Mitochondrial NADH dehydrogenase in cystic fibrosis

(fibroblasts/O₂ consumption/calcium/electron transport)

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ABSTRACT   We have shown that skin fibroblasts from pa-
tients with cystic fibrosis (CF) and from carriers for CF [het-
erozygotes (HZ)] consume more O₂ than do their controls. When
the mitochondrial electron transport inhibitor rotenone was
added to the cells, the relative inhibition of O₂ consumption was
CF > HZ > controls ($P < 0.005$ in both comparisons). Because
rotenone specifically inhibits NADH dehydrogenase, [NADH:
(acceptor) oxidoreductase, EC 1.6.99.3], which is the enzyme of
energy-conserving site 1 of the mitochondrial electron transport
system, activity and kinetics of this enzyme system were studied
in fibroblast homogenates. NADH dehydrogenase activity was
equal in cells from the three genotypes. At pH 8.0, affinity of the
enzyme for its substrate was CF < HZ = controls; at pH 8.6,
affinity was CF > HZ = controls ($P < 0.005$ for the differences).
pH optima for the genotypes were without exception 8.6 (CF),
8.3 (HZ), and 8.0 (control). HZ and control lines were distin-
guished unequivocally in a blind test on the basis of differences
in pH optima. Purified mitochondrial preparations revealed pH
optima identical to those found in whole cell homogenates.
These data suggest that the mutant gene responsible for CF is
expressed in the complex mitochondrial NADH dehydrogenase
system.

Since its identification as a distinct entity, cystic fibrosis (CF)
has been a genetic and pathogenetic enigma. The disease is
transmitted within families as an autosomal recessive trait, af-
fected individuals possessing a double dose of the mutant gene.
Biological parents of subjects with CF possess a single dose of
the mutant gene and, by definition, are obligatory carriers for
the condition. However, carriers are clinically normal and their
detection prior to the birth of an affected child has been pre-
cluded by the absence of detectable effects of the gene in single
dose. Males affected with CF do not reproduce and affected
females reproduce rarely. Despite this reduced ability of in-
dividuals with CF to have children, CF is a uniquely prevalent
lethal single-gene disease in Caucasian populations. The basis
for the cardinal signs of CF—chronic pulmonary obstruction,
gastrointestinal malabsorption, and the sweat chloride abnor-
mality—and their sequelae has been unexplained. The gene
product responsible for these signs and symptoms of CF has
been widely sought but not yet identified (1).

Although CF is considered to be a generalized disease of
exocrine glands (1–4), a number of laboratories including ours
have used skin fibroblasts as a model system for CF studies.
Fibroblasts are not exocrine cells but they do secrete their
products by mechanisms similar to those operating in exocrine
cells (5). In addition, the donor's genotype is retained in cultured
fibroblasts for many generations. For these reasons, skin fi-
broblasts were chosen as a model for CF studies. Using this cell
model, we found that skin fibroblasts from subjects with CF in
culture express premature senescence (6, 7). To this precocious
aging we related increased intracellular Ca²⁺ which occurs in
cells from CF and obligate carriers (6, 8, 9). The site of the al-

tered intracellular Ca²⁺ pool was traced to mitochondria
(10).

Accumulation of Ca²⁺ by mitochondria is a highly complex
and incompletely understood process. Numerous variables are
involved. Perturbations of any one of several systems potentially
could result in the altered mitochondrial Ca²⁺ pools in CF ob-
served by us. One of the systems related to mitochondrial Ca²⁺
influx consists of the terminal steps of oxidation of metabolites
because the uphill accumulation of Ca²⁺ in mitochondria is
thought to be driven by a proton gradient generated during
electron transport (11, 12). Of the many possible explanations
for our previous findings, we reasoned that the increased se-
questration of Ca²⁺ in mitochondria from subjects with CF and
from carriers might be a reflection of increased electron
transport. If this were in fact the case, O₂ consumption would
be expected to be increased in these cells. We therefore exam-
ined O₂ consumption in cells from CF patients and their age-
and sex-matched controls (13).

