NUCLEAR MUTATIONS AFFECTING MITOCHONDRIAL

STRUCTURE AND FUNCTION IN *CHLAMYDOMONAS*

ANDREW WISEMAN, N. W. GILLHAM, and J. E. BOYNTON

From the Departments of Botany and Zoology, Duke University, Durham, North Carolina 27706. Dr. Wiseman's present address is the Division of Biology, California Institute of Technology, Pasadena, California 91125.

ABSTRACT

Wild type cells of the green alga *Chlamydomonas reinhardtii* can grow in the dark by taking up and respiring exogenously supplied acetate. Obligate photoautotrophic (*dark dier, dk*) mutants of this alga have been selected which grow at near wild type rates in the light, but rapidly die when transferred to darkness because of defects in mitochondrial structure and function. In crosses of the *dk* mutants to wild type, the majority of the mutants are inherited in a mendelian fashion, although two have been isolated which are inherited in a clearly nonmendelian fashion. Nine mendelian *dk* mutants have been analyzed in detail, and belong to eight different complementation groups representing eight gene loci. These mutants have been tentatively grouped into three classes on the basis of the pleiotropic nature of their phenotypic defects. Mutants in Class I have gross alterations in the ultrastructure of their mitochondrial inner membranes together with deficiencies in cytochrome oxidase and antimycin/rotenone-sensitive NADH-cytochrome *c* reductase activities. Mutants in Class II have a variety of less severe alterations in mitochondrial ultrastructure and deficiencies in cytochrome oxidase activity. Mutants in Class III have normal or near normal mitochondrial ultrastructure and reduced cytochrome oxidase activity. Eight of the nine mutants show corresponding reductions in cyanide-sensitive respiration.

The biogenesis of mitochondria requires the input of genetic information from at least two genomes, one residing in the nucleus and the other in the mitochondrion itself (26). Mitochondrial genomes of higher animals, including mammals, are small (about 5 μm in length, kinetic complexity of 10^7 daltons), while the mitochondrial genomes of fungi such as *Saccharomyces cerevisiae* (baker's yeast) and *Neurospora crassa*, and of higher plants are considerably larger and have proportionally more informational content (cf. 2, 5).

The limited coding capacity of mitochondrial DNA, especially in mammals, suggests that the mitochondrial genome specifies relatively few mitochondrial proteins and that the nuclear genome must therefore code for a majority of the proteins of this organelle. A number of mendelian (nuclear) mutations affecting mitochondrial function have been characterized in yeast and *Neurospora* (summarized in reference 4). Examples include nuclear mutations affecting cytochrome *c* (43, 44), cytochrome oxidase (13, 48), mitochondrial ATPase (14, 48), malate dehydrogenase (33), aconitase (35), ADP translocation (25, 46), and cyanide-insensitive respiration (15, 16). In addition, nuclear gene products may include mitochondrial peptide chain elongation factors (37), DNA-dependent RNA polymerase (51), and

DNA polymerase (52). Recently, mitochondrial point mutations affecting cytochrome oxidase, cytochrome b, and the F_1 ATPase have also been isolated in yeast (cf. 39).

Wild type cells of the unicellular green alga *Chlamydomonas reinhardtii* can grow either phototrophically by photosynthetically fixing CO_2 as their sole carbon source or heterotrophically by metabolizing exogenously supplied acetate. This alga has a mitochondrial genome of about the same size and informational content as mammalian mitochondrial DNA (38) and a chloroplast genome with an informational content at least ten times as large (3, 50).

In this report we discuss the isolation of a large number of obligate photoautotrophic (*dark dier* or *dk*) mutations in *Chlamydomonas* which are deficient in key mitochondrial functions. Such mutants can grow only under photoautotrophic conditions (light + CO_2) and rapidly die when transferred to heterotrophic conditions (darkness + acetate). The majority of these *dk* mutations are inherited in a mendelian fashion in crosses to wild type. Two *dk* mutations inherited in a clearly nonmendelian fashion are the subject of a separate report (53). In this paper, nine selected mendelian *dk* mutants, representing eight nuclear gene loci, are shown to possess defects in mitochondrial structure and/or respiratory function. Even though each mutant is able to assimilate exogenous acetate, most possess significantly reduced rates of cyanide-sensitive respiration and little or no cytochrome oxidase activity, and some have altered mitochondrial ultrastructure. In addition, three of the *dk* mutants have pleiotropic mitochondrial inner membrane defects accompanied by the reduction of both rotenone- and antimycin-sensitive NADH-cytochrome c reductase activities as well as cytochrome oxidase activity. These respiratory defects presumably result in the inability of the *dk* mutants to grow heterotrophically.

The nine mendelian *dk* mutants have been tentatively grouped into three phenotypic classes on the basis of the pleiotropic nature of their mitochondrial defects. Mutants in Class I have gross alterations in the ultrastructure of their mitochondrial inner membrane together with deficiencies in cytochrome oxidase and antimycin/rotenone-sensitive NADH-cytochrome c reductase activities. Mutants in Class II have a variety of less severe alterations in mitochondrial ultrastructure and deficiencies in cytochrome oxidase activity. Mutants in Class III have normal or near normal mitochon-

drial ultrastructure and reduced cytochrome oxidase activity.

MATERIALS AND METHODS

Culture Conditions

Wild type stocks of both mating types from strain 137c of *Chlamydomonas reinhardtii* were used to derive *dk* mutants. Cells were grown in 300-ml shake cultures at 25°C in high salt (HS) medium (47) or high salt medium supplemented with 0.12% sodium acetate (anhydrous) (HSA) as follows: Phototrophic, HS medium under ~15,000 lx cool white fluorescent light bubbled with 5% CO_2 in air; Mixotrophic, HSA medium under ~15,000 lx cool white fluorescent light; Heterotrophic, HSA medium in darkness using flasks wrapped with black electrical tape. For growth of cells on Petri plates, these liquid media were solidified with 1.5% or 4.0% agar (Difco Laboratories, Detroit, Mich. or Meer Corp., New York) and incubated either under ~6,000 lx cool white fluorescent light or in darkness.

Nitrosoguanidine Mutagenesis

Wild type cells, pregrown to around 1×10^6 cells/ml, were collected in a tabletop centrifuge at room temperature and washed twice with citrate buffer (20.5 ml citric acid, 0.1 M, 29.5 ml sodium citrate, 0.1 M, 50 ml water, final pH 5.5), after which the cells were resuspended to around 2.5×10^6 cells/ml. The cells were then distributed to two 125-ml Erlenmeyer flasks. To one flask, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG, Aldrich Chemical Co., Inc., Milwaukee, Wis.) in a sufficient amount of citrate buffer was added so that the final concentration of MNNG was either 5 μg/ml or 50 μg/ml and the cell concentration was 1.0×10^6 cells/ml. To the other flask, an appropriate volume of additional citrate buffer was added to give 10 ml of cells at 1.0×10^6 cells/ml. Both flasks were gently shaken in a 25°C water bath for 15 min in the dark. The flasks were then brought into the light, the cells transferred to centrifuge tubes, pelleted in a tabletop centrifuge, and washed twice with HS medium. The cells were then diluted to a density of 250 cells/ml for controls, 2,500 cells/ml if 50 μg/ml MNNG was used or 1,500 cells/ml if 5 μg/ml MNNG was used. Aliquots (0.2 ml) of cells were then delivered to 10 plates of HS agar for controls, and to 50-100 plates for the experimentals. Cells were grown phototrophically and allowed to form colonies, after which they were replica-plated to score for the *dk* phenotype. In several mutagenesis experiments, cells were collected on Millipore filters (Millipore Corp., Bedford, Mass.) rather than by centrifugation, according to the method of Lee and Jones (27).

The dk Phenotype

Presumptive *dk* mutations were selected by replica-

plating clones derived from mutagenized cells to HS plates incubated in the light and to HSA plates incubated in the dark. After one week, presumptive *dk* mutants were scored as clones whose dark replicas showed no detectable growth, but whose light replicas had undergone many doublings and were wild type in appearance. Presumptive mutants were repeatedly retested by streaking suspensions of single cells onto HS plates placed in the light and HSA plates placed in the dark. After one week, these populations of single cells of each presumptive *dk* mutant were stringently scored under a dissecting microscope for complete lack of cell division on the dark-incubated plates. Stable *dk* mutants were maintained under phototrophic conditions, i.e., in the absence of acetate, to reduce any selective advantages to wild type revertants.

Genetic Analysis

Standard techniques for crossing, tetrad analysis, and diploid formation were used (11, 18, 28). Gametes were differentiated overnight on N⁻ agar medium in the light (HS without NH₄Cl). Zygotes were matured in the light on N⁻ plates containing 4% agar and germination was induced by returning them to HS plates containing NH₄Cl.[1] This method gave a large increase in zygote viability for crosses involving *dk* mutants, compared to the usual dark maturation technique (28). Allelism was scored in pairwise crosses between *dk* mutants by determining the frequency of zygote clones which contained wild type recombinants. For several pairs of *dk* mutants, spontaneous diploids were selected by plating mating mixtures under restrictive conditions (HSA in the dark) in a fashion analogous to the methods of Harris et al. (19) and Wang et al. (49). Under these conditions, complementing diploids form colonies in 1–2 wk because they are able to grow in the dark on acetate, while *dk* gametes are unable to grow, and zygotes do not germinate.

[¹⁴C]Acetate Assimilation

[¹⁴C]Acetate assimilation was measured essentially by the method of Alexander et al. (1) except that triplicate Whatman no. 3 filters were spotted, soaked in 10% ice cold TCA for 3 min, then in three changes of 5% ice-cold TCA for 10 min. Filters were air dried and counted in a toluene scintillation fluid containing 4 g/l 2,5-diphenyloxazole (Packard Instrument Co., Inc., Downers Grove, Ill.) and 0.1 g/l 1,4-bis[2-(5-phenyloxazolyl)]-benzene (Packard Instrument Co., Inc.).

Whole Cell Respiration

The total and cyanide-sensitive respiration rates of whole cells were measured according to Alexander et al. (1).

Enzyme Assays

Mitochondrial enzyme activities were assayed spectrophotometrically on whole cell homogenates, using either a Zeiss DMR 21 split beam spectrophotometer (Carl Zeiss, West Germany) or an Aminco DW-2 spectrophotometer in the split beam mode (American Instrument Co., Silver Spring, Md.). Enzyme activities were determined at room temperature in crude cell homogenates since we were unable to isolate functional mitochondria from *Chlamydomonas*. Cells growing logarithmically at a density of around 1×10^6 cells/ml were collected, concentrated to a density of 1.0×10^7 cells/ml or 5.0×10^7 cells/ml by centrifugation at 12,000 g for 10 min at 4°C, resuspended in 0.03 M phosphate buffer, pH 7.4, plus 0.1% bovine serum albumin (BSA), and broken twice in a French press (American Instrument Company, Silver Spring, Md.) at 5,000 pounds per square inch. Isocitrate lyase activity (EC 4.1.3.1) was determined by the method of Müller et al. (32). The millimolar extinction coefficient of glyoxylate phenylhydrazone at 324 nm was determined empirically to be 16.6.

