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*INTRACELLULAR MITOCHONDRIAL DIVERSITY IN VARIOUS STRAINS OF SACCHAROMYCES CEREVISIAE**

BY CHARLOTTE J. AVERS, MARTHA W. RANCOURT, AND FU HAI LIN

DEPARTMENT OF BIOLOGICAL SCIENCES, RUTGERS, THE STATE UNIVERSITY, NEW BRUNSWICK, NEW JERSEY

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One indication of the existence of an independent genetic system is the persistence of diverse genotypes in a common nucleocytoplasmic background.[1, 2] Thus, if it could be shown that different mitochondria coexisted in the same cell, it would indicate some autonomous control of organelle phenotype. Since cytochrome *c* oxidase activity is uniquely mitochondrial,[3, 4] we selected this trait to analyze mitochondrial diversity. The mitochondrial population was numerically and cytochemically stable during the 24–48-hr stationary phase period of growth,[5, 6] and therefore developmental variability was minimal. Of the 59 acriflavin-induced mutants collected, 12 isolates were respiratory-competent, presumably due to the presence of 20–40 cytochrome oxidase-positive mitochondria per cell.[7] No cytochrome oxidase reaction was detected in the remaining 47 mutants. The wild-type strain had about 45 reaction-positive mitochondria per cell during stationary phase.[6, 7] The present report describes further evidence derived from electron cytochemistry and from enzymological studies of cell-free extracts.

Materials and Methods.—Diploid cultures of *Saccharomyces cerevisiae* were used exclusively. The wild-type and induced mutants have been described previously.[6, 7] The electron microscopical analysis was performed only for wild-type iso-N, for isolates DP-44, DP-39, DP-33, and DP-31 of the respiratory-competent mutant group, and for DP-62, DP-60, DP-46, and DP-28

of the respiratory-deficient (RD) mutants. All 12 of the respiratory-competent mutants, however, were analyzed manometrically and spectrophotometrically for cytochrome oxidase activity, as were iso-N and the four RD mutants listed above.

Cytochemistry: Inocula from slants were incubated in 100 ml of a semisynthetic medium[8] in 250-ml Erlenmeyer flasks kept on a water-bath shaker at 32°C for 24 hr, with continuous and rapid agitation. A cell pellet was prepared from the culture, and an inoculum of 10^7–10^8 cells was introduced into fresh liquid medium for growth on the shaker, as described above. After 24 hr, the cells were centrifuged, washed with 0.44 *M* sucrose, and sometimes were exposed to partial cell wall digestion[9] by snail enzyme (Glusulase, from Endo Laboratories, Garden City, L.I.).[6, 7] When used, the enzyme solution was decanted, the cells were washed twice with 0.44 *M* sucrose, and the cytochemical incubation mixture was added directly to these cells in the centrifuge tubes. Cells were incubated in a freshly prepared diamine mixture,[10] as described earlier.[6, 7] The product of enzyme activity appeared as brown-black spots in the cytoplasm. The control medium contained the full set of reaction components plus 10 mM potassium cyanide.

Electron microscopy: Cell pellets were prepared from 24-hr shake cultures, as described above, and fixed in unbuffered 5% sodium permanganate for 75–90 min. Then they were dehydrated in an acetone series, embedded in Epon 812,[11] and sectioned with a diamond knife using the Porter-Blum I or the LKB ultramicrotome. Sections of approximately 300–400 A thickness were mounted on formvar-coated titanium or copper grids and examined in the RCA EMU-3G electron microscope at initial magnifications of 16,000–50,000 ×. For cytochrome oxidase activity studies, snail enzyme treatment was omitted because of flocculation problems, and the cytochemical incubations were carried out as usual. The cells then were prepared and examined in the same manner as described for the untreated material. Potassium cyanide controls also were observed ultrastructurally. Some of the preparations were stained with lead hydroxide,[12] but many were left unstained.

