

## Forces, Fluxes and the Control of Microbial Growth and Metabolism

### The Twelfth Fleming Lecture

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#### INTRODUCTION

It is an interesting fact (Macfarlane, 1984) that 58 years elapsed between the publication of the studies of Burdon Sanderson and of Lister showing the antibacterial action of members of the genus *Penicillium* (Burdon Sanderson, 1871; Lister, 1871) and Fleming's somewhat more celebrated paper (Fleming, 1929) to the same effect. Coincidentally enough, a similar period links this latter date with the present year. When seeking to account for Fleming's apparent ignorance of the prior art, Macfarlane (1984) notes that Papacostas & Gaté (1928) had just published a book which contained a 60 page section, headed '*Antibiosis*', on the extensive subject of bacterial inhibition by moulds and by other bacteria, devoted to previously published observations on this phenomenon and with *several hundred* historical references!

The evident moral to be drawn from this tale is that the state of the art as perceived by the collective consciousness (or its foremost representatives) often differs markedly from what has actually been widely recorded in the literature. Thus, given the impossibility of being all-pervasive, one must often, and especially in an overview of the present type, concentrate upon the crucial principles at the expense of the details. I state this by way of an excuse for doing that very thing in what follows, since I shall be seeking, in the most eclectic manner, to summarize my own analysis of several areas of what constitutes a huge field of enquiry: the question of what controls the growth and metabolic rates of bacteria.

Now, the first distinction which we make when we seek to analyse the mechanisms by which bacteria contrive to control the rates at which they grow or metabolize is that between catabolism and anabolism, which are of course linked predominantly by means of the adenine nucleotide system (Fig. 1). Here we see, in a formal sense, the conceptual separation between the provision and utilization of free energy in the living bacterial cell, so that the cell is modelled as a non-equilibrium thermodynamic energy converter. The negative of the free energy change per extent of reaction for the catabolic reactions constitutes the input force (or affinity; see e.g. Prigogine, 1967; Welch, 1985*a*) to the adenine nucleotide system, whilst the output force is represented by the free energy change (per extent of reaction) for the formation of biomass. The relevant fluxes are constituted by the rates at which the catabolic and anabolic processes take place. It is particularly important to note that not all the free energy that is generated by catabolism is actually used to drive anabolism, and this is represented formally as an uncoupled ATP hydrolase reaction. To quantify this contribution in particular, we must define the efficiency of microbial growth.

#### THE THERMODYNAMIC EFFICIENCY OF MICROBIAL GROWTH

In non-equilibrium thermodynamics, the efficiency of an energy converter is given (e.g. Kedem & Caplan, 1965; Stucki, 1980; Westerhoff *et al.*, 1983) by *minus* the product of the output

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This lecture is dedicated to Nancy Kell (1916–1986).

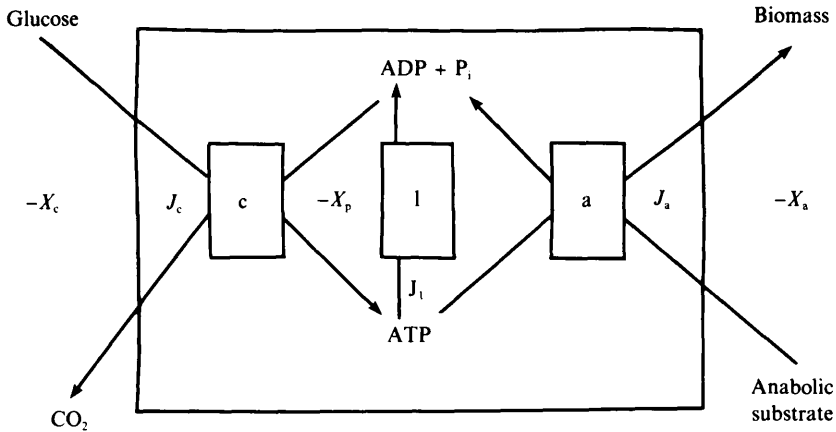


Fig. 1. Simplified formal structure of a prototypical aerobic, heterotrophic microbial cell as a non-equilibrium thermodynamic energy converter. Catabolism is coupled to anabolism by a black box, the cell. The forces and cognate fluxes of the partial reactions are given by the affinities  $X$  and the fluxes  $J$ . The thermodynamic efficiency of a linear energy converter of this type is equal to  $-J_a X_a / J_c X_c$ . Catabolism is coupled to anabolism via the adenine nucleotide system. The coupling between catabolism and phosphorylation is catalysed by an internal black box (and is imperfect), as is the coupling between the ATP system and anabolism. The imperfect couplings are formally collected together as a 'leak' (l) or uncoupled ATP hydrolase activity.

force times the output flux (i.e. rate) divided by the input force times the input flux, in other words by *minus* the output power over the input power. For heterotrophic aerobes growing in a carbon- and energy-substrate-limited chemostat, the relevant free energy changes are those for the formation of biomass by anabolism and of  $\text{CO}_2$  by catabolism of the C-source plus  $\text{O}_2$ . Now as shown by Westerhoff *et al.* (1983), it transpires that in some cases the efficiency of bacterial growth so defined is actually *negative*! This apparently strange result follows from the fact that the free energy change for the production of the appropriate stoichiometry of biomass from the C-source used is such that it is *itself* actually favourable thermodynamically, so that (what is the majority of) the C-source that is used in 'catabolism' is actually in a sense 'wasted'. The appropriate analogy is that of running a car downhill. It will go on its own; all that the engine does is to make it go faster! Indeed, experimental microbial growth yields generally scale monotonically with the degree of reduction of the substrate (Linton & Stephenson, 1978); the changes in chemical potential caused by the oxidation of molecules of various degrees of reduction does provide usable free energy in approximate proportion to the said degrees of reduction. The maximum thermodynamic efficiency of growth that is actually observed in practice, using substrates with a *low* degree of reduction, is some 25% (Westerhoff *et al.*, 1983). In other words, as indicated in Fig. 1 and by many authors (see e.g. Tempest & Neijssel, 1984; Stouthamer & van Verseveld, 1985), the provision of free energy by catabolism is in many cases rather poorly coupled to growth, a fact which is of course indicated by the well-known ability of non-growing, washed cell suspensions of bacteria to glycolyse or to respire at rates that may be little different from those exhibited by growing cultures.

