THE UNIVERSITY OF HULL

Energy transduction in *Propionibacterium acnes*

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SUMMARY

1. Propionibacterium acnes was shown to be sensitive to valinomycin thus enabling the membrane potential ($\Delta\Psi$) generated under a variety of conditions to be measured by determining the distribution of K^+ across the membrane in the presence of valinomycin.
2. Under anaerobic conditions, both anaerobically- and aerobically-grown P. acnes generated a $\Delta\Psi$ of approximately -140 mV. Oxygen stimulated the generation of the $\Delta\Psi$ in both anaerobically- and aerobically-grown cells. The effect of inhibitors of oxidative phosphorylation on the $\Delta\Psi$ generated was studied.
3. Anaerobically- and aerobically-grown P. acnes were capable of uncoupler-stimulated respiration. The effect of various inhibitors on respiration was studied.
4. Respiration-driven H^+ translocation was demonstrated in both anaerobically- and aerobically-grown P. acnes. The $\rightarrow H^+/O$ ratios obtained were consistent with the presence of one coupling site on the electron transport chain. The effect of inhibitors on respiration-driven H^+ translocation was studied.
5. Anaerobically- and aerobically-grown P. acnes contained cytochromes b, a_1, d and o.
6. Attempts to characterize the ATPase from P. acnes and to demonstrate its sensitivity to classical energy-transfer inhibitors were unsuccessful.
7. Using the results obtained from the various approaches, a role for oxygen in the energy transduction process of P. acnes was proposed.

ABBREVIATIONS

The following abbreviations are used throughout this text:

ANSA	1,2,4-amino-napthol sulphonic acid
ATPase	adenosinetriphosphatase
Bicine	(N,N-bis[2-hydroxyethyl]glycine)
CCCP	carbonylcyanide m-chlorophenylhydrazone
DCCD	N,N'-dicyclohexylcarbodiimide
DCPIP	2,6-dichlorophenol indophenol
DDA^+	dibenzyl dimethylammonium
DPPA	diphenyldiphosphoryl azide
Fe/S	iron-sulphur protein
FR	fumarate reductase
Hepes	N-2-hydroxypiperazine-N'-2-ethane suphonic acid
HOQNO	2-heptyl-4-hydroxyquinoline-N-oxide
MK	menaquinone
MOPS	morpholinopropane sulphonic acid
Nbf-Cl	4-chloro-7-nitrobenzofurazan
NOQNO	2-nonyl-4-hydroxyquinoline-N-oxide
PCB^-	phenyldicarbaundecaborane
PCP	pentachlorophenol
PMS	phenazine methosulphate
Quercetin	3,3',4,5,7-pentahydroxyflavone
TCA	trichloroacetic acid
TCS	tetrachlorosalicylanilide
$TPMP^+$	triphenylmethyl phosphonium
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
$\Delta\Psi$	membrane potential
$\Delta\tilde{\mu}_{H^+}$	H^+ electrochemical gradient

CHEMICALS

All chemicals used throughout the course of this work were of highest purity available and where possible 'Analar' grade. DPPA and PCP were purchased from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A.; ATP was purchased from Boehringer Corporation, Lewes, Sussex: DCCD, quercetin, azide and cyanide were purchased from British Drug House, Poole, Dorset; Hepes was purchased from Hopkins and Williams Ltd., Chadwell Heath, Essex; Nbf-Cl was purchased from Serva Feinbiochemica, Heidelberg, W. Germany; CCCP, antimycin A, valinomycin, HOQNO, oligomycin, bovine serum albumin, MOPS, bicine, ANSA, Tris, DCPIP and PMS were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. TCS was a gift from Professor W. A. Hamilton, University of Aberdeen.

[^{3}H] Inositol was supplied by the Radiochemical Centre, Amersham, Bucks, U.K.

ENZYMES

The following enzymes were purchased from the Sigma Chemical Co., St.Louis, Mo., U.S.A.

Carbonic anhydrase (purified powder from bovine erythrocytes), lysozyme (crystallized, dialysed and lyophilized powder from egg white), DNAse II Type VI (salt-free from bovine spleen), RNAse A Type IA (crystallized, from bovine pancreas).

INTRODUCTION

INTRODUCTION.

The investigation described in this thesis is concerned with the mechanism of energy transduction in the facultative anaerobe, Propionibacterium acnes. This organism has been implicated in acne, since bacterial activity appears to be mandatory for this condition to develop. Studies by Leyden et al. (1975) and Holland et al. (1977) showed that there was no correlation between the severity or types of acne with skin bacterial population. However, Holland et al. (1978) suggested that while there may not be a change in the bacterial population, there could however be changes in the physiology of the bacteria in response to micro-environmental changes within the pilosebaceous unit, which could play a role in acne. Their studies with P. acnes showed that there was an increase in growth yield and changes in exoenzyme production were also observed in response to oxygen.

This investigation was initiated primarily to ascertain whether the observed increase in growth yield in the presence of oxygen was brought about by the production of ATP by oxidative phosphorylation. Evidence available to date on the mechanism of energy transduction of propionibacteria has been obtained mainly from studies of molar growth yields and analyses of fermentation patterns. This investigation has involved studying the mechanism(s) by which a protonmotive force was generated in P. acnes. Known inhibitors of oxidative phosphorylation have been used in an attempt to define the process(es) involved. It is therefore necessary to review the current models of oxidative phosphorylation and discuss the role and operation of the electron transport chains, H^+-translocating ATPases, and the various ways by which the protonmotive force may be generated.

P. acnes (previously called Corynebacterium acnes) has only recently been reclassified as a member of the genus Propionibacterium. It is therefore necessary to discuss this classification since it is possible that P. acnes might not be physiologically similar to classical

propionibacteria. In addition, present knowledge of the metabolic pathways and oxidative phosphorylation in propionibacteria is reviewed.

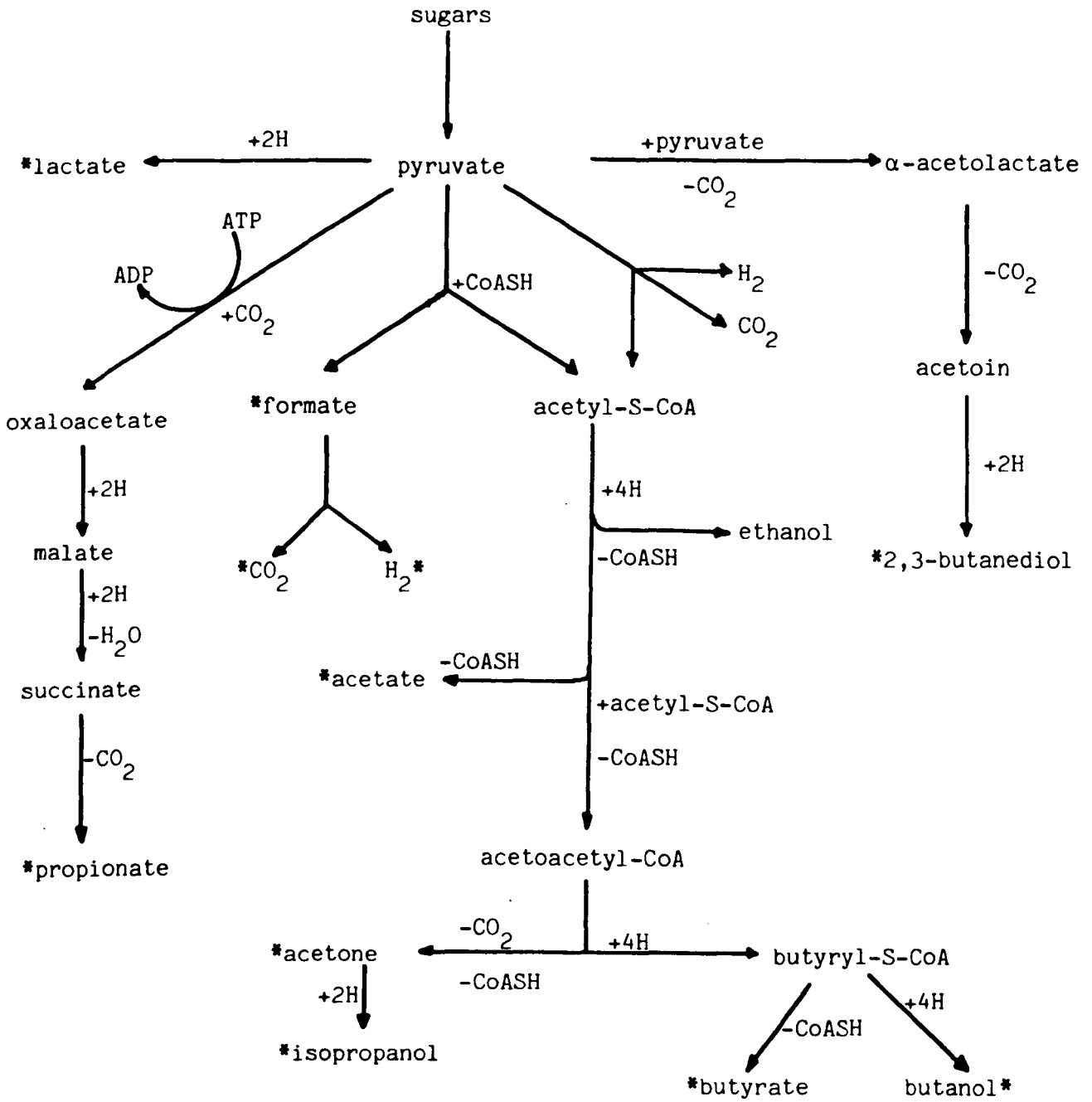
ENERGY TRANSDUCTION IN BACTERIA.

In bacterial energy conservation processes, ATP synthesis occurs by two fundamentally different processes, namely substrate-level phosphorylation and phosphorylation linked to electron transport. In substrate-level phosphorylation, ATP synthesis is catalysed by soluble enzyme systems. It is not within the scope of this introduction to discuss in detail the various energy-yielding pathways present in bacterial systems, though a generalized diagram depicting some of the pathways of fermentation is shown in Fig. 1. The fermentation process in propionibacteria will be discussed in a later section.

Oxidative phosphorylation and photophosphorylation are characterized by the involvement of a membrane-bound electron transport system. The free energy released from electron transfer processes, which can be driven by light (in phototrophs) or by the oxidation of either organic compounds (in organoheterotrophs) or inorganic ions (in chemolithotrophs) linked to the reduction of electron acceptors, is conserved in the form of ATP. Although there are differences in detail, the overall features of electron transport are very similar in bacteria, mitochondria and photosynthetic systems. One feature of oxidative phosphorylation is that it invokes the concept of a 'coupling' process, where the reactions of electron transport are linked to that of ATP synthesis. The actual mechanism by which this occurs is still a subject of major controversy.

HYPOTHESES OF ENERGY COUPLING.

The point of contention in oxidative phosphorylation is the manner in which the free energy released from the oxido-reduction reactions of electron transport is utilized to drive the thermodynamically



*denotes end-product

Fig. 1 : Some fermentation pathways in bacteria.

'uphill' reaction of ATP synthesis. Current models can be divided into three different types, namely, the chemical coupling hypothesis based on the mechanism of substrate-level phosphorylation, the conformational coupling hypothesis and the chemiosmotic hypothesis.

In the chemical coupling hypothesis, expounded by Slater (1953), the precursor for ATP synthesis was postulated to be a high-energy chemical intermediate, X~I, which when phosphorylated forms X~P. ATP production is thus a result of a direct transphosphorylation to ADP. However, despite intensive research, the phosphorylated or non-phosphorylated intermediate was never found. Earlier claims of intermediates (Purvis, 1960; Griffiths, 1963; Bridger et al., 1968) were all later shown to be unfounded. Suggestion of a protein acyl-phosphate as an intermediate (Cross et al., 1970) was subsequently shown to be ATP itself (Cross and Boyer, 1973). Interest in the chemical coupling hypothesis was reactivated with the demonstration by Griffiths and his collaborators that oxidative phosphorylation could be driven by lipoate, oleoyl-S-lipoate and oleoyl-phosphate in the absence of a functional electron transport chain (Griffiths, 1976; Griffiths et al., 1977; Partis et al., 1977), culminating with Griffiths postulating the oleoyl-cycle (Griffiths, 1977). There was however no support for this idea. Indeed it was shown that lipoate-requiring mutants of Escherichia coli were capable of oxidative phosphorylation and various energy-linked functions without being supplemented with lipoate (Singh and Bragg, 1978; 1979; De Chadrarevjan et al., 1979).

The original formulation of the conformational model of energy coupling suggested that the key process in oxidative phosphorylation lay in the strained conformational state of proteins following energization by electron transport processes; energy is transmitted for ATP synthesis by relaxation of the protein conformation (Boyer, 1964). However, the relative insensitivity of $Pi \rightleftharpoons H^{18}OH$ exchange,

believed to result from the dynamic reversal of formation of enzyme-bound ATP from medium Pi, to uncouplers, led Boyer to change this proposal. In its present form, it states that ATP synthesis occurs with little or no energy input; energy being required for the release of formed ATP from the catalytic site (Boyer et al., 1973). The main support for this model are the demonstrations of energy-linked conformational changes in chloroplasts (Ryrie and Jagendorf, 1971; McCarty and Fagan, 1973), and mitochondria (Bertina et al., 1973) and the increased exchangeability of adenine nucleotides firmly bound to the F_1 protein of chloroplasts, mitochondria and bacteria with medium adenine nucleotides (Harris and Slater, 1975). In essence, Boyer does not dispute the involvement of a H^+ electrochemical gradient, which according to Mitchell is the driving force for ATP synthesis (Mitchell, 1966), but sees it as playing a secondary role in that it is required for the release of formed ATP (Boyer et al., 1975).

The third proposed mechanism of energy coupling, the chemiosmotic model, was put forward by Mitchell (1961; 1966) and a variation of the same model was put forward by Williams (1961; 1962; 1969). The central thesis of the chemiosmotic model of energy coupling is that the process of electron transfer causes the vectorial translocation of H^+, creating an electrochemical gradient of H^+ ($\Delta\tilde{\mu}_{H^+}$,Δp or protonmotive force) across the membrane, which when dissipated through the ATPase, catalyses the net synthesis of ATP. The basic difference between Mitchell's and Williams' proposals lies in the location of the H^+ electrochemical gradient. According to Mitchell, translocation of H^+ causes acidification of the surrounding bulk phases, whereas Williams is of the opinion that the effective gradient is largely localized, occurring within the membrane itself. If it is accepted that any intramembrane H^+ electrochemical gradient is likely to be in equilibrium with the bulk phase transmembrane electrochemical gradient,

then the difference between Mitchell's and Williams' proposals lies basically in the question of semantics, though this is not the opinion of the two exponents of the chemiosmotic model of energy coupling (Mitchell, 1977; Williams, 1977).

The chemiosmotic model of energy coupling is best viewed within the framework of Mitchell's proposal. The energy released from the redox reactions of electron transport is conserved as a protonmotive force comprising of a ΔpH and a $\Delta\Psi$ component, which are related and interconvertible, as described by the expression:

$$\Delta\tilde{\mu}_{H^+} = \Delta\Psi - Z\Delta pH.$$

$\Delta\tilde{\mu}_{H^+}$ is the protonmotive force in mV; $\Delta\Psi$ the electrical potential difference (membrane potential) across the membrane; $Z = 2{\cdot}3RT/F$ where R, T and F have their usual meanings, and Z has a numerical value of 59 at $25^{\circ}C$; ΔpH is the pH difference between the interior and the exterior.

The essential features of Mitchell' s chemiosmotic hypothesis can be schematically represented by Fig. 2a, which defines the three basic requirements of this hypothesis, namely (i) the electron transport reactions cause a vectorial translocation of H^+ across the membrane (ii) the membrane in which the components are located is relatively impermeable to most ions including H^+ and OH^- and (iii) the ATPase is a fully reversible proton pump. The fourth requirement of the chemiosmotic hypothesis (not incorporated in Fig. 2a) is that the membrane contains carrier systems able to utilize $\Delta\tilde{\mu}_{H^+}$ or its components for transport purposes. These basic requirements have been found to be experimentally verifiable (reviewed by Greville, 1969; Hamilton, 1975; Haddock and Jones, 1977).

Thus, according to Mitchell, the driving force for ATP synthesis and energy-linked functions is $\Delta\tilde{\mu}_{H^+}$, which can be generated

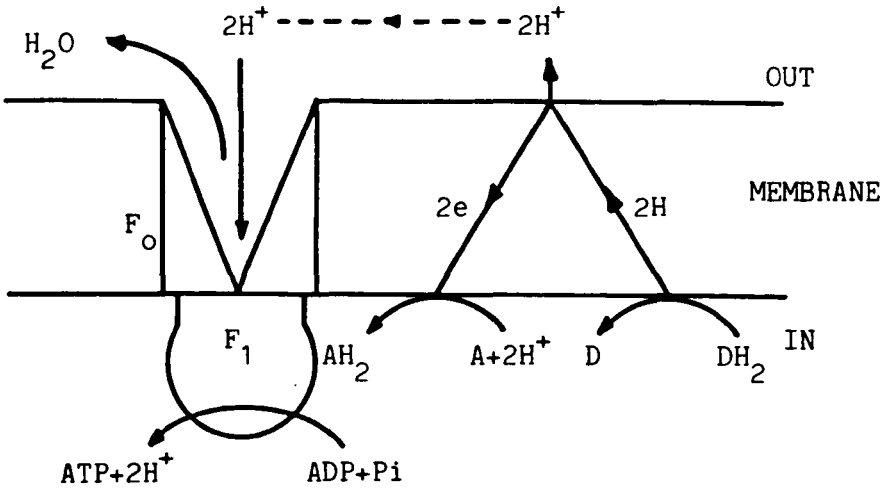


Fig. 2a : Schematic representation of chemiosmotic hypothesis.

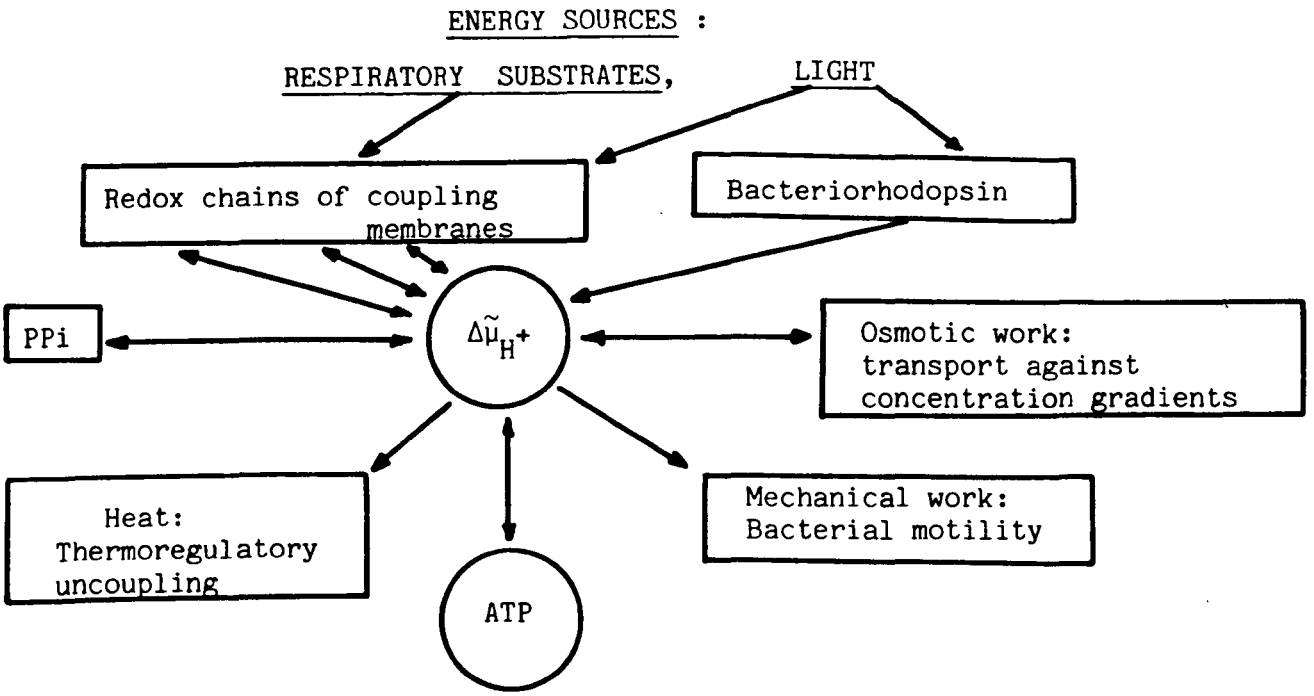


Fig. 2b : The central role of the transmembrane electrochemical potential of H^+ ($\Delta\tilde{\mu}_{H^+}$) in membrane-linked energy transduction.

by either electron transport or ATP hydrolysis catalysed by the ATPase. Electrogenic H^+ extrusion driven by electron transport has been demonstrated in mitochondria (Mitchell and Moyle, 1965), chloroplasts (Neumann and Jagendorf, 1964) and bacteria (Scholes and Mitchell, 1970; Jones et al., 1975; Lawford and Haddock, 1973). Such H^+ movements have been found to generate a $\Delta\tilde{\mu}_{H^+}$ of around -200 mV in mitochondria, bacteria and chloroplasts (Nicholls, 1974; Collins and Hamilton, 1976; Zilberstein et al., 1979; Schuldiner et al., 1972a). To facilitate such H^+ movements, Mitchell postulated that the components of the electron transport chain are arranged to form the 'redox loops' (Mitchell, 1966) comprised of a hydrogen and an electron carrier with ubiquinone acting as the H carrier for the last two segments via the 'protonmotive Q cycle' (Mitchell, 1975). However, the stoichiometry of electron transport and the arrangement of the redox centres as defined by the redox loops is still a subject of intense controversy (Wilkström and Krab, 1979; Mitchell and Moyle, 1979). A consequence of the redox loops is that the redox centres must themselves be arranged across the membrane in order to catalyse the transmembrane protonations and deprotonations necessary to generate the $\Delta\tilde{\mu}_{H^+}$. A large body of evidence supporting this concept of the 'sidedness' of the coupling membrane has been reviewed (Racker et al., 1971; Harmon et al., 1974).

The second mechanism by which a $\Delta\tilde{\mu}_{H^+}$ may be generated is incorporated within the concept of a fully reversible H^+- translocating ATPase, where it is postulated that during ATP hydrolysis, H^+ are translocated away from the site of hydrolysis, hence generating a $\Delta\tilde{\mu}_{H^+}$. Such electrogenic H^+ translocation at the expense of ATP hydrolysis has been demonstrated in mitochondria (Mitchell and Moyle, 1968), submitochondrial particles (Mitchell and Moyle, 1973; Thayer and Hinckle, 1973), chloroplasts (Carmeli, 1970) and bacteria (Hertzberg and Hinckle, 1974; West and Mitchell, 1974; Reenstra et al.,

1980) producing a $\Delta\tilde{\mu}_{H^+}$ of about -150 mV to -200 mV (Reenstra et al., 1980; Nicholls, 1974; Harold and Papineau, 1972a). As predicted by Mitchell, the direction of the H^+ flux is entirely dependent on the orientation of the ATPase complex in the system; ATP hydrolysis in mitochondria leads to the acidification of the medium, while in submitochondrial particles, it leads to alkalinization of the medium. That such H^+ movements are catalysed by the ATPase is demonstrated by its sensitivity to dicyclohexyl-carbodiimide (DCCD) (Harold and Papineau, 1972b), and the inability of mutants lacking a functional ATPase to carry out this process (Hasan et al., 1978). Further unequivocal evidence for the energy-transducing role of the ATPase are the demonstrations of electrogenic, ATP-dependent H^+ translocation by purified ATPase of yeast (Villalobo et al., 1981; Dufour and Tsong, 1982) and bacteria (Kagawa et al., 1976; Sone et al., 1976) reconstituted into proteoliposomes.

The essential role of the $\Delta\tilde{\mu}_{H^+}$ in electron transport linked phosphorylation is supported by the demonstrations of ATP synthesis driven by an artificially imposed $\Delta\tilde{\mu}_{H^+}$ (ie. in the form of ΔpH or $\Delta\Psi$) across the membranes of mitchondria (Reid et al., 1966; Cockrell et al., 1967), chloroplasts (Jagendorf and Uribe, 1966; Schuldiner et al., 1972b) and bacteria (Tsuchiya and Rosen, 1976; Grinius et al., 1975; Maloney and Wilson, 1975; Wilson et al., 1976). Moreover, ATP synthesis was accompanied by a rapid H^+ entry occurring after the 'threshold' $\Delta\tilde{\mu}_{H^+}$ value of approximately -180 mV was reached, below which no ATP synthesis was observed (Maloney, 1977; 1978). A similar observation was earlier made by Thayer and Hinckle (1975a, b) while studying $\Delta\tilde{\mu}_{H^+}$-driven ATP synthesis in submitochondrial particles where the threshold value was found to be approximately -160 mV. In addition, they showed that the rate of ATP synthesis was very similar to that driven by respiration, in accord with chemiosmotic hypothesis.

A reversible H^+-translocating ATPase has important implications in bacterial energy conservation. Among those organisms that are entirely dependent upon fermentation for their source of energy (eg. Streptococci), $\Delta\tilde{\mu}_{H^+}$ is generated by ATP hydrolysis (Laris and Pershadsingh, 1972; Harold and Papineau, 1972a, b). $\Delta\tilde{\mu}_{H^+}$ may then be utilized for energy-linked processes such as transport of metabolites (Kashket and Wilson, 1973; 1974), amino acids (Ashgar et al., 1973) and also for the maintenance of an ionic gradient across the cell membrane. Similarly, in facultative anaerobes, under conditions when electron transport is not functioning, $\Delta\tilde{\mu}_{H^+}$ is generated by ATP hydrolysis, but when electron transport is operational, $\Delta\tilde{\mu}_{H^+}$ is generated by electron transfer processes. Fig. 2b describes the processes that generate and utilize $\Delta\tilde{\mu}_{H^+}$.

The chemiosmotic hypothesis views the reactions of electron transport and the ATPase as essentially separate, linked only by way of circulation of H^+. As such, any perturbations of electron transport or $\Delta\tilde{\mu}_{H^+}$ should be similarly reflected in ATP synthesis. A number of observations have however been reported where the competency of the $\Delta\tilde{\mu}_{H^+}$ as the intermediate is being questioned. These observations will not be described in detail, serving only to show that while the basic principles underlying the chemiosmotic model of energy coupling are based on solid experimental foundation, inconsistencies do exist. For example, decreasing the rate of electron transport is expected to cause a decrease in the $\Delta\tilde{\mu}_{H^+}$ and ATP synthesis. This direct relationship between electron transport, $\Delta\tilde{\mu}_{H^+}$ and ATP synthesis was however not found in bacterial chromatophores (Baccarini-Melandri et al., 1977), submitochondrial particles (Sorgato et al., 1980) and Paracoccus denitrificans vesicles (Kell et al., 1978a), where inhibition of electron transport did inhibit ATP synthesis but was without much effect on $\Delta\tilde{\mu}_{H^+}$. A second example is the apparent lack of correlation between

$\Delta\tilde{\mu}_{H^+}$ and $\Delta G'_p$ (the phosphorylation potential) as demonstrated by Holian and Wilson (1980) and Wilson and Forman (1982). The $\Delta\tilde{\mu}_{H^+}$ may be decreased by titration with uncouplers, but it is not followed by a parallel decrease in $\Delta G'_p$, hence leading to an increase in $\Delta Gp/\Delta\tilde{\mu}_{H^+}$ values at low $\Delta\tilde{\mu}_{H^+}$ (Azzone et al., 1978). Similarly, this lack of correlation was shown by Melandri et al. (1980) where ATP synthesis was observed to cease despite the presence of a substantial $\Delta\tilde{\mu}_{H^+}$.

Another area of contention is the stoichiometry of ATP synthesis in relation to H^+ translocation. Mitchell postulated that $2H^+$ are translocated per coupling site as indicated by the oxygen pulse (Mitchell and Moyle, 1965) and ATP pulse techniques (Mitchell and Moyle, 1968; Thayer and Hinckle, 1973). The work of Lehninger and his collaborators argues that these values are underestimations, due in part to the translocation of various solutes into the matrix in symport with H^+ (Brand et al., 1976). They obtained values of 9 and 6 for NAD-linked and succinate-linked substrates respectively (Brand et al., 1976; Reynafarje and Lehninger, 1977). Thermodynamic measurements (based on the relationship, $\Delta G'_p = [H^+/ATP] \cdot \Delta\tilde{\mu}_{H^+}$) have produced values of $3H^+/ATP$ (Nicholls, 1974; Van Dam et al., 1977; Kell et al., 1978b). Brand (1979) in fact pointed out that the stoichiometry could be variable within the mitochondrial electron transport chain.

These observations do not revoke in total the concept of chemiosmotic energy coupling. The demonstrations of ATP synthesis driven by artifically imposed $\Delta\tilde{\mu}_{H^+}$ in mitochondrial and bacterial systems, and reconstituted proteoliposomes consisting of components from various energy-transducing systems (Winget et al., 1977; Sone et al., 1977; Yoshida et al., 1975a) all point to the key role of the $\Delta\tilde{\mu}_{H^+}$ in oxidative phosphorylation. However, the inconsistencies mentioned might call for a certain amount of flexibility within Mitchell's chemiosmotic model, for example, the incorporation of the

intramembrane $\Delta\tilde{\mu}_{H^+}$ within the working hypothesis.

BACTERIAL ELECTRON TRANSPORT

Within Mitchell's chemiosmotic hypothesis, the primary function of the electron transport chain is to catalyse the vectorial translocation of H^+, generating a $\Delta\tilde{\mu}_{H^+}$. Similarities between mitochondrial, chloroplast and bacterial electron transport processes occur largely in performing this postulated function and in the basic redox carriers they contain. Assessment of the role of redox carriers in bacterial systems involves the application of similar procedures to those used in studying the mitochondrial electron transport chain. Quinones, flavoproteins and iron-sulphur proteins can be determined by methods described by Kröger (1978), Cerletti and Giordano (1971) and Brumby and Massey (1967) respectively. Cytochromes are usually detected spectrophotometrically in either intact cells or cell-extracts. The methodology involved and the characteristics of the various cytochrome types have been described (Smith, 1978; Kamen and Horio, 1970; Horio and Kamen, 1970). In addition to the above methods, mutants of bacteria with defects in the electron transport chain have been of considerable use in elucidating the functional activity of several of the redox components (Cox and Gibson, 1974; Gibson et al., 1979).

Very few species of bacteria possess a respiratory chain similar to that of mitochondria. The constitution of bacterial respiratory chain is usually more variable, particularly in the terminal segment. The composition is usually dependent upon the growth environment, which can cause the induction or repression of the synthesis of certain redox carriers. The versatility of bacterial electron transport can be illustrated by the facultative anaerobe E.coli, which is able to derive energy for growth fermentatively via

glycolysis, and oxidatively via electron transport using oxygen or under anaerobic conditions, using fumarate or nitrate as the terminal electron acceptor. Fig. 3a, b and c represents the electron transport chain of E.coli under aerobic conditions and anaerobic conditions in the presence of fumarate or nitrate respectively. The evidence supporting such arrangements have been reviewed by Haddock and Jones (1977).

In all, E. coli has been reported to synthesize nine different cytochromes ie. 2 c-type cytochromes (C_{550} and C_{548}), 5 b-types cytochromes (b_{556}, b_{558}, b_{562}, $b_{556}^{NO_3^-}$ and o) and cytochromes a_1 and d (Shipp, 1972; Haddock et al., 1976). It must be pointed out that not all cytochromes are involved in the electron transfer processes; c_{550} has been suggested to be a soluble cytochrome not participating in electron transport (Fujita, 1966). E. coli grown under anaerobic conditions with nitrate or fumarate is capable of aerobic electron transport due to the presence of the terminal oxidases o and d (Haddock et al., 1976) though the degree of interaction between the anaerobic and aerobic electron transport is not known.

The efficiency of energy coupling in intact cells can be assessed by measurements of respiration-driven H^+ translocation, intracellular nucleotide content and molar growth yields. Due to the absence of adenine nucleotide translocase in bacteria, techniques developed for studies with mitochondria, like measurements of ADP/O, P/O and reverse electron transport can only be applied to inside-out membrane vesicles.

As discussed earlier, there is ample evidence that respiring bacteria carry out electrogenic H^+ translocation. Assuming $2H^+$ are translocated per coupling site, the value obtained for oxygen-mediated H^+ translocation ($\rightarrow H^+/O$ ratio) would represent twice the number of coupling sites. This technique has been successfully applied to a

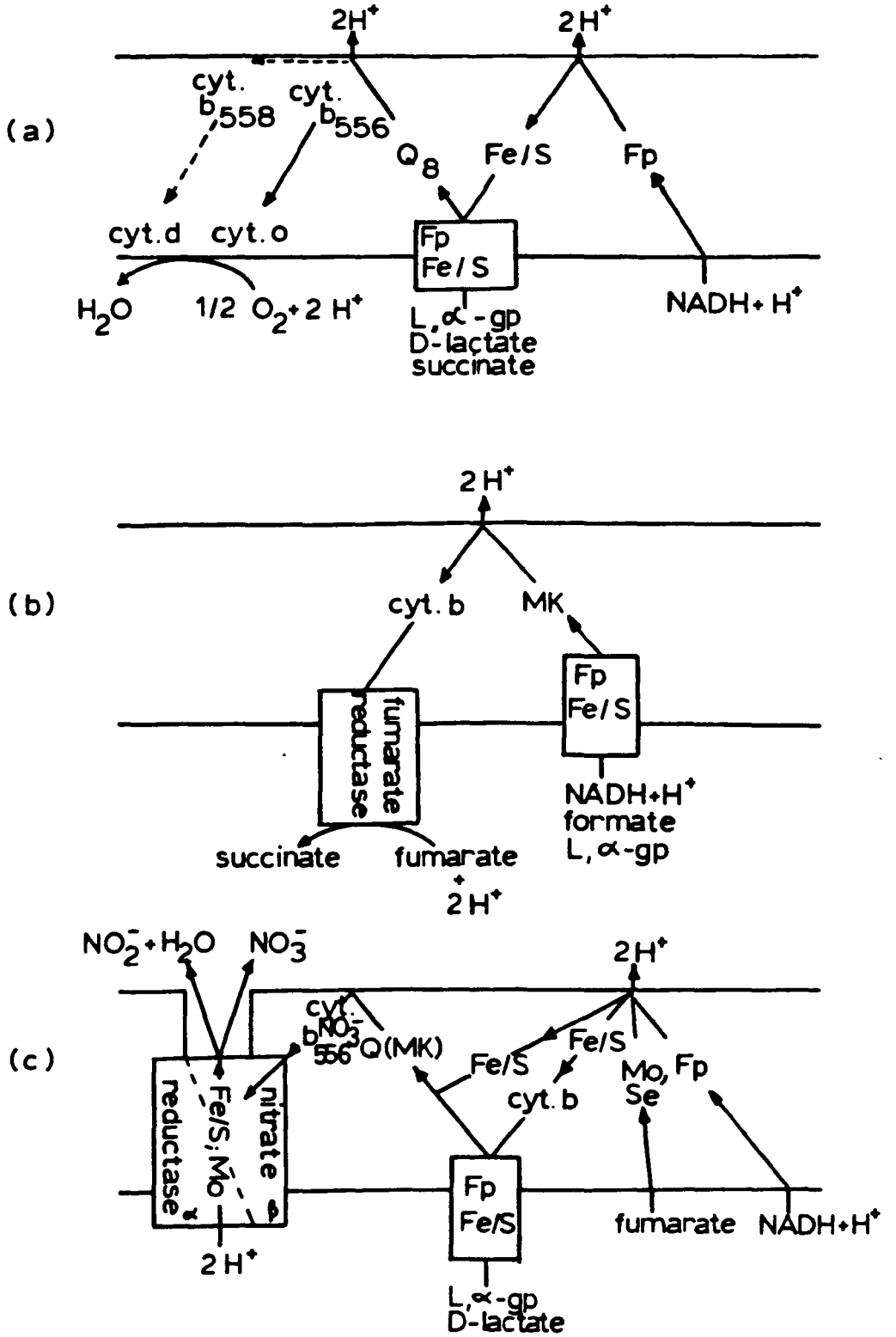


Fig. 3: Electron transport chain of E. coli grown under (a) aerobic conditions (b) anaerobic conditions in the presence of fumarate (c) anaerobic conditions in the presence of nitrate.

number of bacteria (reviewed by Jones, 1977). Generally, it may be said that bacteria containing a functional cytochrome c and cytochrome aa_3 or o have three coupling sites as in the conventional mitochondrial system. This has been shown for a number of bacteria such as P. denitrificans (Scholes and Mitchell, 1970), Alcaligenes eutrophus (Jones et al., 1975) and Paracoccus ovalis chester (Jones et al., 1975). The coupling site associated with the b-c_1 segment is lost in the absence of cytochrome c as shown in E. coli (Lawford and Haddock, 1973), Bacillus megaterium (Downs and Jones, 1975) and Bacillus licheniformis (Jones et al., 1975). Furthermore, the oxygen-pulse technique may also be applied to anaerobic electron transport chains, where H^+ translocation can be induced by pulsing with the terminal electron acceptor such as fumarate in Vibrio succinogenes (Kröger, 1975) and Klebsiella pneumoniae (Brice et al., 1974), nitrate in E. coli (Garland et al., 1975) and nitrite in P. denitrificans (Brogerd et al., 1981).

The assessment of respiratory efficiency by measurement of intracellular nucleotide content (Hempfling, 1970) has been severely criticized on the basis that firstly, the re-reduction of NAD^+ by endogenous substrates can take place and secondly, the oxidation of the quinol pool is not accounted for. Large discrepancies between P/O and P/NADH values were found (van der Beek and Stouthamer, 1973). The other techniques such as ADP/O and P/O measurements can be used for ascertaining whether the oxidation of certain substrates is ATP yielding; the number of phosphorylation sites cannot however be predicted due to the low values usually obtained (Eilermann et al., 1970; John and Whatley, 1970; Jones et al., 1971). Evidence for the presence of an energy coupling site can be reinforced with studies of reverse electron transport (Sweetman and Griffiths, 1971a, b; Bragg and Hou, 1974).

Indirectly, assessment of energy conservation can be achieved by measurements of molar growth yields (Bauchop and Elsden, 1960; Stouthamer, 1969).

BACTERIAL ATPases.

The presence of a H^+-translocating ATPase is a universal feature of energy-transducing membranes. Electron microscopic analyses of membranes from E. coli (Hinckle and McCarty, 1978), Micrococcus lysodeikticus (Gorneva and Ryabova, 1974; Munõz et al., 1968), the thermophilic bacterium PS3 (Kagawa et al., 1976) and a variety of other organisms reveal spheres of about 90Å in diameter attached to the membrane by a stalk similar to that found on mitochondrial inner membranes. The identification of these 90Å particles as the ATPase came largely from the work of Racker and co-workers (Racker et al., 1964; Kagawa and Racker, 1966; Fessenden and Racker, 1966) establishing its functional role in oxidative phosphorylation.

The first report of ATPase localized in the plasma membrane of bacteria came from studies with Streptococcus faecalis (Abrams et al., 1960). Since then, ATPase has been detected in membrane preparations from all the microorganisms so far studied. A common feature of the ATPases is that it is composed of three components, namely the catalytic F_1 headpiece (consisting of 5 sub-units), the hydrophobic F_o component (consisting of 3 to 4 sub-units) and the stalk sector, serving to attach the F_1 to the F_o.

Solubilization of bacterial ATPase can be carried out by a variety of techniques such as aqeous wash procedure (Abrams, 1965), extraction with organic solvents such as n-butanol (Salton and Schor, 1974) or chloroform (Cox et al., 1978), and dispersion with detergents (Hanson and Kennedy, 1973). General techniques for ATPase extraction have been reviewed (Abrams and Smith, 1974; Salton, 1974). Following the release from the membranes, conventional methods for purification

can then be applied to this enzyme. The resultant purified ATPase consists only of the F_1 component; the F_o being lost during the extraction and purification procedure.

Soluble F_1-ATPases generally exhibit characteristics similar to the membrane-bound enzyme. Some major changes do occur on solubilization eg. F_1-ATPase usually exhibits a higher specific activity and a lower K_m, perhaps reflecting the different conformations of the enzyme in the two states. Differences also occur in the sensitivity to various ATPase inhibitors. F_1-ATPases are usually resistant to inhibitors interacting at the level of the F_o, such as DCCD, though DCCD-sensitive F_1-ATPase has also been reported (Evans, 1970). Cold-lability is another property of F_1-ATPases, believed to be due to the dissociation of the sub-units (Kobayashi and Anraku, 1974).

Homogenous preparations of F_1-ATPases have been obtained from a variety of bacteria (Abrams and Smith, 1974; Salton, 1974). They are all relatively large proteins with molecular weights in the region of 300,000 to 400,000, rich in acidic amino-acids and contain approximately 33% hydrophobic residues. In addition, they consist of 5 sub-units, α and β being the major sub-units, with γ,δ and ε as the minor sub-units. Their molecular weights occur in the region of 57,000 (α); 51,000 (β); 30,000 (γ); 20,000 (δ) and 12,000 (ε) (Bragg and Hou, 1972). Thus, in terms of size, bacterial F_1-ATPase is remarkably similar to that of mitochondria and chloroplasts (Penefsky, 1974).

Studies on the stoichiometry and arrangements of the sub-units have been greatly aided by the ability to obtain purified and crystallized F_1-ATPases from bacteria (Yoshida et al., 1975b), beef-heart (Spitzberg and Haworth, 1977) and rat-liver mitochondria (Amzel and Pedersen, 1978). However, there is as yet no unanimity on the stoichiometry of the sub-units. Two main models put forward are

the hexagonal ($\alpha_3\beta_3\gamma\delta\varepsilon$) (Catterall et al., 1973; Bragg and Hou, 1975; Abrams et al., 1976) and the tetragonal ($\alpha_2\ \beta_2\ \gamma_2\ \delta_{1-2}\ \varepsilon_2$) models (Vogel and Steinhardt, 1976; Verschoor et al., 1977; Satre and Zaccai, 1979). These models are represented in Fig. 4a and b respectively. It was suggested by Yoshida et al. (1979) that the discrepancies in the stoichiometry could be due to errors in the estimation of molecular weights of the F_1-ATPases.

DCCD-sensitive F_1F_o complexes capable of catalysing ATP-Pi exchange, ATP-dependent H^+-pumping and $\Delta\tilde{\mu}_{H^+}$-driven ATP synthesis have been isolated from E. coli (Forster and Fillingame, 1979; Friedl et al., 1979), PS3 (Sone et al., 1975) and Rhodospirillum rubrum (Bengis-Garber and Gromet-Elhanan, 1979). The availability of these systems allows the characterization of the ATPase as a H^+ pump as postulated in the chemiosmotic model of energy coupling. The role of the F_o as the H^+ channel was confirmed by the demonstrations of H^+ translocation into K^+-loaded F_o-proteoliposomes upon addition of valinomycin (Okamoto et al., 1977; Negrin et al., 1980). Such H^+ movements are dependent upon F_o concentration and may be inhibited by DCCD and F_1-ATPase. By selective reconstitution of F_o with the purified sub-units of F_1 into proteoliposomes, the 'gate' function of the ATPase was acribed to the γ, δ and ε sub-units (Yoshida et al., 1977). The ATPase thus functions as postulated by the chemiosmotic hypothesis.