We report here that (i) cells from CF patients and obligate
carriers consume significantly more O₂ than do their respective
control cells, (ii) after treatment of the cells with the electron
transport site 1 inhibitor rotenone (14), cells from CF patients
and carriers consume the same amount of O₂ as do controls [i.e.,
cells with the different genotypes respond differentially to the
inhibitor (13)] and (iii) mitochondrial NADH dehydrogenase
[NADH:(acceptor) oxidoreductase, EC 1.6.99.3] energy-con-
serving site 1 of the electron transport system, the target of ro-
tenone, has different properties in cells from CF patients, ob-
ligate heterozygotes, and controls.

MATERIALS AND METHODS

Cell Cultures. Skin fibroblast strains from the upper arm
were obtained from subjects with CF and from their age- and
sex-matched controls and from obligate CF heterozygote par-
ents and their age- and sex-matched controls (8). Cells were
stored in liquid nitrogen at early passages and thawed for use
as needed. In all but a few experiments, strains were passage
matched. For O₂ consumption studies and mitochondrial
preparations, monolayers were grown in 285 × 115 mm roller
bottles (Bellco Glass). For NADH dehydrogenase studies, mo-
nolayers were grown in 75-cm² flasks (Falcon). Monolayers
were cultured in Eagle's minimal essential medium with Earle's
salts plus L-glutamine (K. C. Biologicals, Inc., Lenexa, KA)
supplemented with 10% fetal calf serum and 100 units of
penicillin and 100 μg of streptomycin per ml (GIBCO). The
cultures were maintained at 37°C in 95% air/5% CO₂. Cells
were harvested with trypsin/EDTA [1:250 trypsin plus EDTA
at 0.2 g per liter (GIBCO)].

O₂ Consumption and Rotenone Inhibition. Confluent
monolayers of fibroblasts were harvested by trypsin/EDTA
digestion. Cells were pelleted by centrifugation at 600 × g for

Abbreviations: CF, cystic fibrosis; HZ, obligate heterozygote carriers
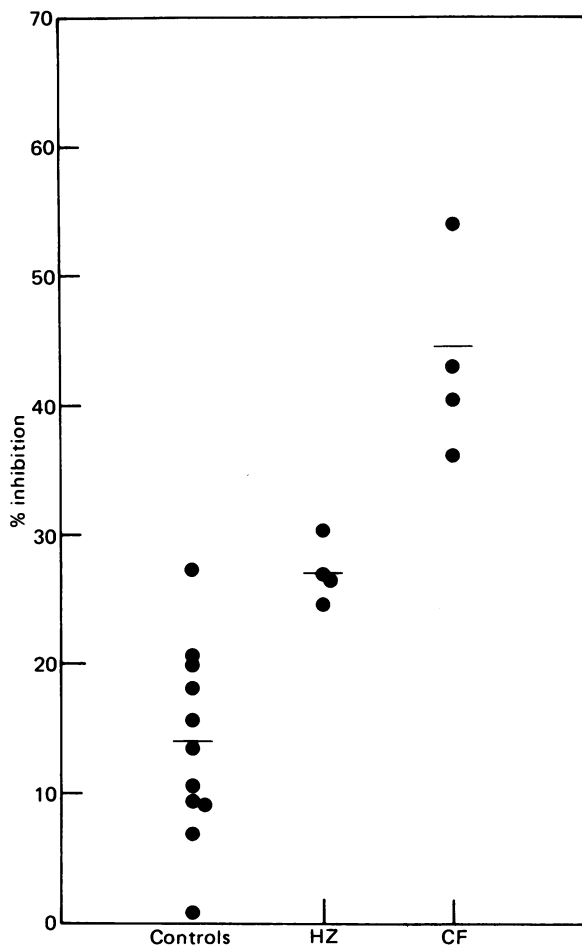for CF.