The homogenates for assays of cytochrome oxidase (EC 1.9.3.1) were prepared in 0.03 M phosphate buffer, pH 7.4, plus 0.1% BSA. Cytochrome oxidase was assayed according to the method of Cooperstein and Lazarow (9) modified by the addition of 1% (final concentration) Triton X-100 (32). Under conditions where substrate was not limiting, the change in absorbance at 550 nm was not linear with time (45). Thus, all activity measurements were calculated from the change in absorbance during the first minute. Cytochrome oxidase activity measured in this fashion was completely inhibited by 1 mM cyanide. Specific activity was calculated using the millimolar extinction coefficient of 19.6 of Yonetani (54). Antimycin- and rotenone-sensitive NADH-cytochrome *c* reductase (EC 1.6.2.1) activities were assayed by the method of Hatefi and Rieske (20). A medium containing 0.05 M Tris, pH 8.0, 0.67 M sucrose, and 1.0 mM histidine (20) was found to be essential to preserve the activity of NADH-cytochrome *c* reductase. Antimycin-sensitive activity was measured by the addition to the reaction mixture (3 ml final volume) of 0.1 ml antimycin solution to give a final concentration of 600 nM antimycin. The antimycin solution was made from a stock solution (10 mg/ml in ethanol) which was diluted to a concentration of 10 μg/ml antimycin in an aqueous solution containing 0.1 M PO₄, pH 8.0, and 0.1% BSA (20). Rotenone-sensitive activity was determined by the addition to the reaction mixture (3 ml final volume) of 0.1 ml rotenone solution to give a final concentration of 850 nM rotenone. The rotenone solution was made by diluting a stock solution (10 mg/ml in ethanol) 1:100 in water. 0.1 ml of a 0.1% ethanol solution was added to the reaction mixture as a control to determine the effect, if any, of the small amount of ethanol present in the antimycin or rotenone solutions on the enzyme activity. Antimycin and rotenone were pur-

chased from Sigma Chemical Co. (St. Louis, Mo.). For protein determinations, homogenates were routinely treated with 80% acetone in water for 20 min to remove chlorophylls, centrifuged, and the pellet was assayed for total protein by the method of Lowry et al. (29).

Electron Microscopy

Samples were prepared for electron microscopy according to Harris et al. (19), except that the fixation buffer contained HS instead of HSA growth medium.

Diaminobenzidine (DAB) was used as a histochemical stain for cytochrome oxidase (24, 42). After samples of each mutant were fixed with glutaraldehyde and washed with phosphate buffer according to the methods of Harris et al. (19), they were resuspended in 4.5 ml of phosphate buffer, 0.1 M, pH 7.4, and 4.5 ml of culture medium, and treated with the following reagents at room temperature for 15 min: DAB, 5 mg (Sigma Chemical Co.), catalase, 1 ml of 20 μg/ml (Worthington Biochemicals Corp., Freehold, N. J.), and H_2O_2, 0.03 ml of a 30% solution. The samples were then processed as previously described (8, 19) except that staining with uranyl acetate and lead citrate was omitted. The DAB staining reaction was completely inhibited in all samples which showed activity by a 10-min pretreatment of the cells with 10 mM cyanide. The staining of mitochondrial cristae by DAB, expressed as the distance of stained cristae per mitochondrial profile or per mitochondrial area, was quantitated on a Hewlett-Packard Model 20 (Hewlett-Packard Co., Palo Alto, Calif.) equipped with a Hewlett-Packard digitizer. Between 29 and 119 mitochondrial profiles per genotype representing between 2.4 and 7.8 μm^2 mitochondrial area were measured on electron micrographs at a magnification of 60,000.

RESULTS

Induction and Selection of dk Mutants

Phototrophically grown wild type cells of *Chlamydomonas* were mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and stable *dk* mutants were isolated according to the protocol outlined in Fig. 1. Those *dk* mutants which die in the dark because they are impermeable to acetate or lack the glyoxylate cycle enzymes necessary for acetate assimilation are readily detected by their lack of sensitivity to fluoroacetate, an acetate analogue and Krebs cycle inhibitor (7, 31). Fluoroacetate-sensitive *dk* mutants assimilate acetate, and thus must die in the dark because of defects in key mitochondrial functions other than those directly involving the Krebs cycle. Mutations which alter mitochondrial function in *Chlamydomonas* could be localized in either the nuclear, mitochondrial, or possibly even the chloroplast genome. Nuclear

mutations should show mendelian inheritance in crosses to wild type, and chloroplast mutations, a nonmendelian, uniparental pattern of inheritance. Mitochondrial mutations might be expected to show a nonmendelian biparental pattern (53) similar to that of the acriflavin-induced minute mutations (1).

Eight MNNG mutagenesis experiments were performed to induce and select *dk* mutants. Details of two of these experiments are presented in Table I. In both, the frequency of presumptive *dk* mutants is strikingly large, whereas the frequency of confirmed stable *dk* mutants is smaller, but still quite high. The many mutants, initially scored as *dark diers*, which were not stable could have been either *dk* phenocopies or possibly nonmendelian *dk* mutants which later vegetatively segregated wild type offspring. With the exception of two nonmendelian *dk* mutants, a total of 62 *dk* mutants initially classified as stable have remained so for several years. The high frequency of stable, confirmed *dk* mutants suggests either that many different gene loci can be mutated to produce the *dk* phenotype, or that a few *dk* loci are very susceptible to MNNG mutagenesis. The fact that only two of the *dk* mutants thus far tested are allelic (see below) suggests that the former interpretation is correct.

All 62 *dk* mutants were tested for sensitivity to 10 mM fluoroacetate in HS solid medium. Wild type and a majority of the *dk* mutants were found to be sensitive to 1 mM fluoroacetate. The six mutants resistant to 10 mM fluoroacetate were probably either acetate permeability mutants or deficient in one or both glyoxylate cycle enzymes, isocitrate lyase, or malate synthetase.

A preliminary genetic analysis of 48 of the *dk* mutants, presented in Table I, revealed that a majority are inherited in a mendelian fashion and are thus mutations of the nuclear genome. Certain other *dk* mutants produced some zoospores with a phenotype intermediate between wild type and *dk*, symbolized *dk*$^{+/-}$, and were classified as having unclear patterns of segregation, although many could be leaky mendelian mutations. Our wild type stocks have been shown to contain a low frequency of *dk*$^{+/-}$ phenotypes (53). Two of the *dk* mutants analyzed were clearly nonmendelian and will be discussed in detail elsewhere (53).

Genetic Analysis of Mendelian Mutants

Nine mendelian *dk* mutants were selected for

Wild type cells in HS liquid phototrophic culture

|

Mutagenesis with nitrosoguanidine

|

Plate 300 cells per plate on HS in light

|

Replica plate

|

Medium and culture conditions	Growth response	
HS (light)	+	+
HSA (dark)	−	+

Obligate photoautotrophs
(*dk* mutants)
select stable mutants

|

Medium and culture conditions	Test for sensitivity to fluoroacetate to eliminate permeability mutants: Growth response	
HS (light)	+	+
HS + fluoroacetate (light)	−	+

Mutants able to   Permeability and
take up acetate   glyoxylate cycle
                   mutants

|

	Genetic analysis	
Pattern of inheritance in crosses to wild type:	$2dk^+$: $2dk$ mendelian mutants	Others nonmendelian mutants?

1. *Additional genetic studies:* Test for allelism and complementation.

2. *Additional biochemical studies:* Measure citric acid cycle and electron transport enzyme activities; cyanide-, antimycin-, and rotenone-sensitive respiration, and uptake of [^{14}C]acetate.

3. *Electron microscope studies:* Investigate whole cell and mitochondrial ultrastructure and test for an active cytochrome oxidase by using diaminobenzidine histochemical stain.

1. *Additional genetic studies:* Do backcrosses and investigate somatic segregation during vegetative growth.

2. *Additional biochemical and electron microscope studies:* None were done as the mutants were found to be unstable.
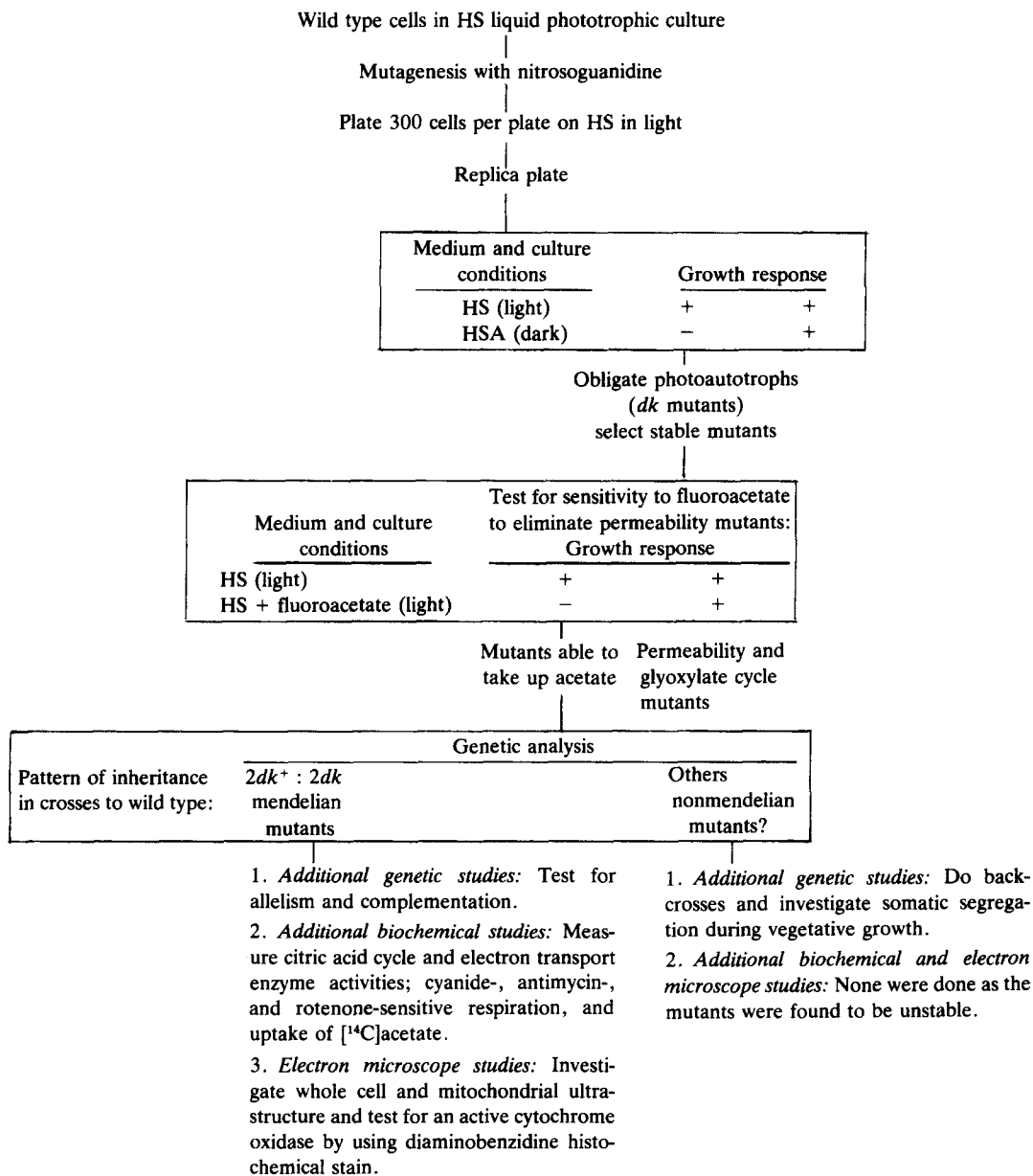
FIGURE 1 Protocol for the selection and analysis of obligate photoautotrophic (*dark dier: dk*) mutants. Wild type (dk^+) cells were pregrown phototrophically in liquid to a density of around 1×10^6 cells/ml.

further study because they were extremely stable and, in preliminary crosses to wild type, had shown clear-cut mendelian inheritance. Additional crosses to wild type, allele testing, and complementation analysis were performed with each of these nine mendelian *dk* mutants. When these mutants are crossed to wild type, the *dk* phenotype in all or a large majority of the tetrads segregates in a mendelian fashion, as does a second mendelian marker, mating type (Table II). The occasional $dk^{+/-}$ progeny recovered in crosses of these mutants as well as in crosses of the wild type controls result in part because there are a few $dk^{+/-}$ cells in the wild type stock and in part because of the variable expression of the *dk* phenotype in occasional *dk* progeny (53).