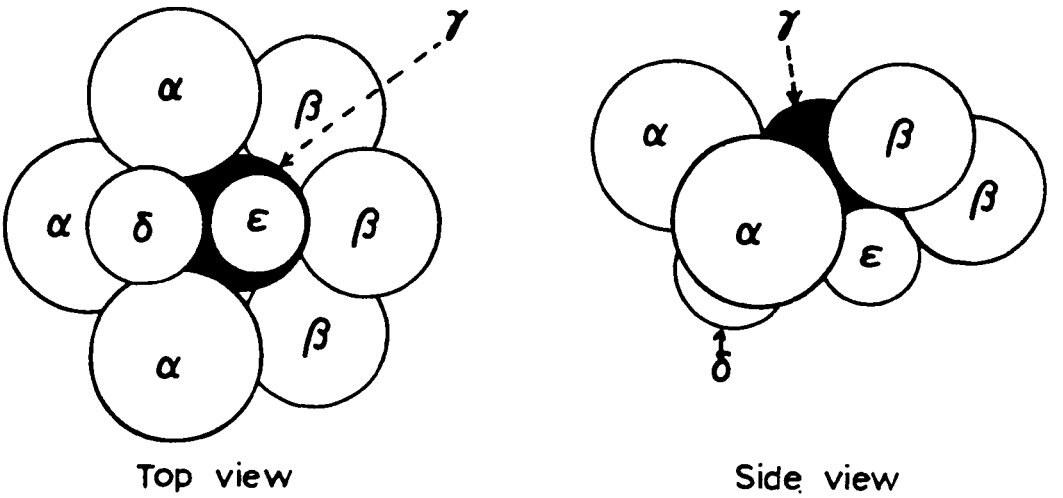
Measurement of $\Delta\tilde{\mu}_{H^+}$.

$\Delta\tilde{\mu}_{H^+}$ is composed of the $\Delta\Psi$, the membrane potential, and ΔpH, related by the equation

$$\Delta\tilde{\mu}_{H^+} = \Delta\Psi - Z\Delta pH$$

Determinations of $\Delta\tilde{\mu}_{H^+}$ would thus require the measurement of these two components.

(a)



(b)

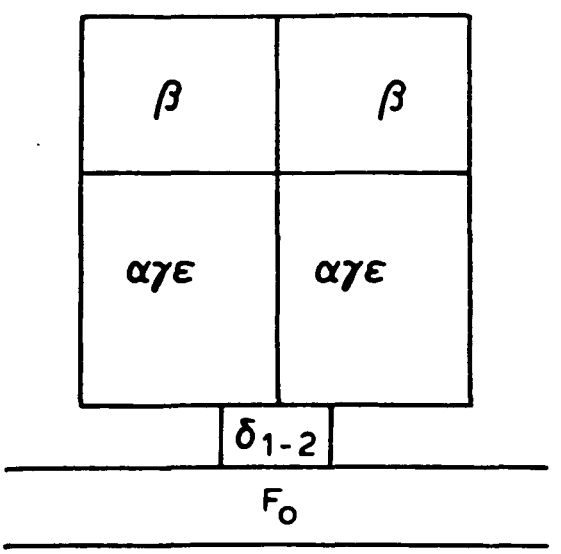


Fig.4: Possible structural organization of the sub-units of the F_1-ATPase of E. coli.
(a) hexagonal model (after Bragg and Hou,1980).
(b) tetragonal model (after Vogel and Steinhardt,1976).

The most widely used method involves monitoring the equilibration of weak acids (or bases) or lipophilic ions across the membrane in response to ΔpH or $\Delta\Psi$ respectively. The concentration gradient of the monitored species can be determined by traditional transport methods such as rapid centrifugation or filtration. These methods, though sensitive, have been criticized on the basis that changes in the environment during the separation stage could lead to underestimations (Ramos et al., 1979). Alternatively, flow dialysis (Colowick and Womack, 1969; Ramos et al., 1979) which is less sensitive, but does not disturb the experimental conditions, can be used.

In the measurement of the $\Delta\Psi$, the choice of lipophilic ions depends on the polarity of the potential; cations such as triphenylmethyl phosphonium ($TPMP^+$) and dibenzyl dimethylammonium (DDA^+) are commonly used to measure $-\Delta\Psi$ (Harold and Papineau, 1972a; Holian and Wilson, 1980) while anions such as SCN^- and phenyldicarbaundecarborane (PCB^-) are used to measure inside-positive $\Delta\Psi$ (Sorgato et al., 1980; Griniuviene et al., 1974).

The distribution of K^+ and related alkali metal ions such as Cs^+ and Rb^+ can be used to measure $\Delta\Psi$ (inside negative) in systems containing membranes made permeable to those ions by the addition of valinomycin. This cyclodepsipeptide has been shown to form a lipid-soluble 1:1 complex with K^+ (Tosteson, 1968), facilitating the electrogenic diffusion across the membranes. Its action is specific to K^+, Cs^+ and Rb^+; Na^+ for example was found to virtually form no lipid-soluble complex with valinomycin (Pressman, 1968).

On the implicit assumption that the distribution of these permeant ions are carrier-free, $\Delta\Psi$ may be calculated by applying the Nernst equation:

$$\Delta\Psi = -2{\cdot}303\ RT/nF \log [M^+]_{in}/[M^+]_{out},\ mV.$$

where $2{\cdot}303\ RT/nF \simeq 59$ at $25^{\circ}C$.

In using lipophilic cations as determinants of the $\Delta\Psi$, any non-specific interactions, shown in the case of $TPMP^+$ (Holian and Wilson, 1980) have to be taken into account. It must also be recognized that the $\Delta\Psi$ is directly affected by the permeable ions; high concentrations of these ions will lead to depolarization of the membrane.

The change in spectral characteristics of chlorophylls (Witt, 1975) and extrinsic probes such as safranine (Schuldiner and Kaback, 1975), 8-anilino-1-napthalene sulphonate (ANS^-) (Sone et al., 1976) and cyanine dyes (Kashket and Wilson, 1974; Laris and Pershadsingh, 1972) offers another non-perturbing means of measuring the $\Delta\Psi$. Relating fluorescence changes to $\Delta\Psi$ values however requires calibration against a reference; the method commonly employed is the diffusion potential of K^+ or Rb^+. In general, though fluorescence changes can be quantitatively related to $\Delta\Psi$, interpretations could be complicated by changes due to surface potential, stacking of probes and fluidity of the membranes which are among some of the factors that are known to cause alterations in fluorescence.

For the measurement of ΔpH, the use of weak acids or bases (choice governed by the internal pH) is on the basis that neutral species can freely cross the membrane while the ions are impermeable. The concentrations of the acids inside and outside can be determined by the methods outlined earlier in the $\Delta\Psi$ measurements (reviewed by Rottenberg, 1979). At equilibrium,

$$\Delta pH = \log [Acid]_{in}/[Acid]_{out}.$$

$$\text{where} \quad [Acid]_{in}/[Acid]_{out} = \frac{1/K_a + 1/H^+_{in}}{1/K_a + 1/H^+_{out}}$$

The $\Delta\Psi$ and ΔpH values can thus be used for calculating $\Delta\tilde{\mu}_{H^+}$.

INHIBITORS OF OXIDATIVE PHOSPHORYLATION.

The use of inhibitors has made a significant contribution to the studies of oxidative phosphorylation and energy-linked reactions. The inhibitors can be divided into three different classes based primarily on their mode of action. These are (i) electron transport inhibitors (ii) ATPase or energy-transfer inhibitors and (iii) uncouplers.

The first of these classes of inhibitors include such compounds as rotenone, antimycin A, piericidin A, 2-n-heptyl-4-hydroxyquinoline-N-oxide (HOQNO), cyanide and azide. Though they all inhibit electron flow, hence the generation of the $\Delta\tilde{\mu}_{H^+}$, they do so at different sites. In the mitochondrial system, rotenone and piericidin A inhibit NADH dehydrogenase, antimycin A and HOQNO inhibit cytochrome b-c_1 segment, and cyanide and azide act on the terminal oxidases (reviewed by Singer, 1979; Slater, 1967). In effect, they inhibit electron transport at sites I, II and III respectively.

Energy-transfer inhibitors may be defined as those compounds that specifically inhibit the terminal stage of oxidative phosphorylation ie. inhibiting only ADP-stimulated electron transport (State 3) and not affecting State 4 . As such, inhibition by energy-transfer inhibitors can be specifically reversed by uncouplers. Some ATPase inhibitors interact directly with the F_1-portion of the ATPase complex. Included in this group are compounds such as aurovertins, 4-chloro-7-nitrobenzofurazan (Nbf-Cl), quercetin and efrapeptin. Others, such as carbodiimides, oligomycins, venturicidins, organotins and Dio 9 interact with the membrane component of the ATPase complex. The characteristics and the mode of action of these inhibitors have recently been reviewed by Linnett and Beechey (1979).

Uncouplers are usually lipophilic compounds that are able to 'dissolve' in the membrane, increasing its H^+ conductance. This would render the membrane freely permeable to H^+ hence dissipating the $\Delta\tilde{\mu}_{H^+}$,

preventing its utilization in ATP synthesis. The action of uncouplers manifests as an acceleration of electron transport as a result of the removal of the constraint imposed by the $\Delta\tilde{\mu}_{H^+}$. Examples of uncouplers are dinitrophenols, salicylanilides, benziimidazoles and carbonyl cyanide phenylhydrazones.

It must be emphasized that the above classification is flexible, due to the non-specific action of some of these inhibitors. DCCD for example, at low concentrations (approx. 1 nmole (mg mitochondrial protein)$^{-1}$) acts as a classical ATPase inhibitor (Beechey et al., 1967) but at high concentrations, interactions with non-specific sites can cause inhibition of electron transport (Gutowsky and Rosenberg, 1976; Casey et al., 1981) and uncoupling of oxidative phosphorylation (Kalra and Brodie, 1971). Similarly, HOQNO at high concentration was found to be an uncoupler (Haas, 1964). In view of the secondary effects of many inhibitors, due care must be taken in the interpretation of data involving the use of inhibitors.

PROPIONIC ACID BACTERIA.

The family Propionibacteriaceae is divided into the genus Eubacterium and Propionibacterium; the latter being the genus to which propionic acid bacteria belong. Members of this group are Gram +ve, non-spore forming, non-motile, anaerobic to aerotolerant bacteria. They are pleomorphic, usually rod-shaped and arranged in pairs of V or Y configuration. They also sometimes occur in short chains or clumps. The principal fermentation products are propionate and acetate.

Eight species of this group are presently recognized (Moore and Holdeman, 1974), a decrease in the previously accepted number of eleven (Breed et al., 1957). This is due to the consolidation of certain closely related species, for example P. shermanii and P. freudenreichii into one, with P. shermanii brought to the sub-species level. Also, certain species, such as those previously recognized as

P. zeae, P. petersonii, P. raffinosaceum and P. technicum are now accepted as being synonymous with P. jensenii, and as such, regrouped into one specie. The inclusion of P. acnes in this genus is somewhat controversial. Originally, this bacterium was placed in the genus Corynebacterium because of its morphological relationship to this group. It was argued that its fermentation pattern and strong preference for anaerobic environment would favour its inclusion in the genus Propionibacterium (Douglas and Gunter, 1946). This suggestion was not accepted probably due to the observed inability of P. acnes to ferment lactate. Fermentation of lactate, a characteristic of Propionibacterium, was later established in P. acnes and ascribed the failure to detect this reaction by Douglas and Gunter (1946) to inefficient anaerobiosis (Moore and Cato, 1963).

P. acnes displays variable characteristics as shown by Tables 1a and b. This could be due in part to the fact that Corynebacterium acnes (as it was then known) consists of two groups, as pointed out by Brzin (1964) and later confirmed by Voss (1970). Groups I and II of Voss (1970) were later identified as P. acnes and P. granulosum respectively (Johnson and Cummins, 1972). Comparison of the characteristics of P. acnes and classical propionibacteria with respect to fermentation and biochemical reactions (Moore and Holdeman, 1974) shows certain variations, though these are no larger between P. acnes and the classical propionibacteria themselves. However, P. acnes consistently produces indole and liquefies gelatin (Moss et al., 1969) while classical propionibacteria do not (Malik et al., 1968). Due to the variations observed, these studies cannot conclusively support the classification of P. acnes into the genus Propionibacterium.

To resolve this taxonomic controversy, cell-wall, lipid and DNA composition of P. acnes were compared with those of classical propionibacteria. The cell-wall of P. acnes was found to show distinct

Table 1a. : Sugar fermentation by P. acnes.

	1	2	3 a,	3 b,	4	5	6*
Arabinose	0/38	4/15	.	.	.	0/37	+
Adonitol	31/38	9/15		.	.	.	-
Cellobiose	0/38	0/15		.	.	.	.
Fructose	38/38	15/15		.	27/27	37/37	+
Galactose	38/38	15/15		.	.	36/37	+
Glucose	38/38	15/15		.	27/27	37/37	+
Glycerol	38/38	15/15		.	27/27	34/37	+
Inositol	0/38	2/15		.	.	.	-
Lactose	0/38	1/15		.	0/27	0/37	.
Maltose	29/38	7/15	0/129	15/17	0/27	1/37	.
Mannitol	35/38	7/15		.	12/27	6/37	.
Mannose	38/38	13/15		.	.	37/37	+
Melibiose	0/38	2/15		.	.	.	.
Raffinose	0/38	4/15		.	.	0/37	.
Rhamnose	0/38	0/15		.	.	.	.
Salicin	0/38	3/15		.	0/27	0/37	-
Sorbitol	21/38	7/15		.	.	.	.
Sorbose	0/38	0/15		.	.	.	.
Sucrose	0/38	7/15	0/129	12/17	0/27	0/37	-
Trehalose	28/38	15/15	0/13	14/15	.	.	+
Xylose	0/38	8/15	.	.	0/27	.	.
Melezitose	.	7/15	0/13	10/15	.	.	.

The figures above represent no. positive to no. tested.

*7 strains tested and results presented collectively as +ve or -ve reactions only.

Refs:

1) Puhvel (1968)

2) Brzin (1964)

3) Voss (1970)

4) Moss et al., (1967)

5) Douglas and Gunter (1946)

6) Zierdt et al. (1968)

Table 1b. : Biochemical characteristics of P. acnes.

	1	2	3 a,	3 b,	4	5	6*
Liquefaction of gelatin	38/38	.	129/129	0/17	27/27	37/37	+
Catalase	38/38	.	.	.	27/27	37/37	+
Indole production	34/38	8/15	119/129	0/17	.	18/37	+
Reduction of litmus	0/38	.	.	.	.	37/37	+
Blood haemolysis	38/38(α)	14/15(β)	.	.	14/57(β)	37/37(β)	.
Nitrate reduction	29/38	8/15	8/11	2/15	27/27	37/37	+
Starch hydrolysis	0/38	3/15	.	.	.	0/37	.
Tyrosine hydrolysis	0/38	.	.	.	.	.	.
Methyl red test	0/38	.	.	.	27/27	.	.
H_2S production	0/38	.	.	.	25/27	.	.
Tributyrin test	38/28	.	.	.	.	.	.
Cytochrome oxidase production	0/38	.	.	.	.	.	.
Citrate ulitilization	0/38	.	.	.	.	.	.
NaCl tolerance 2·5%	38/38	.	.	.	.	.	.
5·0%	0/38	.	.	.	.	.	.
Urease	0/38	.	.	.	0/27	.	.
NH_3 production	38/28	.	.	.	.	.	.

*as in Table 1a

All references are as in Table 1a.

similarities with those of classical propionibacteria, in that it contained combinations of glucose, galactose and mannose as the cell-wall sugars, with alanine, glutamic acid, glycine and L-diaminopimelic acid as the peptidoglycan amino-acids (Johnson and Cummins, 1972). In addition, P. acnes also showed a similar lipid profile as the classical propionibacteria; the principal fatty acids found were the C_{15}-branched chain compounds (Moss et al., 1967; 1969). P. acnes did not contain the long-chain fatty-acids such as corynemycolic acids characteristically found in corynebacteria (Corina and Sesardic, 1980).

Analyses of the DNA base composition of P. acnes gave guanine/cytosine values of 57-60%, while classical propionibacteria had a guanine/cytosine content of 65-67%. The degree of homology between P. acnes and classical propionibacteria, as determined by DNA competition experiments did not provide conclusive evidence to the taxonomic position of P. acnes. While there was good homology between the species of classical propionibacteria, there was very little homology between P. acnes and classical propionibacteria (Johnson and Cummins, 1972). Even though P. acnes is now classified as a Propionibacterium, certain reservations must be made concerning its physiological similarities with classical propionibacteria.

CARBON METABOLISM IN PROPIONIC ACID BACTERIA.

Propionic acid bacteria are characterized metabolically by their ability to ferment carbohydrates to a mixture of propionate, acetate and carbon dioxide. The elucidation of the pathway by which propionate is formed and the characterization of the enzymes involved owes largely to the work of Harland G. Wood and his collaborators (reviewed by Hettinga and Reinbold, 1972). Initially, propionate formation was thought to proceed via the reduction of pyruvate with lactate as the intermediate. There is however no evidence in support of this mechanism. Another

proposed mechanism involves the decarboxylation of a dicarboxylic acid (Werkman and Wood, 1942) as indicated by the earlier observations that propionibacteria could ferment succinate resulting in the formation of propionate (Hitchner, 1934). The major contribution to this proposed mechanism was the discovery of heterotrophic CO_2 fixation in propionibacteria (Wood and Werkman, 1938; 1940). The amount of CO_2 fixed was found to correlate with the amount of succinate formed, with the fixed carbon occurring in the carboxyl group of succinate (Wood and Werkman, 1938; Wood et al., 1941). This therefore indicated that succinate formed was from the union of a C_3 compound with CO_2. The work of Krebs and Eggleston (1941) lent support to the postulated route for succinate formation being by way of oxaloacetate, malate and fumarate. This carboxylation reaction was later shown to be catalysed by carboxytransphosphorylase, converting phosphoenolpyruvate to oxaloacetate in the presence of CO_2 and inorganic phosphate (Siu and Wood, 1962). The decarboxylation of succinate, producing propionate, was demonstrated by Delwiche (1948) and Johns (1951). Studies with cell-free extracts of propionibacteria revealed that this process involved CoA derivatives of succinate and propionate, and that a C_1 compound and not CO_2 is the product (Delwiche et al., 1956; Phares et al., 1956). This low turnover of CO_2 led to the discovery that propionate formation occurred by a transcarboxylation of methylmalonyl CoA (a) (Swick and Wood, 1960) which was formed from the acylation of succinate by CoA transferase (Delwiche et al., 1956), followed by isomerization and racemization reactions catalysed by methylmalonyl isomerase and methylmalonyl racemase respectively (Swick and Wood, 1960; Mazumder et al., 1962). The sequence of the reactions and enzymes involved in propionate formation are summarized in Fig. 5 and Table 2 respectively.

In addition to the above pathway, propionibacteria possess alternative routes for anaerobic metabolism of glucose. This was indicated by the relative insensitivity of glucose metabolism to

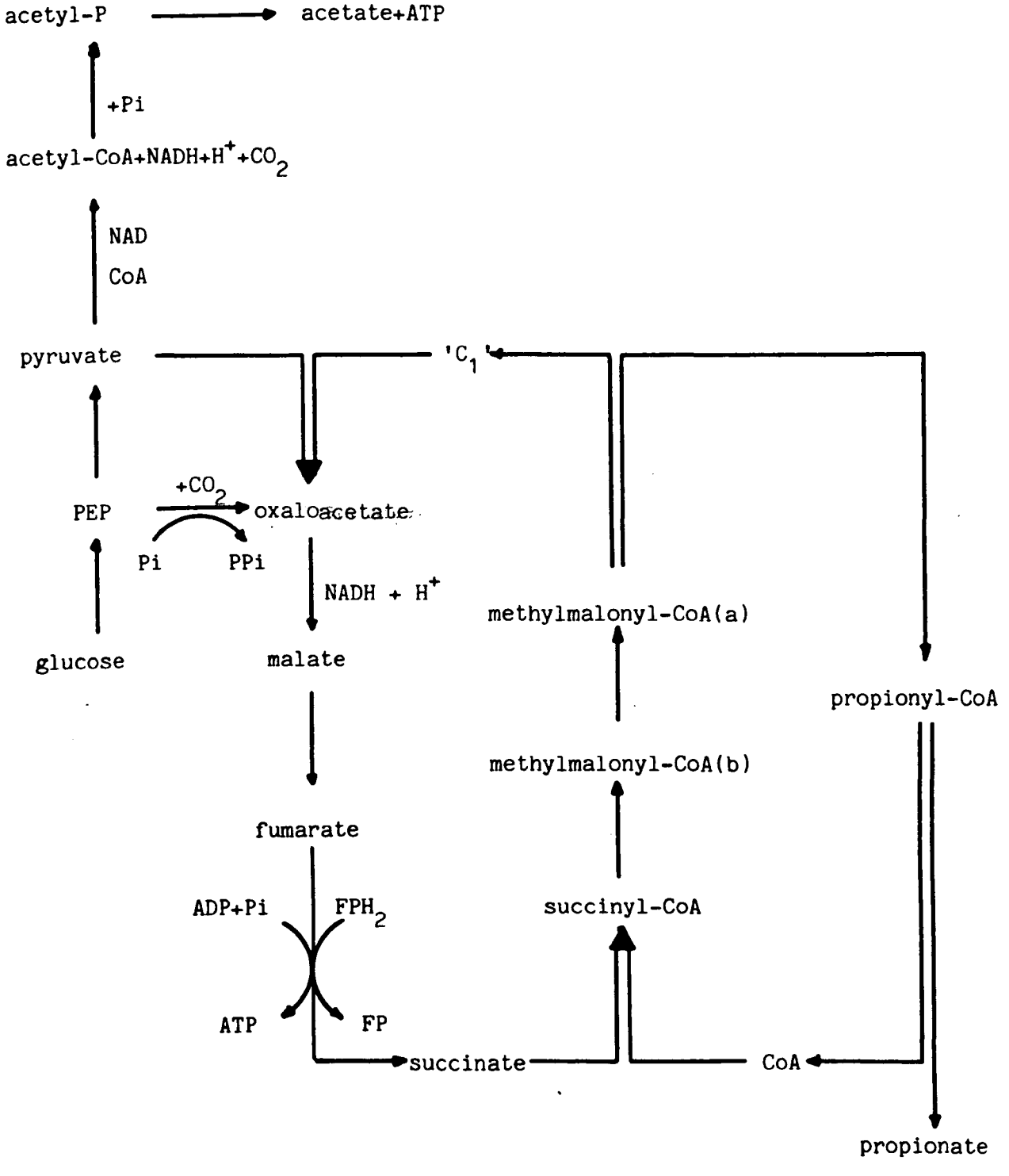


Fig.5 : Pathway for the formation of propionate in propionibacteria.

Reactions:

1. 1·5 glucose + 3 NAD^+ + 3 Pi $\rightleftharpoons$ 3 PEP + 3 NADH + 3 H^+ (Glycolysis).
2. 3 PEP + 3 ADP $\rightleftharpoons$ 3 pyruvate + 3 ATP (Pyruvate kinase).
3. pyruvate + NAD^+ + CoA $\rightleftharpoons$ acetyl-CoA + NADH + H^+ + CO_2 (Pyruvate dehydrogenase).
4. acetyl-CoA + Pi $\rightleftharpoons$ acetyl-P + CoA (Phosphotransacetylase).
5. acetyl-P + ADP $\rightleftharpoons$ acetate + ATP (Acetyl kinase).
6. 2 pyruvate + 2 me-malonyl-CoA(a) $\rightleftharpoons$ 2 oxaloacetate + 2 propionyl-CoA (Transcarboxylase).
7. 2 oxaloacetate + 2 NADH + 2 H^+ $\rightleftharpoons$ 2 malate + 2 NAD^+ (Malic dehydrogenase)
8. 2 malate $\rightleftharpoons$ 2 fumarate + 2 H_2O (Fumarase).
9. 2 NADH + 2 H^+ + 2 Pi + 2 ADP + 2 FP $\rightleftharpoons$ 2 NAD + 2 ATP + FPH_2
10. 2 fumarate + FHP_2 $\rightleftharpoons$ 2 succinate + FP (Fumarate reductase).
11. 2 succinate + 2 propionyl-CoA $\rightleftharpoons$ 2 succinyl-CoA + 2 propionate (CoA transferase).
12. 2 succinyl-CoA $\rightleftharpoons$ 2 me-malonyl-CoA(b) (Methylmalonyl isomerase).
13. 2 methyl-malonyl CoA(b) $\rightleftharpoons$ 2 me-malonylCoA(a) (Methylmalonyl racemase).

Net: 1·5 glucose + 6 Pi + 6 ADP $\rightleftharpoons$ 6 ATP + 2 H_2O + CO_2 + acetate + 2 propionate.

Table 2: Reactions involved in the formation of propionate in propionibacteria.

glycolytic inhibitors (Wiggert and Werkman, 1939; Wood et al., 1937). Further studies with [^{14}C] glucose were consistent with there being more than one pathway of glucose degradation (Wood et al., 1955). The presence of an alternative pathway ie. the pentose phosphate pathway, in propionibacteria was confirmed (Vandermark and Fukui, 1956), and was later found to play an essential role in propionate formation as shown by the studies using [^{14}C] ribose and gluconate (Stjernholm and Flanders, 1962).

The true facultative nature of propionibacteria is reflected by their capability of oxidative degradation of glucose, pyruvate and lactate. The presence of a functional tricarboxylic acid (TCA) cycle was demonstrated in P. pentosaceum (Delwiche and Carson, 1953), P. shermanii and P. petersonii (Bonartseva et al., 1973a). In addition, Bonartseva et al. (1973a) also discovered two key enzymes of the glyoxylate shunt, namely malate synthase and isocitrate lyase.

OXIDATIVE PHOSPHORYLATION IN PROPIONIBACTERIA.

The finding of cytochromes a and b (Chaix and Fromageot, 1942), succinate dehydrogenase (Lara, 1959) and lactate dehydrogenase (Molinari and Lara, 1960) constituted the earliest evidence for an electron transport system in propionibacteria. Its significance in metabolic processes was not recognized until it was demonstrated that the growth yield of P. pentosaceum could not be accounted for solely by substrate-level phosphorylation (Bauchop and Elsden, 1960). Extra energy must therefore be generated by electron transfer processes. Subsequently, it was shown that several species of this genus contain redox components (Table 3), which in terms of types and number of constituents, resemble electron transport chains found in aerobic respiratory chains.

Based on studies of molar growth yields and fermentation balance, de Vries et al. (1973) concluded that anaerobic growth of

SPECIES	COMPONENTS							REFERENCE.
P. shermanii	NADH	MK	b		a_1	d	o FR	de Vries et al., (1972) Bonartseva et al., (1973b) Schwartz, (1973) Schwartz and Sporkenbach, (1975) Pritchard et al., (1977).
P. freudenreichii	NADH	MK	b		a_1	d	o FR	de Vries et al., (1972) de Vries et al., (1973)
P. petersonii	NADH	MK	b	c	a_1	d		Bonartseva et al.,(1973b)
P. pentosaceum	NADH		b		a_1	d	o	de Vries et al., (1972)
P. rubrum	NADH		b		a_1	d	o	de Vries et al., (1972)
P. arabinosum	NADH	MK	b					Sone, (1972) Sone, (1974).

Table: 3 : Components of the electron transport chains in propionibacteria.

P. pentosaceum and P. frendenreichii on glucose, lactate and glycerol is accompanied by phosphorylation linked to electron transport. Further evidence for the operation of an anaerobic electron transport chain was the demonstration of a cytochrone b linked oxidation of NADH, lactate or α-glycerophosphate with fumarate as the terminal acceptor (Sone, 1972; de Vries et al., 1973; 1977). The involvement of cytochrome b is somewhat controversial; Schwartz and Sporkenbach (1975) suggested that electron transport to fumarate does not involve cytochromes. As found with E. coli, anaerobically-grown propionibacteria contain cytochromes a_1, o and d (Table 3). As such, they are capable of aerobic electron transport as indicated by the oxidation of NADH, lactate or α-glycerophosphate with oxygen as the terminal acceptor (Sone, 1972; de Vries et al., 1977; Schwartz and Sporkenbach, 1975).

The role of oxygen in the energy transduction processes of propionibacteria has only been studied in P. freudenreichii, P. shermanii and P. petersonii. In general, a shift from anaerobic to aerobic growth conditions usually results in increased growth yield and a decrease in fermentation products. de Vries et al. (1972) showed that a shift from anaerobic to aerobic growth with P. freudenreichii using lactate as the carbon and energy source resulted in the cessation of acetate and propionate production, with a concomitant accumulation of pyruvate. These observations, coupled with the knowledge that a functional TCA cycle is present in propionibacteria (Delwiche and Carson; 1953) led de Vries et al. (1972) to conclude that oxidative phosphorylation with a P/O ratio of 1 to 2 was the only mode of energy transduction under these conditions. However, Pritchard et al. (1977) did observe continued production of acetate with P. shermanii growing aerobically on lactate, though in agreement with de Vries et al. (1972), there was a decrease in propionate production. The growth yield of the organisms was also found to increase. This would suggest that oxidative phosphorylation was not

the only mode of energy transduction; substrate-level phosphorylation via the acetate kinase reaction was still taking place. The capacity for oxidative phosphorylation in P. shermanii was also reported by Bryukhanacheva et al. (1975).

Oxygen was not found to alter significantly the activity of the glycolytic enzymes in P. shermanii grown on glucose. In addition, a slight decrease in the activity of the TCA cycle enzymes, with the exception of succinate and malate dehydrogenase, where a slight increase was observed (Bonartseva et al., 1973a). This would imply that glycolysis still assumes a major role in P. shermanii under aerobic conditions. These observations with P. shermanii were in contrast with those generally found in other facultative anaerobes like E. coli for example, where oxygen was found to increase the level of the TCA cycle enzymes (Gray et al., 1966a, b), and induce oxidative phosphorylation (Cavari et al., 1968). In P. petersonii however, there was a marked increase in TCA cycle enzymes with the exception of citrate synthase and succinate thiokinase, where a slight decrease was observed. The pathway for glucose degradation was also altered from glycolysis to the pentose phosphate pathway (Bonartseva et al., 1973a).

The level of cytochromes in aerobically-grown P. rubrum, P. pentosaceum, P. shermanii and P. freudenreichii was found to be lower than in aerobically-grown cells (de Vries et al., 1972). This decrease in cytochrome level was ascribed to the inhibition of the δ-amino-laevulinic synthetase and δ-amino-laevulinic dehydratase by oxygen (Menon and Shemin, 1967). However, Pritchard et al. (1977) found that cytochrome synthesis in P. shermanii was only inhibited at low partial pressures of O_2 (pO_2) (10-40 mm Hg) while at higher pO_2 (160-330 mm Hg) the cytochrome level was similar to those found in anaerobically-grown cells. They suggested that the decrease observed by de Vries et al. (1972) was possibly due to an 'oxygen trap' situation, where the oxygen

concentration was sufficient to suppress cytochrome synthesis, but was insufficient to induce increased levels of enzymes and electron carriers required for aerobic metabolism.

From present knowledge, there seem to be variations in the mechanism of energy transduction within the genus Propionibacterium, particularly with respect to the role of oxygen. As discussed earlier, in P. freudenreichii, evidence indicates that oxidative phosphorylation is the only mode of ATP synthesis, though in P. shermanii, substrate-level phosphorylation is still operational. Some of the available data also appears to be conflicting, for example, Bryukhacheva et al. (1975) could not detect oxidative phosphorylation (measured as P/NADH) in P. shermanii grown anaerobically on glucose despite the demonstrations of cytochrome b-linked oxidation of NADH, lactate and α-glycerophosphate by oxygen (Schwartz and Sporkenbach, 1975).

Research on propionibacteria has in the past been concentrated mainly on the pathways of carbon metabolism and studies on energy transduction mechanisms is rather recent. It is hoped that the work described in this thesis will contribute to the available knowledge on energy transducing mechanisms in propionibacteria.

METHODS AND MATERIALS

METHODS AND MATERIALS.

1. General Techniques

i. Weighing of materials.

Materials, depending on the quantity required, were weighed with a Stanton Unimatic CL41 balance or a Thornton table balance.

ii. Measurements of pH.

pH measurements were made with an E.I.L 23A direct reading pH meter.

iii. Spectrophotometric measurements.

For routine absorbance measurements in the visible region where no temperature control was required, a Unicam SP600 was used. For measurements involving continuous monitoring of absorbance changes with the maintenance of a constant temperature, a Unicam SP1800 or SP8100 was used. All spectrophotometric measurements were carried out with cuvettes of 1cm path length.

iv. Sterilization procedures.

Sterilization of growth media, materials used for the production of plates and anaerobic growth vessels was carried out by autoclaving at 121^{o}C for 15 min. Dry sterilization of pipettes was procured at 180^{o}C for 2 h in a suitable oven.

v. Oxygen-free nitrogen.

Oxygen-free nitrogen was obtained by passage of nitrogen gas through three consecutive Dreschel bottles, the first containing Feiser's reagent (KOH, 20 g; sodium anthraquinone-2-sulphonic acid, 2 g; sodium dithionite, 15 g; dissolved in 100 ml of distilled water) and the following two bottles containing distilled water.

2. Analytical methods.

i. Protein estimation.

Protein concentration in cell-extracts was determined by the method of Lowry et al. (1951). Insoluble proteins were first digested by boiling in I.0 M-NaOH for 10 min; portions were then removed for protein estimation. Bovine serum albumin (fraction V) was used as the protein standard.

ii. Determination of inorganic phosphate.

Inorganic phosphate was determined by method of Fiske and SubbaRow (1925).

Reagents:

2·5% (w/v) ammonium molybdate in 1·5 M-H_2SO_4

ANSA reagent (1, 2, 4-amino-napthol sulphonic acid)

prepared as follows:

0·5 g of recrystallized ANSA was dissolved in a solution containing 30 g of $NaHSO_3$ and 6·0 g of $Na_2SO_3.7H_2O$, in a final volume of 250 ml. The solution was filtered and stored in the dark at 4^oC, or in the case of long term storage, at -20^oC.

Recrystallization of ANSA:

15 g of crude ANSA was first dissolved in a litre of hot solution (90^oC) containing 150 g $NaHSO_3$ and 10 g of $Na_2SO_3.7H_2O$. On cooling, the solution was filtered and 10 ml of conc. H_2SO_4 added to crystallize out the ANSA, which was then collected by filtration. The ANSA obtained was first washed with 300 ml of ice-cold distilled H_2O; subsequent washings was carried out with ethanol until the filtrate was colourless. The recrystallized ANSA was then dried and stored in the dark.

Pi assay procedure:

Deproteinized sample (0·5 ml) was added to 2·0 ml of water and

0·3 ml of molybdate solution and was shaken. 0·2 ml of ANSA reagent was then added to the mixture and was again shaken and incubated at room-temperature for 20 min. Absorbance was read at 691 nm against a reagent blank.

The concentration of Pi was determined from a calibration curve constructed for a concentration range of 0 to 1000 nmol Pi.

3. Organisms.

The following organisms were used:

Propionibacterium acnes, laboratory strain P37 (kindly provided by Dr.K.T.Holland, Department of Microbiology, University of Leeds).

Streptococcus faecalis ATCC 9790.

Acinetobacter calcoaceticus NCIB 8250.

i. Growth media.

The complex medium used for culturing P. acnes was Reinforced clostridial medium (RCM; Oxoid) at 38 g l^{-1}, supplemented with 3·4 g l^{-1} KH_2PO_4. The pH was adjusted to 6·9 with 10 M-KOH. RCM plates were made using Oxoid No.1 agar (1%, w/v) as the solidifying agent.

The semi-defined medium used for growing P. acnes contained the following:

	l^{-1}
Tryptone (Oxoid)	20 g
KH_2PO_4	9 g
Biotin (6 mg l^{-1})	2·5 ml
Panthothenate (1·2 g l^{-1})	2·5 ml
Thiamin (1 g l^{-1})	2·5 ml
Nicotinamide (2·4 g l^{-1})	2·5 ml
Pyridoxine HCl	0·025 g
Mineral salts solution	1.25 ml

The pH of the medium was adjusted to pH 7·0 with 10 M-KOH and was then sterilized by autoclaving (Methods section 1(iv)). Glucose was sterilized separately (by autoclaving) and aseptically added to a final concentration of 1·0% (w/v).

The composition of the mineral salts solution was as follows:

	$g\ l^{-1}$
$MgSO_4$	40
$MnSO_4$	2
NaCl	1
$ZnCl_2$	0·5
$CuSO_4$	0·05
$CoNO_3$	0·5

S. faecalis was grown in complex medium containing the following:

	$g\ l^{-1}$
KH_2PO_4	20
Yeast extract (Difco)	10
Tryptone (Difco)	20
Glucose	10
Hepes	12

The pH was adjusted to pH 7·8 with 10 M-KOH. Sterilization was procured by autoclaving.

A. calcoaceticus was grown on nutrient broth (Oxoid) supplemented with 1% glucose (w/v) and KH_2PO_4 (9 $g\ l^{-1}$). The pH was adjusted to pH 7·4 with KOH. The medium was sterilized by autoclaving.

ii. Maintenance of the organisms.

P. acnes was maintained in 10 ml batch cultures in RCM incubated at 37°C in anaerobic jars (Baird and Tatlock Ltd.) in an

atmosphere of H_2/N_2 generated by the 'GASPAK' system. The organism was routinely sub-cultured and plated out at 3 weekly intervals. In addition, P. acnes was also maintained in RCM agar stabs at 4°C and sub-cultured at 6 weekly intervals. A stock of P. acnes was also kept in liquid nitrogen.

S. faecalis and A. calcoaceticus were maintained in 10 ml batches of nutrient broth supplemented with glucose (1%; w/v) and KH_2PO_4 (9 g l^{-1}; pH 7·4) at 37°C respectively. Both organisms were sub-cultured every 48 h. For long term storage, both organisms were maintained as glycerol stabilates in sealed glass ampules at 4°C (by Mrs.A.Robertson and Mrs.G.A.Hardy respectively).

iii. Growth of organisms.

Anaerobic growth.

P. acnes was grown at 37°C in 800 ml batches of semi-defined medium containing 1% glucose (w/v) in 1 l bottles. The medium was made anaerobic by flushing with 95% N_2 and 5% CO_2 overnight prior to inoculating with 20 ml (2·5%, v/v) of an actively growing culture. The culture was harvested after 48-50 h growth; the mean generation time was found to be 8 - 11 h. Cultures of P. acnes were harvested during exponential growth phase.

Aerobic growth.

P. acnes was grown in 800 ml batches in the same medium as used for anaerobic growth, in 3 l Ehlenmeyer flasks at 37°C on a gyrotary shaker (New Brunswick Co.). The flasks were inoculated with 80 ml (10%, v/v) of culture which had been grown for 72 h under static growth conditions (80 ml culture in a 250 ml Ehlenmeyer flask). Cultures were harvested after 72 h growth; the mean generation time was found to be 24-28 h.

S. faecalis was grown at 37°C in 500 ml of the medium described earlier in 1 l Ehlenmeyer flasks. The cultures were grown for 48 h after

being inoculated with 20 ml (4%, v/v) of culture which had been grown for 48 h under static growth conditions.

A. calcoaceticus was grown in 500 ml of medium in 2 l Ehlenmeyer flasks on a gyrotary shaker at 28°C for 16 h with an initial inoculum of 10 ml (2%, v/v) of culture which had been grown for 24 h under static growth conditions.

4. Preparation of washed cell suspensions.

Cultures of organisms were harvested by centrifuging in the MSE Mistral 6L centrifuge at 5,000 $\underset{\approx}{g}$ for 10 min at 4°C. The cells were washed twice and resuspended in the appropriate buffer solution depending on the experiment being performed.

5. Determination of bacterial density.

The quantity of organism was assessed spectrophotometrically by measuring the turbidity at 540 nm of a suitable dilution of bacterial suspension, such that a value of 0·1 to 0·4 was obtained. The value of 0·4 represents the upper limit at which linearity existed in the relationship between extinction and the dry weight of the organism. This relationship was established by measuring the turbidity of a series of dilutions of a bacterial suspension (made with the wash buffer, 20 m$\underset{=}{M}$-KH_2PO_4, pH 7·0). A known volume was then sedimented by centrifuging at 10,000 $\underset{\approx}{g}$ for 10 min on a Sorvall RC5-B centrifuge (SS34 rotor). The supernatant was decanted, the sides of the tube dried and the pellet resuspended to the initial known volume. Portions of this suspension were then dried to a constant weight at 108°C in vials that had been earlier dried to a constant weight. From the data, a calibration curve relating the dry weight and absorbance was obtained.

For P. acnes, an extinction of 1.0 at 540 nm was equivalent to 0·305 mg dry wt ml^{-1}.

6. Measurement of the membrane potential in P. acnes.

Cultures of P. acnes were harvested, washed twice and resuspended in 100 mM-Hepes-Tris (pH 7·0). The cell suspension was kept on ice in Universal bottles (fitted with Subaseal caps) which were continuously flushed with O_2-free N_2.

The reaction vessel consisted of a water-jacketed glass-chamber (volume of 8·7 ml) into which a K^+-sensitive electrode (E.I.L) and a salt-bridge connected to an external reference electrode were fitted. The temperature was maintained at 37°C by a circulating water-bath. Solutions were introduced into the chamber via a pore in the glass-stopper by means of gas-tight microlitre syringes (Hamilton Co.). The K^+ concentration was measured with a Vibret pH meter (Model 3920) and changes in the K^+ concentration were recorded with a Servoscribe chart recorder.

The electrode was calibrated using KCl solutions (0·1 mM to 1·0 mM) made up in 100 mM-Hepes-Tris (pH 7·0). The response of the electrode was linear between K^+ concentrations of 0·1 mM to 1·0 mM. Routinely, the electrode was calibrated with 0·316 mM-KCl, corresponding to a pK (-log $[K^+]$) of 3·5 prior to performing K^+ measurements.

The reaction vessel was completely filled with 100 mM-Hepes-Tris (pH 7·0) and was flushed with O_2-free N_2 for 20 min. The stopper was then lowered into the chamber while simultaneously removing the syringe needle supplying the N_2. Cells (approx. 10 mg dry wt) were introduced into the chamber with the stirrer switched off. With the stirrer switched on, valinomycin (10 μg) was added and the $[K^+]$ was monitored until a steady value was obtained, after which CCCP (final concentration of 23 μM) was added to measure the total K^+ present in the system. All solutions added were deoxygenerated by flushing with O_2-free N_2.

From the data obtained, the $\Delta\Psi$ may be calculated by applying the Nernst equation,

$\Delta\Psi = -60 \log [K^+]_{in}/[K^+]_{out}$, as discussed in the Introduction.

For determining the membrane potential under aerobic conditions, the volume of the reaction mixture was decreased to 6·0 ml and measurements were made with the stopper removed to allow equilibration of the reaction mixture with air.

When the effect of inhibitors on the $\Delta\Psi$ was studied, appropriate solvent controls were performed.

7. Determination of intracellular water.

Intracellular water was measured by using [^{3}H] inositol to label the extracellular water spaces, and the total water content was obtained by drying the cells to a constant weight at 110°C.

[^{3}H] Inositol (1 μCi) was mixed with 10 ml of cell suspension (approx. 100 mg dry wt) in glass centrifuge tubes which had been dried to a constant weight. The cell suspension was then centrifuged at 5,000 $\underset{\approx}{g}$ for 10 min in a swing-out rotor on a MSE Mistral 6L centrifuge. The supernatant was carefully decanted and the sides of the tube dried with tissue paper. Samples (0·1 ml) of this supernatant were removed for scintillation counting (labelled as S_1). The pellet was then resuspended with 10 ml of fresh buffer (100 m$\underset{=}{M}$-Hepes-Tris (pH 7·0) and was recentrifuged at 5,000 $\underset{\approx}{g}$ for 10 min. The supernatant was decanted and the tube again dried. Samples (0·5 ml) of the supernatant were taken for scintillation counting (labelled as S_2). The tube was weighed and then dried to a constant weight at 110°C.

Scintillation counting was carried out using Bray's (1960) scintillation fluid.

From the data obtained, intracellular water may be determined thus:

$$H_2O_{total} = H_2O_{extracellular} + H_2O_{intracellular}$$

$H_2O_{extracellular}$ is given by $[S_{2/S_1} \times 10]$

The intracellular water of P. acnes was found to be 1·47 ml (g dry wt)$^{-1}$.

8. Respiration studies with P. acnes.

Rates of respiration was measured at 37°C in 50 mM-Tris-Cl (pH 7·4) and 10 mM-$MgCl_2$ in a final volume of 3·0 ml with a Clarke-type oxygen electrode (Rank Bros., Cambridge). The changes in oxygen tension were recorded with a Servoscribe or Linseis chart-recorder. The system was calibrated using NADH and PMS as described by Robinson and Cooper (1970). The concentration of oxygen in the medium used in the present investigation was found to be 0·31 mM. Additions were made by means of micro-litre syringes (Hamilton Co.).