FIG. 1. O_2 consumption rate by fibroblasts was measured before and after the addition of 4 μM rotenone. The change in O_2 consumption rate caused by rotenone was recorded as percentage of initial O_2 consumption rate. Each point represents inhibition measured on one cell strain.
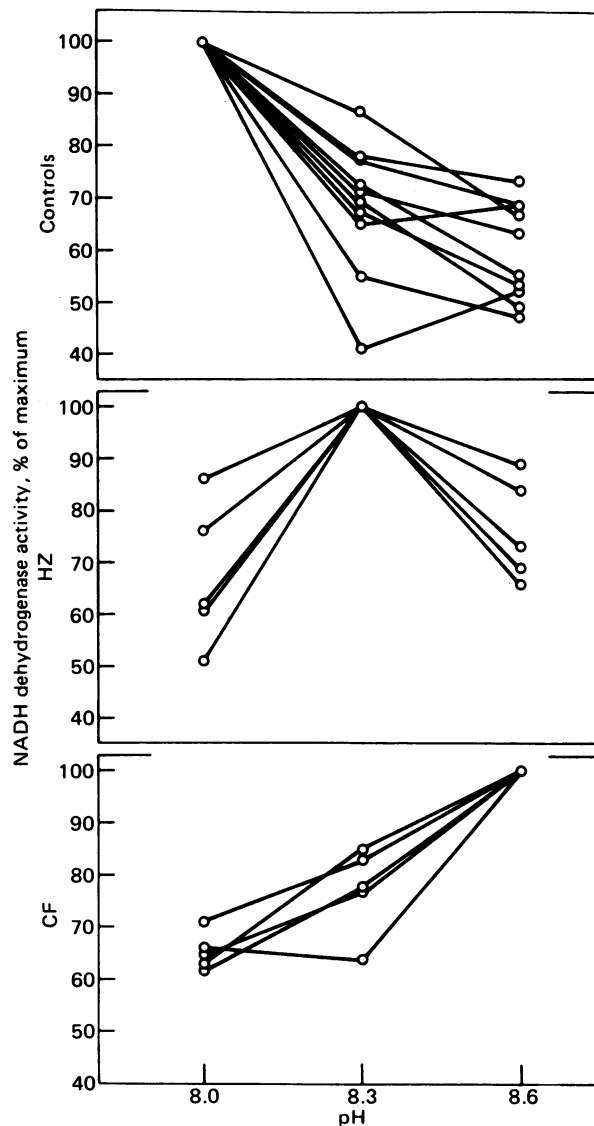

FIG. 2.   pH optima for oxidation of NADH by NADH dehydrogenase from whole cell extract of controls (*Top*), carriers (*Middle*), and CF (*Bottom*) cell strains assayed at room temperature. Potassium ferricyanide was used as an artificial electron acceptor. Each curve represents a different cell strain.

5 min and washed with 10 ml of cold Hanks' balanced salt solution at pH 7.4 containing bovine serum albumin (0.1 mg/ml) and dextrose (10 mg/ml). Aliquots (100 μl) of the washed cell suspension were taken for cell counting in a model ZBI Coulter Counter. The washed cells were again pelleted by centrifugation and resuspended in 1 ml of cold Hanks' solution. O_2 consumption was measured polarographically on a cell suspension of 1×10^7 cells in a final volume of 3 ml by using an O_2 electrode system (Clark model 53, Yellow Springs Instruments) (10). In additional experiments, rotenone (Sigma) was used to specifically inhibit site 1 of the electron transport system. In these experiments the baseline O_2 uptake rate was determined for each cell line. Prior to removal of the cells from the electrode chamber, 100 μl of 100 μM rotenone was added and O_2 consumption was recorded. Preliminary experiments included the addition of 100 μl of 0.1% KCN to assess the extent of inhibition caused by this mitochondrial electron transport inhibitor.