TABLE I

Recovery and Preliminary Characterization of dk Mutants after Nitrosoguanidine Mutagenesis, Fluoroacetate
Sensitivity, and Preliminary Genetic Analysis of Confirmed dk Mutants

Parameter	Exp 1		Exp 2	
Concentration of MNNG in mutagenesis, $\mu g/ml$	0	5	0	5
Cells plated/plate	50	250	50	250
No. of plates	10	60	10	60
Viability, %	50	21	63	30
Killing due to MNNG, %	—	58	—	52
No. of presumptive dk mutants	0	36	0	113
Frequency of presumptive dk mutants (% of total colonies analyzed)	—	0.6	—	1.7
No. of confirmed stable dk mutants	0	7	0	13
Frequency of stable dk mutants (% of total colonies analyzed)	0	0.1	0	0.2

Total stable dk mutants isolated in eight exp: 62

Sensitivity to fluoroacetate	No.	%	Inheritance pattern	No.	%
Sensitive	56	90	Mendelian	34	55
Resistant	4	7	Nonmendelian	2	3
Leaky resistant	2	3	Unclear	12	19
			Not analyzed	14	23

Wild type (dk^+mt^-) cells were pregrown phototrophically in liquid to a density of 1×10^6 cells/ml, and treated with 5 $\mu g/ml$ nitrosoguanidine (MNNG) for 15 min at 25°C, pH 5.5. Fluoroacetate sensitivity was measured by plating suspension of cells on HS agar medium containing 10 mM fluoroacetate which is 10 × the concentration that kills wild type. The pattern of segregation of dk mutants was determined by analyzing 6-8 tetrads from each mutant crossed to wild type.

To ascertain whether any of the nine mendelian dk mutants were allelic, we determined the frequency of recombinant zygote clones from crosses of dk mutants taken in pairwise combination (Table III). Each zygote clone which contained at least one wild type recombinant among the four meiotic products was scored as a recombinant clone, regardless of whether the parental genotypes were also present (as in recombinant clones arising from tetratype tetrads). Thus, the frequency of recombination could exceed 50% and in some cases approached 70%. Although these frequencies do not represent classical map distances, mutants with a frequency of recombinant clones between 50 and 70% are either unlinked or very distantly linked, whereas mutants with a frequency below 1-2% are tightly linked or allelic. The results presented in Table III indicate that dk-32 and dk-34, with fewer than 1% recombinant clones, are tightly linked and may be allelic, while the remaining seven mutants are distantly linked or unlinked. Three of the pairwise combinations, dk-32 × dk-32, dk-32 × dk-76, and dk-105 × dk-105, yielded virtually no viable progeny even after

repeated crosses with various subclones. In five additional combinations, dk-32 × dk-52, dk-32 × dk-80, dk-34 × dk-52, dk-34 × dk-80, and dk-52 × dk-110, the initial cross yielded few or no viable zygotes, although subsequent crosses did produce more viable progeny. The differences in the frequencies of recombinant zygote clones noted for two of these crosses, dk-32 × dk-52 and dk-52 × dk-110, probably result from large variations in zygote viability.

In order to test whether these mutants at eight different gene loci affect the same or different functions, we constructed a complementation matrix by genetically synthesizing diploids containing pairwise combinations of dk mutants, using a standard selection technique employing two tightly linked arginine auxotrophs (11). Those diploids containing two complementing dk mutations were detected by their restored wild type function, scored as the ability to grow heterotrophically. In addition, each mutant was tested for dominance by synthesizing diploids containing only one dk mutation and its wild type allele. The results of this analysis indicate that, with the exception of

dk-32 and *dk-34* which did not complement and are allelic, the *dk* mutants all complement one another (Table IV). Thus, the nine mendelian *dk* mutants can be placed into eight complementation

TABLE II

Crosses of Mendelian dk Mutants to Wild Type (dk+)

Genotype	Pattern of segregation	Tetrads	Tetrads scored for *mt*
dk+	4dk+ : 0dk	11	2
	3dk+ : 1dk+/-	3	2
dk-32	2dk+ : 2dk	16	2
	2dk+ : 1dk+/- : 1dk	2	–
dk-34	2dk+ : 2dk	17	3
	3dk+ : 1dk	1	–
dk-52	2dk+ : 2dk	21	3
	1dk+ : 1dk+/- : 2dk	1	1
dk-76	2dk+ : 2dk	18	4
dk-80	2dk+ : 2dk	13	3
dk-97	2dk+ : 2dk	13	3
	2dk+ : 1dk+/- : 1dk	1	1
	0dk+ : 4dk	2	–
dk-105	2dk+ : 2dk	16	3
	1dk+ : 1dk+/- : 2dk	1	–
	3dk+ : 1dk	1	–
dk-110	2dk+ : 2dk	18	3
	2dk+ : 2dk+/-	2	–
dk-148	2dk+ : 2dk	14	3
	2dk+ : 1dk+/- : 1dk	3	1
	1dk+ : 1dk+/- : 2dk	1	–
	2dk+ : 2dk+/-	1	–

The parental stock of each mutant was crossed to wild type and the progeny tetrads were analyzed for the segregation of the *dk*, *dk+/-* (weak wild type), or *dk+* (wild type) phenotypes. The presence of occasional *dk +/-* progeny in crosses of the *dk* mutants to wild type as well as in the control cross of wild type to wild type is discussed in the text. All tetrads tested showed 2:2 segregation for the mendelian gene mating type (*mt*).

groups. In addition, these data demonstrate that each *dk* mutation is recessive to its wild type allele. Several pairwise combinations of *dk* mutants, noted in Table IV by an asterisk, did not yield any diploids when the arginine auxotroph selection technique was used, although spontaneous complementing diploids were produced by plating mating mixtures directly into the dark on HSA medium. In this manner, complementing diploids can be selected directly because of their restored wild type function. Neither *dk* gametes nor zygotes can grow in the dark and thus do not develop colonies. These genetic experiments indicate that the nine mendelian *dk* mutations map in eight different loci, all of which are functionally distinct from one another.

To understand the nature of the genetic defects which cause each mutant to be an obligate photoautotroph, we have focused our biochemical and ultrastructural analysis on several key parameters of mitochondrial structure and function. The results of these diagnostic tests, outlined in the following sections, reveal the pleiotropic alterations in each individual mutant (see Table VIII), and allow us, as detailed in the Discussion, to group them according to three general phenotypic classes.

Because obligate photoautotrophy could be the result of defects in acetate metabolism, we verified that each of the nine mendelian *dk* mutants could

TABLE III

Allelism Testing of Selected Mendelian dk Mutants

	dk-32	*dk-34*	*dk-52*	*dk-76*	*dk-80*	*dk-97*	*dk-105*	*dk-110*	*dk-148*
dk+	98	97	97	97	98	98	98	100	96
dk-32	*	1	*6, 52	*	*12, 20	56	55	9	50
dk-34		0	*19, 37	8	*1, 11	61	75	12	24
dk-52			0	53	27	69	50	*9, 71	54
dk-76				0	58	21	50	52	47
dk-80					0	27	79	52	61
dk-97						0	57	53	41
dk-105						¹·¹	*	63	58
dk-110								0	56
dk-148									0

Mendelian *dk* mutants were crossed in pairwise combinations and ~500 of the resulting zygote clones were scored for the percent of zygote colonies containing wild type recombinants. Those combinations producing >1-2% recombinants are probably nonallelic and those producing ~50% or more are probably distantly linked or unlinked. Although mating and germination were not strictly monitored, in most crosses they appeared normal.

* Crosses with very low or no mating or zygote germination.

In some cases, when crosses producing few or no viable zygotes were repeated using other clones of the same mutants, the viability was high enough to yield tentative results, although the results obtained from these crosses suggest a variation in zygote viability depending on the pair of subclones used in the cross.

Wild type is symbolized by *dk+*.

TABLE IV

Complementation Analysis Among Mendelian dk Mutants

	dk-32	dk-34	dk-52	dk-76	dk-80	dk-97	dk-105	dk-110	dk-148
dk⁺	+	+	+	+	+	+	+	+	+
dk-32	−	−	+	+	+	+	+	+	+
dk-34		−	+	+	+	+	+	+	+
dk-52			−	+*	+*	+*	+	+	+
dk-76				−*	+*	+*	+	+	+
dk-80					−*	+*	+	+	+
dk-97						−*	+	+	+
dk-105							−	+	+
dk-110								−	+
dk-148									−

Diploids were constructed between dk mutants or a dk mutant and wild type in pairwise combinations using two tightly linked arginine auxotrophs (arg-2 and arg-7) (as detailed in Materials and Methods) and the resulting diploids were tested for complementation. The ability of a particular diploid to grow heterotrophically was considered a positive indication of complementation between the two dk mutants from which it was constructed. Diploids produced by this method were confirmed by extensive mating type testing which showed them to be mt⁻, the mating type of diploid Chlamydomonas (11).

* Crosses which did not produce any diploids by this technique. The data reported for these combinations were obtained by plating mating mixtures of crosses between dk mutants directly into the dark on HSA media. Those colonies which arise must be complementing diploids, because under these growth conditions gametes do not grow and zygotes do not germinate. Those colonies that did arise were confirmed to be diploids by extensive mating type testing which revealed that all were mt⁻.

Wild type is symbolized as dk⁺.

take up acetate and assimilate acetate normally. Wild type and eight dk mutants were sensitive to 1 mM fluoroacetate, while the ninth, dk-52, was resistant to 10 mM fluoroacetate. Incorporation of [¹⁴C]acetate into radioactive material insoluble in cold TCA revealed that all of the mutants are able to assimilate acetate, although several of them, notably dk-52, dk-80, dk-97, and dk-110, do so at reduced rates (Table V). In addition, one mutant, dk-148, incorporates acetate at a much higher rate than wild type.

The activity of isocitrate lyase, one of two key enzymes of the glyoxylate cycle involved in acetate assimilation, was measured in the nine mendelian dk mutants and wild type (Table V). Isocitrate lyase activity is not detectable in wild type cells grown phototrophically, but is induced by exogenous acetate. Thus, isocitrate lyase activity was measured in the mutants after growth in the light in the presence of acetate for 3 days to insure that the enzyme was fully induced. While none of the mutants had activities that were lower than wild type, two of them, dk-34 and dk-52, had activities significantly higher than wild type (Table V). The apparent difference in isocitrate lyase activity between the allelic mutants dk-32 and dk-34 is not statistically significant because of the small sample

sizes. The fourfold increase observed for dk-52 in comparison to wild type may be a response to its reduced rate of acetate incorporation. On the other hand, dk-97, which had similarly low rates of acetate incorporation, had normal levels of isocitrate lyase activity. Thus, all of the mutants are permeable to acetate and can assimilate it into cell metabolites, although four of them do so at reduced rates.

The respiratory chain in Chlamydomonas is a composite of the classical cyanide-sensitive pathway and a second pathway which branches from the main respiratory chain and is insensitive to cyanide and sensitive to salicylhydroxamic acid (SHAM) (22, 23). To assess mitochondrial respiratory function in the nine mendelian dk mutants, we measured cyanide-sensitive and total respiration in phototrophically grown cells utilizing endogenous substrates. The reduced rates of respiration measured in the mutants during the course of 15 min of darkness were presumed to reflect defects in each mutant's respiratory chain rather than secondary effects due to senescence induced by the short exposure to darkness. During the growth of a culture of wild type cells, both the total respiration rate and the cyanide-sensitive rate change in a characteristic and repeatable fashion

Table V

Fluoroacetate Sensitivity, [¹⁴C]Acetate Assimilation, and Isocitrate Lyase Activities of Mendelian dk Mutants and Wild Type (dk⁺)

Genotype	Sensitivity to 10 mM fluoroacetate	Acetate uptake	Isocitrate lyase activity
		$cpm/10^3$ cells per h	μmol glyoxylate produced/mg whole cell protein per min
dk⁺	+	33 ± 11	19 ± 6
dk-32	+	35 ± 22	21 ± 4
dk-34	+	28 ± 11	47 ± 7*
dk-52	−	11 ± 5*	77 ± 9*
dk-76	+	29 ± 11	15 ± 4
dk-80	+	16 ± 8*	12 ± 7
dk-97	+	13 ± 2*	12 ± 2
dk-105	+	21 ± 9	16 ± 5
dk-110	+	17 ± 5*	16 ± 3
dk-148	+	119 ± 19*	23 ± 14

Fluoroacetate sensitivity (+) was measured on cell suspensions plated on minimal media containing 10 mM fluoroacetate. The incorporation of [¹⁴C]acetate into material insoluble in cold TCA was measured as the increase in counts from h 1 to h 2 after addition of acetate at h 0 to phototrophic cultures. The data are corrected for background and are expressed as counts per minute per 10^3 cells. Isocitrate lyase was assayed on crude homogenates of cells grown to a density of around 1×10^6 cells/ml.

