Mitochondria: A Historical Review

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Known for over a century, mitochondria have become during the last three decades an important subject of research within several disciplines of experimental biology. For the cytologist, they represented the ideal test objects for applying electron microscopy to the exploration of cellular ultrastructure and for the elaboration of tissue-fractionation techniques with the aim of isolating cytoplasmic organelles. For the biochemist, the identification of mitochondria as the site of cell respiration and respiration-linked phosphorylation implied a decisive step towards the resolution and reconstitution of these processes at a molecular level and the elucidation of their relationship to cellular membranes. For the physiologist, mitochondria afforded the first opportunity for an experimental approach to structure-function relationships, in particular those involved in active transport, vectorial metabolism, and metabolic control mechanisms on a subcellular level. And for the molecular biologist, the discovery of mitochondrial DNA and protein synthesis and the study of mitochondrial biogenesis opened up a new chapter of eukaryotic gene expression.

The purpose of this review is to give a brief account of these developments by selecting some of the highlights of the long and eventful history of mitochondrial research. Detailed historical accounts are found in numerous monographs (1–7) and review articles (8–12) covering various aspects of the field.

The Beginnings

CYTOLOGICAL OBSERVATIONS: The earliest records on intracellular structures that probably represent mitochondria go back to the 1840s (13–19), only a few years after the discovery of the cell nucleus (20). However, Altmann (21) in 1890 was the first to recognize the ubiquitous occurrence of these structures (Table I). He called them "bioblasts" and concluded that they were "elementary organisms" living inside cells and carrying out vital functions. Altmann would have been greatly satisfied by knowing that his idea of the symbiotic origin of mitochondria would be revived several decades later, based on similarities between mitochondria and bacteria (22). The name mitochondrion was introduced in 1898 by Benda (23), and originates from the Greek "mitos" (thread) and "chondros" (granule), referring to the appearance of these structures during spermatogenesis.

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In 1900, Michaelis (24) found that the redox dye Janus Green B serves as a specific supravital stain of mitochondria. As pointed out by Palade (25) in 1964, this feature became the "official portrait" of mitochondria until 1952, when the first high-resolution electron micrographs of mitochondria were published (26). It is remarkable, in view of Michaelis's active interest in biological redox processes, that he did not relate this finding to a possible role of mitochondria in cellular oxidations. In fact, it took 50 years until Lazarow and Cooperstein (27) demonstrated that the specific staining of mitochondria by Janus Green B is due to their capacity to reoxidize the reduced dye by way of cytochrome oxidase.

Plant mitochondria were first described in 1904 by Meves (28). Four years later, Regaud (29) concluded that mitochondria contain protein and lipid. Both Meves (28) and Regaud (30) suggested a role of mitochondria as "bearers of genes." In 1912, Kingsbury (31) arrived at the foresighted conclusion that mitochondria serve as "a structural expression of the reducing substances concerned in cellular respiration." However, these proposals, like many others put forward during the following 20 years (32–40), were based almost exclusively on morphological observations, without direct chemical evidence. As Cowdry (41) pointed out in 1924, "... it is quite obvious that the investigation of mitochondria will never achieve the usefulness which it deserves as an instrument for advance in biology and medicine until we know much more of their chemical constitution as the only accurate basis for interpretation of our findings. In other words, we must wait upon the slow development of direct, quantitative cellular chemistry."

The first decisive step towards this goal was taken when, in 1934, Bensley and Hoerr (42) described the isolation of a fraction containing globular or rod-shaped structures from guinea-pig liver after homogenization in a physiological salt solution and subsequent centrifugation at 2,000 rpm. Although these granules did not stain with Janus Green B, they most probabaly consisted, at least partly, of mitochondria. This method offered the first opportunity for biochemical analysis of an isolated cytoplasmic fraction, and opened the way to the identification of mitochondria as the site of cell respiration.

EARLY STUDIES ON CELL RESPIRATION AND OXIDATIVE PHOSPHORYLATION: From the early 1910s, beginning with the studies of Battelli and Stern (43) on cell-free preparations of dye-reducing dehydrogenases, it has been recognized that biological oxidations are intimately associated with insoluble cellular structures. In 1913, Warburg (44) reported that in extracts of guinea-pig liver, respiration is linked to particles. He called these particles "grana," and suggested

TABLE I

Some Key Discoveries on Mitochondrial Structure and Function

Year	Discovery	Author(s)	Reference(s)
1890	Description of "bioblasts," a cytoplasmic structure of ubiquitous occurrence, resembling bacteria and functioning as "elementary organisms"	Altmann	21
1912–1922	Recognition of the particulate nature of cell respiration	Battelli and Stern	43
		Warburg	44
		Wieland	46
1925	The cytochrome system is associated with cellular structures	Keilin	47
1934	First attempts to isolate mitochondria by cell fractionation	Bensley and Hoerr	42
1940–1946	First correlated morphological and biochemical studies on isolated mitochondria	Claude	64, 65, 67
1946	Demonstration of the localization of succinoxidase and cytochrome oxidase in mitochondria	Hogeboom et al.	69
1948	Isolation of morphologically well-preserved mitochondria	Hogeboom et al.	72
1948–1951	Mitochondria contain the enzymes of the citric acid cycle, fatty acid oxidation, and oxidative phosphorylation	Kennedy and Lehninger	74
		Schneider and Potter	75
		Green	76
		Lehninger	77
1950–1955	The enzymic complement of mitochondria as revealed by tissue-fractionation studies	Schneider and Hogeboom	73
		Hogeboom and Schneider	105
		de Duve et al.	89, 104
1951–1952	Demonstration of respiratory control, latency of ATPase, and uncoupling effect of dinitrophenol with isolated mitochondria	Lipmann et al.	82, 88
		Rabinovitz et al.	83
		Lardy and Wellman	84, 87
		Kielley and Kielley	86
1952–1953	Early studies on mitochondrial swelling and contraction	Slater and Cleland	138
		Raaflaub	139
1952–1953	First high-resolution electron micrographs of mitochondria	Palade	26, 120
		Sjöstrand	121, 122
1953	"Chemical" hypothesis of oxidative phosphorylation	Slater	179
1952–1955	Localization of coupling sites of the respiratory chain	Lardy and Wellman	84, 87
		Lehninger	78
1952–1956	Introduction of rapid and sensitive physical methods for the study of mitochondrial electron transport. Kinetics and metabolic states of the respiratory chain.	Chance	165, 171
		Chance and Williams	169, 170
1953–1956	Partial reactions of oxidative phosphorylation (P_i-H_2O and P_i-ATP exchange)	Cohn	180
		Boyer et al.	181
		Swanson	182
1953–1957	Demonstration of the membranous localization of the respiratory chain.	Cleland and Slater	52
		Watson and Siekevitz	376
		Siekevitz and Watson	377
1956–1960	Introduction of the use of beef-heart mitochondria for the study of the respiratory chain and oxidative phosphorylation. Demonstration of the participation of ubiquinone, nonheme iron, and metalloflavoproteins as redox carriers of the respiratory chain. Isolation and characterization of electron-transport complexes.	Crane et al.	301, 304
		Singer et al.	302
		Beinert and Sands	309
		Hatefi et al.	310, 312, 315
		Ziegler and Doeg	311
		Kuboyama et al.	318
1957–1961	Demonstration of the reversal of oxidative phosphorylation	Chance and Hollunger	184, 189
		Klingenberg et al.	111, 185–187
		Löw et al.	196, 197
1958–1962	Reconstitution of the respiratory chain	Keilin and King	300
		Hatefi et al.	316
1960	Isolation of mitochondrial ATPase and demonstration of its action as coupling factor (F_1)	Pullman et al.	336
		Penefsky et al.	337
1961	Chemiosmotic hypothesis of oxidative phosphorylation	Mitchell	405
1961–1963	Demonstration of energy coupling in the respiratory chain without the participation of the phosphorylating system	Azzone and Ernster	193
		Ernster	194, 206–208
		Klingenberg and v. Häfen	195

that their role is to enhance the activity of the iron-containing "respiratory enzyme" (*Atmungsferment*) (45). Similarly, Wieland (46), who extended Battelli and Stern's (43) early observations to a generalized concept of cellular dehydrogenases, recognized the particulate nature of these enzymes. Despite diverging views concerning the chemical nature of cell respiration—involving a transfer of oxygen according to Warburg (45), and a transfer of hydrogen according to Wieland (46)—

TABLE I—Continued

Year	Discovery	Author(s)	Reference(s)
1961–1963	Energy-linked uptake of Ca^{2+} and other divalent cations	Vasington and Murphy	225
		DeLuca and Engstrom	226
		Brierley et al.	227
		Chappell et al.	228
		Lehninger et al.	229, 232
		Saris	230
		Chance	233
1962	Discovery of projecting subunits on the mitochondrial membrane	Fernández-Morán	322
1963–1966	Energy-linked transhydrogenase and its use as a tool for the study of mitochondrial energy transduction	Danielson and Ernster	215
		Lee and Ernster	216, 219
		Lee et al.	218
1964	Discovery of the action of valinomycin as K^+ ionophore	Moore and Pressman	241
1965	Conformational hypothesis of oxidative phosphorylation	Boyer	404
1966	Development of the chemiosmotic hypothesis as a general mechanism of oxidative and photosynthetic phosphorylation	Mitchell	406
1966	Discovery of mitochondrial anion translocators	Chappell and Haarhoff	155
1966–1969	Separation and characterization of the inner and outer mitochondrial membranes	Lévy et al.	379
		Schnaitman et al.	380, 381
		Parsons et al.	382
		Sottocasa et al.	81
		Ernster and Kuylenstierna	389
1966–1969	Evidence for chemiosmotic coupling in native membranes	Jagendorf and Uribe	416
		Mitchell and Moyle	413, 415
1966–1976	Isolation and characterization of coupling factors. Resolution and reconstitution of the ATPase complex	Kagawa and Racker	343
		Thayer and Hinkle	351
		Capaldi	352
		MacLennan and Tzagoloff	349
		Racker et al.	347, 348
		Lam et al.	350
1971–1975	Reconstitution of oxidative phosphorylation and related reactions in artificial phospholipid vesicles	Kagawa and Racker	428
		Racker and Kandrach	432
		Ragan and Racker	430
		Hinkle et al.	425
		Skulachev	419
		Leung and Hinkle	426
		Ragan and Hinkle	427
		Rydström et al.	429
		Racker and Stoeckenius	433
1973–1979	Evidence for electron transport-linked proton pumps	Azzone and Massari	235
		Brand et al.	439, 440
		Papa et al.	444
		Guerrieri and Nelson	445
		Höjeberg and Rydström	221
		Wikström	441
		Wikström and Krab	442

they both agreed that the role of the particulate cellular structure may be to enlarge the catalytic surface. Warburg (45) referred to the "charcoal model," and Wieland (46) to the "platinum model," in attempting to explain how this may be achieved.

In 1925, Keilin (47) described the cytochromes, a discovery that led the way to the definition of the respiratory chain as a sequence of catalysts comprising the dehydrogenases on one end and *Atmungsferment* on the other. This resolved the Warburg-Wieland controversy. To achieve this, however, another, equally controversial problem had to be settled, namely, that of the relationship between Keilin's cytochromes and Warburg's *Atmungsferment*. This was not done until 1939, when Keilin and Hartree (48) established the identity between *Atmungsferment* and cytochrome a_3. Keilin's studies were carried out first with the living wax moth and later, in collaboration

with Hartree, with a particulate preparation from mammalian heart muscle. This preparation, which catalyzed the aerobic oxidation of succinate and NADH, was subsequently studied in great detail by Slater (49, 50), especially regarding the catalyst responsible for the interaction of the dehydrogenases and the cytochrome system ("BAL-sensitive factor"). It also became in many laboratories the starting material for the isolation and characterization of various respiratory-chain catalysts.

Keilin and Hartree (51) early recognized the need for a cellular structure for cytochrome activity, pointing out that "it is quite possible that the paramount conditions for existence of this pigment are found in some properties connected with the physico-chemical structure of the cell." In contrast to the charcoal and platinum models, they suggested that the cellular structure may be necessary, not for the activity of the individual

catalysts, but rather for determining their mutual accessibility and thereby the rates of reaction between different members of the respiratory chain. Such a function, according to Keilin and Hartree (51), could be achieved by "unspecific colloidal surfaces." Interestingly, the possible role of phospholipids was not considered in these early studies, and it was not until 1953 that the membranous nature of the Keilin and Hartree heart-muscle preparation and its mitochondrial origin were recognized by Cleland and Slater (52).

During the second half of the 1930s, considerable progress was made in elucidating the reaction pathways and energetics of aerobic metabolism. In 1937 Krebs (53) formulated the citric acid cycle, and Kalckar (54) presented his first observations leading to the demonstration of aerobic phosphorylation, using a particulate system derived from kidney homogenates. Earlier, Engelhardt (55) had obtained similar indications with intact pigeon erythrocytes. Extending these observations, Belitser and Tsybakova (56) in 1939 deduced from experiments with minced muscle that at least two molecules of ATP are formed per atom of oxygen consumed. These results indicated that phosphorylation probably occurs coupled to the respiratory chain. In 1941, Lipmann (57) developed the concept of "phosphate-bond energy" as a general form of energy conservation in cellular metabolism.

In the following years several laboratories reported studies with "washed tissue particles" in which various qualitative and quantitative aspects of the aerobic oxidation of citric acid-cycle metabolites and accompanying ATP synthesis were investigated. A paper of special importance was published in 1943 in Ochoa (58), in which it was concluded that the aerobic oxidation of pyruvate probably gives rise to 3 moles of organically bound phosphate for each atom of oxygen consumed (P/O ratio-3). During these years also the first evidence was presented of the capacity of tissue particles to carry out fatty acid oxidation (59). In 1948-1949 , using a particulate fraction from rat liver and β-hydroxybutyrate or NADH as substrate, Friedkin and Lehninger (60, 61) provided conclusive evidence for the occurrence of respiratory chain-linked phosphorylation. At about the same time, Green and associates (62, 63), in a series of papers, described a particulate system from rabbit kidney which was given the name "cyclophorase" and was shown to catalyze the aerobic oxidation of citric acid cycle metabolites and accompanying phosphorylation. This system displayed certain "organized" properties not observed with earlier-studied particulate systems; for example, it contained a complement of endogenous NAD^+, which was lost upon mechanical damage of the particles. All of these important developments took place before the relationship of these particles to mitochondria was known. The establishment of this relationship had to await the availability of reliable methods for tissue fractionation.

Structure-Function Relationships

ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF MITOCHONDRIA: From the late 1930s, Claude (64) was engaged in a detailed study of the conditions for cell fractionation that was based on the original procedure of Bensley and Hoerr (42). Claude's contributions came to be of fundamental importance for the separation and the morphological and biochemical characterization of cell organelles. He introduced the tissue fractionation technique based on differential centrifugation (65), and worked out basic criteria for the identification and the chemical and enzymic characterization of the fractions obtained. These criteria included examination

of the size, shape, and, whenever possible, the fine structure of the particles recovered in the various fractions, as well as the protein content of each fraction in relation to that of the total homogenate. Most importantly, Claude (66) pointed out that the assessment of the localization of an enzyme or another chemical constituent in a given organelle must be based on quantitative criteria, such as the total recovery of the constituent and its relative concentration in the organelle in question. He was also first to stress the importance of using an isotonic solution as the homogenizing medium, in order to prevent osmotic changes in the organelle structures. Claude's (67) fractionation procedure yielded four fractions: a heavy fraction, consisting of nuclei and cell debris; an intermediate, "large-particle" fraction, containing mitochondria; a light fraction, consisting of "submicroscopic" particles that Claude called "microsomes" (later identified by Palade and Siekevitz [68] as consisting mainly of fragments of the endoplasmic reticulum); and a soluble fraction, including the cell sap.

Through the the the use of the above procedure, Hogeboom, Claude, and Hotchkiss (69) concluded in 1946 that succinoxidase and cytochrome oxidase in rat liver are localized exclusively in the mitochondria. Although the mitochondrial fraction obtained in these studies differed from mitochondria in situ by being round rather than elongate and not being stained by Janus Green B, the size and homogenous appearance of the particles were taken as sufficient evidence to identify them with mitochondria. Significantly, the oxidase activities were highly concentrated in this fraction. Succinoxidase activity earlier had been found (70, 71) in the large-particle fraction isolated by the original procedure of Bensley and Hoerr (43), but in those studies considerable activity was recovered in the small-particle fraction as well.

