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Microbial Energetics [and Discussion]

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Phil. Trans. R. Soc. Lond. B 1980 **290**, 329-339
doi: 10.1098/rstb.1980.0098

Phil. Trans. R. Soc. Lond. B **290**, 329–339 (1980) [329]
Printed in Great Britain

Microbial energetics

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An understanding of the mechanisms by which bacteria derive their energy is clearly important for the prediction of growth yields. Bacteria can synthesize ATP by a variety of routes, by fermentation, by oxidative phosphorylation, and possibly by the excretion of metabolic end products. The bacterium *Escherichia coli* has been studied extensively and a great deal is now known about the different membrane-bound multi-enzyme complexes that are responsible for oxidative phosphorylation. The efficiency of oxidative phosphorylation can vary not only between different bacteria that have adapted to particular ecological niches but also in an individual bacterium grown under different conditions or modified genetically by mutation with respect to its parent. Clearly, the concept that bacteria always grow with maximum thermodynamic efficiency is erroneous and it is important, therefore, to be able to assess the efficiency of energy conversion as well as the biochemical and genetical factors that regulate the physiological expression of energy-yielding reactions if they are to be manipulated by the investigator.

Introduction

The potential for exploiting microorganisms to make contributions in biotechnology, particularly in energy technology, has been the subject of several important proceedings in the last few years (Buvet *et al.* 1977; Schlegel & Barnea 1977; Gysi 1978; Bull *et al.* 1979). It is apparent, however, that, although the potential is considerable, most possibilities are, at this moment, a long way from becoming practical realities. In most microbial processes it is necessary either to maximize or to minimize the growth yield, and to achieve these goals an understanding of microbial energetics is clearly important. Our understanding of the molecular mechanisms whereby microorganisms conserve energy has increased greatly in the last decade or so (Haddock & Hamilton 1977; Haddock & Jones 1977). Indeed, it is now possible to predict and manipulate the efficiency of energy conservation in certain experimental situations.

Attention is here directed towards indicating the routes by which prokaryotes can derive energy and the ways in which the efficiency of oxidative phosphorylation can be manipulated.

Routes for energy conservation

Bacteria can derive the energy that they need for growth from a considerable number o diverse and varied reactions. Operationally, however, these different reactions can be considered as examples of just two general methods for ATP synthesis.

Substrate level phosphorylation

The first of these is the formation of ATP by substrate level phosphorylation; two distinct classes of reaction can be distinguished:

$$\mathrm{ADP} + \mathrm{substrate} \sim \mathrm{P} = \mathrm{ATP} + \mathrm{substrate}; \quad (1)$$

$$\mathrm{ADP} + \mathrm{P_i} + \mathrm{substrate} \sim \mathrm{X} = \mathrm{ATP} + \mathrm{substrate} + \mathrm{X}. \quad (2)$$

Particular examples are described in detail elsewhere (Morris 1975), but it is of interest to note that only a relatively small number of substrate level phosphorylation reactions have been identified, and that all are catalysed by soluble enzymes present in the cell cytoplasm.

Oxidative or photophosphorylation

In this case, ATP synthesis is coupled to electron transport reactions, which 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. These redox reactions are ultimately linked to the reduction of electron acceptors of more positive potential, usually oxygen under aerobic conditions, but a whole host of terminal acceptors, e.g. fumarate (Kröger 1978), NO_3^-, NO_2^-, SO_4^{2-}, CO_2 (Thauer *et al.* 1977), can be utilized by different bacteria under anaerobic conditions. 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 asymmetrically organized in the membrane so as to catalyse vectorial chemical reactions.

