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# Kinetic Control of Solute Accumulation in pmf-dependent Transport Systems

By Dr. W. A. HAMILTON

I should like to begin by congratulating the organisers on their choice of subject area for this workshop. The importance of microbial systems to studies of bioenergetics has for long been a favourite theme of my own, and I am delighted to have been asked to speak on this occasion.

As has already been demonstrated by the previous speakers, microbial systems offer the investigator a great diversity of bioenergetic problems, and their individually unique solutions. By the study of, for example, chemolithotrophic mechanisms or the use of alternative electron acceptors by certain of the anaerobic bacteria, we have gained a much wider understanding of bioenergetic mechanisms and their role in cellular function than would have been possible had we confined our interest to phosphorylation in rat liver mitochondria.

Microbial systems also offer us great experimental power through their capacity for controlled phenotypic and genotypic variation. But especially valuable, I think, is the conceptual insight that derives from the undifferentiated character of the procaryotic bacterial cell with its single non-specialised plasma membrane. Here are located not only the redox and phosphorylation reactions of oxidative phosphorylation, but also the uniport, symport and antiport mechanisms responsible for modulating the relative sizes of the membrane potential ($\Delta\Psi$) and pH gradient ($\Delta pH$), maintaining intracellular pH ($pH_i$) and ionic content, and powering the uptake of organic nutrients. In addition, other energy requiring functions, such as cell motility, are directly associated with the plasma membrane and its attendant protonmotive force (pmf, or $\Delta p$). The interdependence of this array of energy generating, conserving and utilising systems within a single membrane, illustrates dramatically the essential unity of bioenergetic reactions as well as the magnitude of their importance to cell function.

The particular interest of my own group has been in the pmf-dependent transport activities of bacteria; initially with regard to amino acid and sugar uptake, but more recently expanding to take in the control of intracellular pH.

Transport can be considered from a number of viewpoints. As a system for experimental study, it offers both special problems and unique advantages. The problems are largely practical, arising from the difficulties associated with handling integral membrane proteins and the need to develop reconstitution systems in order to attempt the assay of transport activity. These problems arise from the study of membrane transport as an aspect of biochemistry or molecular biology. The peculiar advantages come from the study of transport rather from the point of view of physiology or cell biology. The translocation of a solute, without chemical modification, is a measurable cellular expression of the transmembrane event at the molecular level. As such, bacterial transport studies were the point of origin of the ideas leading ultimately to the chemiosmotic hypothesis and they still form a rich source of its theoretical and experimental verification.

There are two separable components of the transport process; the carrier-mediated facilitated diffusion across the hydrophobic lipid barrier, and the coupling of energy in so-called active transport such that the accumulation of a higher concentration of solute is attained within the cell. Historically, biochemists sought to explain energy coupling in terms of the concepts and techniques, themselves borrowed from enzymology, which were being applied to the study of the translocation process itself. It has only, however, been with the development of chemiosmotic thinking that we have gained any real understanding of the energy coupling mechanism and of its integration with facilitated transport, and so ultimately have achieved an illumination of the whole field of bioenergetics.

One can also subject studies of membrane transport to either kinetic or thermodynamic analysis. The early kinetic models sought to explain the observed phenomena, but had little molecular basis and no heuristic character. Both of these shortcomings are to a large extent overcome in current thermodynamic or chemiosmotic models, but they, in turn, portray an equilibrium or static view of what is, after all, a dynamic system. It is my intention in this lecture to expose this weakness, and to suggest that in the integration of molecular and cell biology studies with kinetic and thermodynamic analyses, bacterial transport can once again take a prominent role in extending our understanding of membrane translocation itself, and of bioenergetics in general.

My group at Aberdeen has been concerned with amino acid transport in *Staphylococcus aureus,* and, more recently, with β-galactoside uptake in *Escherichia coli.* Initially, our studies were qualitative and demonstrated proton fluxes associated with solute uptake, and the accumulation of solute in response to experimentally induced potential and pH gradients in resting cell suspensions.