In 1948, Hogeboom, Schneider, and Palade (72) modified Claude's procedure by using a hypertonic (0.88 M) sucrose solution as the homogenizing medium. This improved the quality of the isolated mitochondria, which now remained elongate and stainable with Janus Green B. In addition, the use of sucrose instead of a salt solution eliminated aggregation of the particles, improving the purity of the fractions. Succinoxidase activity again was localized exclusively in the mitochondria. Later this procedure was further modified (73) by employing isotonic (0.25 M) rather than hypertonic sucrose as the fractionation medium. This modification facilitated the sedimentation of the cell fractions and also eliminated the inhibitory effect of high concentrations of sucrose on certain enzymes. This procedure became the routine method for preparing mitochondria.

The stage was now set for a direct biochemical approach to the elucidation of mitochondrial function. In 1949, Kennedy and Lehninger (74) demonstrated the aerobic oxidation of citric acid cycle metabolites and of fatty acids as well as the accompanying formation of ATP from inorganic phosphate and ADP with rat liver mitochondria prepared in 0.88 M sucrose. Other cell fractions were devoid of these activities, which was to be expected in view of the earlier conclusion by Hogeboom, Claude, and Hotchkiss (69) that cytochrome oxidase is located exclusively in the mitochondria. Similar results were independently reported by Schneider and Potter (75). In 1951, Green (76) concluded that the cyclophorase system consists of mitochondria.

Using isolated mitochondria, Lehninger (77, 78) also confirmed and extended the results with washed particles, which he and Friedkin had obtained (60, 61), that demonstrated the occurrence of phosphorylation coupled to the aerobic oxidation

of externally added NADH. Respiration was accompanied by a phosphorylation with an estimated P/O ratio approaching 3, which appeared to be in agreement with earlier proposals concerning the existence of three sites of phosphorylation in the respiratory chain. At the same time, however, these experiments indicated that mitochondria are impermeable to added NADH and that they possess an "external," nonphosphorylating pathway of NADH oxidation that can be demonstrated in the presence of added cytochrome c. This pathway was later found to differ from that involved in the oxidation of intramitochondrial NADH in being insensitive to antimycin (78), amytal (79), and rotenone (80), and to be associated with the outer mitochondrial membrane (81).

An important advance that was made possible by the isolation of structurally well-preserved mitochondria was the discovery of certain organized features of the mitochondrial enzyme system, not seen in earlier work with washed particles. In 1951-1952, several laboratories (82-84) demonstrated "respiratory control" with isolated mitochondria, an effect consisting of a control of the respiratory rate by the availability of inorganic phosphate and the phosphate acceptor ADP. It was proposed that this phenomenon, which required a certain degree of structural intactness, was a reflection of the capacity of the organism as a whole to adjust its respiration according to the actual need for energy. Respiratory control became an important parameter for the study of mitochondrial energy transduction at both the biochemical and the physiological and pathological levels (85) (see further p. 233s). Another organized feature of intact mitochondria, discovered in 1951 by Kielley and Kielley (86), was the "latency" of the ATPase activity, which was stimulated by agents that damage the mitochondrial structure. An enhanced ATPase activity and abolition of respiratory control was also found (84, 87) to occur with the known uncoupler of oxidative phosphorylation, 2,4-dinitrophenol (88). These observations indicated that the coupling of respiration to phosphorylation requires a structural feature of the mitochondrion in addition to a set of functional enzymes. However, the understanding of the nature of this structural feature had to await better knowledge of mitochondrial ultrastructure and, in particular, of the role of membranes in energy transduction.

ENZYME-DISTRIBUTION STUDIES: Simultaneously with the above developments, several laboratories were actively engaged in studies of enzyme distribution among the various cell fractions as prepared by differential centrifugation (1, 9, 89, 90). These studies led to the recognition of mitochondria as the site of several enzymes in addition to those involved in the citric acid cycle, fatty acid oxidation, respiration, and phosphorylation. Among these enzymes were adenylate kinase (86, 91, 92), glutamate dehydrogenase (93, 94), transaminases (95), pyruvate carboxylase (96), nucleoside diphosphokinase (97), and nicotinamide nucleotide transhydrogenase (98), as well as enzymes involved in the substrate-level phosphorylation linked to the oxidation of α-ketoglutarate (93), the synthesis of porphyrine and heme (99), citrulline (100,101), and phospholipids (102). Mitochondria were also found to contain a part of the cellular hexokinase (103).

An important result of these studies was the discovery that several enzymes in mitochondria were present also in the microsomal fraction or the cell sap. The question arose whether these enzymes really had a dual localization or whether their presence of two cell fractions was due to incomplete separation of the various cell components. In most cases this question could be settled by carrying out a careful quantitative analysis of the various cell fractions, using a refined fractionation technique and a set of "marker enzymes" for the different organelles as devised by de Duve and associates (104). These studies also led to the discovery of a new cell organelle, the lysosome, with a sedimentation rate intermediate between those of mitochondria and microsomes, and characterized by a complement of hydrolytic enzymes.

A much-debated case of an enzyme with dual localization was the NADP$^+$-linked isocitrate dehydrogenase. It was pointed out (105) that since only slightly more than 10% of this enzyme in a liver homogenate is recovered in the mitochondrial fraction, with the remainder in the cell sap, the enzyme may not be a true mitochondrial constituent. On this basis, the part played by mitochondria in the citric acid cycle as a whole was questioned (106). The problem was settled, however, by showing, first, that the small portion of the enzyme found in the mitochondrial fraction fails to react with externally added NADP$^+$ and is thus truly mitochondrial (107); and, second, that the citric acid cycle proceeds via an NAD$^+$-linked isocitrate dehydrogenase (108), which is exclusively mitochondrial (109). A bimodal distribution between mitochondria and cell sap was later found to be characteristic of several enzymes, including malate dehydrogenase and glutamate-oxalacetate transaminase, and to be related to the transfer of reducing equivalents between extra- and intramitochondrial nicotinamide nucleotides (110). The distribution of the nicotinamide nucleotides between the cell sap and mitochondria was first determined by Bücher and Klingenberg in 1957 (111).

Data concerning the gross chemical composition of mitochondria also began to appear in the early 1950s. In 1951, Schneider and Hogeboom (112) estimated that rat liver mitochondria account for about 35% of the total tissue protein. Earlier estimates had indicated that 70-75% of the mitochondrial dry weight consists of protein and the remainder mainly of phospholipid (65). The lipid composition of mitochondria was determined in 1958 by Marinetti et al. (113), who also carried out a quantitative analysis of various phospholipids. That cardiolipin is localized almost exclusively in mitochondria was demonstrated in 1968 by Getz et al. (114).

Isolated, intact mitochondria were found to contain adenine nucleotides (115) as well as a variety of inorganic ions including phosphate, Na$^+$, K$^+$, and Mg^{2+} (116). Mitochondria were also shown to take up and accumulate Ca^{2+} (117) and Mn^{2+} (118). These early observations foreshadowed the occurrence of active ion-transport across the mitochondrial membrane.

MITOCHONDRIAL STRUCTURE AND ITS VARIATIONS: That mitochondria are surrounded by a membrane was suggested on the basis of early observations with the light microscope (18). The first electron micrographs of mitochondria, published by Claude and Fullam (119) in 1945, confirmed this conclusion. However, detailed studies of the mitochondrial ultrastructure became possible only after the development of thin-sectioning techniques in the early 1950s. The first high-resolution electron micrographs were published in 1952-1953 by Palade (26, 120) and Sjöstrand (121, 122), who used osmium-fixed thin sections of various animal tissues (Fig. 1). Palade (26) found that the mitochondrion is surrounded by a membrane, which is folded to form ridges inside the mitochondrion; he named these ridges cristae mitochondriales. Sjöstrand's micrographs revealed a double limiting membrane surrounding the mitochondrion, and a number of double membranes inside the mitochondrion forming divisions, septae, of the inner chamber. The existence of a double limiting membrane was soon confirmed by Palade (120), who concluded
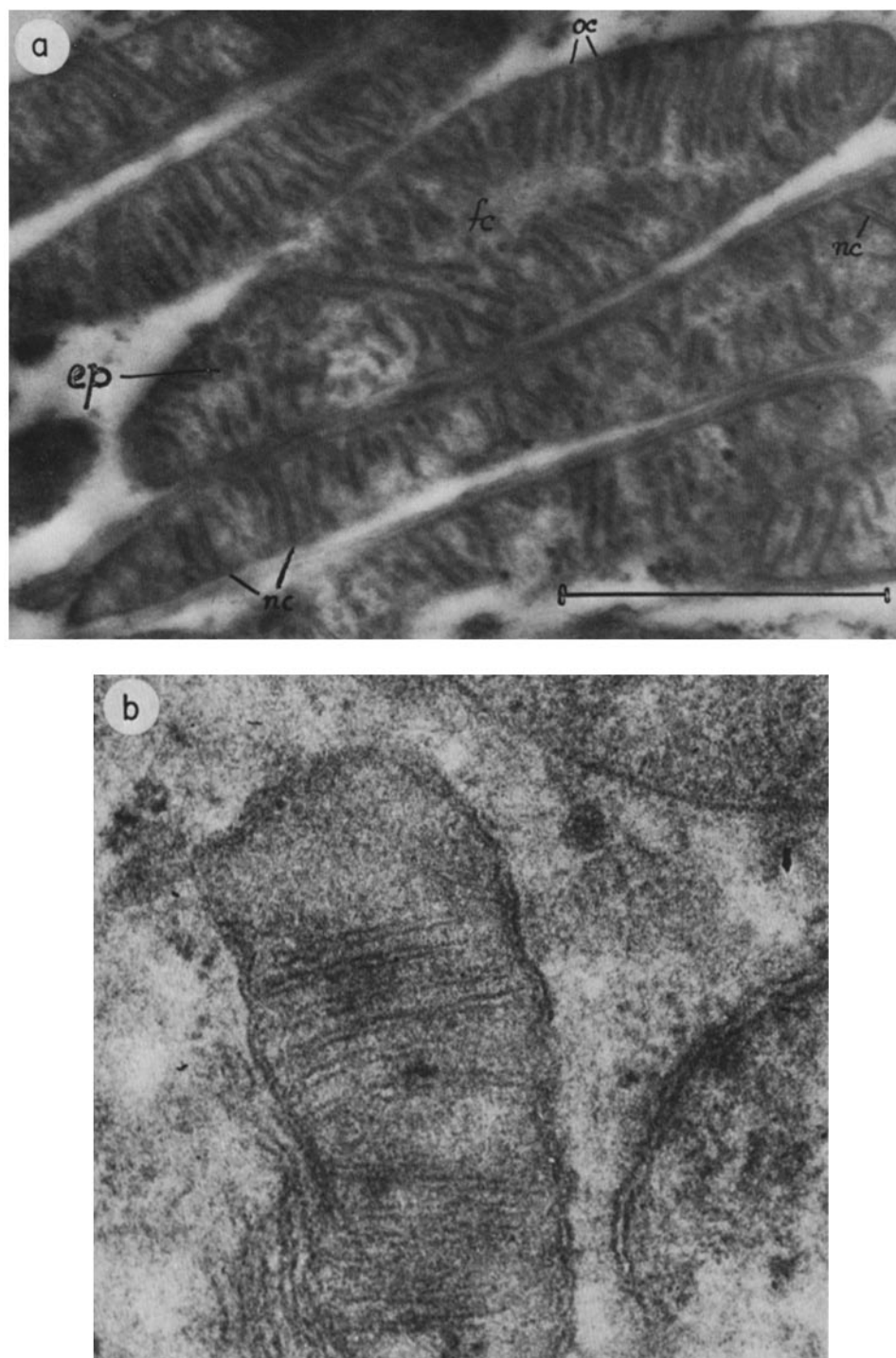
FIGURE 1 Electron micrographs of kidney mitochondria. (a) From Palade, 1953 (120); ×45,700. (b) From Sjöstrand, 1953 (121), × 120,000.

that the cristae are infoldings of the inner membrane. According to Palade's (123) definition, "Two spaces or chambers are outlined by the mitochondrial membranes, an outer chamber contained between the two membranes, and an inner chamber bounded by the inner membrane. The inner chamber is penetrated and, in most cases, incompletely partitioned by laminated structures which are anchored with their bases in the inner membrane and terminated in a free margin after projecting more or less deeply inside the mitochondrion." This definition of the mitochondrial structure has become widely accepted over the years. The space inside the inner membrane is usually referred to as the *matrix*, and the space between the inner and outer membranes as the *intermembrane space* (124).

An explanation of why the cristae seen in electron micrographs of thin sections often lack connection with the inner limiting membrane—an apparent inconsistency with Palade's (123) model—was offered by Whittaker (125), who proposed that this connection may consist of a relatively narrow orifice. That the primary function of the cristae is connected with an increase of the internal surface, rather than a compartmentation

of the inner chamber, is consistent with the early observation that in certain tissues, e.g., adrenal cortex (126), the disc-shaped cristae are replaced by tubular structures, which protrude as fingerlike invaginations from the inner membrane. Tubular cristae are also common in protozoans (127, 128) and have been suggested to represent a phylogenetically basic type of intramitochondrial structure (129). Great variations in the conformation of cristae have been found among different tissues and organisms (4, 5, 9), but the functional implications of these variations are still poorly understood. Irrespective of shape, however, a high respiratory activity seems to be correlated with an abundance of cristae (120). Examples of tissues with mitochondria rich in cristae are insect flight muscle (130), mammalian cardiac muscle (120), and brown adipose tissue (131).

Striking variations are also found in the number, size, shape, and intracellular localization of the mitochondria (4, 5, 9). These variations are clearly related to the specific functions of the tissue. A classic example of specialized mitochondria, described as early as 1871 by Bütschli (16), is the mitochondrial syncytium in the midpiece of sperms (Nebenkern). A "syncytial reticulum" of skeletal muscle mitochondria, which extends in the plane of the I band of the fibers, was observed by Palade 20 years ago (132), and demonstrated recently by three-dimensional reconstruction of electron micrographs carried out by Skulachev and associates (133). A slab-like orientation of mitochondria is also found in insect flight muscle, as Smith described (130). The regularly oriented mitochondria in the distal convoluted tubules, demonstrated by Sjöstrand and Rhodin (134) in 1953, are another striking example of specialized mitochondrial topography.

Mitochondria are highly dynamic structures. As early as 1914–1915, Lewis and Lewis (135) described extensive changes in the position and shape of mitochondria in animal tissue cultures. These observations were later extended by several investigators who used the phase-contrast microscope in combination with time-lapse cinematography. These studies, which were pioneered by Frédéric and Chèvremont (136), revealed striking movements of the mitochondria in various phases of cell activity, e.g., during mitosis, as well as in response to varied physiological, pathological, and experimental conditions. Despite many observations during the past 25 years (4, 5, 9, 137), however, the mechanism and physiological significance of these movements remain largely unexplained. Similarly, many changes that occur in mitochondrial structure after the administration of various drugs or toxic substances to experimental animals have been described (8), but in most cases no causal relationship between these changes and the pharmacological or toxic effects has so far been established.

Shape and volume changes of mitochondria *in vitro*, usually referred to as mitochondrial "swelling" and "contraction," occupy a large chapter in mitochondrial research. Swelling of mitochondria was probably first observed in 1888 by Kölliker (18), who described volume changes of structures isolated from skeletal muscle; these structures were studied in great detail by Retzius (19), who named them sarcosomes. In 1946, Claude (65) demonstrated that mitochondria suspended in a hypotonic medium swell; this was revealed by a decrease in light scattering. From the early 1950s, several laboratories were actively engaged in studies of this phenomenon and its relation to mitochondrial function. It was found that swelling can also occur in an isotonic medium and is promoted by Ca^{2+} (138, 139), inorganic phosphate (140), short-chain fatty acids (141), thyroxine (142), bilirubin (143), and other agents (144, 145); in

some instances, the swelling was dependent on energy in the form of an oxidizable substrate and was inhibited by respiratory inhibitors, anaerobiosis, or by uncouplers (8). During the swelling process, the mitochondria gradually lost their ability to concentrate various ions (116, 146) and nucleotides (115) and to carry out oxidative phosphorylation (115, 147–149); and, simultaneously, they acquired certain hydrolytic activities (86, 140). In most cases, the swelling was prevented by Mg^{2+}, Mn^{2+}, and ATP (147–151), and, as first shown by Raaflaub (138), ATP also was able to reverse the swelling. This effect of ATP was promoted by Mg^{2+} and Mn^{2+}, and was accompanied by a restoration of oxidative phosphorylation and related structure-dependent functions (147–150). The ATP effect in reversing swelling has been compared with muscle contraction (138) and it has been suggested that mitochondria contain a contractile protein similar to actomyosin. Some studies describing such a protein were reported in the early 1960s (152) but were apparently not further pursued. In recent years it has been established that the mitochondrial swelling and contraction concern primarily the inner membrane (153) and are at least partly related to the movements of water across this membrane that accompany the uptake and release of ions by way of specific translocators (154, 155). In fact, the swelling phenomenon has served as an important tool in discovering and characterizing some of these translocators (155) (see the section on "*Ion Translocation*"). It has also played a significant role in the development of methods for the separation of the outer and inner mitochondrial membranes (124), as will be discussed below.