9. Determination of cytochromes in P. acnes.

Reduced-minus-oxidised difference spectra of the cytochromes of P. acnes were determined at 37°C in 50 mM-Tris-Cl (pH 7·4) and 10 mM-$MgCl_2$ in the Unicam SP 8100 at a cell concentration of approximately 7(mg dry wt) ml^{-1}. Reduction and oxidation of the cytochromes were obtained by the addition of dithionite and potassium ferricyanide respectively. To determine the level of the carbon monoxide binding pigment (cytochrome o), a cell suspension that had been reduced by dithionite was gassed with carbon monoxide for 60 s and the carbon monoxide difference spectrum was determined using a dithionite-reduced sample as the reference.

10. Measurements of respiration-driven H^+ translocation in P. acnes.

Cells were harvested, washed twice with 300 mM-KCl and resuspended in the same medium.

The reaction vessel used was an oxygen electrode (Rank Brothers, Cambridge) to which a combined micro-pH electrode (E.I.L) was fitted via a rubber stopper. The temperature was kept constant at 37°C by a

circulating water-bath. Solutions were introduced into the chamber with gas-tight microlitre syringes (Hamilton Co.) through a pore made in the stopper. The pH was measured with a pH meter (built at Biochemistry Department, University of Hull, to a design by Professor P.B.Garland) and pH changes were monitored with a Servoscribe chart recorder.

The reaction vessel was completely filled with 300 mM-KCl or varying concentrations of KCl and KSCN, and was then flushed with O_2-free N_2 for 20 min with the stirrer switched on. With the stirrer off, the stopper carrying the pH electrode was lowered into the chamber while simultaneously removing the nitrogen line. Care was taken to exclude air-bubbles. Cells (at a final concentration of approximately 4·8 mg dry wt ml^{-1}) and carbonic anhydrase (40 μg ml^{-1} final concentration) were introduced into the reaction chamber. The stirrer was then switched on and the cells were allowed to equilibrate for approximately 60 to 90 min until the rate of acid production decreased to approximately 1·5 ng ions (mg dry wt)$^{-1}$ min^{-1} so that a decay in the H^+ pulse may be observed. Oxygen was pulsed as air-saturated 150 mM-KCl at 37°C. To calibrate the scale, a pulse of standard anaerobic 33 mM-HCl in 0·1 M-KCl was added and the pH change was recorded. All inhibitors added were made anaerobic by flushing with O_2-free N_2. Appropriate controls were performed to eliminate the effects of solvents.

11. Preparation of membrane fragments from P. acnes.

Enzymic methods:

1. Lysozyme - EDTA treatment.

The method used was essentially similar to that described by Stephenson (1977). Cells were harvested and washed twice in 50 mM-Tris-Cl (pH 7·4) and 5 mM-$MgCl_2$, and resuspended in buffer containing 50 mM-Tris-Cl (pH 8·0), 5 mM-$MgCl_2$, 20% sucrose (w/v) and 15 mM-EDTA, at a final cell concentration of approximately 10 mg ml^{-1}. Lysozyme (final conc. of 0·5 mg ml^{-1}) and a few crystals of DNAse were added and the suspension

was incubated in a shaking water-bath at 30°C for 30 min. The suspension was then centrifuged at 10,000 g for 10 min at 4°C in a Sorvall RC5-B centrifuge (SS34 rotor). The pellet obtained was resuspended in the original wash buffer.

11. Lysozyme-LiCl treatment.

Lysozyme-LiCl treatment of P. acnes was carried out as described by Stephenson (1977). Cells were harvested and the pellet was resuspended in 0·67 of the original culture volume with a solution containing LiCl (0·59 M), sucrose (0·75 M), Hepes-KOH (10 mM, pH 7·0). $MgSO_4$ (10 mM) and lysozyme (0·5 mg ml^{-1}). The suspension was incubated at 30°C for 1 h on a gyrotary shaker before centrifuging at 10,000 g for 30 min at 4°C on the Sorvall RC5-B centrifuge (GSA rotor). The pellet was resuspended in a solution containing LiCl (0·59 M) and sucrose (0·75 M) to a final volume of 4 ml at 4°C. This suspension was then rapidly added to 200 ml (50 volume) of ice-cold solution containing MOPS-NaOH (10 mM; pH 6·6) and 1 mM-$MgCl_2$ and was then blended in a Waring blender for 60 s. DNAse (20 μg ml^{-1}) and RNAse (10 μg ml^{-1}) was added and the mixture was gently stirred at room-temperature for 30 min. The suspension was then centrifuged at 27,000 g for 30 min at 4°C on the Sorvall RC5-B (GSA rotor).

Isolation of membranes:

The pellet obtained was resuspended by means of a hand-held homogenizer in an ice-cold solution of MOPS-NaOH (0·1 M; pH 6·6) and $MgSO_4$ (10 mM) in a 1:4 (v/v) ratio. Cells and large fragments were removed by centrifuging at 800 g for 30 min at 4°C in MSE Mistral 6L centrifuge. The supernatant obtained was recentrifuged at 30,000 g for 30 min in a Sorvall RC5-B (SS34 rotor). The pellet obtained was resuspended in buffer containing Tris-Cl (50 mM; pH 7·4) and 5 mM-$MgCl_2$.

12. Physical disruption techniques.

i. General technique for membrane isolation.

Cells that had been disrupted (by the methods described below) were gently stirred in the presence of DNAse (0·5 mg ml^{-1}) for 30 min at room-temperature. Unbroken cells and large fragments were removed by centrifuging at 10,000 $\underset{\approx}{g}$ for 10 min at 4°C in a Sorvall RC5-B centrifuge (SS34 rotor). The membrane fragments were then sedimented by centrifuging at 100,000 $\underset{\approx}{g}$ for 60 min at 4°C in a MSE 65 superspeed ultracentrifuge (10 ml x 10 rotor). The pellet thus obtained was resuspended in 50 $\underset{=}{m}M$-Tris-Cl (pH 7 4) and 5 $\underset{=}{m}M$-$MgCl_2$.

ii. Sonication.

Samples (20 ml) of cells (20 mg ml^{-1}) in 50 $\underset{=}{m}M$-Tris-Cl (pH 7·4) and 5 $\underset{=}{m}M$-$MgCl_2$ were sonicated using a Dawe Instruments Sonicator (Model 1130A) for twenty 30 s periods. The temperature was maintained below 10°C by keeping the cell suspension on a mixture of ice and salt.

iii. French pressure cell.

Cells (approx. 10-15 ml; 20 mg ml^{-1}) in 50 $\underset{=}{m}M$-Tris-Cl (pH 7·4) and 5 $\underset{=}{m}M$-$MgCl_2$ were passed 4 times through a French pressure cell operated at 35 MPa. After each press, both the cell suspension and the press were cooled on ice for approx. 10-15 min.

iv. Disruption with glass-beads.

Cell suspensions (10 ml of 20 mg ml^{-1}) in 50 $\underset{=}{m}M$-Tris-Cl (pH 7 4) and 5 $\underset{=}{m}M$-$MgCl_2$ were agitated with 8 ml of acid-washed glass-beads (100 mesh; BDH) in Universal bottles using an MSE cell-homogenizer. The cells were agitated for a total of 5 min in periods of 30 s. The temperature was kept to about 4°C by using an ice-salt-water mixture placed in the attachment reservoir into which the Universal bottles were

nearly fully submerged when fitted to the homogenizer. After treatment, the homogenate was decanted and the glass-beads washed with 5 ml of fresh buffer which was then decanted and pooled with the original homogenate. Membrane fragments were isolated as described previously.

v. Hughes pressure cell.

Cell suspensions (20 ml of approx. 20 mg ml^{-1}) in 50 mM-Tris-Cl (pH 7·4) and 5 mM-$MgCl_2$ were frozen overnight in a Hughes press. Passage of the frozen cell suspension was obtained at a pressure of 127·3 MPa.

vi. Preparation of membrane fragments from S. faecalis.

Membrane fragments from S. faecalis were prepared by two passages through a French pressure cell operated at 35 MPa in buffer containing 50 mM-Tris-Cl (pH 7.4) and 5 mM-$MgCl_2$.

13. Assay of ATPase activity.

Membrane-bound ATPase activity of P. acnes was assayed in acid-washed tubes containing the following:

0·7 ml buffer containing 50 mM-Tris-Cl (pH 7·6) and 7 mM-$MgCl_2$)

0·05 ml of membrane preparation (~ 0·5mg protein)

0·15 ml of H_2O.

The reaction mixture was pre-incubated at 37°C for 10 min before initiating the reaction by the addition of 0·1 ml of 0·1 M-ATP (pH 7·4) The reaction was allowed to proceed for 30 min before being terminated by adding 1·0 ml of ice cold 10% (w/v) TCA. Inhibitors, when added, were similarly pre-incubated with the reaction mixture before the addition of ATP. The volume of water was adjusted accordingly such that the final volume remained at 1·0 ml.

Membrane-bound ATPase activity of S. faecalis was assayed as described for P. acnes except that the volume of the membrane preparation was reduced to 0·02 ml and the reaction was allowed to proceed

for 10 min only. Pi released was assayed as described previously.

ATPase activity of the supernatant fraction (after centrifuging at 100,000 g for 60 min) was assayed in the following reaction mixture:

0·6 ml buffer (containing 50 mM-MOPs-NaOH (pH 7·0) and 12 mM-$MgCl_2$)

0·2 ml supernatant fraction

0·1 ml H_2O

0·1 ml of 0·1 M-ATP (pH 7·4) was added to initiate the reaction after a 10 min pre-incubation as described previously. The reaction was also allowed to proceed for 30 min.

Phosphatase activity was assayed in exactly the same manner as described above except that the substrate used was para-nitrophenyl phosphate instead of ATP.

When using inhibitors, appropriate solvent controls were carried out.

14. Activation of membrane-bound ATPase of P. acnes.

i. Method 1.

Membrane preparation, 0·1 ml (approx. 1 mg protein), was incubated for various time intervals in the following reaction mixture:

0·8 ml buffer (containing 100 mM-Tris-Cl (pH 7·6) and 5 mM-$MgCl_2$)

0·1 ml trypsin (5 mg ml^{-1})

0·05 ml aliquots of the above were extracted and added to tubes containing:

0·02 ml trypsin inhibitor (10 mg ml^{-1})

0·1 ml of 0·1 M-ATP (pH 7·4)

0·83 ml of buffer (containing 50 mM-Tris-Cl (pH 7·6) and 5 mM-$MgCl_2$.

The reaction was allowed to proceed for 30 min before terminating as described previously.

ii. Method 2.

Membrane preparation, 0·05 ml, was incubated at 37°C in tubes containing 0·1 ml trypsin (5 mg ml^{-1}), 0·65 ml buffer (containing 50 mM-Tris-Cl, (pH 7·6) and 7 mM-$MgCl_2$) and 0·1 ml of 0·1 M-ATP. At various time intervals, 0·1 M-ATP (pH 7·4) was added and incubated for a further 30 min. The reaction was terminated as described previously.

iii. Method 3.

Membrane preparation, 0·2 ml (approx. 2 mg protein) was incubated in centrifuge tubes at 64°C for 4 min in the following reaction mixture:

0.2 ml buffer (containing 0·1 M-Hepes-Tris (pH 7.0) and 5 mM-$MgCl_2$).

0·1 ml of 0·1 M-ATP

0·05 ml of 0·1M - dithiothreitol

0·45 ml H_2O

The activation process was halted by cooling the mixture in running water. Samples were withdrawn for ATPase assay as described in section 13.

15. Assay of succinate dehydrogenase.

Succinate dehydrogenase in cell extracts of P. acnes was assayed at 37°C in a reaction mixture containing the following:

	Vol (ml)
Buffer (containing 20 mM-Tris-Cl (pH 7·6). 10 mM-KH_2PO_4, 5 mM-$MgSO_4$ and 0·1 mM-EDTA)	2·3
DCPIP (0·6 mM)	0·2
PMS (20 mM)	0·1
Extract	0·2
Succinate (0·2 M)	0·2

The reaction was initiated by the addition of succinate and decrease in absorption was followed at 600 nm. If the volume of extract used was decreased, the volume was made up to 3·0 ml with water.

RESULTS

1. Studies on the generation of the membrane potential in P. acnes.

The initial approach in studying the process of energy transduction in P. acnes was to examine the mechanism(s) involved in the generation of the membrane potential component ($\Delta\Psi$) of the H^+ electrochemical gradient ($\Delta\tilde{\mu}_{H^+}$). Measurements of $\Delta\Psi$ were performed in a strongly buffered medium to minimize pH changes affecting the electrode response (as described in Methods section 6).

The $\Delta\Psi$ was measured by determining the distribution of K^+ across the membrane in the presence of the ionophore valinomycin (as discussed in the Introduction). Initially, it was necessary to determine whether P. acnes was sensitive to valinomycin. This was carried out by treating P. acnes with CCCP, which itself caused the leakage of K^+ due to the collapse of the $\Delta\tilde{\mu}_{H^+}$. The addition of valinomycin to P. acnes treated in this way rendered the membrane permeable to K^+ resulting in the release of internal K^+ (Fig. 6). The rate of efflux was found to be dependent on the concentration of valinomycin, though at concentrations of 1 μg to 5 μg valinomycin (mg dry wt)$^{-1}$, the rate of efflux was constant. In addition, the extent of K^+ efflux was independent of the valinomycin concentration over the range of 0·5 μg (steady level attained after 870 s) to 5 μg valinomycin (mg dry wt)$^{-1}$. A concentration of 1 μg valinomycin (mg dry wt)$^{-1}$ was thus chosen for all measurements of the $\Delta\Psi$.

The maintenance of the $[K^+]$ gradient across the membrane in the presence of valinomycin was inhibited by protonophores such as CCCP, pentachlorophenol (PCP) and tetrachlorosalicylanilide (TCS) (Table 4). Thus CCCP, PCP and TCS at concentrations of 14·9 μM, 23 μM and 10·3 μM respectively, competely depolarized the membrane. CCCP, at a final concentration of 23 μM was routinely used for measurements of $\Delta\Psi$. Knowing the $[K^+]$ in the medium before and after the addition of CCCP allowed the $\Delta\Psi$ to be calculated by applying the Nernst equation. An example of such a calculation using the following data is shown below:

Legend to Fig. 6.

The experiment was performed essentially as described in Methods section 6, except that CCCP (at a final concentration of 11·5 μM) was added to the cell suspension prior to valinomycin. When the K^+ efflux reached a steady-value, varying concentrations of valinomycin were added.

ΔK^+ represents the difference of the steady values of $[K^+]$ attained after the addition of CCCP and valinomycin.

□ 0·1 μg valinomycin (mg dry wt)$^{-1}$

△ 0·5 "

▲ 1·0 "

○ 2·0 "

● 5·0 "

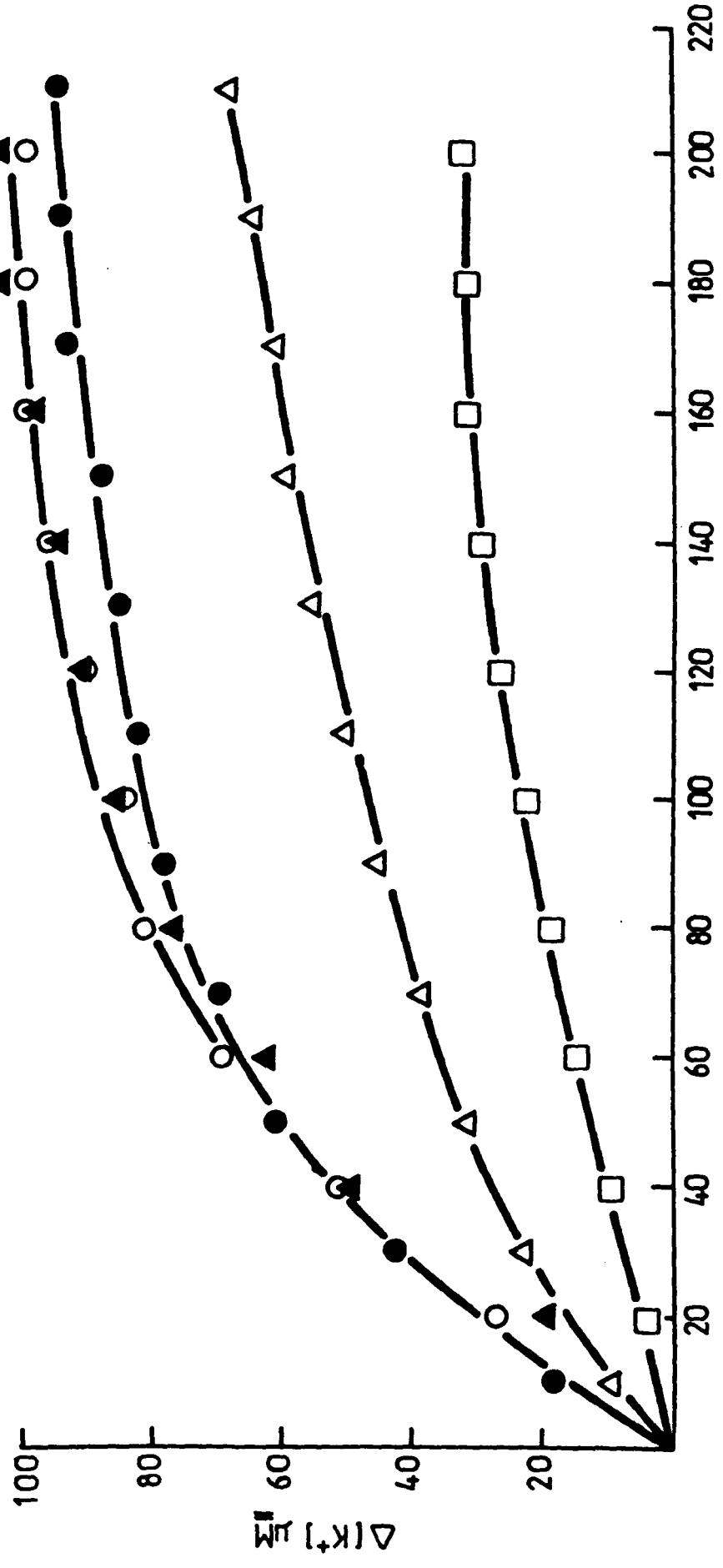


Fig.6: Effect of valinomycin on K^+ efflux from P.acnes treated with CCCP.

Table 4.

Effect of uncouplers on the membrane potential generated by P. acnes.

Compound	% depolarization	Concentration (μM)
CCCP	25	1·2
	50	2·1
	100	14·9
TCS	25	1·2
	50	2·1
	100	10·3
PCP	25	2·3
	50	5·5
	100	23·0

Initial $[K^+]$: 0·2 mM

$[K^+]$ after the addition of CCCP = 0·27 mM

Total cell concentration = 9·76 mg

Intracellular volume = 1·47 µl $(mg\ dry\ wt)^{-1}$

Volume of reaction mixture = 8·7 ml

$$\text{Hence, } [K^+]_{in} = \frac{(0{\cdot}27 - 0{\cdot}2)}{9{\cdot}76 \times 1{\cdot}47} \times 8{\cdot}7 \times 10^3 = 42{\cdot}45 \text{ mM}$$

$$\text{Therefore } \Delta\Psi = -60 \log [K^+]_{in}/[K^+]_{out}$$

$$= -60 \log 42{\cdot}45/0{\cdot}2 = -139{\cdot}6 \text{ mV}.$$

A typical result is illustrated in Fig. 7. On addition of valinomycin, K^+ came into equilibrium with the $\Delta\Psi$ which was subsequently collapsed by the addition of CCCP.

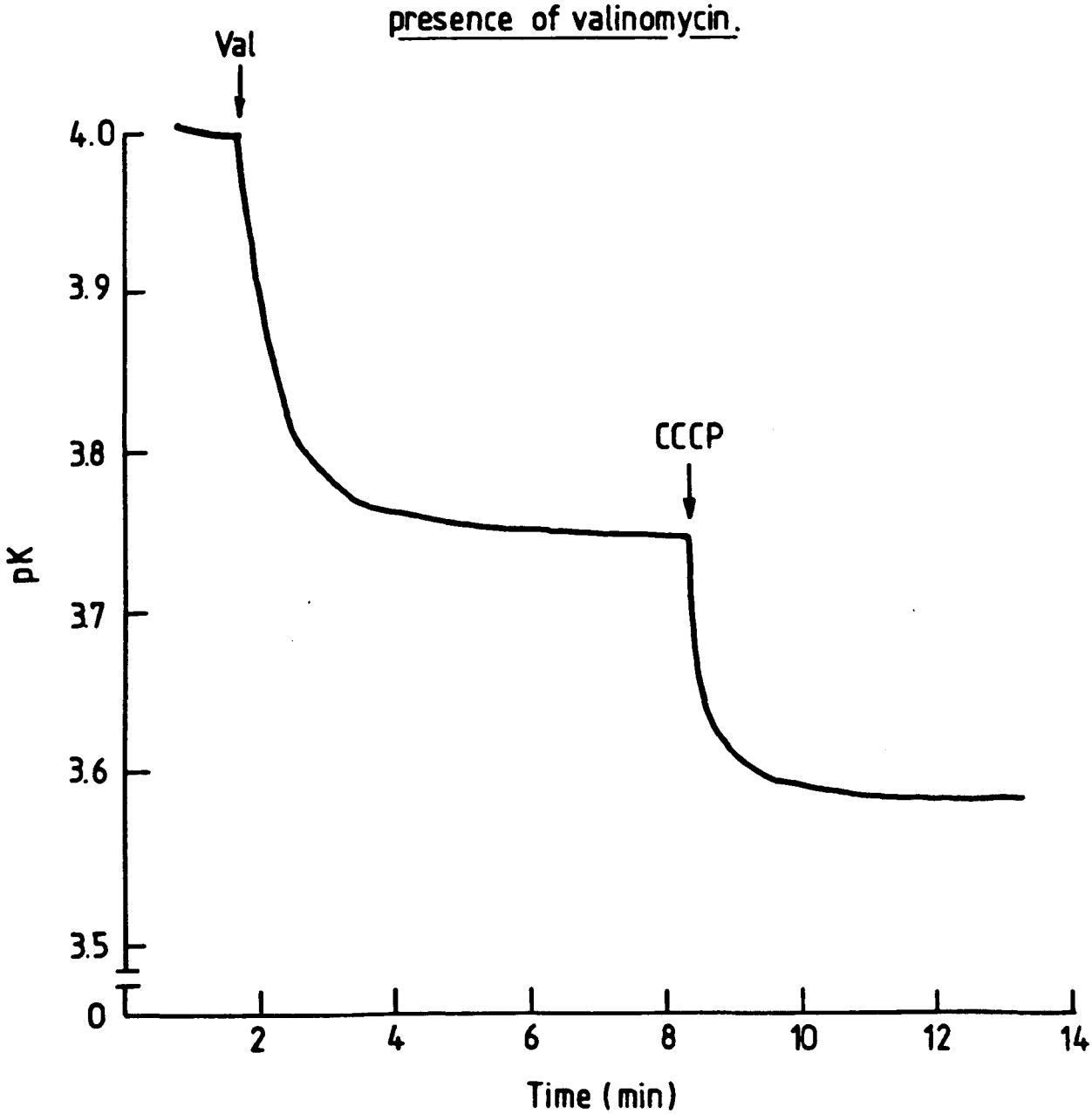
The ratio of $[K^+]_{in}$ to $[K^+]_{out}$ is referred to as the accumulation ratio. The inhibition of the maintenance of the $\Delta\Psi$ will be presented as a percentage decrease in accumulation ratio as it is considered that this provides a better representation of the extent of the depolarization of the membrane. This is because $\Delta\Psi$ is a logarithmic function of the accumulation ratio and as such tends to mask the extent of the depolarization. For example, a decrease in the accumulation ratio from 100 to 10 (corresponding to approximately -120 mV and -60 mV respectively) represents 90% depolarization of the membrane, but in terms of $\Delta\Psi$ this represents a decrease of only 50%. The effect is illustrated in Fig. 8, where the external $[K^+]$ was found to cause a 77% decrease in accumulation ratio, but corresponding to only 23% decrease in $\Delta\Psi$. The sensitivity of the $\Delta\Psi$ to increasing external $[K^+]$ thereby decreasing the accumulation ratio lends support to the validity of using the distribution of K^+ in the presence of valinomycin as a measure of the $\Delta\Psi$. In addition, it also illustrated the major limitation of using this technique in the measurements of the $\Delta\Psi$. In all measurements of the $\Delta\Psi$ generated by P. acnes, the cell

Legend to Fig. 7.

The $\Delta\Psi$ generated by anaerobically-grown P. acnes under aerobic conditions was measured as described in Methods section 6.

The $[K^+]$ in the medium was expressed as its pK, where pK = - log $[K^+]$.

Fig. 7: Measurement of the membrane potential generated by P. acnes by determining the distribution of K^+ across the membrane in the presence of valinomycin.



Legend to Fig. 8.

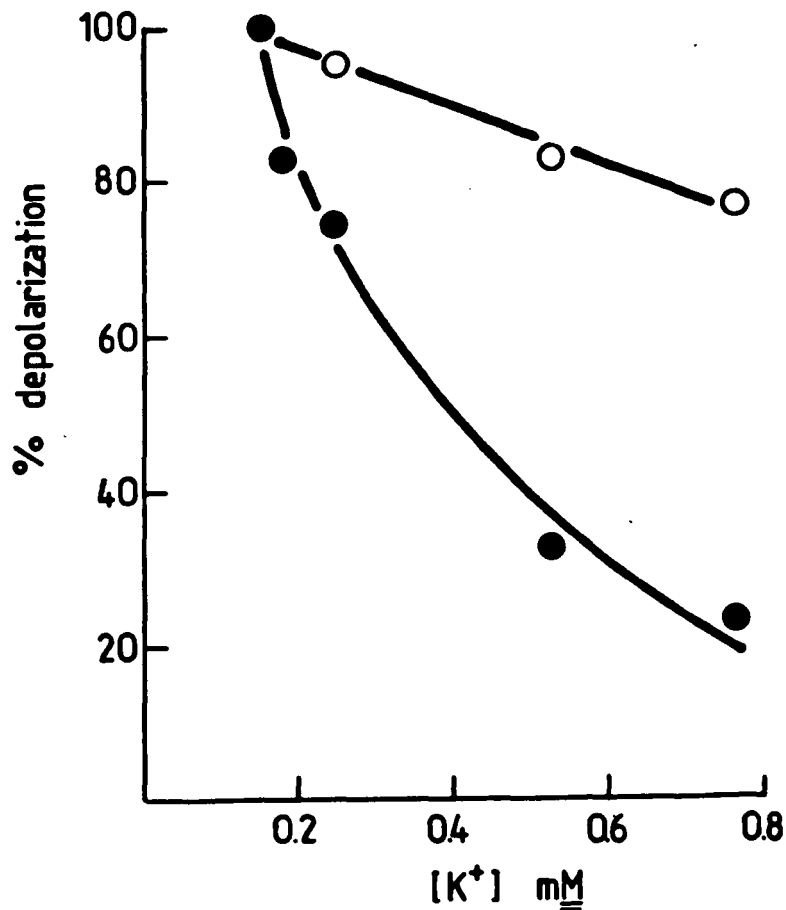
The experiment was performed as described in Methods section 6 except that the cells were pre-treated with valinomycin by incubating the cell suspension (at a concentration of approximately 1·15 mg ml^{-1}) for 20 min with valinomycin (1 μg (mg dry wt)$^{-1}$) at 37°C under anaerobic conditions. The cell suspension was then harvested, washed and resuspended as described in Methods section 4. The external $[K^+]$ was manipulated by addition of aliquots of 10 mM-KCl in 100mM-Hepes-Tris (pH 7·0).

○ $\Delta\Psi$

● accumulation ratio.

The accumulation ratio of the control was 639 corresponding to a $\Delta\Psi$ of -168 mV.

Fig. 8: Effect of $[K^+]$ on the membrane potential generated by P. acnes.



concentration was approximately 1·2(mg dry wt) ml^{-1}. This was specifically chosen because at this cell concentration, the external $[K^+]$ in the presence of valinomycin was found to be approximately 0·3 mM to 0·4 mM. This would imply that all the $\Delta\Psi$ values quoted in the present studies were under estimated by approximately 10%. It is because of this generally low external $[K^+]$ that no special precautions were taken to minimize the depolarizing effect of the external $[K^+]$ on the membrane.

Having established the conditions for measuring the $\Delta\Psi$, the effects of various inhibitors of energy transduction on the generation of the $\Delta\Psi$ were then studied in order to gain information on how the organism generated such a force under anaerobic and aerobic conditions. Energy-transfer inhibitors were found to inhibit the generation of the $\Delta\Psi$ under anaerobic conditions. A typical example is shown in Fig. 9, where Nbf-Cl was found to completely depolarize the membrane. The results obtained from such inhibition curves are summarized in Table 5. It may be seen that all the energy-transfer inhibitors used caused significant depolarization of the membrane. If these inhibitors were regarded as specific and having a similar mode of action in this system as in others, then these observations would indicate that under anaerobic conditions, the $\Delta\Psi$ was generated by an ATPase located on the cell membrane.

The effect of energy-transfer inhibitors on the generation of the $\Delta\Psi$ under aerobic conditions by aerobically-grown cells is shown in Table 6. A comparison of the results presented in Tables 5 and 6 revealed that all the energy-transfer inhibitors, with the exception of oligomycin, almost completely dissipated the $\Delta\Psi$ generated under both conditions. Though there were variations in the concentrations of the inhibitors causing maximum depolarization of the membrane under the two conditions, they all occurred within the same order of magnitude.

Legend to Fig. 9.

The experiment was performed as described in Methods section 6. The average of the accumulation ratio of the uninhibited control was 303·5 corresponding to -148·9 mV.

Fig.9: Effect of Nbf-Cl on the membrane potential generated under anaerobic conditions by anaerobically-grown P.acnes.

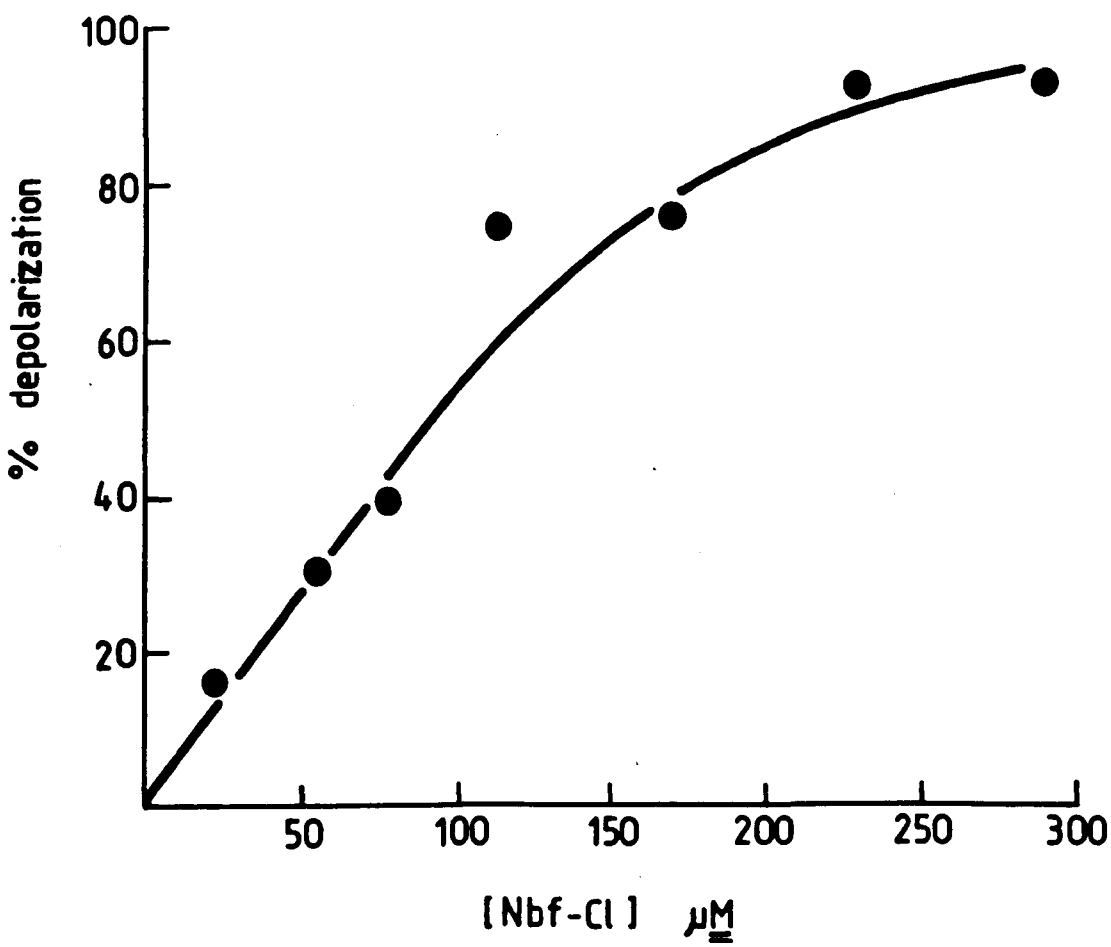


Table 5.

Effect of energy-transfer inhibitors on the membrane potential generated under anaerobic conditions by anaerobically-grown P. acnes.

Compound	% depolarization	Concentration (μM)
Nbf-Cl	25	90
	50	185
	96 (max)	425
DCCD	25	22·5
	50	40
	90 (max)	200
DPPA	25	$1.4 \cdot 10^3$
	50	$3.0 \cdot 10^3$
	88 (max)	$6.8 \cdot 10^3$
Quercetin	25	22·5
	50	40
	80 (max)	175
Oligomycin*	25	1·1
	50	2·2
	90 (max)	8·5

*μg (mg dry wt)$^{-1}$

max = maximum.

Table 6.

Effect of energy-transfer inhibitors on the membrane potential generated under aerobic conditions by aerobically-grown P. acnes.

Compound	% depolarization	Concentration (μM)
Nbf-Cl	25	45
	50	100
	93 (max)	280
DCCD	25	30
	50	110
	90 (max)	400
DPPA	25	530
	50	$1.2 \cdot 10^3$
	72 (max)	$2.5 \cdot 10^3$
Quercetin	25	62·5
	50	150
	64 (max)	325
Oligomycin*	25	2·5
	46 (max)	8·2

*μg (mg dry wt)$^{-1}$

max = maximum.

Hence, at first sight, it would seem that the $\Delta\Psi$ generated by P. acnes was driven primarily by ATPase activity. However, the relatively low percentage of dissipation by oligomycin of the $\Delta\Psi$ maintained under aerobic conditions would question this initial suggestion that the ATPase is the sole mechanism by which the $\Delta\Psi$ is being generated. As will be discussed later (Results, section 3), all the energy-transfer inhibitors used, with the exception of oligomycin, were found to show effects other than those that could be solely attributed to their function as specific ATPase inhibitors. Further studies on the $\Delta\Psi$ generation were performed using only oligomycin as an energy-transfer inhibitor.

As mentioned earlier, oligomycin showed different effects on the $\Delta\Psi$ generated under anaerobic and aerobic conditions by P. acnes. Figs. 10 and 11 represent typical effects of oligomycin on the $\Delta\Psi$ maintained under anaerobic and aerobic conditions by anaerobically- and aerobically-grown P. acnes respectively. With anaerobically-grown P. acnes, it was found that in four different experiments performed with different batches of cells, the generation of the $\Delta\Psi$ under anaerobic conditions was almost completely inhibited at an average oligomycin concentration of approximately 9 μg (mg dry wt)$^{-1}$. With aerobically-grown cells, the concentration of oligomycin required to inhibit the generation of the $\Delta\Psi$ under anaerobic conditions was found to be approximately 10 μg (mg dry wt)$^{-1}$ in four different experiments performed with four different batches of cells. However, under aerobic conditions, at similar oligomycin concentration, the generation of the $\Delta\Psi$ was only partially inhibited in both anaerobically- and aerobically-grown cells. The inhibition values obtained from four different experiments ranged from 34% to 45% at an oligomycin concentration of 10 μg (mg dry wt)$^{-1}$. With aerobically-grown cells, the variation was rather larger; the values obtained from four different experiments ranged from 22% to 50%. It would thus seem that under anaerobic conditions, the $\Delta\Psi$ in both

Legend to Figs. 10 and 11.

$\Delta\Psi$ generated under:

○ anaerobic conditions

● aerobic conditions.

The experiment was performed as described in Methods section 6.

Fig. 10

The average value of the $\Delta\Psi$ maintained under anaerobic conditions in the uninhibited controls were -140 mV, corresponding to an accumulation ratio of 215·4.

Under aerobic conditions, the $\Delta\Psi$ of the uninhibited controls were -158·7 mV corresponding to an accumulation ratio of 441.24.

Fig. 11

The average value of the $\Delta\Psi$ maintained under anaerobic conditions in the uninhibited controls were -140·1 mV corresponding to an accumulation ratio of 216·20.

Under aerobic conditions, the $\Delta\Psi$ of the uninhibited controls were -151·8 mV corresponding to an accumulation ratio of 338·5.

Fig.10: Effect of oligomycin on the membrane potential generated under aerobic and anaerobic conditions by anaerobically-grown P. acnes.

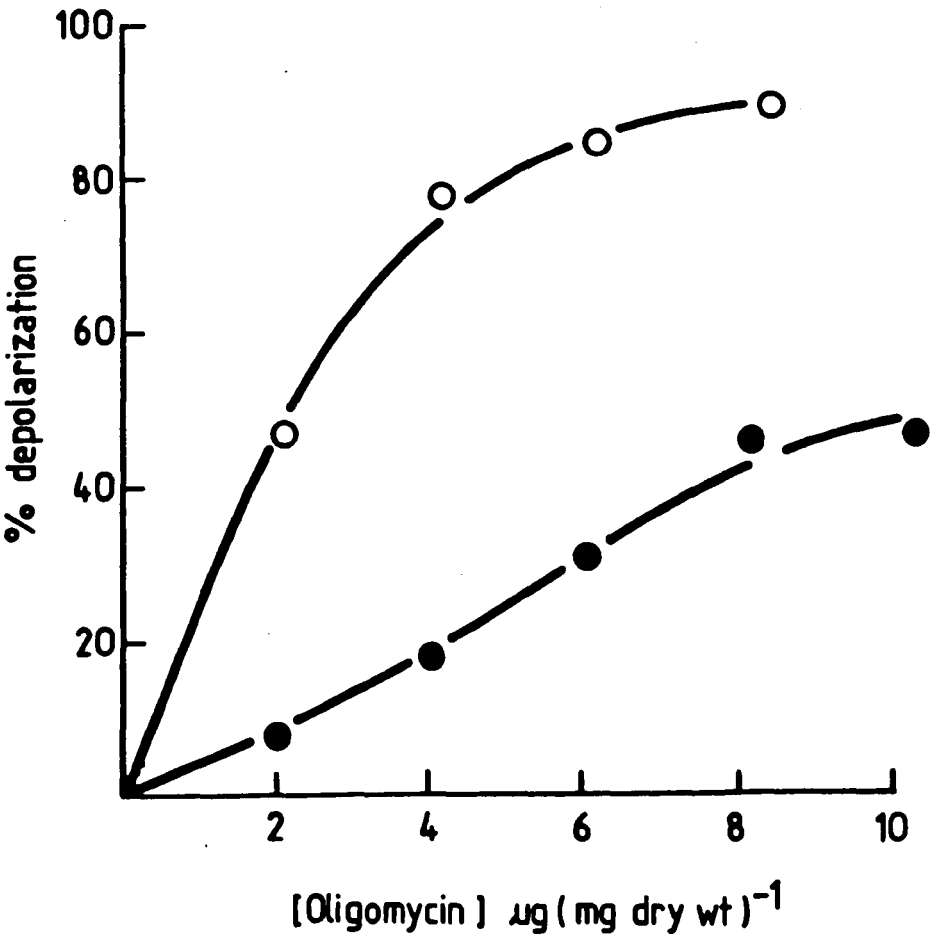
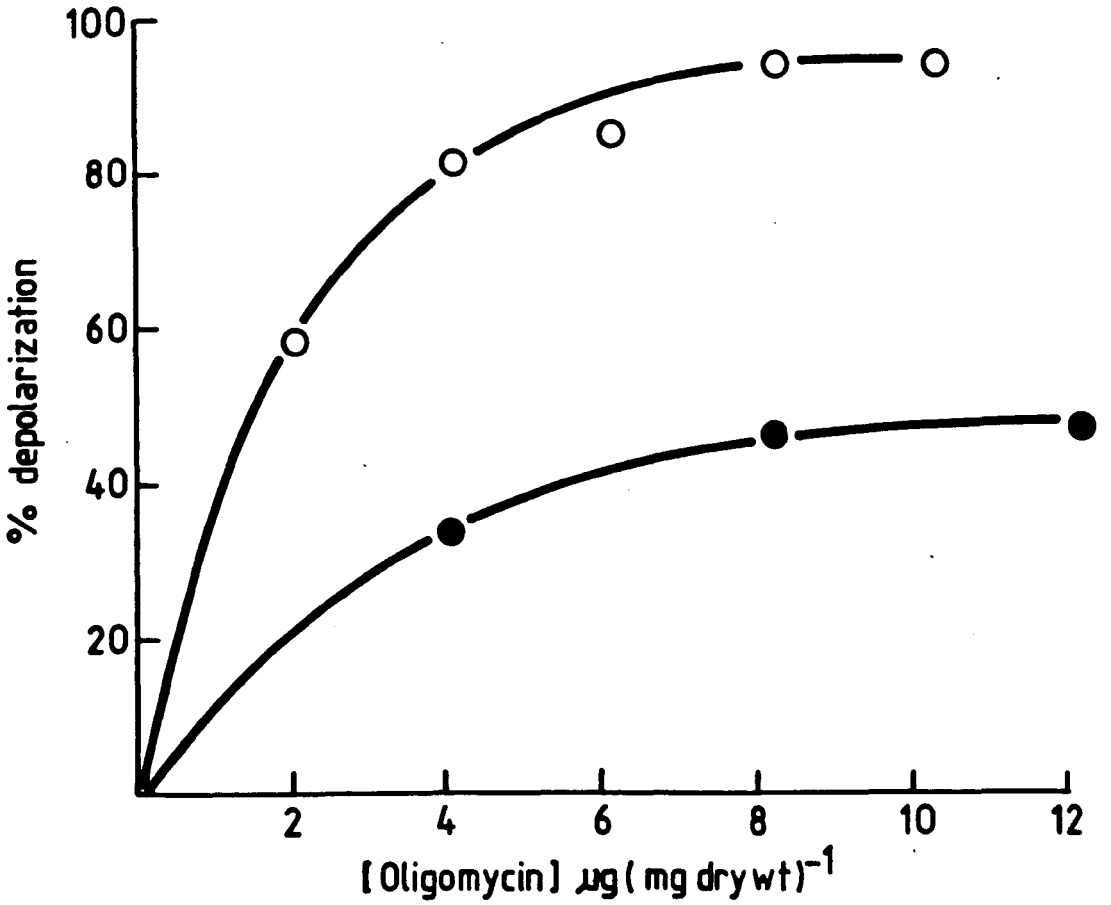


Fig.11 : Effect of oligomycin on the membrane potential generated under aerobic and anaerobic conditions by aerobically-grown P.acnes.



anaerobically- and aerobically-grown P. acnes was generated by ATPase activity. However, under aerobic conditions, the partial sensitivity of the $\Delta\Psi$ to oligomycin would imply that the ATPase was not the only mechanism by which the $\Delta\Psi$ was generated. Electron transport with oxygen as the terminal electron acceptor seemed a likely additional mechanism. To investigate this possibility, the effect of HOQNO, an electron transport inhibitor, on the generation of the $\Delta\Psi$ was studied. It was found that the $\Delta\Psi$ maintained under aerobic conditions in the presence of oligomycin was fully sensitive to HOQNO (Figs. 12 and 13 for anaerobically- and aerobically-grown cells respectively). In each case, the results were reproducible with two different batches of cells, where almost complete dissipation of the $\Delta\Psi$ was observed using the combination of inhibitors.

