BACTERIOLOGICAL REVIEWS, Mar. 1977, p. 47-99 Vol. 41, No. 1
 Printed in U.S.A.

# Bacterial Respiration

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## INTRODUCTION

Bacteria can derive the energy they need for growth from a considerable number of diverse and varied reactions, and the particular reactions utilized by a given organism can change depending upon the growth conditions employed. Operationally, however, these different reactions can be considered as examples of just two general methods for the conservation of energy. The first of these is the formation of adenosine 5'-triphosphate (ATP) by substrate level phosphorylation and two distinct classes of reaction can be distinguished:

(i) ADP + substrate ~ P $\rightleftharpoons$ ATP + substrate
(ii) ADP + $P_i$ + substrate ~ X $\rightleftharpoons$ ATP + substrate + X

where ADP is adenosine 5'-diphosphate, and $P_i$ is inorganic phosphate. Particular examples are described in detail elsewhere (279), but it is of interest to note that only a relatively small number of substrate level phosphorylation reactions have been identified, and all are catalyzed by soluble enzymes present in the cell cytoplasm. The second general method of ATP synthesis in bacteria is by oxidative or photophosphorylation. In this case, ATP synthesis is coupled to electron transport reactions which, in turn can be driven by light (in phototrophs) or by the oxidation of both organic compounds (in organoheterotrophs) and inorganic ions (in chemolithotrophs) of negative redox potential, linked to the reduction of electron acceptors of more positive redox potential. Although there are differences in detail, the overall features of electron transport-dependent ATP synthesis are very similar in bacteria, in mitochondria, and in photosynthetic systems. Thus, in all cases, the enzymes responsible for oxidative phosphorylation are membrane bound and, in addition, are asymmetrically organized in the membrane so as to catalyze vectorial chemical reactions. The mechanism of oxidative phosphorylation, that is, the way in which redox reactions are linked to the synthesis of ATP, has been, and continues to be, a lively topic for debate. To the three general models of energy coupling developed in the 1960s (the chemical theory, the chemiosmotic theory, and the conformational theory) have now been added a number of variations. It is not our intention here to review these various proposals in detail or to argue their relative merits and limita-

tions. These aspects have been covered thoroughly both by the original authors and by others in a number of comprehensive reviews (e.g., references 30, 44, 138, 139, 160, 161, 377). However, it is of importance to discuss one of these theories in some detail, since it is from the chemiosmotic theory proposed by Mitchell (270, 272) that a unifying conceptual framework has emerged to link the various energy-dependent functions in bacteria, mitochondria, chloroplasts, muscle, and nerve.

A characteristic feature of the chemiosmotic theory is the consideration given to the asymmetrical orientation of membrane-bound enzymes catalyzing vectorial reactions that bring about the translocation of molecules, ions, and chemical groups across the membrane. In addition, some of these reactions lead to the separation of electrical charges within and across the membrane, and their recombination underlies the performance of osmotic, chemical, and mechanical work.

In its very simplest form, the chemiosmotic hypothesis requires that a proton-translocating electron transport chain and a proton-translocating adenosine triphosphate (ATPase) coexist in a membrane that is essentially impermeable to most ions, including both $OH^-$ and $H^+$ ions. The end result of either electron transport or ATP hydrolysis is the generation across the membrane of gradients of both pH ($\Delta$pH) and electrical potential ($\Delta\psi$), with the soluble phase on one side of the membrane alkaline and electrically negative relative to the other. The sum of these two components, in electrical units (usually millivolts), is known as the protonmotive force and, although these components are not identical, they are all related and interconvertible as described by the expression:

$$\Delta P = \Delta\psi - Z\Delta pH$$

($\Delta P$ is the proton motive force in millivolts and is a measure of the combined electrical and chemical forces acting on the protons; $\Delta\psi$ is the electrical potential difference across the membrane; $Z = 2.3\ RT/F$ where $R$, $T$, and $F$ have their usual meanings, and $Z$ has a numerical value of 59 mV at 25°C; and $\Delta pH$ is the pH difference between the interior and the exterior). Methods are available for the measurement of transmembrane electrochemical proton gradients, and the results obtained have been reviewed recently (339). It is important to realize that, according to the chemiosmotic theory, $\Delta P$ can be a function of $\Delta pH$ exclusively, of $\Delta\psi$ exclusively, or a combination of the two, but it can only be maintained as long as the membrane forms a topologically closed vesicle. Under suitable conditions and when of the correct magnitude, $\Delta P$ drives a variety of energy-linked processes across the membrane, e.g., reversed electron transport through the respiratory chain, ATP synthesis via the reversible proton-translocating ATPase, and the accumulation of certain solutes via their respective permeases (154).

The essential features of electron transport-dependent ATP synthesis, insofar as they are relevant to the theme of this review, are summarized in Fig. 1. Two protolytic reactions, involving the oxidation of a donor ($DH_2$) and the reduction of an acceptor (A), are catalyzed by an enzyme complex, comprising an alternating sequence of a hydrogen carrier and an electron carrier, which is arranged across the membrane to form a proton-translocating oxidoreduction loop or segment. As drawn and originally described by Mitchell, both donor and acceptor interact with the enzyme complex on the same side of the membrane, and the net result of the reaction is the appearance of 2 $H^+$ on the left of the membrane and the disappearance of 2 $H^+$ from the right of the membrane. However, it must be appreciated that alternative configurations are possible such that the donor and acceptor interact with the enzyme complex on different sides of the membrane (an illustrative example is given in Fig. 5) and also that stoicheiometries other than 2 $H^+$ translocated per redox segment are to be expected for certain reactions (e.g., the oxidation of $NO_2^-$ by *Nitrobacter winogradskyi* [74]).

The nature of the donor and acceptor is not specified in the general scheme shown in Fig. 1. In practice, a variety of both physiological and nonphysiological reductants (e.g., reduced nicotinamide adenine dinucleotide [NADH], succinate, reduced *N,N,N',N'*-tetramethylphenylenediamine) and oxidants [e.g., oxygen, $Fe(CN)_6^{3-}$] can interact with the membrane-bound electron transport chain. In addition, the oxidation of a particular reductant may involve the action of more than one proton-translocating segment. This is illustrated in Fig. 2, which summarizes some current views on the functional organization of the mitochondrial respiratory chain (128, 129, 275, 307, 413). The scheme proposed in Fig. 2 must be considered speculative at this stage; however, it is in accordance with much of the experimental evidence so far available. Most workers agree that the mitochondrial respiratory chain can be operationally divided into three separate regions that are responsible for proton translocation and concomitant ATP synthesis. One of these regions requires a specific orientation of the

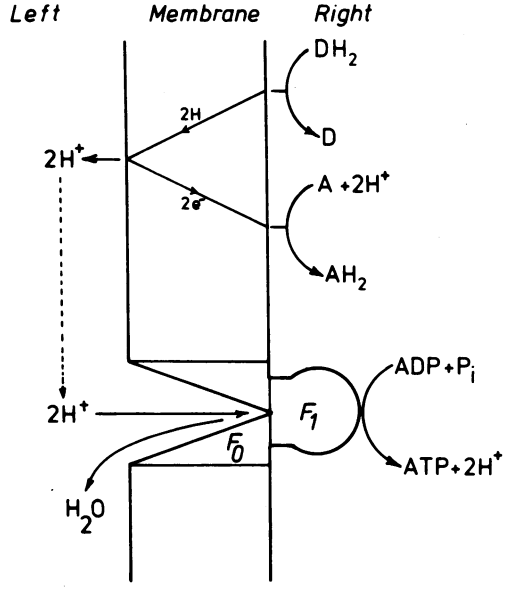


FIG. 1. *Schematic representation of a proton-translocating oxidoreduction segment of the electron transport chain and of a proton-translocating ATP-ase. (Symbols used are defined in the text.)*

NADH dehydrogenase within the membrane, with the proposal that a flavoprotein acts as the hydrogen carrier, and at least two iron sulfur centers act as the electron carriers (129, 307). Studies with yeast mitochondria have shown that the NADH dehydrogenase is modified under certain growth conditions such that it is no longer capable of proton translocation or ATP synthesis and, in some cases, is no longer sensitive to inhibition by the site-specific inhibitors piericidin A and rotenone (73, 75, 129, 140, 147, 307). The remainder of the respiratory chain is responsible for ubiquinol oxidation and is functionally organized into two equivalent potential sites of energy conservation. Recently, Mitchell (275) has proposed the protonmotive Q cycle to describe the organization of the various redox carriers in this region of the respiratory chain. A modified version of these proposals is incorporated in Fig. 2 (128). The important conceptual feature to note is that ubiquinol acts as the hydrogen carrier for two separate electron-carrying limbs, one involving two type *b* cytochromes, the other involving *c* and *a* cytochromes.

Although these suggestions must be considered speculative, and indeed difficult to verify unequivocally, they are largely commensurate with the known properties of the various redox carriers involved (see [128]). However, it should be realized that the experimental evidence for a transmembrane orientation of these carriers as proposed in Fig. 2 is fragmentary. Certainly, the membrane has a defined sidedness, with oxidants and reductants interacting on specific sides of the membrane (for a review, see [160]), and convincing evidence has been presented to suggest that the type *c* and *a* cytochromes form a complex that spans the membrane (176). However, similar evidence for the orientation of the other electron carriers, either the iron-

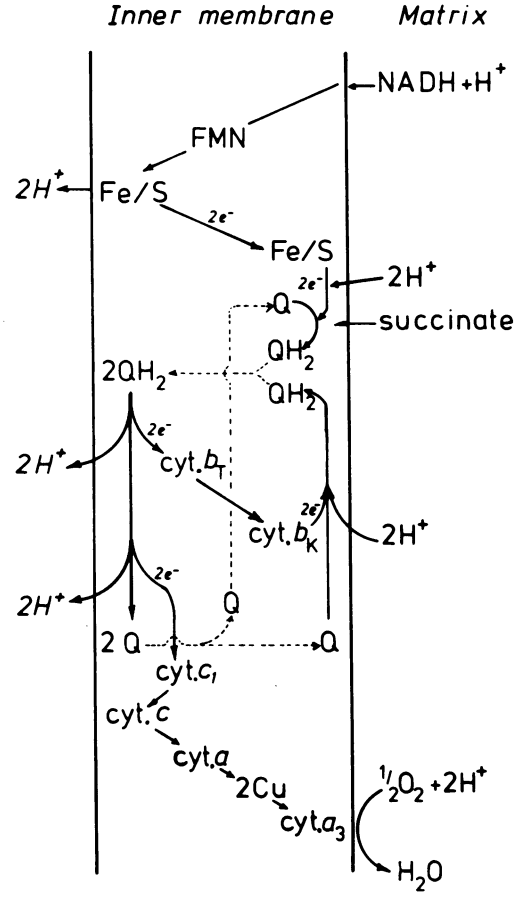


FIG. 2. *Proposed functional organization of the mitochondrial electron transport chain. Abbreviations: FMN, Flavoprotein; Fe/S, iron-sulfur protein; Q, ubiquinone; $QH_2$, ubiquinol; cyt., cytochrome; and Cu, copper-containing redox proteins. Cytochromes $b_K$ and $b_T$ represent the two type b cytochromes differentiated by their spectral and thermodynamic properties (413). The scheme is based upon those proposed previously (e.g., references 128, 129, 307) and is obviously an oversimplification since, for example, it does not take into account the known multiplicity of the iron-sulfur proteins of the electron transport chain (307).*

sulfur proteins of the NADH dehydrogenase (307) or the type *b* cytochromes (128, 413), is not so convincing and is open to alternative interpretation.

According to Mitchell's proposals, as a consequence of electron transport through the respiratory chain, a protonmotive force is generated across the membrane which, in turn, when of the correct magnitude and in the presence of ADP and $P_i$, reverses the direction of a proton-translocating ATPase so as to bring about net synthesis of ATP. It will be noticed that the oxidation of NADH by oxygen results in the net translocation of six protons and that of succinate, for example, of four protons, which can be used for the synthesis of three or two molecules of ATP, respectively. An additional proton-translocating enzyme complex is also found in the inner mitochondrial membrane, the energy-linked nicotinamide nucleotide transhydrogenase, and it has been shown that two protons are translocated per NADPH molecule oxidized by $NAD^+$ (280). Although energy conservation concomitant with transhydrogenase activity has been demonstrated in submitochondrial particles by direct measurement of ATP synthesis (401) or implied by movement of synthetic anions (376), for thermodynamic reasons, it is likely that these are only transient phenomena, not found under physiological conditions, and occur only when the [NADPH]/[$NADP^+$] and [$NAD^+$]/[NADH] ratios are exceptionally high, since the standard redox potentials ($E_0'$) of these couples are so close.

The ATPase complexes of energy-coupling membranes have been the subject of intensive investigation over the past decade and a half. At the beginning of this period, work was directed predominantly towards the mitochondrial and chloroplast systems but, more recently, has been extended to an analysis of the ATPase complex from several bacteria. The information so far available suggests that the ATPase complexes of bacteria, mitochondria, and chloroplasts share many common molecular features (7, 358). In all cases, the ATPase complex can be operationally subdivided into two components (273), $F_0$ and $F_1$, as represented schematically in Fig. 1. In this representation, the hydrophilic $F_1$ component is that part of the enzyme complex whose polypeptides are responsible for adenine nucleotide binding and which is still catalytically active as an ATPase after removal from the membrane. In contrast, the hydrophobic polypeptides that comprise the $F_0$ component are envisaged as forming a pore or well through the membrane so as to allow the passage of $H^+$ and $H_2O$ to and from $F_1$. Overall, the complete membrane-bound enzyme complex catalyzes the vectorial reaction:

$$ATP_R + H_2O_L + 2\ H^+_R \rightleftharpoons ADP_R + P_{i_R} + 2\ H^+_L$$

Although the proposed stoicheiometry is 2 $H^+$ translocated per ATP molecule synthesized (or hydrolyzed) as drawn (281), it is experimentally difficult to determine this $\rightarrow H^+/P$ ratio with precision, due to the presence of charged groups on all chemically reactive substrates and products. Indeed, ATPase complexes from various biological membranes may well have different stoicheiometries (references given in [274]). However, the model defines the general principles and, as discussed in greater detail by Mitchell (274), additional variations and sophistications of this type of model are obviously possible.

An important feature of the ATPase complex is that the enzyme is fully reversible. Thus, it not only acts as an ATP synthetase during oxidative phosphorylation but, in addition, it can catalyze the formation of a protonmotive force at the expense of ATP hydrolysis. The importance of the former reaction to aerobic systems is self-evident. In contrast, the latter reaction is probably little utilized by intact mitochondria or aerobic bacteria in vivo, although it assumes a much greater importance in bacteria such as *Escherichia coli* and *Streptococcus faecalis*, which will grow anaerobically in the absence of added electron acceptors; under such conditions, these organisms conserve energy by substrate level phosphorylation and must therefore effect membrane energization, and hence active transport or reversed electron transfer, via ATP hydrolysis (160, 161, 273, 371).

The mechanism of action of the ATPase complex, that is, how the protonmotive force can be utilized to drive ATP synthesis and conversely how the hydrolysis of ATP brings about the electrogenic movement of protons, is far from certain. Any proposed model for the functional organization of the ATPase complex must take into account additional features of the enzyme discussed in detail elsewhere (313). These features include the various exchange reactions catalyzed by the complex, the presence of bound nucleotides in the enzyme, and the fact that hydrolysis and synthesis of ATP differ both kinetically and in their response to certain inhibitors. A detailed description of the various mechanisms that have been proposed for ATP synthesis is outside the scope of this article, and the reader is referred to Harold's recent review (161) for a critical appraisal of the current hypotheses.

A considerable amount of experimental evi-

dence has now accumulated from studies with bacteria, mitochondria, and chloroplasts in support, at least in principle, of the chemiosmotic theory for oxidative phosphorylation proposed by Mitchell. This is not the place to catalogue all this evidence, but certain key observations are worth noting: (i) there is an invariant correlation between the presence of an energized membrane and the generation of a protonmotive force across that membrane; (ii) ATP synthesis can be supported by an artificially generated protonmotive force; and (iii) isolated proton pumps and ATPase complexes from different sources can be reassembled in membrane vesicles to form a complete system capable of oxidative or photophosphorylation. Indeed, perhaps the greatest single achievement of the chemiosmotic theory has been the stimulus that it has given to the development of new experimental ideas and concepts. There remain, however, certain unresolved questions concerning the ability of the chemiosmotic theory to account for, both qualitatively and quantitatively, oxidative phosphorylation in intact cells and derived organelles. Perhaps the most serious objection to the chemiosmotic interpretation of oxidative phosphorylation in mitochondria is the discrepancy between the maximal phosphorylation potential and the measured protonmotive force. Thus, a comparison of the electrochemical gradient generated across the mitochondrial inner membrane by electron transport, with the chemical potential against which ATP can be formed from ADP and $P_i$, indicates that a stoicheiometry of 2, for the number of protons translocated per ATP molecule synthesized is insufficient to account for the known phosphorylation capacity of mitochondria (304, 339). Recently, a report has appeared to suggest that the number of protons translocated per redox segment of the respiratory chain is at least 3, and the suggestion has been made that the value of 2, obtained previously, was an underestimate caused by the unrecognized masking of $H^+$ ejection by movements of endogenous phosphate (55).

As originally proposed by Mitchell, the chemiosmotic hypothesis requires that the primary event in the energization of a coupling membrane is the translocation of protons from one side of the membrane to the other and the establishment of a protonmotive force across that membrane (Fig. 1). However, as discussed in greater detail, first by Williams (415–417) and then Robertson and Boardman (331), the primary event in energization is more likely to be a charge separation reaction across, but within, the membrane phase. The appearance of $H^+$ in the left aqueous phase is envisaged as a secondary and slower process that is not a necessary requirement for ATP synthesis, since direct proton transfer from the respiratory enzymes to the ATPase can occur directly in the lipid phase. The proposals of Williams and Mitchell are both clearly in accordance with the experimental observation that, under appropriate conditions, the addition of a pulse of air-saturated buffer to an anaerobic suspension of mitochondria results in a transient acidification of the suspending medium. They differ in that, according to Williams, the presence of a transmembrane potential is not a necessary condition for ATP synthesis, and it is the energy of hydration of the "dry" proton produced in the lipid phase of the membrane that drives the synthesis of ATP by the ATPase. The two theories also have different consequences in that, according to Mitchell, electron transport-dependent ATP synthesis and solute transport are exclusively a property of topographically closed vesicles in which two aqueous phases are separated by a lipid phase of limited proton permeability. According to Williams, electron transport-dependent ATP synthesis does not require a closed vesicle system provided that, following the initial charge separation reaction catalyzed by a proton pump, the preferred route for proton movement is through the ATPase enzyme and not dissipatively by release from the membrane into the aqueous phase; naturally, it would only be possible to demonstrate energy-dependent solute accumulation in closed vesicles. These predictions from Williams' theory have some experimental basis in that electron transport-dependent ATP synthesis has been observed with enzyme complexes reassembled in an octane-water interface (e.g., reference 421) and in reputedly nonvesicular membrane fragments (e.g., reference 78).

Clearly, a great deal of controversy and speculation still exists as to the precise mechanism(s) of oxidative phosphorylation and associated energy-linked functions in biological membranes. The generalized picture that is beginning to emerge is as follows. First, the various proteins and their component polypeptides have a defined sidedness and orientation within the membrane and, indeed, some, in addition to their catalytic activity, have a structural role in maintaining membrane integrity (e.g., the $F_1$ component of the ATPase complex, as discussed later). Second, the redox proteins of the electron transport chain are functionally organized in the membrane so as to catalyze some form of charge separation reaction. Third (at this time speculatively), this charge separation reaction can be used directly to synthesize ATP by a series of reactions that occur *within* the

lipid phase of the membrane. Alternatively, the charge separation reaction can be used to establish a protonmotive force *across* the membrane that, in turn, is responsible for the energy-dependent accumulation of solutes across the coupling membrane by the various pathways elaborated by Mitchell (270) and others (154, 160, 161, 371). The concept that ATP synthesis and active transport differ, in that the former is essentially an intramembrane process reflected indirectly by the magnitude of the protonmotive force, whereas the latter is a transmembrane event directly dependent upon the protonmotive force, requires more careful consideration and evaluation. Further information on the functional organization of the various redox carriers into proton-translocating loops and on the stoicheiometry of $H^+$ translocation during respiration and ATP synthesis is obviously required and should be forthcoming in the near future. Also, the significance of protein-protein interactions and conformational changes in the various respiratory complexes and in the ATPase complex needs to be properly evaluated before the mechanism of oxidative phosphorylation can be defined at the molecular level.

## BACTERIAL ELECTRON TRANSPORT CHAINS

It is now firmly established that bacteria possess membrane-bound electron transport chains that are very similar, in general terms, to their counterparts in both mitochondria and photosynthetic systems, in that all result in net proton translocation across a membrane of limited ion permeability during oxidation-reduction reactions. The diversity of the individual membrane-bound redox components found in bacteria, as well as the great variations in the physiological reductants and oxidants utilized by bacteria, are well documented in recent reviews (35, 131, 179, 206, 211, 232, 243, 261, 379, 383, 411). However, comparatively little attention has been directed towards assessing the functional organization of these carriers into proton-translocating electron transport chains, with the notable exception of proposals made for *Micrococcus lysodeikticus* (394). We have chosen a small number of organisms to discuss in detail, partly because they have been studied more extensively than others and partly because they serve to illustrate the general features and properties of the various electron transport chains most commonly found in bacteria. In this analysis, we have chosen to interpret the experimental evidence in accordance with the proposals of Mitchell (e.g., Fig. 3). Therefore, in the various schemes that follow, the redox carriers are arranged in oxidoreduction loops or segments containing an alternate sequence of hydrogen and electron carriers. It should be remembered, however, that the experimental evidence for these proposals on the functional organization of bacterial electron transport chains is even more fragmentary than the equivalent evidence for the mitochondrial or photosynthetic systems. The schemes are presented in the hope that they will encourage future thinking and experiments to be formulated towards understanding the vectorial organization and functional activity of those membrane-bound components responsible for electron transport-dependent ATP synthesis in bacteria.