Next we sought to gain a measure of the pmf developed by respiring cells. The technique we used was essentially that originally developed by MITCHELL and MOYLE (1969) for mitochondrial systems. A lightly buffered suspension of valinomycin-treated cells is incubated at constant temperature under anaero-

bic conditions until an equilibrium has been reached, as indicated by a steady output from the hydrogen and cation selective electrodes showing no, or at least minimal, proton or potassium ion flux. The cells are then energised by oxygen, usually in the form of the addition of a small volume of hydrogen peroxide to the catalase-containing suspension. As is shown in Fig. 1 for *S. aureus,* protons are immediately ejected from the cell and a new steady state is established, characteristic of the respiration-driven $\Delta pH$, inside alkaline. On exhaustion of the oxygen, or quicker with the addition of an uncoupler, the protons flux back into the cell and the deenergised condition of the cells is reestablished. It is from the flux of protons on deenergisation that $pH_i$, and so $\Delta pH$, is calculated, taking into account buffering power of the intra- and extracellular phases, which must also be determined experimentally.

As well as, of course, developing the pH gradient, proton efflux generates the membrane potential, inside negative. With valinomycin-treated cells, the flux of $K^+$ responds to and can be used as a direct measure of this $\Delta\Psi$.

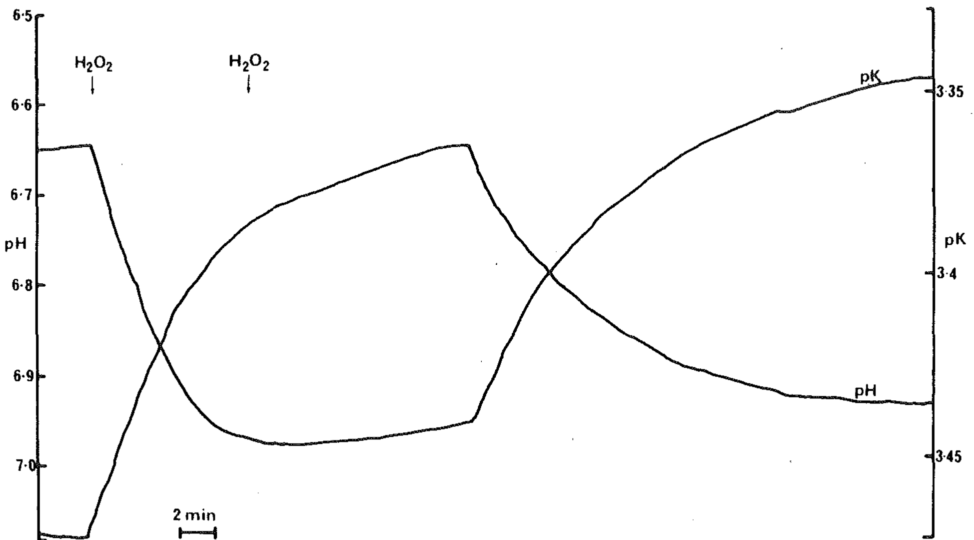


Fig. 1 Changes in pH and pK observed on addition of hydrogen peroxide to resting potassium-permeable cells of *S. aureus* in the presence of catalase. Cells (0.83 mg/ml) were allowed to equilibrate in sucrose-choline chloride. Hydrogen peroxide (100 µl of 2 volume) was added at points indicated by arrows. (Reprinted with permission from J. Bacteriol *126* (1976) 1224)

These experiments were carried out at an extracellular pH ($pH_o$) of 6.45 and for *S. aureus* they gave values of 134 mV for $\Delta\Psi$, 1.3 for $\Delta pH$ and a total pmf of 211 mV. For *E. coli,* the values of these same parameters were, respectively, 132 mV, 1.65 and 230 mV. These early studies have been summarized in a number of papers and in two review articles (COLLINS and HAMILTON, 1976; HAMILTON, 1975, 1977).

tivity of potassium transport systems. Combining the earlier data from $K^+$ efflux on deenergisation with that from the steady state distribution of the ion, it can be said that $\Delta\Psi$ in *S. aureus* lies between 134 and 150 mV and shows no significant increase with increasing values of $pH_o$. The $pH_o$-dependence of $\Delta p$ is also shown in Fig. 2.