The effect of HOQNO on the $\Delta\Psi$ generated under anaerobic conditions by both anaerobically- and aerobically-grown cells was studied to investigate whether the $\Delta\Psi$ was generated solely by ATPase activity. The results obtained were unexpected as the HOQNO partially inhibited the generation of the $\Delta\Psi$ under anaerobic conditions by both anaerobically- and aerobically-grown P. acnes (Figs. 14 and 15). In anaerobically-grown P. acnes, the % depolarization encountered in four different experiments ranged from 30% to 55% at an average HOQNO concentration of 75 $\mu\underline{M}$. The range of values obtained for aerobically-grown cells in four different experiments were very similar, ranging from 33% to 55% at an HOQNO concentration of 70 $\mu\underline{M}$. The $\Delta\Psi$ maintained in the presence of HOQNO was found to be fully sensitive to oligomycin. This was in agreement with the earlier observation that oligomycin almost completely inhibited the generation of the $\Delta\Psi$ under anaerobic conditions in both anaerobically- and aerobically-grown P. acnes (Figs. 10 and 11).

The possible involvement of electron transport in the

Legend to Figs. 12 and 13.

Depolarization of the membrane by:

● oligomycin

○ HOQNO, in the presence of oligomycin.

The experiment was performed as described in Methods section 6.

Fig. 12.

The value of the $\Delta\Psi$ in the absence of inhibitors was -156 mV corresponding to an accumulation ratio of 397·6.

Fig. 13.

The value of the $\Delta\Psi$ in the absence of inhibitors was -161·8 mV corresponding to an accumulation ratio of 496·6.

Fig.12: Effect of oligomycin and HOQNO on the membrane potential generated under aerobic conditions by anaerobically-grown P. acnes.

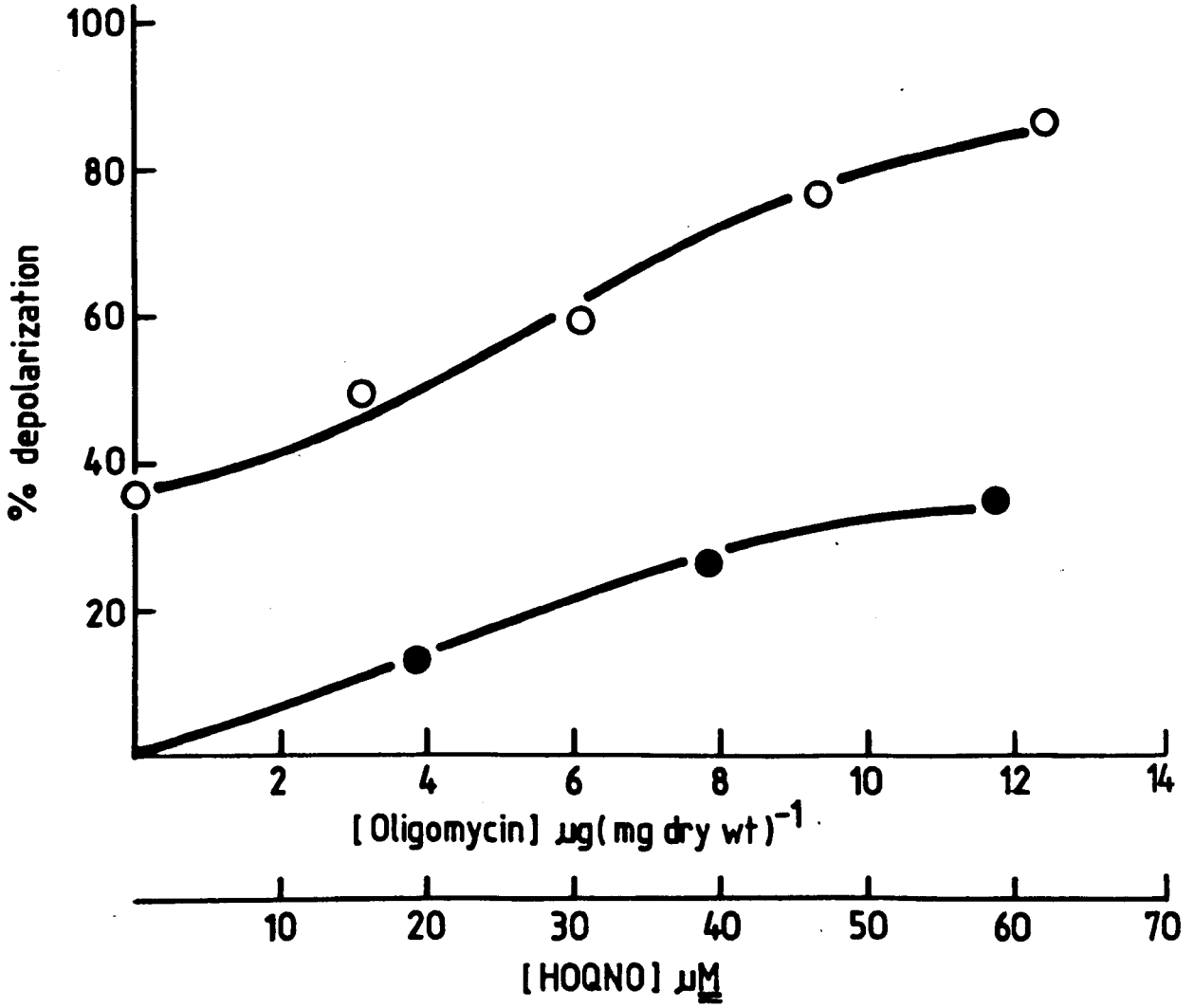
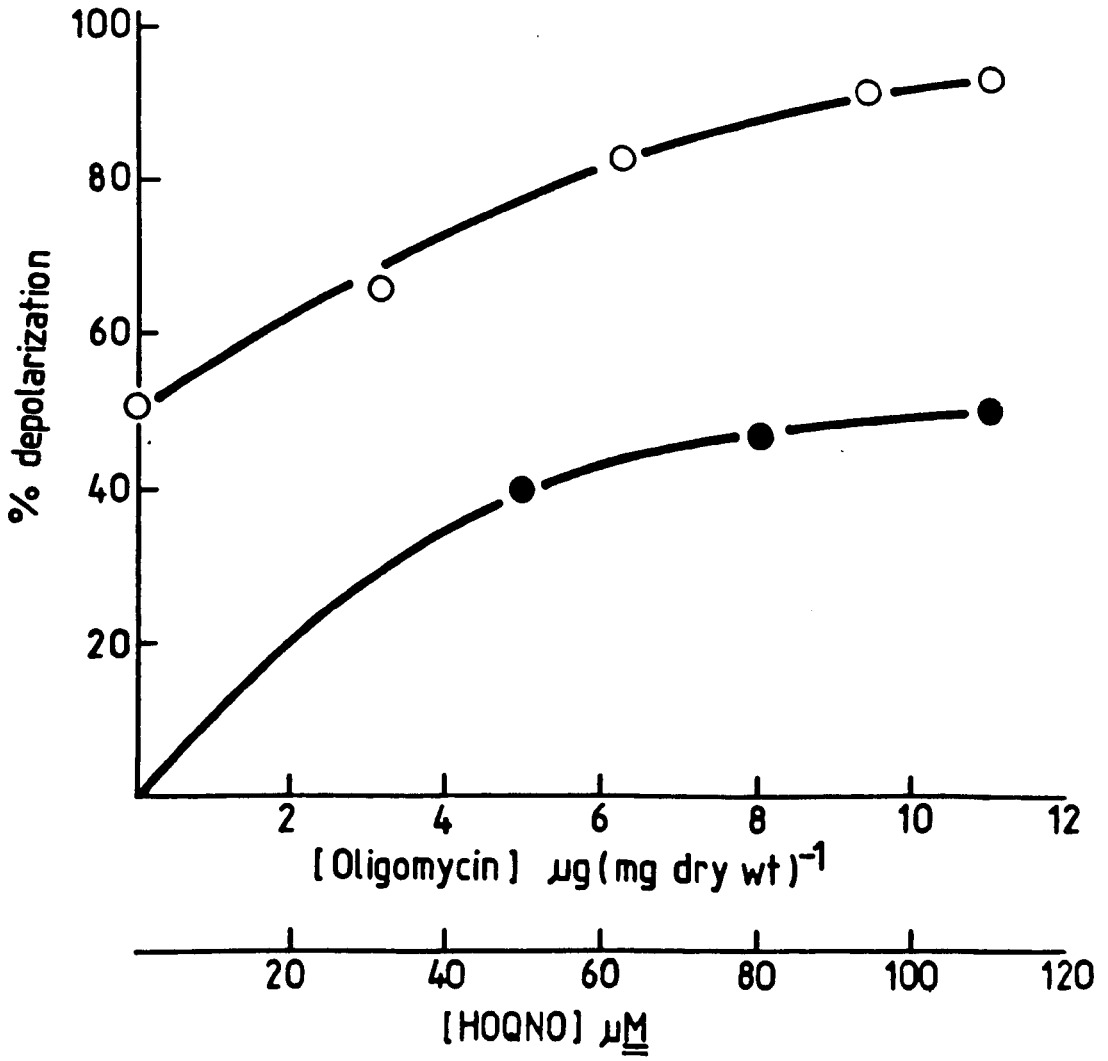


Fig.13 : Effect of oligomycin and HOQNO on the membrane potential generated under aerobic conditions by aerobically-grown P. acnes.



Legend to Figs. 14 and 15.

Depolarization of the membrane by:

● HOQNO

O oligomycin, in the presence of HOQNO

The experiment was performed as described in Methods section 6.

Fig. 14.

The value of the $\Delta\Psi$ in the absence of inhibitors was -138 mV corresponding to an accumulation ratio of 199·8.

Fig. 15.

The value of the $\Delta\Psi$ in the absence of inhibitors was -148.5 mV corresponding to an accumulation ratio of 298·5.

Fig.14: Effect of HOQNO and oligomycin on the membrane potential generated under anaerobic conditions by anaerobically-grown P. acnes.

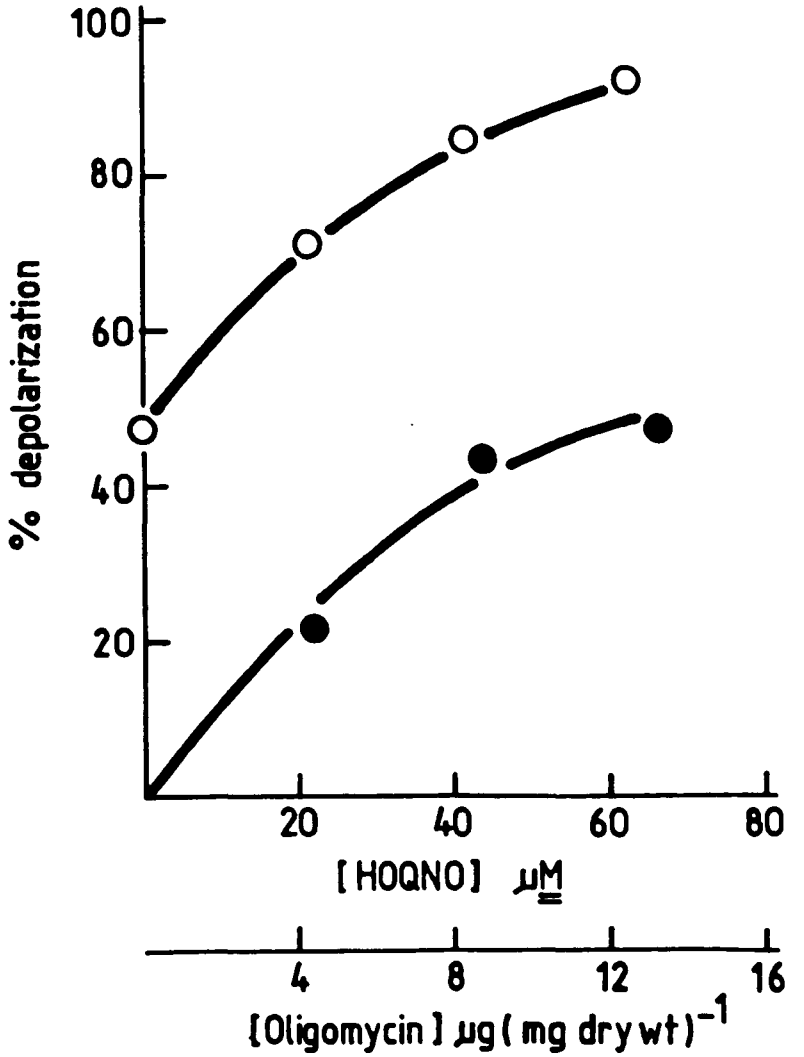
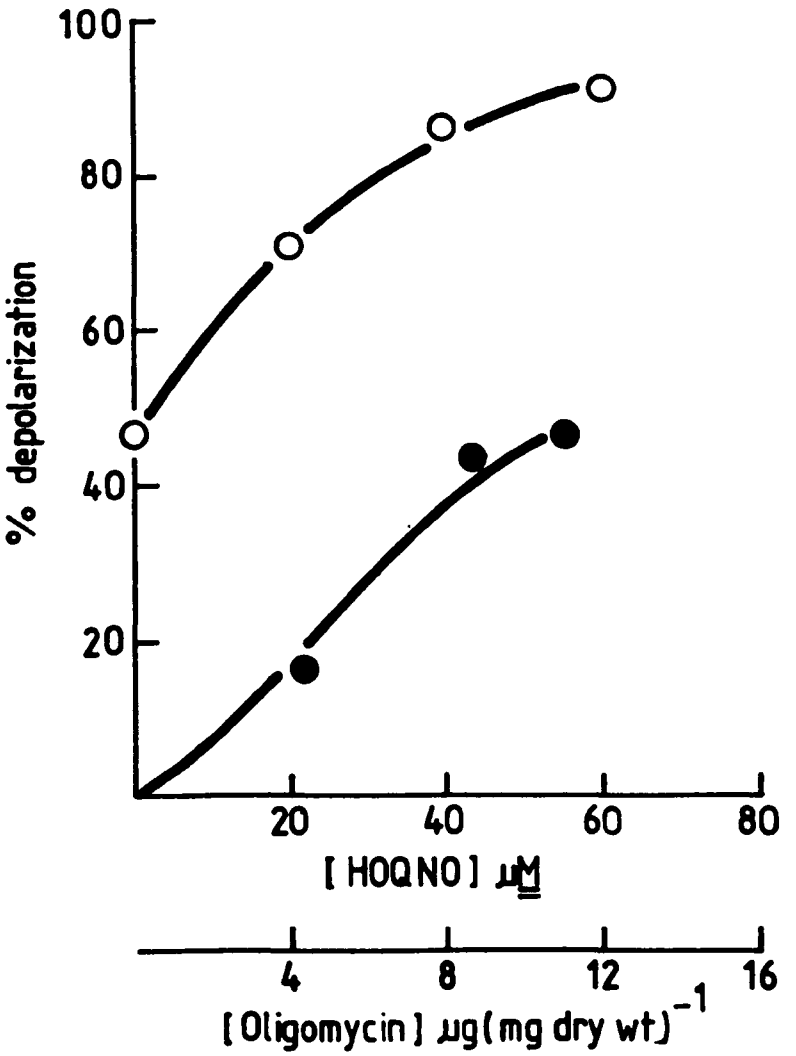


Fig.15: Effect of HOQNO and oligomycin on the membrane potential generated under anaerobic conditions by aerobically-grown P.acnes.



mechanism generating the $\Delta\Psi$ was further supported by the observed increase in the magnitude of the $\Delta\Psi$ generated under aerobic conditions over those generated under anaerobic conditions by both anaerobically- and aerobically-grown cells. Table 7 summarizes the values of the $\Delta\Psi$ generated by P. acnes under anaerobic and aerobic conditions. It may be seen that an increase of approximately 10 mV occurred in the $\Delta\Psi$ generated under aerobic conditions. In the presence of oxygen and glucose, the actual increase was up to 20 mV in the case of aerobically-grown P. acnes. These values represent increases of approximately 7% and 11% respectively. However, in terms of accumulation ratio, these would represent actual increases of 45% and 90% respectively. Fig. 16 illustrates a typical response of the $\Delta\Psi$ generated by P. acnes to oxygen and glucose. Direct evidence for the involvement of electron transport in the mechanism of $\Delta\Psi$ generation under aerobic conditions will be presented later (Results, sections 3 and 4). The addition of glucose however did not cause an increase in the $\Delta\Psi$ generated under anaerobic conditions in both anaerobically- grown (with or without fumarate) and aerobically-grown P. acnes even in the presence of fumarate, an electron acceptor under anaerobic conditions in P. pentosaceum and P. freudenreichii (de Vries et al., 1977). In fact, the $\Delta\Psi$ generated under anaerobic conditions in the presence of glucose showed similar responses to oligomycin and HOQNO as did the $\Delta\Psi$ generated under anaerobic conditions in the absence of glucose. The effect of oligomycin on the $\Delta\Psi$ generated under anaerobic conditions in the presence of glucose by both anaerobically- and aerobically-grown cells is shown in Fig. 17. Oligomycin, at a concentration of 10 μg (mg dry wt)$^{-1}$, was found to inhibit almost completely the maintenance of the $\Delta\Psi$ under these conditions. HOQNO partially inhibited the generation of the $\Delta\Psi$ under anaerobic conditions in the presence of glucose in both anaerobically- and aerobically-grown cells. The $\Delta\Psi$ maintained in the presence of HOQNO was sensitive

Table 7.

The magnitude of the $\Delta\Psi$ generated by P. acnes under anaerobic and aerobic conditions.

Condition	$\Delta\Psi$ (-mV)	
	Anaerobically-grown P. acnes	Aerobically-grown P. acnes
Anaerobic	145 ± 8 (21)	141 ± 10 (19)
Anaerobic + glucose	146 ± 13 (10)	138 ± 11 (6)
Aerobic	155 ± 8 (14)	151 ± 7 (13)
Aerobic + glucose	161 ± 9 (11)	159 ± 8 (9)

Legend to Fig. 16.

The experiment was performed as described in Methods section 6 with anaerobically-grown P. acnes. The $[K^+]$ in the medium was expressed as its pK, where pK = -log $[K^+]$.

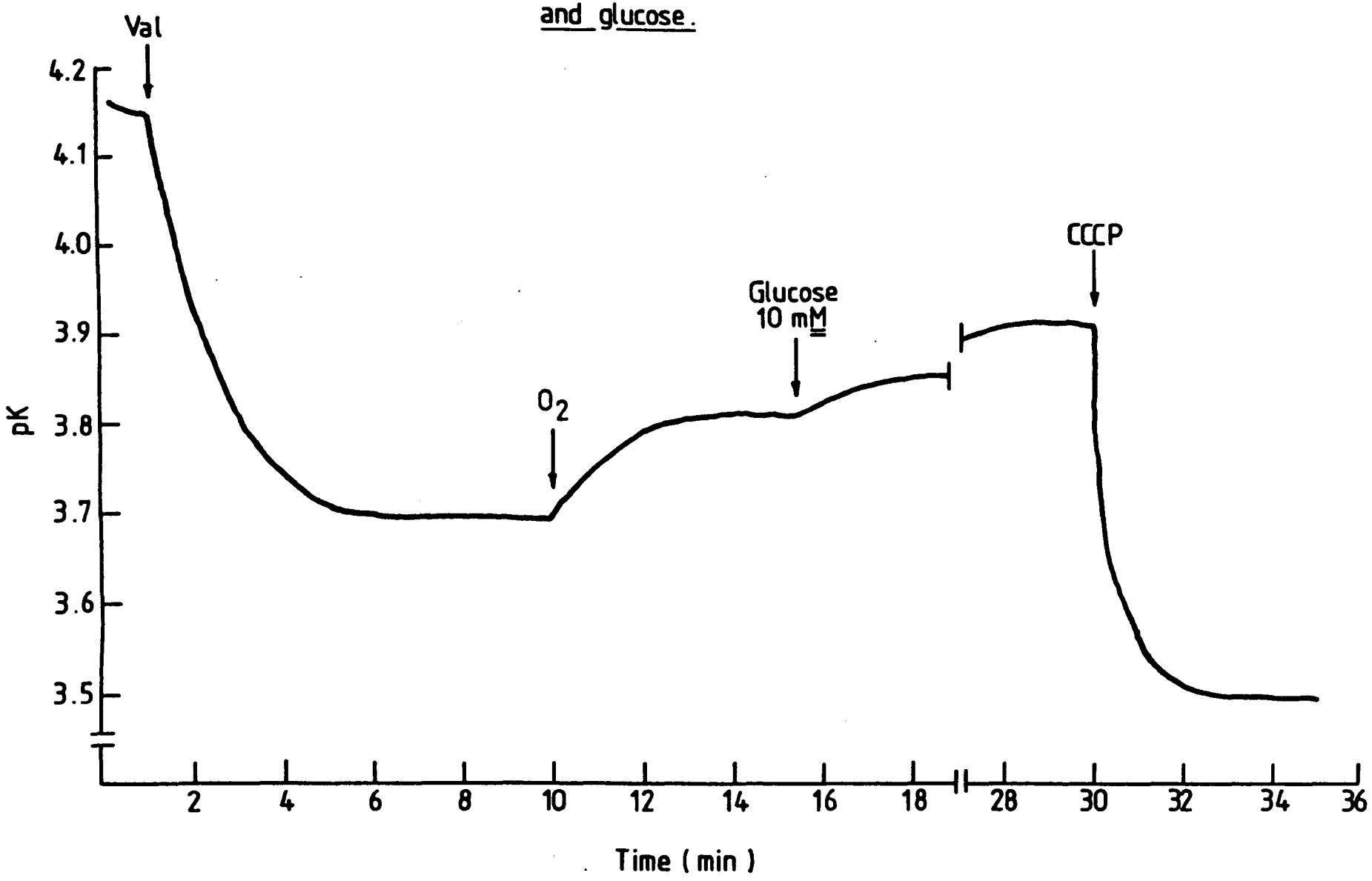
The values of the $\Delta\Psi$ under the various conditions were:

i. under anaerobic conditions: -156 mV.

ii. under aerobic conditions: -164 mV.

iii. under aerobic conditions in the presence of glucose: 177 mV.

Fig.16: Stimulation of the membrane potential generated under anaerobic conditions by oxygen and glucose.



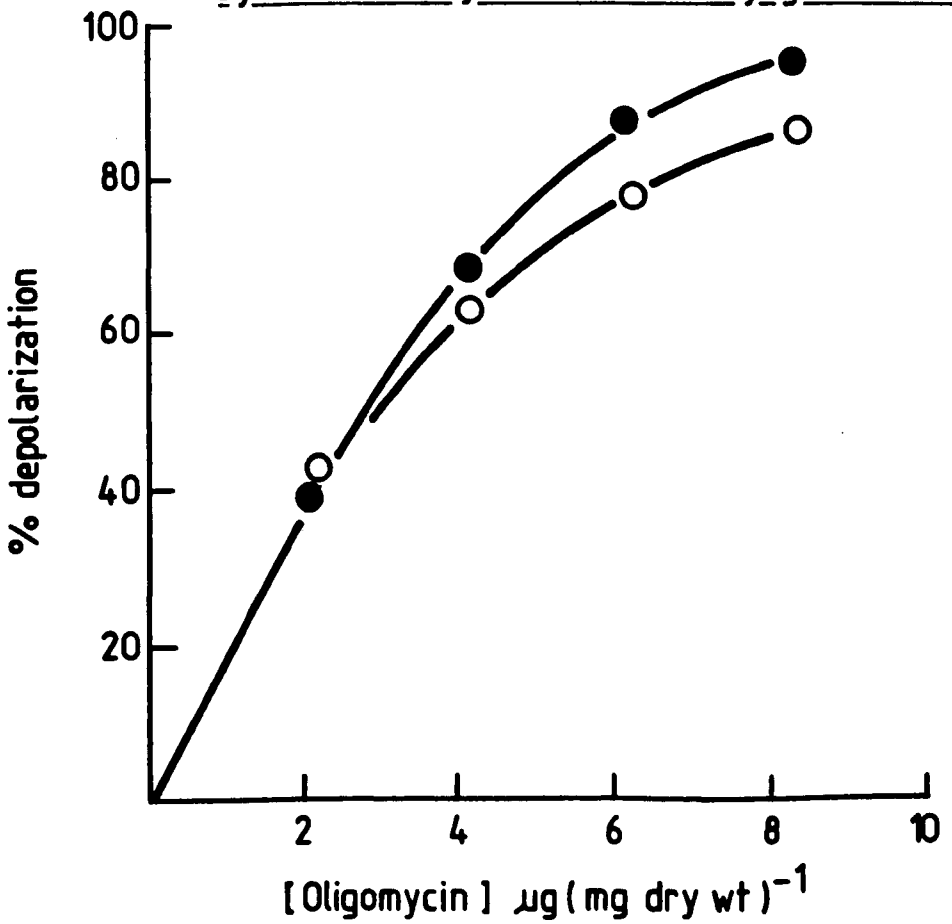
Legend to Fig. 17.

O aerobically-grown P. acnes.

● anaerobically-grown P. acnes.

The experiment was performed as described in Methods section 6. The value of the $\Delta\Psi$ were -147·5 mV and -150·11 mV for anaerobically and aerobically-grown cells respectively. These $\Delta\Psi$ values corresponded to accumulation ratios of 287 and 319 respectively.

Fig.17: Effect of oligomycin on the membrane potential generated under anaerobic conditions in the presence of glucose by anaerobically- and aerobically-grown P. acnes.



to oligomycin (Figs. 18 and 19).

The $\Delta\Psi$ generated by both anaerobically- and aerobically-grown P. acnes under aerobic conditions in the presence of glucose were also found to be partially sensitive to oligomycin, with complete depolarization of the membrane occurring upon adding HOQNO (Figs. 20 and 21). The results obtained were reproducible with two different batches of cells. Indeed, the $\Delta\Psi$ generated under these conditions showed similar responses to oligomycin and HOQNO as the $\Delta\Psi$ generated under aerobic conditions in the absence of glucose (Figs. 12 and 13).

The observations that the $\Delta\Psi$ generated under aerobic conditions in the presence of glucose was sensitive to HOQNO would therefore support the suggestion that electron transport was involved in the generation of the $\Delta\Psi$ under these conditions. Further investigations on the effect of HOQNO on the generation of the $\Delta\Psi$ under aerobic conditions, particularly in the presence of glucose where maximal stimulation of the $\Delta\Psi$ was observed, was warranted. The results obtained are shown in Figs. 22 and 23 for anaerobically- and aerobically-grown P. acnes respectively. HOQNO was not found to inhibit significantly the generation of the $\Delta\Psi$ under aerobic conditions in the presence of glucose by either anaerobically- or aerobically-grown cells. With anaerobically-grown cells, HOQNO, in three different experiments, caused between 30% and 40% dissipation of the $\Delta\Psi$ generated under these conditions. With aerobically-grown cells, in four experiments, HOQNO caused lower levels of inhibition of the maintenance of the $\Delta\Psi$ under these conditions; values obtained ranged from only 12% to 20% inhibition. The maintenance of the $\Delta\Psi$ in the presence of HOQNO was not fully inhibited by the addition of oligomycin. Indeed, the HOQNO-insensitive $\Delta\Psi$ was rather insensitive to oligomycin. These observations were in contrast to those described earlier, where the combination of oligomycin and HOQNO almost totally inhibited the

Legend to Figs. 18 and 19.

Depolarization of the membrane by:

● HOQNO

○ oligomycin, in the presence of HOQNO.

Fig. 18:

The value of the $\Delta\Psi$ in the absence of inhibitions was -137 mV, which corresponded to an accumulation ratio of 192.

Fig. 19.

The value of the $\Delta\Psi$ in the absence of inhibitors was -149 mV, corresponding to an accumulation ratio of 302.

Fig.18: Effect of HOQNO and oligomycin on the membrane potential generated under anaerobic conditions in the presence of glucose by anaerobically-grown P. acnes.

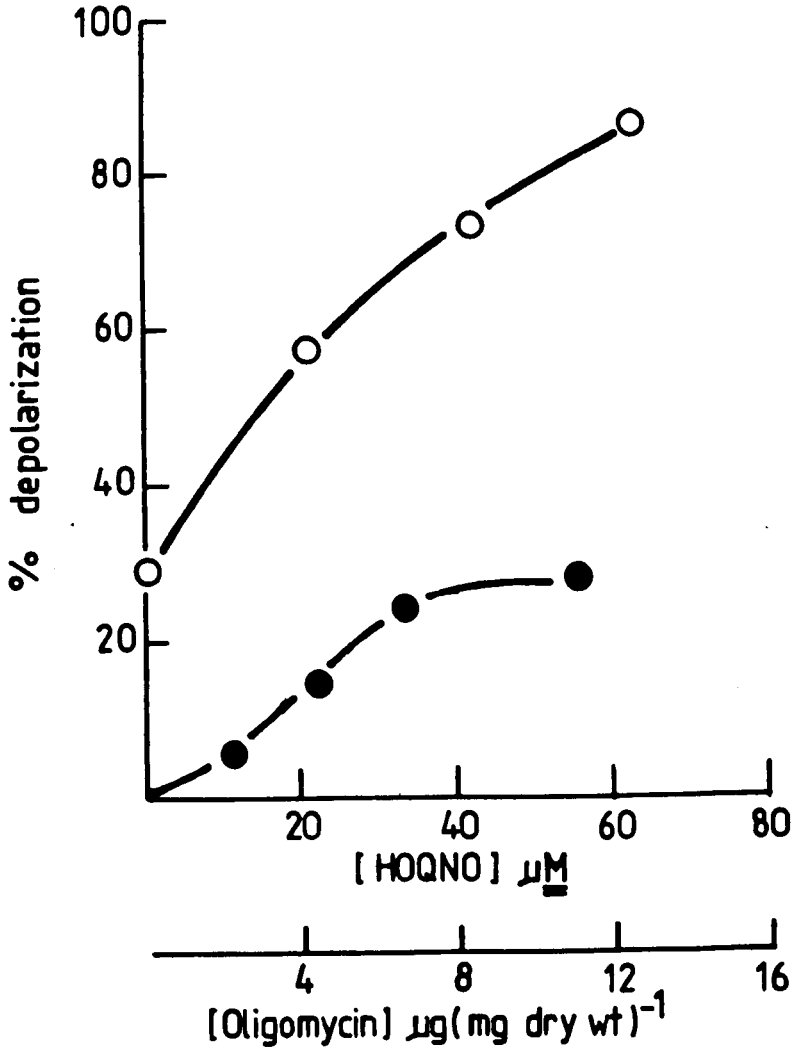
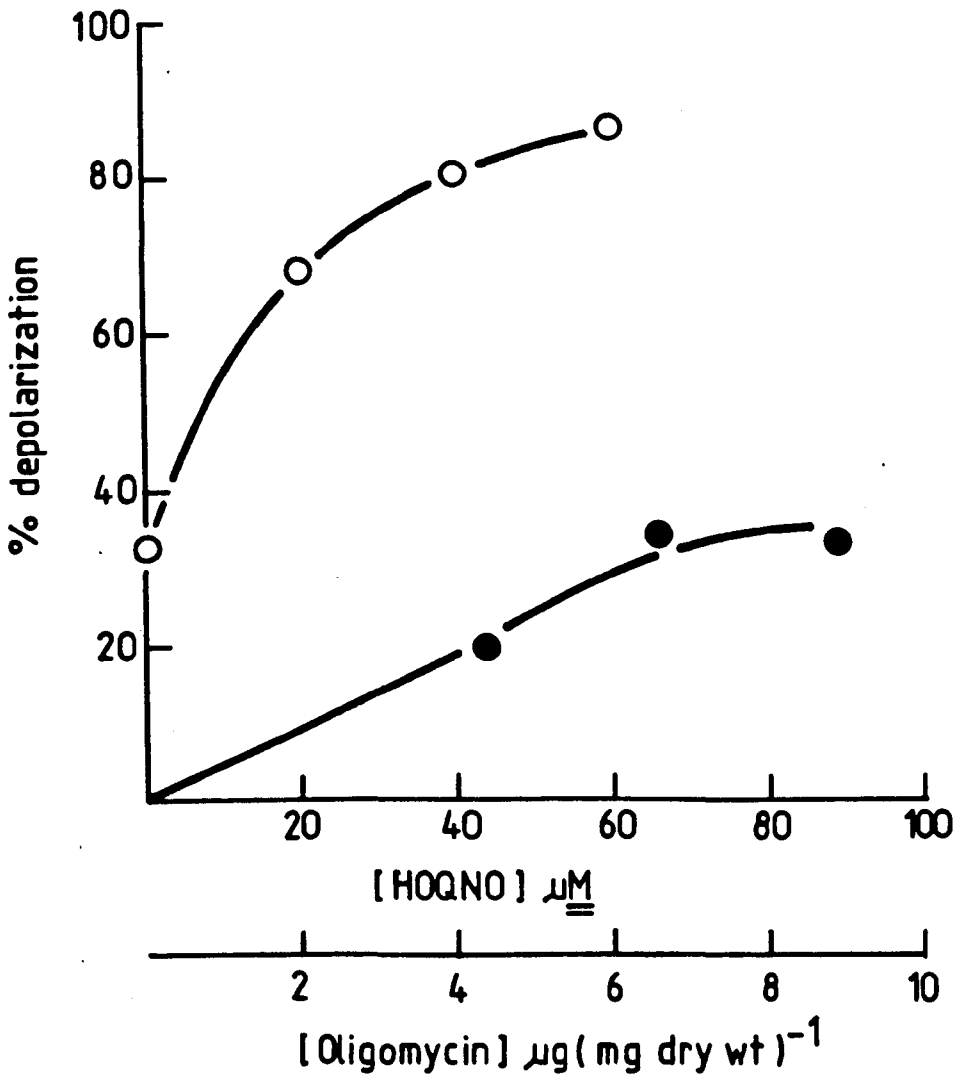


Fig.19: Effect of HOQNO and oligomycin on the membrane potential generated under anaerobic conditions in the presence of glucose by aerobically-grown P. acnes.



Legend to Figs. 20 and 21.

Depolarization of the membrane by:

● oligomycin

O HOQNO, in the presence of oligomycin.

The experiment was performed essentially as described in Methods section 6. Glucose, at a final concentration of approximately 16 mM was added to the cell suspension after a steady level of $[K^+]$ had been attained following valinomycin treatment. Inhibitors were introduced to the cell suspension upon the attainment of a new steady level of $[K^+]$.

Fig. 20.

The value of the $\Delta\Psi$ in the absence of inhibitors was -160·4 mV corresponding to an accumulation ratio of 470·5.

Fig. 21.

The value of the $\Delta\Psi$ in the absence of inhibitors was -155 mV corresponding to an accumulation ratio of 381·5.

Fig.20: Effect of oligomycin and HOQNO on the membrane potential generated under aerobic conditions in the presence of glucose by anaerobically-grown P. acnes.

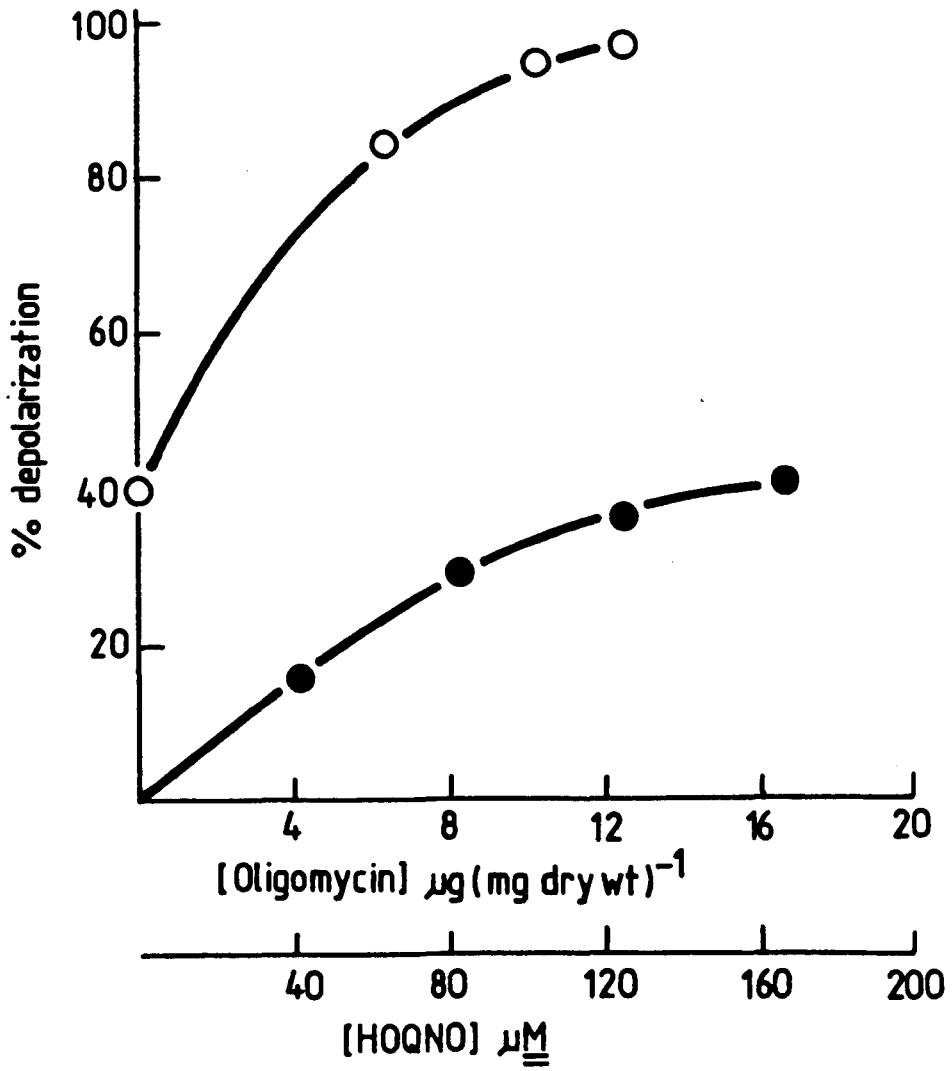
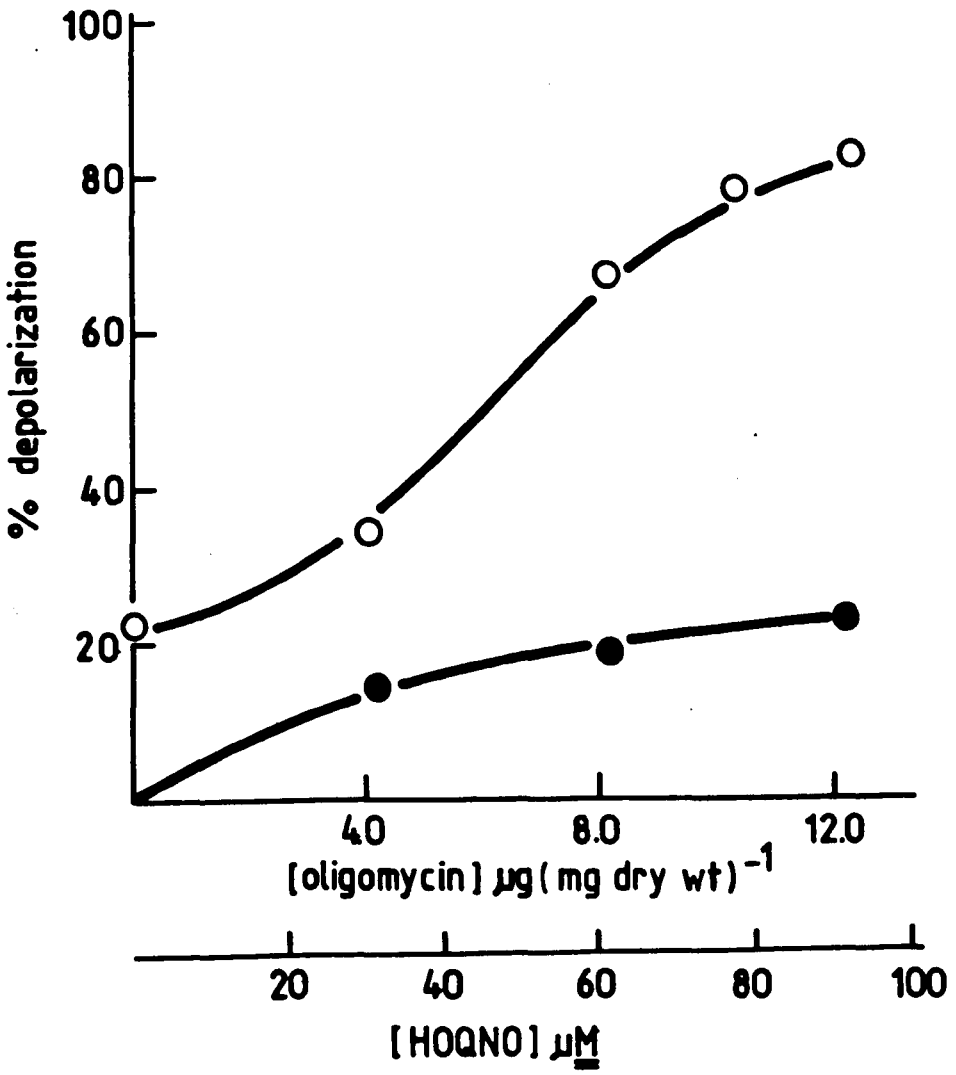


Fig.21: Effect of oligomycin and HOQNO on the membrane potential generated under aerobic conditions in the presence of glucose by aerobically-grown P. acnes.



Legend to Figs. 22 and 23.

Depolarization of the membrane by:

● HOQNO

○ oligomycin, in the presence of HOQNO.

The experiment was performed as described in the legend to Figs. 20 and 21.

Fig. 22.

The $\Delta\Psi$ in the absence of inhibitors was -165·7 mV corresponding to an accumulation ratio of 577·3.

Fig. 23.

The $\Delta\Psi$ in the absence of inhibitors was -162 mV corresponding to an accumulation ratio of 501·7.

Fig. 22: Effect of HOQNO and oligomycin on the membrane potential generated under aerobic conditions in the presence of glucose by anaerobically grown P. acnes.