Besides these large-amplitude volume changes, mitochondria also exhibit a low-amplitude swelling-contraction cycle, which was discovered and studied extensively by Packer (156). This cycle follows the metabolic state of the mitochondria, and is probably related to changes in the prevailing protonmotive force. Also apparently related to the metabolic state of the mitochondria are the so-called "orthodox" and "condensed" conformational states, which were described in 1966 by Hackenbrock (157). These changes are restricted to the conformation of the inner membrane—probably resulting from drastic volume changes of the matrix—whereas the outer membrane is unaltered. Interestingly, the "condensed" mitochondria reveal the existence of multiple points of attachment between the outer and inner membranes. Metabolism-dependent conformational changes of mitochondrial cristae have also been observed by Penniston et al. (158).

The Energy-Transduction System of Mitochondria

Since the early 1950s, an important part of mitochondrial research has been concerned with the mechanism of electron transport and oxidative phosphorylation. It became clear that the mitochondrion was the long sought particulate structure necessary for these processes. However, as Lehninger (2) pointed out, "It was a part of the biochemical *Zeitgeist* that particles were a nuisance and stood in the way of purification of the respiratory enzymes," and this was even more true for membranes. Thus, even when it was proved and generally accepted that the catalysts of the respiratory chain and the phosphorylating system are associated with the inner mitochondrial membrane, it took a long time to begin to understand the role of the membrane in the function of these catalysts. Does the membrane serve as Keilin and Hartree's (51) "colloidal surface," regulating the mutual accessibility of the catalysts?

Do these catalysts form an assembly or do they interact by collisions in the plane of the membrane? Does the membrane serve as a hydrophobic environment protecting labile intermediates? Does it serve as a permeability barrier, and, if so, a barrier to what? Are the catalysts oriented across the membrane in a specific way, and, if so, why? These were some of the questions that governed mitochondrial research from the early 1950s. The general experimental approach included work with intact mitochondria as well as attempts to resolve and reconstitute the components of the respiratory and phosphorylating enzyme system.

OXIDATIVE PHOSPHORYLATION AND ITS PARTIAL REACTIONS. THE "CHEMICAL" HYPOTHESIS: As mentioned above, isolated, well-preserved mitochondria were found to exhibit such organized features as a high degree of respiratory control and a latency of ATPase activity. These findings stimulated interest in assessing the maximal number and the location of phosphorylations in the respiratory chain. One approach was based on determining the P/O (or P/2e$^-$) ratios obtained with mitochondria that were respiring in the presence of various substrates or utilizing artificial electron donors (e.g., ascorbate + cytochrome c [61] or TMPD [159], or menadiol [160]) or acceptors (e.g., ferricyanide [161] or coenzyme Q_1 [162]) in combination with appropriate electron-transport inhibitors. The most commonly used inhibitors were cyanide for cytochrome oxidase (163), antimycin for the cytochrome b-c segment of the respiratory chain (164, 165), and amytal (79) or rotenone (80, 166) for the electron flow between NADH and cytochrome b. Suitable electron-transfer "shunts" were also used to bypass the sites of action of amytal (167) and rotenone (80) and of antimycin (168). To ensure maximal yields of phosphorylation, in most cases the system was supplemented with an efficient "ATP trap" in the form of hexokinase and glucose.

A powerful method for the study of oxidative phosphorylation, which was introduced by Chance and Williams (169, 170), was based on the use of a dual-wavelength spectrophotometer in combination with an oxygen electrode. The redox states of various respiratory-chain components were determined spectrophotometrically, simultaneously with the polarographic measurement of oxygen uptake. Such determinations were carried out in various metabolic states of the mitochondria, e.g., during respiration in the presence of substrate and phosphate, with and without ADP ("State 3" and "State 4") (Fig. 2). The transition between the two states was accompanied by characteristic redox shifts of certain electron-transport carriers, some becoming more reduced, others more oxidized. From the location of these "crossover" points, the sites of phosphorylation in the respiratory chain were determined. At the same time, from the increment in oxygen uptake due to the addition of a given amount of ADP, the ADP/O (P/O) ratio was calculated. This method of Chance and Williams (169, 170) made possible the first quantitative study of the concentrations and kinetics of electron-transport catalysts not only in intact mitochondria but also in other integrated biological systems, including intact cells and tissues (171, 172).

The above lines of investigation constituted the experimental basis for the widely accepted view that the respiratory chain contains three sites of phosphorylation—located between NADH and cytochrome b, cytochrome b and cytochrome c, and cytochrome c and oxygen, respectively—each giving rise to the synthesis of one molecule of ATP from ADP and inorganic phosphate per two electrons transferred. The actual
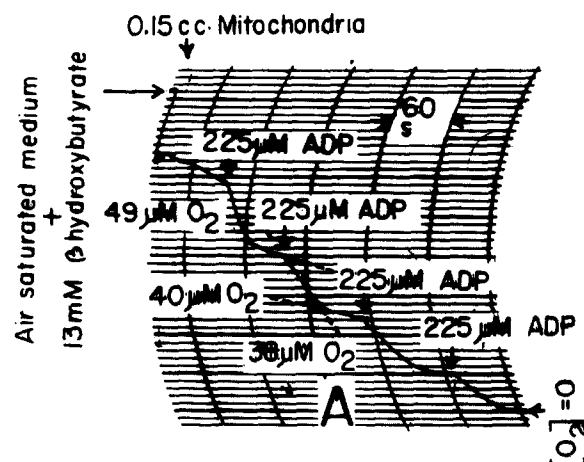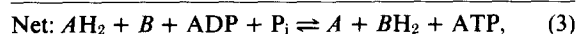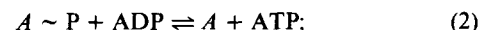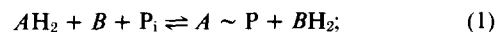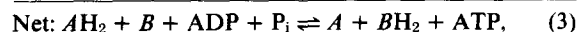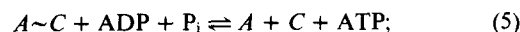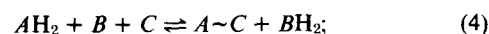
FIGURE 2   Respiratory control: a polarographic recording of the effect of ADP upon the respiration of a suspension of rat liver mitochondria. From Chance and Williams, 1955 (169).

mechanism of these phosphorylations, however, remained unsettled.

In 1946, Lipmann (173) suggested that phosphorylations in the respiratory chain follow a mechanism similar to that of the phosphorylation in glycolysis, involving a phosphorylated derivative of the oxidized electron donor:

$$AH_2 + B + P_i \rightleftharpoons A \sim P + BH_2; \tag{1}$$

$$A \sim P + ADP \rightleftharpoons A + ATP; \tag{2}$$

$$\text{Net: } AH_2 + B + ADP + P_i \rightleftharpoons A + BH_2 + ATP, \tag{3}$$

where A and B are redox carriers. In 1952 Krimsky and Racker (174) demonstrated that the glycolytic phosphorylation proceeds via a thiol ester prior to the formation of the phosphorylated intermediate. One year earlier, Kaufman (175) and Sanadi and Littlefield (176) had shown that the phosphorylation coupled to the oxidation of α-ketoglutarate (discovered by Hunter and Hixon [93] in 1949) also involves a thiol ester, succinyl $\sim$CoA, and in the same year, Lynen and Reichert (177) demonstrated the occurrence of acetyl$\sim$CoA as a product of pyruvate oxidation, after the discovery of coenzyme A by Lipmann (178). Prompted by these developments, several investigators considered the possibility that respiratory chain-linked phosphorylations also may proceed by way of nonphosphorylated high-energy intermediates (1, 78, 179). Slater (179) in 1953 was the first to formulate such a mechanism in general terms:

$$AH_2 + B + C \rightleftharpoons A \sim C + BH_2; \tag{4}$$

$$A \sim C + ADP + P_i \rightleftharpoons A + C + ATP; \tag{5}$$

$$\text{Net: } AH_2 + B + ADP + P_i \rightleftharpoons A + BH_2 + ATP, \tag{3}$$

where C is a hypothetic ligand. Slater's mechanism, which is often referred to as the "chemical" hypothesis of oxidative phosphorylation, constituted a widely accepted framework for designing and interpreting experiments in this field during the following 15 years.

Slater's (179) mechanism appeared to account for a number of findings relating to respiratory chain-linked phosphorylation. For example, the phenomenon of respiratory control

could be explained by assuming that, in the absence of P_i and/or ADP, $A \sim C$ accumulates and this leads to an inhibition of electron transport via A. The proposed mechanism also accounted for the effect of uncouplers in abolishing respiratory control and oxidative phosphorylation and stimulating ATPase activity, by postulating that uncouplers cause a cleavage of $A \sim C$ into A and C. Similarly, structural damage would induce a splitting of $A \sim C$, thus explaining why mitochondrial membrane fragments such as the Keilin-Hartree preparation can respire in the absence of P_i (179).

An important development was the discovery that mitochondria catalyze a number of exchange reactions which represent partial reactions of respiratory chain-linked phosphorylation. One reaction, an oxygen exchange between inorganic phosphate and water, was discovered in 1953 by Cohn (180). Another, described by Boyer et al. (181) and by Swanson (182), involved an exchange of phosphate between inorganic phosphate and ATP. Both reactions were sensitive to uncouplers and proceeded at rates that were higher than the net rate of oxidative phosphorylation. These findings could be explained in terms of Slater's mechanism (179) by assuming that the conversion of $A \sim C$ + ADP + P_i into A + C + ATP (reaction 5) is readily reversible and proceeds via a phosphorylated high-energy intermediate ($C \sim P$), the formation of which involves the splitting of a P-O bond of inorganic phosphate. The occurrence of such an intermediate was also supported by the demonstration of an ADP-ATP exchange by Wadkins and Lehninger (183).

A further extension of the chemical hypothesis was proposed by Chance and Williams (166), who postulated the occurrence of two types of nonphosphorylated high-energy intermediate, one that contained a redox carrier and was individual for each coupling site of the respiratory chain, and a second that contained no redox carrier and was common for the three coupling sites. They also introduced the symbol I instead of Slater's C, (175) to indicate that this ligand inhibits respiration when bound to an electron carrier. Moreover, Chance and Williams (166) concluded that I binds to the reduced, rather than to the oxidized, redox carrier.

One of the most important tenets of Slater's hypothesis (179) and its subsequent extensions was that the energy liberated during electron transport via the respiratory chain can be conserved without the participation of the phosphorylating system. Experimental proof for this concept was obtained during the late 1950s and early 1960s through a series of discoveries, primarily the reversal of respiratory chain-linked phosphorylation, and the phosphorylation-inhibitor oligomycin, which led to the demonstration of energy transfer directly between the coupling sites of the respiratory chain.

ENERGY-TRANSFER BETWEEN COUPLING SITES OF THE RESPIRATORY CHAIN: In 1957, Chance and Hollunger (184) briefly reported that intact mitochondria catalyze an energy-dependent reduction of endogenous NAD^+ by succinate. Similar findings were reported shortly thereafter by Bücher and Klingenberg (111), who used α-glycerophosphate as the reducing substrate. Both laboratories subsequently extended these observations and interpreted them as evidence for a reversal of electron transport through the first energy-coupling site of the respiratory chain (185–189). Initially this interpretation was received with skepticism (190–192), but several laboratories carried out further studies with both intact mitochondria (by use of succinate-linked acetoacetate reduction as the test system [193–195]) and submitochondrial parti-

cles (196, 197) that soon eliminated the objections raised. Continued work, mainly in Chance's (198) and Klingenberg's (195, 199) laboratories, has led to the general concept that electron transport through all three coupling sites of the respiratory chain is reversible and that the reversibility is a reflection of a dynamic equilibrium between the respiratory and phosphorylating systems.

Oligomycin, an antibiotic introduced by Lardy and associates (200) in 1958 as an inhibitor of respiratory chain-linked phosphorylation, has proved to be a most valuable tool for the study of mitochondrial energy transduction. It was shown to inhibit the P_i + ADP-induced stimulation of respiration of tightly-coupled mitochondria (201, 202), but not uncoupled respiration. It was also shown to inhibit the P_i-ATP (200) and P_i-H_2O (202) exchange reactions as well as the ATPase activity induced by uncouplers (202), but not that induced by arsenate (203, 204). These effects were consistent with the interpretation that oligomycin is a specific inhibitor of the phosphorylating system of the respiratory chain—a conclusion that was later confirmed with the isolated mitochondrial ATP synthetase (205).

In 1960, Ernster reported (206) that oligomycin does not inhibit the succinate-linked NAD^+ reduction when the aerobic oxidation of succinate is the source of energy. In fact, oligomycin was able to restore the reaction when it was inhibited by P_i + ADP (206) or by arsenate (207). Furthermore, the reaction was uninhibited in P_i-depleted mitochondria (208). These findings, which are summarized schematically in Fig. 3, demonstrated conclusively that energy can be conserved and utilized by the respiratory chain without the participation of the phosphorylating system (209). Evidence to support this conclusion was reported soon from other laboratories (195, 210–212; see also review 213).

OTHER ENERGY-LINKED FUNCTIONS OF THE RESPIRATORY CHAIN: Another important development that began in the early 1960s was the demonstration of reactions that are capable of utilizing energy derived from the respiratory chain without the participation of the phosphorylating sys-
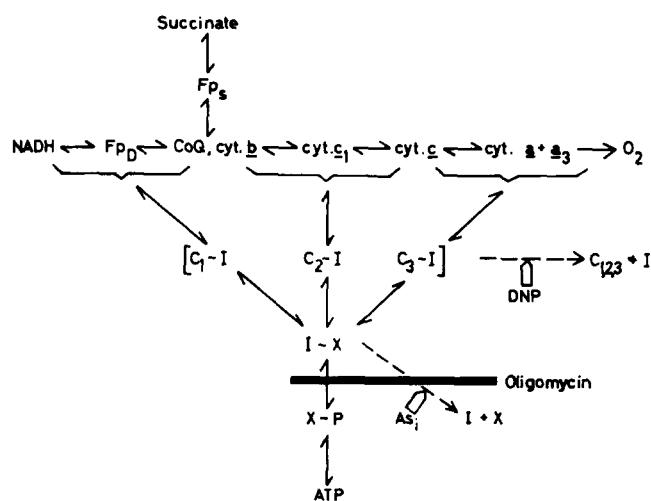

FIGURE 3 Schematic representation of the respiratory chain and the oxidative phosphorylation system. C_1, C_2, C_3 denote electron carriers at the coupling sites of the respiratory chain; I and X denote hypothetic energy-transfer carriers. Open bars indicate probable sites of uncouplind by 2,4-dinitrophenol (DNP) and by arsenate (As_i). From Ernster, 1965 (148).

tem, such as the energy-linked transhydrogenase, various ion translocators, and thermogenesis, as well as some pathological conditions resulting in impaired respiratory control.

Energy-linked Transhydrogenase

In their studies of the energy-linked reduction of endogenous nicotinamide nucleotide in intact mitochondria, Klingenberg and Slenczka (185) observed that both NAD^+ and $NADP^+$ were reduced, the latter to a higher degree than the former. They interpreted this phenomenon as an energy-linked shift of equilibrium of the mitochondrial nicotinamide nucleotide transhydrogenase reaction. Similar findings were reported by Estabrook and Nissley (214).

In 1963 Danielson and Ernster (215) demonstrated an energy-linked transhydrogenase reaction catalyzed by submitochondrial particles. The reaction consisted of an energy-dependent enhancement of the reduction of $NADP^+$ by $NADH$ and resulted, as later shown by Lee and Ernster (216), in about a 500-fold shift of equilibrium of the transhydrogenase reaction towards the formation of $NADPH$ and NAD^+. The energy for the reaction could be supplied either by substrate oxidation through the respiratory chain or by ATP hydrolysis; in the former case the reaction was insensitive to oligomycin, whereas in the latter it was oligomycin-sensitive. In both cases, the reaction was sensitive to uncouplers. From these results it was concluded (215) that the transhydrogenase reaction is functionally linked to the respiratory chain-linked ATP-synthesizing system in such a way that it can utilize energy captured by the energy-conservation mechanisms of the respiratory chain without the participation of the phosphorylating system. In more general terms, this conclusion implied that energy derived from the respiratory chain can be utilized for purposes other than ATP synthesis. An actual competition between the energy-linked transhydrogenase and oxidative phosphorylation was subsequently demonstrated by Lee and Ernster (217). Due to its ability to utilize energy directly from the respiratory chain, the transhydrogenase reaction became a useful tool for studying energy coupling in nonphosphorylating electron-transport systems (218, 219).