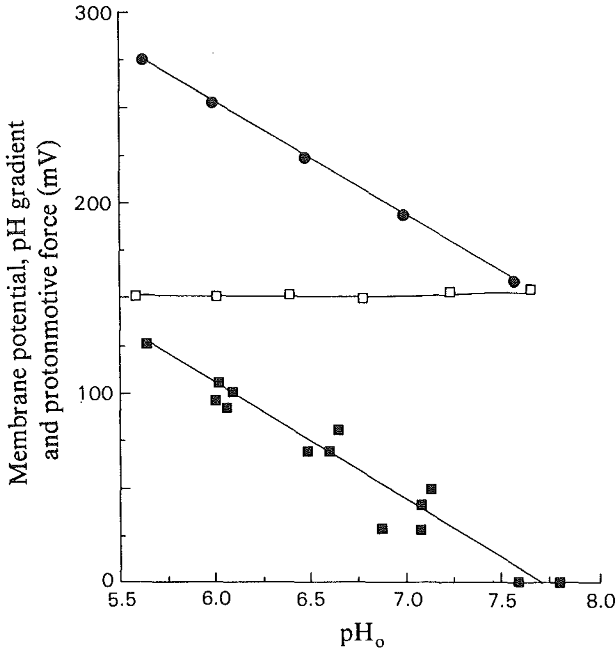


Fig. 2 The protonmotive force in *S. aureus*. Respiring cells were suspended in citric acid/sodium phosphate buffers at a density of 1 mg bacterial dry wt./ml. Membrane potential (□) was measured in the presence of 0.1 mM-KCl, 2 μg of valinomycin/ml and 0.2 μM-[$^3$H]inulin. The results presented are means of nine determinations. The pH gradient [■] was measured in the presence of 54 μM-[$^{14}$C]DMO and 0.12 μM-[$^3$H]inulin. Each point represents a separate experimental determination. The protonmotive force (●) was obtained from the addition of the membrane potential and pH gradient. (Reprinted with permission from Biochem. J. *184* (1979) 441)

A qualitatively similar pattern is found with *E. coli* (Fig. 3), save that here $\Delta pH$ does not appear to decrease to zero, and $\Delta\Psi$ shows significantly increasing values between $pH_o$ 5.9 and 7.0, thereafter remaining constant at 160 mV. Consequently the $pH_o$-dependence of $\Delta p$ is less in *E. coli* than in *S. aureus.*

In seeking to compare solute accumulation with its putative driving force in the form of the pmf, as modulated by the number of protons co-transported (the stoichiometry, n) and the charge on the solute (m), use is made of the equation:

$$Z \log \frac{[S]_i}{[S]_o} = (m+n)\,\Delta\Psi - nZ\Delta pH$$

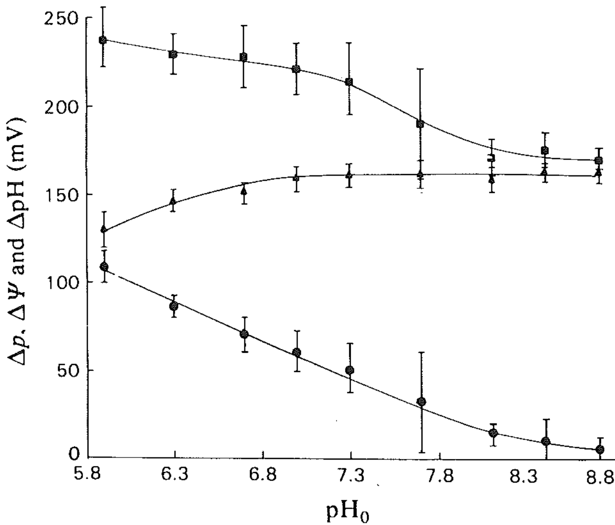


Fig. 3 The protonmotive force in *E. coli*. EDTA/Valinomycin-treated *E. coli* ML 308–225 cells were incubated in Tris/Mes/choline chloride/KCl buffer at the indicated pH. $\Delta pH$ was measured with DMO at an external concentration of 5 µM. $\Delta\Psi$ was determined from the equilibrium distribution of $K^+$.
● $\Delta pH$, ▲ $\Delta\Psi$, ■ $\Delta p$ (Reprinted with permission from Biochem.. *182* (1979) 687)

Z is the factor, equal to 59, which allows transmembrane concentration ratios to be expressed in mV, $[S]_i$ and $[S]_o$ stand for the solute concentration inside and outside the cell, respectively.

More recently we have measured the pmf across a range of $pH_o$ and compared the driving force with the solute accumulation attained; for glutamate in *S. aureus* (MITCHELL, BOOTH and HAMILTON, 1979) and for lactose in *E. coli* (BOOTH, MITCHELL and HAMILTON, 1979).

