proof

Since this paper was submitted for publication, Gregolin and D'Alberton (1964, *Biochem. Biophys. Res. Commun. 14*, 103) have described the isolation of a particle from yeast which catalyzes the oxidation of D-lactate by oxygen at *p*H 6 in the presence of $Mg^{2+}$. We since have confirmed and extended the findings reported by Gregolin using the $R_1$ preparation described by us in the present manuscript. As shown in Table X, there was some oxidation of D-lactate at *p*H 6 without added $Mg^{2+}$ and this rate was increased markedly by the addition of $Mg^{2+}$. However, at *p*H 7.5 with high or low phosphate concentration and at *p*H 6 with low phosphate concentration, there was very little enzymatic activity with $Mg^{2+}$ present in the assay. In contrast, added cytochrome c stimulated enzymatic activity markedly at both *p*H levels and phosphate concentrations and to the same maximal rate obtained with $Mg^{2+}$. Addition of $Mg^{2+}$ and cytochrome c simultaneously did not give rates greater than with cytochrome c alone. The reactions were insensitive to antimycin A (1 $\gamma$/ml). No steady-state reduction of the cytochromes present in the $R_1$ preparation could be demonstrated either with or without added $Mg^{2+}$ at *p*H 6 (high phosphate) upon addition of D-lactate. However, when anaerobiosis was reached the cytochromes (a + $a_3$, c + $c_1$, and b) became completely reduced.

Table X

Stimulation of D-Lactic-Oxidase Activity of the $R_1$ Preparation by $Mg^{2+}$ and Cytochrome c

| Assay Conditions[a] | *p*H 6.0 | *p*H 7.5 |
|---|---|---|
| Low $PO_4$, no addition | None | None |
| Low $PO_4$, 0.01 ml 1% cyt c | 0.05 | 0.03 |
| Low $PO_4$, 0.1 ml 1% cyt c | 0.10 | 0.10 |
| Low $PO_4$, $Mg^{2+}$ (0.033 M) | Trace | Trace |
| High $PO_4$, no addition | 0.01 | None |
| High $PO_4$, 0.01 ml 1% cyt c | 0.02 | None |
| High $PO_4$, 0.1 ml 1% cyt c | 0.09 | 0.03 |
| High $PO_4$, $Mg^{2+}$ (0.033 M) | 0.09 | Trace |

[a] All assays were performed by measurement of oxygen utilization at 38° with an oxygen polarograph. Rates are expressed as $\mu$moles of D-lactate oxidized/min per mg of protein. The assay systems were as follows: high phosphate, 0.25 ml of 0.2 M $PO_4$ of *p*H 6 or 7.5, 0.1 ml of enzyme (2 mg protein), 0.1 ml of 0.05 M D-lactate, and sufficient water to give a final volume of 1 ml; low phosphate, 0.05 ml of 0.2 M $PO_4$ of *p*H 6 or 7.5, 0.1 ml enzyme, 0.1 ml of 0.05 M D-lactate, and water to give a final volume of 1 ml.

Another particle preparation described by Roy (1964, *Nature 201*, 80) catalyzes the oxidation of D-lactate in a mannitol–potassium phosphate–KCl–$MgCl_2$ buffer at *p*H 7.2 without added cytochrome c and with a specific activity 0.5 of that of our $R_1$ preparation. An important paper by Schatz, Tuppy, and Klima (1963, *Z. Naturforsch. 18b*, 145) now demonstrates independently the occurrence of a mitochondria-like fraction and the similarity in amount and localization of the primary dehydrogenases for DPNH and succinate in a respiratory-deficient mutant. Their results together with those of Yotsunayagi (private communication) permit the tentative identification of

our "light" ($R_2L$) and "heavy" ($R_2H$) fraction as mitochondrial fragments (including cristae), and vesicles plus membranes, respectively.

## References


Blair, P. V., Oda, T., Green, D. E., and Fernandez-Moran, H. (1963), *Biochemistry 2*, 756.

Chance, B. (1959), *J. Biol. Chem. 234*, 2421.