The transhydrogenase has subsequently been studied in great detail with respect to its kinetics and reaction mechanism (220), but it is only recently that the enzyme has been purified (221, 222) and the energy-linked reaction has been reconstituted (223).

Ion Translocation

As already mentioned, in the early 1950s several laboratories found that isolated mitochondria take up Ca^{2+} from the suspending medium, and that this causes swelling and uncoupling of oxidative phosphorylation. In 1955 Chance (224) observed that repeated additions of small amounts of Ca^{2+} to respiring mitochondria in the presence of phosphate caused transient enhancements of the rate of oxygen uptake, similar to those found with ADP. In 1962–1963 several laboratories (225–230) independently demonstrated an energy-dependent accumulation of Ca^{2+} and other divalent cations by respiring mitochondria in the presence of phosphate. As first shown by Saris (230), the Ca^{2+} uptake was accompanied by a release of protons. The Ca^{2+} accumulation resulted in a deposition of calcium phosphate as hydroxyapatite-like, electron-dense granules in the matrix (229, 231). The respiration-driven Ca^{2+} uptake was uncoupler-sensitive but insensitive to oligomycin, and was thus

another example of a process capable of deriving energy from the respiratory chain without the involvement of the phosphorylating system. Initially it was believed that phosphate rather than Ca^{2+} was the actively accumulating species (232), but subsequent work in Chance's laboratory revealed that Ca^{2+} uptake occurred also in the presence of other penetrating anions (233) and to some extent even in the absence of added anions (234). The energetic stoichiometry of the anion-linked, massive accumulation of Ca^{2+} was estimated at two atoms of Ca^{2+} taken up per pair of electrons traversing each coupling-site of the respiratory chain (233–235). This stoichiometry and, in general, the mitochondrial transport of Ca^{2+} has been a very active field of research in the last 15 years (235–237). It is now established that the carrier functions as an electrogenic uniporter, mediating an active influx of Ca^{2+} across the inner mitochondrial membrane at the expense of an efflux of protons driven by electron transport or ATP hydrolysis. The carrier also mediates the transport of Mn^{2+}, Sr^{2+}, and Ba^{2+} but not Mg^{2+}. Crompton et al. (238) recently discovered a second Ca^{2+} carrier in mitochondria, which mediates the efflux of Ca^{2+} against an influx of Na^+, and which is found in the mitochondria of heart and other excitable organs. The occurrence of a mitochondrial glycoprotein which might be involved in Ca^{2+} transport has been described by Sottocasa and co-workers (239, 240).

In 1964, Moore and Pressman (241) made the important discovery that the antibiotic valinomycin, earlier described as an uncoupler of oxidative phosphorylation, required K^+ for its uncoupling effect. Closer examination of this phenomenon led to the recognition of valinomycin as a K^+ carrier, which facilitated the energy-linked uptake of K^+ by the mitochondria at the expense of respiratory energy (242). This discovery opened up the new field of ionophores, and had important implications not only for bioenergetics and membrane biology but also for bio-organic chemistry (243) and medicine (242).

By using the swelling of mitochondria as the test, Chappell and Haarhoff (155) discovered in 1967 that mitochondria in the presence of a permeant cation (e.g., NH_4^+, which penetrates the membrane as NH_3) take up various anions including phosphate, malate, and citrate. The uptake of malate was dependent on the simultaneous presence of phosphate, and the uptake of citrate required the presence of both phosphate and malate. From the steric requirements of the anion uptake, it appeared that the transport was mediated by a set of carriers, each specific for a certain anion or combination of anions. These observations set the stage in various laboratories for the demonstration and characterization of a series of translocators that are present in the mitochondrial inner membrane and responsible for the transport of various metabolites into and out of the mitochondrial matrix (Table II) (244–280; cf. reviews 237, 281). Among the metabolic functions of these translocators is to bring pyruvate (the product of glycolysis) and long-chain fatty acids (as the carnitine esters [267]) into the mitochondria, to facilitate amino acid catabolism and urea synthesis, to mediate the transfer of the reducing equivalents between extra- and intramitochondrial nicotinamide nucleotides via various shuttle mechanisms (110), and to provide for the import of phosphate and ADP and the export of ATP in connection with oxidative phosphorylation. The glutamate, aspartate and ADP, ATP translocators are electrogenic, and are responsible for the maintenance of the known disequilibrium that exists in the intact cell between the extra- and intramitochondrial $[NADH]/[NAD^+]$ (111) and $[ATP]/([ADP]\cdot[P_i])$ (282) poten-

tials. The ADP,ATP translocator, which is inhibited by atractylate (275) and bongkrekic acid (276), has recently been purified (277, 278) and its mode of action has been extensively studied in both native and reconstituted membranes (280).

Thermogenesis

Brown fat, discovered in 1551 by the Swiss naturalist Gessner (283), is the organ responsible for nonshivering thermogenesis (284). Heat production by brown-fat mitochondria is another example of a process that derives energy directly from the respiratory chain. For some time, it was believed that the norepinephrine-induced stimulation of respiration and heat production originated from a re-esterification of fatty acids (set free by a hormone-activated lipase), and resulted in a continuous splitting and resynthesis of ATP (285). Subsequent work revealed, however, that brown-fat mitochondria are relatively poor in ATP synthetase (286), and it became clear that the increased heat production is due to the presence of an endogenous "uncoupler," the effect of which is inhibited by certain nucleotides (287). Prompted by these observations, Heaton et al. (288) have demonstrated the occurrence in brown-fat mi-

TABLE II

Mitochondrial Metabolite Translocators

Metabolites	Species translocated (in ⇌ out)	Selected references
Dicarboxylates	malate^{2-} ⇌ phosphate^{2-}	155, 244
Tricarboxylates	citrate^{3-} + H$^+$ ⇌ malate^{2-}	155, 245–248
α-Ketoglutarate	α-ketoglutarate^{2-} ⇌ malate^{2-}	244, 249–251
Phosphate	phosphate$^-$ ⇌ OH$^-$	155, 252–256
Pyruvate	pyruvate$^-$ ⇌ OH$^-$	257–259
Glutamate	glutamate$^-$ ⇌ OH$^-$	260, 261
Glutamine	glutamine ⇌ glutamate$^-$ + H$^+$	262, 263
Ornithine	ornithine$^+$ ⇌ H$^+$	264, 265
Neutral amino acids	neutral amino acids	266
Acyl carnitine, carnitine	acyl carnitine ⇌ carnitine	267–269
Glutamate, aspartate	glutamate$^-$ + H$^+$ ⇌ aspartate$^-$	260, 270, 271
ADP, ATP	ADP^{3-} ⇌ ATP^{4-}	272–280

tochondria of a protein that acts as an unspecific anion channel, and the function of which is blocked by extramitochondrial purine nucleotides (289). Recently, Lin et al. (290) have purified this protein and found that it has some structural resemblance to the ADP,ATP translocator. How norepinephrine regulates the function of this protein is not known, but its effect seems to involve an adenyl cyclase-controlled lipase as well as the mitochondrial Ca^{2+}-Na$^+$ exchange carrier (291).

PATHOLOGICAL CHANGES IN MITOCHONDRIAL RESPIRATORY CONTROL: The discovery of mitochondrial respiratory control stimulated interest in factors and conditions that may control the coupling of respiration to phosphorylation in vivo. In the early 1950s, several laboratories (see reviews 85, 209) proposed that thyroid hormones may exert their physiological effect by uncoupling or loose-coupling respiration from phosphorylation. (The term "loose-coupling" was defined as a state of the mitochondria in which respiratory control is virtually abolished while phosphorylation still can take place in the presence of phosphate and phosphate acceptor.) Early evidence seemed to support this concept by showing that thyroid hormones added to mitochondria in vitro or administered in vivo caused loose-coupling or uncoupling of oxidative phosphorylation and a swelling of the mitochondrial structure (cf. 85, 209). Closer examination of these effects revealed (292, 293), however, that they are observed only at toxic levels of the hormone and thus probably unrelated to the physiological action of thyroid hormones. At the same time, evidence was obtained (292, 293) which indicated that the physiological effect of thyroid hormones is on the synthesis of mitochondrial proteins. This conclusion has received considerable support in recent years.

In 1959, Ernster, Ikkos, and Luft (294), in the course of investigations of skeletal-muscle mitochondria from thyrotoxic patients, discovered a defect of mitochondrial respiratory control in a case of severe hypermetabolism of nonthyroid origin. The mitochondria showed a high degree of "loose-coupling"; they almost completely lacked of respiratory control by P$_i$ and ADP, but could still make ATP from P$_i$ and ADP (Fig. 4). The respiration was insensitive to oligomycin (295, 296). There were also some striking structural changes of the skeletal-muscle mitochondria as well as a large increase in their number and
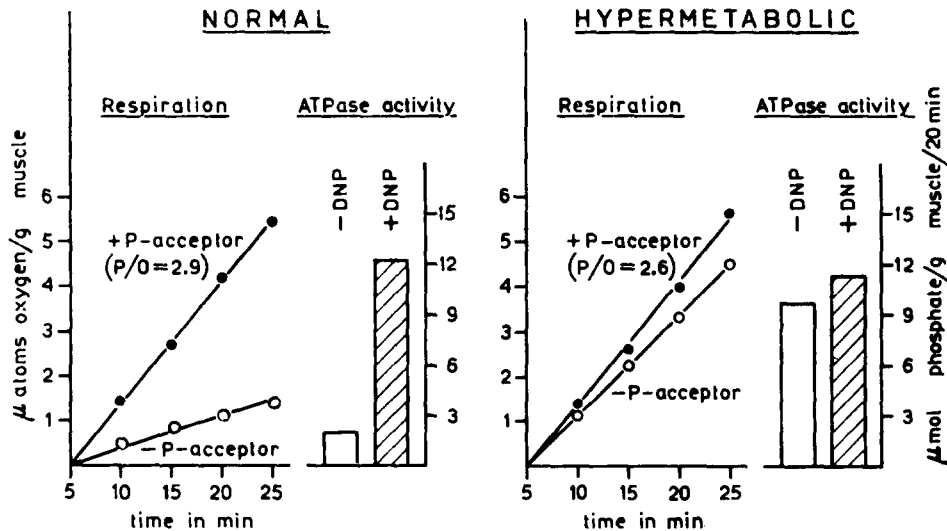
FIGURE 4 "Loose-coupling" of muscle mitochondria from a patient with severe hypermetabolism of nonthyroid origin. From Ernster, Ikkos, and Luft, 1959 (294).

size (295). A second case of this disease, the etiological origin of which is not known, was reported in 1976 by Di Mauro et al. (297).

A comprehensive review of mitochondrial disorders has recently been published by Carafoli and Roman (137).

RESOLUTION AND RECONSTITUTION OF THE ENERGY-TRANSDUCING SYSTEM OF MITOCHONDRIA. MITOCHONDRIAL ENZYME TOPOLOGY: Parallel to the above developments, important progress was made during the 1950s and 1960s towards the resolution and reconstitution of the mitochondrial electron-transport and phosphorylating systems. During the latter half of the 1960s, several laboratories also initiated attempts to separate and characterize the inner and outer mitochondrial membranes and to determine the localization and topology of various mitochondrial catalysts and other chemical constituents.

Resolution and Reconstitution of the Respiratory Chain

A first reconstitution of the respiratory chain was achieved by Keilin (298) in 1930. The oxidation of cysteine by an oxidase preparation was restored by cytochrome c, and all the properties of the reconstituted system were those of "a true respiratory system of the cell." The use of cytochrome c-deficient heart-muscle preparations led in 1940 to the demonstration of the role of cytochrome c in the succinoxidase system (299). In 1958, Keilin and King (300) reconstituted succinoxidase by combining an alkali-treated Keilin-Hartree preparation with solubilized succinate dehydrogenase.

A comprehensive study of large-scale preparations of beef heart mitochondria was begun in the mid-1950s in Green's laboratory (301). Mitochondria from beef heart proved to possess a remarkably high degree of stability; they were capable of withstanding a preparation procedure involving disruption of the tissue by relatively harsh mechanical means, and subsequent storage of the mitochondria in the frozen state for long periods of time. These preparations thus became the material of choice for future studies that aimed at a resolution and reconstitution of the respiratory chain and the phosphorylating system.

In 1956, Singer et al. (302) purified succinate dehydrogenase from beef heart mitochondria and demonstrated that the enzyme is a flavoprotein. The same conclusion was reached simultaneously by Wang et al. (303). A year later Crane and associates (304) reported evidence that ubiquinone is a redox carrier of the respiratory chain between the NADH- and succinate dehydrogenases and the cytochrome system. For some time, the participation of ubiquinone in the respiratory chain was questioned on kinetic grounds (305), but finally was established by reconstitution experiments (306, 307) and by reevaluation of the kinetic data (308), which took into account the fact that the molar amount of ubiquinone in mitochondria is in large molar excess over other respiratory-chain components. In 1960, Beinert and Sands (309) discovered a new type of iron-containing redox catalysts that consisted of nonheme iron proteins. These catalysts, which were later found to contain iron-sulfur centers as their redox groups, were shown to be components of both succinate and NADH dehydrogenase.

In the early 1960s, several groups in Green's laboratory were engaged in the separation and characterization of particulate protein complexes that catalyze partial reactions of the respiratory chain. Four such complexes were isolated: NADH-ubi-

quinone reductase (complex I), containing FMN and nonheme iron (310); succinate-ubiquinone reductase (complex II), containing FAD and nonheme iron (311): ubiquinol-cytochrome c reductase (complex III), which contains cytochromes b and c_1, some bound ubiquinone (312), and, as later shown by Rieske et al. (313), a nonheme iron protein (recently identified with Slater's BAL-sensitive factor [314]); and cytochrome c oxidase (complex IV), containing cytochrome a ($+a_3$) and copper (315). In 1962, Hatefi et al. (316) succeeded in reconstituting NADH oxidase and succinoxidase by combining complexes I, III, and IV and complexes II, III, and IV, respectively, in the presence of cytochrome c. In both cases, the reconstitution required high concentrations of the complexes and resulted in a particulate preparation which did not dissociate upon subsequent dilution.

These results gave rise to the concept (317) that the components of the respiratory chain exist in mitochondria as a fixed assembly ("elementary particles"). Indeed, it was found that cytochrome c can form stable complexes with complex III and complex IV (318) and that mitochondria contain the cytochromes in near-stoichiometric amounts (319). On the other hand, the flavin-containing complexes, and especially complex I, were found to occur in smaller amounts than the cytochromes, and it was suggested that ubiquinone functions as a mobile redox carrier between these complexes and the cytochrome system (308). In recent years it has become evident that this relationship is far more complex than had been anticipated. It is now known (see reviews 320, 321) that both complex I and complex II contain multiple iron-sulfur centers, and that some of these interact with protein-bound ubiquinone in a highly complex fashion, the functional significance of which is not yet fully understood.

In 1962, Fernández-Morán (322) discovered in negatively stained specimens of mitochondria the occurrence of regularly spaced, globular projections on the inner surface of the inner membrane. Initially these projections were believed to correspond to the "elementary particles" (323) and to contain, besides the enzymes of the respiratory chain, a large amount (>50%) of inert "structural protein" (324). However, later work in Racker's and Chance's laboratories (325) led to the conclusion that the globules consist of the coupling factor F_1, which is the catalytic unit of the ATP-synthesizing system of the respiratory chain (see below). The "structural protein" was found to consist mainly of denatured F_1 (326). Whether the projections exist as such in the native mitochondrion or are preparation artifacts has been much debated over the years. In any case, they are found with great regularity in negatively stained mitochondrial preparations and have served as an important landmark for the identification and orientation of the inner membrane. For example, it was on this basis, in addition to biochemical evidence, that the inverted orientation of the membrane of submitochondrial particles prepared by sonication was first proposed (219).

Resolution and Reconstitution of Oxidative Phosphorylation

The first submitochondrial preparation capable of carrying out oxidative phosphorylation was described by Cooper, Devlin, and Lehninger (327) in 1955. By treating rat liver mitochondria with digitonin, they isolated a particulate fraction which catalyzed the oxidation of β-hydroxybutarate and succinate with P/O ratios of 2.4 and 1.5, respectively. The prepa-

rations contained endogenous NAD^+, and external NADH was oxidized by an external type of cytochrome c reductase, similar to that found in intact mitochondria. The size of the particles was estimated to be 1/2,000 of that of mitochondria. Interestingly, these particles, in contrast to preparations obtained by sonication, apparently had the same orientation of the membrane as exists in intact mitochondria.