### *Escherichia coli*

The gram-negative bacterium *E. coli* is a facultative anaerobe that is able to derive en-

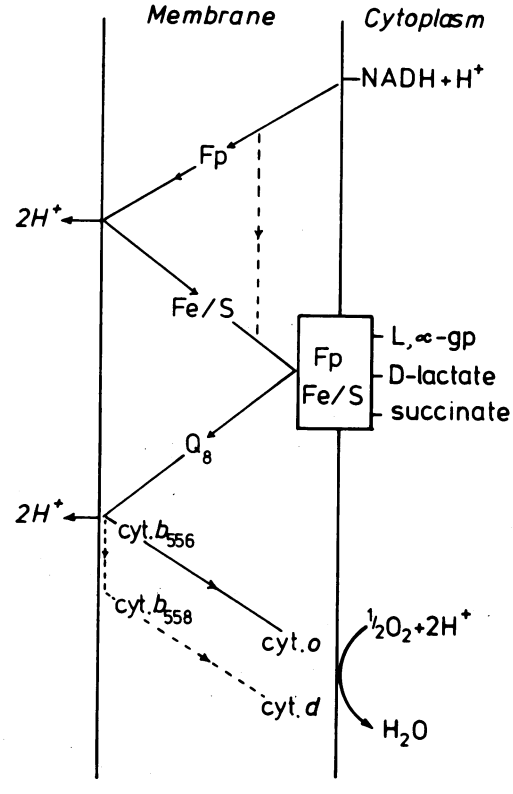


FIG. 3. *Proposed functional organization of the redox carriers responsible for aerobic electron transport in E. coli. The scheme includes the various routes for aerobic electron transport in E. coli, with the dashed lines indicating alternative pathways for reducing equivalents. Abbreviations:* L,α-gp, L-α-*glycerophosphate; otherwise, as in the legend to Fig. 2.*

ergy for growth both fermentatively, via glycolysis, and oxidatively, using either oxygen or, under anaerobic conditions, fumarate and $NO_3^-$ as terminal electron acceptors. The mechanism of electron transport and oxidative phosphorylation in *E. coli* has been the center of considerable attention due largely to the ease with which a variety of mutants can be isolated; the subject has been reviewed recently (79, 133). The mutant strains so far available for the study of electron transport in *E. coli* are summarized in Table 1. The ability to reverse the phenotypic effects resulting from some of these mutations has been of use in elucidating the functional activity of several of the redox components (Table 2).

*E. coli* can synthesize a variety of redox carriers, depending upon the growth phase, the terminal electron acceptor, the carbon source for growth, and the strain. In addition, alterations to the redox components, synthesized under otherwise defined conditions, can be achieved by altering the growth-limiting nutrient in the medium. This technique has not yet been fully exploited, but some relevant examples of its application are summarized in Table 3.

At least nine different cytochromes have been identified in *E. coli*, two type *c* cytochromes (cytochromes $c_{550}$ and $c_{548}$), five type *b* cytochromes (cytochromes $b_{556}$, $b_{558}$, $b_{562}$, $b_{556}^{NO_3^-}$ and *o*), cytochrome $a_1$, and cytochrome *d* (149, 363). Not all of these cytochromes are membrane bound and involved in oxidative phosphorylation. For example, cytochrome $c_{550}$ is present in the periplasmic space, has a midpoint potential ($E_m$, pH 7.0) between −195 and −220 mV (122), and apparently accepts reducing equivalents from a soluble form of formate dehydrogenase (340). A lively controversy ex-

TABLE 1. *Mutants of E. coli deficient in electron transport processes*

| Class of mutant | Gene designation | Comments | References |
|---|---|---|---|
| Ubiquinone deficient | *ubiA-ubiH* | | 132 |
| Menaquinone deficient | *menA,B* | | 298, 427 |
| Heme deficient | *hemA-hemG (pop)* | Isolated as auxotrophs for 5-aminolaevulinic acid or hematin (*hem*) or alternatively as porphyrin-accumulating mutants (*pop*); confusion over gene nomenclatures (see [346]) | 38, 86, 149, 257, 319, 320, 346, 347, 348, 420 |
| Iron deficient | *entA-entG* | Lesions in synthesis of enterochelin | 248, 419, 428 |
| | *fep* | Lesions in transport of ferric-enterochelin complex | 80 |
| | *fesB* | Unable to hydrolyze ferric-enterochelin complex | 238 |
| Nitrate reductase (EC 1.7.99.4) | *chlA-chlG (nar)* | Isolated by three independent methods; some mutants pleiotrophic (*chlA,B,D,E*) involving loss of several of the following activities or components: soluble and particulate formate dehydrogenase (EC 1.2.2.1), hydrogenase (EC 1.98.1.1), cytochrome $c_{550}$, cytochrome $b_{556}^{NO_3^-}$, and nitrate reductase (EC 1.7.99.4) | See 136, 342 |
| Dehydrogenase deficient | *dld* | D-Lactate dehydrogenase (EC 1.1.2.4) | 178, 372 |
| | *sdh* | Succinate dehydrogenase (EC 1.3.99.1) | 89, 247, 382 |
| | *frd* | Fumarate reductase (EC 1.3.99.-) | 247, 381 |
| | *glpA* | L-α-Glycerophosphate dehydrogenase (EC 1.1.99.-); soluble enzyme required for anaerobic growth with fumarate as electron acceptor | 219 |
| | *glpD* | L-α-Glycerophosphate dehydrogenase (EC 1.1.99.5); particulate enzyme required for growth aerobically and used preferentially during anaerobic growth with $NO_3^-$ as electron acceptor | 219, 265 |
| | *nut* | Energy-linked transhydrogenase (EC 1.6.1.1) | Cited in 133 |

TABLE 2. *Phenotypic restoration of functional activity in various mutants of E. coli*

| Genotypic lesion | Phenotypic restoration by: | References |
|---|---|---|
| *ubiB*$^-$ | Addition of Q-1 to membranes | 85, 144, 316 |
| *menA*$^-$ | Addition of MK-1 to membranes | 298 |
| *hemA*$^-$ | Addition of 5-aminolaevulinic acid to nongrowing cells, or hematin + ATP to membranes | 143, 144, 149, 332 |
| *ent*$^-$, *fep*$^-$ | Addition of citrate + $Fe^{III}$ to growth medium | 80 |
| *chlD*$^-$ | Addition of molybdate to anaerobic growth medium containing $NO_3^-$ | 135 |

TABLE 3. *Alterations to electron transport processes in E. coli induced by changes in the growth limiting nutrient*

| Growth-limiting nutrient | Phenotypic effect | References |
|---|---|---|
| Iron (aerobically) | Decreased levels of type *b* cytochrome and nonheme iron | 326 |
| Iron (anaerobically) | Decreased hydrogenase and formate-hydrogen lyase activities | 124 |
| Sulfate (aerobically) | Alteration to iron-sulphur proteins, and synthesis of alternative cytochromes | 317 |
| Molybdate (anaerobically) | Decreased nitrate reductase and formate dehydrogenase activities | 244 |
| Selenite (anaerobically) | Decreased formate dehydrogenase activity | 244 |

ists as to whether reduced cytochrome $c_{550}$, in turn, donates electrons to hydrogenase, constituting an overall formate-hydrogen-lyase activity (76), or alternatively to nitrite reductase (100); neither pathway is coupled to ATP synthesis. An NADPH-sulfite reductase (EC 1.8.1.2), containing siroheme as a prosthetic group, has been isolated and characterized from *E. coli* (286, 368–370). This enzyme also reduces nitrite, and attention has been drawn to the similarities of the *E. coli* NADPH-sulfite reductase and the ferredoxin-nitrite reductase [EC 1.7.7.1] of spinach (287). Clearly, much more work is required to understand the significance of these various soluble redox systems to the metabolic activity of the cell, and they will not be discussed further, but it is important to remember that not all of the cytochrome species found in intact cells are necessarily involved in electron transport-dependent ATP synthesis. The various membrane-bound cytochromes that have defined roles in oxidative phosphorylation are described in detail below; however, it should be noted that the intracellular location and functional significance of other cytochromes, notably cytochrome $c_{548}$ and cytochrome $b_{562}$, is not known at this time. Thus, cytochrome $c_{548}$ is present in low concentration in *E. coli* (363), and its role is uncertain; interestingly, a type *c* cytochrome has been demonstrated in particles derived from cells grown under conditions of sulfate limitation in a chemostat, but has not been characterized (317). Cytochrome $b_{562}$, which has been purified (186), sequenced (187), and extensively studied (e.g., reference 90), although its function remains obscure, is considered to be a soluble protein by some workers (122), yet a considerable amount remains membrane bound during particle isolation (25).

*E. coli* synthesizes both a benzoquinone, ubiquinone-8, and a naphthoquinone, menaquinone-8 (311). In general, a four- to fivefold higher concentration of ubiquinone is found in aerobic cells than in cells grown anaerobically, whereas the content of menaquinone, and its immediate biosynthetic precursor demethylmenaquinone, are present at higher concentration in anaerobically grown cells than in cells grown aerobically (41, 150). However, high concentrations of menaquinone are often, but not always (317), found in aerobically grown cells when the activity of the aerobic respiratory chain(s) is impaired, e.g., in ubiquinone-deficient mutants (85), in heme-deficient mutants (149), and in wild-type cells grown aerobically in the presence of low concentrations of KCN (25). The control mechanisms regulating the synthesis of the two quinone species are not known, but it is possible that each can substitute for the other, at least in part, to support functional electron transport activity (85, 298).

Of the other types of redox carriers typically associated with respiratory chains, there is no convincing evidence for the involvement of copper proteins in electron transport. There is a considerble amount of nonheme iron in electron transport particles, although, as in other systems, presumably only a small fraction is associated with functional iron-sulfur proteins. The presence of several iron-sulfur proteins has been suggested, based upon (i) their differential reactivity with *o*-phenanthroline (46, 218) and the more lipophilic iron chelator bathophenanthroline (88) and (ii) the detection of various $g = 1.94$ signals in electron paramagnetic resonance spectra of membranes and derived preparations at 77°K (153, 169, 303) and at 12°K (317). Molybdenum-containing enzymes play a key role in anaerobic electron transport, as discussed later.

Membrane particles from aerobically grown *E. coli* demonstrate both an energy-dependent and an energy-independent transhydrogenase activity. Energy for the reversal of electron flow from NADH to $NADP^+$ can be derived either by ATP hydrolysis via the proton translocating ATPase (as in mitochondria) or by the oxidation of NADH, succinate, D-lactate, and ascorbate (in the presence of phenazine methosulfate) by the electron transport chain (53, 181, 389) and is a property of vesicles with an inside-out orientation with respect to the original cell (181). The energy-linked transhydrogenase activity has proved a popular assay system for investigating the restoration of functional proton-translocating ATPase activity in various mutants, deficient in such activity, as discussed later. The activity of the energy-dependent transhydrogenase in particles derived from cells grown aerobically on different carbon sources varied markedly (47), though the activity in particles from anaerobically grown cells has not been reported. The enzyme was not subject to catabolite repression, but its synthesis was repressed by various mixtures of amino acids in the growth medium, and it has been suggested that the transhydrogenase has a role in generating NADPH for biosynthesis (47). Although transhydrogenase activity in particles is clearly established, it is of interest that proton translocation associated with NADPH oxidation by whole cells of *E. coli* has not yet been reported.

Evidence has accumulated over the last few years to suggest that the various membrane-bound redox carriers of *E. coli* are functionally organized into several proton-translocating electron transport chains, which serve for respiration-dependent ATP synthesis and for the accumulation of various solutes. We shall discuss the aerobic and anaerobic electron transport systems separately and propose several schemes to describe the functional organization of the various components in the cytoplasmic membrane. It should be remembered, however, that although these schemes are in accordance with the experimental evidence so far available, they are only working models and are presented in the hope that they will serve for the development of more critical experimental tests.

**Aerobic electron transport.** When grown under conditions of vigorous aeration in the presence of a nonfermentable carbon source like glycerol or succinate, the membrane-bound redox carriers include ubiquinone-8 and cytochromes $b_{556}$, $b_{562}$, and *o*. Cytochrome *o* is a type *b* cytochrome that serves as the terminal oxidase and is kinetically competent to support the observed rates of respiration exhibited by cells grown under these conditions (145). It can be identified spectrally as a type *b* cytochrome, absorbing maximally at 556 nm in reduced-minus-oxidized difference spectra (317), with the ability to bind carbon monoxide (65) and, in addition, it exhibits a high affinity for cyanide (321). Further evidence for the presence of an electron transport chain involving only type *b* cytochromes is shown by the ability to restore functional electron transport activity in particles from a *hem*$A^-$-deficient mutant by incubation with hematin and ATP (143, 144, 149, 332).

Assuming that 2 $H^+$ are required per ATP molecule synthesized by the ATPase (410), measurements of the stoicheiometry of respiration-driven proton translocation in intact cells suggest that the electron transport chain is organized into two equivalent energy conservation segments in cells grown under these conditions (57, 197, 241). Furthermore an $\rightarrow H^+/O$ ratio of 4 for L-malate oxidation, and an $\rightarrow H^+/O$ ratio of 2 for the oxidation of succinate, D-lactate, and DL-$\alpha$-glycerophosphate suggests that one of these segments is associated specifically with the NADH dehydrogenase [EC 1.6.99.3] region of the respiratory chain, and the other is located in the region between the junction of the other flavin-linked dehydrogenase with the respiratory chain and the terminal oxidase, cytochrome *o* (241).

Alternative methods exist for assessing the number of energy conservation sites in *E. coli*. Thus, true molar growth yields with respect to oxygen consumption ($Y_{O_2}^{max}$) of $\leq 60$ g of cells per mole of oxygen consumed have recently been determined for glycerol- and glucose-limited continuous cultures of *E. coli* (110, 166) by measuring in situ respiratory activity ($Q_{O_2}$) as a function of dilution rate (= specific growth rate; for a review of this approach, see reference 384). Similar, or slightly lower, values have been determined for *Bacillus megaterium* D440 (101) and *Klebsiella* (*Aerobacter*) *aerogenes* (173, 385), both of which also exhibit only two proton-translocating respiratory segments. During anaerobic growth under glucose-limited conditions, the latter organism exhibited a true molar growth yield with respect to ATP utilization of 14.0 g of cells per mol of ATP consumed (385). Since $Y_{O_2}^{max}$ is equal to the product of $Y_{ATP}^{max}$ and N (the overall efficiency of aerobic energy conservation), then, assuming $Y_{ATP}^{max}$ to be the same under anaerobic and aerobic conditions, N is equivalent to $\leq 4.3$ mol of ATP per mol of oxygen consumed, i.e., the respiratory

systems of *E. coli* and related organisms contain two energy-conserving segments. Furthermore, since each of these segments translocates two protons per pair of reducing equivalents transferred, these results indirectly confirm that ATP synthesis via the reversible, proton-translocating ATPase occurs with an $\rightarrow H^+$/ATP ratio of approximately two. Other investigations employing direct measurements of phosphorylation coupled to NAD(P)H oxidation in whole cells have suggested the presence of three energy-coupling sites in *E. coli* (164), but this approach has been severely criticized (400).

Further evidence for two potential energy conservation sites in the electron transport chain comes from an analysis of energy-linked functions of particles derived from cells grown aerobically on nonfermentable substrates. Thus, in ammonia-treated particles, in which the NADH dehydrogenase activity is inhibited, the oxidation of succinate, D-lactate, and ascorbate (with phenazine methosulfate) can be used to drive the energy-dependent transhydrogenation of $NADP^+$ (53). In addition, an ATP-dependent, uncoupler-sensitive reduction of $NAD^+$ by succinate, D-lactate, and L,$\alpha$-glycerophosphate, has been demonstrated in particles from cells grown under aerobic conditions (316, 388). This energy-dependent reversal of electron flow through the NADH dehydrogenase region of the electron transport chain requires ubiquinone, but not cytochromes (316), a conclusion that is supported by the ability to demonstrate exogenous ubiquinone-dependent proton translocation linked to the oxidation of endogenous substrates in cells of a heme-deficient mutant (146).

A scheme summarizing these various experimental observations and suggesting the functional organization of the membrane-bound redox carriers is given in Fig. 3. At this time, there is no experimental evidence for the relative sidedness of the type *b* cytochromes in the membrane, and the possibility remains that cytochrome $b_{562}$ has a functional role in this electron transport chain (see above).

It is now well established that, under certain growth conditions, *E. coli* synthesizes alternative redox carriers that function during aerobic electron transport. It appears that two, probably separate, modifications can occur to the electron transport chain we have discussed: (i) the NADH dehydrogenase becomes altered such that it is no longer proton-translocating; and (ii) the cytochrome $b_{556} \rightarrow$ cytochrome *o* electron-carrying limb associated with ubiquinol oxidation is replaced by a second electron-carrying limb containing cytochrome $b_{558}$ with an additional kinetically competent (145) oxidase, cytochrome *d* (65), which differs from cytochrome *o* both spectrally and in having a higher $K_i$ for cyanide (321, 322). These additional pathways for electron transport to oxygen are represented schematically by the dashed lines in Fig. 3.

Evidence for the adaptive loss of proton translocation associated with the NADH dehydrogenase region of the electron transport chain has come from measurements of the stoicheiometry of respiration-driven proton translocation in sulfate-limited cells of *E. coli* grown in continuous culture (317), where it was shown that the oxidation of all substrates, including L-malate, was associated with an $\rightarrow H^+/O$ ratio of about 2. Further evidence supporting this conclusion came from the inability to demonstrate energy-dependent reversal of electron transport through the NADH dehydrogenase in particles prepared from such cells (317). These experiments were performed with a low-sulfate-requiring strain of *E. coli* K-12 isolated after prolonged growth of a prototroph under sulfate-limited conditions. More recently, the experiments have been repeated with *E. coli* W grown under sulfate-limited conditions, where it was shown that the oxidation of endogenous substrates and added L-malate was associated with $\rightarrow H^+/O$ ratios approaching 4 (110). The reason for this discrepancy is not known, and although it may well be accounted for on the basis of a strain difference, further work is obviously required. Additional evidence for a non-proton-translocating NADH dehydrogenase has come from measurement of oxygen-dependent proton translocation in anaerobically grown cells where $\rightarrow H^+/O$ ratios of about 2 were obtained for the oxidation of endogenous substrates (57) and added L-malate and D-lactate (J. A. Downie, Ph.D thesis, University of Dundee, Dundee, Scotland, U.K., 1974). Direct evidence for the presence of two separate enzymes catalyzing NADH oxidation in *E. coli* is at present lacking. Although two NADH dehydrogenase activities, differing in their ability to reduce various dye acceptors, have been partially purified from *E. coli* (50, 169), it is not clear whether these are two distinct enzymes or merely two preparations of the same enzyme with different molecular weights. It is possible that the proton-translocating NADH dehydrogenase is converted into a non-proton-translocating form by the loss or modification of one or a few polypeptides that would be difficult to detect experimentally.

The second alteration to the aerobic electron transport chain apparently involves the synthe-

sis of an alternative terminal electron-carrying limb involving cytochromes $b_{558}$ and $d$. Coordinate synthesis of cytochrome $b_{558}$ and $d$ in wild-type cells has been observed under the following growth conditions: (i) during the late-exponential or stationary phase of aerobic batch cultures growing on nonfermentable carbon sources (322, 363); (ii) during aerobic growth in the presence of glucose (149); (iii) during anaerobic growth on either fermentable substrates or on glycerol with fumarate (145, 149); however, in cells grown anaerobically with $NO_3^-$ as terminal electron acceptor, the cytochrome $d$ has altered spectral and kinetic properties (145), possibly the result of $NO_2^-$ binding (B. A. Haddock, unpublished observations); (iv) during growth on a nonfermentable substrate, like succinate, in the presence of low concentrations of cyanide sufficient to inhibit cytochrome $o$, but not cytochrome $d$, oxidase activity (25); and (v) in some strains during sulfate-limited growth conditions in continuous culture (317, but see also reference 110). In addition, during aerobic growth of the respiratory-deficient mutants lacking ubiquinone (85) and functional cytochromes (149) on glucose, high concentrations of (apo)cytochromes $b_{558}$ and $d$ were observed. All these growth conditions also resulted in increased synthesis of cytochrome $a_1$. The role of cytochrome $a_1$ in *E. coli* is a mystery: in certain bacteria, e.g., *Acetobacter* species, cytochrome $a_1$ serves as a terminal oxidase (65, 260), but cytochrome $a_1$ is not kinetically competent to support observed respiration rates in *E. coli* (145). It is also of interest that, under all these growth conditions, except that of sulfate-limited growth in continuous culture (317), the cells contained increased levels of menaquinone. As discussed later, menaquinone has been implicated as an obligatory carrier in fumarate-dependent anaerobic electron transport and its function in, and metabolic significance to, aerobically grown cells has not been determined. Although coordinate synthesis of cytochromes $b_{558}$ and $d$ is observed under a variety of apparently unrelated growth conditions, the obligatory reduction of cytochrome $d$ by cytochrome $b_{558}$ has yet to be established; indeed, there is no evidence for the orientation of the two carriers in the membrane, as proposed in Fig. 3. The experimental evidence so far available suggests that the stoicheiometry of respiration-driven proton translocation associated with ubiquinol oxidation using either cytochrome $b_{556}$ with cytochrome $o$ or cytochrome $b_{558}$ with cytochrome $d$ as the electron-carrying limb is similar and equal to 2 (57, 317).