In fact, it was shown by Stucki (1980) that there is an optimal degree of coupling between the input and output reactions in a linear, symmetrical thermodynamic energy converter, and that this optimal degree of coupling itself depends upon what output reaction it is that one wishes to maximize. These possible output reactions include the output force (namely biomass with the greatest free energy content), output flux (i.e. rate of biomass production), the economic rate of biomass synthesis and the economic power production. The degree of coupling also determines the thermodynamic efficiency of the process. Such a non-equilibrium thermodynamic assessment of the 'efficiency of growth' of heterotrophic bacteria indicates that they have in general evolved to permit a maximum metabolic flux of the C- and energy-source at the expense

of efficiency or yield, so that 'the thermodynamic efficiency of microbial growth is low, but optimal for maximum growth rate' (Westerhoff *et al.*, 1983, but cf. Gnaiger, 1987).

In view of our distinction between the contribution of catabolic, anabolic and 'uncoupling' activities to microbial growth, the next rather general question we may ask is: 'which limits growth rate?', or (more accurately, as we shall see), 'which exerts a stronger control on growth rate under a given set of conditions?'. A recent set of studies by Koch and his colleagues (see Koch, 1985) set out to address this question for the case of *Escherichia coli* cells growing on glucose minimal medium in an otherwise unrestricted batch culture.

#### IS THE RATE OF MICROBIAL GROWTH (MORE) LIMITED BY CATABOLISM OR BY ANABOLISM?

Koch reasoned that the best way to determine the rate-limiting step in a steady-state system is to have all the components present in purified form and to add a small quantity of each to the system one at a time, with the expectation that only the limiting component will augment the rate. Evidently this approach is a bit tricky to implement in a growing culture, and an alternative is to *reduce* the quantity of each component by a small amount. Suitable inhibitors may be used for this, and Harvey & Koch (1980) showed that chloramphenicol behaved as an 'ideal' inhibitor of protein synthesis (i.e. of anabolism) under the conditions chosen; in other words, each molecule added inhibited protein synthesis non-competitively and in proportion to the amount bound, and the drug is bacteriostatic rather than bactericidal. It was found (Harvey & Koch, 1980) that the smallest additions of chloramphenicol decreased the normalized growth rate, so that the conclusions which were drawn were (a) that there were no excess ribosomes under the conditions used and (b) that the absence of a threshold in the titration curve indicated that growth was limited by anabolism rather than catabolism, a conclusion opposite to that divined by Andersen & von Meyenburg (1980) who also worked with batch cultures of the same organism. We shall see that whilst this *general* approach is both sound and powerful, the conclusions relating to (b) are not in fact watertight. To show this, however, we must first digress briefly to consider the concept of a metabolic pool.

#### THE ORGANIZATION OF METABOLIC SYSTEMS: POOLS OR CHANNELS?

Consider a prototypical unbranched metabolic pathway of the form  $A \rightarrow B \rightarrow C \rightarrow D \rightarrow E$ , with each step being catalysed by a particular enzyme  $e_1$  to  $e_4$ . Such a pathway may be organized in two general types of fashion, the extreme versions of which we may refer to as 'pool/delocalized' or 'channelled/localized'. The pool version is one in which each molecule of a given type of intermediate may be used with equal rapidity by any of the enzymes in the cell for which it is a substrate, as one observes with an enzyme exhibiting Michaelis-Menten kinetics *in vitro*. In a perfectly channelled system, however, a particular intermediate (say C) may be utilized only by a *particular* molecule of enzyme  $e_n$ . Evidently, the intermediates must remain protein-associated, and their chemical potential may be very different from what it would be if they were free (Welch, 1977; Ling, 1984). Of course, these types of model represent extreme behaviour, and one may imagine cases in which the behaviour of a metabolic system is intermediate between purely delocalized and purely channelled. Similar comments may be made for reaction-diffusion between membranous enzymes, and Rich (1984) and Ragan & Cottingham (1985) give useful discussions of the relevant considerations and experimental data for respiratory electron transport chains.

Whilst rarely noted in textbooks, one should state that there is a wealth of evidence to the effect that several, if not most, of the primary metabolic pathways of bacteria (and eukaryotes) are organized more according to the channelling model than to the pool model (e.g. Ottaway & Mowbray, 1977; Welch, 1977, 1985*b*; Kell, 1979; Friedrich, 1984; Srere, 1987; Welch & Clegg, 1987). Indeed, there are some cases in which the formal catabolic reactions by which an organism gains its free energy have a free energy change less than that needed to make one molecule of ATP at the prevalent phosphorylation potential. One such is *Syntrophomonas wolfei*, which can

use the reaction  $\text{butyrate} + 2\text{H}_2\text{O} \rightarrow 2 \text{acetate} + \text{H}^+ + \text{H}_2$  to obtain the free energy it requires for growth (McInerney *et al.*, 1981). The free energy change for this reaction under typical conditions *in vivo* is some  $-15 \text{ kJ mol}^{-1}$ , whilst the intracellular phosphorylation potential may be some  $-45 \text{ kJ mol}^{-1}$ , so that (at least) three quanta of free energy from the input reaction must be transferred before a single ATP molecule may be synthesized (Thauer & Morris, 1984). It is not known how this is achieved, but it is implausible (Kell & Walter, 1987) due to the irreversibility and threshold properties of such reactions (Welch & Kell, 1986), that the free energy may be transduced via a pool intermediate. For reasons of space, I can list neither the many criteria nor all the different types of evidence which are generally used to distinguish channelled from pool behaviour; however, I shall mention one particularly germane approach with which we have been involved.