Differences noted in [¹⁴C]acetate incorporation or isocitrate lyase activity between the mutants and wild type were analyzed by t-tests and the results denote the probabilities that the differences are due to chance.

* $P < 0.01$.

The [¹⁴C]acetate and isocitrate lyase data are presented as a mean and a standard deviation.

as the cells increase in number from early log phase (10^5 cells/ml) through stationary phase (10^7 cells/ml) (Fig. 2). Furthermore, the relative proportion of the total rate that is cyanide-sensitive shifts from about 50% during log phase to about 20% at stationary phase.

Because the observed rates of respiraton depend on the growth stage, comparisons between the mutants and wild type could best be made by measuring respiration at various times in a growing culture (Fig. 2). Four of the mutants, dk-80, dk-97, dk-110, and dk-148, have very low cyanide-sensitive respiration rates over most of the growth curve. Concurrent with this, the total respiration rate in each of these mutants is also low. The mutants dk-32, dk-34, dk-76, and dk-105 have intermediate levels of cyanide-sensitive respi-

ration, while that of dk-52 is similar to wild type.

The total respiratory rate of wild type and five dk mutants is inhibited ~15% by 1 mM SHAM, while the respiratory rate of the remaining four mutants, dk-32, dk-34, dk-97, and dk-110, is inhibited ~50%. The total respiratory rate of wild type and each mutant is inhibited 60-70% by 1 mM SHAM plus 1 mM cyanide. Although the concentration of SHAM used does not completely inhibit the remaining cyanide-insensitive respiration, the rates of cyanide-insensitive respiration in the mutants appear to be similar to that of wild type. Thus, each mutant is thought to possess a functional cyanide-insensitive pathway. Except for dk-148, the reduction in total respiration rate in each mutant is largely due to the reduction in cyanide-sensitive respiration. For these mutants, the level of respiratory substrates is probably not greatly reduced, but rather one or more components of the cyanide-sensitive branch of the respiratory chain are not functioning normally. The reduction in both cyanide-sensitive and total respiration rates observed in dk-148 could be due to either reduced levels of respiratory substrates or a defect which affects both branches of the respiratory chain. Although none of the mutants will grow in darkness, Fig. 2 demonstrates that each mutant grows at near wild type rates in the light under strictly photosynthetic conditions.

In agreement with the respiration data (Fig. 2), cyanide-sensitive cytochrome oxidase activity is reduced or absent in each dk mutant when assayed in whole cell homogenates (Table VI). Concurrent with the assays of cytochrome oxidase activity in vitro, the histochemical stain DAB was employed to assess the activity of this enzyme in situ. DAB reacts with cytochrome oxidase (42) in glutaraldehyde-fixed cells in the presence of catalase, and a polymerized oxidation product of DAB is precipitated along the surfaces of the mitochondrial cristae as electron-dense deposits. In addition, DAB reacts with other intermediates involved in cellular oxidation-reduction reactions, resulting in electron-dense deposits on the outer mitochondrial membrane and on the chloroplast lamellae in Chlamydomonas. However, only the reaction with cytochrome oxidase is specifically inhibited by cyanide. Mitochondria from phototrophically grown wild type cells treated with DAB but not contrasted with uranyl acetate and lead citrate are seen in Fig. 3a and b. The mitochondrial cristae "stain" intensely with DAB, whereas cells pretreated with cyanide before the DAB reaction
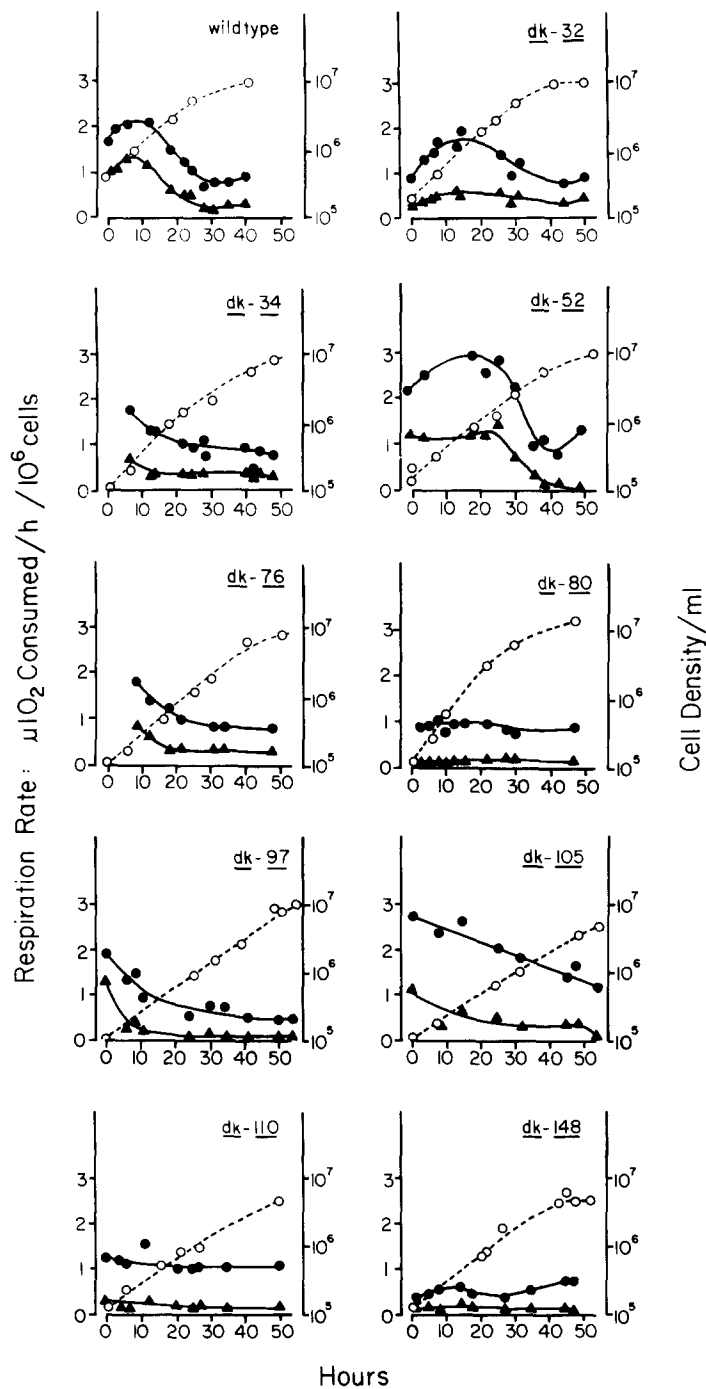
FIGURE 2 Total and cyanide-sensitive respiration of nine mendelian *dk* mutants and wild type. The total (●) and cyanide-sensitive (▲) respiration rates of nine mendelian *dk* mutants and wild type, expressed as μl O_2 consumed per hour per 10^6 cells, were measured with the oxygen electrode. The growth curve (○) is expressed as the cell density in cells per milliliter at various times. These curves demonstrate that both the total and cyanide-sensitive rates of respiration can change during the growth of a culture and that the cyanide-sensitive respiration of many of the mutants is much lower than wild type during log growth.

TABLE VI

Comparison of Cytochrome Oxidase and Antimycin- or Rotenone-Sensitive NADH-Cytochrome c Reductase
Activities of Mendelian dk Mutants and Wild Type (dk⁺)

Genotype	Cytochrome oxidase	NADH-cytochrome c reductase		
		Total activity	Antimycin-sensitive activity	Rotenone-sensitive activity
	μmol cytochrome c oxidized/min per mg whole cell protein	μmol cytochrome c reduced/min per mg whole cell protein × 10^{-2}		
dk⁺	0.35 ± 0.03	6.8 ± 1.8	4.6 ± 1.2	3.8 ± 1.1
dk-32	0.01 ± 0.01*	3.5 ± 0.5*	0.2 ± 0.3*	0.2 ± 0.1*
dk-34	0.01 ± 0.01*	3.7 ± 0.3*	0.1 ± 0.5*	0.7 ± 0.2*
dk-52	0.10 ± 0.02*	7.6 ± 0.2	4.8 ± 0.5	3.6 ± 0.7
dk-76	0.09 ± 0.03*	4.1 ± 1.0*	1.2 ± 0.5*	0.6 ± 0.4*
dk-80	0.01 ± 0.01*	7.7 ± 0.8	5.3 ± 0.5	4.3 ± 0.3
dk-97	0.01 ± 0.01*	9.7 ± 1.9	5.8 ± 0.4	3.5 ± 1.2
dk-105	0.21 ± 0.06*	8.0 ± 0.9	3.3 ± 0.9	3.0 ± 0.2
dk-110	0.002 ± 0.002*	8.1 ± 1.4	3.4 ± 0.6	2.3 ± 0.5
dk-148	0.10 ± 0.04*	5.8 ± 1.8	3.8 ± 1.3	2.2 ± 1.4

Cells were pregrown under phototrophic conditions to a cell density of around 1 × 10⁶ cells/ml. The antimycin- and rotenone-sensitive activities were calculated as the difference between the activities in the presence of the inhibitor and the total activity.

Differences noted in activities between the mutants and wild type were analyzed by t-tests and the results denote the probabilities that the differences are due to chance.

* $P < 0.01$.

The data are presented as the mean and standard deviation of three to five measurements.

have mitochondria with clearly distinguishable but "unstained" cristae (Fig. 3e and f).

The levels of DAB staining of mitochondrial cristae are greatly reduced in six mutants with low cytochrome oxidase activity measured in vitro (Table VII), as can be seen for dk-32 and dk-80 in Fig. 3b and f, respectively. Certain of these mutants, e.g. dk-80 (Fig. 3f) and dk-97, do retain low levels of the DAB reaction which is confined to several heavily stained cristae among many unstained cristae. Apparently, in these mutants, the functional cytochrome oxidase molecules giving the low levels of activity detected in enzyme assays of cell homogenates are clustered in certain cristae rather than being dispersed at a low density over all of the cristae of the mitochondrion. Two of the mutants, dk-105 and dk-148, with moderately reduced levels of cytochrome oxidase activity in vitro appear to have wild type levels of DAB staining (Table VII) which is totally inhibited by cyanide (Fig. 3d and h). While the cytochrome oxidase activity of dk-110 as measured by DAB staining is nearly 40% of the wild type level (Table VII), < 5% of the wild type cytochrome oxidase activity is measured in cell homogenates (Table VI). This may mean that while the DAB-binding site of the enzyme is relatively unaltered, the abil-

ity of the enzyme to catalyze the oxidation of cytochrome c in vitro is greatly reduced.