In simple terms, it can be imagined that *E. coli* synthesizes two oxygen-dependent electron transport chains. The first, shown by the solid lines in Fig. 3, is designed for maximum yield of 2 mol of ATP synthesized per mole of NADH oxidized under conditions of high aeration; the second, shown by the dotted lines in Fig. 3, is designed primarily for the reoxidation of reduced coenzymes, allows for the synthesis of 1 mol of ATP per molecule of NADH oxidized and can operate at lower oxygen tensions. However, at this time, there is no evidence to suggest that the synthesis of a non-proton-translocating NADH dehydrogenase and the additional electron-carrying limb, containing cytochromes $b_{558}$ and $d$, are coordinately controlled and necessarily function together. Clearly, the possession of these various pathways for oxygen-dependent electron transport must endow the bacterium with selective growth advantages under certain conditions, but the nature of these advantages is obscure. It is tempting to speculate that the possession of a proton-translocating NADH dehydrogenase is normally necessary for growth on oxidizable substrates, where all ATP synthesis is generated from electron transport, but that during fermentative growth, when ATP synthesis occurs by glycolysis, a non-proton-translocating, and therefore, non-energy-conserving NADH dehydrogenase would be better suited for coenzyme oxidation. The possibility also exists that, during fermentation, NADH oxidation is linked preferentially to an additional proton-translocating reaction, involving fumarate reduction, by an electron transport pathway that is discussed in the next section. The presence of two terminal electron-carrying limbs with different terminal oxidases that possess different affinities for oxygen, as suggested by their different sensitivity to cyanide, would be useful for adaptive growth at different oxygen tensions.

Little work has been performed to determine the factors regulating either the synthesis or the functional activity of the various redox components that can coexist in the cytoplasmic membrane. Except for the relatively crude data given above there has been no systematic investigation as to the type and amount of quinone synthesized by a particular bacterial strain under a variety of different growth conditions. A variety of mutants blocked in their ability to synthesize both ubiquinone and menaquinone are known (Table 1), though no evidence for control genes regulating the amount of quinone produced has been found. Such information is obviously required before the regulation of quinone synthesis can be discussed in

detail. A regulator gene controlling porphyrin biosynthesis has been identified (Table 1), and cytochrome gene dosage effects in F-*lac* and F-*gal* heterogenotes of *E. coli* suggest that a number of cytochrome genes are clustered in a 2-min-long region of the chromosome between the *purE* and *supE* genes (365). Whether these genes control the amount of heme produced or the amount of apocytochrome synthesized has not been determined; certainly, the apoproteins for cytochromes $b_{556}$, $b_{558}$, *o*, and *d* are synthesized and incorporated into the cytoplasmic membrane in the absence of heme synthesis (149). The synthesis of cytochrome $b_{556}$, and probably *o*, appears to be constitutive, though the presence of cytochrome *o* in all cell types must be confirmed by kinetic analysis rather than by carbon monoxide studies alone, and cytochromes $b_{558}$ and *d* show coordinate synthesis under a variety of at least superficially unrelated growth conditions listed above. Recently, a report has appeared on the stimulation of cytochrome synthesis in a *cya*$^-$ mutant of *E. coli*, grown on glucose, by the addition of cyclic adenosine 3′,5′-monophosphate (AMP) to the growth medium (58). Specific type *b* cytochromes were not identified, and levels of cytochrome *d* were not recorded, but clearly a role for cyclic AMP has been observed and more intensive work with both *cya*$^-$ and *crp*$^-$ mutants should be performed.

An additional control, that of the timing of synthesis during the cell cycle, has again only been briefly studied. Using synchronous cultures, it has been concluded that, for certain membrane proteins, succinate dehydrogenase and cytochrome $b_1$, either the conversion of inactive to active forms, or the coordinate expression of the genes responsible for their synthesis, is controlled by a protein that is only synthesized or activated in turn at a specific phase of the cell cycle (305, 306). Clearly, further work is required to identify the time in the cell cycle at which other well-characterized membrane proteins appear and to differentiate between the timings of apoprotein synthesis and the appearance of holoenzyme with functional activity.

The factors regulating the relative activity of the various routes of electron transport, when alternative pathways coexist in the membrane, are again poorly defined. It appears that ubiquinone is an obligatory redox carrier for oxygen-dependent electron transport, since mutants deficient in their ability to synthesize ubiquinone show negligible oxidase activities (85, 299). Pudek and Bragg have suggested, on the basis of cyanide inhibition studies, that, when both cytochromes *o* and *d* are present, they function simultaneously as oxidases (322), and that the relative flow of reducing equivalents through the two electron-carrying limbs is a function of their pool size in the membrane.

The presence of alternative routes for electron transport in *E. coli* that involve different redox carriers whose relative concentration and functional activity vary with changes in the growth conditions makes the interpretation of some previous work in the literature, where the growth conditions were not rigorously defined or controlled, difficult to interpret. For example, the functional relationship of crystalline cytochrome $b_1$ (95) and of the three thermodynamically characterized type *b* cytochromes, with midpoint potentials at pH 7.1 of about −50, +110, and +220 mV (170), to the proposed redox carriers in Fig. 3 is not known. In addition, the functional significance of various preparations derived from solubilized membranes (e.g., references 29, 49, 50, 142, 168, 169) is difficult to interpret. Indeed, of all the redox proteins associated with these two aerobic respiratory chains, the only one to have been extensively purified and characterized to date, is the D-lactate dehydrogenase (125, 228, 366). These considerations also apply to an evaluation of the scheme proposed earlier for the aerobic electron transport chain of *E. coli* (85). These conclusions were based on experiments performed with a ubiquinone-deficient mutant, grown with glucose as carbon source, whose membranes contain all the cytochromes shown in Fig. 3, and it was proposed that ubiquinone had a double location as a redox carrier, both before and after cytochrome *b*. These results can equally well be interpreted by the schemes proposed in Fig. 3 and by assuming that type *b* cytochromes exist on either side of a quinone pool that contains both ubiquinone and menaquinone (see Fig. 4) in particles from these cells.

The need to control and define the growth conditions of the cell cannot be overemphasized. Glucose is commonly used as the preferred carbon source for growth, since high yields of cell mass are obtained, but it is particularly difficult to characterize and differentiate between the various electron transport chains that are synthesized and operate under these conditions. During aerobic growth in the presence of glucose, cells contain concentrations of cytochromes $b_{558}$ and *d* that are intermediate between those found in anaerobically grown cells and those found in cells grown aerobically on a nonfermentable carbon source. Such cells exhibit a decreased efficiency of oxidative phosphorylation, which can be increased by the ad-

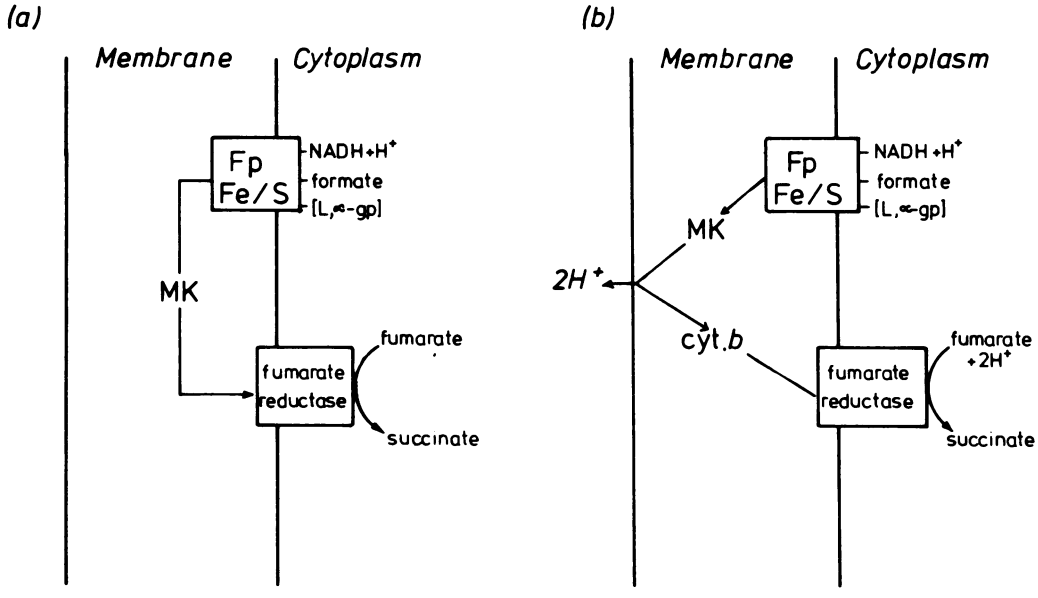


FIG. 4. *Proposed functional organization of the redox carriers responsible for anaerobic electron transport with fumarate as terminal acceptor in E. coli. Scheme (a) allows for the oxidation of reduced coenzymes without concomitant ATP synthesis and occurs in the heme-deficient mutant. In scheme (b) the additional redox components convert this scalar sequence into a vectorial reaction catalyzing net $H^+$ translocation linked to oxidation-reduction. Abbreviations: MK, Menaquinone; otherwise, as in the legend to Fig. 2.*

dition of cyclic AMP to the growth medium (165). In addition, energy-dependent reversal of electron flow through the NADH dehydrogenase region of the respiratory chain could not be demonstrated in particles from glucose-grown cells (316). This suggests that the synthesis of a functional proton-translocating NADH dehydrogenase is sensitive to catabolite repression. However, other growth conditions (e.g., sulfate-limited continuous culture of strain K-12 with glycerol as carbon source) are also known to result in a similar phenotype and, clearly, further work is required to differentiate between those changes that are regulated by catabolite repression and those that are regulated by other mechanisms.

A second poorly defined growth condition is that in which the cells are allowed to become oxygen limited. Although under anaerobic growth conditions the cells produce ATP either by fermentation or by electron transport linked to $NO_3^-$ or fumarate reduction, they apparently synthesize all the cytochromes shown in Fig. 3 and, when supplied with oxygen, demonstrate oxygen-dependent proton translocation and associated ATP synthesis, with an efficiency that suggests the operation of one potential site of energy conservation. Anaerobically grown cells, when incubated under nongrowing conditions in the presence of oxygen, develop oxidase activities and demonstrate efficiencies of oxidative phosphorylation that are similar to aerobically grown cells in a process that requires de novo protein synthesis (70). As described in the next section, fumarate, produced from the catabolism of glucose, can serve as a terminal acceptor for electron transport-dependent ATP synthesis under anaerobic conditions. Cells grown aerobically but with limited oxygen availability synthesize all the components shown in Fig. 3 and have properties which suggest that the NADH dehydrogenase is non-proton translocating, with oxygen as the terminal electron acceptor, but it remains to be established whether these cells have an oxygen-independent pathway for the reoxidation of NADH linked to the reduction of endogenously synthesized fumarate with concomitant ATP synthesis.

**Anaerobic electron transport. (i) To fumarate.** The presence of fumarate as an electron acceptor enables *E. coli* to grow anaerobically on glycerol and L-α-glycerophosphate as the source of carbon. Under these conditions, an anaerobic L-α-glycerophosphate dehydrogenase, distinct from the enzyme already dis-

cussed, and fumarate reductase, distinct from succinate dehydrogenase, are induced (Table 1; 177, 220). So far, the following limited information is available about this electron transport chain: (i) the oxidation of L-$\alpha$-glycerophosphate and formate with fumarate reduction can be used for the accumulation of sugars and amino acids in cells (338, 375) and right-side-out vesicles (42, 266); (ii) NADH-, L-$\alpha$-glycerophosphate-, and formate-induced fumarate-dependent atebrin quenching has been observed in inside-out vesicles (148, 374); (iii) fumarate-dependent proton translocation with an $\rightarrow H^+/2e$ ratio of about 1 has been reported for cells oxidizing endogenous substrates (57); and (iv) an enzyme complex that catalyzes L-$\alpha$-glycerophosphate-dependent fumarate reduction (263) with concomitant ATP synthesis (264, 266) has been isolated from the cytoplasmic membrane, but not extensively characterized. The use of mutants has established that menaquinone is required for fumarate reduction (298) and has shown that this electron transport chain plays a key role in uracil production in cells grown anaerobically on fermentable substrates by allowing the anaerobic oxidation of dihydroorotate to orotate. Although a heme-deficient mutant could grow anaerobically on glycerol with fumarate (374), suggesting that cytochromes were not required for electron transport, further work showed that fumarate-dependent anaerobic active transport could only be demonstrated in cells grown in the presence of 5-aminolaevulinic acid and hence functional cytochromes (375).

The information so far available is summarized in Fig. 4, which shows two schemes to account for these experimental observations. The schemes differ in the proposal that an unidentified cytochrome, possibly with other redox components, converts a membrane-bound electron transport chain catalyzing a scalar sequence of oxidation-reduction reactions (Fig. 4a) into a vectorial reaction sequence (Fig. 4b) in which 2 $H^+$ are translocated across the cytoplasmic membrane for each fumarate molecule reduced. Thus, both pathways are capable of catalyzing the oxidation of NADH and formate, but only the $H^+$ translocating route establishes a protonmotive force that can be used subsequently for ATP synthesis and other secondary energy-requiring processes. In cytochrome-deficient cells, fumarate allows glycolysis to continue by acting as an electron acceptor for the reoxidation of NADH, and only ATP, generated by glycolysis and hydrolyzed by the ATPase, can be used for transport. In cytochrome-sufficient cells, the energy for transport can come from either anaerobic electron transport with fumarate as acceptor or from ATP hydrolysis. Clearly, further work is required to validate these proposals. It is also well established that cells grown anaerobically on glycerol with fumarate demonstrate oxygen-dependent proton translocation and associated energy-linked functions (42, 57, 148, 338, 374, 375). The point at which electron flow from this anaerobic electron transport chain interacts with the aerobic redox carriers and the factor(s) that regulate the relative flow of reducing equivalents down the two pathways, when both oxygen and fumarate are present, have yet to be determined.

**(ii) To nitrate.** As will be seen, this is the best characterized bacterial proton-translocating electron transport chain so far studied, and the various methods used to determine the functional organization of the membrane-bound redox carriers serve as a model for further investigations. When incubated under anaerobic conditions in the presence of $NO_3^-$ and molybdenum, *E. coli* synthesizes a membrane-bound electron transport chain that allows growth on a variety of nonfermentable substrates, like D-lactate, as sole carbon source (e.g., 367). These growth conditions result in the specific induction of two membrane-bound redox carriers cytochrome $b_{556}^{NO_3^-}$ and $NO_3^-$ reductase, whose synthesis is repressed by the presence of oxygen. Interestingly, and unlike *Paracoccus denitrificans*, growth in the presence of high concentrations of $KNO_3$ (0.1 M) does not result in the coordinate induction of nitrite reductase, which is only synthesized at lower concentrations of $KNO_3$ (<10 mM) (77, 418).

If potassium selenite is also included in the growth medium, high levels of a membrane-bound formate dehydrogenase activity are also induced (Table 3). Formate dehydrogenase was isolated from *E. coli* grown under these conditions (107). The purified enzyme had a molecular weight of about 600,000 and contained heme, molybdenum, selenium, nonheme iron, and acid-labile sulfide. The enzyme contained three polypeptides, $\alpha$, $\beta$, and $\gamma$, in approximately a 2:2:1 molar ratio, of molecular weight 110,000, 32,000, and 20,000, respectively; only the $\alpha$-polypeptide contained significant amounts of selenium, but the distribution of the other redox centers in the polypeptides was not reported. It has been argued that formate dehydrogenase is the normal physiological donor of reducing equivalents to $NO_3^-$ reductase (341) and, indeed, formate dehydrogenase and $NO_3^-$ reductase can readily be separated from each other (407); on subsequent recombination, in

the presence of endogenous quinone, an in vitro reconstruction of formate-dependent $NO_3^-$ reduction can be achieved (106). However, other physiological reductants, e.g., NADH, L-$\alpha$-glycerophosphate, and D-lactate, also interact with this membrane-bound electron transport chain via their respective dehydrogenases, and $NO_3^-$-dependent proton translocation in cells (57, 130) and particles (148), as well as $NO_3^-$-dependent solute uptake (42, 338) linked to the oxidation of these compounds, has been demonstrated. Although $NO_3^-$ reductase synthesis is repressed by oxygen, $O_2$-dependent cytochrome oxidation (145), proton translocation (130, 148) and transport (42) can still be demonstrated in cells grown anaerobically in the presence of $NO_3^-$, indicating that aerobic electron transport is still functional in these cells. When oxygen and $NO_3^-$ are added simultaneously to an anaerobic suspension of cells grown under these conditions, oxygen is reduced first, followed by $NO_3^-$ (145).

The nature of the quinone component involved in anaerobic $NO_3^-$ reduction is not known. Under these growth conditions, both ubiquinone and menaquinone are synthesized and, although ubiquinone analogues are more efficient than menaquinone analogues in the in vitro reconstruction experiments described above (106), ubiquinone-deficient mutants grow just as well as parental strains on glucose anaerobically in the presence of $NO_3^-$ (85). It seems likely, therefore, that either ubiquinone or menaquinone functions under these conditions.

The type *b* cytochrome involved in anaerobic electron transport to $NO_3^-$ is specifically induced by $NO_3^-$ (341) and is genetically (341, 342) and kinetically (145) distinguishable from other type *b* cytochromes synthesized by *E. coli*. In addition, work with heme-deficient mutants has demonstrated that, although $NO_3^-$ reductase is synthesized, and in part incorporated into the cytoplasmic membrane during anaerobic growth in the presence of glucose with nitrate, apocytochrome $b_{556}^{NO_3^-}$ is not stable in the absence of heme synthesis (72, 130, 217, 252). Evidence has already been presented to show that the apocytochromes of the aerobic electron transport chain are synthesized and incorporated into the cytoplasmic membrane in the absence of heme synthesis (143, 149, 217, 332) and the results, therefore, indicate that, of all the membrane-bound cytochromes synthesized by *E. coli*, heme synthesis in some way regulates the synthesis (or stability) of apocytochrome $b_{556}^{NO_3^-}$ specifically.

The solubilization and purification of nitrate reductase from the cytoplasmic membrane of *E. coli*, as assayed by the anaerobic, $NO_3^-$-dependent reoxidation of reduced viologen dyes, has been reported by several workers. A summary of the various procedures used and the characteristics of the preparations obtained are given in Table 4. Nitrate reductase is a molybdenum-containing iron sulfur protein composed of two nonidentical subunits designated $\alpha$ and $\beta$. In some preparations, two $\beta$ subunits are seen; however, one of these is apparently a proteolytic degradation product of the other (255; R. A. Clegg, personal communication). The native enzyme can exist as either a monomer ($1\alpha$:$1\beta$) or a tetramer ($4\alpha$:$4\beta$). Active nitrate reductase can also be prepared in association with a third subunit, $\gamma$, which is heme containing and presumably equivalent to cytochrome $b_{556}^{NO_3^-}$ (72, 106, 107, 250); the three subunits appear to be present in a 1:1:1 molar ratio. The precise content and subunit localization of the molybdenum and iron-sulfur center(s) in nitrate reductase have not yet been determined; however, two groups have reported electron paramagnetic resonance spectra of purified enzyme preparations. DerVartarian and Forget (97) obtained complex spectra with one preparation (120) which were interpreted as showing signals due to (i) $Mo^V$-$MO^{III}$ interconversions and (ii) nitrogen hyperfine structure from an NO complex, perhaps with iron, in the enzyme. Neither of these features was observed in subsequent work with a less-degraded enzyme preparation (72), in which two signals due to $Mo^V$ were seen, both signals reducible by sodium dithionite and oxidizable by nitrate; significantly, one of the signals showed interaction of $Mo^V$ with a proton exchangeable with the solvent (56).

$NO_3^-$-dependent proton translocation associated with the oxidation of various substrates added to starved cells has been reported, and the results obtained indicate $\rightarrow H^+/2e^-$ ratios of 4 for L-malate oxidation and 2 for the oxidation of glycerol, succinate, and D-lactate (130). Measurements of the $\rightarrow H^+/2e^-$ ratio with formate as the reductant were complicated by pH changes associated with formate uptake and $CO_2$ production, but it was possible to conclude that the site of formate oxidation is on the inner aspect of the cytoplasmic membrane and the $\rightarrow H^+/NO_3^-$ ratio was greater than 2 (130). Oxygen-dependent proton translocation was also observed in cells grown anaerobically with $NO_3^-$ and $\rightarrow H^+/O$ ratios of 4, for the oxidation of L-malate and formate, and 2, for the oxidation of other substrates were obtained (130).