NADH Dehydrogenase. Monolayers were grown to confluence. At the time of assay they were rinsed with 3 ml of trypsin/EDTA and then 5 ml of trypsin/EDTA was added and the monolayer was incubated at 37°C for 10 min. Cells were removed by gentle shaking and transferred to a centrifuge tube containing 1 ml of fetal calf serum (GIBCO). An aliquot (100 μl) was removed and suspended in 9.9 ml of Isoton II balanced electrolyte solution (CMS, Minneapolis, MN) for cell counting. The cell suspension was centrifuged at 410 $\times$ g for 5 min, the

supernatant was discarded, and the pellet was washed twice with 0.03 M potassium phosphate buffer (pH 7.6) at 0°C. The cells were suspended in 2 ml of 0.12 M potassium phosphate buffer (pH 7.6) at 0°C. After removal of a 150-μl aliquot for protein analysis (15) the cell suspension was homogenized at 0°C at approximately 10,000 rpm in a PT-10 Polytron (Brinkmann). The resulting homogenate was centrifuged at 1400 $\times$ g for 10 min and the supernatant was used for assays. Individual flasks yielded between 1.5×10^6 and 2.5×10^6 cells, the cell and enzyme activity yields from the three genotypes being equivalent.

The oxidation of NADH by homogenates was assayed in an Aminco–Bowman spectrophotofluorometer with potassium ferricyanide as an artificial electron acceptor (16). The excitation and emission wavelengths for NADH were 350 and 470 nm, respectively. The reaction mixture consisted of 200 μl of 10 mM potassium ferricyanide (Sigma), 60 μl of 1 mM NADH (Sigma) in 2 mM potassium phosphate buffer, and 2.64 ml of 0.12 M potassium phosphate buffer. NADH concentrations

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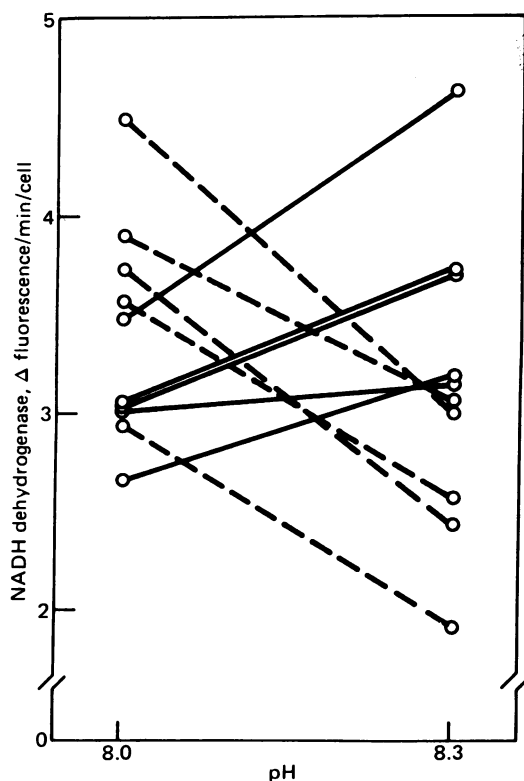*Proc. Natl. Acad. Sci. USA 76 (1979)* 2981

FIG. 3. Oxidation of NADH by NADH dehydrogenase from whole cell extracts of five control (O--O) and five heterozygote (O—O) cell strains was assayed in a blind experiment at pH 8.0 and 8.3. Each line in the graph represents a different cell strain. Without exception, enzyme activity was greater at pH 8.3 than at pH 8.0 in carrier strains and at pH 8.0 than at pH 8.3 in controls. Each strain could be diagnosed blindly as control or CF carrier by this criterion.

FIG. 4. NADH dehydrogenase activity in isolated mitochondrial preparations from CF (O), carrier (△), and control (□) cell strains was assayed at various pH values. [The homogenation and fractionation of mitochondrial preparation has been described (10).] Mitochondrial fractions were frozen and thawed three times to ensure membrane disruption prior to enzyme assay. The control cell strain had its highest activity at pH 8.0, whereas the CF cell strain has its highest activity at pH 8.6. The carrier cell strain showed a broad peak with its optimum at pH 8.3. Purity of the mitochondrial preparation was assayed by transmission electron microscopy and succinate dehydrogenase assay (10). Succinate dehydrogenase activity per mg of protein was greater in the mitochondrial fraction than in the cell homogenate by a factor of 30.