In order to evaluate possible pleiotropic lesions affecting the mitochondrial respiratory chain in each of the mutants, we determined the activity of a second portion of the pathway, antimycin- and rotenone-sensitive NADH-cytochrome c reductase. NADH-cytochrome c reductase is a composite activity comprising the entire respiratory chain from the NADH dehydrogenase to cytochrome c (20). A similar activity, localized on the outer mitochondrial membrane, also utilizes NADH to reduce cytochrome c, but is not sensitive to the respiratory inhibitors antimycin or rotenone (17). Because antimycin inhibits the respiratory chain between cytochromes b and c whereas rotenone inhibits between NADH dehydrogenase and cytochrome b, two distinct respiratory chain enzyme activities can be examined. In three of the nine mutants, dk-32, dk-34, and dk-76, both the antimycin- and rotenone-sensitive NADH-cytochrome c reductase activities are significantly lower than wild type (Table VI).

Whole Cell and Mitochondrial Ultrastructure

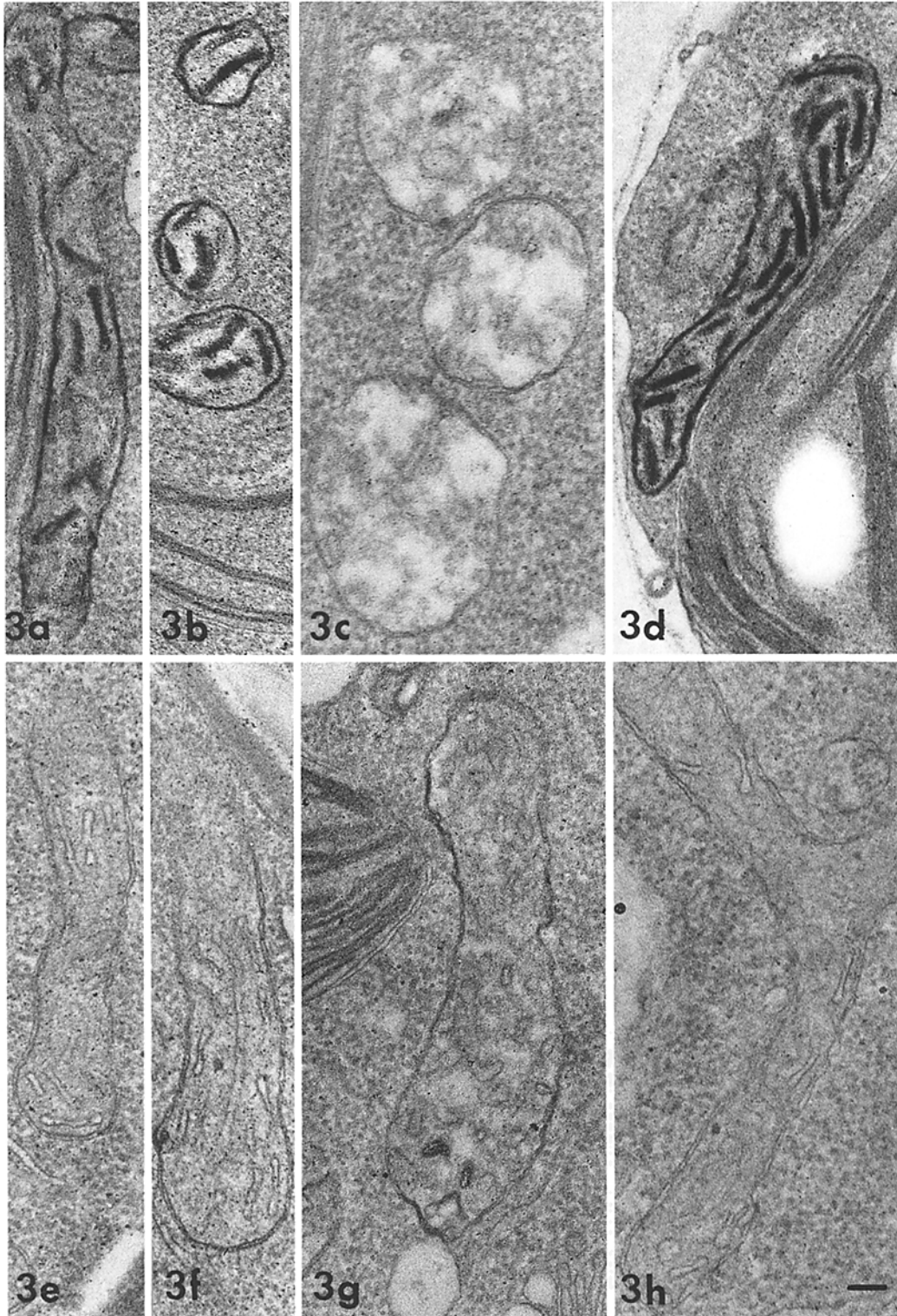Characterization of cell and mitochondrial ul-

FIGURE 3   DAB staining of phototrophically grown wild type and selected *dk* mutants. (*a* and *b*) Wild
type mitochondria stained with DAB. (*e* and *f*) Wild type control mitochondria pretreated with cyanide and
then stained with DAB to demonstrate the inhibition of staining by cyanide, an inhibitor of cytochrome
oxidase. (*c*) Mitochondria from phototrophically grown *dk-32* and (*g*) *dk-80* which have been treated with
DAB showing very low levels of staining of the mitochondrial cristae. (*d*) Mitochondrion of *dk-148* and
(*h*) its cyanide control. Bar, 0.1 μm. × 60,000.

Comparison of DAB Staining of Mitochondria of Nine Mendelian dk Mutants and Wild Type

	DAB-stained cristae per:	
Genotype	Mitochondrion	μm^2 Mitochondrial area $(\times\ 10^{-2})$
	μm	
dk^+	0.46	2.79
$dk^+ + CN$	0.00	0.00
$dk\text{-}32$	0.02	0.05
$dk\text{-}34$	0.06	0.28
$dk\text{-}52$	0.02	0.12
$dk\text{-}76$	0.10	0.46
$dk\text{-}80$	0.04	0.12
$dk\text{-}97$	0.10	0.43
$dk\text{-}105$	1.59	2.91
$dk\text{-}110$	0.15	0.97
$dk\text{-}148$	1.28	2.79

The amount of DAB staining, a histochemical marker for cytochrome oxidase, expressed as the distances of stained cristae per mitochondrion or per mitochondrial area, was quantitated for each mendelian dk mutant and wild type (dk^+) using a Hewlett-Packard Model 20 equipped with a Hewlett-Packard digitizer. Between 29 and 119 mitochondrial profiles per genotype were measured, representing between 2.4 and 7.8 μm^2 mitochondrial area on electron micrographs at $\times$ 60,000. CN, cyanide.

trastructure in each dk mutant permits comparisons of structural alterations in the mitochondria with the observed reductions in cyanide-sensitive respiration, cytochrome oxidase, and antimycin- or rotenone-sensitive NADH-cytochrome c reductase noted for many of the dk mutants. Phototrophically grown wild type Chlamydomonas reinhardtii (Fig. 4a) has a single large cup-shaped chloroplast which surrounds the central nucleus and nucleolus. On the average, 10 elongate mitochondrial profiles per median section are seen dispersed throughout the cytoplasm. The chloroplast comprises around 40% of the total cell area as seen in the median section, while the mitochondria comprise only 1-2% of the total area (6). Wild type mitochondria, shown in Fig. 4b, possess a well-defined double envelope and have a moderate number of fingerlike cristae which project into a rather dark and uniformly staining mitochondrial matrix.

The whole cell and mitochondrial ultrastructure of dk-110 (Fig. 4c and d), dk-76, dk-97 (Fig. 6c and d), and dk-105 are very similar if not identical to wild type despite the fact that each mutant has reduced cyanide-sensitive respiration and cyto-

chrome oxidase activity. The mitochondrial ultrastructure of dk-32 and dk-34, mutants with reduced cyanide-sensitive respiration, cytochrome oxidase, and NADH-cytochrome c reductase activities, is grossly abnormal (Fig. 5 a-d). While the outer mitochondrial membrane of each of these two mutants appears normal, typical cristae are completely absent and are replaced by swollen pockets, sheets, threads, and whorls of inner membrane material. In addition, the matrix does not stain uniformly and matrix material is precipitated adjacent to the abnormal inner membrane. The ultrastructure of the remainder of the cell appears normal despite these extreme mitochondrial defects.

The respiratory deficient mutants, dk-52, dk-80, and dk-148 all have reduced cytochrome oxidase activity and various alterations in mitochondrial ultrastructure, while their NADH-cytochrome c reductase activities are normal. Cells of dk-80 (Fig. 6a and b) contain mitochondria with cristae which appear rounded or "swollen" and not well defined, and the matrix material of dk-80 appears to be precipitated along the cristae surfaces. In addition, these cells contain one or more long, swollen endoplasmic reticula or vacuoles located in the cytoplasm around the periphery of the chloroplast (Fig. 6a). The presence of such swollen endoplasmic reticula or vacuoles is not understood but may be a secondary pleiotropic effect of the defects in mitochondrial structure and function. In dk-148, the mitochondrial matrix material is also precipitated along the surfaces of the cristae, although the morphology of the cristae appears to be normal. In dk-52, the mitochondrial outer membrane and the chloroplast and nuclear envelopes consistently stain less intensely than those of wild type, although the ultrastructure of all organelles appears normal.