#### INHIBITOR TITRATIONS TO DISTINGUISH POOLS FROM CHANNELS

Fig. 1 indicated the importance of free energy transduction via the ATP system in the consideration of the control of bacterial growth and metabolism; many of the studies which I shall discuss have therefore been concerned with the mechanism of ATP synthesis, and, since most of that in Nature is linked to electron transport, with the mechanism of electron-transport-linked phosphorylation. As with Koch's experiment with chloramphenicol, and following the lead of Baum *et al.* (1971), we too have chosen to use inhibitors to approach the question of whether something with the property of a pool acts as an intermediate in bacterial electron-transport-linked ATP synthesis (see e.g. Hitchens & Kell, 1982*a, b*; Westerhoff *et al.*, 1984*b*; Kell & Westerhoff, 1985; Kell, 1986*b*). The reasoning goes as follows.

According to Mitchell's (1966) chemiosmotic coupling hypothesis (e.g. Nicholls, 1982; Harold, 1986), electron transport is coupled to phosphorylation by means of an electrochemical potential difference of protons, the protonmotive force, between the inside and outside of the phase bounded by the bacterial cytoplasmic membrane. The idea is that every redox-linked proton pump can deliver free energy to every ATP synthase molecule in the vesicle of interest, since the protonmotive force, thermodynamically defined as the sum of a bulk-to-bulk phase transmembrane potential and pH gradient, has the property of a delocalized intermediate. One way to assess whether the protonmotive force is the (or a) 'high-energy' intermediate in electron transport phosphorylation is therefore to test whether the intermediate exhibits pool/delocalized behaviour. We have found bacterial chromatophores to be an appropriate system for such studies.

Chromatophores are membrane vesicles formed upon sonication of phototrophically grown photosynthetic bacteria, and have an orientation opposite to that *in vivo*, i.e. they are 'inside-out'. Since ADP is membrane-impermeant, this permits the convenient observation of photophosphorylation. It is germane to mention that the lateral differentiation implicit in the organization of the photosynthetic bacterial membrane into cytoplasmic and intracytoplasmic membranes does not sit easily (e.g. Kell, 1984, 1986*c*) with the idea (Hackenbrock *et al.*, 1986) that the lateral mobility of membrane components is both random and rapid. However, the significance of this for the mechanism of photophosphorylation is not known. Such chromatophores contain a cyclic electron transport chain and the ATP synthase enzymes, *inter alia*. The former may be inhibited at the level of the *b* cytochromes by antimycin A, whilst the latter are inhibited by oligomycin, venturicidin or the covalent modifier dicyclohexylcarbodiimide.

The analysis then goes roughly as follows. One titrates the output flux, here the rate of photophosphorylation, with say antimycin A, and might obtain a hyperbolic titration curve. One then inhibits photophosphorylation by say 50% using an inhibitor of the ATP synthase enzymes. If the intermediate is a pool, the normalized rate of photophosphorylation should now be *less* sensitive to antimycin A, whilst if it is perfectly channelled it should be equally sensitive. The results in chromatophores (Hitchens & Kell, 1982*a, b*), chloroplast thylakoids (Davenport, 1985) and submitochondrial particles (Herweijer *et al.*, 1985) are at first sight in favour of perfect channelling. However, if the rate of phosphorylation was simply controlled by the magnitude of

the protonmotive force, in other words by the concentration of the putative delocalized intermediate, and if each ATP synthase was going at its maximum rate, then if all that the titration with antimycin A did was to decrease the magnitude of the protonmotive force a result such as that obtained might also be expected with a delocalized system under certain conditions (Parsonage & Ferguson, 1982; Davenport, 1985; Ferguson, 1985; Chen, 1986; Pietrobon & Caplan, 1986*a, b*; Pietrobon, 1986). This possibility is decreased, but not in fact absolutely eliminated, by the results of the symmetrical experiment, where similar behaviour is observed; the titre of ATP synthase inhibitor required to inhibit photophosphorylation by a certain amount neither possesses a threshold nor is increased by the presence of a partially inhibitory titre of antimycin A (Hitchens & Kell, 1982*b*; Kell & Hitchens, 1983).