As has been shown by several groups, bacteria place considerable importance on maintaining $pH_i$ within very narrow limits. Consequently $\Delta pH$ decreases as $pH_o$ is increased to more alkaline values. With *S. aureus* (Fig. 2) $\Delta pH$ decreases from 125 mV at $pH_o$ 5.6, to zero at $pH_o$ 7.7. It is worth noting here that in these experiments $pH_i$ was determined from the steady-state distribution of the weak acid dimethyloxazolidinedione (DMO). The value of $\Delta pH$, however, obtained by the earlier ion flux method at $pH_o$ 6.45, was 76.7 mV and is clearly in close agreement with the data obtained from the distribution of DMO.

Fig. 2 also depicts the values of $\Delta\Psi$ estimated from the steady state distribution of $K^+$ in valinomycin-treated cells. Here there is the possibility of an overestimation of intracellular $K^+$ due to ion-binding effects and the likely ac-

I wish to spend some time now considering this equation, and the assumptions implicit in its use in transport studies. Firstly, one must stress that such an equation represents a quantitative description of solute uptake, and as such is dependent upon the numbers and experimental data of which it is composed. The usefulness of the equation, or its validity even, is only as good as the experimentally determined values for the individual parameters. A false low figure for $\Delta\Psi$, for example, could result in an incorrect quantitative or even qualitative conclusion being drawn regarding the mechanism of solute transport and accumulation. This potential danger is all the more invidious since there is no way at present of knowing even how close to correct a particular measurement of $\Delta\Psi$ or $\Delta pH$ may be. All the methods at our disposal employ the use of indicators which are claimed to give an estimate of the size of the parameter under study; such claims are based on assumptions regarding the behaviour of the indicator under the conditions of the assay. Only through comparison of values with different indicators, dependent upon different assumptions, can one gain increased confidence in the absolute values of the parameter being assayed. Also, each indicator should be rigorously tested in order to establish that its qualitative pattern of flux and distribution are fully in accord with the basic assumptions on which its use as an indicator depends. Certainly, figures for $\Delta\Psi$, etc., quoted from a single assay procedure with an indicator that has not been fully tested with respect to its qualitative behaviour, must be treated with some degree of caution.

This point is particularly relevant in the context of this present lecture as the proposal of KABACK and his coworkers that proton: solute stoichiometry is a $pH_o$-dependent variable rest on figures for $\Delta\Psi$ in his vesicles significantly lower than those quoted in this paper and more generally throughout the current literature.

The second problem associated with the use of this equation is conceptual rather than practical, and much broader in its significance. The equation defines a thermodynamic equilibrium in which the driving force is coupled with 100% efficiency to the work done in the form of the solute gradient. Furthermore, use of the equation implies that the direction, extent and control of solute flux are directly responsive to the prevailing pmf, with the carrier itself playing no positive role in the energy coupling process; i.e. an ionophore transport model.

At the risk of labouring the point, I wish to stress that these two caveats at one and the same time affect fundamentally the meaning and usefulness of this equation, while being almost universally ignored by those scientists using the equation in the analysis of their transport data.

Simply, there are two alternative approaches. One can accept the equation as a true statement of a transport steady state, experimentally determine $[S]_i$, $\Delta\Psi$ and $\Delta pH$, and deduce the value of n, the proton: solute stoichiometry. RAMOS and KABACK (1977) have interpreted their findings in this manner and deduced that for β-galactosides in *E. coli*, for example, the stoichiometry is a

$pH_o$-dependent variable increasing from one to two over the $pH_o$ range 5.5–7.5.

Our approach has rather been to obtain experimental values for $[S]_i$, $\Delta\Psi$, $\Delta pH$ and n, substitute these values in the equation, and so test its validity, and the validity therefore of the assumptions on which it is based. We have done this for metabolisable glutamate in *S. aureus* and for non-metabolisable lactose in a $z^-$ strain of *E. coli*. In each case the results lead to the same conclusion, but I will only take time to discuss the lactose data in this lecture.