Submitochondrial particles prepared by sonication and capable of oxidative phosphorylation were first described by Kielley and Bronk (328) through the use of mitochondria from rat liver. In Green's laboratory, it was found that electron transport particles from beef heart mitochondria, when prepared in the presence of media containing Mg^{2+} and ATP (329), or Mg^{2+}, Mn^{2+}, and succinate (330), retained the ability to carry out phosphorylation with relatively high efficiency if either succinate or NADH was used as substrate. These "heavy" electron transport particles (ETP_H), and especially those prepared in the presence of Mg^{2+} and ATP, proved to be valuable for future studies of oxidative phosphorylation and related energy-linked functions. It was, for example, with these particles that a reversal of oxidative phosphorylation was first demonstrated in a submitochondrial system (196); such studies eliminated most of the objections to the conclusions about the mechanism of this process earlier based on experiments with intact mitochondria. With this system it was also first demonstrated (331) that oxidative phosphorylation in submitochondrial particles is not specific for adenine nucleotides—in contrast to intact mitochondria—a phenomenon that was later explained through the discovery of the adenine nucleotide-specific mitochondrial ADP, ATP translocator. Another result of principal importance obtained with phosphorylating sonic particles was the demonstration that energy-linked transhydrogenase driven by the respiratory chain was insensitive to oligomycin whereas that driven by ATP hydrolysis was oligomycin-sensitive (215, 218).

In the late 1950s work was also initiated in Green's laboratory in order to resolve and reconstitute the phosphorylating enzyme system associated with ETP_H. In 1958, Linnane (332) reported that disruption of beef heart mitochondria by sonication in the presence of EDTA resulted in submitochondrial particles ("modified ETP_H") that required a soluble protein fraction for maximal phosphorylation. This factor was purified 8- to 15-fold by Linnane and Titchener (333), who showed that it restored phosphorylation coupled to the oxidation of both succinate and NADH.

After this initial success, Green and associates (334) embarked on a comprehensive study of the individual coupling sites of the respiratory chain. The interpretation of the results became difficult, however, because some of the data, notably those relating to coupling site 3, could not be verified. In the meantime, Racker and associates had begun their work on mitochondrial coupling factors which came to be of fundamental importance for the resolution and reconstitution of the enzyme system involved in electron transport-linked phosphorylation.

Extending an observation reported briefly in 1958 (335), Racker and associates described in 1960 the isolation of an ATPase from beef heart submitochondrial particles (336, 337). The soluble enzyme was cold-labile, which may explain why it escaped detection earlier. The enzyme had the important property of being able to restore oxidative phosphorylation to nonphosphorylating submitochondrial particles. This ability led to the denotion of the ATPase as "coupling factor 1" (F_1).

When F_1 was rebound to the membrane, its ATPase activity again became cold-stable. Furthermore the bound enzyme was sensitive to the phosphorylation-inhibitor oligomycin, whereas the soluble enzyme was oligomycin-insensitive. These findings constituted strong evidence for the notion that the ATPase functions as the terminal enzyme in electron transport-linked ATP synthesis. The binding of F_1 to the membrane of F_1-depleted submitochondrial particles was accompanied by a reappearance of the projecting subunits (325) (Fig. 5).

A much-discussed feature of F_1, observed at an early stage, was that its ability to restore phosphorylation in nonphosphorylating submitochondrial particles actually consisted of a combination of two effects: a catalytic and a "structural" effect (338). It was observed (339) that in certain instances phosphorylation can be restored by catalytically inactive (e.g., chemically modified) F_1. This phenomenon was explained when Lee and Ernster (340) demonstrated that low concentrations of oligomycin can replace F_1 in restoring phosphorylation in certain types of nonphosphorylating particles. These particles contained residual F_1, which was catalytically competent, and the added F_1 served to plug an energy leak—as we now know, a proton leak—that had arisen because of the partial removal of F_1 and caused a dissipation of the energy generated by electron transport. It was this "structural" effect of F_1 that was duplicated by low concentrations of oligomycin (high oligomycin concentrations caused an inhibition of the residual F_1 and thereby an inhibition of phosphorylation). Oligomycin also induced a respiratory control in these particles (219, 341, 342). When particles were completely depleted of F_1, oligomycin was no longer able to replace F_1 in restoring phosphorylation (343).

An important development in the mid-1960s was the demonstration by Kagawa and Racker (205) that the phosphorylation inhibitor oligomycin does not act on F_1 itself but on a segment of the ATPase complex that is imbedded in the membrane. This segment has been denoted F_O (where o refers to oligomycin). F_O consists of several hydrophobic proteins. One of these has been identified by its covalent binding to N,N'-dicyclohexylcarbodiimide (DCCD), which has been shown by Beechey and associates (344) to act as a phosphorylation inhibitor similar to oligomycin. This DCCD-binding protein had been purified from several sources, including mitochondria, chloroplasts, and bacteria; the amino-acid sequences of these proteins show an exceedingly high degree of homology (345).

The discovery of F_1 was followed by extensive efforts in several laboratories to identify and characterize additional coupling factors involved in the mitochondrial ATP-synthesizing system (see review 346). Among the many factors described, two have proved to exhibit well-defined functions: coupling factor F_{c2} (347) (or F_6 [348]), involved in the binding of F_1 to F_O, and OSCP (oligomycin sensitivity-conferring protein) (349), responsible for the conferral of oligomycin sensitivity. Interestingly, both factors have been found thus far only in mitochondria and do not seem to occur in chloroplasts or bacteria. Another coupling factor of continued interest is factor B, which was described by Sanadi and associates (350) and is required for the P_i-ATP exchange reaction that is catalyzed by membrane-bound ATPase (cf. ref. 346).

The whole, oligomycin-sensitive, ATPase complex was first isolated by Kagawa and Racker (205) in 1966, and its action as a reversible proton pump was demonstrated (351) a few years later (see further below). Reconstitution of the ATPase complex
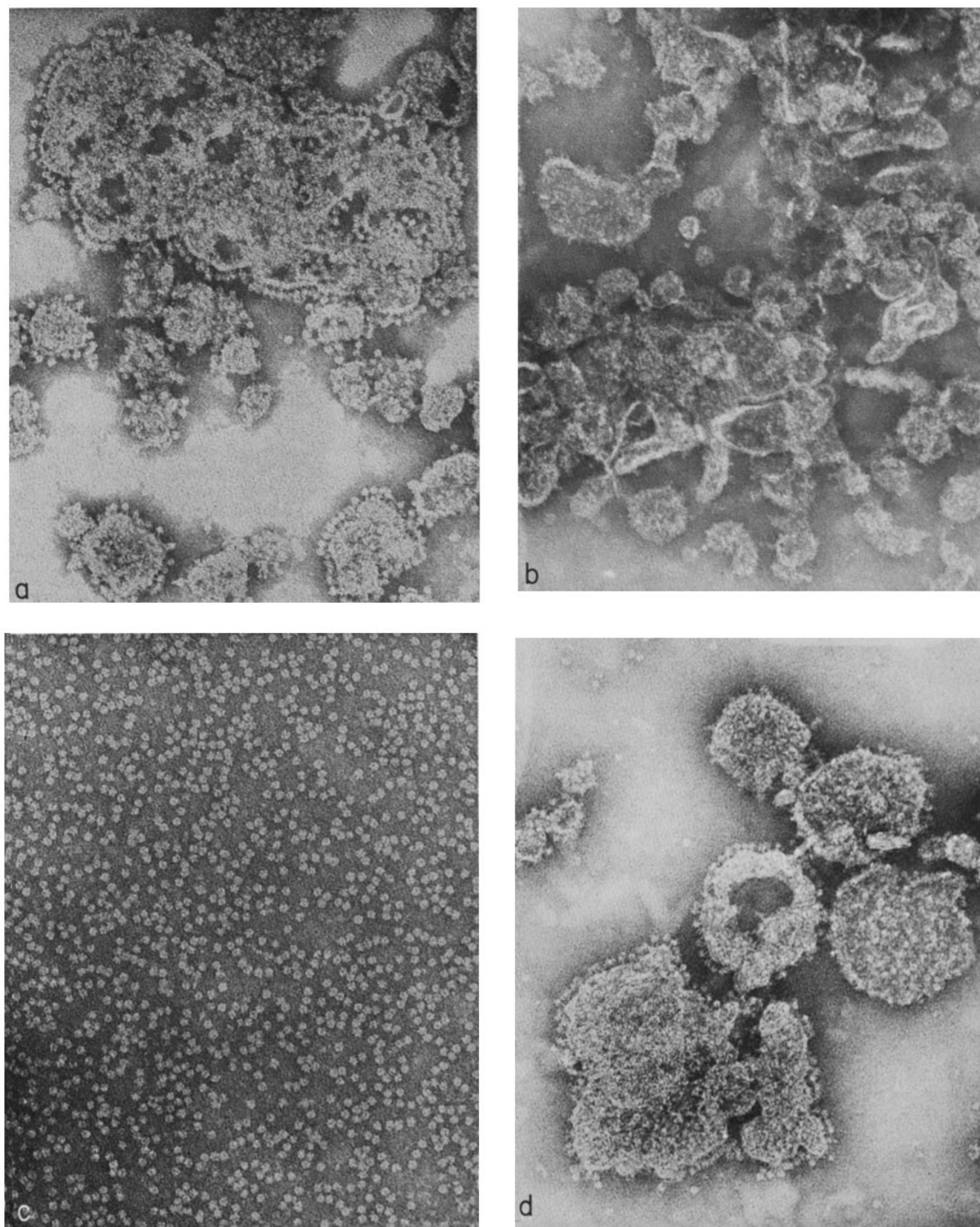
FIGURE 5 Resolution and reconstitution of the projecting subunits of submitochondrial particles from beef heart, containing coupling factor F_1. (a) Native particles. (b) Particles after removal of F_1. (c) Coupling factor F_1. (d) Reconstituted particles. For details, see Racker et al., 1965 (325).

from isolated F_0 and F_1 was first described by Capaldi (352) and its quantitative aspects were investigated by Glaser et al. (353, 354).

Isolated mitochondrial F_1, together with similar proteins from chloroplasts and bacteria, has been the subject of extensive studies (cf. review 346). As first shown by Senior and

Brooks (355, 356), F_1 consists of five subunits (α–ϵ), with molecular weights of 53,000, 50,000, 30,000, 17,000, and 7,500 daltons. Initial estimates, based on Coomassie blue staining of SDS gels, indicated a subunit stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (355, 357), but the validity of these estimates was later questioned on the basis of sulfhydryl-group titrations (358). Recent data, however, seem to support the originally proposed stoichiometry (359, 360). The β-subunit is generally believed to contain the catalytic center of F_1 (cf. ref. 346). The enzyme contains firmly bound adenine nucleotides (361), the role of which is thought to be partly catalytic and partly regulatory. Extensive studies of the reaction mechanism of F_1 have been carried out by means of various exchange reactions as well as by the use of various activators and inhibitors. Among the latter aurovertin (which forms a fluorescent complex with F_1 [326–364]), the covalent inhibitor nitrosobenzofurazan chloride (365), and tris-bathophenanthroline-ferrous chelate (the effect of which is relieved by uncouplers [366, 367]) have been shown to bind to the β-subunit of the enzyme.

A naturally occurring inhibitor of mitochondrial F_1-ATPase was described in 1963 by Pullman and Monroy (368). It consists of a protein of about 10,000 daltons. It has been proposed (369) that the inhibitor acts as a unidirectional regulator of ATP synthesis, inhibiting the backflow of energy from ATP to the respiratory chain. The binding and release of the inhibitor are regulated by the energy state of the mitochondria (370–373). Recent evidence suggests that the inhibitor acts on both the forward and reverse reactions catalyzed by F_1 (374) and that its function may be to regulate ATP synthesis in relation to other energy-linked functions of the respiratory chain, e.g., Ca^{2+} uptake (375).

Isolation and Characterization of the Outer and Inner Membranes. Intramitochondrial Localization of Enzymes

Although the existence of two mitochondrial membranes had been established in the early 1950s, it was not until the second half of the 1960s that methods for their separation were developed. Perhaps the main reason for this long interval was that interest in mitochondrial research was focused primarily on studies of the respiratory chain and oxidative phosphorylation. The association of respiration with the cristae was indicated by early morphological evidence (26), and, thus, when it was later found that the Keilin and Hartree heart-muscle preparation (52) or a deoxycholate-solubilized succinoxidase from liver mitochondria (376, 377) consisted of membranes, it was plausible to assume that these membranes were derived from the inner mitochondrial membrane. Support for the association of the respiratory chain with the inner mitochondrial membrane was also obtained by histochemical techniques (378).

In the mid-1960s several laboratories independently reported the successful separation of mitochondrial inner and outer membranes (see review 124). Lévy et al. (379) and Schnaitman et al. (380) used low concentrations of digitonin to detach the outer membrane of liver mitochondria, after which they separated the outer and inner membrane fractions by differential centrifugation. The rationale for this method is that the outer membrane is rich in cholesterol, which binds digitonin. Later Schnaitman and Greenawalt (381) refined this method to allow the preparation of "mitoplasts," i.e., mitochondria without an outer membrane and with a relatively intact inner membrane and matrix. Another procedure, worked out by Parsons et al.

(382) and Sottocasa et al. (81), took advantage of the selective shrinking of the inner membrane following exposure of liver mitochondria to a swelling-contraction cycle. The distended outer membrane ruptures either spontaneously or, more efficiently, after gentle sonication, and can be separated from the inner-membrane fraction by density-gradient centrifugation.

Striking differences between the two mitochondrial membranes were found in regards to osmotic behavior and permeability. As already mentioned, the inner membrane readily unfolds and refolds in response to changes in osmotic pressure. In contrast, the outer membrane shows no reversible response to such changes, and the distention and rupture during mitochondrial swelling may be interpreted as a passive process following the unfolding of the inner membrane. Extensive studies (383), based on measurements of the space occupied by various substances present in the medium in relation to the total water space of the mitochondria, and correlated with morphological observations, have led to the conclusion that the inner membrane is practically impermeable to most substances except uncharged molecules of a molecular weight not greater than 100–150. The majority of charged molecules of physiological significance pass through the inner membrane by way of specific translocators associated with this membrane (see Table II). In contrast, the outer membrane seemed to be permeable to a wide range of substances, both charged and uncharged, with molecular weights up to about 5,000. Parsons et al. (382) observed in negatively-stained specimens of isolated outer membranes the occurrence of pore-like structures. Recently, two laboratories (384, 385) reported the isolation from the mitochondral outer membrane, of a protein with a molecular weight of about 30,000 and the ability to make phospholipid bilayers permeable to a variety of substances. A similar "pore protein" ("porin") had been described previously in the outer membrane of Gram-negative bacteria (386).

Chemically the most striking quantitative difference between the mitochondrial inner and outer membranes is in the relative contents of protein and lipid; the outer membrane contains, on a protein basis, two to three times more phospholipid than the inner membrane (382). Like energy-transducing membranes in general, the inner membrane is particularly rich in proteins deeply embedded in the membrane, a circumstance strikingly revealed by freeze-fracture techniques (387) (Fig. 6). A conspicuous qualitative difference in phospholipid composition is the virtually exclusive localization of cardiolipin in the inner membrane (382). Cholesterol is found predominantly in the outer membrane (388).