Cell-free extracts: Homogenates were prepared from cells grown under vigorous aeration for 40–48 hr at 28°C on a rotary shaker. About 10^8–10^9 cells were inoculated into 2-liter Erlenmeyer flasks containing 1 liter of semisynthetic medium. After 40–48 hr, the cells were centrifuged at 1,000 × *g* for 10 min, washed with distilled water, and centrifuged again at 1,000 × *g* for 10 min. The 10–12 gm of cells harvested after the second centrifugation were suspended in 10 ml of 0.5 *M* sucrose and broken in the French pressure cell at 16,000 psi. This mixture was centrifuged twice, for 10–15 min each time at 1,000 × *g*, to remove intact cells and debris. The supernatant fluid of the centrifugation was fractionated further by centrifuging at 29,000 × *g* for 20 min. The pellet was suspended in 20 ml of 0.25 *M* sucrose and centrifuged at 29,000 × *g* for 20 min. The pellet recovered after this was kept at 0°C until 1–4 hr later in the cytochrome *c* oxidase activity assays. All extract manipulations were carried out at 0–4°C.

Enzyme activity assay: The cytochrome *c* oxidase (EC 1.9.3.1) of the pellet was solubilized using a synthetic Zeolite 3A powder[13] (Linde Co., Tonowanda, N.Y.). The aqueous extract of the enzyme preparation was added to the sample cuvette to initiate the reaction at the appropriate time. The method used was essentially that of Wainio and co-workers.[14] The sample cuvette contained 0.1% ferrocytochrome *c*, 1 ml; 0.1 *M* Tris-HCl buffer of pH 7.4, 1 ml; distilled water, 0.9 ml; and a 10^{-2} dilution of the aqueous enzyme extract, 0.1 ml. The enzyme extract contained 20–50 μg of protein per cuvette. The rate of oxidation of mammalian ferrocytochrome *c* at 27°C was measured by recording the decrease in absorbancy at 550 mμ using 3-ml cuvettes having a 1-cm pathlength in a Beckman DB spectrophotometer. Readings were taken from automatic tracings using a Photovolt-43 recorder attached to the spectrophotometer, for at least 5 min. Activities were calculated from the initial constant rates and were expressed as the first-order rate constant (sec^{-1}) per mg protein in the test. At least three readings were made for each extract to check reproducibility, and at least three different extracts were assayed for each strain.

Oxygen uptake: Rates of oxygen uptake were measured using conventional Warburg manometers at 37°C in the presence of air. Cells were suspended in twice their volume of 0.1 *M* sodium phosphate buffer, pH 7.4, and were broken in the French pressure cell at 16,000 psi. The entire mixture was used as the enzyme source. The reaction mixture contained 0.1 ml cell extract (50-mg cells) added to 0.1 *M* sodium phosphate buffer, pH 7.4, 1 ml; 3.12 mg horse-heart cytochrome *c;* 157 μg aluminum chloride; 6.7 mg sodium ascorbate; and distilled water to make a total volume of 3 ml. The center well contained 40 mg KOH. After 10 min for equilibration, the

enzyme source was tipped in, and readings were taken at 10-min intervals for at least 2 hr. The rate of reaction was expressed as μl O_2 per mg cells per hr.

Protein content: Total mitochondrial protein was measured by the Folin method,[15] using crystalline bovine serum albumin as the reference standard.

Sporulation: Cells from vigorously aerated 24-hr shake cultures grown in semisynthetic medium were plated on sporulation agar for 5–7 days at 28°C. The sporulation medium contained 1% potassium acetate, 0.1% dextrose, 0.25% yeast extract, 2% Bacto-agar, in distilled water. Colonies were examined microscopically and those with mature asci were prepared for spreading on plates. Colonies developed from random samples of spores on nutrient agar supplemented with 1% dextrose, for 5 days at 28°C. Tetrazolium agar overlay[7, 8] was used to determine the frequency of respiration-competent colonies (red color develops in 1–3 hr) and respiration-deficient petites (remain white).