A further set of these experiments, which collectively have come to be known as dual-inhibitor titrations, was carried out with uncouplers and ATP synthase inhibitors (Hitchens & Kell, 1983*a, b*). Uncouplers have the property that whilst they do not directly inhibit the activity of electron transport or ATP synthase enzymes *per se*, they inhibit the coupling between them. The textbook view is that uncouplers are protonophores and, more important, act *thereby* by decreasing the magnitude of the protonmotive force by catalysing electrogenic proton backflux across coupling membranes, probably in preferential locations (Kell & Westerhoff, 1985). The most active uncouplers, such as those used by us herein, are 'substoichiometric', i.e. the titre necessary for full uncoupling is less than the number of redox chains or ATP synthase enzymes present, and this is important to the present analysis since it sets the boundary condition that all uncoupler molecules may be working all of the time (e.g. Kell, 1986*d*). The expectation here was that if phosphorylation was limited by the magnitude of the protonmotive force, and uncouplers decreased it, then they should not do so more efficiently (in a delocalized model) if a partially inhibitory titre of ATP synthase inhibitor was present, since this would, if anything (and see later), be expected to raise the 'starting' protonmotive force. By contrast, if coupling was localized, and the rate-limiting step was uncoupling itself and not diffusion to the site of action, one might imagine that substoichiometric uncouplers could work *more* potently when the partially inhibitory titre of ATP synthase inhibitor was present, since uncouplers can, by definition, only uncouple something which could be coupled in the first place! In practice (e.g. Hitchens & Kell, 1983*a, b*; Davenport, 1985; Herweijer *et al.*, 1986), the latter behaviour may indeed be observed. All these results have been reproduced with many uncoupler/inhibitor combinations in several systems by a number of different groups, and although again at first sight clear-cut, the conclusions turn out not, on this evidence *alone*, to be watertight. To see why, we must briefly mention the metabolic control analysis developed by Kacser & Burns (1973) and Heinrich & Rapoport (1974). This formalism is now enjoying something of a renaissance of interest, for the very good reason that it provides a rigorous and exact approach to the treatment of steady-state metabolic systems (see e.g. Groen *et al.*, 1982; Westerhoff *et al.*, 1984*a*; Derr, 1985, 1986; Porteous, 1985; Keleti & Vértessy, 1986; Kell & Westerhoff, 1986*a, b*; Kacser & Porteous, 1987; Westerhoff & Kell, 1987*a, b*).

#### METABOLIC CONTROL ANALYSIS: ITS GENERAL SCOPE AND ITS USE IN DISTINGUISHING POOLS FROM CHANNELS

In our prototypical metabolic scheme  $A \rightarrow \dots \rightarrow E$ , we may as before ask which enzyme (or group of enzymes) is catalysing the rate-limiting step or portion of the pathway. Using the general approach described above, then, we may, as a *gedanken* experiment (Kell & Westerhoff, 1986*a*), add small amounts of each of the enzymes and observe the effect upon the pathway flux. One may imagine at least three possible outcomes. Two are, one may say, more conventional. In the first case there is no measurable change in flux, and we would say that the enzyme was not rate-limiting, whilst in the second case the flux is changed *in direct proportion* to the amount of extra enzyme, so one would say that the enzyme *was* rate-limiting. In each case we consider only the steady-state behaviour. The third possibility, however, lies between the other two: the flux is increased but not in direct proportion to the change in enzyme concentration. Thus, a qualitative, two-valued logic system (rate-limiting/non-rate-limiting) is inadequate, and a

quantitative measure of flux control is required. By normalizing the fluxes and the amounts of enzyme, so that we consider the fractional changes in each, we obtain such a quantification; it is known as the *flux-control coefficient* and ascribes to *each* enzyme in (or affecting) a pathway a number, usually (though not always) between 0 and 1, which defines the extent to which it controls the flux.

According to the flux-control summation theorem, *and on the assumption of pool behaviour of the intermediates*, the flux-control coefficients of all the enzymes in or affecting a metabolic pathway add up to 1. This theorem has been proved rigorously (Kacser & Burns, 1973), and it should be noted that for branched pathways the enzymes in the branch other than that of primary interest will tend to have *negative* flux-control coefficients, since stimulating them will *decrease* the flux of interest. Flux-control coefficients are not constants for particular enzymes but will tend to change with conditions; a particular enzyme may be more or less flux-controlling depending upon the values of parameters including the relative activities of the other enzymes, and Walsh & Koshland (1985) give an elegant example in *E. coli*.

The *distribution* of control in a metabolic pathway is obviously a function of the properties of the individual enzymes that it contains (or of those which, by changing the concentrations of allosteric modifiers, otherwise affect the flux), and the control analysis refers to these enzymic properties as elasticity coefficients or *elasticities*. These are defined as the fractional change in enzyme turnover number divided by the fractional change in the concentration of the effector or substrate of interest, under conditions in which all other parameters and variables are held constant. Any enzyme has as many elasticities as there are molecules present which significantly affect its activity, and simple connectivity theorems, which will not concern us here, serve to express flux-control coefficients in terms of elasticities alone. In general, enzymes with low elasticities towards their substrates (and which are therefore saturated) have high flux-control coefficients. It should be obvious, then, that the control analysis, which contains a number of other theorems, is *the* way to approach the question of what regulates metabolic fluxes, and also therefore provides, of course, a rational approach to the optimization of microbial fermentations used for the production of substances of commercial interest (Kell & Westerhoff, 1986*a, b*; Westerhoff & Kell, 1987*a*). Because of the assumption of pool behaviour embodied in the control analysis, however, the analysis also provides a rigorous *test* of pool behaviour, since systems exhibiting channelling behaviour will violate the summation theorem when studied by means of inhibitors (Kell & Westerhoff, 1985; Kell & Walter, 1987; Petronilli *et al.*, 1987; Westerhoff & Kell, 1987*b*). This is because removing individual molecules of *any* enzyme in a (non-leaky) 'supercomplex' (which may be functional rather than structural) will serve to inhibit the activity of the entire pathway in direct proportion (i.e. the flux-control coefficient will be 1), and this will be true for *each* enzyme.

The availability of tight-binding and specific inhibitors permits a facile estimation of the flux-control coefficients, since the extrapolated titre for full inhibition gives the number of target enzyme molecules present, and the lack of threshold (i.e. a hyperbolic titration curve) means, indeed, that the flux-control coefficient for the target enzyme will be 1. Artefacts such as the presence of inactive binding sites for inhibitors will always *lower* the apparent flux-control coefficient. Hyperbolic curves of the stated type were observed in the experiments of Harvey & Koch (1980) on the control of growth by anabolism, and in our own work (Hitchens & Kell, 1982*a, b*) for both electron transport and ATP synthase enzymes, so that in the latter case the flux-control summation theorem would indeed seem to be violated and pool behaviour disproved.