The availability of procedures for the separation of the two mitochondrial membranes opened the way to a determination of the intramitochondrial distribution of enzymes. Such data soon became available regarding almost all enzymes known to be present in mitochondria (124, 389). The outer membrane was found to contain a diversity of enzymes including a rotenone-insensitive NADH-cytochrome c reductase (81) (similar to, but not identical with, the microsomal NADH-cytochrome b_5 reductase-cytochrome b_5 system), monoamine oxidase (380, 381), as well as various enzymes involved in phospholipid metabolism (390). The intermembrane space was identified as the location of adenylate kinase (124, 381, 389, 391), nucleoside mono- and diphosphokinases (381, 389), sulfite oxidase (392) and yeast cytochrome c peroxidase (393). The inner membrane proved, as expected, to be the site of the catalysts of the respiratory chain and respiratory chain-linked phosphorylation. Also found to be associated with the inner membrane were the nicotinamide nucleotide transhydrogenase
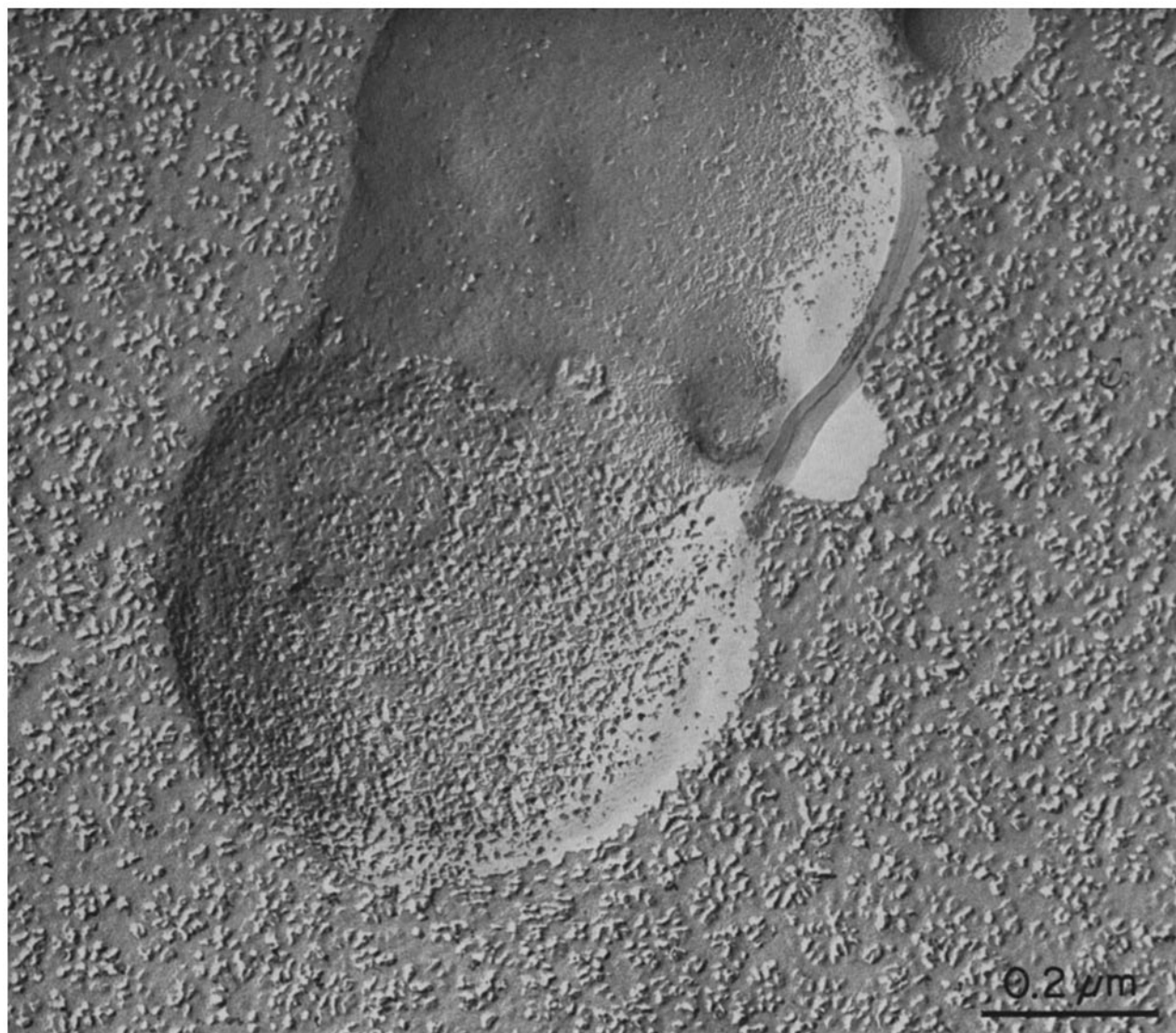
FIGURE 6   Freeze-etch preparation of glutaraldehyde-fixed mitochondrion showing the two membrane faces exposed by fracture. From Wrigglesworth and Packer, 1970 (387).

(389), β-hydroxybutyrate dehydrogenase (381, 390), fatty acyl carnitine transferase (394), and two enzymes involved in heme synthesis (ferrochelatase [395, 396] and δ-aminolevulinic acid synthetase [396]). Enzymes localized in the matrix include those involved in the citric acid cycle (except succinate dehydrogenase) and related processes such as substrate-level phosphorylation, pyruvate and phosphopyruvate carboxylation, glutamate oxidation, transamination, citrullin synthesis, and fatty acid oxidation (see review 124). The localization of some of these enzymes in the matrix was questioned by Green and associates (cf. 124, 397 and refs. therein), who claimed that the enzymes of the citric acid cycle and fatty acid oxidation are associated with the outer membrane. However, this controversy was successfully settled through a joint effort between two of the laboratories involved (398).

Enzyme Topology of the Inner Membrane

In the 1960s information also began to be available concerning the enzyme topology of the inner mitochondrial membrane. As membrane proteins in general, the enzymes associated with the inner membrane of mitochondria proved to possess a well-defined orientation in relation to the plane of the membrane—a transverse topology, or "sidedness," that was of special interest in connection with the chemiosmotic hypothesis (see below).

A first striking illustration of this sidedness was the demonstration that the coupling factor F_1 resides in the projections on the inside surface of the inner mitochondrial membrane. More general criteria used for the assessment of the transverse topology of various enzymes included accessibility to proteases, antibodies, and other macromolecules as well as nonpenetrant substrates, effectors, and protein reagents (see review 399). The inverse relationship in membrane orientation between intact mitochondria (or mitoplasts) and sonic submitochondrial particles was also exploited. The results could be verified further by reconstitution experiments using isolated components. The available information (see reviews 308, 399) may be summarized as follows (Fig. 7).

The flavin prosthetic groups of NADH- and succinate-ubiquinone reductase face the matrix (M) side of the membrane. Ubiquinone and the cytochrome b moiety of ubiquinol-cytochrome c reductase are probably inaccessible to either side of the membrane, whereas cytochrome c_1 faces the cytosolic (C)
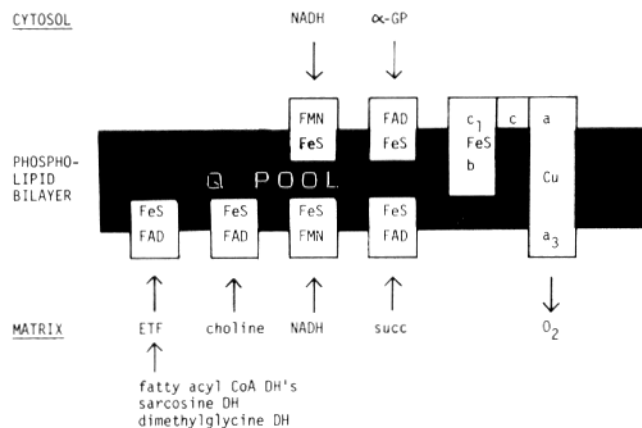
FIGURE 7 Possible topology of various FeS-flavoproteins, the Q pool and the cytochrome system in the inner mitochondrial membrane. Abbreviations: α-GP, α-glycerophosphate; ETF, electron-transferring flavoprotein; succ, succinate; DH, dehydrogenase. From Ernster, 1977 (538).

side. Cytochrome c is located on the C side of the membrane, which is its site of interaction with both cytochrome c_1 and cytochrome a. Cytochrome c oxidase is thought to span the membrane, with its O_2-reactive site (cytochrome a_3) oriented toward the M side. Also facing the M side of the membrane are the catalytic sites of nicotinamide nucleotide transhydrogenase and ATPase as well as β-hydroxybutyrate dehydrogenase, choline dehydrogenase, and "ETF dehydrogenase," the enzyme serving as the catalytic link between the electron-transferring flavoprotein (ETF) and the cytochrome system. The flavoenzymes glycerol phosphate dehydrogenase and an "external" NADH dehydrogenase found in yeast and plants react with their substrates on the C side of the membrane. It should be pointed out that all these localizations of the various enzymes concern their catalytic sites and that the enzyme as a whole spans the membrane in many cases. This is the case with the proton-translocating energy-transducing units (complexes I, III, IV, ATPase, transhydrogenase) and probably with several other enzymes as well.

SEARCH FOR CHEMICAL HIGH-ENERGY INTERMEDIATES. ALTERNATIVE HYPOTHESIS: Despite the vast amount of information that accumulated during the 1950s and early 1960s concerning the pathways and components of mitochondrial energy transduction, relatively little progress was made towards elucidating the mechanism of electron transport-linked phosphorylation. Although most data were consistent with the predictions of a "chemical" mechanism, which, as formulated by Slater (179) and others, involved "high-energy intermediates" as energy transducers between electron transport and ATP synthesis, there was no success in isolating or identifying such intermediates, in spite of efforts in many laboratories. There is hardly any component of the respiratory chain that has not been proposed as part of such an intermediate (400). An involvement of Ca^{2+} (234) and of K^+ (+ valinomycin) (241) in high-energy intermediates has also been considered in connection with the active uptake of these cations by respiring mitochondria. None of the proposed or, in some cases, allegedly demonstrated intermediates could be verified experimentally. A promising exception was a protein-bound phosphohistidine, described in Boyer's laboratory (401), which, however, turned out to be an intermediate of the succinyl thiokinase reaction (402). As could be expected, the search for

chemical "high-energy" intermediates gradually decreased in intensity from the mid-1960s (although new proposals still appear occasionally [403]).

In 1965, Boyer (404) proposed that the energy-yielding oxidoreduction steps in the respiratory chain might give rise to "energized" conformational states of the catalysts involved. These conformational states of the redox catalysts would induce the formation of a covalent high-energy bond in an adjacent ATPase molecule, with the result that the latter now would be able to synthesize ATP from ADP and P_i. While this mechanism provided an alternative explanation of how energy may be conserved by electron-transport catalysts or ATP-synthesizing enzymes, it did not readily account for the mechanism by which energy may be transferred *between* such enzymes. By the middle of the 1960s there was evidence that in mitochondria energy can be transferred between a relatively large number of energy-transducing units, including the three coupling-sites of the respiratory chain, the ATP-synthesizing system, the nicotinamide nucleotide transhydrogenase, and various ion translocators. It appeared unlikely that all these enzymes and translocators would transfer energy by direct molecular collision within the membrane.

A solution to this problem was opened in 1961 when Mitchell (405) advanced his chemiosmotic hypothesis, which he subsequently developed to represent a general mechanism for energy-coupling in respiratory and photosynthetic phosphorylation (406) (Fig. 8). According to this hypothesis energy conservation in the oxido-reduction chain proceeds by way of a proton gradient across the coupling membrane; the gradient is brought about through the action of alternating hydrogen and electron carriers, which form loops across the membrane in such a way that hydrogen ions are taken up on one side of the membrane and given off on the other side. It was further proposed that the same membrane contains a proton-translocating reversible ATPase, which can utilize the proton gradient generated by the oxido-reduction chain for the synthesis of ATP from ADP and P_i. The same year, Williams (407) proposed a mechanism which also involves a flux of protons generated by the respiratory chain as the driving force for ATP synthesis. However, instead of establishing a gradient *across* the membrane, as in the case of chemiosmotic hypothesis, this proton flux was assumed to give rise to a proton activity in the lipid phase *within* the membrane, localized so that it can bring about a dehydration around the active center of the ATPase and thereby promote the condensation of ADP and P_i to ATP (408). The chemiosmotic mechanism elicited especially great interest, since it provided a rational explanation for the role of the membrane in oxidative and photosynthetic phosphorylation and constituted a common link between these processes and active transport.

Historically, the concept of vectorial transport of protons originated from the suggestion by Lundegårdh (409) in 1945 that if oxido-reduction through the cytochrome system were anisotropically organized across the membrane, protons would be produced on one side and consumed on the other. In their studies of acid secretion by gastric mucosa, Davies and Ogston (410) concluded in 1950 that proton secretion may involve the parallel operation of two mechanisms: (1) a redox-linked proton pump; and (2) a proton pump driven by the hydrolysis of a labile phosphate compound (e.g., creatine phosphate). They pointed out (410) that "this is an interesting possibility, because, each mechanism being reversible, mechanism 1 could drive mechanism 2 backwards, leading to phosphorylation of crea-
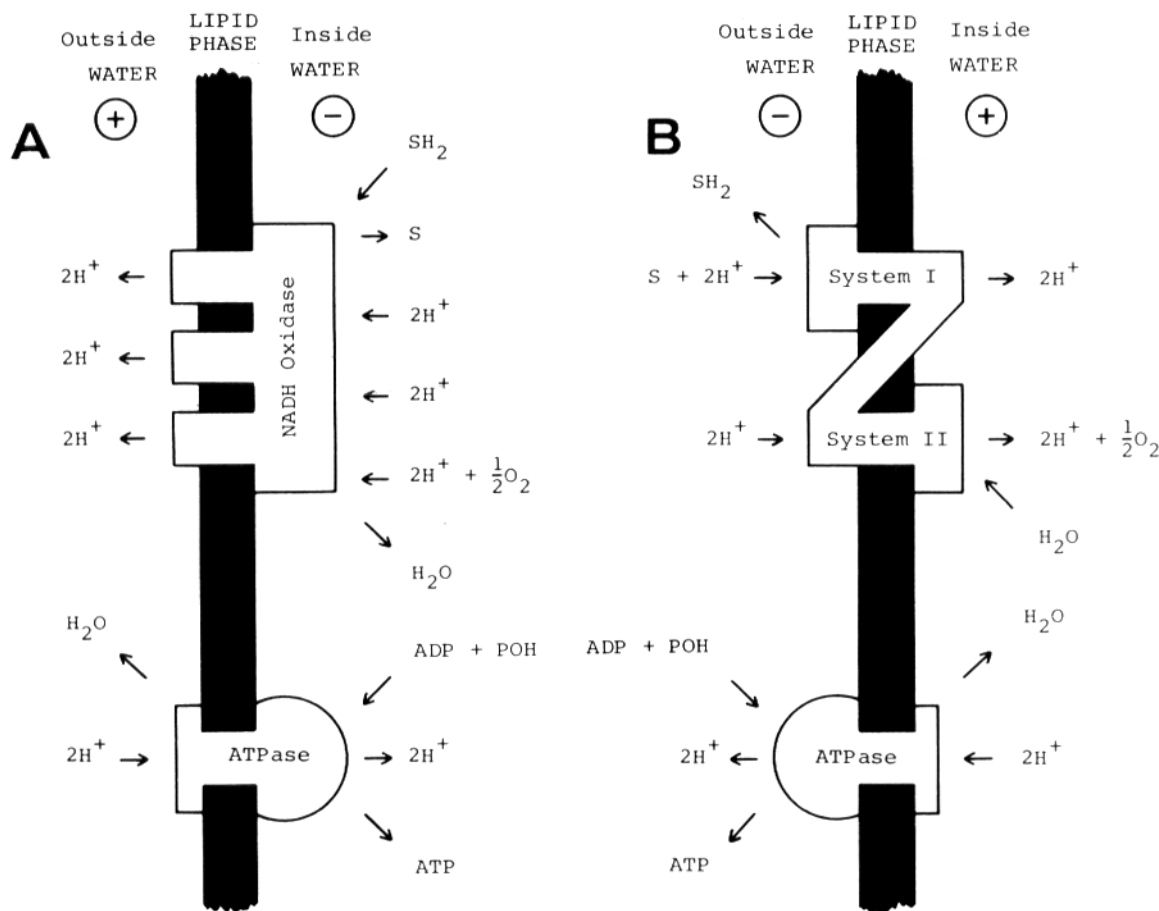
FIGURE 8   Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. From Mitchell, 1970 (539).

tine and this might explain how oxidation in the cytochrome system at high E_h' could bring about phosphorylation." However, they discarded this possibility in view of Friedkin and Lehninger's (60) demonstration of phosphorylation linked to the oxidation of NADH in a liver-particulate preparation, pointing out (410) that "it is doubtful whether such a degree of organization as would be required by our mechanism could have survived in such a preparation."

Mitchell's interest in chemiosmotic mechanisms arose in the early 1950s from studies of bacterial membrane transport. He noted that some of these processes resembled enzyme-catalyzed reactions and, furthermore, that bacterial membranes contained both electron-transferring and phosphorylating enzymes. These observations gave rise to the idea that certain transport processes across membranes might be catalyzed by vectorial group-translocating enzymes that may be related to oxidative phosphorylation. In a lecture published in 1954, Mitchell (411) stated: "... in complex biochemical systems, such as those carrying out oxidative phosphorylation, the osmotic and enzymic specificities appear to be equally important and may be practically synonymous."