Results.—As described earlier,[7] counts of colored reaction deposits in the cytoplasm revealed different numbers for the various strains. Except for 12 of the isolated 59 mutants, all others consistently gave negative reactions in the cytochemical test for cytochrome oxidase activity. The current tests yielded spot counts which were not significantly different from earlier counts. To answer the question of whether one "spot" equals one mitochondrion, ultrastructural analysis was used. Detailed studies were made of selected strains which showed a gradation of counts, from 45 in wild-type iso-N to about 20 in the mutant DP-33, as well as of RD strains. A comparison of brightfield and electron microscopical counts (Table 1 and Fig. 1) clearly showed a direct relationship between the numbers of mitochondria (colored "spots") seen at a resolution of 2,000 A with the light microscope and the numbers of mitochondrial profiles per cell section with electron-dense deposits seen at a resolution of about 20 A. None of the RD mutants contained detectable reaction product at either level of resolution (Fig. 2). In addition, it was clear that the four respiration-competent strains had at least two kinds of mitochondria per cell, cytochrome oxidase-positive and cytochrome oxidase-negative. These strains contained approximately the same numbers of mitochondrial profiles per cell section as did iso-N (Table 1), but there were significantly fewer profiles with associated reaction product per cell section when compared with iso-N (Figs. 3–7). Potassium cyanide controls invariably lacked enzyme reaction product (Fig. 8).

Only those strains which were positive in the cytochemical tests also gave spectrophotometric and manometric evidence of cytochrome oxidase activity (Table 1).

TABLE 1

COMPARATIVE DETERMINATIONS OF RESPIRATORY COMPETENCY IN WILD-TYPE AND MUTANT STRAINS

		Cytochrome Oxidase Activity Assays				
			Electron Microscopy			
Phenotype	Strain	Brightfield cytochemical counts per cell[8] ±S.E.	Mean no. reacted mitochondria per cell section ±S.E.	Mean no. total mitochondria per cell section ±S.E.	First-order rate constant per mg mitochondrial protein	μl O_2 per mg cells per hr
Wild type	iso-N	44.4 ± 0.7	10.1 ± 0.5	11.1 ± 0.5	0.47	10.3
Respiration-competent	DP-39	40.3 ± 0.9*	9.0 ± 0.5	11.2 ± 0.6	0.39	9.7
	DP-31	34.2 ± 1.0*	7.2 ± 0.3*	11.2 ± 0.6	0.27	9.9
	DP-44	27.8 ± 1.1*	7.0 ± 0.3*	10.5 ± 0.4	0.36	7.2
	DP-33	20.2 ± 0.9*	5.2 ± 0.3*	10.9 ± 0.5	0.33	5.3
Respiration-deficient	DP-62, DP-60, DP-46, DP-28	0.0*	0.0*	3.8 ± 0.3*	0.00	0.5

* Significantly different from iso-N (P = <0.01).

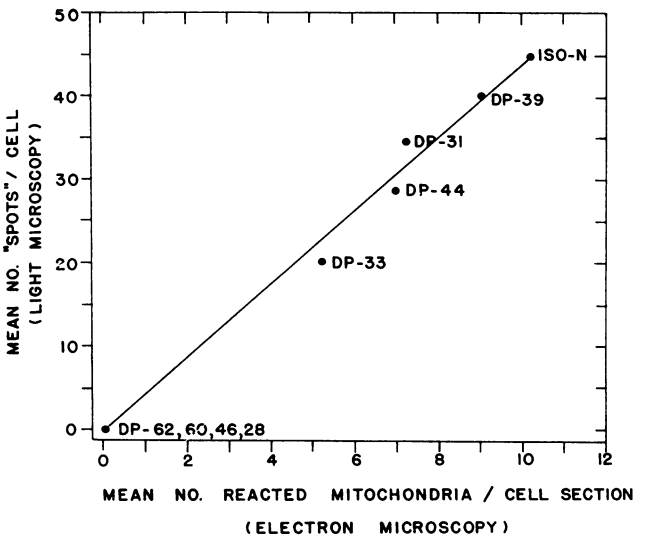


FIG. 1.—Relationship between counts of reacted mitochondria seen as colored "spots" after light microscopy of cytochemically incubated cells and as electron-dense mitochondrial profiles per cell section after electron microscopy. For each strain, a minimum of 150 cells from at least three replicate tests provided the mean brightfield values, and 40–100 whole cell sections from at least three different preparations were tabulated for the electron microscopical counts. Details are given in Table 1.

But there was little correspondence between the manometric and spectrophotometric values or between either of these and the cytological counts. Even greater inconsistencies were apparent when all 12 respiration-competent mutants were examined than for the particular strains in Table 1.