Actually even this analysis has forgotten one crucial point: as with microbial growth, the electron-transport-linked phosphorylation system is itself not perfectly coupled, and as before must be modelled at the minimum as a *branched* pathway in which what we may call the energy 'leak' is a side-branch. In other words, the leak will have a *negative* flux-control coefficient towards the phosphorylative flux and if its magnitude were as great as  $-1$  then the flux-control summation theorem (and the assumption of pool behaviour) would be inviolate. This might be the case, for instance, if the system was extremely poorly coupled or leaky, so that one should be aware of the elasticities of each of the enzymes towards each of the 'metabolites' present.

However, it turns out from the relevant analyses, which are mathematically rather involved (Pietrobon & Caplan, 1986*a, b*; Petronilli *et al.*, 1987; Westerhoff & Kell, 1987*b*), that no coupling scheme involving pool behaviour can accommodate the data when they are considered *in toto*. All of this then serves to make the points (i) that the description of what controls metabolic fluxes is not as simple as once believed, but *may* be addressed rigorously and quantitatively, and (ii) that some systems, such as those involved in electron-transport-linked phosphorylation, are not coupled by means of pool or delocalized intermediates. Especially in the case of energy-coupling systems, this latter point raises thermodynamic difficulties of a philosophical nature, which have not been resolved to date (Blumenfeld, 1981, 1983; Welch & Kell, 1986; Kell, 1987*a*), although it is interesting to note that there is an isomorphism between the flux-control coefficients of the metabolic control analysis and the thermodynamic parameters used in the phenomenological non-equilibrium thermodynamic analysis of linear energy converters (Stucki, 1983).

#### CONTROL ANALYSIS OF THE ROLE OF ANABOLISM VERSUS CATABOLISM IN DETERMINING MICROBIAL GROWTH RATES IN UNRESTRICTED BATCH CULTURE

I mentioned that the measurement of anabolism alone suggested that it limited growth in batch cultures, and it is clear now that this view is at best a partial truth. A recent study from this laboratory (Walter *et al.*, 1987) concerned the mechanism by which media of superoptimal osmolality inhibited the growth of certain anaerobic bacteria; was it due to a low water activity (as is usually assumed) or to other factors, and what were the target sites at which growth inhibition was exerted? When the rate of growth and glycolysis of *Clostridium pasteurianum* are titrated with the relatively membrane-impermeant solute xylitol under similar, though I must stress unfortunately not identical, conditions, it is observed that both are decreased, though not in strict parallel (Walter *et al.*, 1987). From a similar analysis to that given earlier, we see that at least some control on the growth rate is associated with catabolism in this organism. The effect of a number of other solutes on growth strongly suggested that membrane-permeant substances were far more inhibitory than membrane-impermeant ones, and mechanistically, it transpired (Walter *et al.*, 1987) that a major target of growth inhibition was provided by the membrane-located glucose phosphotransferase enzyme. Nevertheless, by measuring the glycolytic flux and the enzyme turnover under the same conditions, the flux-control coefficient of this enzyme on glycolysis was found not to exceed 0.2 (Walter *et al.*, 1987). A particular advantage of this approach is that the use of membrane-impermeant inhibitors automatically selects for the carrier molecules implicated in the uptake of the substrate of interest, and thus provides the specificity which is otherwise lacking chemically. I know of no study to date which has sought systematically to address the question of the relative contributions of catabolism, anabolism and 'leak' to the control of microbial growth rate under defined conditions by the use of the control analysis, and it is obvious that such studies are urgently needed. The control analysis does, for instance, provide a ready explanation of why micro-organisms conform more to Blackman than to Monod kinetics (Dabes *et al.*, 1973), although the use of the control analysis in exponentially growing cultures on a long time-scale deems that such cultures be considered to be in an expanding stationary state (Kacser, 1983). One of the particular strengths of the control analysis lies in the fact that it seeks to relate the behaviour of a system (the cell or a metabolic pathway) to the properties of its components, the enzymes, and I would like to digress to stress one point concerning enzymic properties which has thus far not apparently been considered in the microbial literature.

#### VISCOSITY, AND THE MAGNITUDE OF ENZYME CATALYTIC RATE CONSTANTS

It is now well known that the molecular evolution of individual enzymes is generally constrained so as to provide them with properties suitable for their function *in vivo*. In particular, it is now becoming appreciated that the values of both  $K_m$  and  $k_{cat}$  of a 'soluble' enzyme are generally strongly affected by the prevailing viscosity (e.g. Beece *et al.*, 1980; Somogyi *et al.*, 1984; Welch, 1986*a*). Since it is, from a physical standpoint, mainly the *viscosity* which

represents or controls at the molecular level the forces with which a protein interacts with its environment, and the viscosity is for instance strongly temperature-dependent, it is to be assumed that molecules have evolved to attain a degree of conformational flexibility (and hence catalytic rate constant; see Welch, 1986*b*) consistent with the conditions of 'viscosity' *in vivo* (Alexandrov, 1977; Somero, 1978). Whilst *viscotaxic* organisms have been described (Petrino & Doetsch, 1978; Daniels *et al.*, 1980; Murvanidze *et al.*, 1982), I know of no search for a 'viscophilic' micro-organism (or indeed the use of this term, despite the many 'extremophiles' whose physiology is now under intense study). It is at least plausible that some of these organisms, especially amongst the so-called xerophiles or osmophiles but perhaps also amongst the thermophiles, are actually viscophiles. Given that the  $k_{\text{cat}}$  of an enzyme is often a strong function of the viscosity *in vitro*, it is reasonable to suppose that those wishing to increase the catalytic rate constants of industrial enzymes, which are often woefully low (Kell & Westerhoff, 1986*b*), might seek out a viscophilic micro-organism as a source of enzyme and then run the reaction at a much lower viscosity. Obviously the 'viscosity' of the bacterial cytoplasm is a heterogenous and uncertain quantity, but no more so than that of the eukaryotic cytoplasm, where substantial progress is being made towards an understanding of its meaning (e.g. Clegg, 1984).