These various experimental results are sum-

TABLE 4. *Summary of data on the purification and properties of nitrate reductase (EC 1.7.99.4) from E. coli*

| Characteristic | Enzyme prepared by: | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Alkali-acetone precipitation of membranes, solubilization with sodium deoxycholate (Forget [120]) | Alkaline-heat treatment (MacGregor et al. [255]; See also [390]) | Precipitation from Triton X-100 extracts of cells by antibody to $NO_3^-$ reductase (MacGregor [250]) | Solubilization with deoxycholate (Enoch and Lester [106, 107])[a] | | | Triton X-100 solubilization (Clegg [72])[b] | | |
| | | | | I | II | III | I | II | III |
| Native enzyme | | | | | | | | | |
| Mol wt | | | | | | | | | |
| Gel filtration | 300 K | 720 K | | | I | | 740 K | | 230 K |
| Electrophoresis | 320 K | | | | | | | | |
| Ultracentrifuge | | 773.6 K | | 498 K | | | 880 K | c500K | 220 K |
| Isoelectric point | pH 4.2 | | | | More acidic than I | | | | |
| Metal content (atoms/mol) | Mo (1.5); Fe (20); S (20) | Mo (3.2); Fe | | | | | | | |
| Heme content (nmol/mg protein) | | | | 6.7 | 3.2 | | | 15 | |
| Subunits mol wt | | | | | | | | | |
| $\alpha$ | | 142 K | 142 K | 155 K | 155 K | 155 K | 150 K | 150K | 150K |
| $\beta_1$ ($\beta_2$) | | 58 K (60 K) | 60 K | 63 K | 63 K | 63 K | 67 K (65K) | 67K (65K) | 67K (65K) |
| $\gamma$ | | none | 19.5 K | 19K | 19 K | None | None | 20K | None |
| Molar ratio subunits $\alpha$:($\beta_1$+$\beta_2$):$\gamma$ | | 1:1:0 | 1:1:2 | | Less $\gamma$ subunit than I | | 4:4:0 | 2:2:2 | 1:1:0 |

[a] Preparations I and II were separated by electrophoresis and chromatography; preparation III was isolated from preparation I after alkaline-heat treatment.

[b] Various preparations, isolated from sucrose density gradients, were analyzed.

marized in Fig. 5, and proposals are made for the functional organization of the redox carriers in the cytoplasmic membrane. The essential features of the scheme are that (i) the polypeptide components of both NADH and formate dehydrogenase are organized in the membrane to allow for the translocation of 2 $H^+$ per quinone molecule reduced and (ii) quinol (H carrier) and cytochrome $b_{556}^{NO_3^-}$ with $NO_3^-$ reductase ($e^-$ carriers) are similarly arranged to give proton translocation during oxidation-reduction. In the presence of oxygen, presumably cytochromes $b_{556}$ and $o$ or cytochromes $b_{558}$ and $d$ (Fig. 3) serve as electron carriers in place of cytochrome $b_{556}^{NO_3^-}$ and nitrate reductase. There are several unresolved questions concerning this scheme including: (i) the nature of the type $b$ cytochrome involved in the proton-translocating formate dehydrogenase activity; (ii) whether this type $b$ cytochrome is required for proton translocation associated with the NADH dehydrogenase; (iii) the relationship between the proton-translocating NADH and formate dehydrogenase activities found in cells grown anaerobically with $NO_3^-$ and the corresponding activities found in cells grown both aerobically and also anaerobically with fumarate as terminal electron acceptor.

Because of the relative simplicity of the terminal electron-transporting limb involving cytochrome $b_{556}^{NO_3^-}$ and $NO_3^-$ reductase and the ease with which the various polypeptides can be isolated and identified, considerable attention has been directed towards assessing the spatial orientation of the cytochrome $b_{556}^{NO_3^-}$-nitrate reductase complex in the cytoplasmic membrane. That the site of $NO_3^-$ reduction, and presumably of binding to $NO_3^-$ reductase, cannot be on the cytoplasmic face of the membrane (130) is suggested by the following experimental observations: (i) the observed rate of reduced benzylviologen-dependent $NO_3^-$ reduction in whole cells or protoplasts is three orders of magnitude higher than the observed rate of passive diffusion of $NO_3^-$ into protoplasts suspended in isosmotic $KNO_3$ containing valinomycin; (ii) the failure to detect antiport systems for $NO_3^-$ with $NO_2^-$ or $OH^-$; (iii) the lack of effect of a transmembrane pH gradient on the competitive inhibition by azide of $NO_3^-$ reductase in whole cells (see reference 310); and (iv) the absence of $NO_3^-$ accumulation and, in fact, $NO_3^-$ exclusion in a hemeless mutant of *E. coli* in which membrane-bound $NO_3^-$ reductase was, nevertheless, active with artificial donors (217). Using (i) closed-vesicle preparations of *E. coli* with a defined sidedness, protoplasts (same orientation as the original cell) and vesicles

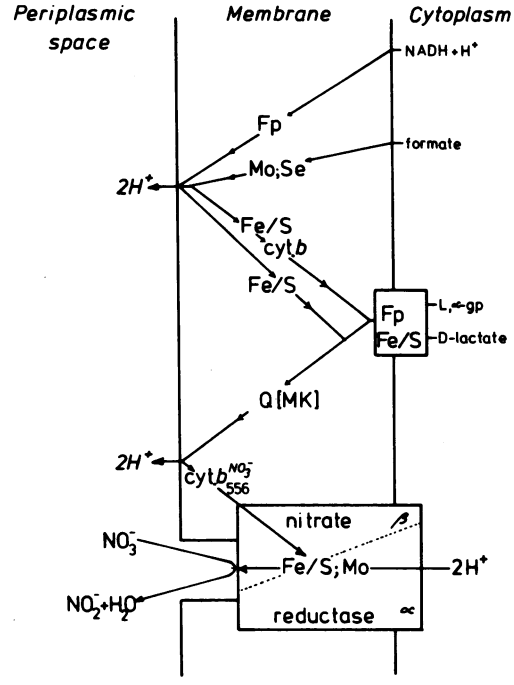


FIG. 5. *Proposed functional organization of the redox carriers responsible for anaerobic electron transport with $NO_3^-$ as terminal electron acceptor in E. coli. Note the separate polypeptide components of the proton-translocating NADH- and formate-dehydrogenases; it is not known whether some of the components are common to both segments. The scheme is modified from Garland et al. (128). Abbreviations: Mo, Molybdenum-containing polypeptide; Se, selenium-containing polypeptide; α, β, subunits of nitrate reductase; otherwise as in the legends to preceding figures.*

prepared by sonication (inside-out with respect to the original cell), (ii) a specific nonpenetrant label for tyrosine residues in exposed proteins (lactoperoxidase/$H_2O_2$-mediated incorporation of $^{125}I$) and (iii) the knowledge that antibody to $NO_3^-$ reductase specifically precipitates the polypeptides of cytochrome $b_{556}^{NO_3^-}$ (γ) and $NO_3^-$ reductase (α + β), Boxer and Clegg were able to show that the γ subunit was located on the periplasmic side of the membrane, and the α subunit was located on the cytoplasmic side of the membrane; the relative sidedness of the β subunit could not be determined with the same certainty (43). In the light of these experimental observations and in the absence of any direct evidence that $NO_3^-$ reductase is exposed on both surfaces of the cytoplasmic membrane, it has been proposed (128) that $NO_3^-$ approaches the active center of $NO_3^-$ reductase via a cleft or well, as shown in Fig. 5. An important consequence of this proposal is that $NO_3^-$ reductase must catalyze a vectorial reaction in conveying both $e^-$ and $H^+$ across the membrane. Clearly, further work is required to test the validity of these proposals and to determine whether $NO_3^-$ reductase does indeed have a transmembrane orientation by using, for example, permeant and nonpermeant analogues of viologen dyes as reductants of $NO_3^-$ reductase in vesicle preparations of known sidedness.

Mutants defective in $NO_3^-$ reductase activity have been widely used in an attempt to characterize the factors regulating the synthesis and assembly of this anaerobic electron transport chain. As indicated in Table 1, such mutants have been isolated by three general methods: (i) resistance to chlorate during anaerobic growth conditions, (ii) differential ability to grow on nonfermentable substrates aerobically and anaerobically with $NO_3^-$, and (iii) inability to reduce $NO_3^-$ to $NO_2^-$, as judged by a dye overlay technique. Seven genetic loci have been identified to date of which four, the *chlA*, *chlB*, *chlD*, and *chlE* genes, are pleiotropic mutations involving not only the loss of membrane-bound formate-dependent $NO_3$ reductase activity but also the loss of formate hydrogenlyase activity (formate dehydrogenase with hydrogenase), which is apparently located in the periplasmic space (Table 1). All of these mutants are able to grow normally under aerobic conditions with both fermentable and nonfermentable carbon sources. Considerable attention has been directed towards assessing the gene products defective in these various mutants. Four general methods have been used: (i) the phenotypic restoration of functional activity by changing the growth conditions of the cell (135; Table 2); (ii) the identification of missing polypeptides in Triton-solubilized membranes from cells by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using point mutants (253) and deletion mutants (334), although a direct causal relationship between a missing or an altered polypeptide and the loss of functional activity is difficult to demonstrate unambiguously; (iii) in vitro complementation assays involving the mixing of soluble extracts, i.e., nonsedimentable on prolonged centrifugation, from the various mutants and testing for the restoration of particulate $NO_3^-$ reductase activity as developed by Azoulay and co-workers (e.g., references 26, 27, 256, 288, 329; but see also reference 254) and (iv) the specific identification of missing polypeptides from the cytochrome $b_{556}^{NO_3^-}$-$NO_3^-$ reductase complex by challenging Triton extracts of cells with antibody to purified $NO_3^-$ reductase (249).

Interpretation of the data provided by these different methods is difficult and further com-

plicated by the possibility that individual *chl* loci may be functionally subdivisible. Thus, Venables has shown by fine-structure genetic analysis of *chl* loci that *chlA*, *chlB*, and *chlE* are divisible into two, three, and two complementation groups, respectively, which, in the cases of *chlA* and *chlE* may represent distinct genes (404). Indeed, *chlE* mutants exhibit a variety of phenotypes and, of three mutants assayed for their ability to produce $NO_3^-$ reductase specifically, using the antibody precipitation technique, two made reduced amounts of precipitable enzyme and one made none (249). In addition, the variable amounts of $NO_3$ reductase, formate dehydrogenase, and type *b* cytochrome that are retained by *chlC* mutants, as well as the possibility that these amounts are related in a polarized manner to the map position of the *chlC* gene involved, have led to the suggestion (141, 342) that *chlC* may comprise an operon specifying these three components, but evidence for genetic complementation between *chlC* mutants is, unfortunately, lacking. An attempt has been made to summarize the postulated primary lesions in those individual *chl* mutants that have been extensively characterized in several different laboratories (Table 5). However, it should be remembered that detailed biochemical investigations have usually only been performed with a single mutant strain thought typical of a particular *chl* locus. As mentioned above, the evidence so far available indicates that this is an oversimplification and clearly a more detailed genetic analysis of each individual locus by fine-structure genetic mapping, as well as a rigorous biochemical analysis of different isolates reputedly characteristic of a single locus, are required before the proposals made in Table 5 can be accepted without reservation.

A heme-deficient mutant has also been used to study the factors regulating the synthesis of functional $NO_3^-$ reductase. When this mutant is grown anaerobically in the presence of $NO_3^-$ and absence of heme synthesis, apocytochrome $b_{556}^{NO_3^-}$ is not present in the membrane, and the $\alpha$ and $\beta$ subunits of $NO_3^-$ reductase are over produced and chiefly located in the cytoplasm, though some enzyme is still membrane bound and susceptible to proteolytic attack (251). If heme synthesis is allowed to proceed, by the addition of 5-amino-levulinic acid to the growth medium, then the $NO_3^-$ reductase present in the cytoplasm is incorporated into the membrane in a stable form in parallel with the formation of a functional type *b* cytochrome (252).

TABLE 5. *Proposed primary lesions in various chl$^r$ mutants of E. coli*

| Gene | Suggested primary lesion | References |
|---|---|---|
| *chlA* | Synthesis of Mo-cofactor | 249, 254 |
| *chlB* | Association factor ($F_A$) required for attachment or insertion of Mo-cofactor into nitrate reductase | 249, 254, 329 |
| *chlC* | Structural gene for nitrate reductase, possibly $\alpha$ subunit | 141, 249 |
| *chlD* | Processing of Mo (transport, redox level, incorporation into Mo-cofactor) | 135 |
| *chlE* | Structural gene for nitrate reductase, possibly $\gamma$ subunit | 249, 253 |
| *chlF* | Structural gene for formate dehydrogenase(?) | 136 |
| *chlG* | Not known | 136 |

Therefore, the following information is so far available concerning the factors regulating the synthesis and assembly of a functional formate-dependent $NO_3^-$ reductase activity: (i) the molybdenum-containing enzymes, soluble and particulate formate dehydrogenase and $NO_3^-$ reductase, require the gene products of the *chlD* gene (probably for the transport of Mo), the *chlA* gene (for the synthesis of a Mo-cofactor), and the *chlB* gene (for the attachment or insertion of Mo-cofactor into the apoenzymes) for the synthesis of functional enzymic activity, though apoprotein synthesis apparently occurs in the absence of the Mo-processing machinery; (ii) apocytochrome $b_{556}^{NO_3^-}$ synthesis requires concomitant heme synthesis, possibly the product of the *chlE* gene, and probably occurs in the cytoplasmic membrane; (iii) apo-$NO_3^-$ reductase synthesis requires the product of the *chlC* gene, occurs in the cytoplasm, and functional cytochrome $b_{556}^{NO_3^-}$ is required for correct binding and insertion into the membrane; (iv) only when membrane bound and functional is $NO_3^-$ reductase protected from proteolytic attack.

Although *E. coli* has been studied extensively by molecular biologists in the past, it is only comparatively recently that attention has been directed towards elucidating the mechanism of oxidative phosphorylation in this bacterium. The experimental evidence indicates that the various redox carriers synthesized by *E. coli* are functionally organized in the membrane into several proton-translocating oxido-reduction segments. Those redox segments that have so far been identified and, in part, characterized include the following.

(i) A proton-translocating NADH-ubiquinone oxido-reductase activity, produced and utilized under aerobic growth conditions, which is apparently cytochrome independent, requires flavoprotein and iron-sulfur protein(s) and, at least superficially, resembles the equivalent mitochondrial enzyme. Under certain growth conditions, this protein is either modi-

fied or replaced by an alternative enzyme such that the associated redox reactions are no longer linked to net proton translocation (Fig. 3).

(ii) A proton-translocating NADH fumarate oxidoreductase activity produced and utilized under anaerobic growth conditions in the presence of fumarate, which is apparently cytochrome dependent and requires menaquinone in addition, presumably, to flavoprotein and iron-sulfur protein(s) (Fig. 4b). A proton-translocating NADH dehydrogenase is also synthesized and functional during anaerobic growth in the presence of nitrate. It is not known, at this time, if this activity is the result of a cytochrome-independent route (as shown in Fig. 3 and 5) or a cytochrome- and menaquinone-dependent route (equivalent to the scheme shown in Fig. 4b without fumarate reductase) for electron transport; indeed, it is possible that both of these proton-translocating redox segments for NADH oxidation can function simultaneously during anaerobic electron transport to nitrate and, indeed, under certain conditions during aerobic electron transport.

(iii) A proton-translocating formate dehydrogenase activity that requires, as minimal components, iron-sulfur protein(s), molybdenum, selenium, and a type *b* cytochrome. Although menaquinone has been implicated as a carrier in formate-dependent fumarate reduction (Fig. 4b), it remains to be established whether this carrier is an obligatory component of the proton-translocating formate dehydrogenase activity produced and functional during anaerobic growth in the presence of nitrate (Fig. 5).

(iv) A proton-translocating ubiquinol-nitrate oxidoreductase activity (Fig. 5).

(v) Two proton-translocating ubiquinol-oxygen oxidoreductase activities that possess different electron-carrying limbs (Fig. 3).

The following general conclusions can be reached concerning the functional organization of the various redox carriers in these proton-translocating redox segments. First, as minimal components in those redox segments that result in the direct reduction of an exogenous acceptor (e.g., nitrate, fumarate, or oxygen), the hydrogen-carrying limb contains a quinone species, and the electron-carrying limb contains two components, a type *b* cytochrome and a terminal reaction center (nitrate reductase, fumarate reductase, cytochromes *o* and *d*) capable of interacting with and reducing the acceptor. Second, during oxygen- and nitrate-dependent electron transport, an additional energy conservation site is available to the bacterium through the activity of NADH and formate dehydrogenase, which results in the reduction of an endogenous membrane-bound acceptor, presumably ubiquinone. The individual redox carriers present in the hydrogen- and electron-carrying limbs of these dehydrogenases remain uncertain and, presumably, alternative components function in different enzymes, or in different forms of the enzyme, depending upon the control mechanisms that regulate their synthesis and activity as the growth conditions and metabolic demands on the cell vary. Third, although electron transport in *E. coli* may appear superficially to be more complex than in mitochondria, this apparent complexity is due to the presence of alternative and parallel pathways for electron transport in this bacterium.

If each of the proposed proton-translocating redox segments is considered as a distinct building block, then each block can function either separately (e.g., in NADH-dependent fumarate reduction or D-lactate-dependent nitrate reduction) or sequentially, such that reducing equivalents can pass from a block of low redox potential (e.g., the NADH dehydrogenase) to a block of high redox potential (e.g., ubiquinol-oxygen oxidoreductase), through carriers of similar redox potential at the junction of the two blocks, and thence to the terminal electron acceptor. Thus, under any particular growth conditions, alternative and parallel pathways can exist in the membrane for the reoxidation of reduced coenzymes. For example, NADH oxidation, by oxygen, could proceed simultaneously through one of the two proton-translocating redox segments (either menaquinone dependent or menaquinone independent) or through the non-proton-translocating route and then, via ubiquinone, through either the cytochrome $b_{556}$ with *o* or cytochrome $b_{558}$ with *d* electron-carrying limbs to oxygen. Previous schemes in which the various redox carriers were arranged in a linear sequence (85) are obviously too simple to account for the diversity of pathways available for electron transport in *E. coli*. The fourth general point to emerge from these considerations is the need to identify and characterize the redox carriers that are synthesized by, and are functionally active in, a particular strain of *E. coli* grown under controlled and defined conditions, before attempting to interpret their metabolic significance to the cell.

Although much further work is obviously required, in the last few years sufficient progress has been made, from a combined biochemical and genetic approach, to suggest that *E. coli* has many advantages for studying the molecular mechanism of respiratory driven proton translocation and associated energy-linked

functions that are not found with classical mitochondrial systems. It is of interest that the various redox carriers, which are synthesized by *E. coli* and which are functionally active during aerobic electron transport, are organized in the membrane into no more than two potential energy conservation sites. Although *E. coli* can synthesize two terminal electron-carrying limbs for the oxidation of ubiquinol by oxygen, it appears that this bacterium has not developed an electron-carrying limb containing types *c* and *a* cytochromes, which is necessary for the functional activity of the additional potential site of energy conservation available to mitochondria and *P. denitrificans* (see below).

### *Paracoccus denitrificans*

The gram-negative bacterium *Paracoccus denitrificans* is a chemoorganotroph and facultative chemolithotroph, capable of using the oxidation of molecular $H_2$ (116, 223, 233) and methanol (87) as sole course of energy for autotrophic growth. Many different organic compounds serve as sole carbon source for growth, but the metabolism is respiratory, never fermentative. Molecular $O_2$, or $NO_3^-$, and $NO_2^-$, under anaerobic conditions (192, 234), serve as terminal electron acceptors. $NO_3^-$ is reduced to nitrous oxide and, ultimately, to molecular $N_2$ under anaerobic conditions.

**Aerobic electron transport.** When grown aerobically, *P. denitrificans* synthesizes an electron transport chain which, in its components and their functional organization, more closely resembles the electron transport chain of the inner mitochondrial membrane than that of any other bacterium (for a review, see reference 193). The essential features that characterize the aerobic electron transport chain of this bacterium, which is located in the cytoplasmic membrane (357), are summarized in Fig. 6a and include: an energy-dependent nicotinamide nucleotide transhydrogenase activity (23); an NADH dehydrogenase activity that shows energy-dependent reversal (24), sensitivity to rotenone (183) and piercidin A (240) at low concentration, and an iron-sulfur center(s) with a characteristic g = 1.94 signal in electron paramagnetic resonance spectroscopy at 77°K, decreased in content under conditions of iron-limited growth (184); ubiquinone-10 as the sole functional quinone of the respiratory chain (357) and at least two kinetically and spectrally distinguishable type *b* cytochromes, possibly two type *c* cytochromes, with cytochromes $a + a_3$ serving as the terminal oxidase, although cupric copper, associated with the mitochondrial cytochrome oxidase, was not present in *P. denitrificans* particles (184, 240, 357, 364, 405, 406). In addition, electron transport is sensitive to low concentrations of antimycin A (357) and carboxin (396). All these features are typical of the mitochondrial inner membrane, and indeed the cytoplasmic membrane of *P. denitrificans* shows further similarities to this membrane in its content of phospholipids and fatty acids (193). It has been claimed that cytochrome *o*, a type *b* cytochrome that binds carbon monoxide in the original terminology and definition of Castor and Chance (65), serves as a terminal oxidase in *P. denitrificans*, as it does in certain mitochondria (349). However, there is no evidence from kinetic data, using a stopped-flow spectrophotometer, to suggest that a type *b* cytochrome is acting as a terminal oxidase in *P. denitrificans* (240).