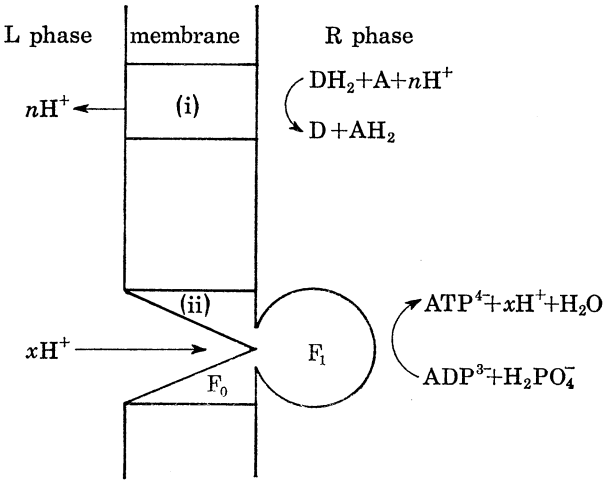

FIGURE 1. Schematic representation of a proton-translocating oxido-reduction segment of the electron transport chain, and of a proton-translocating ATPase. The pair of parallel vertical lines represent the cytoplasmic membrane, generally impermeable to ions except via specific translocases. The R phase represents the cytoplasmic space, the L phase represents the extracellular space, including the periplasmic space. The oxido-reduction segment (i) is catalysing the oxidation of a donor (DH_2) with concomitant reduction of an acceptor (A) and translocation of protons (nH^+). The ATPase (ii) is depicted as two components, an integral membrane-bound F_0 complex and a membrane associated F_1 complex, that serve to synthesize ATP and to translocate protons (xH^+) as shown.

The search for the mechanism that couples electron transport-dependent oxido-reductions to ATP synthesis and solute transport has produced numerous hypotheses. The most successful and useful of these is Mitchell's chemiosmotic hypothesis (Mitchell 1961, 1966, 1968, 1976). The hypothesis comes in four parts and, in short, it postulates (1) a reversible, proton-translocating ATPase, (2) a reversible, proton translocating respiratory or photosynthetic oxido-reduction chain, (3) a set of translocases that facilitate the transport of anions and cations, and (4) an ion-impermeable membrane incorporating the components of the previous three systems. A diagrammatic representation of the various postulates relevant to this discussion is presented in figure 1.

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The end result of either electron transport or ATP hydrolysis is the generation, across the membrane, of gradients of both pH (Δ 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 (ΔP) and, although these components are not identical, they are interconvertible and, if $\Delta\psi$ is expressed in millivolts and Δ pH in pH units, then at 30 °C they are related by the expression

$$\Delta P = \Delta\psi - 60\Delta \text{ pH}. \quad (3)$$

Under suitable conditions and when of the correct magnitude, ΔP drives a variety of energy-linked reactions, e.g. reversed electron transport through the respiratory chain, ATP synthesis via the reversible proton-translocating ATPase, the accumulation of certain solutes, and cell mobility.

Clearly, this is a simplistic view of the hypothesis, and 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 various properties of the membrane illustrated in figure 1 are experimentally demonstrable, and their uniquely chemiosmotic significance lies only in the proposal that the respiratory oxido-reductions drive ATP synthesis by a proton flow or circuit. As discussed in greater detail by Williams (1969, 1975), the primary event in energization is more likely to be a charge separation reaction across, but within, the membrane phase. The appearance of protons 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. However, confinement of this proton flow within the physical limits of the membrane phase would give coupling of ATP synthesis to oxido-reductions by a mechanism that could be called cryptically chemiosmotic.