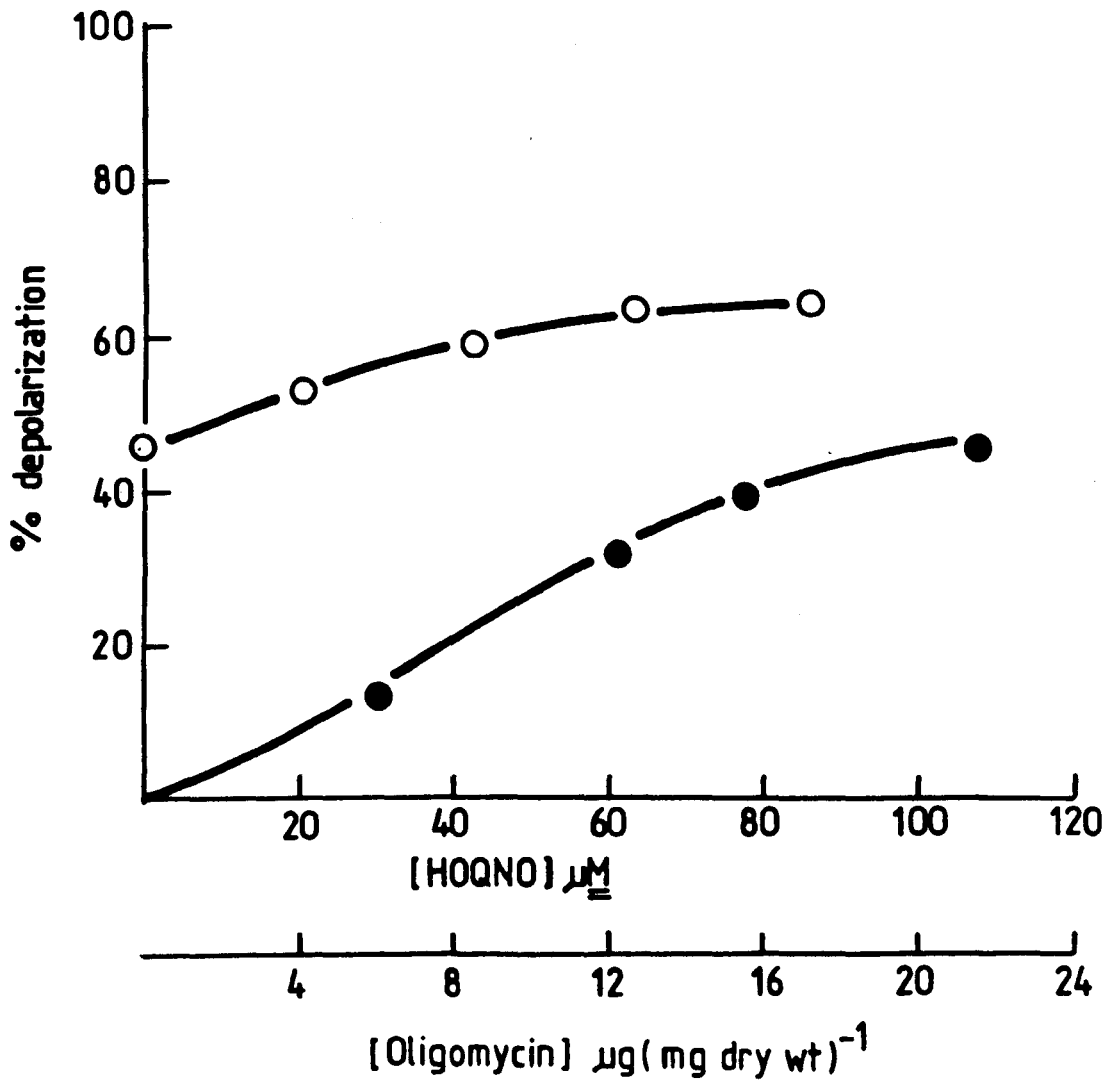
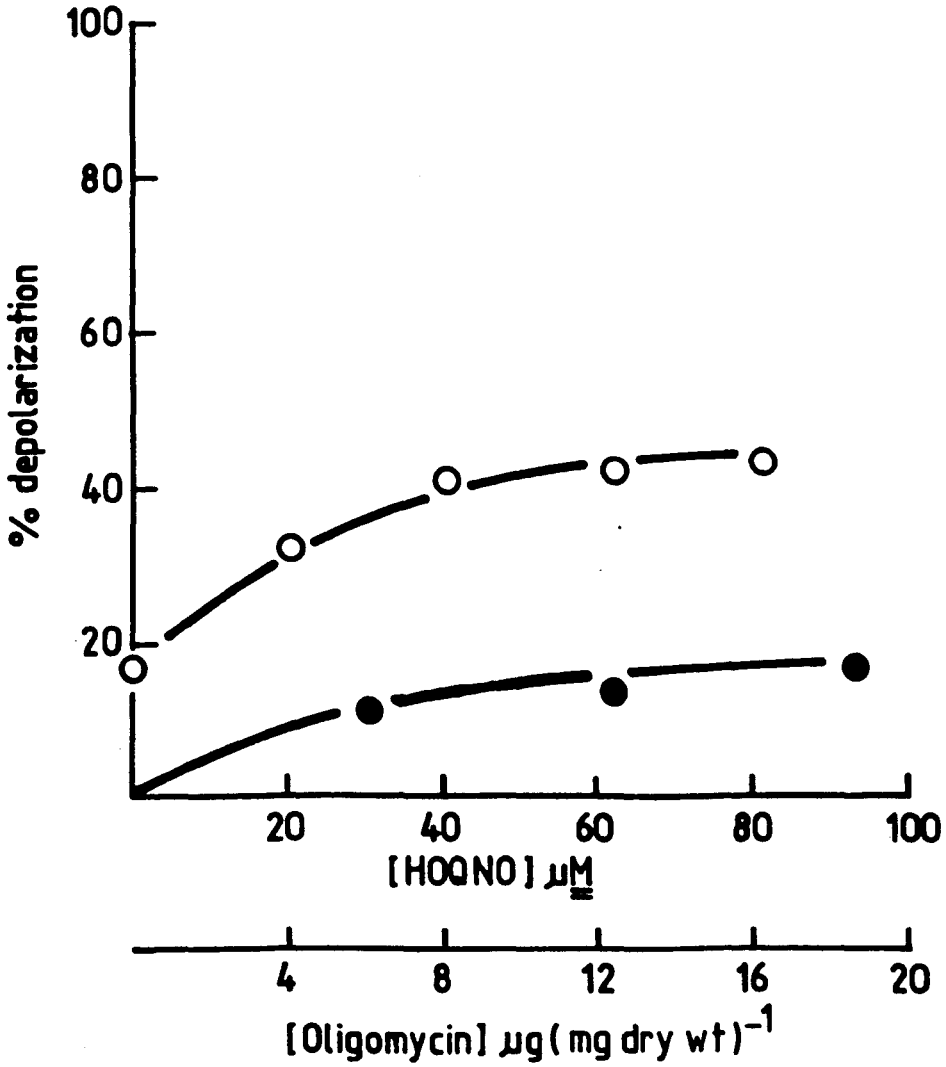


Fig.23: Effect of HOQNO and oligomycin on the membrane potential generated under aerobic conditions in the presence of glucose by aerobically-grown P. acnes.



generation of the $\Delta\Psi$ under aerobic conditions in the presence of glucose (Figs. 20 and 21). The inhibition of the generation of the $\Delta\Psi$ under aerobic conditions in the presence of glucose by HOQNO and oligomycin was usually slightly more extensive in anaerobically-grown cells than in aerobically-grown cells. With anaerobically-grown cells, the % inhibition by the combination of HOQNO and oligomycin ranged from 55% to 74% in three different experiments. However, with aerobically-grown cells, the values encountered in four different experiments ranged from 23% to 46%. This anomalous behaviour was observed in batches of cells which showed complete sensitivity to oligomycin and HOQNO if added in that order. In the absence of glucose, a similar response of the $\Delta\Psi$ to HOQNO and oligomycin was observed in anaerobically- and aerobically-grown cells (Figs. 24 and 25).

Due to the above observations, it was decided to introduce another electron transport inhibitor, antimycin A, into the present studies involving the generation of the $\Delta\Psi$ under aerobic conditions. This was to test whether the HOQNO-insensitive $\Delta\Psi$ was actually generated by a second or branched electron transport chain. The effects of antimycin A and HOQNO on the $\Delta\Psi$ generated under aerobic conditions by anaerobically- and aerobically-grown cells are shown in Figs. 26 and 27 respectively. In both anaerobically- and aerobically-grown cells, antimycin A depolarized the membrane by approximately 40%. The residual $\Delta\Psi$ was almost completely sensitive to HOQNO. In the presence of glucose, a similar response by anaerobically- and aerobically-grown cells was observed (Figs. 28 and 29 respectively). This would support the suggestion that an electron transport system with different sensitivites to HOQNO and antimycin A was present.

Legend to Figs. 24 and 25.

Depolarization of the membrane by:

● HOQNO

O oligomycin, in the presence of HOQNO.

The experiment was performed as described in Methods section 6.

Fig. 24.

The $\Delta\Psi$ in the absence of inhibitors was -155·1 mV corresponding to an accumulation ratio of 384.

Fig. 25.

The $\Delta\Psi$ in the absence of inhibitors was -156·5 mV corresponding to an accumulation ratio of 405·5.

Fig. 24: Effect of HOQNO and oligomycin on the membrane potential generated under aerobic conditions by anaerobically-grown P. acnes.

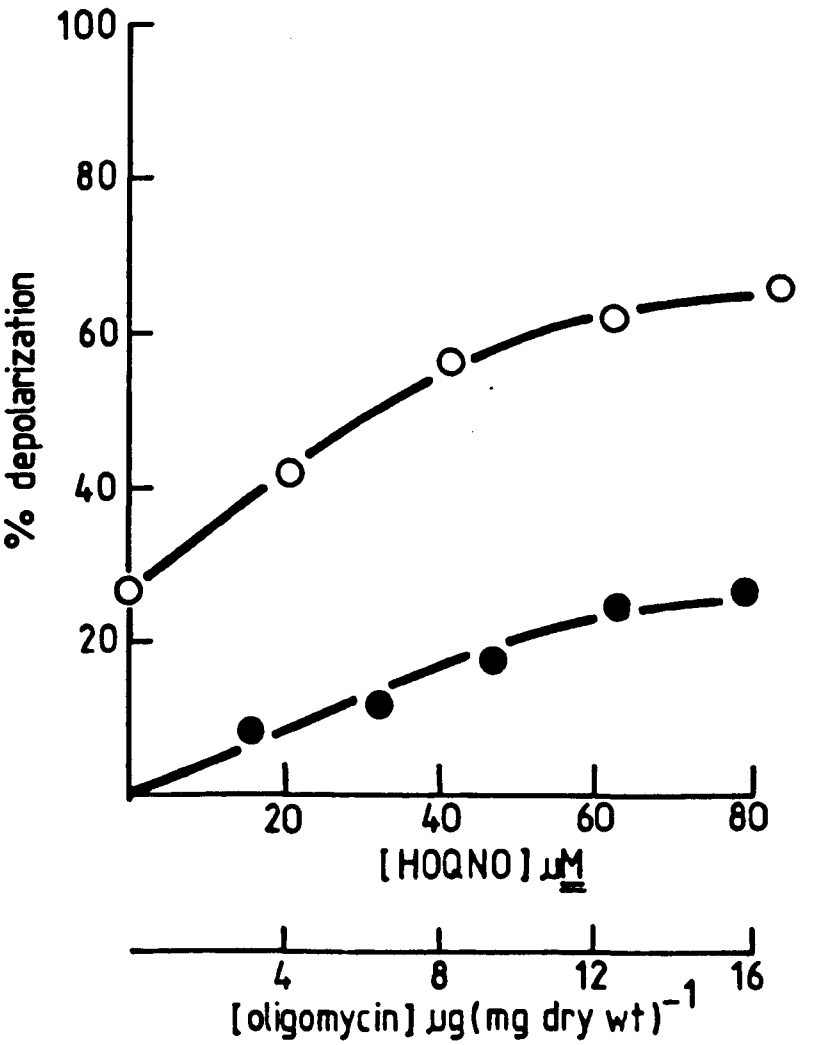
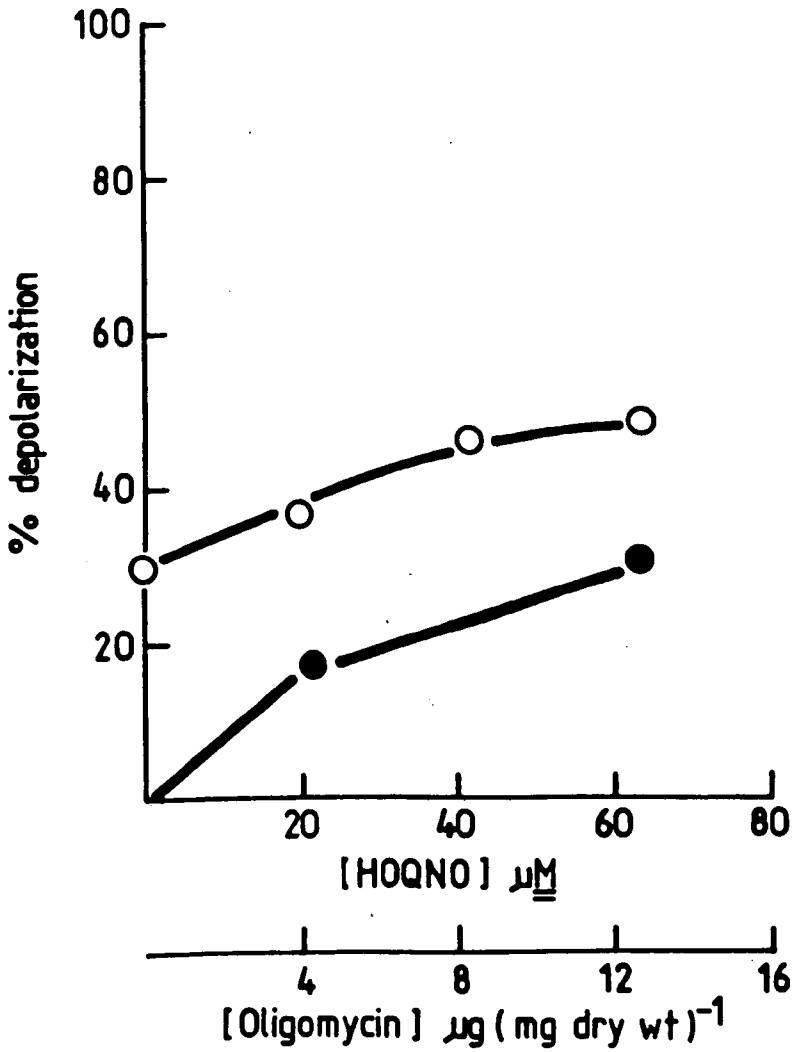


Fig.25: Effect of HOQNO and oligomycin on the membrane potential generated under aerobic conditions by aerobically-grown P.acnes.



Legend to Figs. 26 to 29.

Depolarization of the membrane by:

● Antimycin A

○ HOQNO, in the presence of antimycin A.

The experiment was performed as described in Methods section 6.

The values of the $\Delta\Psi$ in the following figures were:

Fig.	$\Delta\Psi$ (-mV)	Accumulation ratio.
26	148·3	296·1
27	144·19	253
28	155·4	388·4
29	161·2	486·2

Fig.26: Effect of antimycin A and HOQNO on the membrane potential generated under aerobic conditions by anaerobically-grown P. acnes.

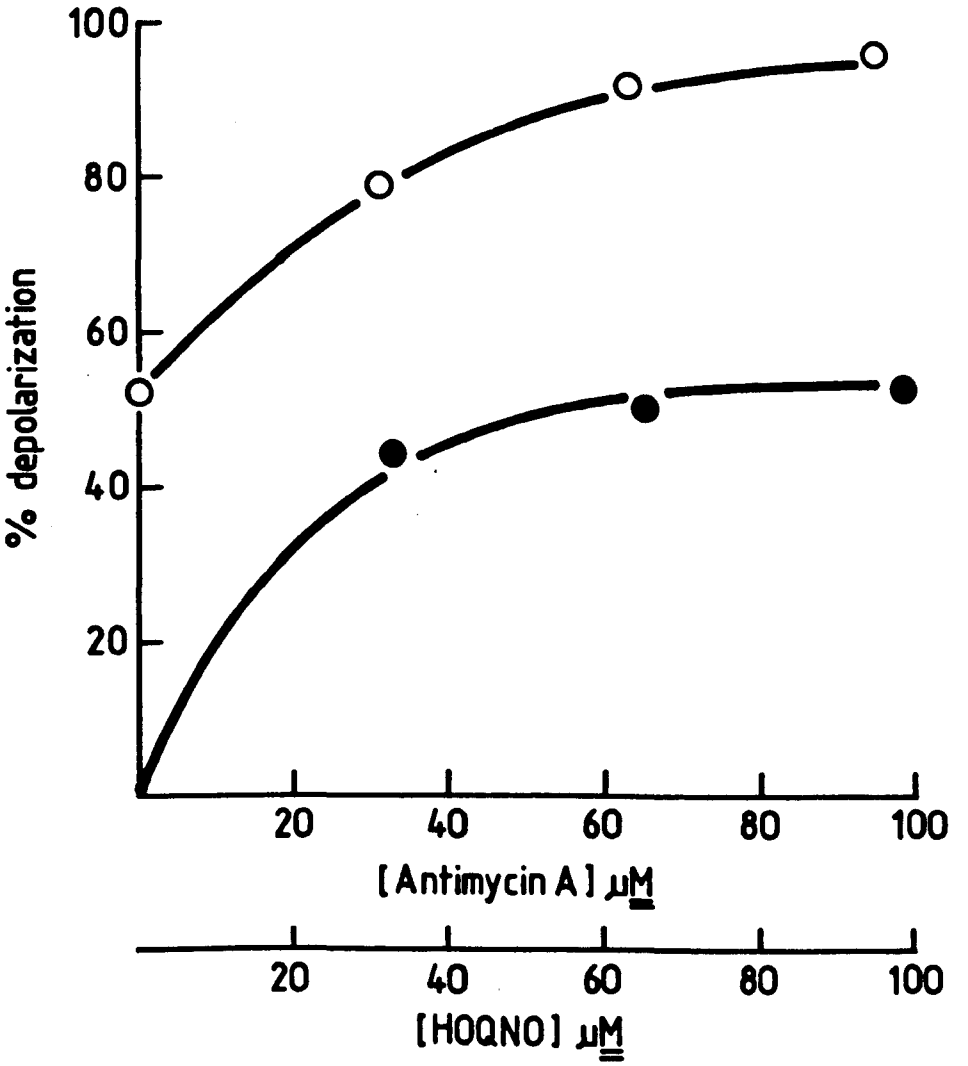


Fig. 27: Effect of antimycin A and HOQNO on the membrane potential generated under aerobic conditions by aerobically-grown P. acnes.

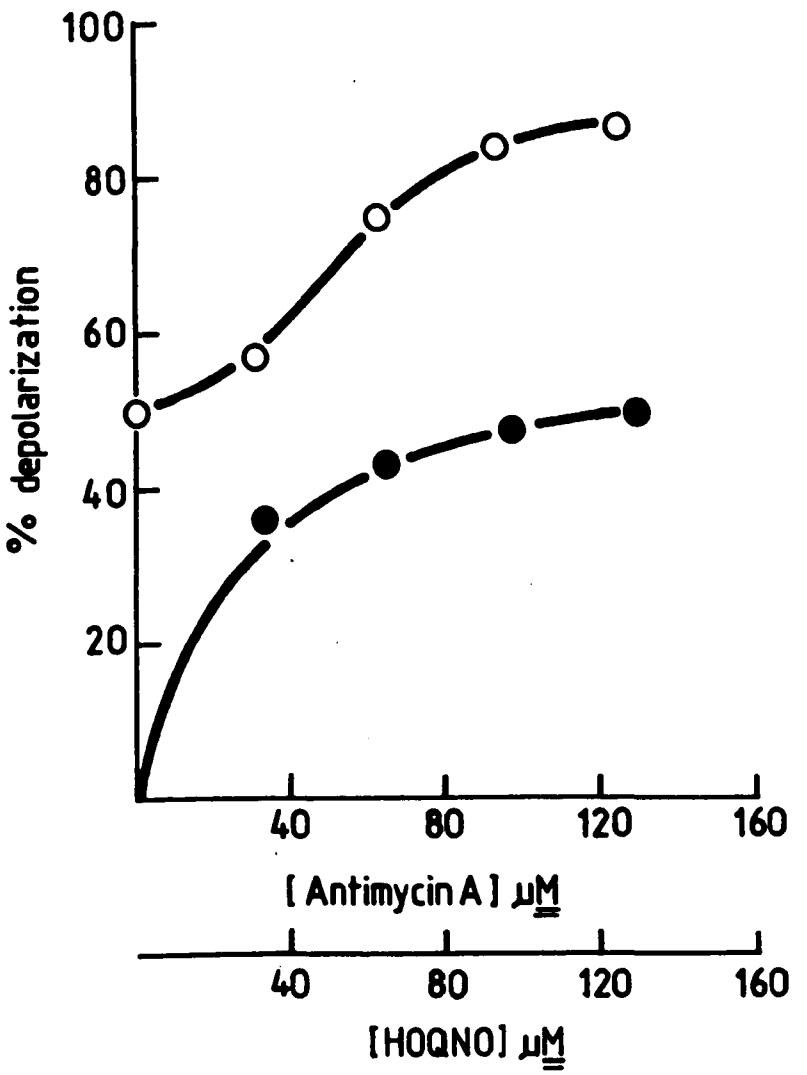


Fig. 28: Effect of antimycin A and HOQNO on the membrane potential generated under aerobic conditions in the presence of glucose by anaerobically-grown P. acnes.

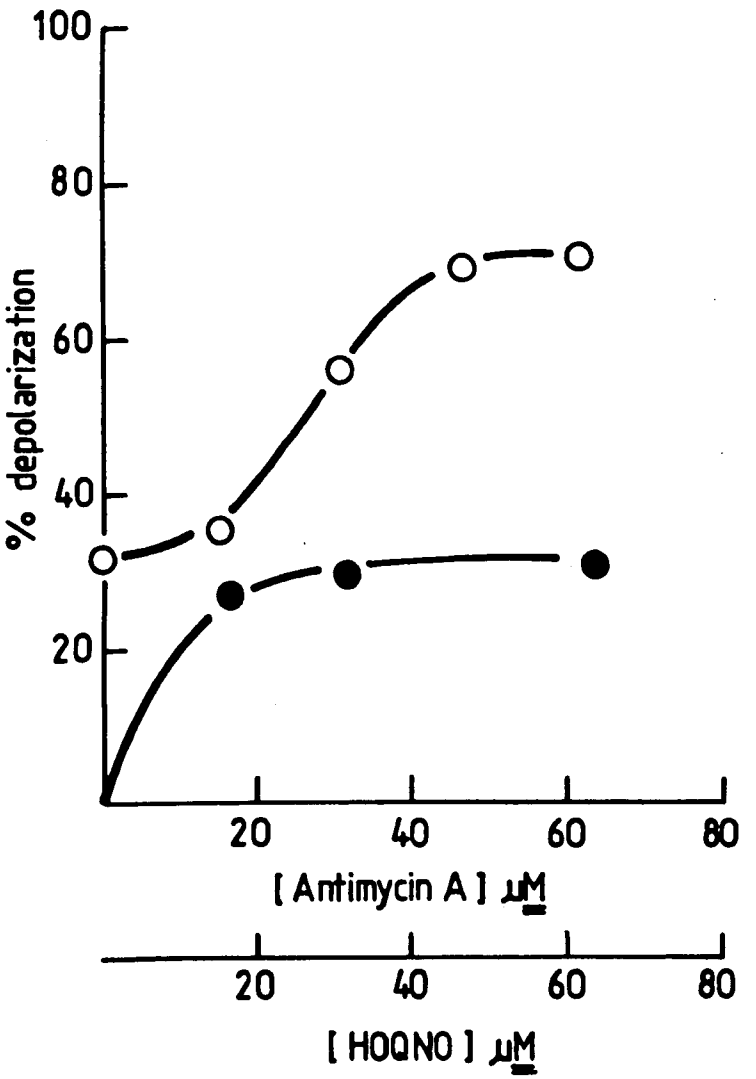
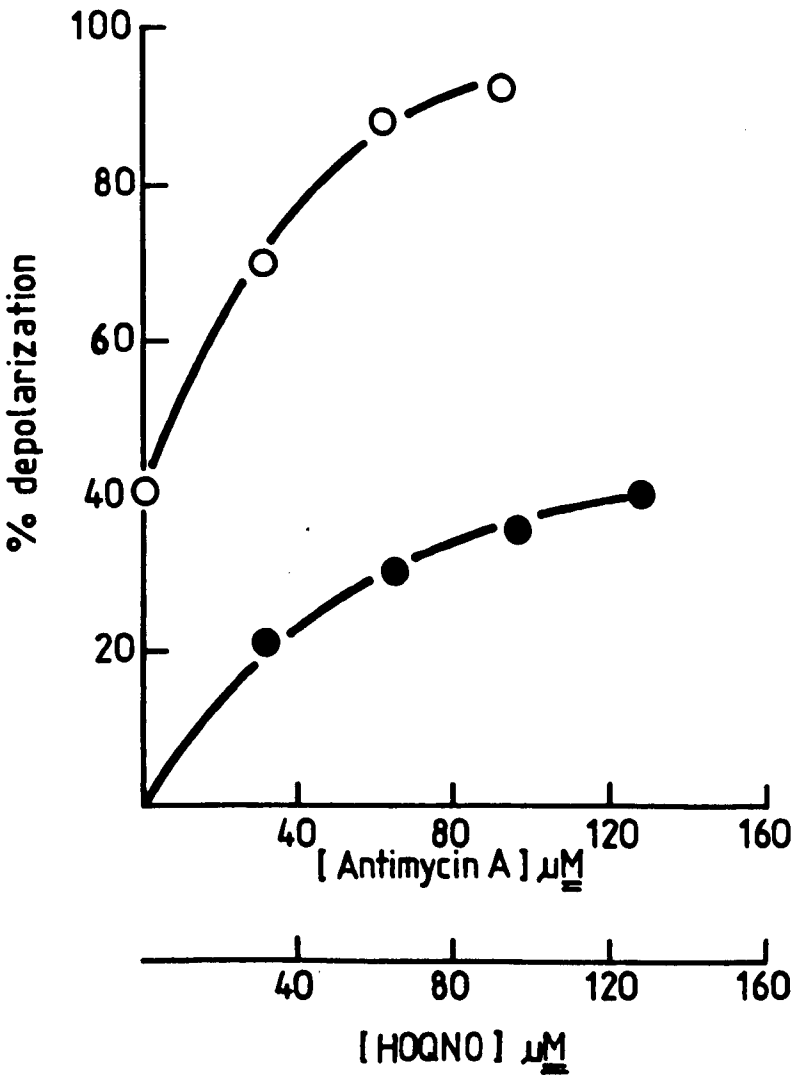


Fig. 29: Effect of antimycin A and HOQNO on the membrane potential generated under aerobic conditions in the presence of glucose by aerobically-grown P. acnes.



2. Studies on the ATPase of P. acnes.

The interpretation of the data obtained from studies on the generation of the $\Delta\Psi$ in P. acnes suggested the presence of a membrane-bound energy-transducing ATPase sensitive to the various energy-transfer inhibitors used. Such an enzyme had not been studied in Propionibacterium and since it could play a fundamental role in the process under study, a characterization of the ATPase was therefore attempted.

In studying a membrane-bound enzyme like the ATPase, a reliable technique for the preparation of membrane fragments was required. Conventional techniques for cell-disruption such as enzymic lysis, passage through French and Hughes pressure cells, sonication and grinding with glass-beads (described in Methods section 11 (i, ii), 12 (iii, v, ii and iv) respectively) were attempted. The main criterion of cell-breakage by the various disruption techniques used was based on the amount of protein released into the supernatant obtained after centrifuging the disrupted cells at 10,000 g for 10 min (cell-free extract), on the assumption that 60% of the total dry weight is made up of proteins.

Initially, the search for a convenient and reliable method of cell-disruption was performed with cells grown on complex medium (Reinforced clostridial medium, Oxoid). However, cells grown on complex medium were found to be extremely resistant to the disruption techniques used, possibly due to the presence of a thick exopolysaccharide layer. This suggestion is based on the observation that the pellet obtained upon harvesting P. acnes grown on complex medium was 'slimy' as compared with those grown on the semi-defined medium.

A number of studies on the ATPase of Gram +ve and Gram -ve organisms have successfully used enzymic techniques, particularly

lysozyme digestion followed by the disruption of the spheroplasts or protoplasts by exposure to hypotonic medium. This yields membrane preparations possessing high membrane-bound ATPase activity (Abrams et al., 1960; Evans, 1969). This technique also minimizes damage to the ATPase that could be incurred by physical disruption techniques. However, P. acnes was found to be resistant to lysozyme action in the presence of EDTA. Incubating P. acnes with lysozyme and EDTA (as described in Methods section ii(i) for up to 90 min did not cause lysis of the cells. Prior treatment with a high concentration of LiCl also proved unsuccessful.

The application of physical disruption techniques showed varying degrees of success. Sonication brought about the release of 37% of the cell protein into the cell-free extract. The release of protein was accompanied by the appearance of ATPase and succinate dehydrogenase activities, the latter being used as a membrane marker (Fig. 30).

Passage of P. acnes through a French pressure cell operated at 35·5 MPa caused the release of approximately 60% protein into the cell-free extract. The degree of breakage was relatively consistent, with values of protein released varying from 45% to 60% with different batches of cells. Approximately 70% of the total protein in the cell-free extract was soluble and 20% was insoluble protein as judged by their ability to sediment at 100,000 $\underset{\approx}{g}$ centrifugation for 60 min (Fig.31a). As can be seen in Fig. 31(a), P. acnes was a relatively difficult organism to disrupt; the total protein released did not plateau even after 4 passages through the French press. The release of protein was accompanied by ATPase and succinate dehydrogenase activities (Fig. 31b). However, as shown in Fig. 31(b), the ATPase activity was largely solubilized with approximately 20% of the total activity occurring in the membrane-bound form.

Legend to Fig. 30.

○ ATPase activity

● % protein released into cell-free extract.

▲ succinate dehydrogenase activity.

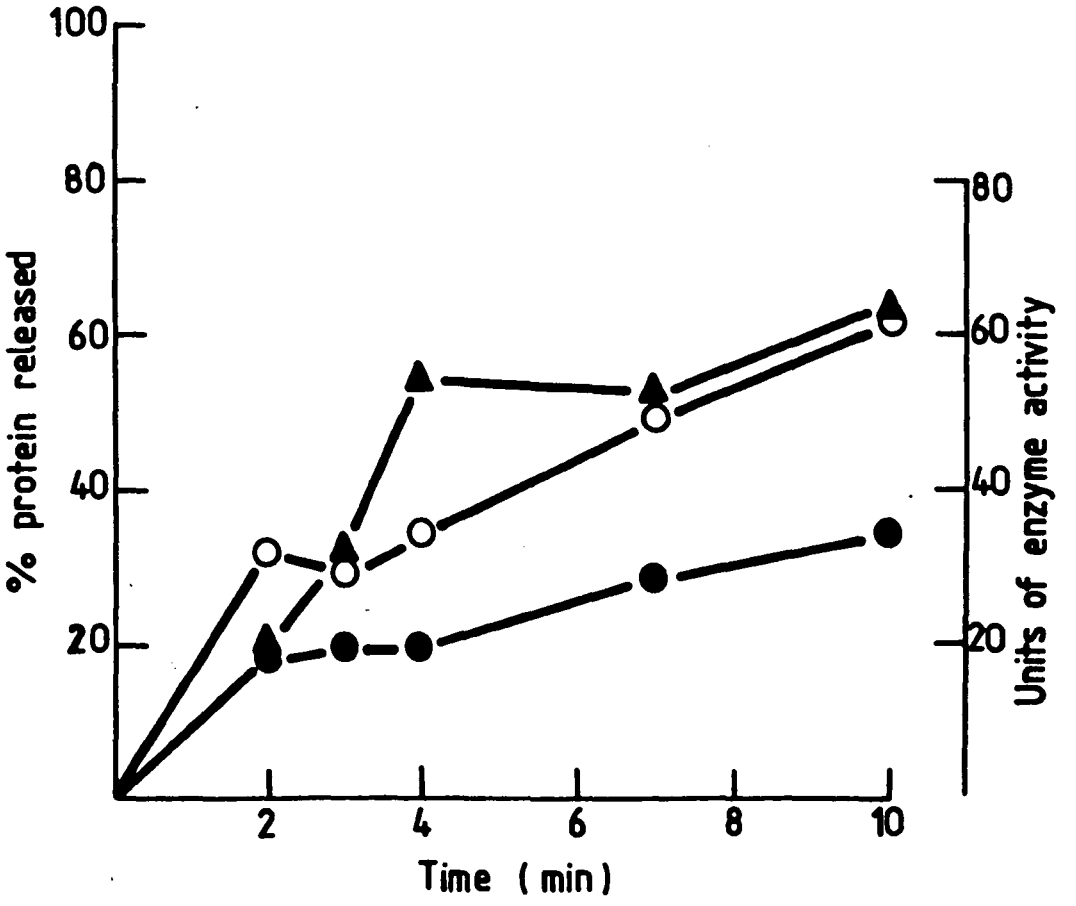
Cell-disruption by sonication was performed as described in Methods section 12(ii). ATPase and succinate dehydrogenase activities were assayed as described in Methods section 13 and 15 respectively.

1 unit of ATPase activity represents 1 μmol Pi released mg protein^{-1} h^{-1}. Total units = Unit x total protein.

1 unit of succinate dehydrogenase activity represents a decrease of 1 OD unit (at 600 nm) (mg protein)$^{-1}$ h^{-1}.

Total units : Units x total protein.

Fig. 30: Disruption of P. acnes by sonication.



Legend to Fig. 31.

a) ○ % protein released into cell-free extract.

● % protein in the supernatant obtained after centrifuging the above cell-free extract at 100,000 $\underset{\approx}{g}$ for 60 min.

▲ % protein in the pellet obtained by centrifuging the above cell-free extract at 100,000 $\underset{\approx}{g}$ for 60 min.

b) ● total ATPase activity of cell-free extract.

○ total ATPase activity obtained in the supernatant fraction obtained after centrifuging the cell-free extract at 100,000 $\underset{\approx}{g}$ for 60 min.

▲ total ATPase activity of the pellet obtained after centrifuging the cell-free extract at 100,000 $\underset{\approx}{g}$ for 60 min.

□ total succinate dehydrogenase activity of cell-free extract.

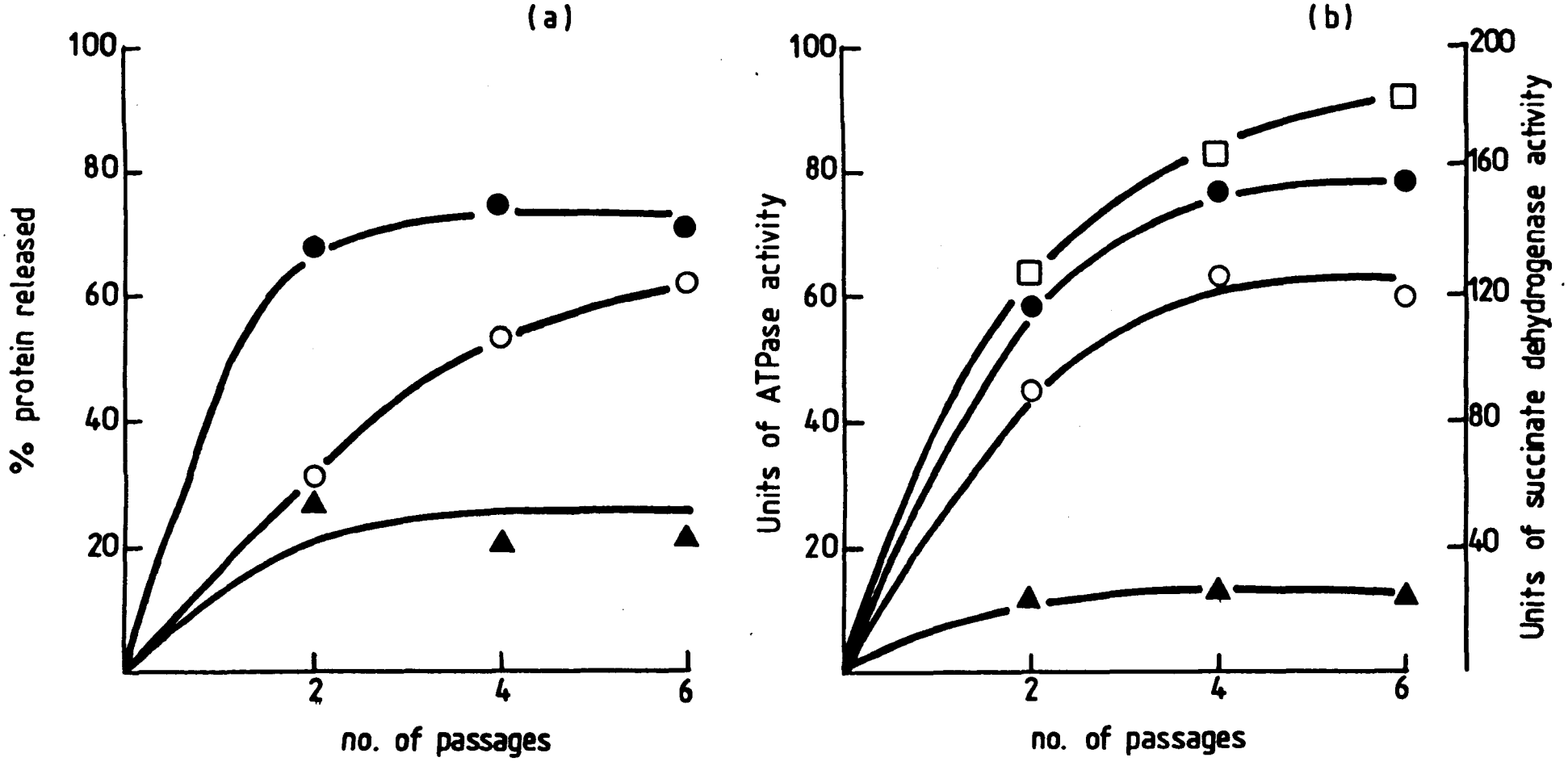
Disruption of P. acnes by passages through a French press was carried out as described in Methods section 12(iii). ATPase and succinate dehydrogenase activities were assayed as described in Methods sections 13 and 15 respectively. 1 unit of ATPase activity represents 1 μmol Pi released (mg protein)$^{-1}$ h^{-1}.

Total : Units x total protein.

1 unit of succinate dehydrogenase activity represents a decrease of 1 OD unit at (600 nm) (mg protein)$^{-1}$ h^{-1}.

Total units : units x total protein.

Fig. 31: a) Disruption of P. acnes by passages through a French pressure cell.
b) Distribution of the ATPase activity in the cell-free extract obtained.



Passage of P. acnes through the Hughes press gave very similar results to those obtained for the French press. As shown in Table 8, only 46% of the protein was released into the cell-free extract. The ATPase activity was also largely solubilized, with only 18% occurring as the membrane-bound form.

Agitation with glass-beads was another technique used for disrupting P. acnes. The time-course for protein release showed that a plateau was reached after 5 min treatment, resulting in the release of 40% protein into the cell-free extract (Fig. 32a). As with other techniques used, it was consistently found 80% of the total protein released occurred as soluble proteins with only 20% occurring in the insoluble fraction. The ATPase activity in the cell-free extract did not reach a maximum until 9 min agitation as shown in Fig.32(b). However, the ATPase activity associated with the insoluble fraction reached a maximum after 6 min treatment. It may be inferred that prolonged agitation caused solubilization of the ATPase as noted by the continued increase in the ATPase activity of the soluble fraction. Because of this, the agitation process was limited to 5 min only.

Of the various techniques used for the preparation of membrane fragments from P. acnes, passage through the French pressure cell was the most effective technique for disrupting the cells. However, of all the methods used, agitation with glass-beads (5 min) was found to consistently give a higher % of membrane-bound ATPase activity, ranging from 25% to 35% of the total ATPase activity of the cell-free extracts. As discussed in the Introduction, soluble and membrane-bound ATPases exhibit differences with respect to sensitivity to various energy-transfer inhibitors. Soluble ATPase, in most cases, is not sensitive to those inhibitors whose site of action is believed to be the membrane portion of the ATPase (F_o), such as DCCD and oligomycin. To relate meaningfully the sensitivity of the ATPase and the mechanism responsible

Table 8: Disruption of P. acnes by a passage through a Hughes press and the distribution of ATPase activity in the cell-free extract.

Fraction	% protein released	Units of ATPase activity	% of Total ATPase activity
Cell-free extract	46	29·2	100
Supernatant of 100,000 $\underset{\approx}{g}$ x 60 min centrifugation	88	25·3	88
Pellet of 100,000 $\underset{\approx}{g}$ x 60 min centrifugation	4	5·4	18

Legend to Table 8:

Cell-free extract of P. acnes was prepared by passage through a Hughes press as described in Methods section 12(v). ATPase activity was assayed as described in Methods Section 13.

A unit of ATPase activity is defined as 1 μmol Pi released (mg protein)$^{-1}$ h^{-1}.

Legend to Fig. 32.

a) ● % protein released into cell-free extract

▲ % protein in the supernatant obtained after centrifuging the cell-free extract at 100,000 $\underset{\approx}{g}$ for 60 min.

O % protein in the pellet obtained after centrifuging the cell-free extract at 100,000 $\underset{\approx}{g}$ for 60 min.

b) ● Total units of ATPase activity in cell-free extract.

O Total units of ATPase activity in the supernatant obtained after centrifuging the cell-free extract at 100,000 $\underset{\approx}{g}$ for 60 min.

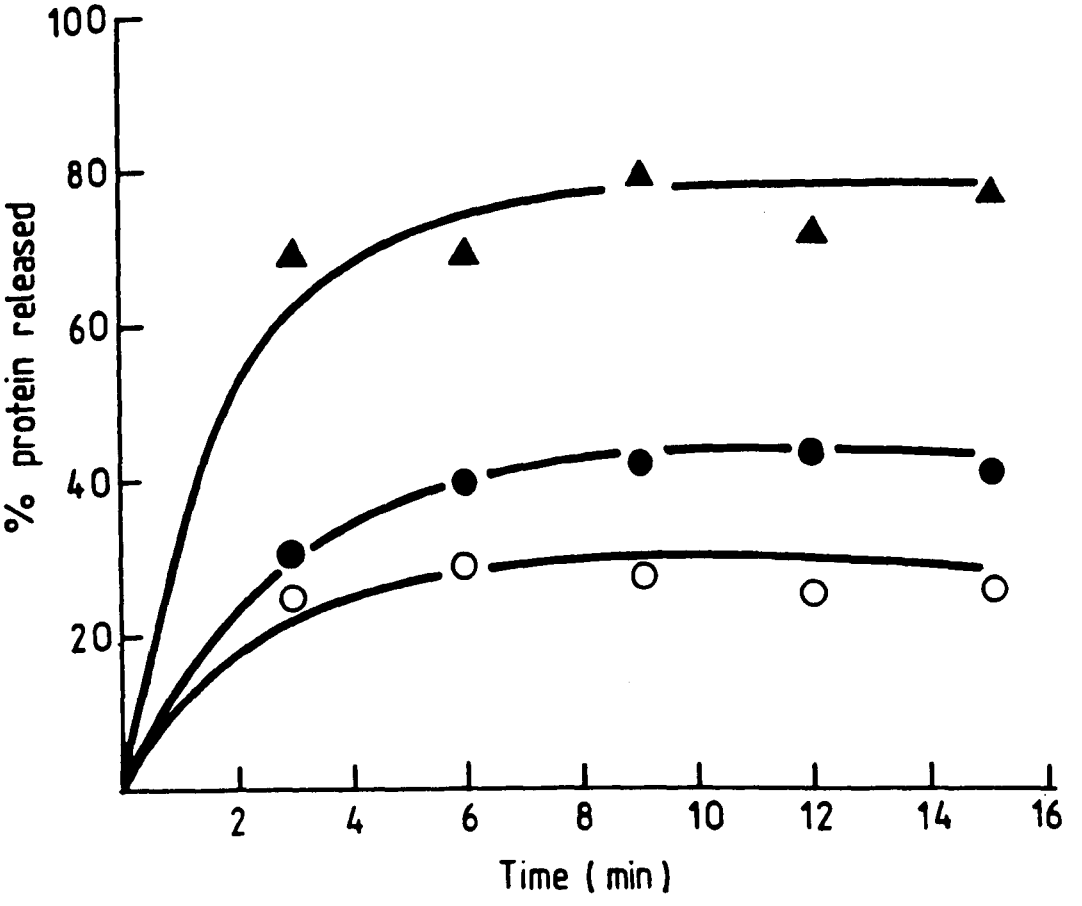
▲ Total units of ATPase activity in the pellet obtained after centrifuging the cell-free extract at 100,000 $\underset{\approx}{g}$ for 60 min.

Disruption of P. acnes by agitation with glass-beads was performed as described in Methods section 12(iv). ATPase activity was assayed as described in Methods section 13.