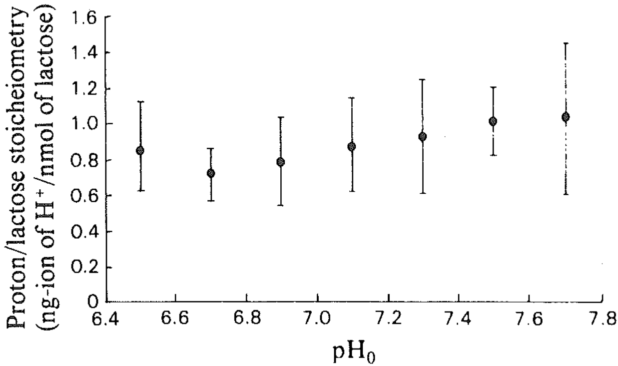


Fig. 4 Effect of $pH_O$ on proton: lactose stoichiometry in *E. coli* ML 308–225. Cells were resuspended in 150 mM KCl weakly buffered with glycylglycine and incubated anaerobically in the presence of iodoacetate. Lactose (1 mM) was added at zero time. (Reprinted with permission from Biochem. J. *182* (1979) 687)

Fig. 4 describes our findings for the proton: lactose stoichiometry across a range of $pH_o$ values. The data are obtained from the comparison of initial rates of uptake of $H^+$ and lactose under deenergised conditions. These experiments are technically difficult to perform and subject to considerable experimental variation, as can be seen from the error bars in this figure. It is quite clear, however, that these data are consistent with a value of $n = 1$ across the $pH_o$ range studied, and give no indication of an increase to $n = 2$. ZILBERSTEIN, SHULDINER and PADAN (1979) also showed the proton: lactose stoichiometry to be 1 at two individual values of $pH_o$, 6.0 and 8.0.

For the neutral sugar lactose, therefore, $m = 0$, $n = 1$, and the equilibrium equation simplifies to:

$$Z \log \frac{[lac]_i}{[lac]_o} = \Delta\Psi - Z\Delta pH = \Delta p$$

As shown on Fig. 5, however, at all values of $pH_o$ tested, the work done does not equal the driving force; the pmf is always in excess of the solute accumulation achieved. That is, there is not a thermodynamic equilibrium with 100%

coupling efficiency between the driving force and the driven solute translocation, and therefore the implicit assumption that the direction, extent and control of solute flux responds directly to changes in $\Delta p$ is also disproven. Consequent upon this analysis, any deduction, such as variable stoichiometry, based on the thermodynamic equation must also be invalid; a point amply reinforced by the experimental finding that the stoichiometry is $pH_o$-independent with a value of 1.

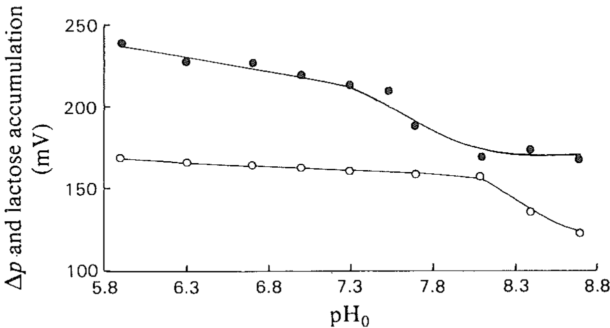


Fig. 5 Comparison of $\Delta p$ (●) in *E. coli* with lactose accumulation, expressed in mV (○), at various values of $pH_o$. (Reprinted with permission from Biochem. J. *182* (1979) 687)

These findings led us to reconsider solute transport rather as a dynamic steady state; in line with earlier kinetic models, but with the extra insight resulting from our present understanding of the proton co-transport mechanism. It has been known, for example, since the work of the Pasteur Institute group in the mid-fifties that the relationship of intracellular to extracellular solute concentrations is hyperbolic rather than linear. This and related findings gave rise to the "pump and leak" model (Kepes, 1960), which had its most sophisticated treatment at the hands of Maloney and Wilson (1973). These authors described transport and accumulation as a kinetic steady state defined by the equation:

$$v_{in} = v_{ex} + D[S]_i$$

where $v_{in}$ and $v_{ex}$ are the steady state rates of carrier-mediated influx and efflux, and $D[S]_i$ is the rate of efflux due to passive diffusion through a carrier-independent route with diffusion coefficient D. At the low solute concentrations employed in most transport assays, the inwardly-directed diffusion, or "leak", pathway is vanishingly small and can be omitted from the left hand side of the kinetic equation.