Other areas of controversy are the precise stoichiometries of respiratory-driven (n in figure 1) and ATP-driven (x in figure 1) proton translocation for any particular system (see, for example Brand 1977). Certainly, the difference in redox potential between the donor (DH_2) and acceptor (A) will determine the potential number of ATP molecules synthesized by any specific redox reaction, but the redox carrier composition also seems important. Thus, if one considers NADH oxidation by molecular oxygen, those bacteria that can synthesize a high potential cytochrome *c* (e.g. *Paracocccus denitrificans* or *Rhodopseudomonas capsulata*) generate 3 moles of ATP per mole of NADH oxidized, as do mitochondria, whereas those bacteria that, because of their genetics, cannot synthesize such a cytochrome (e.g. *Escherichia coli*) only synthesize 2 moles of ATP per mole of NADH oxidized (Jones 1977). Where studied, oxidative phosphorylation in chemolithotrophs has also provided some interesting variations, e.g. NO_2^- oxidation by *Nitrobacter winogradskyi* (Cobley 1976), Fe^{2+} oxidation in an acid environment by *Thiobacillus ferrooxidans* (Ingledew *et al.* 1977), and sulphate respiration by *Desulfovibrio* species (Wood 1978). Indeed, although, in general, respiratory-energy conservation systems of bacteria and mitochondria are similar in principle, it can be predicted that there will be differences in detail, as discussed by Garland & Haddock (1977).

Energy conservation by the excretion of metabolic end products

As mentioned above, under anaerobic conditions many facultatively anaerobic bacteria like *E. coli* can generate ΔP in a way similar to that under aerobic conditions, i.e. via electron

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transport using fumarate or nitrate as terminal electron acceptor. In strictly fermentative bacteria lacking a proton translocating respiratory chain, e.g., *Streptococcus faecalis*, the fermentation of 1 mole of glucose yields only 2 moles of ATP. In these organisms the generation of ΔP by ATP hydrolysis would consume a considerable fraction of the ATP produced. This led Michels *et al.* (1979) to suggest that the excretion of fermentation products, such as D-lactate, together with a proton could serve to generate an electrochemical proton gradient that, under fermentative conditions, would contribute significantly to the metabolic energy of cells. Although these suggestions have yet to be tested in detail, they are certainly worth further consideration.

Methods for assessing the efficiency of energy conservation in bacteria

The literature abounds with information on the composition and organization of bacterial respiratory chains; however, much less is known about the efficiency with which energy is conserved. The main reasons for this are undoubtedly the experimental difficulties encountered when assaying oxidative phosphorylation in bacterial cells and in derived membrane preparations. Oxidative phosphorylation in whole cells is generally assayed by measuring (1) molar growth yields (Stouthamer & Bettenhaussen 1973), (2) respiration-induced changes in the composition of endogenous adenine nucleotide pools (Hempfling 1970), or (3) the stoichiometry of respiration-linked proton ejection (Scholes & Mitchell 1970). In membrane preparations, the problem is more difficult because of the heterogeneous nature of the material. Any membrane preparation contains a proportion of right side out (i.e. same orientation as the original cell) and inside out closed vesicles, as well as non-vesicular membrane fragments. Various experimental assays are possible, such as (1) respiration driven esterification of ADP with inorganic phosphate, (2) reversed electron transport, (3) respiratory control, or (4) the quenching of various fluorescent probes. Jones (1977) has presented a critical evaluation of the use of these different experimental approaches to study the efficiency of energy conservation in bacteria. It is important to appreciate, however, that none are completely satisfactory and results obtained with particles are, at best, qualitative indicators that energy conservation is taking place and give little information on the efficiency of the process.

Oxidative phosphorylation in *E. coli*

The most extensively studied bacterium is undoubtedly *E. coli*, this being because it is a facultative anaerobe that can derive energy for growth from a variety of metabolic routes. Thus, it can grow fermentatively via glycolysis, and oxidatively, using either oxygen or, under anaerobic conditions, fumarate and nitrate as terminal electron acceptors. So *E. coli* affords an ideal system for the study of the effects of genotypic and phenotypic modifications to the efficiency of oxidative phosphorylation. The relevant data have been reviewed previously (Cox & Gibson 1974; Haddock & Jones 1977; Haddock 1977) and only recent reports are covered here.