varied between 2.5 and 25 μM. Reaction mixture pH varied between 7.9 and 8.8. Reagents were mixed and incubated for 5 min at room temperature. Nonenzymatic reaction was recorded first, and then 100 μl of whole cell extract was added to the reaction mixture to assay the oxidation of NADH. The reaction was assayed at room temperature. Activity of NADH dehydrogenase was expressed as Δ fluorescence (F)/min per mg of protein or cell. In some experiments, NADH dehydrogenase obtained from isolated mitochondria (10) was assayed.

RESULTS

O$_2$ Consumption. KCN completely abolished measurable O$_2$ uptake in all cell lines tested. Mean ($\pm$SEM) O$_2$ uptake (nmol O$_2$/min per 10^8 cells) was greater in fibroblasts from CF subjects (328.7 $\pm$ 28.2, n = 6) than in their controls (199.5 $\pm$ 22.8, n = 6) and in those from heterozygote subjects (227.7 $\pm$ 17.7, n = 8) than in their controls (156.3 $\pm$ 15.6, n = 8 (P < 0.005, Student's t test in both comparisons).

At the dosage used, rotenone inhibited O$_2$ consumption in all cell strains tested but the inhibitory effect was CF > HZ > controls (P < 0.005 in both comparisons, Student's t test). The inhibitory effect of rotenone on O$_2$ consumption in the three genotypes is shown in Fig. 1.

NADH Dehydrogenase. Maximal enzyme activity in cell homogenates occurred at pH 8.6 in CF strains and pH 8.0 in control strains (Fig. 2). The clear and consistent difference in pH optima for NADH dehydrogenase in CF and control strains occurred at all passages tested (Table 1). In strains from obligate
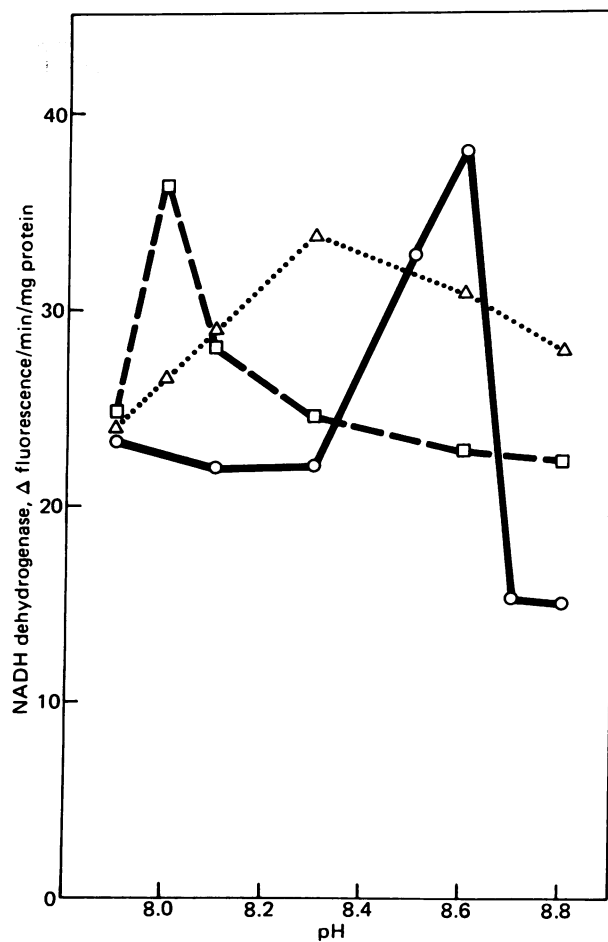
heterozygotes and their controls, optimum pH was 8.3 in the carriers and 8.0 in their controls (Fig. 2).

pH optima repeatedly were different in the three genotypes. In a blind experiment, carriers could be distinguished from their controls on the basis of this variable alone (Fig. 3).