It may be noted that enzyme reaction product was distributed discontinuously along the mitochondrial membranes (Figs. 3–7). Such disjunct deposits would not usually be resolved with the light microscope. Since the average yeast mitochondrion is approximately $0.1 \times 0.3\ \mu$, it is unlikely that separate deposits would occur more than $0.2\ \mu$ apart. Since this is the maximum resolution of the light microscope, disjunct deposits probably would appear as a single aggregate per mitochondrion.

The failure of diploid RD strains to sporulate has been credited to their inability to carry on aerobic respiration.[16] Other factors also must obtain since there are intensely aerobic asporogenous yeasts.[17] None of the RD mutants sporulated. Except for DP-67, which sporulated poorly, the other 11 respiration-competent mutants sporulated as readily as iso-N. The frequencies of RD spores (Table 2) ranged from 0.7 per cent for DP-44 to 30.4 per cent for DP-29.

Discussion.—The absolute correlation among mutant strains of cytochrome *c* oxidase activity measured both spectrophotometrically and manometrically, and positive cytochemical reactions observed by both light and electron microscopy validates the cytochemical test as a qualitative measure of cytochrome oxidase in this system. Person and co-workers[18] also provided evidence that the diamine cytochemical reagent was oxidized via cytochrome *c*, which then was oxidized by cytochrome oxidase.

The localization of reaction product only in mitochondria may preclude the identity of the enzyme as cytochrome *c* peroxidase, which was reported to be active

only in nonparticulate fractions in yeast.[19] The close correspondence between brightfield "spot" counts and the electron microscopical data also favors the specific mitochondrial location of the enzyme activity, especially since we observed the same correspondence for each mutant strain.

TABLE 2

FREQUENCIES OF COLONIES WHICH DID NOT REDUCE TRIPHENYLTETRAZOLIUM IN AGAR OVERLAY TESTS

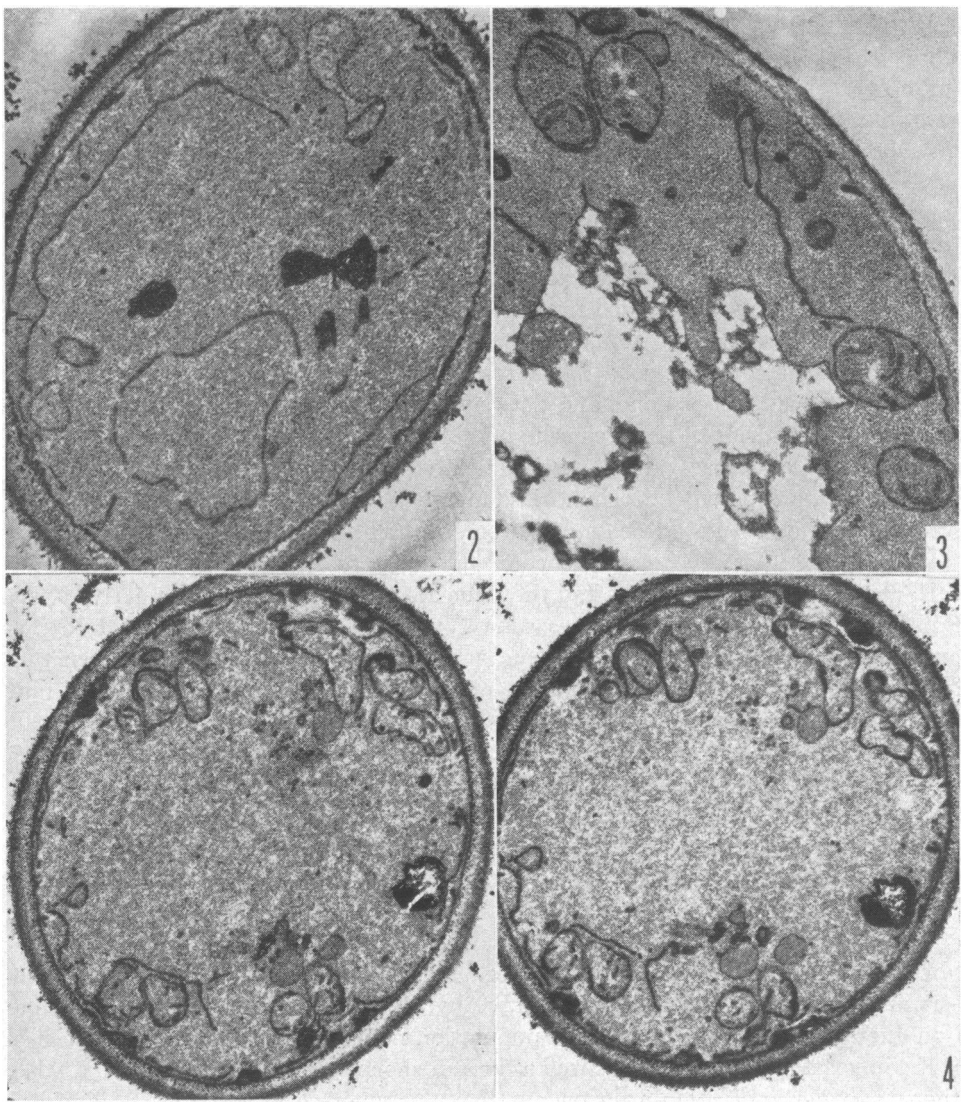
Strain	Percentage "Petites" from: Vegetative cells ($2n$)	Spores (n)
iso-N	0.4	1.1
DP-39	0.4	9.8
DP-31	0.3	7.6
DP-44	0.5	0.7
DP-33	0.2	19.4
DP-62	100.0	—*
DP-28	100.0	—*