Electron transport

It is important to appreciate that not all the membrane-bound redox enzymes synthesized in *E. coli* are necessarily involved in energy conservation. Many serve simply for the reoxidation of reduced coenzymes, the removal of potentially toxic metabolic products, or the reduction of

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intermediates required for biosynthetic reactions. The energy-conserving reactions can be divided operationally into low and high potential segments that serve separately or together to generate a proton gradient across the cytoplasmic membrane.

Low potential segments

E. coli can synthesize, under appropriate conditions, an energy-dependent membrane-bound transhydrogenase activity. It appears that this enzyme is required for NADPH synthesis and, for thermodynamic reasons, cannot be energy-conserving (Jones 1977). Its synthesis is repressed by amino acids, particularly leucine (Gerolimatos & Hanson 1978), and mutants with defects in their ability to produce the enzyme have been described (Zahl *et al.* 1978).

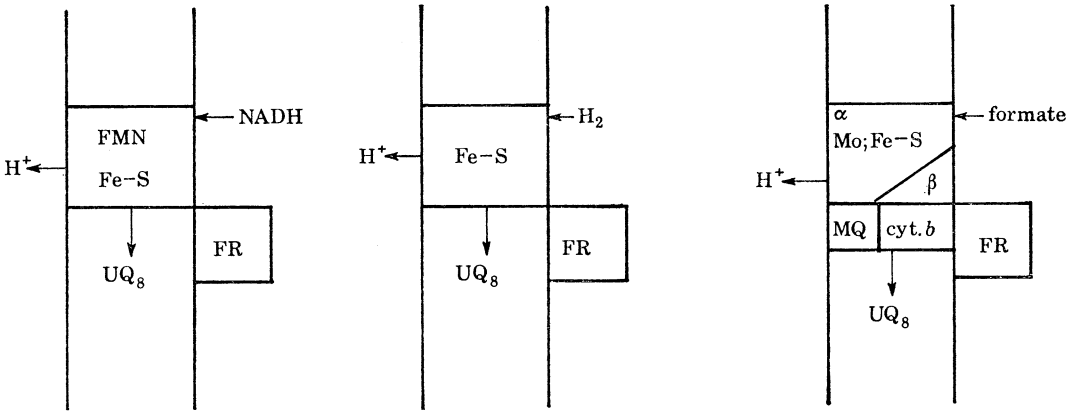

FIGURE 2. Low potential energy-conserving complexes in the cytoplasmic membrane of *E. coli*. The vertical lines represent the cytoplasmic membrane; the various donors, NADH, H_2 and formate, are shown to interact on the cytoplasmic face of the membrane. Abbreviations used: Fe–S, iron–sulphur centre; UQ_8, ubiquinone$_8$; cyt., cytochrome; MQ, menaquinone; FR, fumarate reductase; and Mo, molybdenum. Note that the stoichiometry of proton translocation is not specified.

Three reductants, NADH, hydrogen and formate, reduce either ubiquinone (under aerobic and anaerobic conditions) or fumarate (under anaerobic conditions) and, in so doing, translocate protons across the cytoplasmic membrane, as shown in figure 2.

NADH dehydrogenase has been purified from the cytoplasmic membrane as a single polypeptide containing FMN and an iron–sulphur centre (Dancey *et al.* 1976). Mutants with defects in the structural gene have been described (Young & Wallace 1976); the gene has been cloned (Young *et al.* 1978) and partially sequenced (I. G. Young, personal communication). Under sulphate-limited growth conditions aerobically or under anaerobic growth conditions (Poole & Haddock 1975), the NADH dehydrogenase becomes non-proton-translocating and, therefore, non-energy-conserving. It is not clear whether there are two distinct enzymes produced under different growth conditions or whether the proton-translocating NADH dehydrogenase is converted, by modification, into a non-proton-translocating form. There is certainly immunological evidence for two forms of the enzyme in membrane preparations of *E. coli* (Owen & Kaback 1979*a*, *b*).