The cytoplasmic membrane of *P. denitrificans* is known to have a low proton conductance (355), and Scholes and Mitchell (354) have shown that protons are ejected through the cytoplasmic membrane after the addition of a small amount of oxygen to a stirred anaerobic suspension of cells. Limiting $\rightarrow H^+/O$ quotients of 8 were obtained with cells oxidizing endogenous substrates (354). This suggests that the electron transport chain of *P. denitrificans*, like that of mitochondria, is arranged in three proton-translocating segments that can be used to drive ATP synthesis, plus a fourth, the transhydrogenase, that probably cannot do so under physiological conditions. This was confirmed in subsequent experiments using starved cells oxidizing known added substrates, where it was shown that the oxidation of succinate or glycerol resulted in the translocation of 4 $H^+$ per O consumed, and the oxidation of L-ascorbate, via *N,N,N',N'*-tetramethyl-*p*-phenylene diamine (TMPD), was associated with the translocation of about 2 $H^+$ per O consumed (240). It was also shown in this work that, in cells harvested in the early-exponential phase of batch growth, the oxidation of L-malate was associated with an $\rightarrow H^+/O$ ratio of about 8, which decreased to a value closer to 4 in cells harvested in the stationary phase of growth; the sensitivity of L-malate oxidation to piercidin A inhibition was similar in cells harvested from all phases of the growth cycle (240). This suggests that proton translocation associated with both the transhydrogenase and the NADH dehydrogenase can be modified under certain growth conditions. The data also indicate that sensitivity towards piercidin A and the occurrence of proton translocation associated with the NADH dehydrogenase are distinct and separate features of this region of the respiratory chain; this is a point of

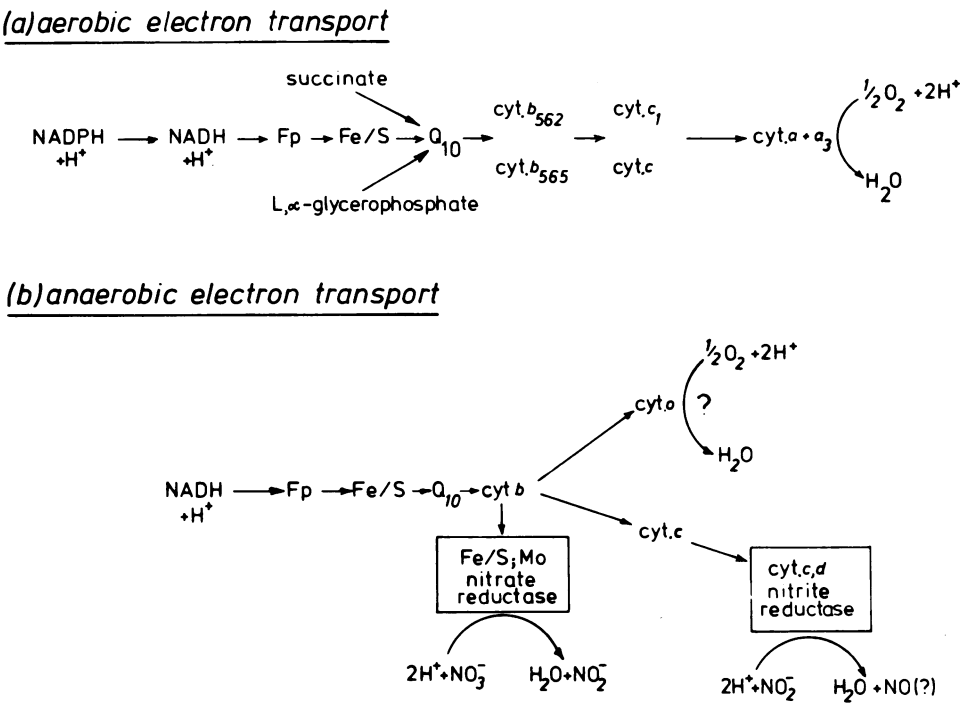


FIG. 6. *Proposed electron transport chains in Paracoccus denitrificans. Scheme (a) is the proposed linear sequence of redox carriers associated with aerobically grown cells. Although there is no experimental evidence available at present, it is proposed that these carriers have a similar functional organization in the cytoplasmic membrane to those of the inner mitochondrial membrane (shown in Fig. 2) and are so arranged as to allow the synthesis of 3 mol of ATP per mol of NADH oxidized. Scheme (b) is a summary of the known redox carriers found in cells grown anaerobically in the presence of $NO_3^-$. During these growth conditions, there is an increase in the synthesis of cytochrome o (see text) which presumably can act as a terminal oxidase, yet its significance to the metabolic activity of the cell during anaerobic growth remains obscure. Nothing is known of the functional organization of these redox carriers in the membrane; indeed $NO_2^-$ reductase is not membrane bound, and the specific identity of the type b cytochrome has not been established. In addition, the number of potential sites of energy conservation associated with anaerobic electron transport has not been determined. The schemes are modified from John and Whatley (193) and the abbreviations used are defined in the legend to Fig. 2.*

some controversy in analogous studies with mitochondria derived from the yeast *Candida utilis*, in which it has been claimed that these phenomena are either independent (147, 183) or interdependent (75, 140) properties. Of particular interest is the fact that particles prepared from cells grown under iron-limited conditions, though containing only a very small amount of nonheme iron, had normal NADH oxidase activity and performed NADH dehydrogenase-dependent ATP synthesis by oxidative phosphorylation as effectively as normal preparations; unfortunately, the sensitivity of NADH oxidase activity to rotenone or piercidin A was not reported (184).

A $Y_{O_2}^{max}$ of approximately 73 g of cells per mol of oxygen consumed has recently been determined for glycerol-limited continuous cultures of *P. denitrificans* (C. Edwards and C. W. Jones, unpublished data). This value is considerably higher than has been reported by other workers for carbon-limited cultures of this organism (403) and is also very much higher than has been obtained with organisms whose NADH oxidases contain only two proton-translocating segments (101, 110, 166, 385), thus suggesting that in *P. denitrificans* the terminal cytochrome $c \rightarrow aa_3$ limb contributes towards ATP synthesis.

One of the advantages of working with *P. denitrificans* is the relative ease with which subcellular particles capable of oxidative phosphorylation can be isolated (182, 192, 223). Indeed, phosphorylating particles, prepared from the cytoplasmic membrane of *P. denitrificans*, show a mitochondrial type of respiratory control in oxygen electrode experiments that is rarely observed in other phosphorylating bacte-

rial preparations (190, 191; see also references 105, 195, 196). These particles necessarily have an orientation that is the reverse of that of the cytoplasmic membrane of the intact cell, that is, they are inside-out vesicles. Recently, right-side-out vesicles, in which the vesicle membrane has the same orientation as the cytoplasmic membrane of the intact cell, have been prepared (60, 412) and, clearly, a comparative study of these two preparations will be of use in elucidating the relative sidedness of the various redox components in the cytoplasmic membrane.

**Anaerobic electron transport.** *P. denitrificans* can grow anaerobically using either $NO_3^-$ or $NO_2^-$ as a terminal electron acceptor, the ultimate product being $N_2$ gas (234). Under anaerobic growth conditions in the presence of $NO_3^-$, several alterations occur to the aerobic respiratory chain which include: (i) the disappearance of cytochrome $a + a_3$; (ii) increased synthesis of type *b* and *c* cytochromes, including cytochrome *o*, although the additional cytochromes have not been characterized extensively; (iii) the appearance of a membrane-bound $NO_3^-$ reductase activity; and (iv) the synthesis of a soluble $NO_2^-$ reductase activity (192, 234, 349, 357). The various results obtained by these investigators and the interrelationships between the different redox carriers are summarized in Fig. 6b.

The respiratory $NO_3^-$ reductase has been solubilized and purified from the cytoplasmic membrane (119, 235) and shown to be a molybdenum-containing iron-sulfur protein (121). $NO_2^-$ reductase has also been purified from *P. denitrificans*. The enzyme is a two-heme cytochrome, containing both a type *c* (with an unusual double $\alpha$-band) and a *d*-like heme and, in addition to its $NO_2^-$ reductase activity, it also exhibits an oxidase activity; the enzyme is not membrane bound (236, 297). Interestingly, a "blue"-copper protein co-purifies with $NO_2^-$ reductase in the early stages of enzyme isolation, though the functional involvement of the "blue" copper protein has not been determined (296).

Although cytochromes $a + a_3$ are no longer present in cells grown anaerobically with $NO_3^-$, the apparent oxidase activity of such cells is similar to that of aerobically grown cells. It has been suggested, on the basis of a carbon monoxide-binding assay, that cytochrome *o* synthesis is increased under anaerobic growth conditions and could presumably serve as the terminal oxidase (349); a kinetic analysis is required to support this proposal. If oxygen and $NO_3^-$ are added simultaneously to an anaerobic suspension of cells, grown anaerobically in the presence of $NO_3^-$, the oxygen is used first followed by the $NO_3^-$ (71). The control mechanism(s) that regulates the relative flow of reducing equivalents down the various pathways to the different terminal electron acceptors has yet to be determined.

Comparatively little attention has been directed towards assessing the efficiency of oxidative phosphorylation associated with anaerobic electron transport. In cells grown anaerobically with $NO_3^-$, ATP synthesis from NADH-, but not succinate-, dependent $NO_3^-$ reduction has been demonstrated in phosphorylating particles (192), and $NO_3^-$-dependent proton translocation has been observed in starved whole cells oxidizing unspecified endogenous substrates (H. G. Lawford and J. C. Cox, unpublished observations). There are no reports, to our knowledge, of oxidative phosphorylation associated with $NO_2^-$ reduction; this is due, in part, to the experimental difficulties of growing cells anaerobically in the presence of $NO_2^-$, which is toxic above a certain concentration, and in part to the fact that the soluble nitrite reductase is lost during the preparation of phosphorylating particles (192).

An attempt has been made to determine the factors influencing the synthesis of the various redox components and it appears that: (i) oxygen represses the synthesis of both $NO_3^-$ reductase and $NO_2^-$ reductase; (ii) $NO_3^-$, but not $NO_2^-$, induces the synthesis of $NO_3^-$ reductase; (iii) $NO_2^-$, either added exogenously or derived from $NO_3^-$ via $NO_3^-$ reductase, induces the synthesis of $NO_2^-$ reductase, so that cells grown anaerobically with $NO_3^-$ contain both $NO_3^-$ reductase and $NO_2^-$ reductase activity; and (iv) cytochrome $a + a_3$ synthesis is oxygen dependent (234, 249).

As can be seen from the above brief review, the electron transport chains of *P. denitrificans* have not, as yet, been explored in depth, but already sufficient information is available (e.g., similarity to mitochondrial electron transport chain, variability of redox carriers and efficiency of oxidative phosphorylation with growth conditions, conspicuous lack of work with mutants) to indicate the profitability of more extensive investigations.

### *Azotobacter vinelandii*

In contrast to *E. coli* and *P. denitrificans*, *Azotobacter vinelandii* (like its close relatives *A. chrococcum* and *A. beijerinckii*) is a nitrogen-fixing, obligate aerobe; this organism can therefore use only molecular oxygen as a terminal oxidant for respiration. The energy-dependent, reductive assimilation of atmospheric ni-

trogen (174, 318, 423) is catalyzed by an enzyme complex, nitrogenase, comprising two iron-sulfur proteins—one of which contains molybdenum (61, 424). Purified nitrogenase from *A. vinelandii*, like that from various obligate or facultative anaerobes, is readily inactivated by high concentrations of molecular oxygen. Thus, during growth, the organism is faced with the problem of maintaining its intracellular oxygen concentration at a level that is too low to inhibit nitrogen fixation, but which is high enough to allow adequate ATP synthesis. The respiratory system of *A. vinelandii*, therefore, has a dual function, viz., the conservation of energy via oxidative phosphorylation and the protection of nitrogenase via the removal of excess oxygen.

**Respiratory chain energy conservation.** The respiratory system of *A. vinelandii* is located in the extensively invaginated cytoplasmic membrane (174, 308) and is characterized by an extremely rich complement of redox carriers. These include highly active, flavin-dependent NADH, NADPH, and malate dehydrogenases (9, 200, 204), of which the former is known to contain iron-sulfur centers (96), ubiquinone Q-8 as the sole quinone component (200, 387), and at least six spectroscopically detectable cytochromes, viz., $b_{560}$, $c_4$, $c_5$, $a_1$, *o*, and *d*, plus cytochromes $c_{551}$ and $c_{555}$, which have been detected in low concentrations and are probably subunits of the normally dimeric cytochromes $c_4$ and $c_5$, respectively (200, 201, 386, 395).

In addition, the particulate respiratory chain interacts with a soluble nicotinamide nucleotide transhydrogenase (399) and also with various soluble $NAD^+$- and $NADP^+$-linked dehydrogenases (34, 200, 203, 204).

Respiration by *A. vinelandii* membranes is remarkably insensitive to classical inhibitors of mitochondrial electron transfer such as rotenone or antimycin A, but is readily inhibited by 2-*n*-alkyl-4-hydroxyquinoline-*N*-oxide (167, 201, 205) and, to a variable extent, by cyanide (194, 201, 214, 215, 216). The ability of the latter to inhibit the oxidation of the artificial electron donors ascorbate-TMPD and ascorbate-2,6-dichlorophenol indophenol (DCPIP) much more readily than the oxidation of physiological substrates ($K_i$ = 0.5 $\mu$M versus $\leq$115 $\mu$M (194); see also references 32, 201, 205, 207, 216, 386) has led to the now generally accepted concept of a branched respiratory system in *A. vinelandii* (Fig. 7). This is fully supported by the different sensitivities of the two branches towards carbon monoxide (201), the photodissociation patterns of the cytochrome oxidase-carbon monoxide complexes (108), and the strikingly different oxidation-reduction kinetics of the type *b* and *c* cytochromes (201, 216). The cytochrome $b \rightarrow d$ branch thus resembles the terminal limb of the *E. coli* system (following the growth of that organism under oxygen-limited conditions; see Fig. 3), whereas the $b \rightarrow c_4, c_5 \rightarrow a_1$ *o* branch is similar to the terminal cytochrome systems of *P. denitrificans* (substituting $a_1$ for $aa_3$; see Fig. 6) and of several major families of bacteria (199, 261). The qualitative response of this latter pathway to cyanide has led to the suggestion (194) that cytochrome oxidases *o* and $a_1$ may oxidize different type *c* cytochromes; since there is good evidence that cytochrome *o* is functionally associated with $c_4$ (216), cytochrome $a_1$ would therefore accept electrons from $c_5$. However, recent claims that *A. vinelandii* particles devoid of cytochrome $a_1$ still readily oxidize reduced TMPD and DCPIP (215, 282) and that this cytochrome may not be competent as a terminal oxidase in some bacterial systems (145) have cast doubts upon the involvement of cytochrome $a_1$ in the cytochrome *c*-linked pathway.

In contrast, Kauffman and van Gelder have

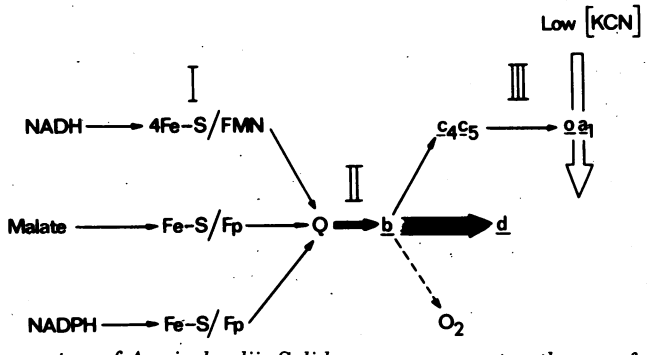


FIG. 7. *Respiratory system of A. vinelandii. Solid arrows represent pathways of electron transfer, the broken arrow represents the cyanide-insensitive autooxidation of cytochrome b, and the open arrow represents the site of action of low concentrations of cyanide. I, II, and III are the approximate sites of energy coupling (proton translocation) (after Downs and Jones [102]).*

recently provided convincing kinetic and spectral evidence that confirms the role of cytochrome *d* as the oxidase of the major terminal pathway (216). It has been proposed that the oxidized form of the enzyme exists in two different conformations, viz., an inactive conformation that absorbs at 648 nm and a more open, active conformation ($d_x$) to which cyanide binds preferentially, the binding being greatly enhanced under conditions in which the enzyme is turning over rapidly (214–216).

A third terminal pathway to oxygen has recently been proposed (216); this branch, which is characterized by its extreme insensitivity to cyanide, carries only about 5% of the total electron flux from NADH and many involve autooxidation of the type *b* cytochrome.

The dehydrogenase end of the *A. vinelandii* respiratory chain is particularly interesting, since it furnishes a rare example of an apparently allosteric NADPH dehydrogenase (9). The latter is highly active, shows sigmoidal saturation kinetics with respect to NADPH concentration, and is competitively inhibited by both adenine nucleotides (AMP > ADP > ATP: no loss of sigmoidicity) and $NAD^+$ (with reversion to Michaelis-Menten kinetics). The $[S]_{0.5}$ of the enzyme is severalfold higher than the $K_m$ values of the other membrane-bound dehydrogenases and thus ensures that the respiratory chain rapidly oxidizes NADPH only when the latter is present in abundance, a property that is probably of major importance to the mechanism of respiratory protection (see below).

Carefully prepared respiratory membrane preparations from *A. vinelandii* exhibit electron transfer-linked energy conservation, as evidenced by their abilities to catalyze either ATP synthesis (10, 104), the quenching of atebrin fluorescence (103), or the active transport of glucose (31, 32). It was concluded from these studies that a maximum of three energy-coupling sites are present, viz., at the level of NADH dehydrogenase (site I), in the central Q $\rightarrow$ *b* region of the chain (site II) and on the cytochrome $c_4$, $c_5 \rightarrow a_1$ *o* terminal branch (site III); the cytochrome $b \rightarrow d$ terminal branch and the flavin-linked malate and NADPH dehydrogenases show no evidence of energy coupling. Classical respiratory control is detectable at sites I and II, but not at site III (105, 195, 196), thus reflecting the low energy conservation efficiency of site III in these membrane preparations. In contrast, the activity of the uncoupled NADPH dehydrogenase is apparently modulated by the ambient energy charge (9).

The recent measurement of respiration-linked proton ejection by starved cells of *A. vinelandii* oxidizing added substrates (e.g., $\beta$-hydroxybutyrate, malate, or reduced TMPD and DCPIP) has indicated the presence of three proton-translocating respiratory segments (102) at locations similar to those proposed for energy conservation in isolated membranes. Furthermore, concentrations of cyanide sufficient to block the $c_4$ $c_5 \rightarrow a_1$ *o* branch (but not the $b \rightarrow d$ pathway) slightly decreased the $\rightarrow H^+/O$ ratios that were observed during the oxidation of physiological substrates, thus confirming that the cytochrome $b \rightarrow d$ pathway does not conserve energy and reinforcing the concept that this branch constitutes the major, terminal route to oxygen. This latter conclusion is fully supported by the P/O ratios of approximately 2 for the oxidation of $NAD^+$-linked substrates that have been determined by direct measurement of ATP synthesis after the addition of oxygen to anaerobic whole-cell suspensions (225; but see also reference 28). It is clear from these results that the abilities of the terminal branches of the *A. vinelandii* respiratory system to translocate protons are dependent upon their redox carrier composition, particularly with respect to type *c* cytochromes. This conclusion is supported by the observations that a third proton-translocating segment is present in the respiratory system of *P. denitrificans*, but not *E. coli*, and by the results of comparative studies on a variety of bacterial respiratory systems (197).

**Respiratory protection of nitrogenase.** The growth of *Azotobacter* under nitrogen-fixing conditions in the presence of a high $pO_2$ is characterized by a higher respiratory activity ($Q_{O_2}$) and a lower molar growth yield ($Y_{O_2}^{max}$ or $Y_{substrate}^{max}$) than during growth under conditions of oxygen limitation (11, 91, 92, 174, 198, 289). Since both of these parameters are proportional to the overall efficiency of cellular energy conservation (predominantly respiratory chain phosphorylation in this obligate aerobe), the above changes must reflect energy wastage by the cell. The latter may occur either via the complete or partial failure of the respiratory system to couple electron transfer to the formation of the energized state (and subsequently to the synthesis of ATP) or via the operation of metabolic "slip reactions" (290, 291), which spill excess energy by ATP hydrolysis (either directly via ATPase, or indirectly via futile metabolic cycles).

The branched nature of the *Azotobacter* respiratory system is fully compatible with the former possibility, since it would allow substantial variation in the efficiency of energy conser-

vation according to the exact route of electron transfer. Thus, the exposure of oxygen-limited populations of *Azotobacter* to excess oxygen causes a sudden cessation of nitrogen fixation and elicits a rapid increase in $Q_{O_2}$, together with mobilization of reserve storage materials such as polysaccharide and poly-$\beta$-hydroxybutyrate, which are accumulated under oxygen-limited conditions (91, 188, 360). These changes are accompanied by the de novo synthesis both of cytochrome *d* and of the NADH and NADPH dehydrogenases (99, 198), as well as the rapid loss of respiration-linked proton translocation and ATP synthesis at the level of NADH dehydrogenase (102, 198; C. W. Jones, unpublished data). It is clear, therefore, that these changes would tend to lower the overall efficiency of respiratory chain energy conservation and thus produce an increase in respiratory activity. Since the second proton-translocating segment of the respiratory chain is still retained under excess oxygen conditions (102), the efficiency of energy conservation would fall by a maximum of two-thirds, which would thus lead to a threefold increase in $Q_{O_2}$ (provided that the growth rate is unaltered). However, since the sudden exposure of nitrogen-fixing populations of *Azotobacter* to excess oxygen leads both to a cessation of growth and to at least a threefold increase in $Q_{O_2}$ (91, 198), it must be acknowledged that the observed changes in energy-coupling efficiency cannot alone account for the increase in respiratory activity. The hypersensitivity of phosphate-limited cultures of *A. chroococcum* to oxygen (242) suggests that a large adenine nucleotide pool is a prerequisite for efficient respiratory protection and, thus, lends some weight to the idea that as yet unidentified ATP-spilling reactions may comprise part of the overall mechanism of respiratory protection.

## BACTERIAL ATPase COMPLEXES

The $Mg^{2+}$-dependent, proton-translocating ATPase complexes of bacteria have been intensively investigated in the last few years. Thus, the pioneering work of Abrams and his colleagues on the ATPase complex of the normally fermentative bacterium *Streptococcus faecalis* has recently been extended to aerobes and facultative anaerobes, principally *Micrococcus lysodeikticus* (*luteus*) and *E. coli* (for reviews, see references 7, 343, 371).