DISCUSSION

Obligate photoautotrophy is a common feature of many species of algae (10) and probably results from an impermeability to exogenous carbon sources, because of the lack of specific permeases, or an inability to assimilate them, on account of the absence of key enzymes (34). On the other hand, nine mendelian obligate photoautotrophic (dk) mutants in Chlamydomonas reinhardtii are capable of assimilating acetate into TCA-insoluble material at 50% or more of the wild type rate, and each possesses wild type or slightly elevated levels of the glyoxylate cycle enzyme, isocitrate lyase.
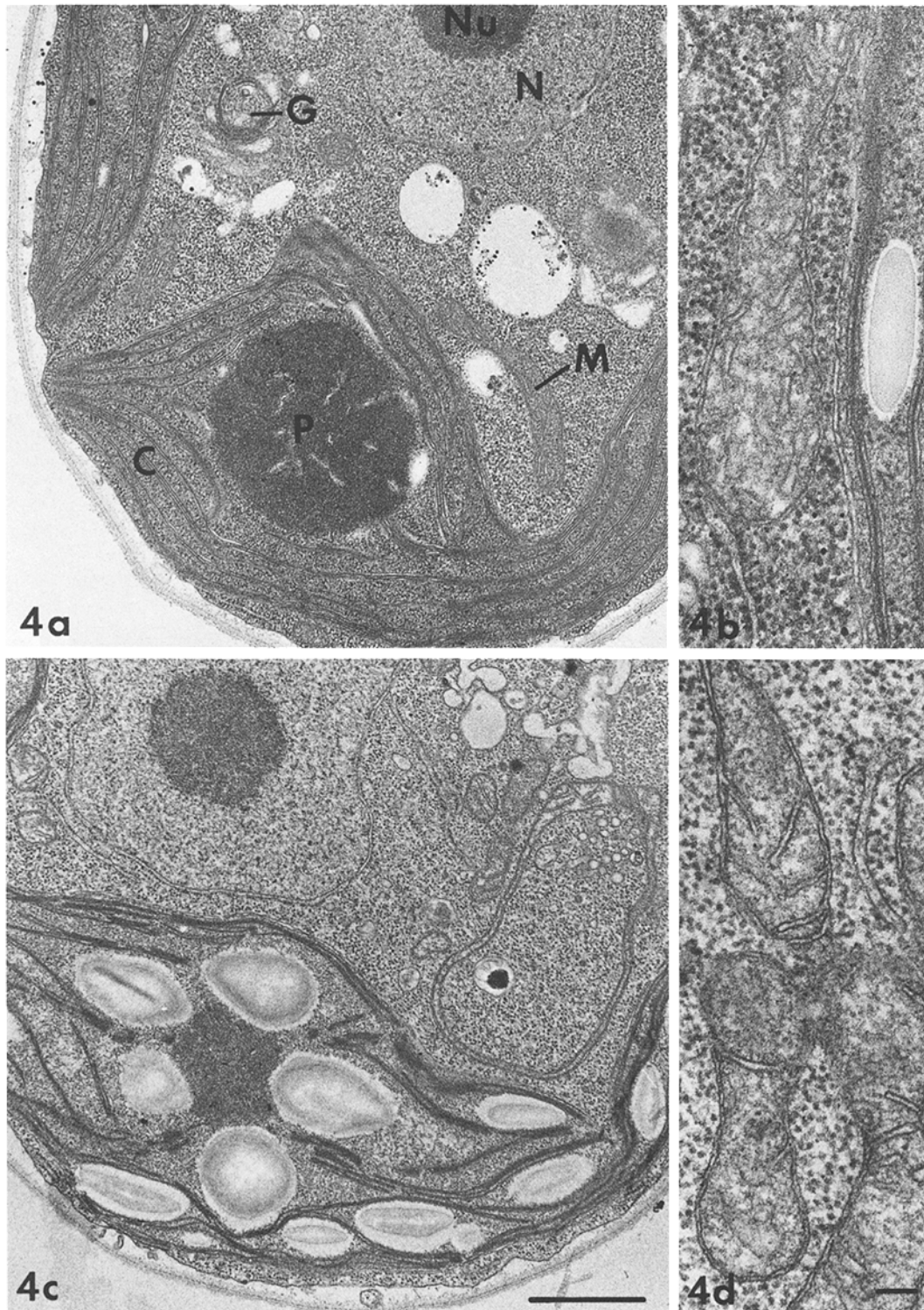
FIGURE 4 Ultrastructure of phototrophically grown wild type and *dk-110*. (*a*) Median section through a phototrophically grown wild type *mt⁻* cell showing the central nucleus (*N*) and nucleolus (*Nu*), mitochondrion (*M*), Golgi apparatus (*G*), and the peripheral chloroplast (*C*) containing a pyrenoid (*P*). × 18,000. (*b*) Higher magnification showing a wild type mitochondrion with well-defined cristae and a uniformly staining matrix. × 60,000. (*c*) Median section through a phototrophically grown *dk-110 mt⁺* cell. Bar, 1 μm. × 18,000. (*d*) Higher magnification showing mitochondria with normal morphology. Bar, 0.1 μm. × 60,000.
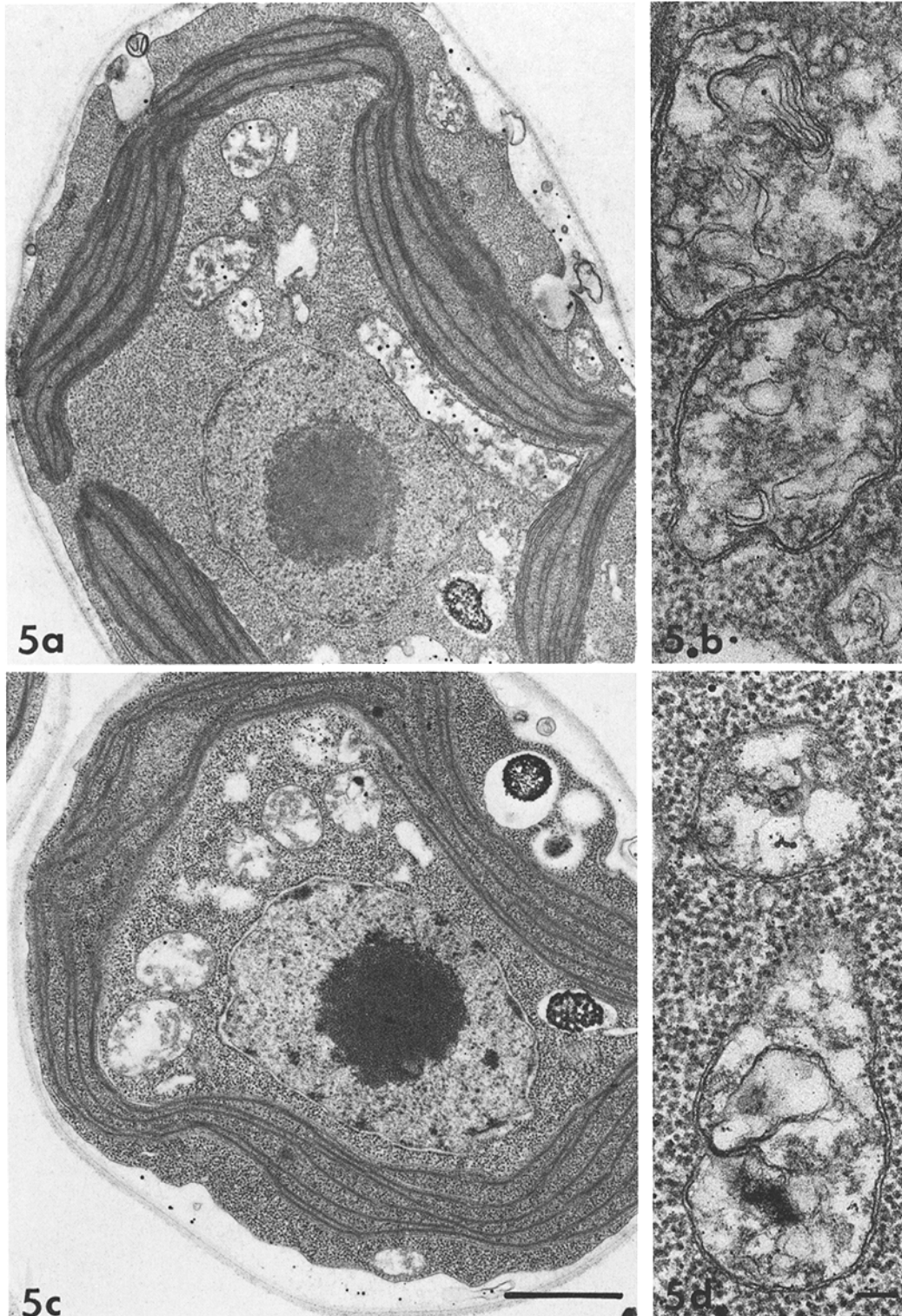
Figure 5 Ultrastructure of phototrophically grown *dk-32* and *dk-34*. (*a* and *c*) Median sections through phototrophically grown *dk-32 mt⁺* and *dk-34 mt⁺*. Bar, 1 μm. × 18,000. (*b* and *d*) Higher magnifications of mitochondria showing the grossly disorganized cristae in each mutant and the ultrastructural similarities between the two mutants. Bar, 0.1 μm. × 60,000.
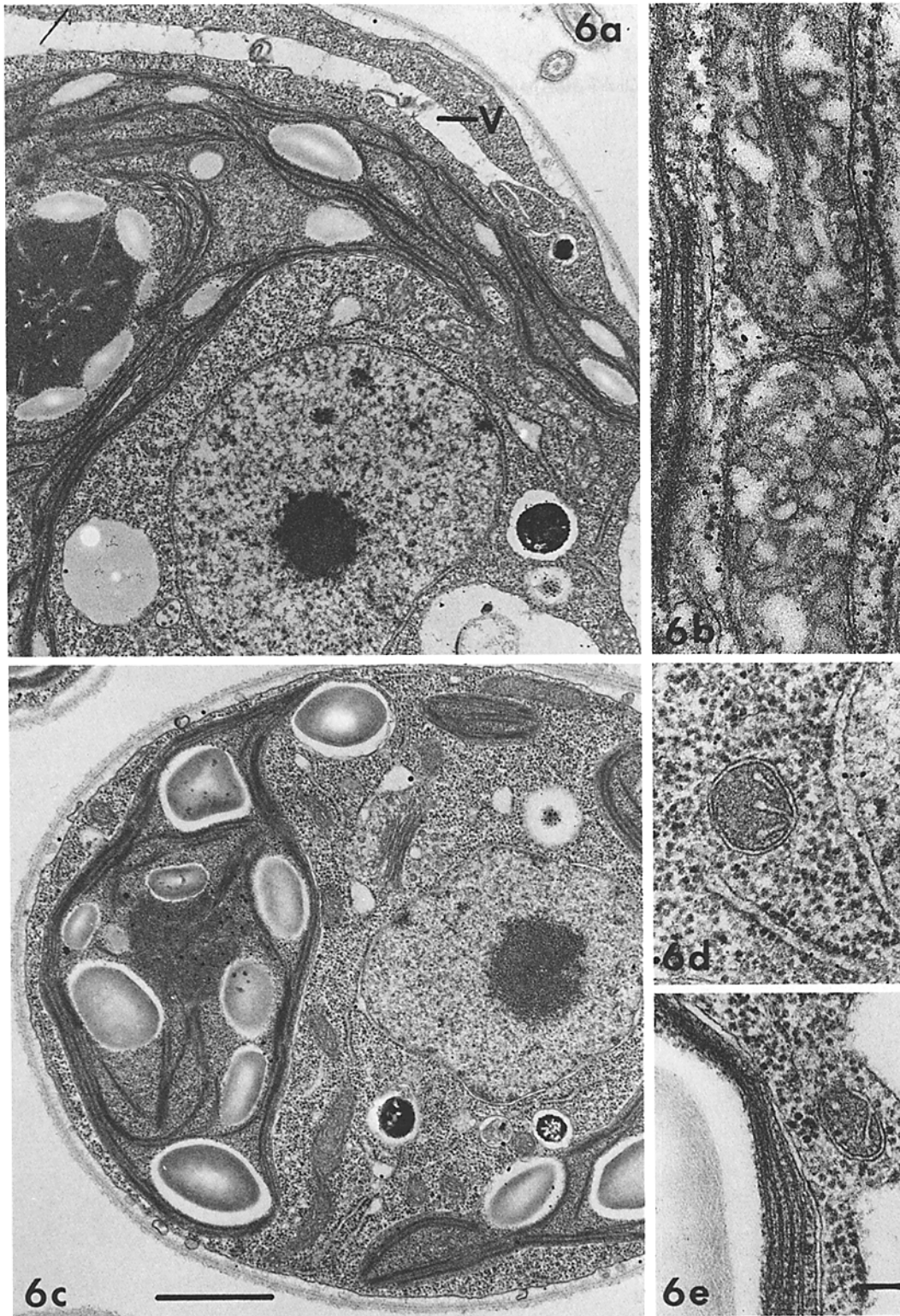
FIGURE 6 Ultrastructure of phototrophically grown *dk-80* and *dk-97*. (*a*) Median section through a phototrophically grown *dk-80 mt⁻* cell showing a large peripheral vacuole (*V*) or swollen endoplasmic reticulum. × 18,000. (*b*) A higher magnification showing distorted saclike cristae. × 60,000. (*c*) Median section through a phototrophically grown *dk-97 mt⁻* cell. Bar, 1 μm. × 18,000. (*d* and *e*) Higher magnifications showing small but normal mitochondria. Bar, 0.1 μm. × 60,000.

Because each mutant grows at near wild type rates in the light, a reduction in the activity of the Krebs cycle, the major source of respiratory substrates, seems unlikely. Because of the central role of the Krebs cycle in intermediary metabolism, any significant reduction in its activity should affect not only cyanide-sensitive respiration and acetate assimilation, but phototrophic growth rates as well. Each mutant appears to have a normal cyanide-insensitive pathway, based on the sensitivity of its respiration to SHAM alone or in combination with cyanide. The cyanide-insensitive pathway in *dk* mutants with significantly reduced cyanide-sensitive respiration may provide the essential function of oxidizing respiratory substrates, especially NADH produced via the Krebs cycle. In the absence of the cyanide-insensitive pathway, mutants with defective respiratory chains and significantly reduced respiration rates might be lethal because oxidized respiratory substrates which could accept electrons from Krebs cycle intermediates would rapidly become depleted.