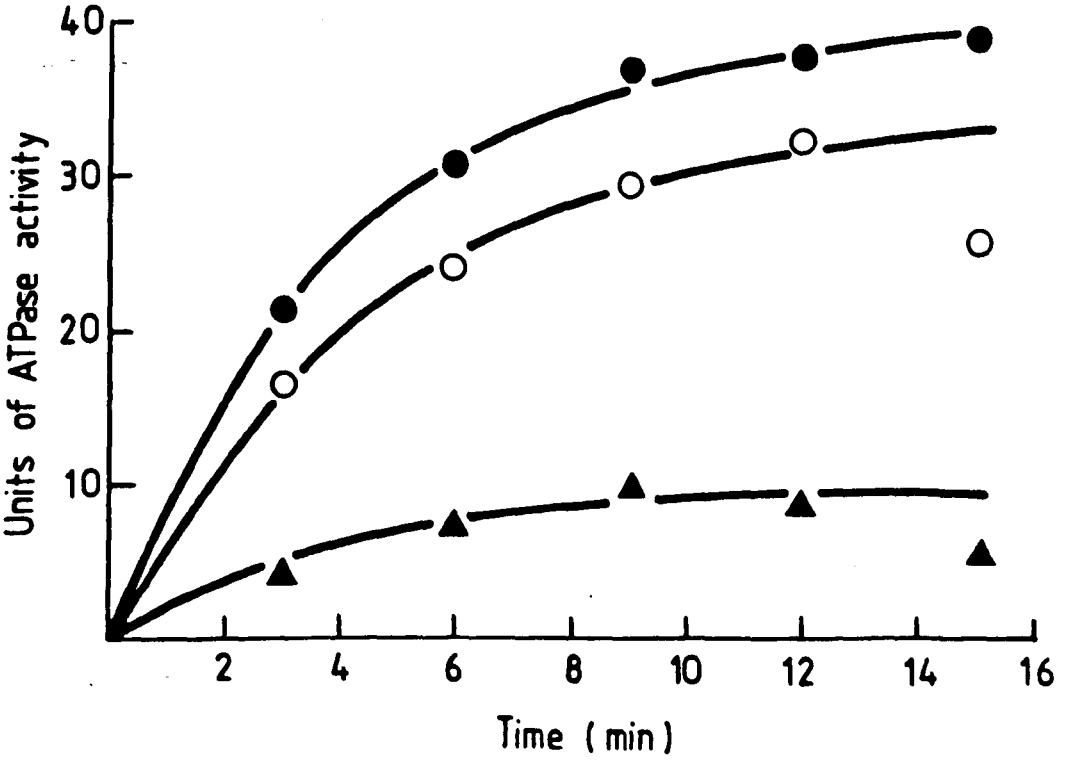
A unit of ATPase activity is defined as 1 μmol Pi released $(\text{mg protein})^{-1}\ h^{-1}$.

Total units : Unit x total protein.

Fig. 32: a) Disruption of P. acnes by agitating with glass-beads.



b) Distribution of the ATPase activity in the cell-free extract obtained.



for generating the $\Delta\Psi$ to the various energy-transfer inhibitors used, studies were required with the membrane-bound ATPase. Therefore, disruption with glass-beads was adopted for routine preparations of membrane fragments. As shown earlier, the yield of membrane-bound ATPase was very low. Conditions for the preparation of the membrane fragments were studied in order to maximize the yield of the membrane-bound ATPase.

Preparations of membrane fragments exhibiting ATPase activity from organisms such as E. coli (Evans, 1969), S. faecalis (Abrams, 1965) and M. lysodeikticus (Munõz et al., 1969) all showed a requirement for Mg^{2+} in the medium used for disrupting the organisms, usually at a concentration of 5 mM. Solubilization of the membrane-bound ATPase can be carried out by repeated washing of the membranes in a medium of low ionic strength in the absence of Mg^{2+} (discussed in the Introduction). The Mg^{2+} concentration in the medium used for preparing the membrane fragments was therefore varied from 5 mM to 20 mM, and the ionic strength increased using NaCl to a final concentration of 0·2 M. There was however no increase in the total membrane-bound ATPase activity.

The possibility of there being components present in the medium used for preparing the membrane fragments which was causing solubilization of the ATPase was investigated using S. faecalis as the control. Membrane fragments prepared from S. faecalis in medium containing 50 mM-Tris-Cl (pH 7·4) and 5 mM-$MgCl_2$ showed high ATPase activity as shown in Table 9. The presence of membrane-bound ATPase activity was indicated by its sensitivity to DCCD; soluble ATPase from S. faecalis is not sensitive to DCCD (Abrams and Smith, 1974). The ATPase activity was inhibited by approximately 60% at 0·1 mM-DCCD (Table 9).

The soluble and membrane-bound ATPase from P. acnes showed

Table 9.

ATPase activity in cell-free extract of S. faecalis.

	Activity[1]	% inhibition
No addition	0·28	0
+ oligomycin (100 μg)	0·28	0
+ oligomycin (200 μg)	0·28	0
+ DCCD (0·1 mM)	0·12	57·1
+ DCCD (0·2 mM)	0·11	60·7

Legend to Table 9.

Cell-free extract of S. faecalis was prepared as described in Methods section 12(vi). ATPase activity was assayed as described in Methods section 13. Each assay contained 0·2mg protein.

[1]Activity was expressed as μmol Pi released (mg protein)$^{-1}$ min^{-1}.

different characteristics with respect to pH and Mg^{2+} requirement. As shown by Fig. 33(a) and (b), the pH optimum for soluble ATPase was pH 7·0 and 7·5 for the membrane-bound ATPase respectively. Both soluble and membrane-bound ATPases showed a specific requirement for Mg^{2+} as shown in Fig. 34. In those requirements, the ratio of Mg^{2+}:ATP was 0·5 : 1 for membrane-bound ATPase and 1 : 1 for soluble ATPase. Neither ATPase was activated by Ca^{2+}.

Having established the conditions for assaying the ATPase activity of the soluble and particulate fractions of the cell-free extract from P. acnes, the effect of energy-transfer inhibitors on the ATPase was then investigated. These studies were however rather unsuccessful as both the soluble and membrane-bound ATPase were found to be relatively resistant to the various energy-transfer inhibitors used. A typical response of the membrane-bound ATPase to the energy-transfer inhibitors used is shown in Table 10. Only Nbf-Cl and quercetin showed significant inhibition ie. 40% and 43% respectively at a concentration of 0·4 mM. Oligomycin and DCCD did not show a significant effect on the membrane-bound ATPase despite the fact that both inhibited the generation of the $\Delta\Psi$ under anaerobic conditions (Results, section 1). A similar response to the above inhibitors was obtained with soluble ATPase (results not shown).

One notable feature of both ATPases was the consistently low activity obtained. Generally, a value of 15 to 25 nmol Pi released $(mg\ protein)^{-1}\ min^{-1}$ was observed for both ATPase activities. The likelihood that these ATPases were latent, as observed for certain ATPases such as those from M. lysodeikticus (Munõz et al., 1969) and chloroplasts (Petrack et al., 1965), was investigated. Known methods for activating the ATPases involving trypsin digestion (Munõz et al., 1969) and heat-treatment in the presence of dithiothreitol (Lien and Racker, 1971) were therefore attempted (Methods, section 14).

Legend to Fig. 33.

● 100 mM-MES-NaOH

○ 100 mM-MOPS-NaOH

▲ 100 mM-Bicine-NaOH

ATPase activity of the soluble and particulate fractions obtained after grinding P. acnes with glass-beads was assayed as described in Methods section 13. Variation of the pH of the assay medium was obtained by using the buffers described above.

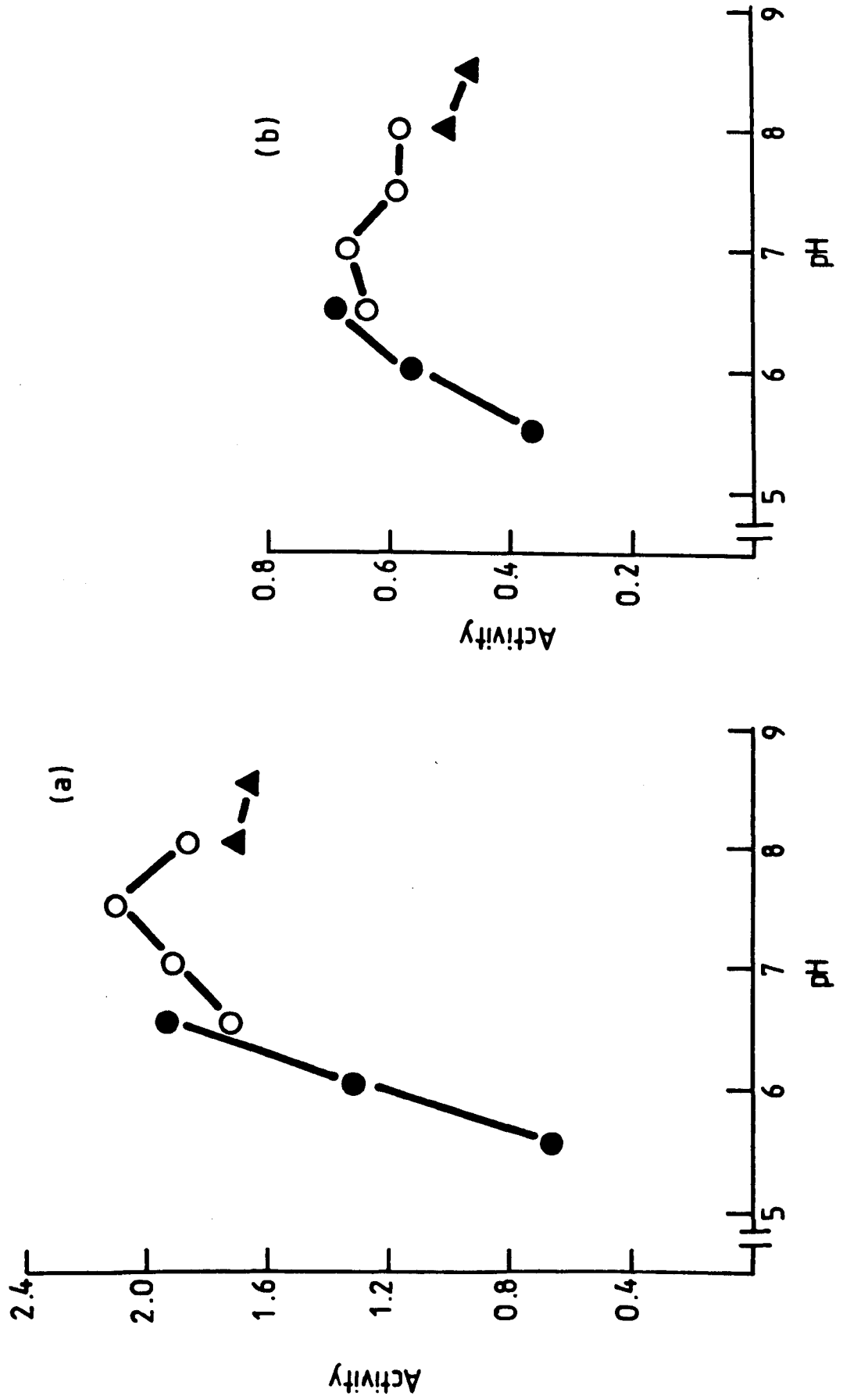


Fig.33: Effect of pH on the ATPase activity of P.acnes.
a) membrane-bound ATPase.
b) soluble ATPase.

Legend to Fig. 34.

ATPase activity of soluble and particulate fractions of P. acnes obtained by disruption with glass-beads was assayed as described in Methods section 13.

● ATPase activity of the particulate fraction in the presence of $MgCl_2$

○ ATPase activity of the particulate fraction in the presence of $CaCl_2$

▲ ATPase activity of the supernatant fraction in the presence of $MgCl_2$

△ ATPase activity of the supernatant fraction in the presence of $CaCl_2$

Fig.34: Dependance of ATPase activity on $MgCl_2$ and $CaCl_2$ on particulate and soluble preparations of P.acnes.

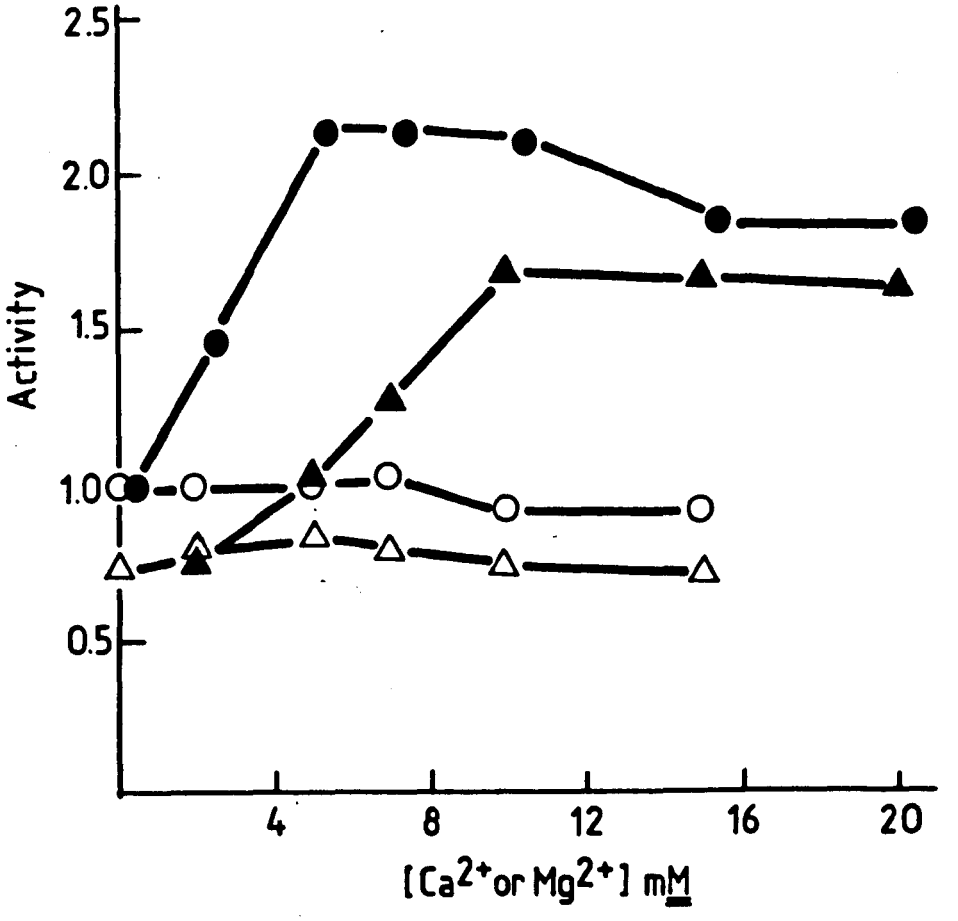


Table 10.

Effect of energy-transfer inhibitors on the membrane-bound ATPase activity of P. acnes.

	Activity[1]	% inhibition
no addition	1·9	0
+ DCCD (0·2 mM)	1·9	0
+ DCCD (0·4 mM)	1·9	0
+ Nbf-Cl (0·2 mM)	1·3	34·4
+ Nbf-Cl (0·4 mM)	1·2	40·1
+ oligomycin (100 μg)	1·8	5·7
+ oligomycin (200 μg)	1·8	5·7
+ Quercetin (0·2 mM)	1·3	32·3
+ Quercetin (0·4 mM)	1·1	43·2

Legend to Table 10.

Membrane fragments were prepared by disruption with glass-beads as described in Methods section 12(v). ATPase activity was assayed as described in Methods section 13. Each assay contained 0·8 mg membrane protein.

[1]Activity expressed as μmol Pi released (mg protein)$^{-1}$ h^{-1}.

These attempts were however, unsuccessful.

The likelihood of there being an endogenous ATPase inhibitor in the membrane preparations of P. acnes was also investigated. This was carried out by assaying the ATPase activity of membrane fragments of S. faecalis in the presence of membrane fragments from P. acnes. As shown in Table 11, it was found that the ATPase activity of the membrane fragments of S. faecalis was not significantly inhibited by the presence of P. acnes membrane fragments.

Table 11.

Effect of the membrane fragments of P. acnes on the ATPase activity of S. faecalis.

	Activity[1]
S.F membranes	0·84
P.A membranes	0·03
S.F + P.A. membranes	0·71
S.F. membranes + DCCD (0·2mM)	0·21

Legend to Table 11.

S.F and P.A represents S. faecalis and P. acnes membrane fragments respectively. In the above experiment, the concentration of S. faecalis membrane fragments was 45 μg protein, and the P. acnes membrane fragments was 244 μg protein.

[1]Activity was expressed as μmol Pi released h^{-1}.

3. Studies on the respiration of P. acnes.

The $\Delta\Psi$ generated by both anaerobically- and aerobically-grown P. acnes was stimulated upon exposure to oxygen and a further stimulation occurred upon the addition of glucose. It was important to ascertain whether the observed increase was due to oxidative phosphorylation, ie. whether oxygen was acting as a terminal electron acceptor whereby in its presence, electron transport was functional, hence generating a greater H^+ flux which thus increased the $\Delta\Psi$. Respiration studies could provide vital information on the possible role of oxygen in the energy transduction process of P. acnes.

The earlier attempts to characterize the ATPase with respect to its sensitivity to energy-transfer inhibitors failed to provide a definite answer as to whether the depolarization of the membrane under aerobic conditions was actually due to the inhibitors interacting with the ATPase rather than at a site other than the ATPase. Thus respiration studies could provide an indication as to the specificity of the energy-transfer inhibitors used in this study.

Respiration studies were carried out as described in Methods section 8. A comparison of the respiration rates of anaerobically- and aerobically-grown cells revealed that both had a similar capacity to carry out respiration. Generally, an endogenous respiration rate of approximately 10 nmol O_2(mg dry wt)$^{-1}$ min^{-1} was consistently observed in a number of experiments for both anaerobically- and aerobically-grown cells. In the presence of glucose, the respiration rate was increased by nearly 100% to approximately 20 nmol O_2 (mg dry wt)$^{-1}$ min^{-1}.

Initially, the effect of energy-transfer inhibitors such as DCCD, Nbf-Cl, quercetin, DPPA, and oligomycin on endogenous and glucose-stimulated respiration was studied in anaerobically-grown P. acnes. All the above compounds inhibited both endogenous and glucose-stimulated respiration to varying degrees. Nbf-Cl, quercetin

and DCCD in particular caused severe inhibition of endogenous and glucose-stimulated respiration. A typical response of respiration of anaerobically-grown cells to DCCD is shown in Fig.35, where at 66 $\mu\underline{M}$, endogenous and glucose-stimulated respiration were inhibited by 55% and 70% respectively. A summary of the effects of the energy-transfer inhibitors on anaerobically- and aerobically-grown P. acnes are presented in Tables 12 and 13 respectively. In both anaerobically- and aerobically-grown cells, oligomycin caused only partial inhibition of both endogenous and glucose-stimulated respiration.

Respiration studies also allow the effect of electron transport inhibitors such as KCN and NaN_3 to be studied. In the previous experiments on the generation of the $\Delta\Psi$, it was not possible to study the effects of KCN and NaN_3 due to the fact the K^+-sensitive electrode was also sensitive to Na^+. In addition, increasing the external $[K^+]$ brought about the depolarization of the membrane (Fig.8). However, respiration by both anaerobically- and aerobically-grown P. acnes was totally insensitive to KCN up to a concentration of 10 $\underline{mM}$. NaN_3, as shown in Tables 12 and 13, stimulated respiration which possibly indicated that it was uncoupling oxidative phosphorylation. HOQNO and antimycin A both partially inhibited endogenous and glucose-stimulated respiration in both anaerobically- and aerobically-grown P. acnes.

The effect of the uncoupler CCCP on respiration is shown in Fig.36. The rate of respiration in the presence of glucose was further stimulated upon the addition of CCCP. The extent of the stimulation by CCCP ranged from 26% to 50% with four different batches of cells.

Energy-transfer inhibitors, if sufficiently specific, will only inhibit coupled electron transport, and the inhibition may be reversed by uncouplers. This would therefore represent another method for ascertaining the specificity of the energy-transfer inhibitors used.

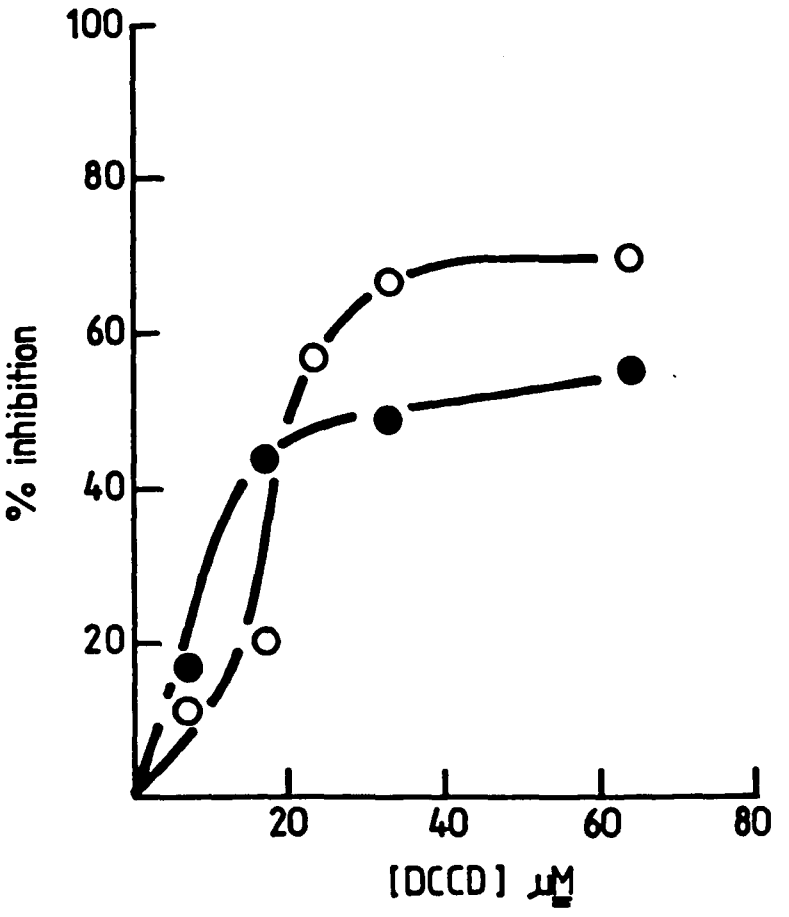
Legend to Fig. 35.

● endogenous respiration.

O glucose-stimulated respiration.

Respiration of anaerobically-grown P. acnes was measured as described in Methods section 8, at a cell concentration of 1·4(mg dry wt) ml^{-1}. The average rates of endogenous and glucose-stimulated respiration in the absence of DCCD were 10·2 and 19·7 nmol O_2 min^{-1} (mg dry wt)$^{-1}$ respectively.

Fig.35: Effect of DCCD on respiration of P. acnes.



Legend to Tables 12 and 13.

The table was compiled from a series of experiments such as that presented in Fig. 35. The figures in parentheses denote the maximum inhibition obtained.

For glucose-stimulated respiration, the values quoted refer to the effect of the various inhibitors on the total respiration rate in the presence of glucose.

Table 12.

Effect of energy-transfer and electron transport inhibitors on respiration of anaerobically-grown P. acnes.

Compound	% inhibition	Concentration (μM)			
		Endogenous		+ glucose	
Nbf-Cl	25	20		20	
	50	40		42	
	maximum	160	(65%)	160	(80%)
DCCD	25	10		18	
	50	30		29	
	maximum	66·7	(55%)	66·7	(70%)
Quercetin	25	60		30	
	maximum	133·3	(39%)	66·7	(38%)
Oligomycin*	maximum	6·67	(17%)	5	(22%)
DPPA	25	$8·3·10^3$		$10·10^3$	(20%)
	maximum	$11·6·10^3$	(30%)	-	
HOQNO	maximum	128	(20%)	128	(17%)
Antimycin A	25	23·3		42	
	maximum	100	(45%)	133	(46%)
Azide	stimulation	not determined		$10·10^3$	(17%)

*μg (mg dry wt)$^{-1}$

Table 13.

Effect of energy-transfer and electron transport inhibitors on respiration of aerobically-grown P. acnes.

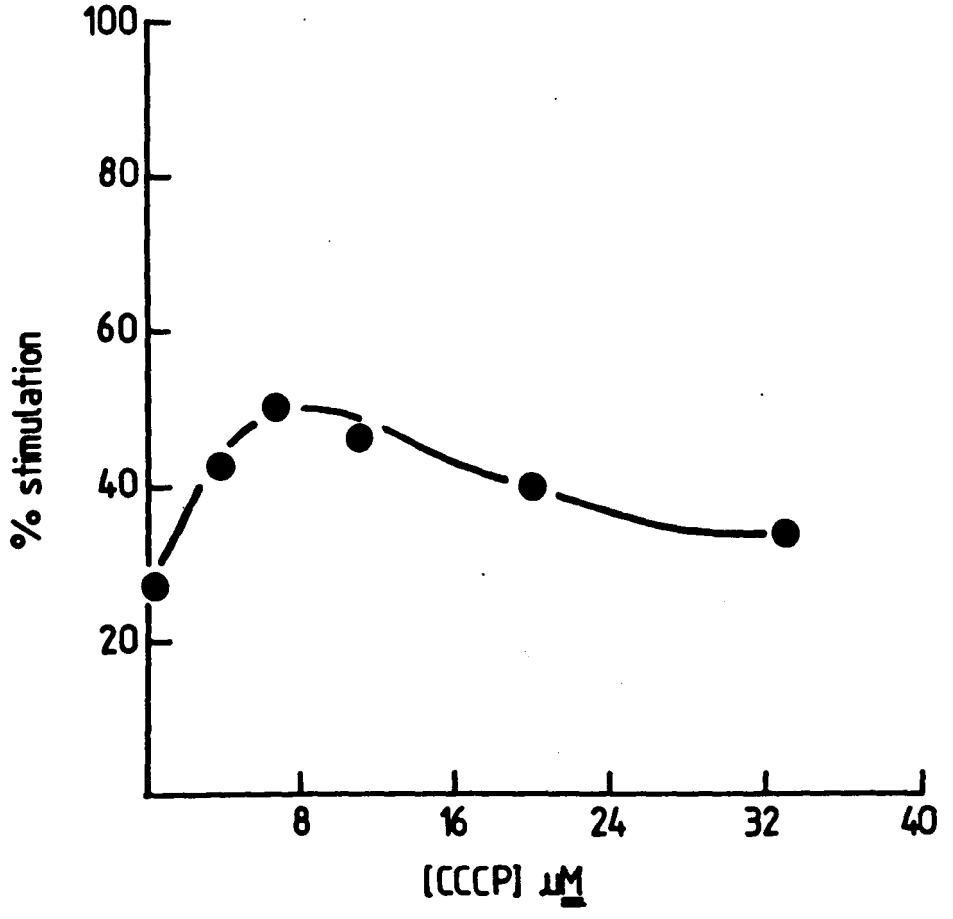
Compound	% inhibition	Conc. (μM)			
		Endogenous		+ Glucose	
Nbf-Cl	25	45		30	
	50	75		56	
	maximum	200	(78%)	130	(88%)
DCCD	25	58		30	
	50	80		48	
	maximum	100	(64%)	90	(80%)
Quercetin	25	80		70	
	maximum	133·3	(34%)	133·3	(40%)
Oligomycin*	maximum	3·5	(17%)	3·5	(10%)
DPPA	25	$10 \cdot 10^3$	(22%)	$6 \cdot 10^3$	
	maximum	-		$10 \cdot 10^3$	(35%)
HOQNO	maximum	96	(22%)	120	(16%)
Antimycin A	25	20	(25%)	20	
	maximum	-		66·7	(48%)
Azide	stimulation	not determined		$10 \cdot 10^3$	(20%)

*$\mu g\ (mg\ dry\ wt)^{-1}$

Legend to Fig. 36.

Respiration in the presence of glucose (final concentration of 10 mM) was measured as described in Methods section 8. The experiment was performed with aerobically-grown cells at a cell concentration of 1 mg ml^{-1}. The rate of glucose-stimulated respiration in the absence of CCCP was 18 nmol O_2 (mg dry wt)$^{-1}$ min^{-1}.

Fig.36: Effect of CCCP on respiration of P.acnes.



The experimental system used is described in Fig.37, where the uncoupled endogenous respiration was first stimulated by the addition of glucose prior to the introduction of the inhibitor Nbf-Cl. An analogue of glucose, methyl α-glucoside, at a concentration of 10 mM, was not found to stimulate the uncoupled endogenous respiration. The results obtained from such experiments are presented in Table 14, where it may be seen that all the energy-transfer inhibitors used, with the exception of oligomycin, caused partial inhibition of uncoupled respiration. Nbf-Cl and DCCD, at 166·7 μM brought about almost complete inhibition of uncoupled respiration in both anaerobically- and aerobically-grown P. acnes. Both quercetin and DPPA caused partial inhibition of uncoupled respiration, hence their specificities as energy-transfer inhibitors would be questionable.

Both HOQNO and antimycin A only partially inhibit uncoupled respiration, though this would be expected for electron transfer inhibitors. Azide did not have any effect, which would support the earlier suggestion that it might well be uncoupling oxidative phosphorylation at the concentrations used.

Legend to Fig. 37.

a) Anaerobically-grown cells at a cell concentration of 1(mg dry wt) ml^{-1}.

b) CCCP, at a final concentration of 8·3 μM.

c) Glucose, at a final concentration of 10 mM.

d) Nbf-Cl, 33 μM.

e) Nbf-Cl, 66 μM.

f) Nbf-Cl, 166·7 μM.

Respiration was measured as described in Methods section 8. The above additions were made at the positions indicated by the arrows.

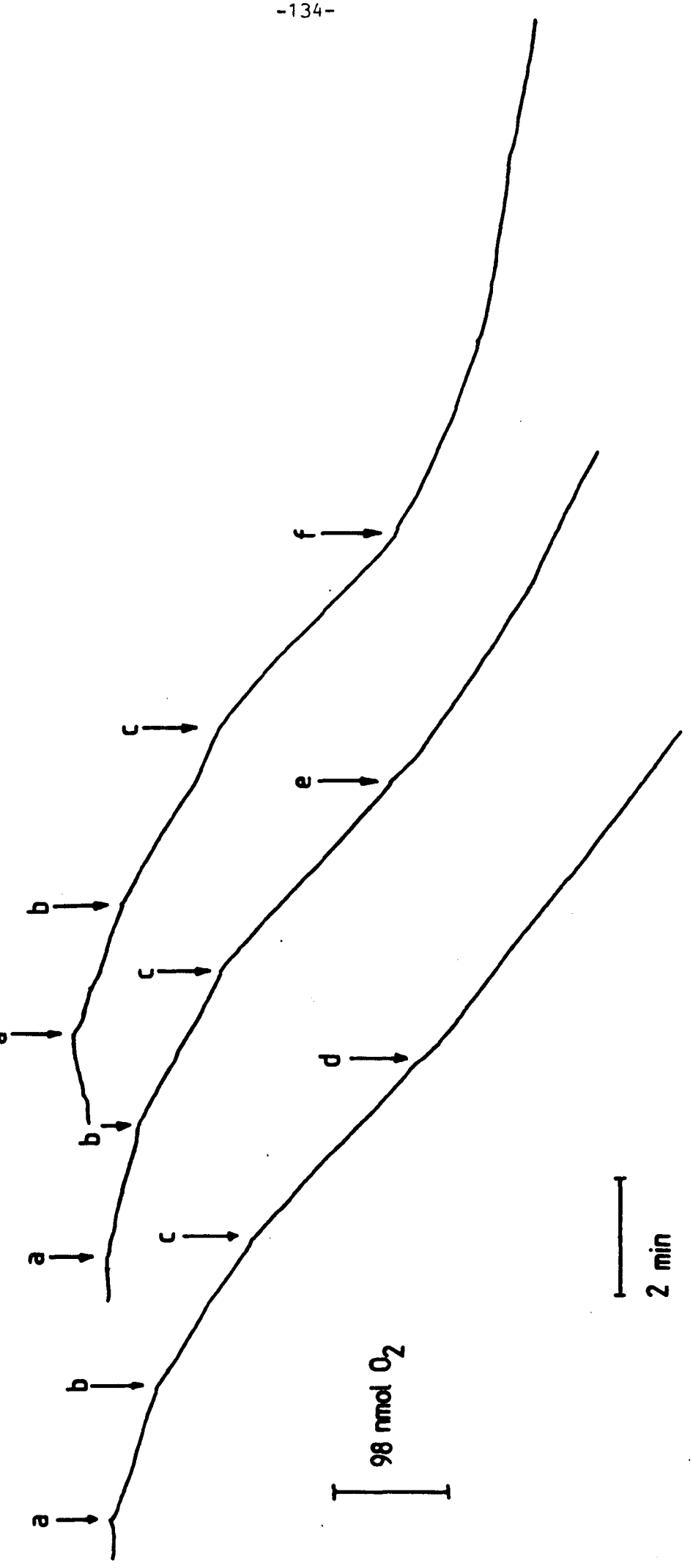


Fig. 37: Effect of Nbf-Cl on uncoupled respiration of P.acnes.

Table 14.

Effect of energy-transfer and electron transport inhibitors on uncoupled respiration of P. acnes.

Compound	Concentration (μM)	% inhibition	
		Anaerobically-grown P. acnes	Aerobically-grown P. acnes
Nbf-Cl	33·3	13·6	19·2
	66·7	31	43·3
	166·7	50	77
DCCD	33·3	86·5	9·6
	66·7	87	19·2
	166·7	92	84·0
Quercetin	33·3	26·7	15·4
	66·7	32·7	25
	100	45	30·4
DPPA	$3·3 \cdot 10^3$	9·7	9·1
	$6·6 \cdot 10^3$	15·3	18·2
	$10 \cdot 10^3$	34	22·2
Oligomycin*	1	0	0
	5	0	0
	10	0	0
HOQNO	32·1	16·1	11·5
	64·3	39·1	19
	96·4	40·6	19·3
Antimycin A	33·3	40	18
	66·7	36	27·5
	100	35·1	28
Azide	$1·7 \cdot 10^3$	0	0
	$5 \cdot 10^3$	0	0
	$10 \cdot 10^3$	0	0

*μg (mg dry wt)$^{-1}$

4. Studies on respiration-driven proton translocation in P. acnes.

In the previous sections, it was shown that both anaerobically- and aerobically-grown P. acnes responded in a similar manner to oxygen. $\Delta\Psi$ formation was stimulated by exposure to oxygen in both anaerobically- and aerobically-grown P. acnes. In addition, similar rates of endogenous and glucose-stimulated respiration were noted for the two growth conditions. The fact that CCCP stimulated respiration suggested that classical oxidative phosphorylation was occurring rather than oxygen merely serving as an electron sink. A direct way of confirming the occurrence of oxidative phosphorylation was to look for respiration-driven translocation. The respiration-driven acidification of the medium (ΔpH_o) on the addition of known quantities of oxygen-saturated KCl solution to anaerobic suspension of P. acnes was compared with the pH displacement resulting from the addition of known amounts of acid. This permitted the number of H^+ translocated per oxygen atom reduced ($\rightarrow H^+/O$ ratio) to be calculated, and also gave some measure of the number of coupling sites in operation.

In measurements of respiration-driven H^+ translocation in organisms such as E. coli (Lawford and Haddock, 1973) and P. denitrificans (Scholes and Mitchell, 1970) pulses of oxygen were introduced into the cell suspension after a suitable period of incubation during which anaerobiosis had been attained and the acid-drift low, which is important for accurate determination of $\rightarrow H^+/O$ values. As described in Methods section 10, respiration-driven H^+ translocation in P. acnes was measured in an oxygen electrode and as such, attainment of anaerobiosis was directly ascertained. Acid-drift was however a major problem encountered with P. acnes; cell suspensions which had been incubated for up to 150 min still showed significant rates of acid production. A typical rate of acid production obtained after 150 min incubation was 1·5 ng ions $(mg\ dry\ wt)^{-1}\ min^{-1}$. All $\rightarrow H^+/O$ values calculated were

corrected for the acid-drift by projecting the rate of acid production, assuming that this rate of acid production was unchanged during the H^+ release and subsequent decay of the acid-pulse. A typical example of a respiration-driven H^+ translocation profile obtained with P. acnes is shown in Fig. 38(a). A semi-logarithmic plot of the decay of the ΔpH_o after an oxygen pulse is shown in Fig. 38(b), which also illustrated that the $\rightarrow H^+/O$ ratio obtained without correcting for the acid-drift was slightly lower than the drift-corrected value ie. a value of 2·5 as compared to 3·0 was obtained.

The extent of the H^+ movement was limited by the electrical capacity of the membrane and the degree to which charge compensation occurred. To determine the optimal compensation required, varying concentrations of the permeant ion, thiocyanate, were added to the medium. The decay of the acid-pulse in the presence of thiocyanate is shown in Fig. 39. It was found that the decay of this pulse was dependent on the concentration of the thiocyanate present. In the absence of KSCN, the introduction of an oxygen pulse did cause H^+ movements but no decay was however observed. As shown in Fig. 39, even at 100 mM-KSCN, there was no decay of the acid-pulse. The $\rightarrow H^+/O$ ratio obtained showed a maximum at 200 mM-KSCN, where a value of 3·0 was obtained as shown in Fig. 40.

The extent of the H^+ movement was also dependent on the concentration of KCl as shown in Fig. 41. The decay of the H^+ translocated showed that a minimal concentration of 200 mM-KCl was required, suggesting that K^+ may have been acting as a counter-ion. The $\rightarrow H^+/O$ value obtained was approximately 2·0 at a concentration range of 200 mM to 400 mM-KCl as shown in Fig. 42.

The requirements for thiocyanate would indicate that the H^+ translocation that occurred upon pulsing anaerobic suspensions of P. acnes with oxygen was an electrogenic process. Pulsing with 150 mM-KCl

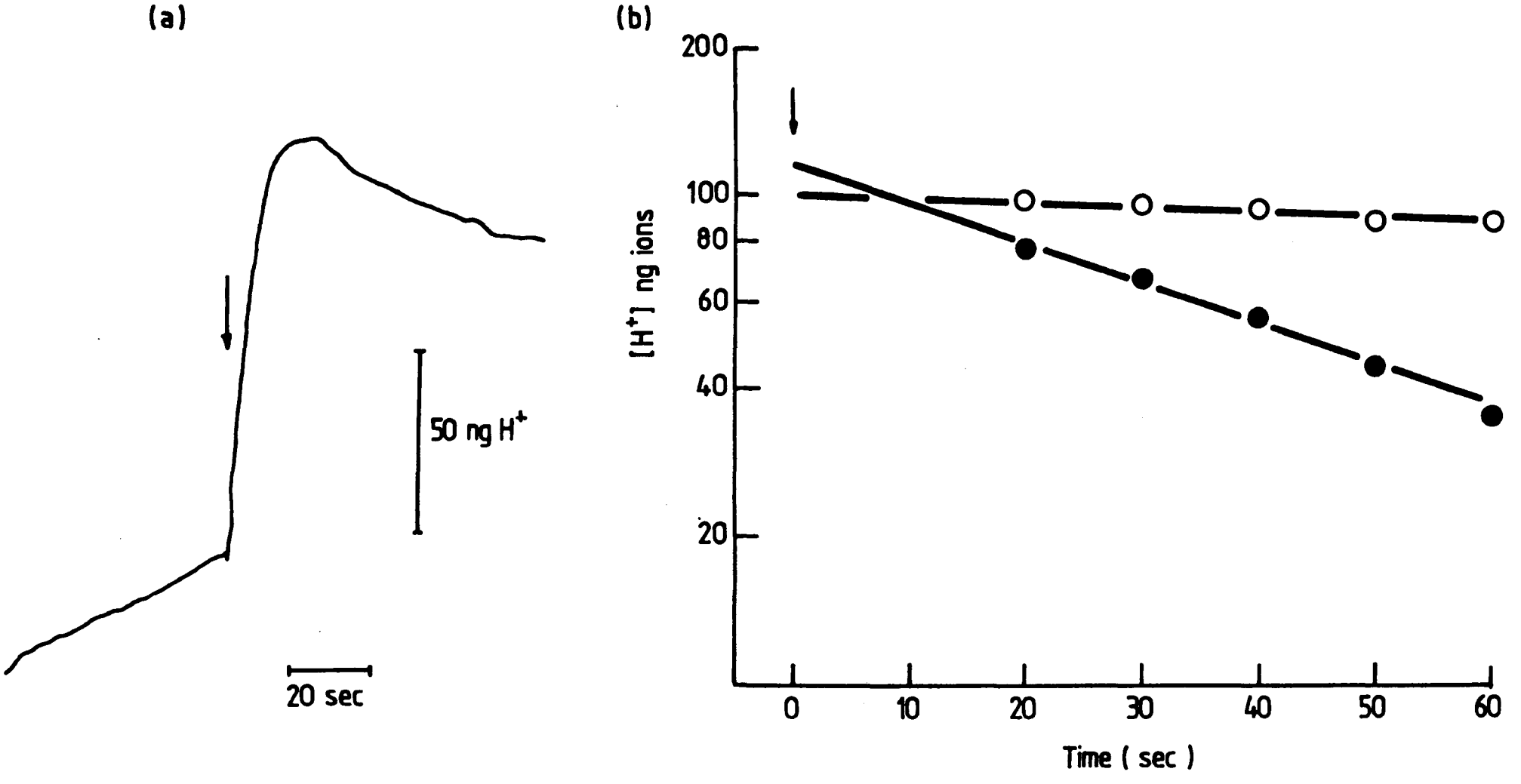
Legend to Fig. 38.

Respiration-driven H^+ translocation by anaerobically-grown P. acnes was measured as described in Methods section 10, in 225 mM-KSCN and 200 mM-KCl. Oxygen (40 ng atoms) was added as air-saturated 150 mM-KCl at the time indicated by the arrow.

○ uncorrected for acid-drift.

● corrected for acid-drift.

Fig.38: a) Time course of respiration-driven H^+ translocation by P. acnes.
b) Semilogarithmic plot of the decay of ΔpH_o.



Legend to Fig. 39.

Respiration-driven H^+ translocation by anaerobically-grown P. acnes was measured in a medium containing 300 mM-KCl and the following concentrations of KSCN.

□ No KSCN

■ 100 mM-KSCN

○ 200 mM-KSCN

● 300 mM-KSCN

△ 400 mM-KSCN

▲ 500 mM-KSCN

Oxygen (40 ng atoms) was added as air-saturated 150 mM-KCl at the time indicated by the arrow (↓).

Fig.39: Effect of SCN^- on respiration-driven H^+ translocation by P. acnes.