The effect of pH on NADH dehydrogenase activity in relatively pure mitochondrial preparations is shown in Fig. 4. A sharp peak occurred at pH 8.0 in the control strains and at pH 8.6 in the CF strain. Peak activity in the heterozygote strain was at pH 8.3, but the peak was broad. When these experiments were repeated several times with different cell strains they gave consistent results. Under the conditions used V_{max} was equal in the three genotypes. Apparent affinity of the enzyme for its substrate (K_m) was determined for each cell line at pH 8.0 (the control optimum) and at 8.6 (the CF optimum). No difference in K_m between control and HZ strains occurred at either pH. At pH 8.0, K_m for the CF strains was significantly greater and at pH 8.6 it was significantly lower than in the HZ and control strains (P < 0.005 in all comparisons, Student's t test) (Table 1).

Table 1.   Fibroblast strain source and NADH dehydrogenase kinetics

Group	Strain	Donor Sex	Donor Age, yr	pH optimum	Passage(s)	At pH 8.6 K_m, μM	At pH 8.6 Passage	At pH 8.0 K_m μM	At pH 8.0 Passage
CF	46	M	16	8.6	5, 6, 10	9.8	12	27.9	6
CF	69	M	17	8.6	4, 10	8.5	4	25.3	6
CF	70	M	21	8.6	4	9.8	12	34.3	6
CF	71	M	7	8.6	4	11.7	12	37.2	6
CF	72	F	20	8.6	10, 11	11.4	4	36.3	6
HZ	73	F	54	8.3	10	19.2	13	18.9	14
HZ	75	F	35	8.3	9, 11	20.8	13	22.7	14
HZ	76	F	30	8.3	9, 11, 12	14.5	13	22.6	14
HZ	77	M	60	8.3	10, 12	17.2	13	19.9	14
HZ	78	F	48	8.3	11, 12	19.1	13	17.9	14
C(H)	81	F	32	8.0	9, 11	20.8	13	22.6	14
C(H)	82	F	30	8.0	10, 12	19.2	13	18.1	14
C	83	F	22	8.0	4, 10, 11	17.7	12	23.2	7
C	84	M	20	8.0	4, 10	18.3	4	21.2	7
C	85	M	6	8.0	5, 10	20.2	4	18.4	7
C(H)	87	F	54	8.0	10	16.7	13	20.7	14
C(H)	88	F	43	8.0	9, 11	20.0	13	18.7	14
C(H)	89	M	58	8.0	10, 11	18.2	13	21.7	14
C	90	M	20	8.0	4	17.1	12	24.2	7
C	91	M	20	8.0	4	17.0	12	25.9	7

DISCUSSION

Because previously reported findings suggested that increased intracellular Ca^{2+} pools and O_2 consumption occurred in mitochondria from CF and HZ strains and because cell strains of the three genotypes responded differently to rotenone, an inhibitor specific to site 1 of the mitochondrial electron transport system, we examined kinetics of mitochondrial NADH dehydrogenase, the site 1 enzyme. Consistent and significant kinetic differences in NADH dehydrogenase from CF, HZ, and control cell strains were observed. Enzyme from relatively pure mitochondrial preparations revealed the same pH optima differences in the three genotypes as was found in whole cell homogenates. pH optima in microsomal preparations (data not shown) did not coincide with those from whole cell extracts and did not differ among the three genotypes. We conclude, therefore, that the kinetic differences observed reflect mitochondrial derived enzyme.

Alterations in K_m and pH optima of an enzyme ordinarily suggest protein variants (17) such as those usually associated with Mendelian traits. The NADH dehydrogenase system of mitochondria, however, is so complex, "perhaps the most complex of all flavoproteins" (18), that analogy to other proteins may be inappropriate. In the preparations used here, no effort was made to remove membrane phospholipids which are integral to functioning mitochondrial NADH dehydrogenase (19). Theoretically, therefore, the kinetic differences observed could be a reflection of altered amounts or types of mitochondrial phospholipid in cells of the three genotypes. In any case, the consistent difference in pH optima for NADH dehydrogenase activity between HZ (clinically normal in every way) and their controls precludes any pathological or therapeutic effect of CF as being responsible for the differences reported.