To place the nine mendelian *dk* mutants in perspective with wild type and with one another, we have summarized in Table VIII all of the basic biochemical and ultrastructural findings of the investigation reported here. This summary demonstrates that *dk-32* and *dk-34* are biochemically and ultrastructurally similar and supports the conclusion, based on genetic evidence, that these two

mutants are alleles. Differences in both biochemical and ultrastructural parameters for each of the remaining seven *dk* mutants demonstrate that each possesses a unique phenotype and support the genetic observations that these mutants are nonallelic and affect separate functions.

This investigation demonstrates that eight of nine mendelian *dk* mutants have diminished cytochrome oxidase activity, and three of these also have reduced antimycin- or rotenone-sensitive NADH-cytochrome *c* reductase activity.

Seven of the eight *dk* mutants with low levels of cytochrome oxidase activity both in vitro and *in situ* also have greatly reduced cyanide-sensitive respiration rates. These findings suggest that in many of the mutants the reduced respiratory activity results from defective respiratory chains incapable of oxidizing respiratory substrates at normal rates. In addition, four mutants possess defects in mitochondrial ultrastructure including grossly disorganized mitochondrial cristae and/or the precipitation of matrix material along the surfaces of the cristae.

These observations suggest that the primary genetic lesion in many of the *dk* mutants is not in a gene coding for specific cytochrome oxidase peptides but rather in a gene that indirectly reduces cytochrome oxidase activity either by limiting its capacity to integrate functionally into the mitochondrial membrane or by altering the machinery

TABLE VIII

Summary of Biochemical and Ultrastructural Properties of Nine Mendelian dk Mutants and Wild Type (dk⁺)

Genotype	Fluoroacetate sensitivity	Acetate incorporation	Isocitrate lyase activity	Cyanide-sensitive respiration	Cytochrome oxidase activity	Antimycin- or rotenone sensitive NADH-cytochrome c reductase	Whole cell ultrastructure (except mitochondria)	Mitochondria Ultrastructure	DAB staining
dk⁺	yes	+	+	+	+	+	normal	normal	+
dk-32	yes	+	+	±	−	−	normal	grossly altered	−
dk-34	yes	+	+	±	−	−	normal	grossly altered	−
dk-52	no	±	+	+	±	+	reduced electron density of membranes	reduced electron density of membranes	−
dk-76	yes	+	+	±	±	±	normal	normal	−
dk-80	yes	±	+	−	−	+	swollen endoplasmic reticulum	swollen cristae	−
dk-97	yes	±	+	−	−	+	normal	normal	−
dk-105	yes	+	+	±	±	+	normal	normal	+
dk-110	yes	±	+	−	−	+	normal	precipitated matrix material	±
dk-148	yes	+	+	−	±	+	normal	precipitated matrix material	+

The biochemical and ultrastructural properties of nine mendelian *dk* mutants are summarized from Tables V to VII and Figs. 2 to 5, as well as unpublished observations. The symbol +, indicates >75% of wild type levels of activity, the symbol ±, ~25-75% of wild type levels of activity, and the symbol −, <25% of wild type levels of activity.

necessary for the transcription or translation of the enzyme or membrane peptides.

On the basis of their pleiotropic phenotypes as summarized in Table VIII, each *dk* mutant with reduced cytochrome oxidase activity can be provisionally assigned to one of three possible phenotypic classes. Mutants in Class I may affect genes which code for components involved directly or indirectly in mitochondrial protein synthesis, including those genes which regulate the biosynthesis of enzymes such as cytochrome oxidase. Mutants in Class II may affect genes which code for nonenzymatic proteins of the inner mitochondrial membrane that are essential for the proper organization of the membrane as a whole or the proper integration into the membrane of other enzymatic proteins such as cytochrome oxidase or the ATP synthetase. Mutants in Class III may alter genes which code for peptides of the enzyme cytochrome oxidase.

Mutants with altered mitochondrial protein synthesis, or mutants affected in membrane biogenesis, particularly the biogenesis of the inner mitochondrial membrane, would likely possess the broadest syndrome of defects in mitochondrial structure and function (Class I). *dk-32*, *dk-34*, and possibly *dk-76* with grossly abnormal mitochondrial structure and/or function and reduced cytochrome oxidase activity most likely exemplify such mutants. The allelic mutants *dk-32* and *dk-34* possess grossly altered inner mitochondrial membranes which appear to be completely disorganized with the membrane components forming random sheets, threads, and amorphous clusters. That these two mutants have virtually no cytochrome oxidase activity and reduced antimycin- and rotenone-sensitive NADH-cytochrome *c* reductase activities is not surprising.

The mutant *dk-76* has moderately reduced antimycin- and rotenone-sensitive NADH-cytochrome *c* reductase and cytochrome oxidase activities and cyanide-sensitive respiration but normal mitochondrial ultrastructure. This mutant is likely to be a leaky mutant of Class I that makes functional respiratory chains, but in reduced amounts, and assembles them into morphologically normal mitochondria.

Mutants such as *dk-52*, *dk-80*, and *dk-148* with structural and/or functional abnormalities of the mitochondrial inner membrane and reduced cytochrome oxidase activity may contain altered nonenzymatic membrane proteins which affect the assembly or function of cytochrome oxidase pep-

tides (Class II).

The mitochondrial cristae of *dk-80* are distorted and swollen, the mitochondrial matrix material is unevenly distributed along these cristae surfaces, and cytochrome oxidase activity is only 2-4% of the wild type level. The mutant *dk-148*, which also contains precipitated matrix material along the surfaces of the cristae, has about 30% of normal cytochrome oxidase activity, but very low total and cyanide-sensitive respiration. While the low respiration rate may be due to a lack of respiratory substrates, this is unlikely because the mutant has very high levels of acetate assimilation. More likely, *dk-148* has a defect in its respiratory chain at a site that has not been studied, possibly cytochrome *c* or succinate dehydrogenase, and this may result in the observed precipitation of matrix material along the mitochondrial cristae.

The mitochondrial membranes of *dk-52* are stained very poorly by both uranyl acetate and lead citrate, which suggests the generalized loss of one or more membrane components. In addition, while the mutant's cytochrome oxidase activity in vitro and *in situ* is at most 30% of normal, its total and cyanide-sensitive respiration rates are as high as wild type's. The reason for this apparent anomaly is not clear but may be due to an uncoupling of respiration from oxidative phosphorylation which is known to increase the respiratory rate (30).

The third class of mutants, those in which only cytochrome oxidase is affected, include *dk-97*, *dk-105*, and *dk-110*. All three mutants have reduced cytochrome oxidase activity, which may account for their reduced rates of cyanide-sensitive respiration, but normal levels of antimycin- and rotenone-sensitive NADH-cytochrome *c* reductase. Although *dk-110* has some precipitated matrix material along its cristae, the morphology of the mitochondrial cristae of these three mutants appears normal, especially when compared to the more extreme defects in mitochondrial ultrastructure in *dk-32*, *dk-34*, or *dk-80*.

The simplest explanation of why the *dk* mutants die in the dark is that they produce insufficient ATP via mitochondrial oxidative phosphorylation for growth and survival, although we have not as yet demonstrated this directly. The nine mutants with reduced or very low cytochrome oxidase activities most likely produce less ATP, at least at the third coupling site. The three *dk* mutants which also have reduced antimycin- or rotenone-sensitive NADH-cytochrome *c* reductase activities may also synthesize reduced amounts of ATP at

sites one and two. The cyanide-sensitive respiration rates of many of the mutants are sufficiently low that even if ATP were made at each coupling site, the total rate of respiratory ATP synthesis would only be 20–50% of the wild type rate, possibly not sufficient to sustain growth in the dark. The gross lesions observed in the inner mitochondrial membranes of dk-32 and dk-34 support the argument that coupled ATP synthesis may be reduced or absent in these mutants.

If these dk mutants die in the dark because of reduced mitochondrial ATP production, then a priori, chloroplast ATP production plus cyanide-insensitive respiration must account for each mutant's ability to grow in the light. For chloroplast photophosphorylation to compensate for the deficiency in ATP production of the mutant mitochondria by supplying all or nearly all of the ATP for phototrophic growth, an excess of ATP must be synthesized over what is required for photosynthetic dark reactions and other chloroplast functions. The ability of chloroplasts to export ATP to the cytoplasm is difficult to assess in vivo, although chloroplasts have been shown to be permeable to both ATP and ADP (41). Heber and Krause (21) have suggested that ATP produced in the chloroplast could be effectively transported to the cytoplasm by way of a shuttle involving triosephosphates such as 3-phosphoglycerate and dihydroxyacetone phosphate, both of which can readily pass across the chloroplast membrane, coupled with a shuttle of reducing agents involving oxaloacetate and malate. Santarius and Heber (40) have shown that illumination of Elodea results in a parallel increase in the level of ATP in both the cytoplasm and chloroplast, and they infer that the increased cytoplasmic ATP is a product of chloroplast photophosphorylation. Raven (36) suggests that the contribution of ATP produced by photophosphorylation to the overall growth of algae may actually exceed the production of ATP by mitochondrial oxidative phosphorylation. Whereas these observations certainly indicate that chloroplasts can produce ATP in excess of what is needed for chloroplast functions, we have not yet shown directly in these dk mutants of Chlamydomonas reinhardtii that a lack of mitochondrial ATP synthesis results in the dk phenotype, and that chloroplast photophosphorylation supports their growth in the light.

Evidence from several genetic and biochemical studies constitutes the present understanding of the interaction of the nuclear and mitochondrial genomes in the biogenesis of the mitochondrion. Ebner et al. (12) isolated 18 different nuclear respiratory deficient mutants in yeast belonging to seven complementation groups. Subsequently, Tzagoloff et al. (48) have isolated 55 more nuclear respiratory deficient mutants which fall in 29 complementation groups. Whether the two sets of complementation groups overlap to any extent is not known. Three of the complementation groups identified by Ebner et al. (12) and 10 identified by Tzagoloff et al. (48) contain mutants specifically deficient in cytochrome oxidase. Several of the other complementation groups identified by Ebner et al. (12) are highly pleiotropic and contain mutants which, in addition to being cytochrome oxidase deficient, show a number of other defects in mitochondrial function. Similar pleiotropic mutants were isolated by Tzagoloff et al. (48) but not characterized in detail. Ebner et al. (13) also demonstrated that the synthesis or integration of mitochondrially translated subunits of cytochrome oxidase can be prevented by nuclear mutations.

The results of the present investigation, as well as those of Ebner et al. (12) and Tzagoloff et al. (48) discussed above, indicate that many mutations which affect the assembly of the mitochondrial inner membrane will also affect the activity of cytochrome oxidase which is an integral part of this membrane. We have discussed three phenotypic classes of mutants that affect mitochondrial biogenesis, all of which while initially having only a single primary defect, probably lead to a pleiotropic phenotype which involves the decreased activity of cytochrome oxidase. The responsible genes may include those coding for (a) components involved directly or indirectly in mitochondrial protein synthesis, including those that regulate the biosynthesis of enzymes such as cytochrome oxidase, perhaps represented by mutants in Class I; (b) nonenzymatic proteins of the inner mitochondrial membrane that are essential for the integration or enzymatic activity of other proteins, possibly represented by Class II mutants; and (c) peptides of the enzyme cytochrome oxidase, which may include some or all of the Class III mutants. Assignment of mutants to these three genetic categories is based on our present knowledge of their biochemical and ultrastructural phenotypes and will serve to define the most reasonable direction for further biochemical investigations to establish the nature of the primary genetic lesion in each mutant. Additional studies of mutations that affect mitochondrial structure and func-

tion in both *Chlamydomonas* and yeast should clarify the role of both nuclear and mitochondrial genetic systems in the biogenesis of the mitochondrion, particularly with respect to key components of the respiratory chain such as cytochrome oxidase and the ATPase.

REFERENCES

1. Alexander, N. J., N. W. Gillham, and J. E. Boynton. 1974. The mitochondrial genome of *Chlamydomonas*. Induction of minute colony mutations by acriflavin and their inheritance. *Mol. Gen. Genet.* **130:**275-290.