* Does not sporulate.

We found no quantitative relation between numerical data obtained from the several procedures. But, should we expect to find such correlations? The cytological evidence is all-or-none, and does not indicate amount of activity per competent mitochondrion. If we expected a correlation between the cytological and the spectrophotometric values, we would have to assume that each milligram of protein from competent mitochondria had the same activity rate, and that only protein from defective mitochondria would contribute to variation in rate constants. Also, a direct correspondence would rest upon the further assumption that each competent mitochondrion had the same amount of protein, since activity was expressed on this basis. Such an assumption would be invalid in view of the appreciable range of mitochondrial size and membrane area. Luck[20] has shown that *Neurospora* mitochondria with different phospholipid to protein contents still may be functionally equivalent, as determined by their Q_{O_2}'s and specific enzyme activities, expressed on a protein basis.

Individual differences within or between mitochondria in any single extract probably would be obscured because the measurements express only the average activity of a homogenate. Although the cytological observations lack the degree of quantitation of the other methods, they refer to whole mitochondria of single cells and populations. This resolution is not possible using cell-free extracts.

The persistence of enzyme-positive and enzyme-negative mitochondria in equilibrium mixtures through thousands of cell generations must be due to specific, conserved intramitochondrial information. Any extramitochondrial genetic information should affect all the organelle population equally.[1, 21] There is evidence of DNA in yeast mitochondria[22] as well as in mitochondria of various species.[23] Also, there is evidence of a DNA-dependent RNA polymerase in log phase *Neurospora* mitochondria.[24] Acriflavin and fluorouracil mutagenesis studies have implicated both DNA and RNA in the genetics of vegetative petite mutants in yeast.[25] The physical basis for mitochondrial phenotype control thus has been demonstrated for mitochondria as well as for the nucleus[26] in yeast. Various alternatives might be offered to explain mitochondrial diversity, such as changes in membrane permeability, availability of cofactors and metabolites, structural alterations in membranes, or developmental variability. Each of these possibilities seems implausible in view of the persistence of specific, constant proportions of mitochondrial phenotypes in the mutant strains. Any extrinsically controlled variable should affect all mitochondria alike, or, perhaps different proportions of the organelle population at one time or another in the history of a culture. We have not found such variation within any of these mutant strains.