Using this equation, we have suggested (Booth et al., 1979) that the difference between $\Delta p_{lac}$ and $\Delta p$ depicted in Fig. 5 might be due to the effect of the carrier-independent leak pathway. Accordingly we have recently sought to measure this leak pathway for lactose in *E. coli* by measuring the exit of sugar

from cells treated with p-chloromercurobenzoate sulphonate (PCMBS): this reagent is known to inhibit carrier-mediated processes. Our finding, however, is that there is no passive diffusion leak of lactose from preloaded cells. (Interestingly this result is β-galactoside-specific as identical experiments with thiomethyl β-galactoside [TMG] gave evidence for two distinct routes of efflux; carrier-mediated PCMBS-sensitive and non-carrier-mediated PCMBS-resistant. Both exit pathways demonstrated first-order kinetics with respect to intracellular TMG concentration.)

For the transport of lactose by *E. coli,* therefore, the kinetic equation simplifies to:

$$V_{in} = V_{ex}$$

That is to say, the level of accumulation attained must depend exclusively on the relative rates of the carrier-mediated influx and efflux processes, and it is only through these that the cell can respond to variations in $[S]_o$, $[S]_i$ $\Delta\Psi$, $\Delta pH$ and $pH_i$. In direct contradiction to the discredited equilibrium model, therefore, we conclude that the carrier responds directly to the pmf through modulation of its kinetic parameters, and that this is the mechanism which ultimately leads to the observed levels of intracellular accumulation.

Interestingly, the groups of TANNER and KOMOR in Regensburg, OVERATH in Tübingen, KONINGS in Groningen and even KABACK in New Jersey, are all currently engaged on experimental studies concerned with the effects of variation of the pmf on the kinetic parameters of various influx and efflux processes in whole cell and vesicle systems.

One is still left with the problem of how to visualize the inefficiency of energy coupling to transport, and its $pH_o$-dependence as shown in Fig. 5. In this regard it is interesting to look at the findings of LANYI (1978) with the Na-linked amino acid transport in vesicles of *Halobacterium halobium.* LANYI was able to manipulate his experimental conditions such that he could get transport driven either by the membrane potential or by the chemical gradient of $Na^+$ ($\Delta\mu_{Na^+}$). He was able to show that where there was a linear increase in amino acid accumulation with increasing $\Delta\Psi$, solute uptake appeared to be coupled to the exclusively chemical gradient with less than maximal efficiency, in that an increase in $\Delta\mu_{Na^+}$ did not result in a proportionate increase in the amino acid concentration ratio.

From our own studies (Fig. 5) we can see that the efficiency of energy coupling to lactose transport in *E. coli* appears to be least at $pH_o$ 5.9 where $\Delta pH$ is at the greatest, while at $pH_o$ 8.1 where $\Delta pH$ is very small, $\Delta p$ and $\Delta p_{lac}$ are closest to being equal. ZILBERSTEIN and his colleagues (1979) even find in their work that at $pH_o$ 8.0, $\Delta pH$ is zero and the pmf is coupled to lactose accumulation with 100% efficiency. In *E. coli* cells, therefore, there is also evidence for a difference in the efficiency of the coupling of the electrical and the chemical potentials to solute translocation. It will be extremely interesting to subject this novel idea to rigorous experimental examination.

While it is not yet possible then to give an accurate description of the molecular mechanisms, whereby the pmf and its component parts $\Delta\Psi$, $\Delta pH$ and $pH_i$ modulate the kinetic parameters of carrier-mediated solute flux, clearly this is the direction of present and future studies which aim to elucidate the mechanism of energy coupling in pmf-dependent transport systems.

I should like to acknowledge the collaboration of several colleagues over the period of years covered by this lecture; Dr. D.F. NIVEN, Dr. R.E. JEACOCKE, Mr. T.R. HORNE, Dr. A.W. JARVIS, Dr. S.H. COLLINS, Dr. W.J. MITCHELL, and most particularly Dr. I.R. BOOTH, who is directly responsible for many of the ideas put forward. The work of my laboratory has been supported by grants from the Science Research Council.

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## Note added by the authors:

For experimental details of the work presented in this lecture the articles by BOOTH I.R. et al. (1979), COLLINS S.H. and HAMILTON W.A. (1976) and MITCHELL W.J. et al. (1979) should be consulted.

*Address of the author:*
Dr. W.A. HAMILTON, University of Aberdeen, Dept. of Microbiology, Marischal College, Aberdeen, Scotland GB.