Hydrogen has been shown to support the growth of *E. coli* in the presence of fumarate under anaerobic conditions (Bernard & Gottschalk 1978; Yamamoto & Ishimoto 1978), and the reaction has been shown to be proton-translocating (Jones 1979). The enzyme has been

purified from *E. coli* (Adams & Hall 1978), and mutants of the structural gene responsible for its synthesis obtained (M. A. Mandrand & B. A. Haddock, unpublished observations).

Formate dehydrogenase has been purified from *E. coli* membranes and shown to consist of three polypeptides of relative molecular masses 110000, 32000 and 20000. As isolated, the complex contains molybdenum, iron–sulphur centre(s) and haem (Enoch & Lester 1975). Genetically, the assembly of a functional formate dehydrogenase is complex. Certain mutants, designated *chlA*, *chlB* and *chlD* are pleiotropic in that they result in the inability of the cell to synthesize a functional nitrate reductase (see later) as well as a functional formate dehydrogenase (see Begg *et al.* 1977). The *chlA*, *chlB* and *chlD* gene products are required for the insertion of molybdenum into the respective apoproteins. Other mutants have been described that specifically lack formate dehydrogenase activity (Mandrand-Berthelot *et al.* 1978).

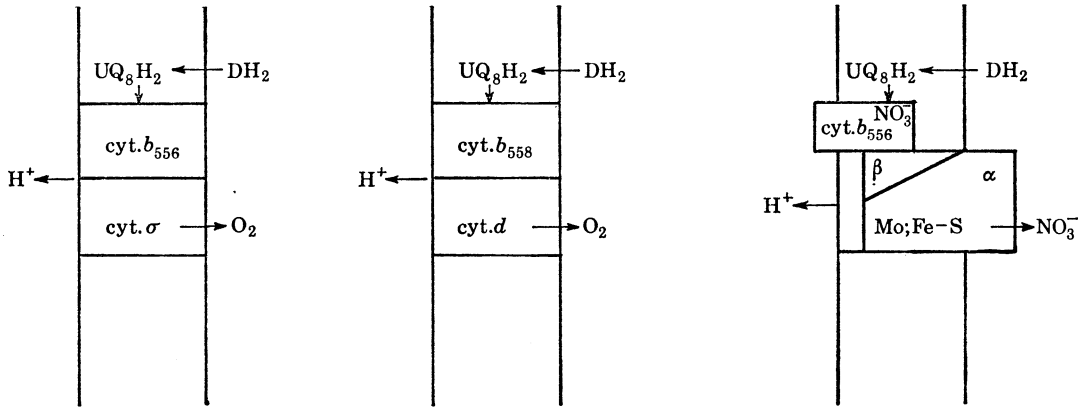

FIGURE 3. High potential energy conserving complexes in the cytoplasmic membrane of *E. coli*. The vertical lines represent the cytoplasmic membrane, and the donors DH_2 (D-lactate or succinate, for example) are shown to interact on the cytoplasmic face of the membrane. UQ_8H_2, ubiquinol$_8$; other abbreviations as for figure 2.

Molybdenum-limited growth inhibits the formation of a functional formate dehydrogenase and nitrate reductase (Lester & DeMoss 1971). In addition, it has been shown that the presence of tungstate in the growth medium also leads to the production of inactive formate dehydrogenase and nitrate reductase (Scott & DeMoss 1976) and that these inactive enzymes can be converted to their active forms by incubation of cell suspensions with molybdate in the presence of chloramphenicol (Scott *et al.* 1979).

These three proton-translocating dehydrogenases reduce the quinone pool in the membranes, ubiquinone under aerobic growth conditions, and either ubiquinone or menaquinone under anaerobic conditions. In turn, the quinol can be reoxidized by fumarate via fumarate reductase under anaerobic conditions, with the concomitant synthesis of 1 mole of ATP per mole of donor oxidized. The structural gene for fumarate reductase has been identified (Spencer & Guest 1973) and amplified (Cole & Guest 1979) in *E. coli*. Alternatively, the quinol can be reoxidized by three high potential proton-translocating segments, with the concomitant synthesis of 2 moles of ATP per mole of initial donor oxidized.