The energized state is viewed by the chemiosmotic hypothesis as a transmembrane protonmotive force. In the previous section, we have described how this force can be produced through the action of proton-translocating respiratory chains; in this section, we will discuss the properties of bacterial ATPase complexes to see to what extent these are compatible with their postulated role as reversible, proton-translocating systems.

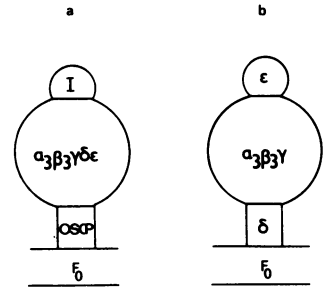


FIG. 8. *Diagrammatic representation of the various types of membrane-bound, energy-transducing ATPases. (a) Mitochondrial; (b) bacterial; $\alpha,\beta,\gamma,\delta$ and $\epsilon$ are the $F_1$ subunits, and I is the ATPase inhibitor protein.*

### Membrane-Bound ATPase Complexes

**Morphology and location.** Repeating structures of similar shape and size to the ATPase complex of the mitochondrial inner membrane have been observed in electron micrographs of negatively stained plasma membrane preparations from *M. lysodeikticus* (137, 283) and from several other species of bacteria (2, 21, 202). These structures were identified as ATPase complexes by their ability to form microscopically detectable conjugates with ferritin-labeled antibodies to purified ATPase. In addition, exposure of the membranes to low-ionic-strength buffers yielded smooth membranes which no longer reacted with these antibodies and which were essentially devoid of ATPase activity (309). The observations that protoplasts from *M. lyosdeikticus* failed to bind ferritin-labeled antibodies, whereas membrane vesicles concentrated them on one surface only, strongly supported the concept that in whole bacteria, as in mitochondria, the ATPase is located on the inner surface of the coupling membrane.

It is now generally accepted that intact bacteria, or spheroplasts and protoplasts prepared from them, do not readily hydrolyze ATP or utilize ADP as a phosphoryl acceptor for oxidative phosphorylation. However, when spheroplasts of *E. coli* were lysed by treatment with low concentrations of detergents or made more permeable by exposure to toluene, ATPase activity was substantially increased and was almost totally sensitive to inhibition by ATPase-specific antibodies (126, 402). These results clearly suggested that the ATPase is located on

the inner surface of the coupling membrane which, in contrast to the mitochondrial inner membrane, appears to lack an adenine nucleotide translocase.

In mitochondria, the latter effects the inward, electrogenic transport of one molecule of ADP concomitant with the ejection of one molecule of ATP (222), thus balancing the requirement of the ATP synthetase for a phosphoryl acceptor against that of the cytoplasm for energy. The absence of this transport system from the bacterial plasma membrane is clearly in accordance with the relatively self-contained existence of these predominantly free-living organisms, which are able to exchange nutrients and waste products, but not essential cofactors, with the surrounding medium.

If the orientation of the bacterial ATPase complex across the coupling membrane is indeed asymmetric, then it should be possible to isolate two pure species of topologically closed vesicles analogous to submitochondrial particles resulting from digitonin and sonic treatment (324), viz., right-side-out vesicles (in which the orientation of the membrane is the same as that present in the intact cell, such that the ATPase remains on the inner surface and is thus inaccessible to exogenous adenine nucleotides) and inside-out vesicles (in which the orientation of the membrane has become inverted, such that the ATPase is fully exposed). Both types of vesicles have now been detected in preparations of bacterial membranes, and there is also some evidence for the presence of scrambled vesicles, i.e., those in which the membrane of a single vesicle exhibits areas of right-side-out and inside-out orientation (160, 371).

In 1971, Kaback reported a method for the isolation of predominantly right-side-out vesicles from *E. coli*, in which spheroplasts prepared by lysosyme-ethylenediaminetetraacetic acid treatment were subsequently subjected to osmotic shock in the presence of potassium phosphate buffer (208). The resultant relatively large vesicles were osmotically sensitive and were thus judged to be essentially intact (209). Freeze-cleave electron microscopy indicated that the vesicles were almost completely in the right-side-out orientation (17, 209), although their relatively high sensitivity to agglutination by antibodies to purified ATPase has recently thrown some doubt on this conclusion (159). However, the predominantly right-side-out orientation of these vesicles was supported by their ability to exhibit respiration-linked proton ejection (328) and to drive the uptake of various nutrients at the expense of electron transfer to oxygen and other acceptors (40, 209); because of this latter property, they are often referred to as active transport vesicles. The observations that these vesicles usually hydrolyzed ATP rather slowly (221, 402, 410; but see reference 126), failed to utilize ATP as a source of energy for nutrient uptake (209), and did not catalyze oxidative phosphorylation (221) supported the concept that the ATPase was located on the inner surface of the vesicle membrane. Further support was added to this conclusion by the ability of Triton X-100 and other detergents to stimulate ATPase activity by destroying the membrane permeability barrier towards adenine nucleotides, thus allowing ATP to reach the inner surface (402, 410). In addition ATP-dependent amino acid uptake could be induced by osmotically shocking ATP into the vesicles (402). The results of similar experiments with osmotically (prepared vesicles from *Bacillus subtilis* (156, 230, 231) and protoplast ghosts from *Mycobacterium phlei* (21, 22) have suggested that these structures are also predominantly, but by no means completely, right-side-out and that the ATPase is located on the inner surface. A similar location for the ATPase in whole cells of *M. lysodeikticus* has also been deduced from studies on heterogeneous populations of right-side-out, inside-out, and scrambled vesicles from this organism (137, 309).

Disruption of whole cells, spheroplasts, or right-side-out vesicles of *E. coli* by sonication or by exposure to shear forces (e.g., in a French pressure cell or Ribi cell fractionator) yields a population of relatively small closed vesicles (17, 181, 259, 410); on the basis of evidence from freeze-cleave electron microscopy, these vesicles are predominantly in the inside-out configuration (17, 105). Biochemical examination indicated that they did not catalyze energy-dependent amino acid uptake at a significant rate (181, 259), although they readily catalyzed respiration-driven proton uptake (171), oxidative phosphorylation (259), ATP hydrolysis (181), and ATP-dependent transhydrogenation (157, 181); the latter two reactions were almost completely inhibited by antibodies to ATPase (157, 181). In contrast to right-side-out vesicles. ATPase activity was not stimulated by exposure to detergents (181). These results, together with those of less comprehensive experiments on similarly prepared vesicles from other bacteria, convincingly show that the ATPase is located on the outer surface of inside-out vesicles and thus confirm its location on the inner surface of the coupling membrane in whole cells.

**Proton-translocating properties.** There is

considerable experimental evidence to suggest that, in mitochondria and heterotrophic bacteria, two protons are extruded per pair of reducing equivalents transferred through each oxidation-reduction segment of the respiratory chain, although this stoicheiometry can be decreased by the use of certain artificial electron donors and acceptors, and it may also be low in some chemolithotrophs. Thus, in order to comply with the P/O ratios that have been observed with intact mitochondria, two protons must be retranslocated inwards for every molecule of ATP synthesized. Since the ATPase is reversible, the same stoicheiometry (but of opposite direction) should be observed during ATP hydrolysis. In accordance with this prediction, $\rightarrow H^+/P$ ratios ($=\rightarrow H^+/ATP$) of approximately 2 g ion of $H^+$ per mol of ATP hydrolyzed have been determined for mitochondria (276, 281, 391) and chloroplasts (63). Transmembrane proton translocation concomitant with ATP hydrolysis, albeit of unknown stoicheiometry, has also been detected in chromatophores from photosynthetic bacteria (356) and in reconstituted membrane vesicles from the thermophilic bacterium PS3 (380, 425).

The quantitative measurement of $\rightarrow H^+/P$ ratios associated with ATP hydrolysis is a technically difficult operation, since it is necessary to distinguish between that part of the total pH change that simply reflects ionization changes resulting from the chemical hydrolysis of ATP (up to 0.8 $H^+$ per molecule of ATP [15]) and that part which results from transmembrane proton movement and thus reflects the anisotropic properties of the ATPase. With bacterial systems, this problem is compounded by the absence of an ATP-ADP translocase (126, 159, 402), which makes the use of inside-out vesicles obligatory.

West and Mitchell (410) recently reported that ATP hydrolysis by inside-out vesicles of *E. coli* was accompanied by acidification of the external medium. However, when correction was made for the ionization effects of chemical hydrolysis, it was clear that the reaction was also accompanied by inwardly directed proton translocation (equivalent in intact cells to inward translocation during ATP synthesis). By comparing the initial rates of ATP hydrolysis and proton uptake, an $\rightarrow H^+/P$ ratio of approximately 0.6 g ion of $H^+$ per mol ATP hydrolyzed was obtained. Since this value was not corrected for the undoubted presence of imperfectly sealed vesicles (which would allow translocated protons to escape), the true $\rightarrow H^+/P$ ratio is likely to be considerably higher. Indeed, an $\rightarrow H^+/P$ ratio that is approximately equal to the $\rightarrow H^+/2e$ ratio for one oxidation-reduction segment (i.e. 2 g ion of $H^+$ per mol of ATP hydrolyzed) can be predicted from comparisons of molar growth yields of *E. coli* under aerobic and anaerobic conditions.

The inherent proton conductance of energy-coupling membranes is generally low (189, 270, 354), although it can be increased substantially by the addition of uncoupling agents or by the initiation of known proton-translocating events (e.g., the hydrolysis or synthesis of ATP and the transport of certain nutrients). It has been proposed that the transmembrane movement of protons concomitant with ATPase or ATP synthetase activity occurs via a specific channel in the $F_0$ complex of the coupling membrane (273). Removal of $F_1$, either by physically stripping it from the membrane or by genetic deletion, would therefore be expected to increase proton conductance by exposing the entrance to the proton-conducting channel; this would lead to a concomitant decrease in the efficiency of respiration-linked energy-dependent reactions. This prediction has been confirmed experimentally using $F_1$-depleted membrane vesicles from beef heart mitochondria (175, 271, 312); when the vesicles were subsequently treated with oligomycin of *N,N'* dicyclohexylcarbodiimide (DCCD) or reconstituted by the addition of $F_1$, their proton-conducting and energy-transducing properties were substantially repaired. Thus, these results support the idea that $F_1$ has a structural, as well as a catalytic, role in the membrane-bound ATPase; in the absence of $F_1$, this structural role can apparently be mimicked by oligomycin and DCCD which, by binding to the oligomycin-sensitivity-conferring protein (OSCP) and the DCCD-sensitivity-conferring protein (DSCP), respectively, presumably block the proton-translocating channel, which is postulated to be present in $F_0$, and thus prevent dissipation of the protonmotive force.

Similar conclusions have also been drawn from the results of analogous experiments using bacterial membranes which, as a result of genetic manipulation, have been depleted of $F_1$ or contain structurally defective of $F_1$ or $F_0$ components (see below). In this respect, it is interesting to note that the addition of purified $F_1$ from *S. faecalis* to artificial phospholipid bilayers causes a large increase in electrical conductance, which is claimed to be compatible with the formation of discrete, but relatively wide, aqueous-filled channels (327).

## Purification and Properties of the $F_1$-ATPase

**Isolation and general properties.** Bacterial ATPases, like those of mitochondria and chloro-

plasts, can be released from the coupling membrane with relative ease (the various methods are reviewed in detail in references 7 and 343). The most commonly used release procedure has been a shock-wash process in which L-forms of whole bacteria, spheroplasts, protoplasts, or isolated membrane preparations (3, 51, 94, 185, 226, 267, 269, 278, 284, 333, 409) were washed several times in $Mg^{2+}$-free, low-ionic-strength buffers [e.g., 2 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.2] (226), such that the ATPase was dissociated from the coupling membrane by the lack of cations. Alternative, and considerably more drastic, methods of release have involved extraction of the plasma membrane with organic solvents such as *n*-butanol (which caused the enzyme to accumulate in the aqueous phase) (344, 345) or dissociation of the membrane with detergents such as Triton X-100 or sodium dodecyl sulfate (109, 157). The solubilized enzyme was then purified by relatively standard procedures involving combinations of ammonium sulfate fractionation, ion exchange or exclusion chromatography, density gradient centrifugation, and polyacrylamide gel electrophoresis (51, 54, 226, 227, 269). The resultant purified ATPase was the headpiece ($F_1$) component of the original, membrane-bound ATPase complex, the membrane ($F_0$) components having been lost during the purification procedure.

Homogeneous preparations of purified $F_1$ have now been obtained from *S. faecalis* (5a, 352, 353), *B. megaterium* (267, 268, 269), *M. lysodeikticus* (283, 285, 344), *Bacillus stearothermophilus* (151), *Alcaligenes faecalis* (14), *Salmonella typhimurium* (54), the thermophilic bacterium PS3 (425), *M. phlei* (172), and from several strains of *E. coli* (54, 127, 134, 157, 294). They are all relatively large, multi-subunit proteins with molecular weights in the range 250,000 to 400,000 (Table 6) and are thus similar in size to the solubilized ATPases of mammalian and yeast mitochondria (molecular weight, 340,000 to 360,000 [69, 237, 362, 397]) and green plant chloroplasts (molecular weight, 325,000 [111]).

Analysis of $F_1$ from *S. faecalis* (353), *B. megaterium* (269), *B. stearothermophilus* (151), and *M. lysodeikticus* (343) showed that they contained very similar amino acid contents; all were fairly rich in acidic amino acids, and their content of hydrophobic residues was strikingly constant: approximately 32%. Kobayashi and Anraku (227) reported that *E. coli* $F_1$ contained 8 mol of phosphorus per mol of protein, of which only a small proportion could be attributed to phospholipids; the remainder probably reflected the presence of other tightly bound, phosphorous-containing compounds such as adenine nucleotides. ATP, ADP, and inorganic phosphate (in the molar ratios 1.0:1.0:0.1 per mol of enzyme) also appeared to be associated with the $F_1$ from *S. faecalis* (6). In this respect, these two bacterial enzymes resemble their counterpart from beef heart mitochondria,

TABLE 6. *Subunit size and composition of purified ATPases ($F_1$)*[a]

| Source | Mol wt | Subunit mol wt ($\times 10^{-3}$) | | | | | Subunit composition | References |
|---|---|---|---|---|---|---|---|---|
| | | $\alpha$ | $\beta$ | $\gamma$ | $\delta$ | $\epsilon$ | | |
| Rat liver mitochondria | 340-380 | 62.5 | 57 | 36 | 12 | 7.5 | $\alpha_3\beta_3\gamma\delta\epsilon$ | 68, 69 |
| | | 53 | 50 | 28 | 12.5 | 7.5 | $\alpha_3\beta_3\gamma\delta\epsilon$ | 237 |
| Beef heart mitochondria | | 53 | 50 | 25 | 12.5 | 7.5 | $\alpha_3\beta_3\gamma\delta\epsilon$ | 361, 362 |
| | | | | | | | $\alpha_2\beta_x\gamma_z\delta y\epsilon_2$ | 359 |
| Yeast mitochondria | 340 | 58 | 54 | 38 | 31 | 12 | | 397 |
| Spinach chloroplasts ($CF_1$) | 325 | 59 | 56 | 37 | 17.5 | 13 | $\alpha_2\beta_2\gamma\delta\epsilon_2$ | 39, 111, 245, 246, 293 |
| *Escherichia coli* | | 56.8 | 51.8 | 32 | 20.7 | 13 | $\alpha_3\beta_3\gamma\delta\epsilon$ | 54 |
| | 400 | 54 | 52 | 33 | | 11 | | 226,227 |
| | 360 | 60 | 56 | 35 | | 13 | | 157 |
| | 340 | 56 | 52 | 32 | 21 | 11.5 | $\alpha_2\beta_2\gamma_2\delta_{1-2}\epsilon_2$ | 408 |
| *Salmonella typhimurium* | | 56.8 | 51.8 | 30.9 | 21.5 | 13.2 | $\alpha_3\beta_3\gamma\delta\epsilon$ | 54 |
| *Alcaligenes faecalis* | 350 | 59 | 54 | 43 | | 12 | | 12, 13, 14, (see also [313]) |
| Thermophilic bacterium PS3 ($TF_1$) | 380 | 56 | 53 | 32 | 11 | 15.5[c] | | 380, 425 |
| *Micrococcus lysodeikticus* | | 62 | 60 | —[b] | | | | 344 |
| | 345 | 52.5 | 47 | 41.5 | 28.5 | | $\alpha_3\beta_3\gamma\delta$ | 19, 20 |
| *Streptococcus faecalis* | 385 | 60 | 55 | 37 | 20 | 12 | $\alpha_3\beta_3\gamma\delta\epsilon$ | 5a, 352, 353 |
| *Bacillus megaterium* | 399 | 68 | 65 | | | | $\alpha_3\beta_3$ | 268, 269 |

[a] The purified ATPases from *B. stearothermophilus* (molecular weights, 280,000 [151]) and *M. phlei* (molecular weight, 250,000 [172]) are not included in this table, since little is known of their subunit composition.

[b] Several additional, minor bands are present in shock-wash preparations which are not present after extraction with *n*-butanol (123, 344).

[c] The authors refer to this subunit as $\delta'$ rather than $\epsilon$ (425).

which contains 3 mol of tightly bound ATP and 2 of ADP per mol of enzyme (163); it is possible that these bound nucleotides play an important role during ATP synthesis (45). Some controversy exists over the phospholipid content of purified $F_1$; phospholipids were detected in the enzyme from *E. coli* (315) but not in that from *M. lysodeikticus* (285). In the former case, removal of phospholipid by centrifugation or ion exchange chromatography caused a decrease in ATPase activity, which could be partly restored by the addition of a phospholipid extract from the whole organism.

The ATPase activities of bacterial $F_1$ preparations were extremely variable, although they were generally higher than those of the membrane-bound ATPase complexes from which they were derived. All of the purified ATPases were found to be dependent upon divalent metal ions for maximum activity; this requirement was usually satisfied by either $Mg^{2+}$ or $Ca^{2+}$, although, in the case of *S. faecalis* $F_1$, only $Mg^{2+}$ was effective (3). $Na^+$ or $K^+$ appeared to exert little stimulatory action upon the solubilized ATPases (94, 226), although they occasionally stimulated the membrane-bound enzymes to a variable extent (3, 152, 409).

The ability of bacterial $F_1$ to hydrolyze nucleotides is not limited to ATP. Indeed, all of the purified $F_1$ preparations so far examined showed substantial activity with guanosine 5'-triphosphate (GTP) and also, to a lesser extent, with uridine 5'-triphosphate (UTP), and cytidine 5'-triphosphate (CTP). *B. megaterium* $F_1$ was unusual in that it exhibited as high an activity with inosine 5'-triphosphate (ITP) as with ATP (267); the *E. coli* enzyme hydrolyzed both ITP and ADP at a significant rate (94, 157), and the *S. faecalis* $F_1$ hydrolyzed ITP and deoxyATP (7). None of the other purified bacterial enzymes showed significant activity with ITP, ADP, AMP, or $P_i$ (see reference 343). All of the purified ATPases investigated exhibited Michaelis-Menten kinetics with respect to ATP concentration. There is some evidence that both bacterial and mitochondrial $F_1$ preparations have significantly lower $K_m$ values for ATP than do the corresponding membrane-bound complexes (155, 157, 277, 333, 352, 353), which suggests that the two forms exist in different conformations; this conclusion is supported by evidence from electron microscopy. Those $F_1$ preparations that have been investigated for evidence of product inhibition all showed a loss of activity with increasing concentrations of ADP and $P_i$. The enzymes from *S. faecalis* (353), *E. coli* (157, 226, 333), and *M. phlei* (172) were all inhibited competitively by ADP and were inhibited either competitively or noncompetitively by $P_i$.

In striking contrast to the membrane-bound, bacterial ATPase complexes, most of the $F_1$ preparations examined so far have been found to be cold labile (i.e., activity was rapidly lost after storage at 0 to 4°C (157, 227, 267); this property is shared with motochondrial $F_1$ (314), but not with the purified ATPases from PS3 and *M. phlei* (150, 153). The precise molecular mechanism of cold-lability is not known, although there is good evidence that the ATPases from *E. coli* (157, 227, 408) and beef heart mitochondria (314) undergo dissociation into smaller fragments with, in the latter case, concomitant release of the bound adenine nucleotides. The cold-lability of the *E. coli* $F_1$ can be prevented by storage in the presence of high concentrations of glycerol or methanol, which presumably help to maintain the quaternary structure of the enzyme (226, 227, 408); this protective action is nullified by high salt concentrations (e.g., 0.5 M Tris-hydrochloride or 2 mM Tris-hydrochloride plus 0.5 M NaCl) (227).

Further differences between purified and membrane-bound ATPases are exemplified by their differential sensitivities to inhibitors and uncoupling agents. Thus, the ATPase activity of bacterial $F_1$ preparations, in contrast to that of the membrane-bound enzymes, is neither stimulated by uncouplers nor inhibited by DCCD (134, 162, 226, 333), although it remains sensitive to azide, since the latter binds to the headpiece of the enzyme complex rather than to a component of the membrane-located $F_0$; neither the purified or membrane-bound bacterial ATPases are inhibited by oligomycin. This phenomenon, in which a purified soluble enzyme exhibits a whole range of different properties compared with its membrane-bound counterpart, is called allotopy (324) and is apparently common to all energy-transducing ATPases.