The official debut of the chemiosmotic hypothesis took place in a short paper in *Nature* in July, 1961 (405); one of the reasons given for the proposal of the hypothesis was "to acknowledge the elusive character of the $C \sim I$ intermediates by admitting that they may not exist." Shortly afterwards, Mitchell (412) reported evidence that uncouplers of oxidative phosphorylation act as proton conductors through the membranes of mitochondria and bacteria. However, the real breakthrough of the hypothesis did not begin until 1966 when

Mitchell (406) published a book entitled: *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation.* In this book he outlined his hypothesis in detail, specifying its basic postulates and defining the "protonmotive force" as the sum of an electric and a chemical component. At the same time, experimental evidence for these concepts began to appear (413–416). As a result, work was initiated in many laboratories in order to evaluate Mitchell's hypothesis, and constituted the principal task in bioenergetics for the next decade.

THE CHEMIOSMOTIC DECADE: The chemiosmotic hypothesis was based upon four postulates (406): (*a*) a proton-translocating ATPase; (*b*) a proton-translocating oxido-reduction chain; (*c*) exchange-diffusion systems, coupling proton translocation to that of anions and cations and permitting entry and exit of essential metabolites; and (*d*) an ion-impermeable membrane, in which systems 1, 2, and 3 reside. The protonmotive force, Δp (or $\Delta \tilde{\mu}_{H^+}$, as designated by many investigators), was defined (406, 414) as the sum of the electric potential ($\Delta \psi$) and the pH difference (ΔpH) across the membrane, according to the equation

$$\Delta p (\text{or } \Delta \tilde{\mu}_{H^+}) = \Delta \psi - Z\Delta pH,$$

where Z stands for $-2.303RT/F$ (-59 at 25° when Δp [or $\Delta \tilde{\mu}_{H^+}$] and $\Delta \psi$ are expressed in mV and ΔpH in pH units). Early evidence reported by Mitchell and Moyle (413, 415) showed that the addition of oxygen to anaerobic mitochondria in the presence of an NAD$^+$-linked substrate resulted in an ejection of protons with a stoichiometry of 6H$^+$/O (Fig. 9 *top*), and that the proportion of the electrical and chemical compo-

nents of the protonmotive force varied with the experimental conditions (Fig. 9 *lower panel*), all in accordance with the predictions of the chemiosmotic hypothesis. An early piece of evidence that acquired great attention was the "acid-bath" experiment of Jagendorf and Uribe (416), in which it was shown that a proton gradient imposed across the thylakoid membrane of chloroplasts gave rise to ATP synthesis.

Further progress in this area was greatly dependent upon technical developments that made possible the demonstration and quantitation of the protonmotive force and its electric and chemical components (see reviews 417, 418). The availability of sensitive pH and ion-specific electrodes, in combination with suitable ionophores, and of various optical probes was of great significance. Another important development was the introduction by Skulachev, Liberman, and their associates (419) of a series of synthetic organic anions and cations, among them several boron compounds, which proved very useful for determining $\Delta\tilde{\mu}_{H^+}$. A number of elegant physicochemical techniques that facilitated the measurement of transient phases of the electrochemical events, especially those accompanying light-induced electron transport and phosphorylation, were developed in Witt's laboratory (420). Another crucial technical development, initiated in Racker's laboratory (421, 422) and pursued and further developed by Skulachev's group (423, 424), was the elaboration of methods to incorporate energy transducing systems—electron transport catalysts, ATPases, and ion translocators—into artificial phospholipid vesicles.
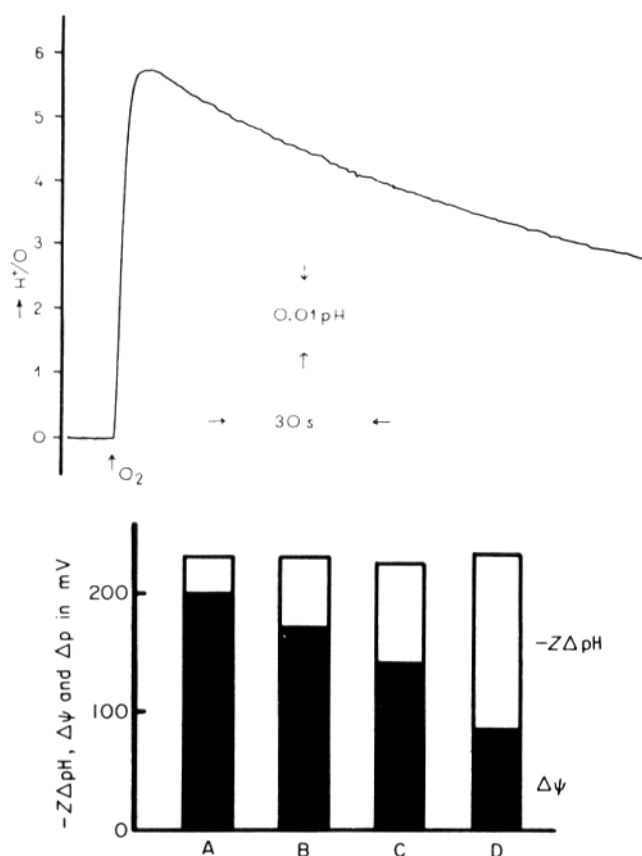

FIGURE 9   Demonstration and measurement of protonmotive force in respiring mitochondria. (*Top*) Proton pulse driven by β-hydroxybutyrate oxidation. From Mitchell and Moyle, 1967 (413). (*Bottom*) Electrical (Δψ) and chemical (−ZΔ pH) components of the protonmotive force (Δp) in mitochondria respiring under different conditions. From Mitchell and Moyle, 1969 (415).

Due to these techniques, and to a large amount of experimental data collected in many laboratories, the four basic postulates of Mitchell's chemiosmotic hypothesis were essentially proven by the middle of the 1970s. It was established that mitochondria and submitochondrial particles can form a transmembrane proton gradient concomitant to electron transport or ATP hydrolysis, and that the generation of a proton gradient by the former can be utilized for ATP synthesis by the latter. It was also shown that the mitochondrial inner membrane contains a substrate-specific exchange diffusion carrier system (cf. Table II), and that the membrane itself has a low permeability to protons and other ions. Similar results were obtained with chloroplasts and bacteria. Incorporation of the isolated respiratory-chain complexes representing the three coupling sites of the chain (425–427), of ATPase (428), and of transhydrogenase (221, 429) into liposomes was shown to result in an ability of these catalysts to bring about transmembrane proton gradients. Furthermore, when one of the three electron-transport complexes and ATPase were incorporated into the same liposome, the system was capable of exhibiting oxidative phosphorylation (430–432). Recently, ATP-driven transhydrogenase was also reconstituted (223). In a spectacular reconstitution experiment performed in 1974, Racker and Stoeckenius (433) incorporated bacteriorhodopsin and mitochondrial ATPase in the same liposome and demonstrated light-induced phosphorylation.

Over the years, the chemiosmotic hypothesis has been the subject of much debate. Some of the criticism (408, 434, 435) addressed the basic principles of the hypothesis and gave rise to lively rebuttal (436). Other criticism (e.g., 437) concerned details of Mitchell's mechanism and often led to modifications of the hypothesis. This debate is still going on (see below). An overview of the field (see e.g., 438), however, shows that the basic idea of the chemiosmotic hypothesis:

$$\text{electron transport} \rightarrow \Delta\tilde{\mu}_{H^+} \rightarrow \text{ATP synthesis}$$
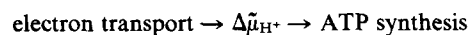
was widely accepted by the middle of the 1970s as the "central dogma" of membrane bioenergetics.

PRESENT STATE OF THE ART: For the last five years, research in mitochondrial energy transduction (and in membrane bioenergetics in general) has been centered mainly on three questions (cf. 438): (*a*) the mechanism of proton translocation by various energy-producing units; (*b*) the possible role of conformational changes of proteins as the primary events in energy transduction; and (*c*) the possible involvement of localized (intramembrane) electrochemical mechanisms in energy transfer between energy-transducing units.

A controversial issue relating to the mechanism of proton translocation that has been actively pursued concerned the number of protons that are translocated across the mitochondrial inner membrane for each pair of electrons transferred through the individual coupling sites of the respiratory chain. According to Mitchell (406) this number is 2, in accordance with the concept of redox loops. Estimates from other laboratories (235, 439, 440) indicate higher values, probably 3 or 4, which suggests the occurrence of proton pumps. Evidence for a proton pump linked to cytochrome *c* oxidase has been reported by Wikström (441) and others (see review 442) but is questioned by Mitchell (443). The possibility that cytochrome *b* functions as a proton pump has also been considered (444, 445) with reference to Chance's concept of "membrane Bohr effect" (446). Alternatively, the generally observed $H^+/2e^-$ stoichiometry of 4 of the ubiquinol-cytochrome *c* reductase

may be explained in terms of Mitchell's "Q-cycle" (447). There is strong evidence for a proton pump linked to transhydrogenase (221), in agreement with earlier predictions (409, 448). It is also generally agreed that the ATPase functions as a proton pump, but the H^+/ATP ratio is not yet settled (cf. 438). The possibility that different energy-coupling sites may have different proton stoichiometries has led to a reevaluation of the concept of integral $P/2e^-$ and P/O ratios; various current proposals are listed in Table III (449).

Another controversial issue is the mechanism of interaction between the proton-translocating (F_O) and catalytic (F_1) moieties of ATPase. According to Mitchell (443), protons conducted by F_O interact directly with the active site of F_1 in the course of the catalytic event. Boyer (450) and Slater (451) have proposed an indirect mechanism, according to which proton conduction via F_O alters the conformation of F_1 so as to release bound ATP; the synthesis of the latter from bound ADP and P_i would take place at the expense of energy stored in the "energized" conformational state of the enzyme. Later Boyer and associates (452) extended this hypothesis to involve catalytic cooperativity between identical subunits of the enzyme, where the binding of ADP and P_i to one subunit is accompanied by a release of ATP from another subunit. This "alternate-site" or "binding-change" mechanism has received significant experimental support in recent years (453). In general, there is growing evidence (cf. 438) that both the ATPase and various energy-transducing electron-transport catalysts are capable of undergoing energy-linked conformational changes and that proton gradients may serve not only as energetic links but also as effectors of these catalysts.

Several laboratories are pursuing the important problem of whether energy-transducing catalysts located in the same membrane interact merely by way of a bulk proton gradient or whether there may be more localized interactions, e.g., electrochemical activities along or within the membrane. Indications for the latter type of interaction have been obtained by using the ATP-driven transhydrogenase of mitochondria as the test system (371, 372, 454) as well as with various membrane probes (455–458). Evidence for a localized energy transfer has also been obtained with photosynthetic systems (459–463).

The final answers to these questions will have to await more detailed knowledge of the chemical structure of the individual energy-transducing catalysts. A great deal of information has accumulated during the last few years concerning the subunit composition and topology of both the ATPase complex (cf. 346) and various electron-transfer complexes (464–467). For the latter, information of great interest has been obtained from X-ray studies of two-dimensional membrane crystals followed by image reconstruction (Fig. 10); such information is now available concerning the shape and membrane topology of

TABLE III

Proposed H^+/site, $P/2e^-$ and P/O Ratios

Author(s), year (ref.)	H^+/site					$P/2e^-$			P/O
	Site 1	Site 2	Site 3	ATP synth.*	ATP transp.‡	Site 1	Site 2	Site 3	Sites 1–3
Mitchell, 1966 (406)	2	2	2	2	0	1	1	1	3
Brand et al., 1976 (439)	4	4	4	3	1	1	1	1	3
Wikström and Krab, 1978 (442)	3	2	4	2	1	1	0.67	1.33	3
Brand et al., 1978 (440)	2	2	4	2	1	0.67	0.67	1.33	2.67
Hinkle and Yu, 1979 (449)	2	2	2	2	1	0.67	0.67	0.67	2

* H^+/site used for ATP synthesis.
‡ H^+/site used for ATP transport via the ADP, ATP translocator.
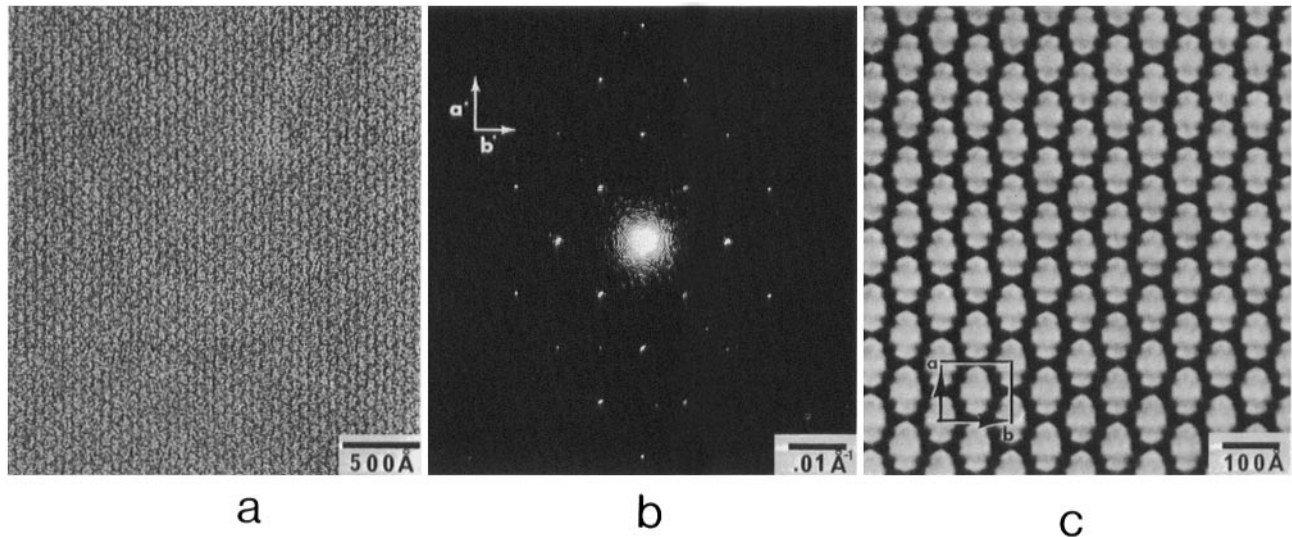
FIGURE 10   Structure of negatively stained crystalline cytochrome c oxidase. (a) Electron micrograph of crystalline cytochrome c oxidase stained with 1% uranyl acetate. (b) Optical diffraction pattern of (a). Reciprocal space lattice vectors (a˙ and b˙) are indicated. (c) Optically filtered image of (a). Reconstruction is based on the information at or around the reciprocal lattice points of the optical diffraction pattern (b). One unit cell is outlined and lattice vectors (a and b) are indicated. From Frey, Chan, and Schatz, 1978 (470).

cytochrome *c* oxidase (468–470) and ubiquinol-cytochrome *c* reductase (467). The primary structures of several subunits of cytochrome *c* oxidase (466) and of the DCCD-binding protein of the ATPase complex (345) have recently been determined. Progress in this area has been greatly accelerated by the advent of DNA-sequencing techniques and by the rapid advance of the field of mitochondrial genetics.

In an introduction to a symposium on the Biochemistry of Mitochondria 15 years ago, Slater (471) ended his lecture with the following words: "I suspect, however, that it will be a long time before we understand the mechanism of oxidative phosphorylation. It is an open bet whether it will be the enzymologist, the membranologist or the protein chemist who will give the answer." Today it appears that the answer will come from all three, in a relay, and that the bâton has just passed from the membranologist to the protein chemist.