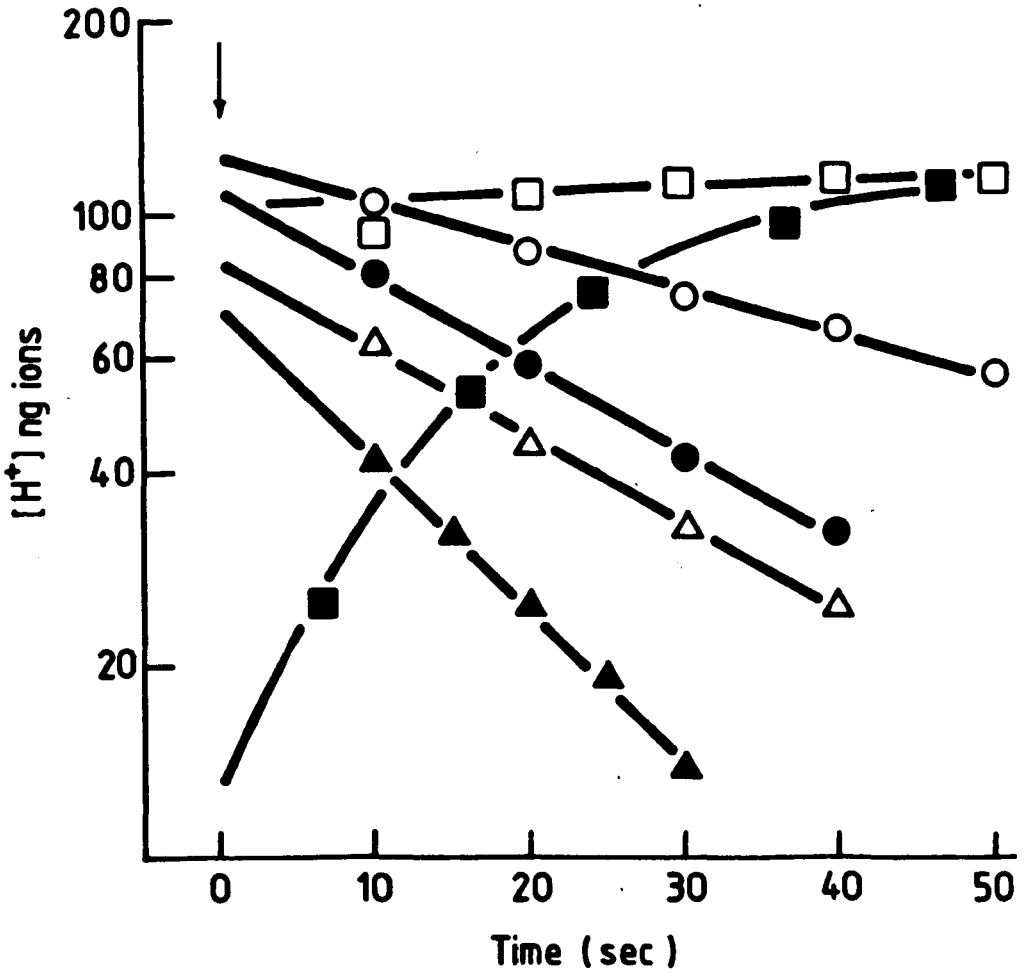
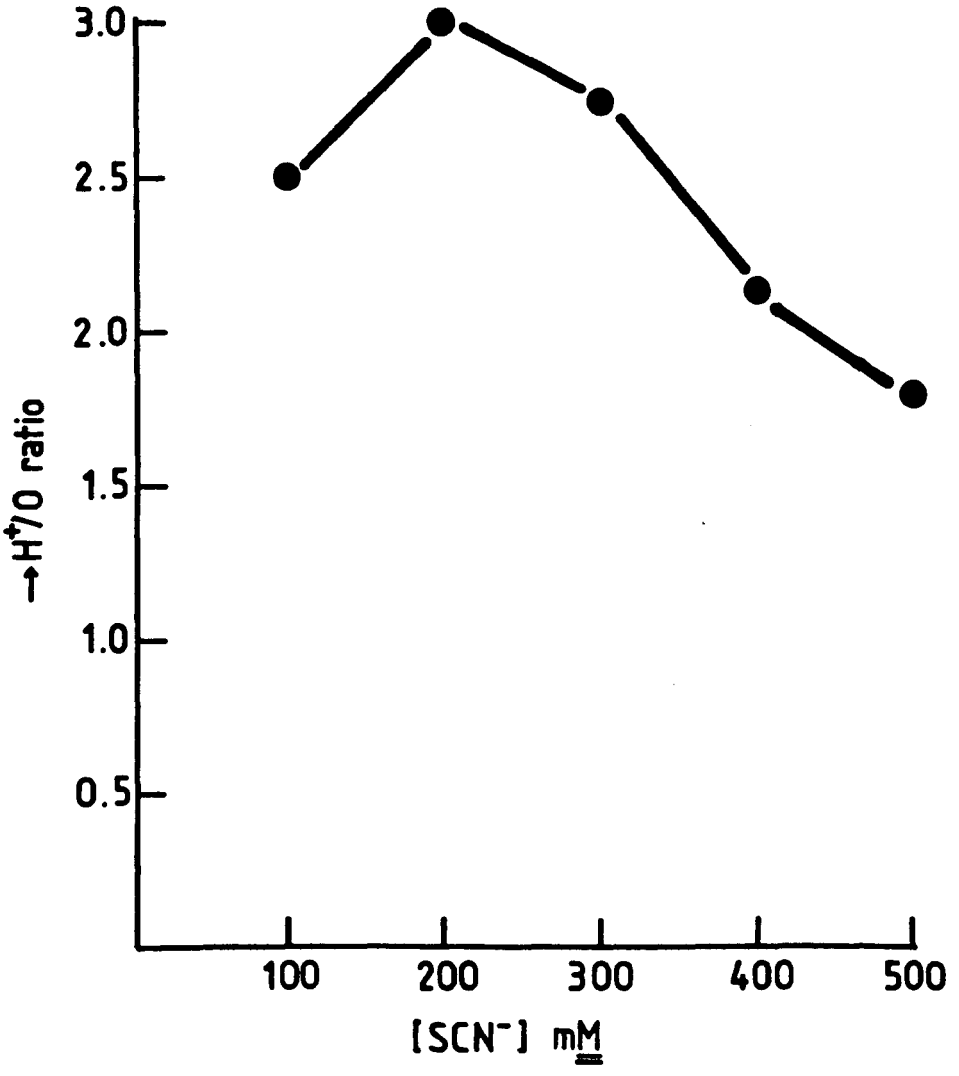


Fig.40: Variation of $\rightarrow H^+/O$ ratio with SCN^- concentration.



Legend to Fig. 41.

Respiration-driven H^+ translocation by anaerobically-grown P. acnes was measured as described in Methods section 10, in a medium containing 225 mM-KSCN and the following concentrations of KCl:

▲ 100 mM-KCl

● 200 mM-KCl

○ 300 mM-KCl

△ 400 mM-KCl

Fig.41: Effect of KCl on respiration-driven H^+ translocation by P. acnes.

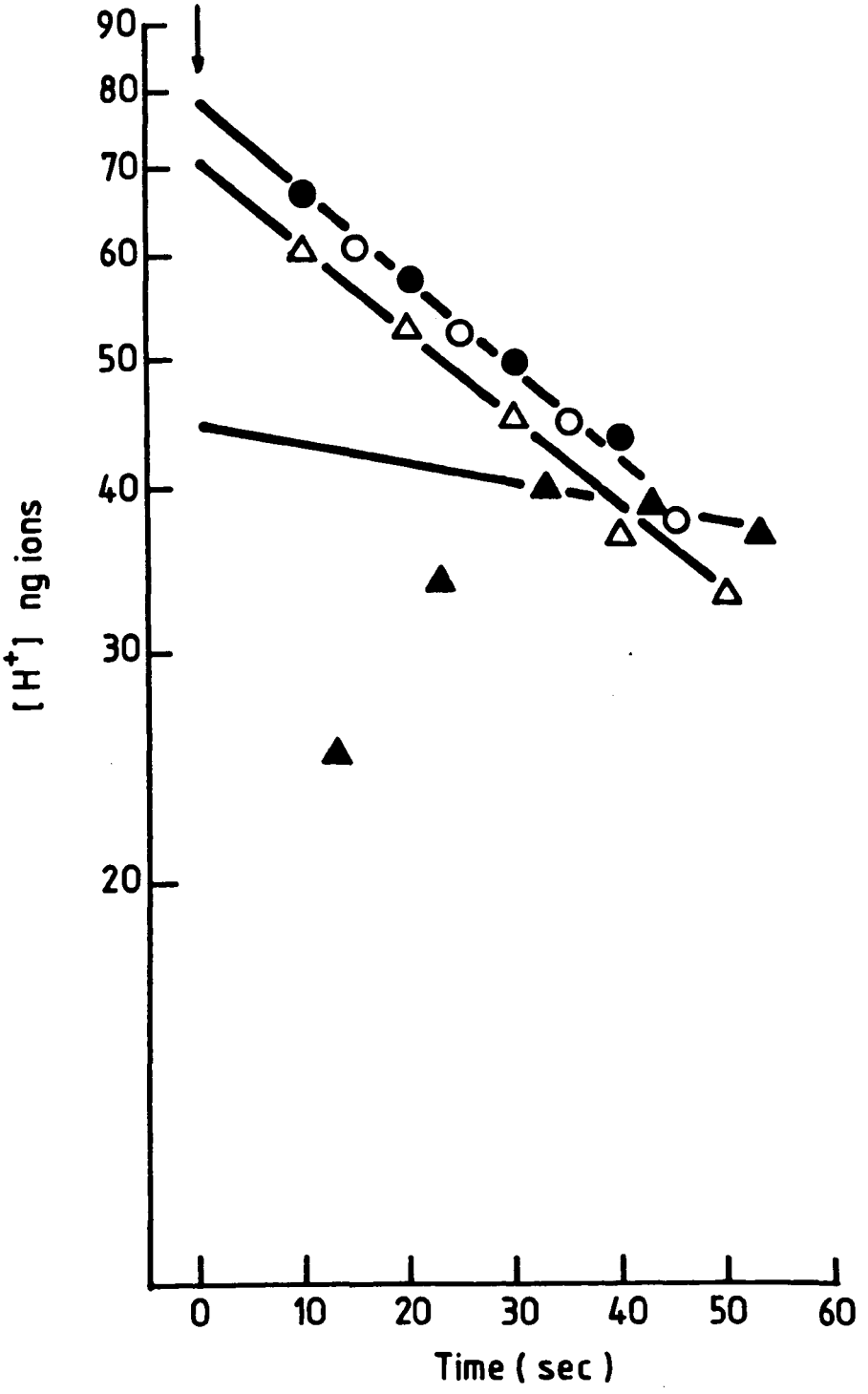
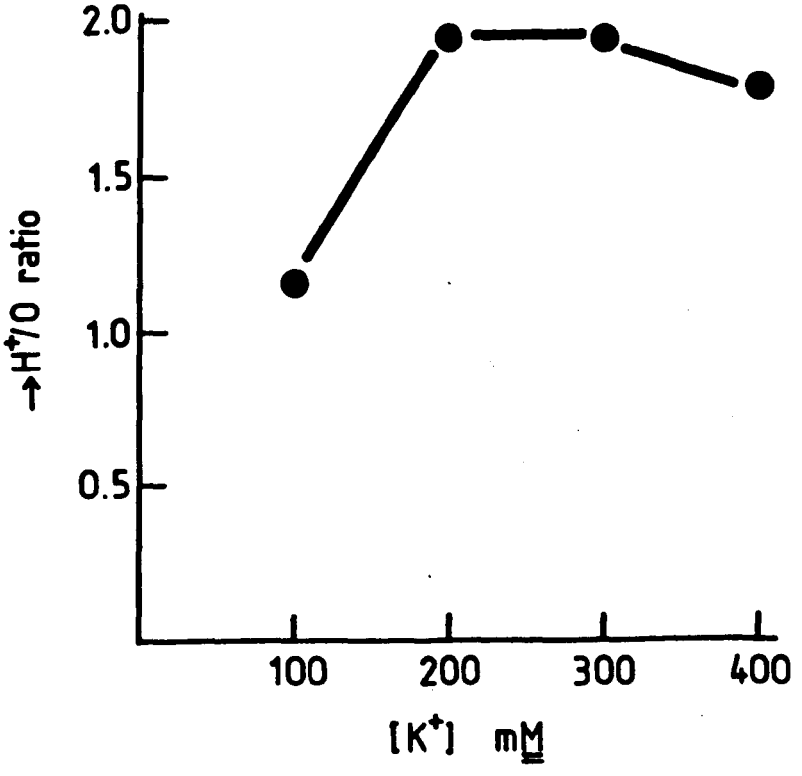


Fig.42: Variation of $\rightarrow H^+/O$ ratio with KCl concentration.



made anaerobic by flushing with O_2-free N_2 did not produce H^+ movements. That the movement is an electrogenic process was further verified by the action of the uncoupler CCCP. As shown in Fig. 43, the addition of CCCP caused rapid decay of ΔpH_o; an observation consistent with the postulated role of uncouplers in collapsing the electrical barrier of bioenergetic membranes. A plot of the half-time of the decay of the H^+ movement ($t_{\frac{1}{2}}$) against the concentration of CCCP showed that at approximately 10 μM-CCCP, the $t_{\frac{1}{2}}$ was less than 5 s as compared with 40 s in the absence of CCCP (Fig. 44).

Both anaerobically and aerobically-grown P. acnes were found to show similar $\rightarrow H^+/O$ values. The $\rightarrow H^+/O$ ratios obtained were 2·72 ± 0·39 (15 measurements) and 2·58 ± 0·38 (11 measurements) for anaerobically and aerobically-grown P. acnes respectively. The possibility that these values were underestimated due to the oxygen pulses being at a saturating concentration was investigated. Routinely, oxygen was introduced as 100 μl of 150 mM-KCl at 37°C, which corresponded to 40 ng atoms of O_2 (Chappell, 1964). Fig.45 shows that the concentration of H^+ translocated was directly proportional to the amount of oxygen added.

A control carried out with A. calcoaceticus, a strict aerobe, revealed that the system employed was capable of measuring $\rightarrow H^+/O$ ratios greater than those values obtained for P. acnes. Measurements of $\rightarrow H^+/O$ ratio for A. calcoaceticus were not complicated by prolonged acid-drifts as encountered with P. acnes. After only 30 min incubation, the acid-drift was almost negligible. The time-course of respiration-driven H^+ translocation in A. calcoaceticus is shown in Fig. 46(a). The value of $\rightarrow H^+/O$ ratio calculated by extrapolating the decay of the ΔpH_o gave a value of 5·2 as shown in Fig. 46(b).

The effects of electron transport inhibitors, namely HOQNO, antimycin A, NaN_3 and KCN on respiration-driven H^+ translocation by

Legend to Fig. 43.

Respiration-driven H^+ translocation by anaerobically-grown P. acnes was measured as described in Methods section 10, in a medium containing 225 mM-KSCN and 200 mM-KCl. Oxygen (40 ng atoms) was added as air-saturated 150 mM-KCl at the time shown by the arrow.

○ control

● + 3·21 μM-CCCP

△ + 6·42 μM-CCCP

▲ + 9·63 μM-CCCP

Fig.43: Effect of CCCP on respiration-driven H^+ translocation by P. acnes.

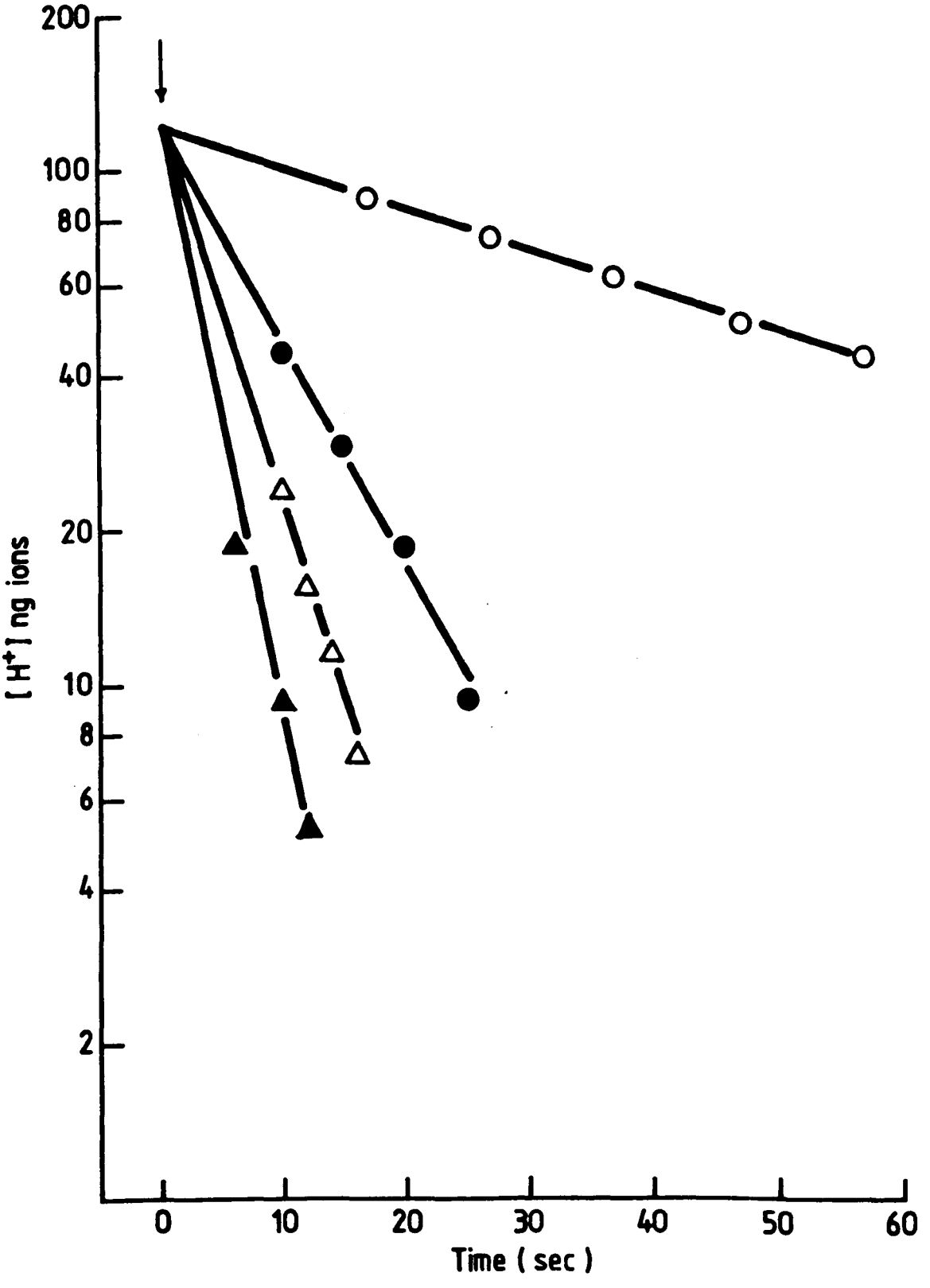
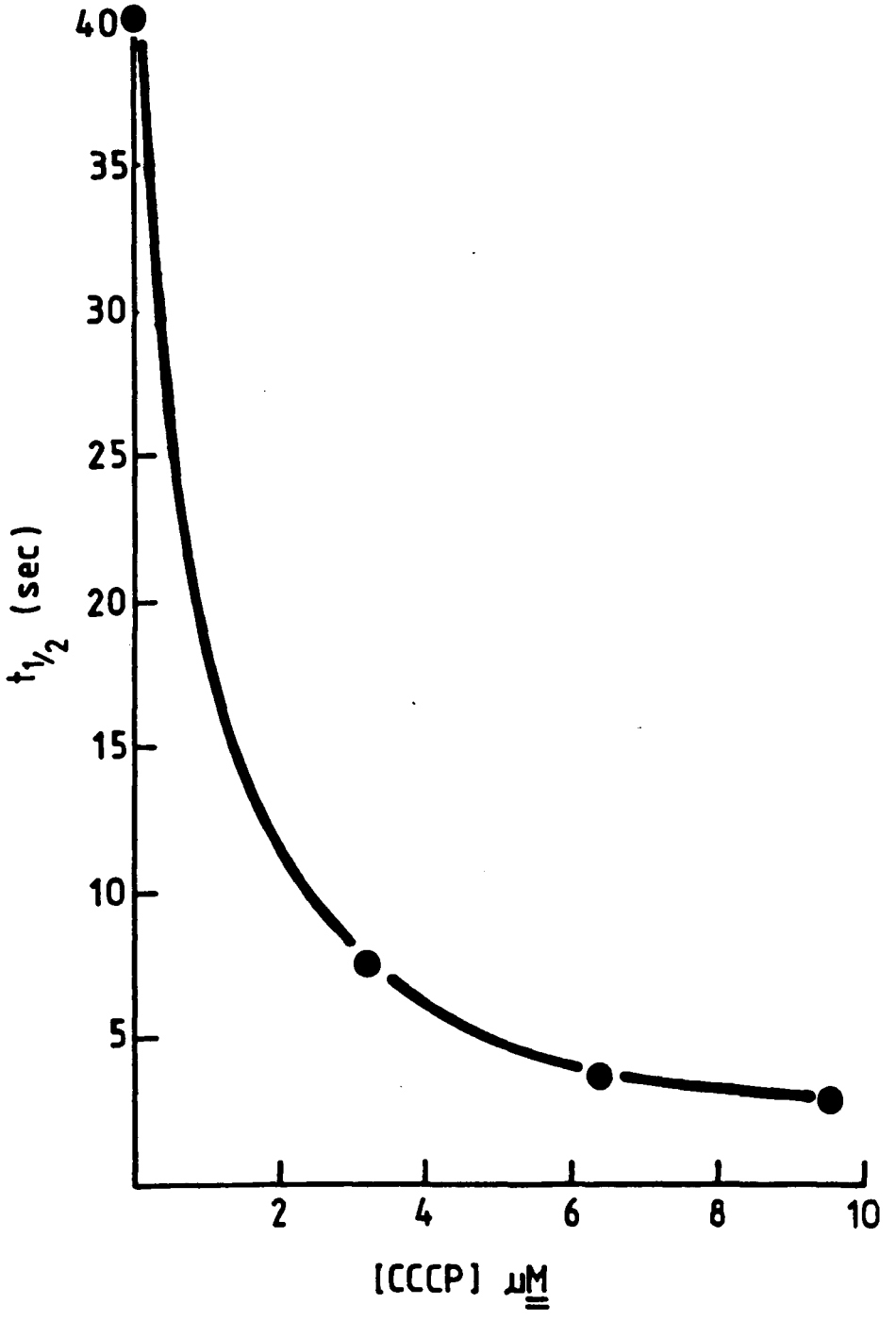


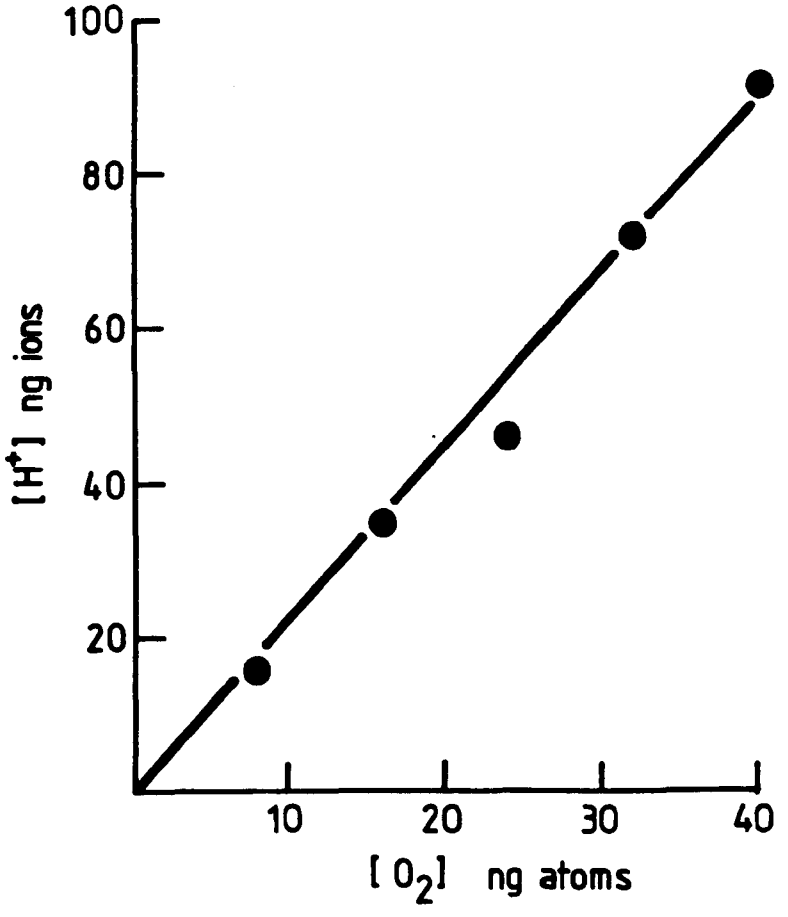
Fig.44: Effect of CCCP on the half-time of decay of respiration-driven proton translocation by P.acnes.



Legend to Fig. 45.

Respiration-driven H^+ translocation by aerobically-grown P. acnes was measured as described in Methods section 10, in a medium containing 225 mM-KSCN and 200 mM-KCl. Variations in oxygen pulses were obtained by adding 20 to 100 µl of 150 mM-KCl at 37°C.

Fig.45: Proportionality of H^+ extruded to oxygen during respiration-driven H^+ translocation by P. acnes.

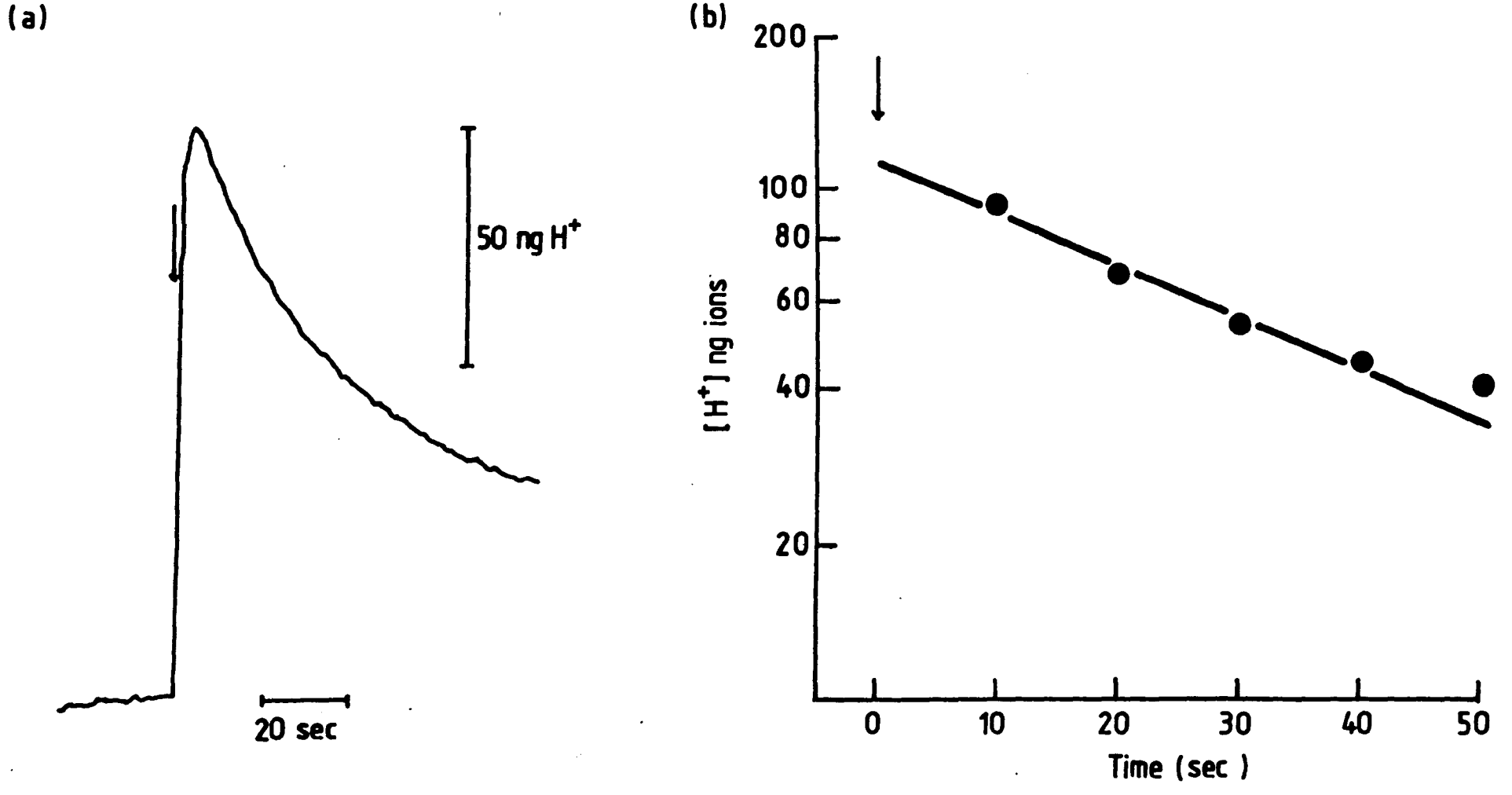


Legend to Fig. 46.

Respiration-driven H^+ translocation was measured as described in Methods section 10, in 150 mM-KSCN and 150 mM-KCl at 30°C. Oxygen (22 ng) was added as air-saturated 150 mM-KCl (at 30°C) at the time indicated by the arrow.

Fig.46: a) Time course of respiration-driven H^+ translocation by A.calcoaceticus.

b) Semilogarithmic plot of the decay of ΔpH_o.



P. acnes was studied. It was found that HOQNO affected respiration-driven H^+ translocation in very much the same way as it did $\Delta\Psi$ and respiration in both anaerobically and aerobically-grown P. acnes. Fig. 47 shows the typical effect of HOQNO on respiration-driven H^+ translocation in anaerobically and aerobically-grown P. acnes. With four different batches of cells, the range of values encountered were 30% to 50% and 30% to 45% for anaerobically- and aerobically-grown cells. Antimycin A too only partially inhibited respiration-driven H^+ translocation in both anaerobically- and aerobically-grown P. acnes (Fig. 48), where a maximal inhibition of approximately 30% was observed. This was reproducible with two different batches of cells for both anaerobically- and aerobically-grown cells. NaN_3, in contrast, significantly inhibited respiration-driven H^+ translocation as shown in Fig. 49. KCN, at concentrations of up to 10 mM did not affect respiration-driven H^+ translocation in either anaerobically or aerobically-grown P. acnes.

Based on data obtained from respiration studies, it was suggested that energy-transfer inhibitors such as DCCD inhibited oxidative phosphorylation by interacting with sites other than the ATPase only. DCCD, at concentrations that inhibited the generation of the $\Delta\Psi$ under aerobic conditions was also found to inhibit respiration-driven H^+ translocation. Oligomycin, however, did not affect respiration-driven H^+ translocation, which was to be expected if it were to interact solely with the ATPase.

The earlier observation that the $\Delta\Psi$ maintained under anaerobic conditions was partially sensitive to HOQNO suggested the possible involvement of an anaerobic electron transport system, with fumarate possibly acting as the terminal electron acceptor. Respiration-driven proton translocation with fumarate as the terminal electron acceptor was therefore investigated. However, attempts to demonstrate such an effect with fumarate were negative.

Legends to Figs. 47, 48 and 49.

○ aerobically-grown P. acnes

● anaerobically-grown P. acnes.

Respiration-driven H^+ translocation was measured as described in Methods section 10, in a medium containing 225 mM-KSCN and 200 mM-KCl.

The values of the $\rightarrow H^+/O$ ratio in the absence of inhibitors were:

Fig. 47: anaerobically-grown cells: 3·08
aerobically-grown cells: 2·5

Fig. 48: anaerobically-grown cells: 3·0
aerobically-grown cells: 2.5

Fig. 49: anaerobically-grown cells: 2·85
aerobically-grown cells: 2·5

Fig.47: Effect of HOQNO on respiration-driven H^+ translocation by P. acnes.

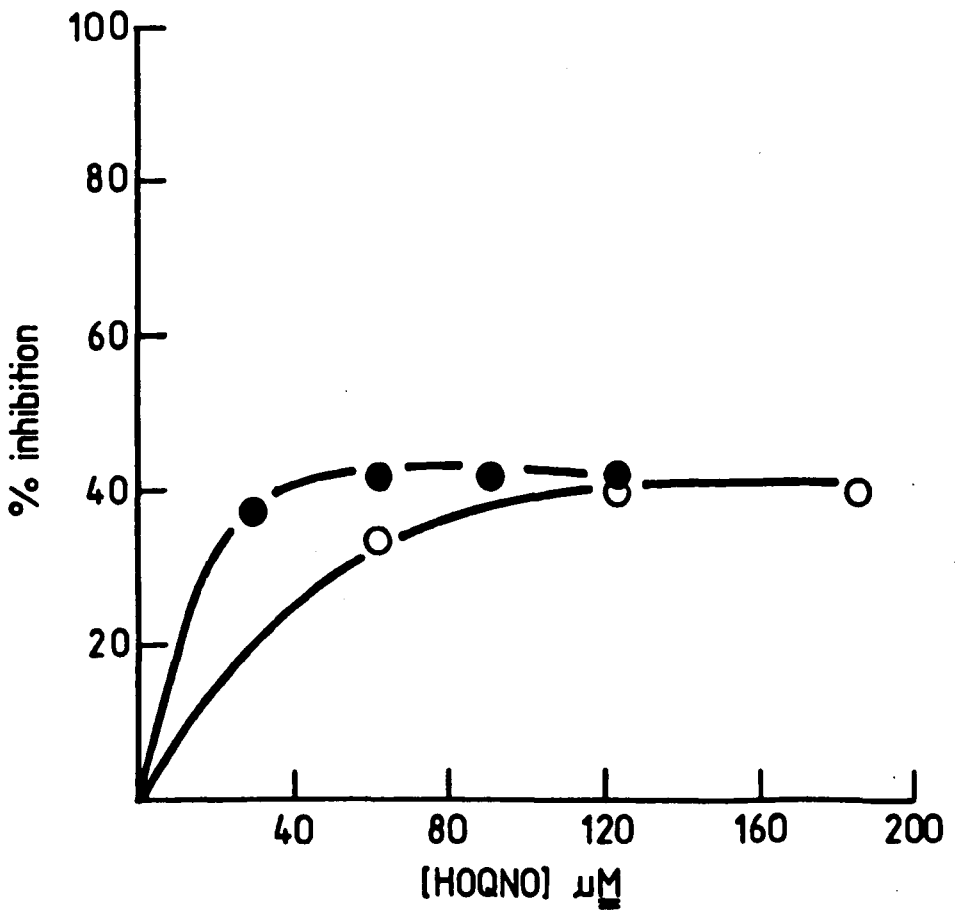


Fig.48: Effect of antimycin A on respiration-driven H^+ translocation by P.acnes.

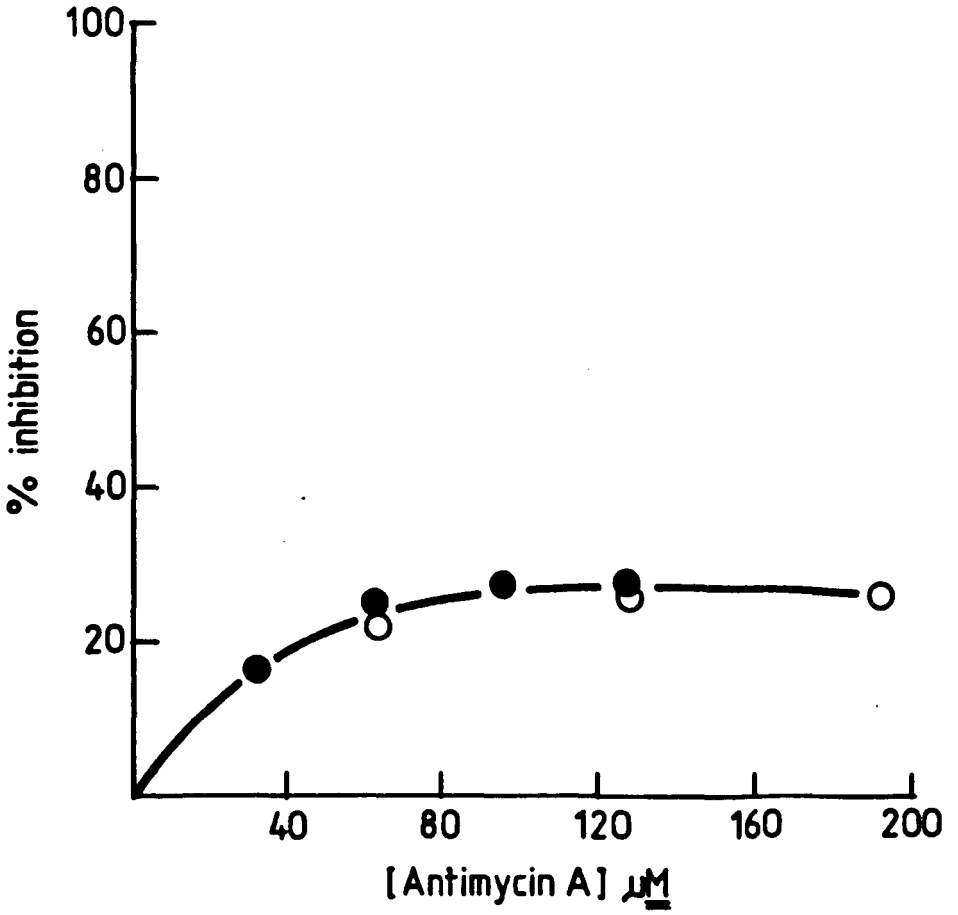
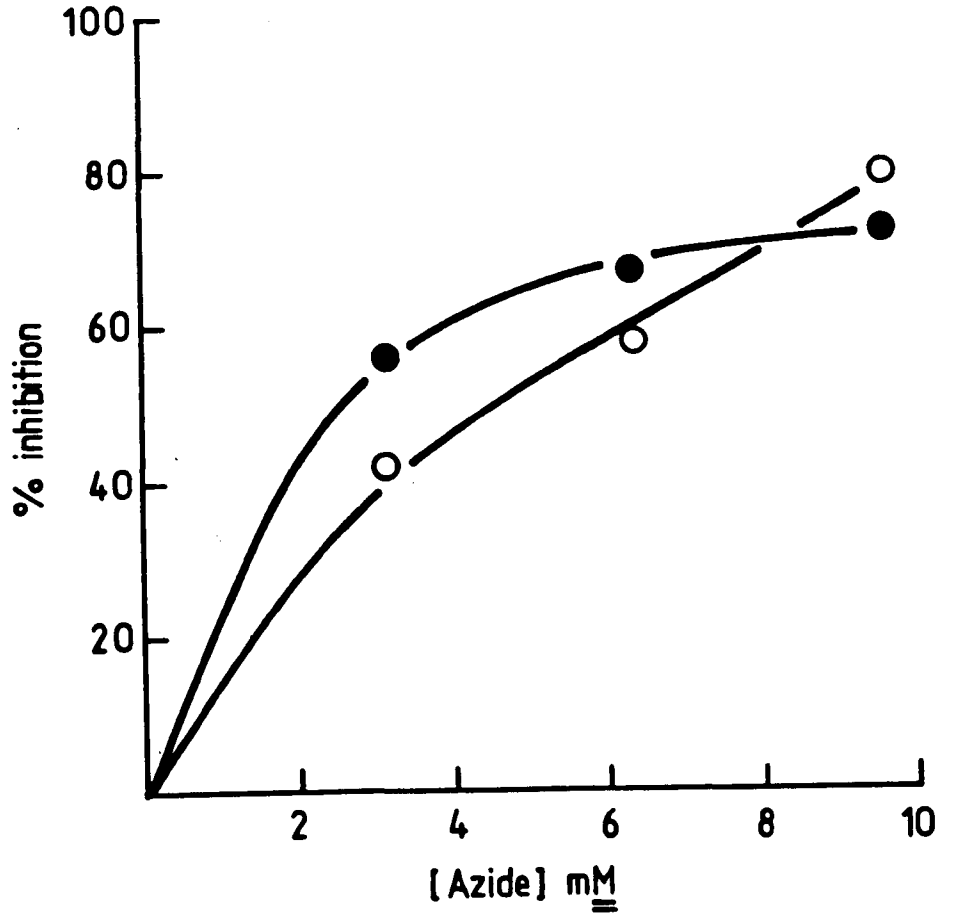


Fig.49: Effect of azide on respiration-driven proton translocation by P.acnes.



Studies on the cytochromes of P. acnes.

The investigations described earlier showed that both anaerobically- and aerobically-grown P. acnes were capable of generating a $\Delta\tilde{\mu}_{H^+}$ by the operation of electron transport with oxygen as the terminal electron acceptor. Studies on the cytochromes of both anaerobically- and aerobically-grown P. acnes were performed to gain information on the components of the electron transport chain, in particular to determine whether similar terminal oxidases were present in P. acnes grown under different physiological conditions.

Typical reduced-minus-oxidised difference cytochrome spectra of anaerobically- and aerobically-grown P. acnes are shown in Fig. 50. It can be seen that both spectra were dominated by the α-peak of cytochrome b which occurred at 560 nm. Three other peaks, occurring at 630 nm, 600 nm and 530 nm were identified at the α-peaks of cytochromes d and a_1 and the β-peak of cytochrome b respectively. Thus both anaerobically- and aerobically-grown cells contain cytochromes a_1 and d which could possibly act as terminal oxidases though in bacterial systems such as E. coli and A. vinelandii, the ability of cytochrome a_1 to function as a terminal oxidase is in doubt (Haddock et al., 1976; Kaufman and van Gelder, 1973). In addition to cytochromes a_1 and d, both anaerobically- and aerobically-grown cells were found to contain cytochrome o, identified by the trough and peak occurring at 430 nm and 417 nm respectively in the reduced +CO minus reduced difference spectrum as shown in Fig. 51. It would thus seem that anaerobically- and aerobically-grown P. acnes contain similar cytochrome components. It was however recognised that there could be different cytochrome components present in both anaerobically- and aerobically-grown P. acnes with very similar absorption maxima which were not resolved by the method used. With the exception of cytochrome o, the relative

Legend to Fig. 50.

——— aerobically-grown *P. acnes*

------- anaerobically-grown *P. acnes*

Dithionite-reduced minus ferricyanide-oxidised difference spectra were obtained as described in Methods section 9. The cell concentration for both anaerobically- and aerobically-grown cells was 7 (mg dry wt) ml^{-1}.

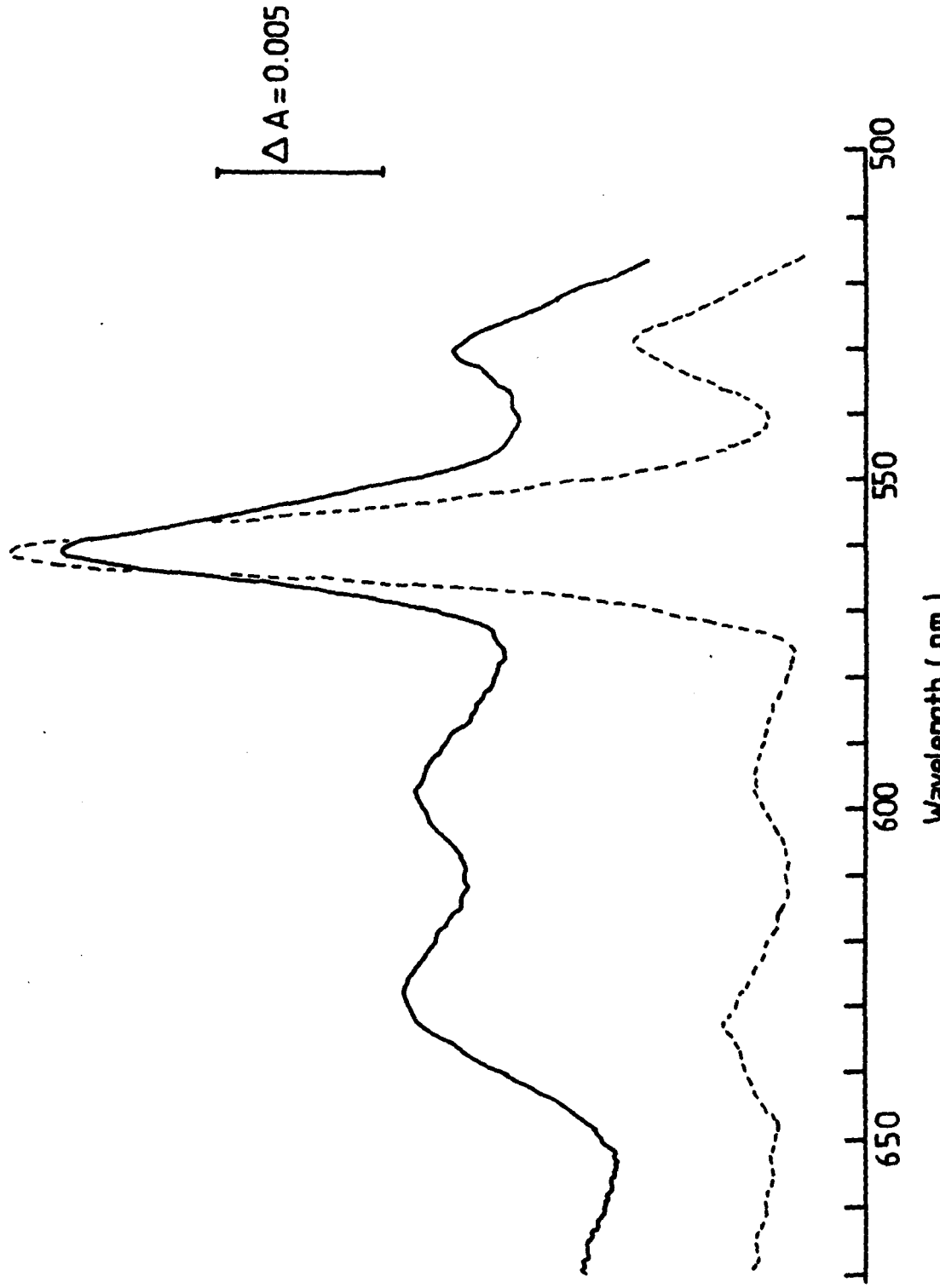


Fig.50: Reduced minus oxidized difference spectra of P.acnes.