**Subunit composition.** All the purified bacterial ATPases that have so far been examined can be dissociated into at least two nonidentical subunits after exposure to polyacrylamide gel electrophoresis in the presence of low concentrations of sodium dodecyl sulfate (see example, reference 227). Subunit stoicheiometry is subsequently determined either by colorimetric analysis of the stained gels or by radioactive analysis of the gels after electrophoresis of the $^{14}C$-labeled enzyme.

(i) ***E. coli* and *S. typhimurium*.** It has recently been reported that the purified ATPases from *E. coli* (54, 127, 408) and *S. typhimurium* (54) contain five different subunits (termed $\alpha$, $\beta$, $\gamma$, $\delta$, and $\epsilon$, in order of decreasing molecular

weight; see Table 6); other reports have concluded that the enzyme lacks a δ subunit (157, 227). These differences have been resolved by Futai et al. (127), who showed that, by slightly modifying the isolation-purification procedure that normally yielded an ATPase containing four different subunits, it was possible to produce an enzyme that also contained the δ subunit. However, it has been claimed that different strains of *E. coli* consistently yield purified ATPases with either four or five subunits, regardless of the extraction and purification procedures employed (371). The presence or absence of the δ subunit in the purified enzyme, therefore, probably reflects the strength with which the polypeptide is bound to the remainder of the ATPase molecule. Since the δ subunit appears to be necessary for energy coupling (see below), there seems to be little question that the $F_1$ from these organisms is comprised of five different subunits in vivo (Fig. 8).

Until very recently, it appeared that the $F_1$ from *E. coli*, *S. typhimurium*, and mammalian mitochondria exhibited similar subunit stoicheiometries, viz., $\alpha_3\beta_3\gamma\delta\epsilon$ (54, 68, 69, 237, 361, 362). However, after a quantitative analysis of the sulfhydryl groups and disulfide bonds found in beef heart $F_1$ and its constituent subunits, Senior has recently suggested the stoicheiometry $\alpha_2\gamma_2\epsilon_2$ *plus* unknown proportions of the β and δ components (359). Furthermore, the stoicheiometry $\alpha_2\beta_2\gamma_2\delta_{1-2}\epsilon_2$ has been proposed for *E. coli* $F_1$ (408). It is possible, therefore, that these enzymes are structurally even more closely related to chloroplast $CF_1$ (which is aggregated in the subunit ratio $\alpha_2\beta_2\gamma\delta\epsilon_2$ [293]) than was originally thought likely, but this obviously requires more extensive investigation. The purified ATPase from the thermophilic bacterium PS3 also has five subunits (α, β, γ, δ, and δ'), albeit of unknown stoicheiometry; the fifth subunit is referred to as the δ' component, since it is rather larger than the ε subunit of other ATPases. Because of its thermal stability (temperature maximum, 70°C), the ATPase of PS3 is usually referred to as $TF_1$ (380, 425).

It is clear that both purified and membrane-bound ATPase from various sources exhibit some degree of latency, i.e., their ATP-hydrolyzing activities can be stimulated by a variety of chemical or physical treatments. Thus, the ATPase activity of coupling membranes from eukaryotes appears to be at least partly controlled by a trypsin-sensitive component that may or may not be part of the purified ATPase, depending upon the isolation procedure employed. In chloroplasts, this role appears to be played by the ε subunit of $CF_1$ (295), whereas in mitochondria an additional, loosely bound component (the ATPase inhibitor protein) is probably responsible (180, 323, 325, 361; but see also reference 224). Evidence is now accumulating that *E. coli* $F_1$ also contains a trypsin-sensitive ATPase inhibitor. Thus, Bragg and Hou (54) observed that maximum activation of *E. coli* $F_1$ occurred when trypsin had digested all of the δ and ε subunits, together with over 70% of the γ subunit; the α and β components were little affected. It was concluded from the results of this and similar experiments (294) that one or more of the smaller subunits (γ, δ, or ε) partially masks the ATPase activity of the enzyme, whereas the larger α and β subunits have a catalytic role. Further support for the presence of an ATPase inhibitor in *E. coli* $F_1$ has been provided by Nieuwenhuis et al. (300), who reported that the activity of a solubilized ATPase from this organism was irreversibly inhibited by high concentrations of urea (probably as a result of the complete dissociation of the enzyme into its constituent subunits) and that the inactivated enzyme strongly inhibited the ATPase activity of both untreated and trypsin-activated $F_1$. These workers also isolated a crude protein fraction (molecular weight, 12,000) from *E. coli* $F_1$ which potently inhibited the ATPase activity of the intact enzyme. Although it has not been unequivocably identified as such, circumstantial evidence suggests that the active component of this fraction is the ε subunit. Thus, the ATPase inhibitor protein from *E. coli* appears to more closely resemble its counterpart in chloroplasts rather than in mitochondria. This view is reinforced by the observation that the ATPase inhibitor can be removed from submitochondrial particles by passage over a Sephadex G-50 column (325), whereas the inhibitor proteins of the *E. coli* and chloroplast ATPases are resistant to this treatment and are therefore presumably bound relatively strongly to the remainder of the enzyme.

The postulate that the α and β subunits of *E. coli* $F_1$ have a catalytic function is supported by the work of Gutnick and his colleagues (213, 294), who observed that antibodies prepared against the combined α and β components of *E. coli* $F_1$ completely inhibited ATP-linked transhydrogenation (without having any effect on the respiration-linked reaction), ATP hydrolysis, and ATP-$P_i$ exchange in *E. coli* membranes. Antibodies against the α subunit alone were also strongly inhibitory, but those prepared against the β or γ subunits were much less effective. It is likely, therefore, that the α and β subunits of *E. coli* $F_1$, like those of mito-

chondrial $F_1$ (229) and chloroplast $CF_1$ (98), play a central catalytic role in ATP-dependent energy-coupling reactions, as well as in oxidative phosphorylation.

It is clear that the $\delta$ subunit of the *E. coli* ATPase is not required for ATP hydrolysis, since its removal from the enzyme (after treatment of the latter with trypsin) was accompanied by an increase rather than a decrease in hydrolytic activity (54). On the other hand, there is reasonably good evidence that the $\delta$ subunit facilitates the binding of $F_1$ to the coupling membrane and is therefore required for oxidative phosphorylation and ATP-dependent energy transduction; this is based upon the observations that only the five-subunit $F_1$ was able to reconstitute ATP-dependent transhydrogenation when added to $F_1$-deficient membranes (48, 127, 294) and that the inactive four-subunit preparation could be reactivated by the addition of a combined $\delta$, $\epsilon$ subunit fraction (378). It appears, therefore, that the $\delta$ subunit of the *E. coli* ATPase is functionally analogous to the OSCP of mitochondria (in the sense that it acts as the stalk component of the entire complex), although it does not, of course, confer sensitivity to oligomycin. In contrast, the $\delta$ subunit of chloroplast $CF_1$ does not appear to have a binding function (295) and the role of this subunit in eukaryotic ATPases is unclear.

Little is known about the function of the $\gamma$ subunit, although it has been suggested that in mitochondrial $F_1$ it serves to bind the $\delta$ and $\epsilon$ subunits together to form a composite, "super-subunit" of a size comparable with each of the $\alpha$ and $\beta$ subunits (229). There is some evidence from the use of an *E. coli* mutant which contains an ATPase with a defective $\gamma$ subunit, that the presence of the latter is obligatory for effective energy transduction in this organism, although it is apparently not essential for ATPase activity (48) (see below).

(ii) ***A. faecalis.*** The purified ATPase from *A. faecalis* appears to contain only three subunits ($\alpha$, $\beta$, $\gamma$) plus a fourth component which, solely on the basis of its very low molecular weight, must be classed as the $\epsilon$ rather than $\delta$ subunit (14); the relative proportions of the constituent subunits are not known. It is possible, by analogy with *E. coli,* that in vivo the enzyme also contains a weakly bound $\delta$ subunit which is lost during the purification procedure, but there is currently no experimental evidence to support this. The hydrolytic activity of the *A. faecalis* enzyme is strongly enhanced by trypsin, thus supporting the concept that one of the subunits acts as an ATPase inhibitor.

(iii) ***M. lysodeikticus.*** The properties and subunit composition of the $F_1$ from *M. lysodeikticus* appear to depend very much upon the procedures employed for its extraction and purification. Thus, the enzyme exhibited the stoicheiometry $\alpha_3\beta_3$ when isolated using *n*-butanol (344), but the gentler shock-wash procedure yielded a preparation that contained two additional, smaller polypeptides (19, 20, 123, 344), viz., a loosely bound component ($\gamma$) and an integral subunit ($\delta$), to give the overall stoicheiometry $\alpha_3\beta_3\gamma\delta$; no $\epsilon$ subunit was detected in either of these preparations. The observations that both membrane-bound and crude, soluble ATPases from *M. lysodeikticus* could be activated by trypsin, whereas both preparations of pure $F_1$ were insensitive to this enzyme (19, 20, 239, 285), suggested that the trypsin-sensitive component was lost during purification; it is likely that this is the $\epsilon$ subunit. Similarly, since only the ATPase purified from shock-wash extracts was capable of binding to respiratory membranes, it is possible that either the $\gamma$ or, more probably, the $\delta$ subunit attaches the $F_1$ to the membrane in vivo.

(iv) ***S. faecalis.*** Until very recently, the $F_1$ from *S. faecalis* was thought to consist only of $\alpha$ and $\beta$ subunits, plus an additional polypeptide called nectin (Latin, *nectere* = to bind) (4, 33, 353). However, it has now been reported that two minor subunits ($\delta$ and $\epsilon$) are also present such that the enzyme has the overall composition $\alpha_3\beta_3\gamma\delta\epsilon$ (5a) and is thus similar to the $F_1$ from most other bacterial and mitochondrial sources. Interestingly, when the enzyme is isolated and purified in the absence of magnesium ions, it appears to lack the $\delta$ subunit and is no longer capable of binding to $F_1$-depleted membranes. It has been concluded therefore that $Mg^{2+}$ is probably an integral component of the enzyme, since it is apparently required for the attachment of the $\delta$ subunit to the remaining polypeptides. Furthermore, it is likely that the $\delta$ subunit is involved in binding the enzyme to the receptor site on the coupling membrane (either alone or in association with $Mg^{2+}$); this subunit thus has a similar role in the $F_1$ from both *S. faecalis* and *E. coli*. The earlier report that nectin is involved in the binding of $F_1$ to the membrane (33) has been rationalized by the claim that it may be a functionally active dimer of the $\delta$ subunit (5a).

(v) ***B. megaterium.*** The ATPase that Mirsky and Barlow (269) purified from *B. megaterium* after shock-wash treatment of cytoplasmic membranes contained only $\alpha$ and $\beta$ subunits (in the ratio $\alpha_3\beta_3$) and was apparently devoid of additional polypeptide components. However, in view of the complex subunit composition of

all the other bacterial ATPases so far investigated, it is likely that minor polypeptides are present in *B. megaterium* $F_1$ in vivo but are lost during the purification of the enzyme.

**Functional organization of subunits.** The chemical properties and intermolecular arrangement of the constituent subunits of bacterial $F_1$ are currently receiving increasing experimental attention, since only by resolving these problems can the mode of action of these complex enzymes be properly understood.

Central to these studies is the use of chemical-modifying agents that inhibit ATPase activity by forming covalent linkages with specific amino acid residues and thus yield information on the possible involvement of these residues in the active site of the enzyme or in the binding adjacent subunits. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) has been used extensively for this purpose by Radda and his colleagues (113–115). This reagent reacts with the phenolic oxygen of tyrosine and with the sulfhydryl group of cysteine; the two reactions can be distinguished from the different absorption spectra of the resultant adducts (1, 115) or by comparing the observed inhibition with that caused under similar conditions by *N*-ethyl maleimide or iodacetamide, both of which react only with cysteine (113).

NBD-Cl readily inhibited the ATPase activity of $F_1$ from beef heart mitochondria (115) and *E. coli* (294). In both cases, the inhibition was overcome by treating the covalent enzyme-inhibitor complex with the sulfhydryl reagent dithiothreitol to remove the inhibitory group. There is good evidence that NBD-Cl reacts with a tryosine residue of mitochondrial $F_1$ (114, 115), and this is probably also true for the *E. coli* enzyme; in the latter case, the site of inhibition was identified as the $\beta$ subunit through the use of $^3$H-labeled NBD-Cl (294). This reagent also inhibited ATP hydrolysis and ADP-dependent respiratory control by respiratory membranes from *P. denitrificans* (113); modification of a tyrosine residue again appeared to be responsible. Since these effects were reversed by the relatively nonpenetrating sulfhydryl reagent glutathione (as well as by dithiothreitol), it was concluded that the reactive tyrosine residue(s) was not buried within the ATPase. These experiments thus strongly implicate tyrosine in the active site of the *P. dentrificans* ATPase.

The problem of determining the arrangement of the subunits in bacterial ATPase has been tackled principally via electron microscopy and, more latterly, through the use of protein cross-linking reagents. The former has been applied with some success to purified $F_1$ from *M. lysodeikticus* (283), *B. megaterium* (185), and *S. faecalis* (353), all of which appear as planar hexagonal arrangements of six subunits that surround any other polypeptides that might be present. In contrast, Bragg and Hou have recently used protein cross-linking reagents, particularly dithiobis (succinimidyl propionate; DSP), to examine the architecture of the purified, five-subunit ATPase from *E. coli* (54). This reagent cross-links the $\epsilon$-amino groups of lysine residues and thus can be used to investigate the proximity and location of such groups within the same and adjacent subunits; very usefully, the cross-linkage can subsequently be cleaved by reduction with mercaptoethanol or dithiothreitol. Exposure of *E. coli* $F_1$ to DSP resulted in the rapid loss of ATP-hydrolyzing activity, the partial disappearance of the $\alpha$ and $\beta$ subunits, and the concomitant formation of a new, high-molecular-weight component (Y) plus traces of other, even larger components; the $\gamma$, $\delta$ and $\epsilon$ subunits disappeared completely (54). Treatment of Y with dithiothreitol regenerated $\alpha$ and $\beta$ subunits in equal proportions, whereas treatment of the larger adducts yielded $\gamma$ subunits in addition to the $\alpha$ and $\beta$ components. These results suggested that the latter were arranged within the enzyme in such a manner that they could easily be cross-linked without the concomitant formation of $\alpha\alpha$ or $\beta\beta$ dimers, and they therefore support the concept of alternating subunits arranged in the form of a hexagon. The ability of the $\gamma$ subunit to cross-link with $\alpha$ and $\beta$ subunits supports its location in the central hole of the hexagon. Furthermore, the fact that an *E. coli* ATPase can be isolated which is deficient in the $\delta$ subunit (48, 157, 294, 336) indicates that the latter is not necessary for attaching the $\gamma$ and $\epsilon$ components to the remainder of the molecule, although it is clearly required to attach $F_1$ to the coupling membrane.

Two models have recently been proposed by Kozlov and Mikelsaar (229) for the structure of $\alpha_3\beta_3\gamma\delta\epsilon$ ATPases. The first envisages that alternating $\alpha$ and $\beta$ subunits form the outer hexagonal structure and that the $\gamma$, $\delta$, and $\epsilon$ subunits comprise the center of the hexagon; the second suggests that a planar trimer of $\beta$ subunits is packed above a similar arrangement of $\alpha$ subunits, and that the remaining components comprise a stalk that presumably assists in the attachment of the enzyme to the coupling membrane. A slightly modified version of the first model is fully compatible with the chemical and morphological properties of *E. coli* $F_1$ with the subunit ratio $\alpha_3\beta_3\gamma\delta\epsilon$ (54) (Fig. 9a). In contrast,

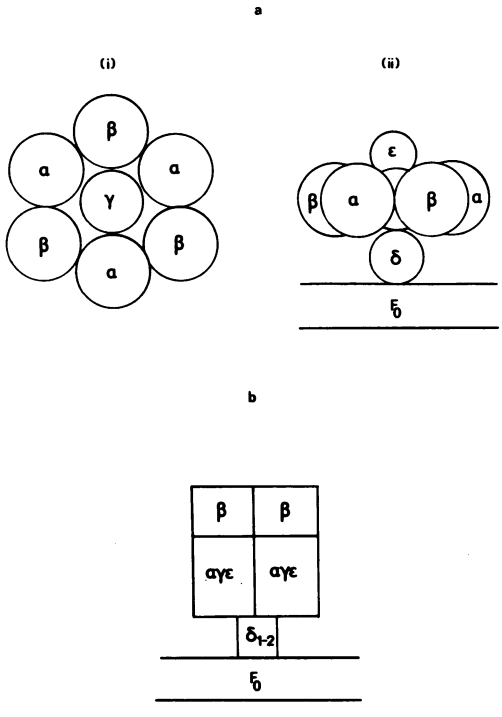


FIG. 9. *Possible arrangement of subunits in the $F_1$ of E. coli. (a) Assuming the structure $\alpha_3\beta_3\gamma\delta\epsilon$, (i) transverse section through the $\alpha_3\beta_3$ hexagon (ii) side view (after Bragg and Hou [54]); (b) assuming the structure $\alpha_2\beta_2\gamma_2\delta_{1-2}\epsilon_2$ (after Vogel and Steinhart [408]).*

neither model can explain the behavior of *E. coli* $F_1$ as reported by Vogel and Steinhart (408). This enzyme was dissociated by freezing and thawing into two major subunits of dissimilar size, namely, $I_A$ (molecular weight, 100,000; subunits $\alpha\gamma\epsilon$) and II (molecular weight, 54,000; subunit $\beta$), but could be reconstituted to show ATPase activity by mixing $I_A$ and II in equimolar proportions. These results confirmed the theory that both $\alpha$ and $\beta$ subunits were required for catalytic activity and were considered to be commensurate with the stoicheiometry $\alpha_2\beta_2\gamma_2\delta_{1-2}\epsilon_2$, the subunits being arranged as shown in Fig. 9b.

## Purification and Properties of the $F_0$-$F_1$ Complex

Although the membrane-bound ATPases from mitochondria and bacteria are readily inhibited by DCCD and other carbodiimides, purified $F_1$ preparations are insensitive to this type of inhibitor (36, 162, 333). This observation indicates that the DCCD-sensitive component is not one of the $F_1$ subunits, but is presumably a membrane component of the ATPase complex that is eliminated during the solubilization-purification procedure. In confirmation of this, Beechey and his colleagues have shown that DCCD binds covalently to a proteolipid component, DSCP (molecular weight, 10,000), of the mitochondrial $F_0$ complex (36, 66, 67, 330). A second major component of mitochondrial $F_0$, OSCP, is apparently absent from bacterial ATPases and its structural role, at least in the more complex bacterial enzymes, is probably taken over by the $\delta$ subunit of $F_1$ (see above). Resolution of the component proteins of $F_0$, and of the structural relationship between $F_0$ and $F_1$, are fundamental prerequisites to a proper understanding of the mode of action of reversible ATPases, particularly since $F_0$ is probably involved in the transfer of protons across the coupling membrane and is thus intimately associated with the mechanism of energy transduction.

Several attempts have recently been made to isolate a DCCD-sensitive ATPase complex (i.e., an $F_0$-$F_1$ complex) from bacterial systems. By solubilizing respiratory membranes of *E. coli* with low concentrations of cholate or deoxycholate, Neiuwenhuis et al. (302) isolated a low-activity ATPase that showed some sensitivity to DCCD. This crude enzyme preparation, which still contained a substantial amount of cytochrome, was activated severalfold by the addition of soybean phospholipids, which also rendered it almost completely sensitive to DCCD. Hare (158) reported generally similar results using a partially purified, deoxycholate-solubilized ATPase complex from the same organism and attributed the stimulatory and sensitizing properties of the added phospholipids (of which phosphatidyl choline, phosphatidyl serine, and phosphatidyl ethanolamine were the most potent) to their abilities to replace those that were lost during the solubilization procedures. It is interesting to note that phospholipids are also required for maximal ATPase activity in mitochondrial and other bacterial $F_0$-$F_1$ preparations (210, 380, 425) and possibly also in purified $F_1$ (315).

Detailed investigations of the composition of the partially purified, DCCD-sensitive ATPase complex from *E. coli* indicated the presence of 12 polypeptide components, 5 of which were identified as the $\alpha$ to $\epsilon$ subunits of $F_1$, since they comigrated with the purified subunits on sodium dodecyl sulfate-polyacrylamide gels and were immunoprecipitated by $F_1$-specific antibody (158). The latter also precipitated two other polypeptides, $\zeta$ (molecular weight, 29,000)

and $\eta$ (molecular weight, 9,000), of which $\eta$ was identified as the DCCD-sensitivity-conferring protein (DSCP) through its ability to form a covalent adduct with $^{14}$C-labeled DCCD (117, 118, 158); other workers have claimed a molecular weight of 12,000 to 13,000 for DSCP (18). The nature and function of the five remaining components are unclear.

A DCCD-sensitive ATPase complex has also been purified from the thermophilic bacterium PS3 after extraction of its membranes with Triton X-100 (380, 425). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of this complex yielded only eight subunits, five of which corresponded to the subunits of $TF_1$; the three remaining $TF_0$ components were hydrophobic and exhibited molecular weights of 19,000, 13,500, and 5,400. No attempt was made to determine which of the $TF_0$ subunits was responsible for binding DCCD. The addition of phospholipids to the $TF_0$-$TF_1$ complex stimulated its ATPase activity and also caused the formation of inside-out membrane vesicles. The latter exhibited ATP-dependent energization (as measured by the quenching of 1-anilinonaphthalene-8-sulfonate fluorescence) and also catalyzed the uptake of protons concomitant with ATP hydrolysis; both of these reactions were abolished by DCCD and uncoupling agents, and neither reaction was carried out by the nonvesicular $TF_0$-$TF_1$ complex. It was later shown (426) that vesicles reassembled from phospholipids, $TF_0$-$TF_1$ and purple membranes (bacteriorhodopsin) from *Halobacterium halobium* exhibited light-dependent ATP synthesis that was sensitive to DCCD and uncoupling agents. These elegant experiments thus show that a relatively simple, eight-subunit ATPase complex can catalyze a two-way translocation of protons across the coupling membrane that is sufficient to drive ATP synthesis in one direction and membrane energization at the expense of ATP in the other.