Biogenesis

GENETIC EVIDENCE FOR SEMIAUTONOMOUS REPLICATION: Research on mitochondrial biogenesis is one of the youngest areas of "mitochondriology" (Table IV). This is not surprising since the problem of how mitochondria are formed could only be attacked after the morphology, composition, and function of the organelles had become reasonably well known. About 30 years ago it was widely held that mitochondria arise *de novo* by transformation of other cellular structures. For example, experiments by Harvey with sea urchin eggs (472) and by Zollinger with mouse kidney tubules (473) seemed to indicate that these cells could regenerate their mitochondria after all preexisting mitochondria had been removed or destroyed. Also, several light- and electron micrographs that were made of animal- and plant cells in the

TABLE IV

Some Key Discoveries on Mitochondrial Biogenesis

Year	Discovery	Author(s)	Reference(s)
1950–1952	Mitochondrial formation in yeast and *Neurospora crassa* is controlled by "non-Mendelian" genetic factors	Ephrussi	479
		Mitchell and Mitchell	480
1958	Mitochondria synthesize protein	McLean et al.	482
1963–1964	Mitochondria contain DNA	Nass and Nass	493
		Schatz et al.	494
		Luck and Reich	495
1964	Mitochondria synthesize RNA	Wintersberger and Tuppy	498
1966	Mitochondrial biogenesis in vivo is blocked by some antibiotics	Clark-Walker and Linnane	484
1966	The extrachromosomal "petite" mutation in yeast alters mitochondrial DNA	Mounolou et al.	501
1966	Iso-1-cytochrome *c* is coded by a nuclear gene	Sherman et al.	492
1967	Mitochondria contain ribosomes	Küntzel and Noll	500
1967–1968	Mitochondrially inherited drug-resistant yeast mutants	Linnane et al.	505
		Thomas and Wilkie	506
1969–1970	Yeast mitochondria are not formed *de novo*	Plattner et al.	478
1968–1970	Methods for mapping mitochondrial genes	Thomas and Wilkie	508
		Gingold et al.	509
		Coen et al.	510
1972–1973	Identification of mitochondrially synthesized polypeptides	Tzagoloff and Meagher	489
		Weiss	490
		Mason and Schatz	488
1974–1975	Mitochondrially-inherited yeast mutants with specific phenotypic lesions (mit⁻ mutants)	Flury et al.	512
		Tzagoloff et al.	513
1975–1976	Genetic map of yeast mitochondrial DNA	Molloy et al.	535
		Schweyen et al.	515
1976	Physical map of mitochondrial DNA from various species	Sriprakash et al.	536
		Sanders et al.	520
		Morimoto et al.	520
		Bernard and Küntzel	520
		Attardi et al.	520
		Terpstra et al.	520
		Dawid et al.	520
1977	Mitochondrially-translated polypeptides are coded by mitochondrial DNA	Douglas and Butow	534
		Sebald et al.	516
		Cabral et al.	517
		Claisse et al.	518
		Mahler et al.	519
1978	Some mitochondrial genes are "interrupted"	Bos et al.	524
		Slonimski et al.	521
		Haid et al.	522
		Mahler et al.	519
1979	Identification of larger precursors to cytoplasmically synthesized mitochondrial proteins	Maccecchini et al.	531
		Neupert et al.	533
1979–1980	Nucleotide sequences of mitochondrial genes. The mitochondrial genetic rode has some unique features	Hensgens et al.	525
		Macino and Tzagoloff	526
		Fox	528
		Barrell et al.	529

1950s and early 1960s appeared to show formation of mitochondria from other cellular membranes, in particular the nuclear outer membrane (e.g., 474). *De novo* formation of mitochondria was still debated as recently as 11 years ago: two laboratories claimed that anaerobically-grown yeast cells (which lack respiratory enzymes) were devoid of mitochondria, yet could reform them upon oxygenation (475, 476). *De novo* formation of mitochondria ceased to be taken seriously only in the late 1960s when it was shown that, during anaerobic growth, the mitochondria of yeast are not lost but are merely dedifferentiated into respiration-deficient promitochondria which are difficult to detect by conventional electron microscopy (477, 478). In retrospect, it is not easy to understand how the *de novo* formation of mitochondria could have been considered seriously after the discovery of mitochondrial DNA. However, initially, the genetic system of mitochondria was often compared to that of bacteriophages, which suggested the possibility that a "master copy" of mitochondrial DNA could be transiently incorporated into nuclear DNA under "mitochondria-free" growth conditions.

Modern research on mitochondrial biogenesis was started in the early 1950s by Ephrussi (479) and Mitchell and Mitchell (480), who found that the properties of certain respiration-deficient *Saccharomyces cerevisiae-* and *Neurospora crassa* mutants were not inherited according to Mendelian laws (Table IV). Whereas earlier studies, particularly with higher plants, had suggested that hereditary determinants may reside outside the nucleus (481), the properites of these new microbial mutants suggested that "extrachromosomal genetic factors" might reside in, or even be identical with, mitochondrial organelles. This set the stage for a merger between genetics and mitochondrial physiology, but this merger was slow in coming. In our opinion, a major hurdle was the reluctance of most "mitochondriologists" to accept the fact that yeast and *Neurospora* cells possessed *bona fide* mitochondria. Interest in these mutants thus remained confined to a few specialized laboratories and research on mitochondrial formation was unnecessarily delayed.

MITOCHONDRIAL PROTEIN SYNTHESIS: It is particularly fortunate that the next development occurred with rat liver mitochondria which, at that time, were the best characterized mitochondrial species. When McLean et al. (482) reported in 1958 that isolated rat liver mitochondria could incorporate labeled amino acids into protein, their paper immediately attracted widespread attention and raised hopes that a thorough study of this process would quickly provide insight into the mechanism of mitochondrial formation. However, disappointment was not far behind. Amino acid incorporation by isolated mitochondria proved to be very slow and critically dependent on a seemingly endless list of trivial parameters. Even more frustrating was the search for labeled protein product(s): none of the matrix enzymes or cytochromes appeared to be labeled by isolated mitochondria. Instead, most of the label was found in a highly insoluble, ill-defined protein fraction which, for a while at least, was viewed as a "structural protein" for the mitochondrial inner membrane (483). In the early 1960s, many workers in the field had begun to doubt that mitochondria could synthesize any functional polypeptide. Finally, Clark-Walker and Linnane broke the impasse; they discovered in 1966 (484) that the formation of respiring mitochondria in intact yeast cells could be blocked by chloramphenicol—precisely the antibiotic that had been shown several years before to specifically inhibit protein synthesis by isolated

mitochondria but not by microsomes (485). This convinced most workers that mitochondrial protein synthesis was a biological reality and that it was necessary for the biogenesis of respiring mitochondria. Because yeast and animal cells grown in the presence of chloramphenicol proved to be deficient in cytochrome c oxidase and cytochrome b (12), it seemed likely that these mitochondrial components were made at least partly within mitochondria. Direct proof came from several technical advances. In 1969, two laboratories independently showed that the mitochondrially-made proteins could be specifically labeled *in vivo* by pulse-labeling cells in which the extramitochondrial ribosomes had been blocked with cycloheximide (486, 487). The labeling rates of these in vivo systems were one or two orders of magnitude higher than those attainable with isolated mitochondria. Another crucial step was the decision to isolate labeled products by immunoprecipitation rather than by conventional fractionation and to analyze them by the newly developed technique of SDS-polyacrylamide gel electrophoresis. By 1973 it had become clear that mitochondria synthesize three (out of the seven) subunits of cytochrome c oxidase (488), at least two (out of the approximately ten) subunits of the oligomycin-sensitive ATPase complex (489), and one (out of the six to nine) subunits of the cytochrome bc_1 complex (490, 491). The main conclusion from these studies is still valid today: mitochondria do not synthesize complete enzymes, but only some subunits of oligomeric enzymes; the remaining subunits of these enzymes are manufactured outside the mitochondria under the direction of nuclear genes. The involvement of nuclear genes in mitochondrial formation had already been implicated in 1950 by the discovery of respiration-deficient yeast mutants which, unlike the mutants mentioned above, exhibited classical Mendelian inheritance (491). In 1966, Sherman et al. (492) had analyzed one of these "Mendelian" mutants in detail and had furnished the first rigorous evidence that a mitochondrial protein (iso-1-cytochrome c) is coded by a typical nuclear gene. The identification of mitochondrially-made subunits of cytochrome c oxidase, the cytochrome bc_1 complex, and the ATPase complex now opened the way for studying the interplay between the mitochondrial and the nuclear-cytoplasmic genetic systems at the level of relatively simple and well-defined enzyme complexes.

THE MITOCHONDRIAL GENETIC SYSTEM. Where are the genes coding for the mitochondrially-translated polypeptides? Now we have to backtrack by almost a decade and recount the discovery of mitochondrial DNA. Late in 1963, Nass and Nass (493) startled the biological community with electron micrographs which indicated that the mitochondria of chick embryo cells contained threadlike structures that could be digested by DNAse, but not by RNAse. A few months later, DNA was detected and quantitated in highly purified yeast mitochondria by biochemical procedures (494). Then DNA was extracted from *Neurospora* mitochondria and shown to differ from the bulk of *Neurospora* DNA by its buoyant density in CsCl gradients (495). From the very beginning, it was expected that this "mitochondrial DNA" would turn out to be the "extrachromosomal factor" that, more than a decade before, had been reported to control mitochondrial formation in lower eukaryotes. Just as the discovery of mitochondrial protein synthesis had prompted many classical "mitochondriologists" to take a closer look at mitochondrial formation, so the discovery of mitochondrial DNA brought to the field an ever increasing number of molecular biologists. Now things started to happen fast. In 1966, van Bruggen et al. (496) and Nass (497)

reported that mitochondrial DNA from many higher eukaryotes is a circular double-stranded molecule of which the size may differ between different species. In 1965, Wintersberger and Tuppy (498) isolated RNA from yeast mitochondria and separated it into two ribosomal-type RNA species and a 4S fraction with the functional properties of tRNA. They also showed that isolated yeast mitochondria could synthesize RNA from nucleoside triphosphates by a process that was partly sensitive to actinomycin. Three years later, DNA-RNA hybridization experiments by Wintersberger and Viehhauser (499) indicated that mitochondrial RNA was homologous to, and thus probably transcribed from mitochondrial DNA. In 1967, Küntzel and Noll (500) isolated ribosomes from *Neurospora* mitochondria and demonstrated that these ribosomes were similar in many respects to bacterial ribosomes. Thus, although the evidence available in 1968 was still incomplete, it left little doubt that mitochondria contain a typical genetic system capable of replicating, transcribing, and translating genetic information.

But how could one prove that this genetic information was in any way related to mitochondrial biogenesis? The first clear indication for this was a report by Mounolou et al. (501) that the overall base composition of mitochondrial DNA was altered in some of the "extrachromosomally-inherited" respiration-deficient yeast mutants that had been discovered in the early 1950s. (Because of their small colony size on certain media, these mutants are generally referred to as "petite" mutants). Subsequent analysis of these abnormal "petite" mitochondrial DNAs suggested that they had suffered massive deletions and that the extent and the nature of these deletions differed between different petite mutants (502). In 1970, two laboratories reported that some petite mutants are totally devoid of mitochondrial DNA (503, 504). However, because all petite mutants had lost the same mitochondrial proteins and neither reverted nor complemented each other, they were unsuited for unraveling the genetic content of mitochondrial DNA. Clearly, more restricted mitochondrial mutations were needed that could be identified by their specific phenotype and mapped by recombinational analysis or other reliable genetic methods.

Such mutants were first isolated by Linnane et al. (505) and Thomas and Wilkie (506) in 1968 and from then on, yeast has remained the organism of choice for studying mitochondrial biogenesis. The newly discovered mutants differed from wild-type yeast cells in that their mitochondrial protein synthetic system had become resistant to chloramphenicol or erythromycin. Later, mutants resistant to other "antimitochondrial" antibiotics such as oligomycin, paromomycin, or antimycin were discovered (507). These mutants showed "non-Mendelian" inheritance, had specific and easily measurable phenotypes, could be readily obtained by appropriate selection and, most important, were amenable to some of the classical genetic tests. Soon procedures for studying recombination between mitochondrial mutations were worked out (508–510) and, in 1969, the field of "mitochondrial genetics" was formally inaugurated at a meeting in Canberra (511). The first circular genetic map of yeast mitochondrial genome was published in 1975 (535). However, additional mitochondrial markers were badly needed. It was thus a major breakthrough when, in 1974-1975, many mitochondrially inherited yeast mutants became available that were not simply resistant to a particular antibiotic, but had suffered specific lesions in their oxidative phosphorylation system (512, 513). These "mit⁻-mutants" initiated

a particularly fruitful and exciting period in research on mitochondrial biogenesis for they led to the discovery of several new loci on the mitochondrial genome (514, 515). Because these mutants had lost individual mitochondrial components, they made possible the identification of the structural genes for mitochondrially translated polypeptides on mitochondrial DNA (516–519), which settled the decade-old question of whether the mRNAs for these proteins are imported into the mitochondria from the nucleus (Fig. 11). In 1976, several laboratories used the new tool of restriction enzyme analysis to work out the physical map of mitochondrial DNA from several species including yeast (520). As a consequence of these parallel developments, the structure of several mitochondrial genes is now being studied by genetic (521–523), electron microscope (524), or DNA sequencing methods (525–529), with many surprising results (537). For example, mitochondrial genes in yeast are usually separated from each other by long AT-rich sequences and, in some instance, contain intervening sequences. In contrast, human mitochondrial genes appear to lack intervening sequences and are so closely packed on the genome that the terminal codon of one gene can be immedi-
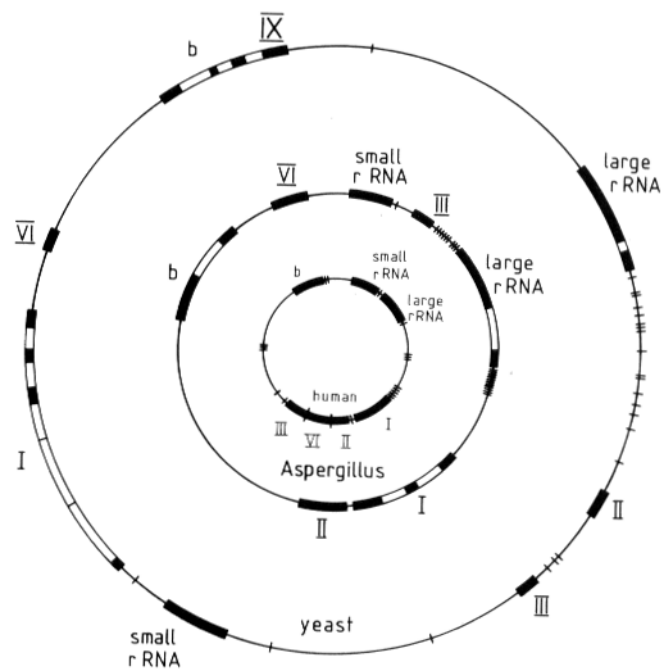

FIGURE 11 Genetic and physical maps of mitochondrial DNAs from yeast (*Saccharomyces cerevisiae*), *Aspergillus nidulans*, and humans. The different sizes of the rings are roughly proportional to the molecular weights of these mitochondrial DNAs. The blocks on the rings signify genes for proteins or ribosomal RNAs, the short thin lines pointing towards the center signify genes for transfer RNAs. Shaded areas of the blocks stand for exons, open areas for introns. The exact number of transfer RNA genes on each of the three mitochondrial DNAs is still uncertain, as in the evidence for some of the indicated introns. (*b*) Cytochrome *b*. (I, II, and III) Subunits I, II and III of cytochrome *c* oxidase. (VI, IX) Subunits VI and IX of the ATPase complex (subunit IX is often referred to as the dicyclohexylcarbodiimide-binding protein). Yeast mitochondrial DNA contains an additional gene between the subunit IX gene and the large rRNA gene; this additional gene controls the expression of a polypeptide associated with the small mitochondrial ribosomal subunit. Because it is not clear whether this gene codes for any protein (537), it has been omitted from this simplified map. Adapted from refs. 537, 540, and 541.

TABLE V

Some Unique Features of the Mitochondrial Genetic Code

Codon	"Normal" codon assignment	Codon assignment in mitochondria from		
		yeast	Neuro-spora	humans
UGA	stop	trp	trp	trp
CUN	leu	thr	leu	leu
AUA	ile	?	?	met
AGA or AGG	arg	arg	?	stop

See ref. 537 for details.

ately succeeded by the first codon of the following gene. Finally, the genetic code in mitochondria differs in several respects from that which, up to now, has been taken to be "universal" (Table V). More exciting findings are emerging almost by the month, but now our narrative deals no longer with history but the present.

All this new information has shattered the view that the mitochondrial genetic system is "bacteria-like." This view was quite popular in the past two decades because ribosomes, initiation factors, and RNA polymerase of mitochondria from most species are sensitive to inhibitors, which also are specific for the analogous components from bacteria (12). Although this fact is certainly in harmony with the hypothesis that mitochondria evolved from free-living prokaryotes (22), it must now be weighed against equally striking "eukaryotic" features of the mitochondrial genetic system such as "split" genes and mRNA-splicing events. Indeed, present information suggests that the mitochondrial genetic system should be considered as unique.

What is the future of the field? We suspect that the mitochondrial genetic system will soon have yielded its most important secrets and that research will shift to the interaction between the mitochondrial and the nucleo-cytoplasmic genetic systems. Almost certainly, new recombinant DNA technologies and our recent ability to transform eukaryotic cells with defined pieces of DNA (530) will help us to decipher the language through which the nucleus and the mitochondria "talk" to each other. On another front, evidence has accumulated that nuclearly-coded proteins are imported into mitochondria via post-translational, energy-dependent steps that frequently involve proteolytic processing of larger precursors (531–533). In order to unravel these processes, we will finally be forced to take a closer look at the mitochondrial outer membrane which, for much too long, has remained a stepchild of mitochondrial research. Possible interactions between mitochondria and the cytoskeleton are another promising hunting ground for future researchers. Clearly, the merger between mitochondrial research and cell biology has just begun.

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