Chance, B., and Hess, B. (1959), *J. Biol. Chem. 234*, 2986.

de Duve, C., Berthet, J., and Beaufay, H. (1958), *Progr. Biophys. Biophys. Chem. 9*, 325.

Estabrook, R. W. (1956), *J. Biol. Chem. 223*, 781.

Gregolin, C., and Ghiretti-Magaldi, A. (1961), *Biochim. Biophys. Acta 54*, 62.

Gregolin, C., and Singer, T. P. (1961), *Biochem. Biophys. Res. Commun. 4*, 189.

Gregolin, C., and Singer, T. P. (1963), *Biochem. Biophys. Res. Commun. 6*, 201.

Hebb, C. R., Slebodnik, J., Singer, T. P., and Bernath, P. (1959), *Arch. Biochem. Biophys. 83*, 10.

Kuff, E. L., Hogeboom, G. H., and Dalton, A. J. (1956), *J. Biophys. Biochem. Cytol. 2*, 33.

Linnane, A. W., and Still, J. L. (1956), *Austral. J. Sci. 18*, 165.

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

Minakami, S., Ringler, R. L., and Singer, T. P. (1962), *J. Biol. Chem. 237*, 569.

Nygaard, A. P. (1961), *J. Biol. Chem. 236*, 920.

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

Singer, T. P., and Kearney, E. (1957), *Methods Biochem. Analy. 4*, 307.

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

Somlo, M. (1962), *Biochim. Biophys. Acta 65*, 333.

Utter, M. F., Keech, D. B., and Nossal, P. M. (1958), *Biochem. J. 68*, 431.

Vitols, E., North, R. J., and Linnane, A. W. (1961), *J. Biophys. Biochem. Cytol. 9*, 689, 701.


# Biochemical Correlates of Respiratory Deficiency. II. Antigenic Properties of Respiratory Particles*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Antibodies against respiratory subparticles of yeast produce a differential inhibition of various particulate enzymic activities in the order: succinic dehydrogenase < DPNH dehydrogenase ≃ cytochrome c oxidase < DPNH oxidase < DPNH-cytochrome c reductase. Studies with this antibody-antigen complex have shown that the combining sites for reduced and oxidized cytochrome c cannot be identical, and that the role of the cytochrome in stimulating DPNH-oxidase activity is not that of a mobile carrier between the reductase and the oxidase portion of the chain. "Respiratory" subparticles from *petite colonie* mutants contain a cross-reacting material capable of binding (or reacting with) the anti-DPNH-cytochrome reductase of sera prepared against particles from wild-type cells. This cross-reacting material is not identical with the primary dehydrogenase and is not found in the bulk of the "mitochondrial" fraction of the mutant.


As part of a continuing research program in our three laboratories designed to elucidate the genetic and biochemical basis of respiratory deficiency (Ephrussi, 1953; Slonimski, 1953) in *Saccharomyces cerevisiae*, we have in the present investigation (Mahler *et al.*, 1964a,b) established that wild-type and cytoplasmic mutant strains are similar with regard to: (a) the presence of primary dehydrogenases for D-lactate, L-lactate, succinate, and DPNH; (b) the localization of characteristic enzymatic activities in mitochondria and respiratory particles therefrom derived; (c) the distribution of protein among soluble and particulate fractions; and (d) the qualitative and quantitative pattern of unsaponifiable lipids in the intact cell. They differ most notably with regard to (a) the amount of cytochromes (*including* cytochrome c + $c_1$) tightly bound to respiratory particles and (b) the ability of these cytochromes to participate in all the enzymatic activities characteristic of a functional respiratory chain (i.e., DPNH- and succinoxidase, DPNH- and succinate-cytochrome c reductase, and cytochrome c oxidase). In the present communication we wish to report on some immunochemical properties of the respiratory particles which have permitted us to unravel certain additional details of electron transport in the wild type, and to demonstrate a cross-reacting material (CRM)[1] in the mutant capable of neutralizing the anti-DPNH-cytochrome reductase of sera prepared against particles isolated from the wild type.


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## Experimental

The various particle preparations used, the enzymological assay procedures, and the source of the

[1] Abbreviation used in this work: CRM, cross-reacting material.