DCCD-resistant mutants of *E. coli* (RF-7) and *S. faecalis* (sf-dcc-8) have recently been isolated after selection for growth on plates containing growth medium supplemented with normally lethal concentrations of DCCD (8, 117). Both mutants were unimpaired with respect to energy transduction, as evidenced by their normal growth characteristics and by their capacity for catalyzing ATP-dependent membrane reactions, but their membrane-bound ATPases were up to 100-fold less sensitive to inhibition by DCCD than were those from the wild-type organisms.

Abrams and his colleagues (5, 162) have reported that the inhibition of the membrane-bound ATPase of *S. faecalis* by DCCD and other carbodiimides probably occurs via the formation of a covalent compound between the inhibitor and an undissociated carboxyl group on a so-called carbodiimide-sensitizing factor (CSF), which was tentatively identified as the $F_1$-binding protein, nectin (33). However, in later experiments, the location of CSF was more precisely identified by measuring the sensitivity to DCCD of hybrid ATPase complexes reassembled from $F_1$, nectin, and depleted membranes isolated from mutant and wild-type cells (8). It was observed that the ATPase complex that had been reassembled from mutant $F_1$, mutant nectin, and wild-type membranes was sensitive to DCCD, whereas any complex that contained mutant membranes was insensitive to this inhibitor. The results of these elegant experiments thus indicated that DCCD sensitivity was a property of the coupling membrane, rather than of nectin as previously claimed, and that the mutation was probably expressed as a defective CSF.

By similarly reassembling ATPase hybrids of *E. coli* using components isolated from the wild-type and DCCD-resistant (RF-7) strains, Fillingame showed that the sensitivity of this organism to DCCD was also a property of its membrane fraction (117, 118). Furthermore, since membranes from this mutant and from *E. coli* mutant K-12DG7/1 (18) both contained a DSCP that was no longer capable of binding [$^{14}$C]DCCD, it was concluded that DCCD resistance was caused by an alteration in the structure of this proteolipid such that its functional groups were no longer accessible to the inhibitor. The mutation in RF-7 was mapped at approximately 73.5 min on the chromosome and was 90% cotransducible with the *uncA* gene (see below).

There is, therefore, general agreement that DCCD sensitivity requires the presence of a competent, DCCD-binding proteolipid (DSCP, CSF) in the $F_0$ complex of the membrane-bound ATPase, although preliminary experiments with some energy-transduction mutants of *E. coli* suggest that a second component may also be required (213). In the light of these results, the observed sensitivities of the solubilized (but not purified) ATPases of *Proteus* $P_{18}$ L-form (278) and *E. coli* (109) to DCCD implies that they also contain associated membrane proteins which presumably include DSCP.

### Mutants Defective in Energy Transduction

The problem of determining the physiological functions of the individual polypeptide compo-

nents of $F_1$ and $F_0$ has recently been approached through the isolation and analysis of mutant strains that are defective in energy transduction. Because of its ready susceptibility to genetic manipulation, *E. coli* has been used almost exclusively in these studies, and two general classes of mutants have been isolated from this organism which appear to be useful in this respect: *unc*$^-$ ATPase$^-$ and *unc*$^-$ ATPase$^+$ (317). Phenotypically, both classes grow aerobically on glucose but not on nonfermentable carbon sources such as succinate; aerobic molar growth yields with respect to glucose consumption ($Y_{glucose}$) are midway between those exhibited by the wild-type organism under aerobic and anaerobic conditions. These growth patterns are compatible with genetic lesions resulting in defective ATPase complexes such that both classes of mutants are uncoupled, i.e., they are defective in oxidative phosphorylation, but their capacity for respiration is unimpaired.

The properties of energy-coupling membranes prepared from these mutants have been investigated mainly by detailed analysis of the following reactions: (i) ATP hydrolysis (ATPase activity); (ii) ATP synthesis via oxidative phosphorylation; (iii) transhydrogenation or active transport at the expense of energy derived from ATP hydrolysis or respiration; (iv) energization of the membranes by ATP hydrolysis or respiration, as measured by the quenching of fluorescent dyes such as 9-amino-6-chloro-2-methoxyacridine (ACMA) or atebrin. Additional information has also been obtained by measuring the sensitivities of the above reactions to inhibition by DCCD and from attempts to reconstitute hybrid ATPase complexes from mutant and parental components. However, not all of these assays have been applied to each *unc*$^-$ mutant, and many of these strains are as yet incompletely characterized with respect to their energy-transducing properties. As a result, it is often extremely difficult to make a useful comparison of closely related mutants or to relate with any confidence their phenotypic properties to a defect in an individual polypeptide subunit of the ATPase complex.

***unc*$^-$ ATPase$^-$ mutants.** Several mutants of *E. coli* that fall into the *unc*$^-$ ATPase$^-$ category have been investigated in some detail: *uncA* (strain AN 249) and *unc-405* (strain AN 285) (79, 81, 82), various MDA strains (93), *unc-17* (393, 422), $N_{144}$ (212, 301), DL-54 (16, 52, 359), NR-70 (335, 336), NR-76 (337) and *uncA103c* (350). Generally speaking, membrane vesicles prepared from these mutants failed to catalyze ATP hydrolysis, oxidative phosphorylation, or ATP-dependent reactions such as transhydrogenation or active transport. On the other hand, respiration and respiration-linked active transport or transhydrogenation were generally unimpaired, although there were a number of exceptions to this. Thus, respiration-linked active transport was defective in membrane vesicles from DL-54, $N_{144}$, and NR-70 (16, 335, 336), transhydrogenation was of only low activity in DL-54 (52), and membrane energization as measured by the quenching of ACMA fluorescence was defective in $N_{144}$ (301); however, in all cases, the defective reactions could be largely restored by the addition of DCCD or normal $F_1$ to the membranes.

It is clear from these observations that *unc*$^-$ ATPase$^-$ mutants cannot use ATP as a source of energy for membrane-associated reactions, although they can still, with some exceptions, use the high energy state that is produced at the expense of electron transfer. This is nicely illustrated by the reports that whole cells of *unc-17*, maintained under anaerobic conditions in the presence of glucose, failed to exhibit their expected motility or to catalyze either active transport or DNA synthesis, unless also supplied with an electron acceptor such as oxygen or nitrate (258, 393, 422). Thus, the growth of *unc*$^-$ ATPase$^-$ strains on glucose (but not succinate) is possible because substrate-level phosphorylation provides ATP, whereas electron transfer to oxygen or nitrate furnishes the energized state that is necessary for driving essential energy-dependent membrane functions such as active transport. *Unc*$^-$ ATPase$^-$ strains will not grow anaerobically on glucose in the absence of an added electron acceptor.

Much of the work that has been carried out with these mutants has been directed towards determining the nature of the ATPase$^-$ lesion: e.g., do these mutants lack the ability to hydrolyze ATP because the ATPase is absent from the coupling membrane or because the enzyme, although bound to the membrane, is defective in catalysis? In this respect, Gibson and his colleagues have obtained compelling evidence that the *uncA* lesion is expressed as a defective membrane-bound $F_1$, since agarose-gel chromatography of a low-ionic-strength wash from *uncA* membranes yielded a protein peak that was characteristic of $F_1$ yet showed no ATPase activity (79). Furthermore, both oxidative phosphorylation and various ATP-dependent reactions could be reactivated in ATPase-depleted *uncA* membranes by the addition of $F_1$ from the wild-type organism or from an *unc*$^-$ ATPase$^+$ mutant (79, 81). Since $F_1$ from either of these two sources failed to reactivate intact *uncA*

membranes, it was concluded that the defective $F_1$ was still attached to these membranes and occupied the available binding sites. In contrast, the low-ionic-strength wash from membranes of *unc-405* contained no protein peak corresponding to $F_1$, and both washed and intact membrane preparations could be reactivated by the addition of parental $F_1$; it was concluded, therefore, that this mutant had probably synthesized an altered $F_1$ that was no longer capable of binding to the coupling membrane (79, 82). Finally, neither intact or washed membranes from mutant $N_{144}$ could be reactivated by the addition of parental $F_1$, an observation which suggested that this mutation had produced a catalytically defective $F_1$ that was more firmly bound to the membrane than the wild-type enzyme (301). The $MDA_1$, $MDA_2$, and $MDA_3$ mutants isolated by Kepes and his colleagues exhibited ATPase-binding and reactivation properties which suggested very close similarities with the *uncA*, *unc-405*, and $N_{144}$ mutants, respectively (93).

It is likely that the genetic lesion in the *uncA* and $MDA_1$ mutants has led to the synthesis of a defective catalytic subunit ($\alpha$ or $\beta$ or both), while leaving the binding capacity of the $F_1$ unimpaired (which is expressed via the $\delta$ subunit). On the other hand, the ability of $F_1$ to bind to the coupling membranes is apparently decreased in the *unc-405* and $MDA_2$ mutants (and probably also in DL-54 and NR-70), which suggests that the affinity of the $\delta$ subunit of $F_1$ for its binding site of the membrane may be diminished. The evidence suggests that the $MDA_3$ and $N_{144}$ mutants are also characterized by a point mutation in the $\delta$ subunit, but one that has pleiotropic effects, i.e., it enhances the binding of $F_1$ to the membrane but, in doing so, eliminates its catalytic properties.

Although the mutations in DL-54, NR-70, NR-76, and $N_{144}$ clearly decreased the efficiency with which membranes prepared from these strains utilized the energized state produced by respiration, this defect could be repaired by the addition of DCCD (16, 52, 301, 335–337). Furthermore, membrane vesicles prepared from NR-70, NR-76, and DL-54 were much more permeable to protons than were parental vesicles and, in both cases, the defect could again be repaired by the addition of DCCD (16, 336). It must be concluded, therefore, that the stalk component of $F_1$ plays an important role in maintaining the integrity of the coupling membrane, presumably by preventing the useless dissipation of the transmembrane proton gradient. It has been suggested that the effect of a mutation in which the $F_1$ is either physically deformed or missing from the membrane (e.g., in mutants DL-54, NR-70, NR-76, and $N_{144}$, but not *uncA*) is to cause the internal exit of the proton-translocating channel in $F_0$ to become exposed, with the resultant loss of respiration-linked energization (16). The ability of DCCD to repair these membranes suggests that the DCCD-binding protein is located in $F_0$ such that it forms a transmembrane proton-translocating channel; DCCD would thus inhibit oxidative phosphorylation in the intact ATPase complex and restore energization to the $F_1$-defective complex by preventing the passage of protons through $F_0$. The molecular weight of DSCP is fully compatible with a transmembrane location, but the ionophoric properties of the purified protein have yet to be investigated.

It is clear that the various *unc*$^-$ ATPase$^-$ mutants that have been isolated from *E. coli* are not the expression of a single genetic defect, although they all map between 73 and 74 min on the chromosome and are partly cotransducible with the *ilv* locus. Instead, they all probably reflect point mutations in a cluster of structural genes that code for the five polypeptide components of $F_1$.

***unc*$^-$ ATPase$^+$ mutants.** The most intensively studied *E. coli* mutants of this genotype are *unc-B* (AN-283) (79, 81), $B_{V4}$, $A_{144}$ and $K_{11}$ (212, 301), MDB (93), *uncD* (392), BG-31 (373) *etc-15* (48, 68). Membrane vesicles prepared from these mutants exhibited ATP hydrolysis, yet failed to catalyze either oxidative phosphorylation or ATP-dependent membrane energization; their capacity for using respiration to generate a utilizable energized state was extremely variable. However, many of these mutants were capable of growing anaerobically on glucose with the addition of exogenous electron acceptors. This paradox was subsequently resolved by the observation that these mutants contained a highly active fumarate reductase and thus presumably generated an energized membrane state via anaerobic respiration to fumarate produced by endogenous metabolism (338); genetic deletion of fumarate reductase eliminated this capacity for anaerobic growth.

The ATPase activity of membranes from mutants *uncB*, $B_{V4}$ $A_{144}$, and $K_{11}$ was at least 50% of that exhibited by parental membranes but, whereas most of the cellular ATPase activity of the parent and *uncB* strains was associated with the coupling membrane, over 90% of the total activity of strains $B_{V4}$, $K_{11}$, and $A_{144}$ was located in the supernatant fraction (213). These apparently contradictory figures are explained by the observation that the total ATPase activity of each of these latter three mutants was up

to fivefold higher than in the parent strain. Furthermore, the $F_1$ was so weakly attached to the coupling membrane that it could be removed simply by recentrifuging the particles in 50 mM Tris-sulfate buffer (pH 7.8) + 10 mM $MgSO_4$, i.e., it was not necessary, as was the case with parental membranes, to lower the ionic strength and $Mg^{2+}$ concentration of the wash medium in order to effect the release of the ATPase.

The addition of solubilized ATPase from *uncB* membranes to depleted membranes from the parent or *uncA* strains brought about restoration of both oxidative phosphorylation and ATP-dependent transhydrogenation; in contrast, depleted membranes from the *uncB* mutant could not be reactivated by parental $F_1$ (79, 81). Similarly, crude solubilized ATPase preparations from mutants $K_{11}$ and $A_{144}$ were able to restore both the ATP- and respiration-dependent quenching of ACMA fluorescence when added to depleted parental membranes, whereas the addition of parental supernatants to depleted mutant membranes was again without effect. In addition, the response of sodium dodecyl sulfate-treated membranes from $K_{11}$ and $A_{144}$ to antibodies prepared against the $\alpha$, $\beta$, $\alpha + \beta$, and $\gamma$ subunits of parental $F_1$ was identical to that of similarly treated parental membranes (213, 301). It was concluded that mutants *uncB*, $K_{11}$, and $A_{144}$ contain a normal $F_1$ and that the genetic lesion in each of these organisms is expressed as a defect in an as yet unidentified component of the membrane-bound $F_0$-$F_1$ complex (possibly DSCP) that leads to inefficient energy transduction; in $K_{11}$ and $A_{144}$, this defect is also expressed as a weak binding of $F_1$ to the coupling membrane.

The ATP-dependent, energy-transducing properties of mutant $B_{V4}$ were generally similar to those of $K_{11}$ and $A_{144}$; in contrast, $B_{V4}$ was uniquely capable of using respiration to drive the active transport of amino acids (213). Hybridization experiments of the type described above indicated that $B_{V4}$ membranes were apparently normal (since they could be reconstituted by parental ATPase) and suggested that the genetic lesion was probably expressed as a defective ATPase (since solubilized ATPase from $B_{V4}$ failed to reconstitute depleted parental membranes). The defect in the ATPase was clearly one that did not abolish its capacity for ATP hydrolysis or alter its immunodiffusion profile, although it did affect the ability of the ATPase to act as a coupling factor and to bind with the membrane. The properties probably reflect a relatively small structural alteration in the $\gamma$ or $\delta$ subunit of $F_1$ rather than in either of its catalytic subunits. It has been concluded that mutant MDB may also have a defective subunit in the stalk region of its ATPase, since the phenotype of this mutant is very similar to that of $B_{V4}$ (93).

The ATPase activities of membranes prepared from mutants $B_{V4}$, $K_{11}$, and $A_{144}$ were markedly resistant to DCCD but, whereas addition of this inhibitor to membranes from $K_{11}$ and $A_{144}$ restored defective respiration-linked reactions, it had no similar restorative effect on membranes from $B_{V4}$. However, the various ATP-dependent reactions that could be reconstituted via the formation of active ATPase hybrids from membrane and supernatant fractions were all sensitive to DCCD. These observations have led to the suggestion that conferral of sensitivity to DCCD is dependent upon the presence of two polypeptides: DSCP (the component of $F_0$ that binds the inhibitor) and a second, as yet unidentified, component of the ATPase complex which is involved in the binding of $F_1$ to the coupling membrane and which appears in the supernatant fraction of these mutants (213).

Strain BG-31 contained a highly active, DCCD-resistant ATPase, but was capable of membrane energization only at the expense of respiration (373). This lesion was located in the $F_0$ region which appeared to lack a polypeptide (molecular weight, 54,000) that was present in the parent organism. In this case it was concluded that DCCD resistance was probably a reflection of an alteration in the binding of $F_1$ to the coupling membrane, such that it prevented access of DCCD to its binding site, rather than to a lesion in DSCP per se.

Mutant *etc-15* exhibited slightly lowered ATPase activity, but was strikingly deficient in its ability to catalyze active transport and transhydrogenation at the expense of either respiration or ATP (48, 178). Since electrophoresis of the mutant $F_1$ indicated that the genetic lesion was expressed as a defective $\gamma$ subunit (48), it was concluded that this subunit was not essential for ATPase activity, although it was necessary for efficient energy transduction. In the latter context, the $\gamma$ subunit may need to be present in order for the $F_1$ to bind most efficiently to the coupling membrane and thus block the wasteful dissipation of the energized state produced by respiration.

The *uncD* mutant is a unique member of the *unc*$^-$ ATPase$^+$ class in that its ATPase activity, in contrast to that of the wild-type organism, was stimulated much more strongly by $Ca^{2+}$ than by $Mg^{2+}$ (392). However, the addition of $Ca^{2+}$ to membrane vesicles did not restore their

capacity for either oxidative phosphorylation or ATP-dependent transhydrogenation. It was tentatively concluded that the lesion leads to an alteration in a subunit of $F_1$ which is responsible for imposing divalent ion specificity and which is also necessary for energy transduction to and from ATP.

All of the *unc*$^-$ ATPase$^+$ mutants that have been examined resemble the *unc*$^-$ ATPase$^-$ and DCCD-resistant mutants, insofar as they map at approximately 73.5 min on the *E. coli* chromosome and are partly cotransducible with the *ilv* and *asn* loci. Since some of these mutants are defective in components of $F_0$, whereas others probably contain altered subunits of $F_1$, it is clear that the structural genes for the various polypeptides that comprise these two coupling factors lie close together on the chromosome. In contrast, Cox et al. (83) have described a possible regulatory mutant of *E. coli* (AN-295) which maps at approximately 77 min and is partly cotransducible with the *argH* gene. Membranes prepared from this mutant catalyzed ATP-dependent transhydrogenation and contained an apparently normal ATPase with an activity that was several times higher than that of wild-type membranes; $F_1$ purified from the mutant successfully reconstituted ATP-dependent transhydrogenation in depleted *uncA* membranes. Genetic evidence indicated that the high ATPase activity reflected derepression of the enzyme, but is is not known whether this is the direct result of a mutation in a regulator gene or whether it is merely an indirect effect of a mutation elsewhere.

## CONCLUSIONS

The work described in this review allows us to draw several general conclusions regarding bacterial oxidative phosphorylation. Thus, it is clear that, in spite of the enormous diversity that is encountered in bacterial respiratory chains, there exist striking similarities between proton-translocating redox segments from different systems. In contrast to mitochondria, however, the number of potential energy conservation sites available to, and utilized by, different bacteria vary widely. Indeed, for any one bacterium the number of such sites can change, depending upon the growth conditions employed. It should also be apparent that, with appropriate experimental techniques, oxidative phosphorylation in bacteria can be studied as easily, specifically, and conveniently as in mitochondria. There are many advantages in studying oxidative phosphorylation in bacterial systems; for example, the availability of a wide range of mutants and the possibility of effecting alterations to the various components synthesized by the cell in response to changes in the growth conditions. The ability to vary the components responsible for oxidative phosphorylation at the discretion of the investigator is a technique not generally available in equivalent studies with mitochondria. Yeast and other fungi can be used, but interpretation of the results obtained is complicated by the fact that certain mitochondrial enzyme complexes contain polypeptides synthesized from the mitochondrial, as well as the nuclear, DNA. The potential use of these approaches in bacteria has by no means been fully exploited in the analysis of the control mechanisms that serve to regulate the synthesis and functional activity of different electron transport chains. In addition, their application to the more fundamental problem of the mechanism of oxidative phosphorylation remains a challenge for the future.

It should also be remembered that certain bacteria, for example, chemolithotrophs, alkalophiles, and acidophiles, have had to develop unusual enzymes and reaction pathways in order to exploit a wide variety of potential electron donors and acceptors and, thereby, adapt to life in somewhat exotic ecosystems. The identification and characterization of these novel components and their role in oxidative phosphorylation should prove of great scientific interest.

The final point we should like to consider briefly is the possible significance of the increasing order of complexity of the electron transport chains, described in this review, to the evolutionary development of more efficient pathways for the conservation of energy. Thus, examples have been given of electron transport chains in which the oxidation of one molecule of NADH is linked to the synthesis of one (in the presence of fumarate, Fig. 4) two (in the presence of oxygen, Fig. 3, or nitrate, Fig. 5), three (in the presence of oxygen, Fig. 2 and 6), or between one and three (according to the oxygen concentration, Fig. 7) molecules of ATP. The complexity of biological mechanisms has increased gradually through time and, although it would be foolhardy to assume any direct evolutionary significance in the examples given in this review, a comparative study of the branched respiratory chains of bacteria, in terms of their individual redox components and functional activity, will clearly be informative in unravelling the evolutionary development of the mitochondrial respiratory chain and its ability to conserve energy.

## ACKNOWLEDGMENTS

We would like to extend our thanks and appreciation to our many colleagues and co-workers who have helped in a variety of ways to make this review possible. Our work described in this review was financed by the Science Research Council, United Kingdom, through grants B/RG/58518 to (B.A.H.) and B/SR/59102 (to C.W.J.).

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