2. Ashwell, M., and T. W. Work. 1970. The biogenesis of mitochondria. *Annu. Rev. Biochem.* **39:**251-290.

3. Bastia, D., K.-S. Chiang, H. Swift, and P. Siersma. 1971. Heterogeneity, complexity and repetition of the chloroplast DNA of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U. S. A.* **68:**1157-1161.

4. Beck, J. C., J. H. Parker, W. X. Balcavage, and J. R. Mattoon. 1971. Mendelian genes affecting development and function of yeast mitochondria. *In* Autonomy and Biogenesis of Mitochondria and Chloroplasts. N. K. Boardman, A. W. Linnane, and R. M. Smillie, editors. North-Holland Publishing Co., Amsterdam. 194-204.

5. Borst, P. 1972. Mitochondrial nucleic acids. *Annu. Rev. Biochem.* **41:**333-376.

6. Boynton, J. E., N. W. Gillham, and J. F. Chabot. 1972. Chloroplast ribosome deficient mutants in the green alga *Chlamydomonas reinhardi* and the question of chloroplast ribosome function. *J. Cell Sci.* **10:**267-305.

7. Brady, R. O. 1955. Fluoroacetyl coenzyme A. *J. Biol. Chem.* **217:**213-224.

8. Conde, M. F., J. E. Boynton, N. W. Gillham, E. H. Harris, C. L. Tingle, and W. L. Wang. 1975. Chloroplast genes in *Chlamydomonas* affecting organelle ribosomes. Genetic and biochemical analysis of antibiotic-resistant mutants at several gene loci. *Mol. Gen. Genet.* **140:**183-220.

9. Cooperstein, S. J., and A. Lazarow. 1951. A microspectrophotometric method for the determination of cytochrome oxidase. *J. Biol. Chem.* **189:**665-670.

10. Droop, M. R. 1974. Heterotrophy of carbon. *In* Algal Physiology and Biochemistry. W. D. P. Stewart, editor. Blackwell Scientific Publications, Ltd., Oxford. 530-559.

11. Ebersold, W. T. 1967. *Chlamydomonas reinhardi*: heterozygous diploid strains. *Science (Wash. D. C.).* **157:**447-449.

12. Ebner, E., L. Mennucci, and G. Schatz. 1973. Mitochondrial assembly in respiration-deficient mutants of *Saccharomyces cerevisiae*. I. Effect of nuclear mutations on mitochondrial protein synthesis. *J. Biol. Chem.* **248:**5360-5368.

13. Ebner, E., T. L. Mason, and G. Schatz. 1973. Mitochondrial assembly in respiration-deficient mutants of *Saccharomyces cerevisiae*. II. Effect of nuclear and extrachromosomal mutations on the formation of cytochrome *c* oxidase. *J. Biol. Chem.* **248:**5369-5378.

14. Ebner, E., and G. Schatz. 1973. Mitochondrial assembly in respiration-deficient mutants of *Saccharomyces cerevisiae*. III. A nuclear mutant lacking mitochondrial adenosine triphosphatase. *J. Biol. Chem.* **248:**5379-5384.

15. Edwards, D. L., and F. Kwiecinski. 1973. Altered mitochondrial respiratory chain in a chromosomal mutant of *Neurospora crassa*. *J. Bacteriol.* **116:**610-618.

16. Edwards, D. L., and E. Rosenberg. 1976. Regulation of cyanide-insensitive respiration in *Neurospora*. *Eur. J. Biochem.* **62:**217-221.

17. Ernster, L., and B. Kuylenstierna. 1970. Outer membrane of mitochondria. *In* Membranes of Mitochondria and Chloroplasts. E. Racker, editor. Van Nostrand Reinhold Co., New York. 172-212.

18. Gillham, N. W. 1963. Transmission and segregation of a non-chromosomal factor controlling streptomycin resistance in diploid *Chlamydomonas*. *Nature (Lond.).* **200:**294.

19. Harris, E. H., J. E. Boynton, and N. W. Gillham. 1974. Chloroplast ribosome biogenesis in *Chlamydomonas*. Selection and characterization of mutants blocked in ribosome formation. *J. Cell Biol.* **63:**160-179.

20. Hatefi, Y., and J. S. Rieske. 1967. The preparation and properties of DPNH-cytochrome *c* reductase (complex I-III of the respiratory chain). *Methods Enzymol.* **10:**225-231.

21. Heber, U., and G. H. Krause. 1971. Transfer of carbon, phosphate energy and reducing equivalents across the chloroplast envelope. *In* Photosynthesis and Photorespiration. M. D. Hatch, C. B. Osmond, and R. O. Slatyer, editors. John Wiley & Sons, Inc., New York. 218-225.

22. Henry, M.-F., and E.-J. Nyns. 1975. Cyanide-

insensitive respiration. An alternative mitochondrial pathway. *Sub-Cell. Biochem.* **4:**1-65.

23. IKUMA, H. 1972. Electron transport in plant respiration. *Annu. Rev. Plant Physiol.* **23:**419-436.

24. KEYHANI, E. 1972. Localisation cytochimique de la cytochrome oxidase chez le levure *Candida utilis. J. Microsc. (Paris).* **15:**343-352.

25. KOLAROV, J., J. SUBIK, and L. KOVAC. 1972. Oxidative phosphorylation in yeast. IX. Modification of the mitochondrial adenine nucleotide translocation system in the oxidative phosphorylation deficient mutant op_1. *Biochim. Biophys. Acta.* **267:**465-478.

26. KROON, A. M., and C. SACCONE, editors. 1974. *In* The Biogenesis of Mitochondria, Transcriptional, Translational, and Genetic Aspects. Academic Press, Inc., New York. 551 pp.

27. LEE, R. W., and R. F. JONES. 1973. Induction of mendelian and nonmendelian streptomycin resistant mutants during the synchronous cell cycle of *Chlamydomonas reinhardtii. Mol. Gen. Genet.* **121:**99-108.

28. LEVINE, R. P., and W. T. EBERSOLD. 1960. The genetics and cytology of *Chlamydomonas. Annu. Rev. Microbiol.* **14:**197-216.

29. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193:**265-275.

30. MAHLER, H. R., and E. H. CORDES. 1971. Biological Chemistry. Harper and Row Publishers, New York. 1009 pp.

31. MORRISON, J. F., and R. A. PETERS. 1954. Biochemistry of fluoroacetate poisoning: The effect of fluorocitrate on purified aconitase. *Biochem. J.* **58:**473-479.

32. MÜLLER, M., J. F. HOGG, and C. DEDUVE. 1968. Distribution of tricarboxylic acid cycle enzymes and glyoxylate cycle enzymes between mitochondria and peroxisomes in *Tetrahymena pyriformis. J. Biol. Chem.* **243:**5385-5395.

33. MUNKRES, K. D., and F. M. RICHARDS. 1965. Genetic alteration of *Neurospora* malate dehydrogenase. *Arch. Biochem. Biophys.* **109:**457-465.

34. NEILSON, A. H., O. HOLM-HANSEN, and R. A. LEWIN. 1972. An obligately autotrophic mutant of *Chlamydomonas dysosmos*: a biochemical elucidation. *J. Gen. Microbiol.* **71:**141-148.

35. OGUR, M., L. COKER, and S. OGUR. 1964. Glutamate auxotrophs in *Saccharomyces.* I. The biochemical lesion in the glt_1 mutants. *Biochem. Biophys. Res. Commun.* **14:**193-197.

36. RAVEN, J. A. 1974. Photosynthetic electron flow and photophosphorylation. *In* Physiology and Biochemistry. W. D. P. Stewart, editor. Blackwell Scientific Publications, Ltd., Oxford. 391-423.

37. RICHTER, D. 1971. Production of mitochondrial peptide-chain elongation factors in yeast deficient in

mitochondrial deoxyribonucleic acid. *Biochemistry.* **10:**4422-4425.

38. RYAN, R. S., K.-S. CHIANG, and H. SWIFT. 1974. Circular DNA from mitochondria of *Chlamydomonas reinhardtii. J. Cell Biol.* **63**(2, Pt. 2): 293*a*. (Abstr.).

39. SACCONE, C., and A. M. KROON, editors. 1976. *In* The Genetic Function of Mitochondrial DNA. North Holland Publishing Co., Amsterdam. 354 pp.

40. SANTARIUS, K. A., and U. HEBER. 1965. Changes in the intracellular levels of ATP, ADP, AMP and P_i and regulatory function of the adenylate system in leaf cells during photosynthesis. *Biochim. Biophys. Acta.* **102:**39-54.

41. SANTARIUS, K. A., U. HEBER, W. ULLRICH, and W. URBACH. 1964. Intracellular translocation of ATP, ADP and inorganic phosphate in leaf cells of *Elodea densa* in relation to photosynthesis. *Biochem. Biophys. Res. Commun.* **15:**139-146.

42. SELIGMAN, A. M., M. J. KARNOVSKY, H. L. WASSERKRUG, and J. S. HANKER. 1968. Nondroplet ultrastructural demonstration of cytochrome oxidase activity with a polymerizing osmiophilic reagent, diaminobenzidine (DAB). *J. Cell Biol.* **38:**1-14.

43. SHERMAN, F., J. W. STEWART, M. JACKSON, R. A. GILMORE, and J. H. PARKER. 1974. Mutants of yeast defective in iso-1-cytochrome *c*. *Genetics* **77:**255-284.

44. SHERMAN, F., J. W. STEWART, J. H. PARKER, E. INHABER, N. A. SHIPMAN, G. J. PUTTERMAN, R. L. GARDISKY, and E. MARGOLIASH. 1968. The mutational alteration of the primary structure of yeast iso-1-cytochrome *c*. *J. Biol. Chem.* **243:**5446-5456.

45. SMITH, L. 1955. Spectrophotometric assay of cytochrome *c* oxidase. *Methods Biochem. Anal.* **2:**427-434.

46. SOMLO, M. 1970. Respiration-linked ATP formation by an "oxidative phosphorylation mutant" of yeast. *Arch. Biochem. Biophys.* **136:**122-133.

47. SUEOKA, N. 1960. Mitotic replication of deoxyribonucleic acid in *Chlamydomonas reinhardi. Proc. Natl. Acad. Sci. U. S. A.* **46:**83-91.

48. TZAGOLOFF, A., A. AKAI, and R. B. NEEDLEMAN. 1975. Characterization of nuclear mutants of *Saccharomyces cerevisiae* with defects in mitochondrial ATPase and respiratory enzymes. *J. Biol. Chem.* **250:**8228-8235.

49. WANG, W.-Y., W. L. WANG, J. E. BOYNTON, and N. W. GILLHAM. 1974. Genetic control of chlorophyll biosynthesis in *Chlamydomonas.* Analysis of mutants at two loci mediating the conversion of protoporphyrin-IX to magnesium protoporphyrin. *J. Cell Biol.* **63:**806-823.

50. WELLS, R., and R. SAGER. 1971. Denaturation and the renaturation kinetics of chloroplast DNA from

Chlamydomonas reinhardi. *J. Mol. Biol.* **58:**611–622.

51. WINTERSBERGER, E. 1965. Protein Synthese in isolierten Hefe-mitochondrien. *Biochem. Z.* **341:**409–419.

52. WINTERSBERGER, E. 1970. DNA-dependent RNA polymerase from mitochondria of a cytoplasmic "petite" mutant of yeast. *Biochem. Biophys. Res.*

Commun. **40:**1179–1184.

53. WISEMAN, A., N. W. GILLHAM, and J. E. BOYNTON. 1977. The mitochondrial genome of *Chlamydomonas*. II. Genetic analysis of two non-Mendelian obligate photoautotrophic mutants. *Mol. Gen. Genet.* **150:**109–118.

54. YONETANI, T. 1967. Cytochrome oxidase: beef heart. *Methods Enzymol.* **10:**332–335.