#### ANOTHER APPROACH TO AN ASSESSMENT OF THE ROLE OF THE PROTONMOTIVE FORCE IN ELECTRON-TRANSPORT-LINKED PHOSPHORYLATION

From the inhibitor titrations mentioned above, I cast doubt on the view that any delocalized intermediate, which includes the protonmotive force, could be a major energetic intermediate in electron-transport-linked phosphorylation. However, since (generally rather modest) pH gradients and a reasonably homeostatic degree of pH regulation are well known facets of microbial bioenergetics (Booth, 1985), and many studies reviewed by Kell (1986*a*) have shown that a very large *artificial* protonmotive force can drive phosphorylation in all the relevant systems investigated, logic demands, and *direct* estimation indicates (Giulian & Diacumakos, 1977; Tedeschi, 1980, 1981; Ferguson, 1985; but cf. Felle *et al.*, 1980), that the finger of suspicion be pointed at the putative transmembrane electrical potential. Is it energetically significant or not? Well, a study which I shall now summarize, and which was based upon the classical O<sub>2</sub>-pulse technique, suggests that it cannot be.

In a typical O<sub>2</sub>-pulse experiment (Scholes & Mitchell, 1970), one adds a pulse of a known amount of O<sub>2</sub>, as air-saturated KCl, to a well-stirred, weakly buffered suspension of bacteria, and observes the pH changes in the extracellular phase which accompany the reduction of the O<sub>2</sub>. From calibration experiments and from the excursion of the pH trace, one can then obtain the stoichiometry of proton ejection, known as the  $\rightarrow\text{H}^+/\text{O}$  ratio. Three general and qualitatively different types of trace are obtained for a given size of O<sub>2</sub> pulse (Scholes & Mitchell, 1970; Gould & Cramer, 1977; Gould, 1979; Kell & Hitchens, 1982; Hitchens & Kell, 1984). Since the cells and the amount of O<sub>2</sub> added are in each case the same, the question obviously arises as to what is different! The first type of trace is the standard trace observed when cells are suspended in 150 M-KCl at pH 6.5: the apparent  $\rightarrow\text{H}^+/\text{O}$  ratio is rather small and the observable backflux of protons across the cytoplasmic membrane of the bacterium is almost immeasurably small. The second type of trace is observed in the presence of a 'magic molecule' such as valinomycin or the thiocyanate ion (SCN<sup>-</sup>); both the observable  $\rightarrow\text{H}^+/\text{O}$  stoichiometry and the rate of proton backflux are significantly increased. The final type of trace is that observed (in the absence or presence of SCN<sup>-</sup>) when an (appropriate amount of) uncoupler is present, and serves to show that all the protons otherwise observed were vectorial, i.e. pumped across the membrane, and not the result of some uninteresting scalar reaction. What then is the magic molecule doing?

The classical chemiosmotic explanation of such data is that the energy coupling membrane has a relatively low electrical capacitance (we shall return to this later), so that the electrogenic translocation of even a small number of protons across the membrane between phases in equilibrium with the bulk aqueous phases on either side of it leads to the generation of a substantial transmembrane potential difference. This either inhibits further proton

translocation by causing 'slippage' or drives a backflux of protons from the bulk external aqueous phase that is more rapid than the response time of the pH electrode. The membrane-permeant thiocyanate ion can dissipate this membrane potential, leading to the manifestation of a greater  $\rightarrow\text{H}^+/\text{O}$  ratio. The uncoupler, as a protonophore, catalyses a very rapid backflux of protons across the membrane. Whilst the fact that the observable backflux of protons is greater in the presence of  $\text{SCN}^-$  when the protonmotive force is expected to be smaller is not obviously consistent with this explanation (Ferguson, 1985; Kell, 1986*a*), direct tests of this explanation of the role of  $\text{SCN}^-$  are possible.

If the apparently low  $\rightarrow\text{H}^+/\text{O}$  ratio observed in the absence of  $\text{SCN}^-$  is caused by the buildup of a substantial bulk-to-bulk phase transmembrane potential, then an increase in the amount of  $\text{O}_2$  added should be accompanied by no increase in the number of  $\text{H}^+$  observed (and thus to a decrease in the  $\rightarrow\text{H}^+/\text{O}$  ratio), since the membrane potential should be saturated at its highest possible value. What happens when one does the experiment (Gould & Cramer, 1977; Gould, 1979; Kell & Hitchens, 1982; Hitchens & Kell, 1984)? The answer is that the number of protons observed increases in strict proportion to the amount of  $\text{O}_2$  added over a wide range. So whatever it is that is stopping us seeing the right number of protons, it is not a chemiosmotic membrane potential. Nonetheless, we have *observed* vectorially ejected protons in the outer bulk aqueous phase, so they or at least the overwhelming majority of them, must obviously have arrived there electroneutrally, either in symport or antiport with an appropriately charged ion. This would be consistent with the stimulation by  $\text{SCN}^-$  of the observable rate of proton backflux (Ferguson, 1985; Taylor & Jackson, 1985*a, b*).