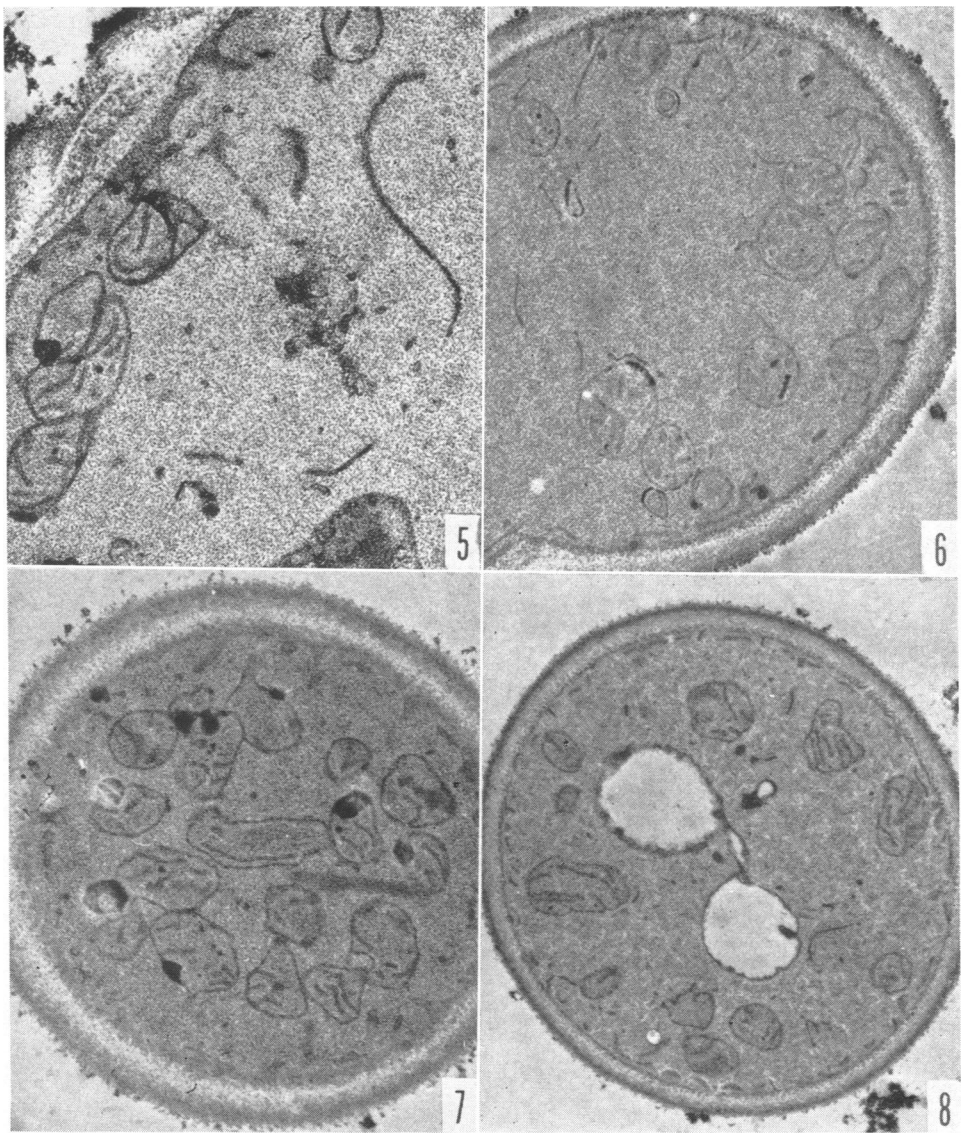
FIGS. 2–4.—Electron micrographs of wild-type and mutant cells after the cytochemical test for cytochrome oxidase activity.

FIG. 2.—Respiration-deficient DP-60, with no reaction product visible in the four mitochondrial profiles. Magnification × 29,000.

FIG. 3.—Respiration-competent mutant DP-44 showing reaction product along membranes of two of the four obvious mitochondria in the cytoplasm. Magnification × 45,000.

FIG. 4.—Two sections in a series of a wild-type iso-N cell showing reaction product associated with each mitochondrion. Magnification × 26,000.

One of the commonest interpretations of intracellular diversity is based upon the stage of development during growth. Cytochrome *c* oxidase activity obviously varies during development of multicellular organisms[27] and of unicellular populations.[6, 28] While we cannot rule out this idea altogether without long-term studies of synchronized cultures, our previous study[6] makes this explanation unlikely. Judged on the basis of growth kinetics, cytochemical analysis, and ultrastructural



FIGS. 5–8.—Electron micrographs of respiration-competent mutant cells after the cytochemical test for cytochrome oxidase activity.