High potential segments

The three high potential segments illustrated in figure 3 reoxidize quinol, which, in turn, has been reduced by either the low potential segments, as described above, or, alternatively, by

other donors, such as succinate and D-lactate, via their respective dehydrogenases. In the latter case, clearly, only 1 mole of ATP is produced per mole of donor oxidized.

Two of the high potential segments utilize molecular oxygen as the terminal electron acceptor. The factors regulating the synthesis of these two routes are not known, but coordinate synthesis of cytochromes b_{558} and *d* occurs under a variety of growth conditions (see Haddock & Jones 1977), generally under conditions of low oxygen tension or in the presence of inhibitors of cytochrome *o* function, e.g. low concentrations of cyanide (Ashcroft & Haddock 1975). The experimental evidence suggests that the stoichiometry of respiration-driven proton translocation is similar via each the two routes. So far, no mutants have been described with specific lesions in their ability to synthesize specific apocytochromes, but mutants unable to synthesize haem have proved useful in studies of the assembly of functional cytochromes (Haddock & Schairer 1973; Haddock 1973; Rockey & Haddock 1974). Attempts to isolate the different cytochromes from the membrane in pure form have been rather unsatisfactory, but a recent report (Kita *et al.* 1978) encourages optimism for the future.

Under anaerobic conditions and in the presence of nitrate, *E. coli* synthesizes a respiratory nitrate reductase. This enzyme has been extensively studied, both biochemically and genetically (Haddock 1977; Haddock & Jones 1977; Begg *et al.* 1977). Four lines of evidence show that the proton translocating oxido-reduction of ubiquinol by nitrate is achieved by a chemiosmotic mechanism. They are (1) location of the respiratory carriers in the membrane, as judged by covalent labelling, or by (2) access to permeant and non-permeant dye reductants, (3) location of the protolytic reactions on opposite sides of the membrane, and (4) measurements of H^+/e ratios (Jones *et al.* 1978). As isolated from the cytoplasmic membrane, nitrate reductase is a molybdenum, iron–sulphur protein composed of two, non-identical subunits, α (relative molecular mass, 155 000) and β (relative molecular mass, 63 000). A third polypeptide, the γ-subunit (relative molecular mass, 19 000) or cytochrome $b_{556}^{NO_3^-}$ is required for functional ubiquinol-dependent nitrate reductase activity. Nitrate reductase synthesis occurs only in the absence of oxygen and presence of nitrate, and, when fully induced, can account for up to 15 % of the total cytoplasmic membrane protein. The *chlC* gene is the structural gene for the α-subunit of nitrate reductase, as indicated from an analysis of membrane-bound proteins of a *chlC* mutant by means of specific antisera and from the isolation of temperature-sensitive *chlC* mutants. The immediate product of the *chlC* gene has no known enzymatic activity, and conversion to the functional holoenzyme requires the expression of at least three other genes required for molybdenum processing (*chlA*, *chlB* and *chlD*), none of which in turn requires the presence of nitrate for derepression (or induction), as discussed above. Since the immediate product of the *chlC* gene has no known enzymic activity, study of the genetic control of its expression has proved difficult. However, the isolation of gene fusion strains in which β-galactosidase synthesis and growth on lactose only occur under anaerobic conditions in the presence of nitrate, implying fusion of the structural genes for lactose catabolism to the promoter-operator region regulating the *chlC* locus, makes detailed analysis now possible (Fimmel & Haddock 1979). Two other genes are known to affect the synthesis of nitrate reductase. It has been suggested that the *chlE* gene product is apocytochrome $b_{556}^{NO_3^-}$, but, to date, it has not proved possible to define a gene–product relation for the *chlG* gene (Jenkins *et al.* 1979). The availability of mutants, the abundance of the enzyme in the membrane, and its amenability to biochemical investigation make nitrate reductase one of the best understood proton motive respiratory segments at this time.