NADH Dehydrogenase. This system, the first of several energy-conserving sites in mitochondrial electron transport, has been extensively studied and reviewed (18–20). We are not aware of a previous study of it in human fibroblasts. The enzyme system has been studied thoroughly in preparations from beef heart mitochondria and, to a lesser extent, in yeast (19). Despite the considerable literature on this system, the mechanism of NADH oxidation, the functional organization of

complex I in the inner mitochondrial membrane, and its molecular structure remain obscure (20). Ubiquinone is the natural acceptor for electrons released in the oxidation of NADH, and ferricyanide is the most efficient artificial electron acceptor. The particulate preparation of the enzyme which includes mitochondrial inner membrane phospholipid is thought to most closely resemble the *in vivo* system and has been called complex I of the mitochondrial electron transport system (19). Complex I contains also FMN, iron-sulfur groups, and substantial amounts of ubiquinone 10 (21) and has a molecular weight of approximately $8–8.5 \times 10^5$ (19). The number of different proteins comprising complex I has been controversial. Hatefi and Stiggall (19) consider complex I to be a multienzyme complex. Singer and Edmondson (18) and Ragan (20) suggest that complex I is a multisubunit enzyme.

Despite the complexity and controversy that exist there are several important points of agreement concerning mitochondrial NADH dehydrogenase: (*i*) it is the site of entry of NADH into the respiratory chain; (*ii*) it catalyzes the dehydrogenation of NADH generated through oxidation of numerous NAD^+-linked dehydrogenase reactions; (*iii*) it is the first of three energy-conserving sites where ATP is formed; (*iv*) it is one of three sites of uphill Ca^{2+} influx into mitochondria; and (*v*) it is specifically inhibited by several agents including rotenone (18–21).

Pathogenesis of CF. The earliest lesion in exocrine tissues in CF is the microobstruction of glandular tissue by cellular products that are less soluble than those produced by normal cells (1–4). Dilatation of ducts (the "cysts") and fibrosis ensue. These cystic and fibrotic lesions in the pancreas lead to failure of delivery of digestive enzymes to the gut and, in the lung, to chronic obstruction and infection. The potential role of Ca^{2+} in the production of exocrine products of decreased solubility has been suggested and demonstrated (22). The source of this increased Ca^{2+}, however, has not been identified. Data from this laboratory suggest that cells from subjects with CF sequester greater amounts of Ca^{2+} in their mitochondria, the major Ca^{2+} sequestering organelle of many cell types (12, 23, 24). Mitochondrial Ca^{2+} stores are thought to be the most important

source of cytosolic Ca^{2+} in eukaryotic cells (23). Secretory granules in exocrine glands have been shown to accumulate and export from cells significant amounts of Ca^{2+} in conjunction with their secretory products (25, 26). Furthermore, it has been reported that parotid acinar cells from patients with CF have significantly higher Ca^{2+} concentrations than do cells from controls (27). It appears reasonable to us to conclude that significantly increased mitochondrial Ca^{2+} could be the source of increased Ca^{2+} in exocrine secretions in CF and that incorporation of increased Ca^{2+} in secretory granules is the basis of decreased solubility of otherwise normal secretory products. Data reported here suggest also that the basis for increased sequestration of Ca^{2+} in mitochondria in CF may be anomalous functioning of NADH dehydrogenase, the first of three sites in the respiratory chain associated with Ca^{2+} accumulation by these organelles.

Conclusion. These rationalizations about CF pathogenesis aside, we have traced increased mitochondrial Ca^{2+} and O_2 consumption in CF and HZ fibroblasts to NADH dehydrogenase. We have shown that cells from CF subjects and obligate CF carriers consistently and clearly differ from controls in kinetic characteristics of this enzyme system. We believe that an alteration in a portion of this complex enzyme system may be a fundamental expression of the genetic abnormality in this prevalent lethal disease.

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