FIG. 5.—Mutant DP-39. Magnification × 50,000.

FIG. 6.—Mutant DP-33 with reaction product present in a minority of the mitochondrial profiles. Magnification × 37,000.

FIG. 7.—Mutant DP-31. Magnification × 36,000.

FIG. 8.—Potassium cyanide control of DP-31 showing no reaction product after 2.5 hr incubation in the complete cytochemical mixture. Magnification × 24,000.

aspects of the chondriome,[29] each strain was in stationary phase when examined. If the reduced numbers of enzyme-positive mitochondria were due to developmental variations, then we should have found a wide fluctuation in counts typical of the growth cycle.[6] No significant differences in cytochemical counts were detected in cultures sampled at 4-hr intervals between 24 and 48 hr of the stationary growth

phase,[5] in contrast with variability in the earlier phases.[6] It seems most unlikely that precisely the same number of undeveloped mitochondria would occur at every period of sampling during 24 hr of stationary phase, and that this number would be consistently different from strain to strain. More probably, maximum numbers of competent mitochondria had been produced, and existed in stable equilibrium mixtures throughout the stationary growth phase.

It is quite clear that cytological data alone cannot provide the whole basis for the postulate that mitochondria contain specific, functional genetic material. But supporting evidence has come from mutagenesis experiments,[25] from DNA localizations,[22-24] and from kinetic studies of thymidine-labeled mitochondria.[30] Genetic analysis is required to establish the postulate more firmly, and such studies currently are in progress in this laboratory.

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*SUBUNIT EXCHANGE AND LIGAND BINDING: A NEW HYPOTHESIS FOR THE MECHANISM OF OXYGENATION OF HEMOGLOBIN**

BY RUTH E. BENESCH, REINHOLD BENESCH,† AND GEORGE MACDUFF

DEPARTMENT OF BIOCHEMISTRY, COLLEGE OF PHYSICIANS AND SURGEONS, COLUMBIA UNIVERSITY

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The mechanism of the reaction between hemoglobin and oxygen has been a matter of active discussion for over half a century. The realization that the binding of oxygen to hemoglobin follows a sigmoid course in contrast to the exponential saturation curve of myoglobin, has given rise to a number of attempts to account for this phenomenon. Douglas, Haldane, and Haldane[1] first suggested that the experimental data could be accounted for if it were assumed that deoxyhemoglobin is more aggregated than oxyhemoglobin, and that only unaggregated molecules are capable of binding oxygen. Although this hypothesis had to be discarded later in the case of mammalian hemoglobin when Adair found the molecular weight to be 67,000,[2] it is extremely interesting that such a reversible polymerization has actually been shown to accompany the deoxygenation of lamprey hemoglobin.[3] Adair's discovery that hemoglobin has four oxygen combining sites per molecule then led him to propose his well-known mechanism of stepwise oxygenation and the concept of heme-heme interaction as the reason for the initial resistance to oxygenation and the subsequent steep rise in oxygen affinity.[4] However, direct interaction between the hemes seems highly unlikely in view of their wide separation in oxyhemoglobin[5, 6] and still wider separation in deoxyhemoglobin.[7, 8] Furthermore, the oxygenation curve of hemoglobin H is not sigmoid, although it contains four hemes per molecule attached to identical polypeptide chains.[9]

The impressive progress which has been made in recent years in unraveling the detailed structure of the hemoglobin molecule has made this protein an ideal candidate for the study of structure/function relationships. This has, of course, also reopened the question of the precise mechanism by which hemoglobin is transformed from the deoxygenated to the oxygenated state.

It should be remembered that long before the dramatic developments in the structural field, Wyman and Allen proposed that the characteristic ligand binding curve of hemoglobin could be explained on the assumption that this protein exists in two distinct conformational states corresponding to the oxygenated and deoxygenated forms, respectively.[10] Some experimental evidence for such a view had, in fact, been presented by Haurowitz as long ago as 1938.[11] In the last few years, the difference in conformation between oxy- and deoxyhemoglobin has been confirmed in a number of different ways,[12–21] culminating in the direct demonstration of the change in subunit arrangement with oxygenation.[8]