The addition of venturicidin, an ATP synthase inhibitor, to this type of system, permits a further test of the theory. Under quasi-stationary conditions, the available degrees of freedom by which a chemiosmotic coupling model can account for a variable  $\rightarrow\text{H}^+/\text{O}$  ratio are that a high protonmotive force induces either redox slip (i.e. the passage of electrons without concomitant pumping of protons) or non-ohmic leak (i.e. a backflux of protons whose rate is greatly increased as the protonmotive force increases). The effect of venturicidin (Hitchens & Kell, 1984) is to raise the  $\rightarrow\text{H}^+/\text{O}$  ratio, which excludes redox slip (see also Cotton *et al.*, 1981), whilst this reagent also *increases* the initial rate of return of protons from the outer aqueous phase, possibly through its weak action as a  $\text{Cl}^-/\text{OH}^-$  exchanger. Together, these results show us that, within experimental error, all (i.e. the overwhelming majority) of the protons observed in the bulk are electroneutral and are thus not contributing to the generation of a membrane potential. The next question then is, *are any* of them?

The way to answer this is to turn the above type of experiment on its head. The equation for the charging of a parallel plate capacitor with a capacitance of  $C$  Farads is  $Q = CV$ , where  $V$  is the voltage generated between its plates when  $Q$  Coulombs of electrical charge are passed across it. Since  $Q$  is for us given by the number of protons *times* the elementary electrical charge, then provided that we know the membrane capacitance per unit area and the cell number we may calculate the *maximum* membrane potential which could possibly be built up on the most charitable assumptions (Kell, 1986*a*) (i) that *every* proton observed is electrogenic and (ii) that the cells are spheres of their observable diameter with no membrane invaginations. By decreasing the cell: $\text{O}_2$  ratio, we may make the membrane potential arbitrarily small, so that if what is stopping us seeing the correct number of protons is a membrane potential then by making the maximum possible value of the latter small, which means less than  $kT$ , we should see an upturn in the  $\rightarrow\text{H}^+/\text{O}$  ratio at very low membrane potentials (Gould & Cramer, 1977; Kell & Hitchens, 1982; Taylor & Jackson, 1985*a, b*). I will delay a description of how we measure the membrane capacitance per unit area, and simply state that, as with other biological membranes (Cole, 1972), it is approximately  $1 \mu\text{F cm}^{-2}$ . The data show (Gould & Cramer, 1977; Kell & Hitchens, 1982; Taylor & Jackson, 1985*a, b*) that even at very low values of the putative membrane potential the  $\rightarrow\text{H}^+/\text{O}$  ratio is still very much less than its true, limiting value, and the conclusion yet again is that whatever is stopping one seeing the proper number of protons in the external aqueous phase it really does not seem possible that it can be an energetically significant membrane potential. I am well aware that this conclusion may appear extremely heterodox to a great many readers; however, it is one that is gaining an increasing acceptance, and has the

merit of accounting simply for the observable protonmotive activity and the rapid phosphorylation driven by a *large* protonmotive force on the one hand and with the many observations which on the other hand do not seem to favour a delocalized chemiosmotic coupling model. Stimulated in particular by the poorly recognized *inability* of co-reconstituted phosphorylating systems to display an adequate turnover number *in vitro*, one is directed to a consideration of the dynamic lateral organization of the bacterial cytoplasmic membrane, which remains poorly understood. Additional and specific mechanistic proposals, implicating the involvement of 'protonural' proteins distinct from those polypeptides in the generally recognized respiratory chain and ATP synthase complexes, have been outlined elsewhere (Kell *et al.*, 1981; Kell & Morris, 1981; Kell & Westerhoff, 1985).

#### DETERMINATION OF THE ELECTRICAL CAPACITANCE OF THE BACTERIAL CYTOPLASMIC MEMBRANE

Since the estimation of the membrane capacitance is so important for the experiments described in the previous paragraph, and one cannot measure it *directly* by sticking a microelectrode inside a coccus of 1  $\mu\text{m}$  diameter, the question obviously arises as to how we *can* measure it. The answer is that we pass alternating current into the suspension via two (or more) *extracellular* electrodes. The macroscopic electrical properties of such an arrangement are encompassed (see e.g. Schwan, 1957; Grant *et al.*, 1978; Pethig, 1979; Harris & Kell, 1985*a*; Kell & Harris, 1985*a, b*; Kell, 1986*b*; Pethig & Kell, 1987) in the electrical admittance of the system, which consists of the complex vector sum of the observed conductance and the susceptance, the susceptance being equal to the angular frequency of the field *times* the capacitance. These are extensive properties of the system, and depend on the electrode size and geometry. The relevant *intrinsic* or *dielectric* properties of the system are the conductivity and permittivity, which take the electrode geometry or 'cell constant' into account.

What we find when we measure the dielectric permittivity of a cell suspension is that it is strongly frequency-dependent. The frequency dependence of the dielectric properties of a system is known as dielectric dispersion, and most biological cells possess at least three major dispersions, referred to as the  $\alpha$ -,  $\beta$ - and  $\gamma$ -dispersions (Schwan 1957). Our interest is focused on the  $\beta$ -dispersion, which occurs in the radio-frequency range of the electromagnetic spectrum, say between 0.1 and 100 MHz. Physically, the explanation of this dispersion is that at high frequencies the membrane capacitance is short-circuited whilst at low frequencies it may be fully charged up by ion migration to the interfaces. Fricke (1925) gave the equation which relates the capacitance of the membrane per unit area to the macroscopic permittivity  $\epsilon'_L$  of a suspension of (spherical) membrane-enclosed cells at a frequency that is suitably 'low' relative to that of the  $\beta$ -dispersion:

$$\epsilon'_L = \epsilon'_{\infty} + 9PrC_m/4\epsilon_0 \quad \text{eq. (1)}$$

where  $\epsilon'_{\infty}$  is the high-frequency permittivity,  $P$  the volume fraction of the suspended phase (i.e. that enclosed by the cytoplasmic membrane),  $r$  the cell radius,  $C_m$  the membrane capacitance per unit area and  $\epsilon_0$  the permittivity of free space ( $= 8.854 \times 10^{-12} \text{ F m}^{-1}$ ). In other words, given a knowledge of the number and radius of the cells, we can obtain  $C_m$  simply by measuring  $\epsilon'_L$  and  $\epsilon'_{\infty}$ . This approach is of historical significance in that it was Fricke (1925) who first determined that biological membranes should be of molecular thickness; he used the equation and electrical approach described to obtain a value for the erythrocyte membrane capacitance of  $0.81 \mu\text{F cm}^{-2}$ , and on the assumption that the permittivity of membranes was between 3 and 10, derived a value for the thickness of 3–10 nm. The value calculated for the capacitance of the cytoplasmic membrane of *Paracoccus denitrificans* using this approach is again in the range  $0.5$ – $1 \mu\text{F cm}^{-2}$  (Harris & Kell, 1985*a*).

Now all of this may seem pretty *recondite*, and we are constantly being urged by our supposed paymasters (the Government) to do so-called relevant research. Equally, it will not come as news to most readers that the most interesting discoveries invariably come, as did Fleming's penicillin, rather *directly* from 'pure', unfettered, non-goal-oriented research. I therefore wish to end this overview by illustrating that this remains true in an area of microbiology somewhat different from recombinant DNA work.

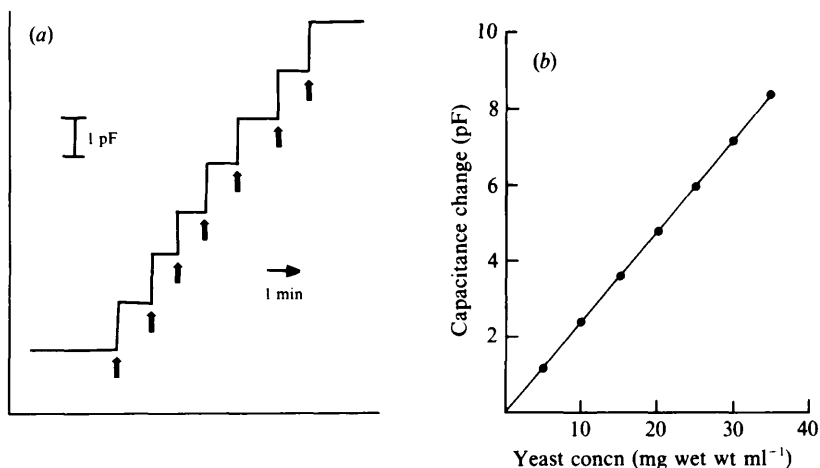


Fig. 2. Real-time estimation of microbial biomass by determination of the radio-frequency capacitance of cell suspensions. Measurements were made at 0.3 MHz using the four-terminal instrument described previously (Harris *et al.*, 1987; Kell, 1987*b*; Kell *et al.*, 1987) except that the probe was constructed of PTFE (polytetrafluoroethylene) and stainless steel and had a cell constant of 2.9 cm<sup>-1</sup>. The reaction mixture consisted of 100 ml 0.1 M-potassium phosphate, pH 7.0, and the capacitance value was backed off to read 0 pF when buffer alone was present. (a) Time-dependent behaviour. At the arrows, small volumes of a concentrated stock suspension of *Saccharomyces cerevisiae* in the same buffer were added so as to increase the biomass content of the suspension by 5 mg wet wt ml<sup>-1</sup>. (b) Capacitance of the suspension plotted versus the biomass content, using the data in (a).

#### A REAL-TIME SENSOR FOR MICROBIAL BIOMASS

We have seen some of the ways in which micro-organisms control their own activities, but as fermentation technologists we would generally wish to control them *exogenously*, and preferably on-line (e.g. Kell, 1980; Clarke *et al.*, 1982, 1985; Carleysmith & Fox, 1984). To do so we must measure the properties of our fermenter broths, and one of the more obviously important variables is the microbial biomass concentration, something which has been notoriously difficult to measure (Harris & Kell, 1985*b*; Meyer *et al.*, 1985; Clarke *et al.*, 1986). Now we saw from equation (1) that if we knew the volume fraction of the cells we could estimate the membrane capacitance per unit area by measuring the dielectric permittivity of the suspension. Similarly, the volume fraction of the membrane-enclosed phase, which obviously equates to biomass, is a direct and monotonic function of the permittivity at low radio frequencies (Harris & Kell, 1983), so that measuring the latter provides the basis for what amounts to an almost 'ideal biomass probe'. It responds in real time, is non-destructive, the metal electrodes are autoclavable and may be cleaned by electrolysis (Dhar, 1985) *in situ*, it is adequately sensitive to membrane-enclosed cells, is essentially insensitive to non-cellular particulate matter, and the RF permittivity is linear with biomass content up to extremely high volume fractions. In collaboration with Dulas Engineering in Wales and with ICI Biological Products at Billingham we have therefore built an electronic box of tricks (the 'Bug Meter') to make such measurements (Harris *et al.*, 1987; Kell, 1987*b*; Kell *et al.*, 1987). For technical reasons to do with electrode polarization (Kell, 1986*b*), four electrodes, two for current and two for voltage, are used, and Fig. 2 shows a typical trace to illustrate the effects of biomass on the RF permittivity (capacitance) of the system.

It is perhaps worth noting, in view of the current financial and intellectual climate, that this novel solution to the problem of microbial biomass estimation during fermentations followed rather directly from 'purely' academic studies of the organization of the cytoplasmic membrane of bacteria.

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