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The ATP*ase*

A membrane-bound ATPase concerned with energy metabolism appears to be associated with all living cells. The ATPase complex has the same general structure in all organisms and consists of an aggregate of at least eight polypeptides. Five of these polypeptides ($\alpha, \beta, \gamma, \delta, \varepsilon$) constitute a complex ($F_1$) that can be readily solubilized from the cytoplasmic membrane of prokaryotes, has all the ATPase activity and contains bound adenine nucleotides. The ATPase components remaining in the membrane (F_0) after solubilization of the F_1 complex have been less well characterized biochemically. The complete F_0/F_1 complex can be isolated from membranes only with detergent.

Recent work on prokaryotic ATPases, including the enzyme from *E. coli*, has been concerned with the dissociation of F_1 into its individual subunits, purification of these subunits and attempts to reconstitute a functional complex. Most success in this area has been achieved by using ATPase preparations from a thermophilic bacterium in which the subunits are particularly resistant to denaturation. By means of various combinations of purified subunits of the F_1 complex from this organism, it has been possible to gain some insight into the functions of the individual subunits. Some progress has also been achieved along these lines with F_1 from *E. coli*, but the genetic analysis of various mutants unable to synthesize a functional proton-translocating ATPase in this organism has proved more rewarding. To date, the genetic characterization of five genes (*uncA*, *uncB*, *uncC*, *uncD*, and *uncE*) has been reported in the literature. These genes have been shown to form part of an operon on the *E. coli* chromosome mapping near the origin of replication. Gene–polypeptide relations have been established for several of these genes (for a recent review, see Downie *et al.* 1979). Recently, specialized transducing phage has been isolated containing some of the structural genes for the ATPase complex (Kanazawa *et al.* 1979), and the cloning of these genes, shortly, is to be expected.

Concluding comments

The respiratory systems of bacteria exhibit great diversity, not only in their redox carrier composition, but also in the number of potential energy conservation sites that they contain. In addition, of course, some bacteria cannot generate energy via oxidative phosphorylation. There now exists the technology to investigate the various systems, at the molecular level, both biochemically and genetically. Although attention has been focused primarily on *E. coli*, the genetics and biochemistry of photosynthetic bacteria, for example, are also well advanced. Factors regulating the synthesis, assembly and functional expression of specific energy-conserving multi-enzyme complexes are, at the moment, less well understood but the topic of investigation.

For the future, one may speculate on the possibility of increasing or decreasing, by genetic manipulation, the efficiency of oxidative phosphorylation. For example, it might prove possible to transfer the structural genes from a bacterium that can synthesize 2 moles of ATP per mole of ubiquinol oxidized into a bacterium that normally can only synthesize 1 mole of ATP in the equivalent reaction. Would these genes be expressed and would the molar growth yield be increased? Alternatively, it may be possible to decrease the bacterial growth yield of a particular organism by cloning in structural genes for the ATPase in an attempt to lower the intracellular ATP concentration.

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It would seem that further development of a combined biochemical and genetic approach to microbial energetics will make significant contributions to our understanding of energy transformations in living cells, as well as providing well characterized and perhaps genetically 'constructed' cells for biotechnology.

The work of the authors was generously supported through research grants from the S.R.C., the M.R.C. and N.A.T.O.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Discussion

B. Khosrovi (*Cetus Corporation*, 600, *Bancroft Way*, *Berkeley*, *California* 94710, *U.S.A.*). I have investigated the ATP yield of a certain strict anaerobe and found that the yield was threefold higher when growth took place in a H_2 atmosphere than in a N_2 atmosphere. This is despite the fact that there was no utilization of the hydrogen.

B. A. Haddock. This phenomenon could be explained by hydrogen inhibition of certain of the respiratory enzymes, but, without knowing more about the organism and its physiology, I cannot provide an adequate explanation.

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