Legend to Fig. 51.

——— anaerobically-grown P. acnes

- - - - - aerobically-grown P. acnes

Reduced +CO minus reduced difference spectra was obtained as described in Methods section 9. The cell concentrations for anaerobically- and aerobically-grown cells were 5·4 (mg dry wt) $^{-1}$ and 6·9 (mg dry wt) ml^{-1} respectively.

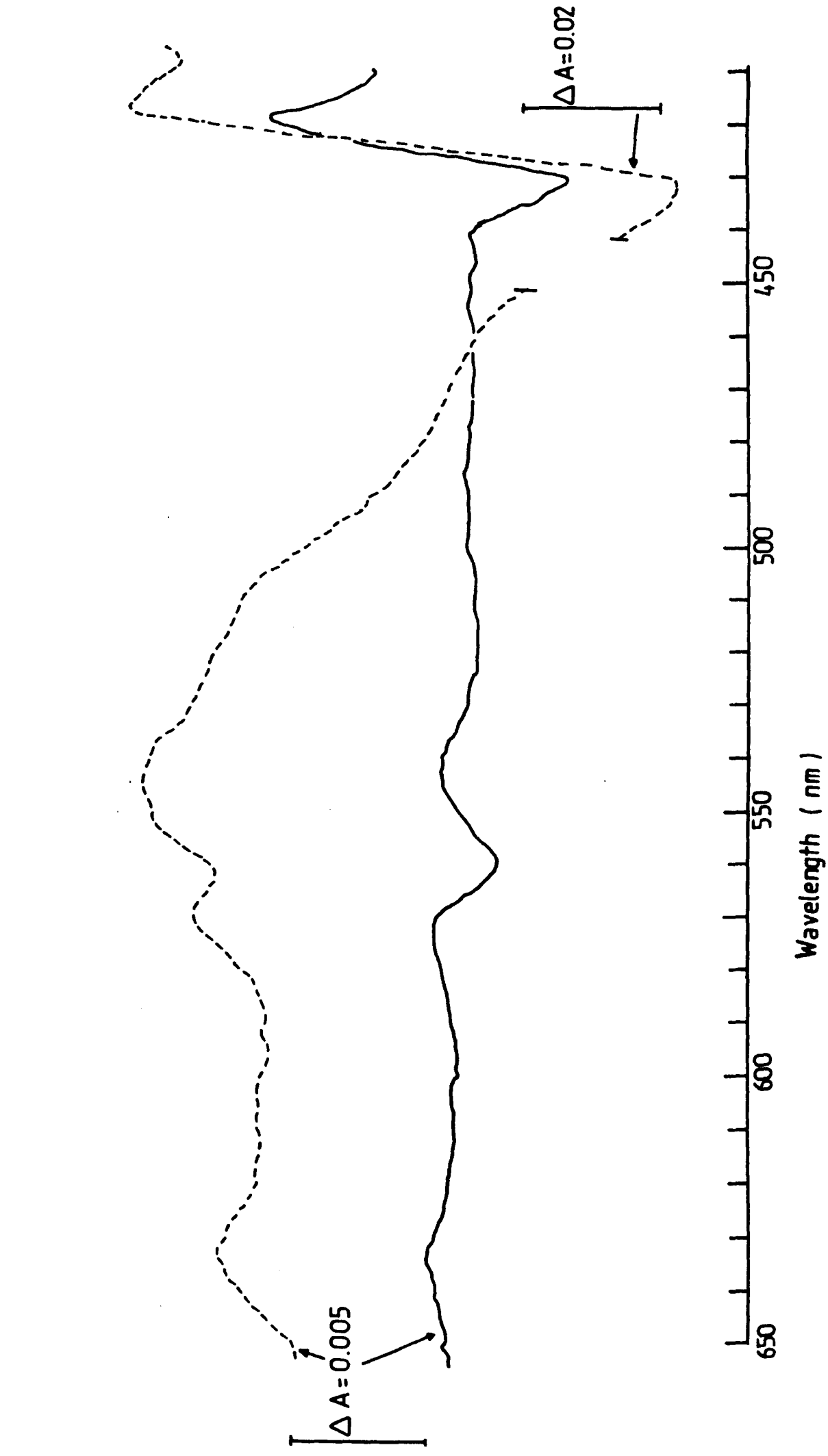


Fig. 51: Reduced+CO minus reduced difference spectra of P. acnes.

concentrations of the various cytochromes were very similar in anaerobically- and aerobically-grown P. acnes (Table 15). Aerobically-grown cells were found to have a higher cytochrome o content than anaerobically-grown cells. The values presented in Table 15 represent the average relative concentrations of the various cytochromes as variations were frequently observed with different batches of cells. This was however to be expected with batch-grown cells as the cytochrome content of micro-organisms is dependent on their stage of growth.

Table 15.

Concentrations of the cytochromes found in anaerobically- and aerobically-grown P. acnes.

Cytochrome	Concentration*	
	Anaerobically-grown cells	Aerobically-grown cells
b	0·76	0·6
a_1	0·06	0·07
d	0·13	0·13
o	0·6	1·8

Legend to Table 15.

*Concentration was expressed as Δ absorbance $(g\ cells)^{-1}$ using the following wavelength pairs.

Cytochrome b : 560 - 575

Cyrochrome a_1 : 600 - 615

Cytochrome d : 630 - 650

Cytochrome o : 416 - 430

DISCUSSION

DISCUSSION.

This investigation was initiated primarily to study the role of oxygen in the energy-transducing processes of the facultative cutaneous propionibacterium, P. acnes. It has been shown that exposure of P. acnes to oxygen resulted in an increase in the growth yield, indicating that oxygen was in some way involved in the energy-yielding processes of this organism (Holland et al., 1978).

The problem was approached using Mitchell's chemiosmotic hypothesis as the central dogma ie. the $\Delta\tilde{\mu}_{H^+}$, which may be generated by either electron transport or ATP hydrolysis via a H^+-translocating ATPase, was postulated to play a fundamental role in energy transduction in all bioenergetic systems so far studied. This investigation has attempted to distinguish between the two major possibilities by which oxygen may interact with the organism to generate a $\Delta\tilde{\mu}_{H^+}$. Firstly, oxygen may interact directly as a terminal electron acceptor for a H^+-translocating electron transport chain which thus operates to generate a $\Delta\tilde{\mu}_{H^+}$ which may be used for either ATP synthesis (ie. oxidative phosphorylation) or to drive transport processes, or indeed, both. In E. coli for example, the $\Delta\tilde{\mu}_{H^+}$ generated by electron transport with oxygen as the terminal electron acceptor (Lawford and Haddock, 1973) may be used to drive ATP synthesis (Tsuchiya and Rosen, 1976) or transport processes (Hirata et al., 1973).

Secondly, oxygen may act as an electron sink, allowing normal pathways of fermentation which are mainly constrained by the necessity to generate terminal electron acceptors, to be diverted so that more ATP can be made by substrate-level phosphorylation. Thus, the fermentation pathway changes and a $\Delta\tilde{\mu}_{H^+}$ has to be generated by some other process, for example, ATP hydrolysis via a H^+-translocating ATPase. If this mechanism operates, interaction with oxygen may occur either via soluble

oxidases such as those demonstrated in S. faecalis (Jacobs and Van Denmark 1960; Hoskins et al., 1962), or via a non-energy-transducing segment of the electron transport chain as found in E. coli grown under sulphate-limited conditions (Poole and Haddock, 1975) and A. vinelandii grown under highly aerobic conditions (Jones et al., 1973).

The two mechanisms outlined above may be represented by the following equations:

Scheme I : $O_2 \xrightarrow[\text{transport}]{\text{electron}} \Delta\tilde{\mu}_{H^+} \rightleftharpoons^{\text{ATPase}} ATP$

Scheme II : $O_2 \xrightarrow[\text{phosphorylation}]{\text{substrate-level}} ATP \rightleftharpoons^{\text{ATPase}} \Delta\tilde{\mu}_{H^+}$

A variety of experimental approaches has been used to distinguish between these two mechanisms. Some of the approaches adopted have yielded information that requires a minimum of assumptions in its interpretation and these will therefore be considered first of all.

1. Studies on respiration of P. acnes.

Studies on respiration of P. acnes showed that both anaerobically- and aerobically-grown cells were capable of oxygen utilization. The respiration rate of both anaerobically- and aerobically-grown cells in the presence of glucose was approximately 20 nmol O_2 (mg dry wt)$^{-1}$ min^{-1}. This rate was comparable to that of P. freudenreichii oxidizing lactate, pyruvate, succinate or acetate where a respiration rate ranging from 5 to 42 nmol O_2 (mg dry wt)$^{-1}$ min^{-1} was observed (de Vries et al., 1972). However, these values were low when compared with the respiration rate of P. shermanii using lactate as the substrate, where cells grown at a partial pressure of oxygen (pO_2) of 20 mm Hg was found to respire at a rate of 133 nmol O_2 (mg dry wt)$^{-1}$ min^{-1}. P. shermanii grown at a pO_2 of 160 mm Hg was found to have a respiration

rate of 70 nmol O_2 (mg dry wt)$^{-1}$ min^{-1} (Pritchard et al., 1977). It was significant that anaerobically-grown P. acnes exhibited a similar respiratory capacity as aerobically-grown P. acnes. This would indicate that anaerobically-grown cells possess the full complement of components required for aerobic metabolism. Indeed anaerobically-grown P. shermanii, P. freudenreichii and P. pentosaceum had been shown to contain terminal oxidases associated with aerobic electron transport (Schwartz and Sporkenbach 1975; Pritchard et al., 1977; de Vries et al., 1973; Bonartseva et al., 1973b). However, the ability to P. acnes to utilize oxygen does not necessarily indicate that electron transport to oxygen (ie. Scheme I) was occurring since it could also be incorporated in the scheme put forward for the 'electron sink' mechanism. It has been shown that S. faecalis grown under aerobic conditions in the absence of haematin where an electron transport chain is absent, has the capacity to respire mediated by soluble oxidases (Pritchard and Wimpenny, 1978; Pugh and Knowles, 1982).

Electron transport inhibitors, if sufficiently specific in their mode of action, provided a means of ascertaining the mechanism responsible for oxygen utilization. In S. faecalis for example, the membrane-bound but not the soluble NADH oxidase, derived from cells grown under conditions where cytochrome synthesis was induced (ie. aerobic conditions in the presence of haematin), was sensitive to HOQNO and antimycin A (Pugh and Knowles, 1982). This would indicate that oxygen uptake in S. faecalis grown under aerobic conditions in the presence of haematin was largely mediated by cytochrome-linked electron transport. Both HOQNO and antimycin A only partially inhibited respiration of anaerobically- and aerobically-grown P. acnes (Tables 12 and 13). The degree of inhibition by HOQNO found in the present studies was low compared with those reported for the inhibition of aerobic oxidation of glycerol 3-phosphate in P. pentosaceum (de Vries et al., 1977) and NADH, lactate and succinate by 2-n-nonyl-4-hydroxyquinoline-N-oxide (NOQNO)

in P. shermanii (Schwartz and Sporkenbach, 1975). Both HOQNO and NOQNO were reported to inhibit the activities measured by up to 80% except for succinate oxidation, where complete inhibition was obtained. The inhibition obtained using antimycin A in the present studies was comparable to those found to inhibit NADH, lactate and succinate oxidations in P. shermanii (Schwartz and Sporkenbach, 1975). The relatively low sensitivity of respiration to HOQNO and antimycin A was not unique to P. acnes since P. arabinosum was also insensitive to both inhibitors despite the fact that it had been shown to contain cytochrome b, the postulated interaction site for both inhibitors (Sone, 1972).

Respiration by P. acnes was insensitive to cyanide up to a concentration of 10 mM. If respiration in P. acnes was due to electron transport, this would indicate that the major role of the terminal oxidase was assumed by cytochrome d which, in other bacterial systems, was shown to be the functional cytochrome oxidase responsible for the manifestation of cyanide-insensitive respiration (Pudek and Bragg, 1974; Arima and Oka, 1965; Jones, 1973). Similar insensitivity to cyanide was also observed in P. shermanii (Bonartseva et al., 1973b; Schwartz and Sporkenbach, 1975) though respiration by P. arabinosum (Sone, 1972) was completely sensitive to cyanide. Pritchard and Asmundson (1980) however found that cyanide did inhibit NADH and lactate oxidations by membrane particles of P. shermanii at concentrations of approximately 20 mM. Azide, another possible inhibitor which interacts with terminal oxidases, stimulated respiration. This would suggest that azide was acting as an uncoupler; a suggestion supported by the observation that azide had no effect on uncoupled respiration of either anaerobically- or aerobically-grown P. acnes (Table 14). In other propionibacteria such as P. shermanii, P. petersonii and P. arabinosum, azide had very little effect on respiration (Bonartseva et al., 1973b; Sone, 1972).

The relative insensitivity of respiration of P. acnes to

electron transport inhibitors could suggest that oxygen uptake was not linked to electron transport but mediated by oxidases via the 'electron sink' mechanism presented earlier. However, this conclusion rests entirely on the assumption that electron transport, if present, must be totally sensitive to the inhibitors used. As discussed earlier, variations in sensitivities to these inhibitors ie. HOQNO, antimycin A, cyanide and azide, do occur within the genus Propionibacterium. Therefore, these studies involving the use of inhibitors could not conclusively discriminate between the two possible ways in which oxygen may interact with this organism.

Interestingly, respiration of P. acnes was stimulated by the uncoupler CCCP, an action that is consistent with the existence of respiratory control exerted by the $\Delta\tilde{\mu}_{H^+}$. In a classical system such as the mitochondria, oxidizing substrates such as NADH or succinate for example, the stimulation by CCCP may be construed to be due to the removal of the constraint imposed upon electron transport by the $\Delta\tilde{\mu}_{H^+}$. However, in the present system, such action by CCCP does not necessarily signify that oxygen uptake was linked to a $\Delta\tilde{\mu}_{H^+}$-generating electron transport chain. This is because if oxygen was promoting ATP synthesis via the mechanism outlined in Scheme II and that the $\Delta\tilde{\mu}_{H^+}$ was maintained by ATP hydrolysis, it could be envisaged that collapsing the $\Delta\tilde{\mu}_{H^+}$ by CCCP could stimulate oxygen uptake due to the necessity to synthesize the ATP required to maintain the $\Delta\tilde{\mu}_{H^+}$. However, in S. faecalis grown under aerobic conditions in the absence of haematin, respiration in the presence of glucose was partially inhibited by CCCP whereas a stimulation was observed in cells grown aerobically in the presence of haematin (Pritchard and Wimpenny, 1978). The inhibition observed in the case of cells grown in the absence of haematin where an electron transport chain was absent could be due to the depletion of ATP hence decreasing the rate of glucose utilization. However, in cells grown in the presence of

haematin, the role of maintaining the $\Delta\tilde{\mu}_{H^+}$ could be assumed by electron transport and the ATP required for glucose utilization was generated by substrate-level phosphorylation hence the stimulation of respiration by CCCP. If the action of CCCP on S. faecalis, grown under conditions where the formation of an electron transport chain was not induced, was taken to be characteristic for the operation of Scheme II (ie. oxygen was acting as an electron sink), then this would indicate that the uncoupler-stimulated respiration of P. acnes was due to electron transport (Scheme I). However, this does not preclude a concerted process, ie. Schemes I and II operating simultaneously with the mechanism outlined in Scheme II not involved in $\Delta\tilde{\mu}_{H^+}$ generation, as found in E. coli grown under sulphate-limited conditions where site I was inoperative (Poole and Haddock, 1975). In fact, Pritchard et al. (1977) suggested that exposure of P. shermanii to oxygen resulted in an increase of substrate-level phosphorylation via the acetate kinase reaction with oxidative phosphorylation with one coupling site occurring simultaneously.

2. Studies on the cytochromes of P. acnes.

The major cytochrome components found in anaerobically- and aerobically-grown P. acnes were cytochromes b, a_1, d and o. As shown in Table 15, there was only a slight variation in the concentrations of the cytochromes in anaerobically- and aerobically-grown cells. These cytochromes are associated with H^+-translocating electron transport chains of a number of bacterial systems (reviewed by Jones, 1977). The presence of cytochromes usually associated with aerobic electron transport accounts for the ability of anaerobically-grown P. acnes to respire, assuming that oxygen uptake was mediated by electron transport. These cytochromes have also been found in other propionibacteria such as P. freudenreichii (de Vries et al., 1977) and P. shermanii (Pritchard et al., 1977; Schwartz and Sporkenbach, 1975).

In view of the total insensitivity of respiration and respiration-

driven H^+ translocation to cyanide, it would seem that the major role of the terminal oxidase was assumed by cytochrome d. However, studies by Pritchard and Asmundson (1980) showed that the inhibition of NADH and lactate oxidations by cyanide in P. shermanii was due to the formation of cyano-cytochrome d, with cytochrome o unaffected. This would be in contrast to the observations in other organisms where cyanide-sensitivity is usually associated with cytochrome o (Haddock and Jones, 1977). The present studies do not allow any conclusions concerning which of the cytochromes was functioning as the active terminal oxidase. However, the presence of these cytochromes in P. acnes grown under aerobic and and anaerobic conditions was consistent with the presence of an energy-transducing electron transport system (Scheme I) capable of utilizing oxygen as the terminal electron acceptor.

3. Studies on respiration-driven proton translocation in P. acnes.

Studies on respiration-driven H^+ translocation have been successfully used to determine the energy-transducing capability and efficiency of electron transport systems using various terminal electron acceptors in a number of bacterial systems (Scholes and Mitchell, 1970; Jones et al., 1975; Brice et al., 1974; Brogerd et al., 1981). Pulsing anaerobic suspensions of anaerobically- and aerobically-grown P. acnes with oxygen resulted in the acidification of the medium. The requirement of KSCN and the sensitivity of the decay of ΔpH_o to the uncoupler CCCP constituted evidence that this process was electrogenic.

The values of the $\rightarrow H^+/O$ ratios obtained for anaerobically- and aerobically-grown P. acnes were 2·7 and 2·6 respectively. The stoichiometry of the number of H^+ translocated per coupling site is still a matter of controversy (Brand et al., 1976; Garland, 1977). However, if the number of H^+ translocated per coupling site was assumed to be either 2 or 3, the $\rightarrow H^+/O$ values obtained would indicate the presence of one coupling site on the electron transport chain of P. acnes.

The demonstration of respiration-driven H^+ translocation constituted evidence for the occurrence of an electrogenic electron transport system in P. acnes with oxygen acting as the terminal electron acceptor as outlined in Scheme I. However, it may be argued that the oxygen introduced into the cell suspension was utilized by a mechanism such as that described in Scheme II and the H^+ translocation observed was a result of the ATP produced being hydrolysed via the H^+-translocating ATPase. This is considered unlikely because in S. faecalis, for example, grown under aerobic conditions in the absence of haematin where it is known that a mechanism such as that presented in Scheme II was operating, no respiration-driven H^+ translocation was observed (Clarke and Knowles, 1980). As pointed out in the discussion of studies on respiration, a concerted process involving Schemes I and II where Scheme II is not involved in the generation of a $\Delta\tilde{\mu}_{H^+}$ such as the occurrence of a non-energy-transducing segment along the electron transport chain is a possibility. Indeed the $\rightarrow H^+/O$ ratio obtained was consistent with there being only one coupling site on the electron transport chain of P. acnes despite the demonstrations of two coupling sites in other bacteria containing similar cytochrome components (Jones, 1977). It is also a possibility that the values of the $\rightarrow H^+/O$ ratio obtained were underestimated due to oxygen utilization that is not linked to electron transport. For this to occur, it must be postulated that a constant percentage of the oxygen pulse was consumed by this mechanism since it was shown that the amount of H^+ translocated was directly proportional to the oxygen concentration (Fig. 45).

The effect of electron transport inhibitors such as antimycin A and HOQNO on respiration-driven H^+ translocation was very similar to that shown on respiration, where a partial inhibition by both inhibitors was observed. This observation could not be readily explained as site-specific electron transport inhibitors were expected to cause complete

abolition of coupling sites associated with an inhibitor-sensitive electron transport chain. Piericidin A for example was shown to inhibit completely respiration-driven H^+ translocation in P. denitrificans with malate as the substrate but had no effect when succinate was used as the substrate (Lawford et al., 1976). Respiration-driven H^+ translocation with succinate as the substrate was however inhibited by antimycin A. Therefore on the assumption that P. acnes could possibly have a maximum of two coupling sites, it would be expected that both HOQNO and antimycin A, which interacted at cytochrome b, to inhibit completely respiration-driven H^+ translocation. In fact, in the presence of HOQNO, both anaerobically- and aerobically-grown P. acnes were still capable of performing respiration-driven H^+ translocation with $\rightarrow H^+/O$ ratios of 1·85 and 1·50 respectively. Similarly, in the presence of antimycin A, $\rightarrow H^+/O$ ratios of 2·1 and 1·75 were obtained for anaerobically- and aerobically-grown cells respectively. The values were indeed large enough to suggest the presence of a coupling site even in the presence of these inhibitors, therefore questioning the use of these compounds for studying electron transport processes in P. acnes.

Respiration-driven H^+ translocation, like respiration, was not sensitive to cyanide up to a concentration of 10 mM probably indicating that the role of the primary terminal oxidase was assumed by cytochrome d.

4. The generation of the membrane potential by P. acnes.

The $\Delta\Psi$ component of the $\Delta\tilde{\mu}_{H^+}$ generated by P. acnes was estimated by measuring the distribution of K^+ across the membrane in the presence of valinomycin after it was established that the organism was valinomycin-sensitive. In the present studies, all the $\Delta\Psi$ measurements were performed in a strongly buffered medium at pH 7·0. It was therefore considered that the $\Delta\Psi$ provided a valid representation of the $\Delta\tilde{\mu}_{H^+}$ since, at this pH, it would be expected that the $\Delta\Psi$ would

be the dominant component of the $\Delta\tilde{\mu}_{H^+}$. This was based on the assumption that the internal pH of P. acnes was in the region of 7·0 to 8·0 as found in other organisms such as E. coli, S. faecalis and Clostridium pasteurianum (Collins and Hamilton, 1976; Harold et al., 1970; Riebelling et al., 1975). The collapse of the $[K^+]$ gradient by protonophores such as PCP, TCS and CCCP (Table 4) constituted evidence that the parameter being measured was maintained by the electrogenic translocation of H^+, resulting in the generation of a $\Delta\tilde{\mu}_{H^+}$.

Both anaerobically- and aerobically-grown P. acnes was shown to generate a substantial $\Delta\Psi$ under anaerobic conditions; the magnitude being -145 mV and -141 mV respectively. The exposure of valinomycin-treated P. acnes incubated under anaerobic conditions to oxygen caused the uptake of K^+ from the medium, resulting in the attainment of a new equilibrium position corresponding to an increase in the $\Delta\Psi$. This directly demonstrated that oxygen played a key role in the generation of the $\Delta\Psi$ in both anaerobically- and aerobically-grown P. acnes. In the presence of oxygen, glucose further stimulated the generation of the $\Delta\Psi$ (Fig. 16). The $\Delta\Psi$ generated under anaerobic conditions by cells grown anaerobically either with or without fumarate was not stimulated by glucose even when fumarate was present. In classical propionibacteria such as P. freudenreichii, P. pentosaceum and P. arabinosum, fumarate has been shown to be an anaerobic electron acceptor (de Vries et al., 1977; Sone, 1972).

The values of the $\Delta\Psi$ measured are presented in Table 6. However, due to the sensitivity of the $\Delta\Psi$ to the external $[K^+]$ (Fig. 8), the values would represent an underestimate of approximately 10%. Under ideal conditions where the external $[K^+]$ was approaching zero concentration, the extrapolated values of the $\Delta\Psi$ generated by anaerobically-grown P. acnes under anaerobic, aerobic and aerobic

conditions in the presence of glucose would be -160 mV, -170 mV and -177 mV respectively. Similarly, the $\Delta\Psi$ generated by aerobically-grown P. acnes would be -155 mV, -166 mV and - 175 mV respectively. These values were comparable to the $\Delta\Psi$ generated by the anaerobe S. faecalis (Laris and Pershadsingh, 1972) and the facultative anaerobe E. coli (Felle et al., 1980; Collins and Hamilton, 1976) and S. aureus (Collins and Hamilton, 1976). Based on the assumptions made earlier (ie. the pH of the medium remained at 7.0 and the internal pH was between 7.0 to 8.0), the $\Delta\tilde{\mu}_{H^+}$ generated by P. acnes (the average for anaerobically- and aerobically-grown cells) under anaerobic, aerobic and aerobic conditions in the presence of glucose may be estimated to be in the region of -157 to -217 mV, -168 to -228 mV and -176 to -236 mV respectively. Thus the estimated $\Delta\tilde{\mu}_{H^+}$ generated by P. acnes was similar to those generated by other energy-transducing systems such as bacteria, (Collins and Hamilton, 1976), mitochondria (Mitchell and Moyle, 1969) and chloroplasts (Schuldiner et al., 1972a), and thus energetically competent to drive ATP synthesis, provided an ATPase was present.

The studies on the generation of the $\Delta\Psi$ in itself could not distinguish between Schemes I and II. However, on the basis of the results discussed earlier, it was reasonable to suggest that the increase in the $\Delta\Psi$ in the presence of oxygen was due to the increase in the H^+ flux resulting from the occurrence of electron transport with oxygen as the terminal electron acceptor. The stimulation of the $\Delta\Psi$ generated under aerobic conditions upon the addition of glucose would be consistent with electron transport being the primary mechanism generating the $\Delta\Psi$ since glucose also stimulated respiration in both aerobically- and anaerobically-grown P. acnes.

An assessment as to which of the two mechanisms presented in Schemes I and II was responsible for generating the $\Delta\Psi$ under aerobic

and anaerobic conditions could be made if specific energy-transfer and electron transport inhibitors were available. It was to this end that studies involving the use of these inhibitors on the generation of the $\Delta\Psi$ were performed.

5. Effect of inhibitors on the generation of the membrane potential in P. acnes.

Inhibitors of oxidative phosphorylation have been used successfully in studies on energy transduction in a number of systems. In the present studies, two classes of inhibitor, namely energy-transfer inhibitors (such as Nbf-Cl, DCCD, oligomycin, DPPA and quercetin) and electron transport inhibitors (HOQNO and antimycin A) were used in an attempt to define the mechanism reponsible for generating the $\Delta\tilde{\mu}_{H^+}$ in P. acnes. under anaerobic and aerobic conditions. In theory, by employing members of these two classes of inhibitor, the roles of the electron transport chain and the ATPase in energy transduction processes could be investigated independently. Energy-transfer inhibitors should only dissipate the $\Delta\Psi$ if it was generated by the ATPase for example as shown by the action of DCCD on C. pasteurianum (Riebelling et al., 1975) and S. faecalis (Harold and Papineau, 1972b) and oligomycin on the photosynthetic organism Rhodospirillum rubrum (Isaev et al., 1970). Likewise, electron transport inhibitors had been shown to inhibit the generation of the $\Delta\Psi$ by electron transport processes (Isaev et al., 1970; Hirata et al., 1973). Essentially, if the mechanism described in Scheme I was responsible for generating the $\Delta\tilde{\mu}_{H^+}$, the $\Delta\Psi$ should be dissipated by electron transport inhibitors only. However, if Scheme II was operating, the $\Delta\Psi$ should be sensitive to energy-transfer inhibitors only. The success of such an approach is entirely dependent on the specificity of the inhibitors used. Therefore it was imperative that the specificity of the action of these inhibitors on P. acnes be ascertained in order that a meaningful interpretation of the data obtained involving the use of the inhibitors could be made.

The demonstration of uncoupler-stimulated respiration, which available evidence suggests was due to electron transport, provided an experimental system whereby the specificity of the inhibitors used could be ascertained. Energy-transfer inhibitors by definition, inhibit only coupled electron transport and the inhibition can be relieved by uncouplers. As shown in Table 14, all the energy-transfer inhibitors used, with the exception of oligomycin, inhibited uncoupled respiration and as such, the inhibition by these compounds could not be attributed solely to interactions with the ATPase. The non-specificity of some of the inhibitors used ie. interactions with sites other than the ATPase has been documented in other systems. DCCD, for example, has been shown to inhibit electron transport and the transport of certain metabolites such as α-ketoglutarate, fumarate and malate in E. coli (Gutowsky and Rosenberg, 1976; Singh and Bragg, 1974). Nbf-Cl is also a very reactive compound and due to it being a thiol reagent, it would not be unreasonable to postulate that it could well be inhibiting glycolysis by interacting with the thiol group at the active site of glyceraldehyde 3-phosphate dehydrogenase.

The results obtained in the studies on the effect of energy-transfer inhibitors on uncoupled respiration provided only a qualitative assessment of the specificity of the inhibitors used. DCCD, for example, can be said to cause inhibition of electron transport by interacting with sites other than the ATPase but the concentration at which it was specifically acting on the ATPase (if indeed it was) could not be ascertained from the results obtained. This would also apply to Nbf-Cl, quercetin and DPPA. Oligomycin however, inhibited respiration but had no effect on uncoupled respiration. It was solely on this evidence that oligomycin appeared to be a specific ATPase inhibitor in this system. With the exception of the photosynthetic organisms Rhodospirillum rubrum

(Horiuti et al., 1968) and Rhodospirillum capsulata (Melandri and Baccarini-Melandri, 1971), the ATPase of most other bacteria is insensitive to this compound. Oligomycin has not been reported to cause inhibition of oxidative phosphorylation other than by interaction with the ATPase. Without supporting experimental evidence, such as a direct demonstration of an inhibition of ATPase activity in membrane preparations, the use of oligomycin as a specific energy-transfer inhibitor in P. acnes has obvious drawbacks.

In general, the studies on the effects of inhibitors on the generation of the $\Delta\Psi$ in P. acnes were inconclusive. The results obtained were not readily explicable on the basis of a simple scheme and depended entirely on which of the inhibitors was assumed to be specific. The $\Delta\Psi$ generated by anaerobically- and aerobically-grown P. acnes under anaerobic conditions was fully sensitive to oligomycin (Figs. 10 and 11) and partially sensitive to HOQNO (Figs. 14 and 15). If oligomycin was assumed to be a specific ATPase inhibitor on the basis of its uncoupler-sensitive inhibition of respiration, this would then indicate that under anaerobic conditions, the $\Delta\Psi$ was generated by ATPase activity. This would also assume that partial depolarization of the membrane by HOQNO was due to interactions at sites other than the electron transport chain unless it is suggested that there is an HOQNO-sensitive electron transport chain involved in fermentation, allowing enhanced substrate-level phosphorylation. HOQNO has in fact been shown to uncouple oxidative phosphorylation (Haas, 1964) though there was no evidence to support such action in P. acnes. In any case, if HOQNO was acting as an uncoupler, it would be expected to dissipate completely the $\Delta\Psi$. However, if it were assumed that HOQNO was specifically inhibiting electron transport this would then suggest that anaerobic electron transport played a role in generating the $\Delta\Psi$ under these conditions. Upon inhibition of anaerobic

electron transport by HOQNO, the role of maintaining the $\Delta\Psi$ was assumed by the ATPase, hence the sensitivity of the residual $\Delta\Psi$ to oligomycin (Figs. 14 and 15). This explanation necessitates the assumption that the dissipation of the $\Delta\Psi$ maintained under anaerobic conditions by oligomycin was due to interactions at sites other than the ATPase. However, there is currently no evidence for the occurrence of anaerobic electron transport in P. acnes. On this basis, and the fact that oligomycin dissipated the $\Delta\Psi$ generated, it would seem that under anaerobic conditions, the ATPase was responsible for maintaining the $\Delta\Psi$.

It must be emphasized that during ATP synthesis, the H^+ flux is inwardly directed through the ATPase while during ATP hydrolysis, it is outwardly directed. Therefore, under conditions when electron transport is driving ATP synthesis, electron transport and ATPase activity cannot simultaneously function to generate the $\Delta\tilde{\mu}_{H^+}$.

Under aerobic conditions, the $\Delta\Psi$ generated by anaerobically- and aerobically-grown P. acnes was partially sensitive to both oligomycin (Figs. 12 and 13) and HOQNO (Figs. 24 and 25). In the presence of glucose, the response of the $\Delta\Psi$ to both inhibitors was essentially similar. Using similar lines of argument to that presented for the effect of oligomycin and HOQNO on the $\Delta\Psi$ generated under anaerobic conditions, the use of these inhibitors could not completely define whether it was electron transport (ie. Scheme I) or ATPase activity (ie. Scheme II) which was responsible for generating the $\Delta\Psi$ under aerobic conditions. If it was accepted from previous evidence that Scheme I was operating in the presence of oxygen, the partial dissipation of the $\Delta\Psi$ generated under aerobic conditions by oligomycin suggested that it was acting at a site other than the ATPase hence the earlier suggestion that under anaerobic conditions the $\Delta\Psi$ was generated by ATPase activity.

It was curious that while HOQNO only partially dissipated the $\Delta\Psi$ generated under aerobic conditions, in agreement with its partial

effect on respiration, it completely dissipated the oligomycin-insensitive $\Delta\Psi$. In addition, the combination of oligomycin and HOQNO (in that order) completely dissipated the $\Delta\Psi$ while HOQNO and oligomycin (in that order) only partially dissipated the $\Delta\Psi$ generated under similar conditions. In fact, the HOQNO-insensitive $\Delta\Psi$ was relatively resistant to oligomycin. The only evidence from the studies involving the use of inhibitors that could indicate that under aerobic conditions the $\Delta\Psi$ was generated by electron transport was the dissipation of the $\Delta\Psi$ by the combination of antimycin A and HOQNO. This would however, involve postulating that P. acnes has either two or branched electron transport chains with different sensitivities to antimycin A and HOQNO. Without other experimental evidence, this remains a speculation. It is therefore evident that the results obtained from the studies involving the use of inhibitors could not reliably refute or support the schemes put forward for the mechanism responsible for generating the $\Delta\Psi$ based on the evidence discussed earlier.

6. Studies on the ATPase of P. acnes.

The operation of the mechanisms described in Scheme I (to utilize the $\Delta\tilde{\mu}_{H^+}$ generated by electron transport for ATP synthesis) or Scheme II (to generate a $\Delta\tilde{\mu}_{H^+}$ by ATP hydrolysis) requires the presence of a functional H^+-translocating ATPase, hence the desire to demonstrate the presence of this enzyme in P. acnes. The initial problem encountered with studies on the ATPase of P. acnes was the difficulty in disrupting the organism. A similar difficulty was also experienced by another group working with P. acnes (Holland, personal communication). A survey of the various disruption techniques revealed that agitation with glass-beads produced the highest yield of membrane-bound ATPase. This characteristic was considered essential to allow a direct comparison of the inhibitor sensitivity of the ATPase in vivo and in vitro.

The ATPase activity associated with the membrane fragments

of P. acnes was sensitive only to Nbf-Cl and quercetin but insensitive to oligomycin and DCCD (Table 9). Nbf-Cl was postulated to cause inhibition of the ATPase by interacting with the β sub-unit of the F_1-ATPase in mitochondria (Holowka and Hammes, 1979) and the α and β sub-units of bacteria (Verheijen et al., 1978). Similarly, quercetin also interacted with the F_1-portion of the ATPase complex (Futai et al., 1974). This would explain the equal sensitivity of the soluble and the membrane-bound ATPases observed in the present studies to these two inhibitors. Unlike Nbf-Cl and quercetin, oligomycin and DCCD were believed to interact with the F_o-portion of the ATPase complex. Both inhibitors were however without effect on the membrane-bound ATPase activity of P. acnes.

Because of the low ATPase activity associated with cell-free extracts of P. acnes, and the insensitivity to DCCD, the likelihood that ATP hydrolysis found was due to experimental artifacts must be considered. Combinations of enzymes such as hexokinase and a phosphatase would manifest as ATPase activity, though this would presumably be glucose-dependent. However, phosphatase activity was not detected under the conditions used for assaying the ATPase. In addition, such enzymes would be expected to be associated only with the soluble fraction of the cell-free extract and not the particulate fraction.

It is difficult to accept that P. acnes has no ATPase because this enzyme has been found in all the energy-transducing systems so far studied, though in some cases it was latent and required activation for its presence to be detected. Attempts to 'activate' the ATPase in P. acnes were, however, unsuccessful. Because of the universal distribution of the ATPase in microorganisms, it seems probable that the ATP hydrolysis activity associated with the membrane fragments of P. acnes, albeit low, was a manifestation of an energy-transducing ATPase. However, there is little compelling evidence to support such a claim

and the question of the existence of a classical energy-transducing ATPase in this system remains open.

7. Concluding discussion.

From the results obtained, ie. the demonstration of respiration-driven H^+ translocation, uncoupler-sensitive respiration and the presence of cytochromes which in other bacterial systems had been shown to be associated with electrogenic electron transport, the observed increase in the $\Delta\Psi$ generated by P. acnes under aerobic conditions can be attributed to the occurrence of electron transport with oxygen as the terminal electron acceptor. Therefore, under aerobic conditions it is proposed that Scheme I was operating. This would correlate the present findings with those of Holland and co-workers. They found that in the presence of oxygen, there was almost a 100% increase in the growth yield of P. acnes grown on either complex (Reinforced clostridial medium; Oxoid) (Holland et al., 1978) or semi-defined media using amino-acids as carbon and energy-source (Cove et al., 1983; submitted for publication), In complex media, the increase in growth yield occurred at an oxygen tension of 10 to 90 mm Hg while in the semi-defined medium, it only occurred between 8 and 32 mm Hg above which very little increase in growth yield occurred, despite the fact that oxygen was not lethal to the organism (Cove et al., 1983; submitted for publication). They also reported that the growth rate decreased even at oxygen tensions at which a stimulation in the growth yield occurred. It is therefore evident that oxygen was exerting other limits on the growth of P. acnes despite the increase in efficiency of energy transduction as indicated by the increase in growth yield. In the present studies, aeration for aerobic growth was procured by shaking the growing cultures in air. The oxygen tension present during growth was not determined but since respiration rate of

P. acnes was low, this suggests that the oxygen tension of the cultures would be near saturation. If this were the case, even at this oxygen tension where there was no increase in growth yield, the results obtained from the present studies would suggest that electron transport with oxygen as the terminal electron acceptor was still the primary mechanism generating the $\Delta\tilde{\mu}_{H^+}$, hence ATP, assuming the presence of an energy-transducing ATPase.

In P. acnes, a shift from anaerobic to aerobic growth conditions caused a drastic decrease in the rate of propionate and acetate production (Cove and Holland, personal communication). This would indicate that under aerobic conditions, the fermentation pathway was diverted from the propionate generating cycle (Fig. 5) to probably the TCA cycle, which has been shown to be present in propionibacteria (Delwiche and Carson, 1953; Bonartseva et al., 1973a). In addition, the decrease in acetate production suggested that ATP synthesis via the acetate kinase reaction was no longer a major route for ATP synthesis. These observations would support the suggestion that under aerobic conditions, oxidative phosphorylation was the major mechanism for ATP synthesis. Similar observations and conclusions have been made from studies on P. freudenreichii (de Vries et al., 1972). However, it was reported that exposure of P. shermanii to oxygen led to a decrease in propionate but an increase in acetate production, indicating that ATP generation via the acetate kinase reaction was still of major significance (Pritchard et al., 1977).

The conclusion that oxidative phosphorylation occurs in P. acnes requires the presence of a functional energy-transducing ATPase which, as discussed earlier, was not conclusively demonstrated. If it is assumed that such an enzyme was indeed absent from P. acnes, it may be argued that the $\Delta\tilde{\mu}_{H^+}$ generated under aerobic conditions was used only for transport purposes. If this were so, it would not be expected that such a large increase in growth yield would be observed

because, according to the calculations of Stouthamer (1977), only approximately 25% of the total energy requirement of the cell is used for transport purposes. Hence, if this was met by the $\Delta\tilde{\mu}_{H^+}$ generated, one would expect an increase in growth yield of approximately 25% only. Therefore, the increase in growth yield could not be accounted for on the basis that the $\Delta\tilde{\mu}_{H^+}$ generated under aerobic conditions was used solely for transport purposes. Thus the $\Delta\tilde{\mu}_{H^+}$ was also used for ATP synthesis hence requiring the presence of a functional H^+-translocating ATPase.

Apart from the possible action of a H^+-translocating ATPase, which as discussed above is necessary for oxidative phosphorylation to occur, the results obtained from the present investigation do not allow any major conclusions to be made concerning the mechanism by which the $\Delta\tilde{\mu}_{H^+}$ was generated under anaerobic conditions. Attempts to demonstrate the presence of an anaerobic electron transport system with fumarate as a possible electron acceptor were unsuccessful. However, without further experimental evidence the question concerning the absence or presence of an anaerobic electron transport system remains open.

If P. acnes does not have either a functional energy-transducing ATPase or an anaerobic electron transport chain, other mechanisms by which a $\Delta\tilde{\mu}_{H^+}$ may be generated under anaerobic conditions must be considered. One such mechanism was put forward by Michels et al. (1979) who suggested that the efflux of a metabolic end-product in symport with H^+ could generate a $\Delta\tilde{\mu}_{H^+}$. They calculated that the $\Delta\tilde{\mu}_{H^+}$ generated from the efflux of lactate produced from homolactic fermentation of glucose could increase the energy-yield by up to 30%. Experimental evidence for this mechanism was provided by ten Brink and Konings (1980) who demonstrated an uncoupler-sensitive transport of proline into E. coli vesicles loaded with lactate upon exposure of the vesicles to lactate-free medium.

The mechanism described above could in fact be applied to explain the results obtained from studies on respiration and respiration-driven H^+ translocation if it was postulated that oxygen was actually stimulating the formation and excretion of an end-product in symport with H^+. The increase in respiration rate by uncouplers could therefore be due to the collapse of the $\Delta\tilde{\mu}_{H^+}$ generated by this process hence stimulating oxygen uptake which was required for further production of the end-product. Equally, respiration-driven H^+ translocation could be the result of the H^+ movements in symport with the end-product which was produced in direct proportion to the amount of oxygen introduced into the cell suspension. Essentially, this mechanism is similar to that described in Scheme II except that in Scheme II oxygen was suggested to promote the synthesis of ATP via substrate-level phosphorylation and that $\Delta\tilde{\mu}_{H^+}$ was generated by the hydrolysis of ATP. In the present mechanism ATP synthesis could still occur as described in Scheme II but an ATPase is not required for the generation of a $\Delta\tilde{\mu}_{H^+}$.

As mentioned earlier, the decrease in acetate and propionate production upon exposure of P. acnes to oxygen (Cove and Holland, personal communication) would result in the loss of two ATP-generating sites (Fig.5). In order to explain the large increase in growth yield, the mechanism whereby oxygen was postulated to stimulate excretion of an end-product would not only have to compensate for the loss of these ATP-generating sites but also to generate more ATP required to account for the increased growth yields. It is therefore considered that the operation of the mechanism described in Scheme I which, assuming the presence of a functional energy-transducing ATPase which could drive ATP synthesis via oxidative phosphorylation, would provide a more plausible explanation for the observed growth yield increase of P. acnes in the presence of oxygen.

From the present investigation, it must be concluded that oxygen

plays a major role in the energy transduction process of the cutaneous propionibacterium, P. acnes, by promoting the occurrence of electron transport. The mechanism of energy transduction under anaerobic conditions is somewhat less clear though P. acnes does not seem to have an anaerobic electron transport linked to fumarate reductase. Under anaerobic conditions, excretion of metabolic end-products could well be a mechanism by which a $\Delta\tilde{\mu}_{H^+}$ is generated, assuming the absence of a H^+-translocating ATPase. It would therefore have been useful to study the effect of oligomycin on fermentation. On the assumption that P. acnes does have a functional ATPase, then under anaerobic conditions, the $\Delta\tilde{\mu}_{H^+}$ may be generated by the hydrolysis of ATP produced from substrate-level phosphorylation. Under aerobic conditions, the $\Delta\tilde{\mu}_{H^+}$ is generated by electron transport which could then drive the synthesis of ATP by oxidative phosphorylation.

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