ORGANIZATION AND CONTROL OF ENERGY METABOLISM IN ANAEROBIC MICROORGANISMS

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INTRODUCTION AND SCOPE

A recurrent question, which dates from the very origins of modern biochemistry itself (see Schlenk, 1985), and which constitutes a major theme of the present conference, concerns the degree of relatedness between the organization and activities of the enzymes of cellular energy metabolism *in vivo* and their behavior *in vitro*. At one level, two extreme types of viewpoint, which we may refer to as "holistic" and "reductionist", may be discerned.

The reductionist school would hold that the ability to reconstruct a biochemical pathway, using isolated enzymes *in vitro*, at a rate, and with a sensitivity to effectors, similar to that obtained *in vivo*, provides the evidence necessary and sufficient to define the system operating *in vivo*. Any inability to achieve such a reconstruction may be ascribed to technical difficulties (e.g. denaturation) or to the loss of unidentified enzymes or cofactors required for the pathway.

The holistic view, which is enjoying a renaissance of philosophical interest in the field of quantum physics (e.g. Bohm, 1981; Primas, 1981; Wolf, 1981; Wheeler and Zurek, 1983; Garden, 1984), would hold firstly that any attempt even to measure the properties of a system can have the effect of seriously modifying the "observed" system, and secondly (and especially) that the attempt to extrapolate measurements *in vitro* to describe a biochemical system *in vivo* must of necessity fail, for the "disruption" to the real cellular *organization* occasioned upon enzyme isolation is very severe (Clegg, 1984). In particular, it would be argued, the properties of a system are specified not only by the *types* and *numbers* of molecules present but by the way in which the *individual* molecules of a given type are organized functionally and physically, so that it is the physical organization, as well as the chemical composition, *which is different in vitro from that in vivo*, and which must be defined if one wishes to understand "the organization of cell metabolism". This is not to mean, of course, that the reductionist view does not permit a rather sophisticated spatiotemporal organization and integration of metabolic pathways; indeed, mathematical networks of fairly simple reaction-diffusion equations incorporating feedback loops

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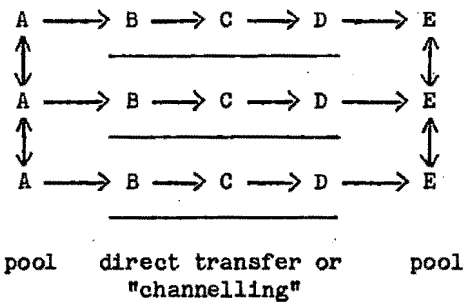
can exhibit highly non-linear behavior (e.g. Stucki, 1978; Hess et al., 1978; Hess, 1983; Hess et al., 1984), without any need, beyond the simple diffusion of enzyme and substrate molecules, to invoke collective or coherent properties (Froehlich and Kremer, 1983) to describe the organization of the cellular matrix and its associated metabolism. However, this is not what we have in mind here.

Four our present purposes, we would argue (Welch and Kell, 1986), as have others (Westerhoff and Chen, 1985), that the fundamental distinctions between the two types of viewpoint lie in the recognition that, as far-from-equilibrium systems, the types of metabolic pathway with which we are here concerned do not conform to the ergodic principle, and that if their organization is of a microscopic nature any macroscopic treatment must be regarded as inappropriate. What do we mean by "microscopic" in this context?

Consider a generalized metabolic pathway:

$$A \xrightarrow[E_1]{} B \xrightarrow[E_2]{} C \xrightarrow[E_3]{} D \xrightarrow[E_4]{} E \qquad \text{..... Scheme 1}$$

Here A, ..., E represent substrate molecules and E_1, ..., E_4 enzymes catalyzing specific reactions. If one makes measurements, as is usual, of the rate of production under steady-state conditions of E from A, one will, of course, be measuring the ensemble behavior of numerous enzyme and substrate molecules of the "same" type. Two extreme organizational modes are then possible; we will refer to them as bulk (or delocalized) and microscopic (or localized). In the bulk case, any molecule of (say) B produced by an individual E_1 molecule may diffuse at a rate sufficiently rapid to ensure that it is freely available to any individual molecule of E_2 in the ensemble of interest, such that, apart from the usual statistical fluctuations (which should be negligible), molecules of B (i) possess a concentration which has a sharp value, (ii) exhibit pool behavior and (iii) may adequately be treated macroscopically. In the localized case, no such pool behavior exists; a molecule of B produced by a given E_1 molecule may act as substrate only for a specific E_2 molecule. The concentration of B seen by an enzyme is no longer equal to the number of molecules divided by the total volume of the appropriate compartments within the reaction vessel, and neither the standard chemical potential nor the chemical activity coefficient of B is remotedly independent of the state, nature and free energy of the protein matrix (Welch, 1977; Somogyi et al., 1984; Welch and Kell, 1986; Berry et al., 1985). In other words, the metabolic pathways are in this case organized, at least functionally, as a "supercomplex", and the system may be said to exhibit "channelling" behavior:

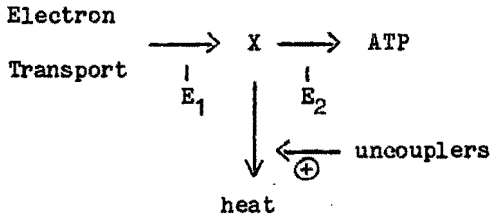


..... Scheme 2

In the present article, our considerations of this question will be directed mainly to three areas, with reference to some recent experiments which have been carried out in this laboratory: (i) are the free-energy-transducing enzymes of electron transport-linked phosphorylation organized in a "localized" fashion?, (ii) can the so-called double-inhibitor titration method effect a rigorous distinction between localized and pool behavior? and (iii) does localization also occur in "soluble" pathways of energy metabolism such as glycolysis? We will also mention some applied or biotechnological aspects of this question, and will outline certain fundamental difficulties which arise in the description of systems exhibiting localized behavior. However, for reasons of space, we shall limit our primary scope, in so far as it is possible, to systems derived from anaerobic microorganisms.

ORGANIZATION OF ELECTRON TRANSPORT-LINKED PHOSPHORYLATION

As may be gleaned from any biochemistry textbook or relevant monograph (e.g. Nicholls, 1982), we may write the macroscopic process of electron transport-linked phosphorylation (ETP) using the type of shorthand given in Scheme 1, as follows:



..... Scheme 3

Here E_1 and E_2 represent membranous redox enzymes and ATP synthase enzymes respectively; and drugs called uncouplers can act to inhibit the overall process of ETP, not by inhibiting E_1 or E_2 but by stimulating the decay of "X", a "high-energy intermediate", into heat. Whilst there are many lines of circumstantial evidence which lead one to invoke the presence of additional enzymes (which we have called "protoneural proteins") necessary for ETP (Kell and Morris, 1981; Kell et al., 1981; Kell and Westerhoff, 1985), the above scheme serves as a minimal and widely accepted model. Arguably, the central problem of membrane bioenergetics concerns the nature of this energized intermediate X and the question of whether or not it is constituted, as in the chemiosmotic coupling hypothesis (Mitchell, 1966, 1968; Nicholls, 1982), by the macroscopic proton electrochemical potential difference across a topologically closed vesicle, in the membrane of which are embedded ensembles of E_1 and E_2 molecules (e.g. Kell, 1979; Ferguson and Sorgato, 1982; Kell and Hitchens, 1983; Westerhoff et al., 1984b; Ferguson, 1985; Kell and Westerhoff, 1985; Kell, 1986).

This proton electrochemical potential difference, or protonmotive force (pmf, Δp), is, in the chemiosmotic paradigm, taken to be in equilibrium with that between the bulk aqueous phases that the coupling membrane serves to separate; and thus, within the framework of Scheme 3 above, if X is the protonmotive force then, by definition, such an energy coupling scheme should exhibit classically bulk, delocalized (or pool) behavior. We should like to obtain a rigorous set of criteria by which one might discern whether or not it does so, so as to provide a test of the chemiosmotic coupling hypothesis. However, it is first necessary to mention a somewhat circular argument which has a tendency to creep into

discussions of this rather controversial problem.

This argument stems from the well-known finding (e.g. Thayer and Hinkle, 1975; Smith et al., 1976; Hangarter and Good, 1982; Maloney, 1982; Schmidt and Graeber, 1985) that an artificially applied protonmotive force can drive phosphorylation at rates similar to those implicated in native ETP; and, naturally enough, this has widely been taken to constitute powerful support for the veracity of Scheme 3, with the place of X taken by Δp. However, what the data in this type of experiment (op. cit.) actually show in every case is that there is no phosphorylation below a (non-thermodynamically defined) threshold of the applied pmf equal to approximately 150 mV. Thus, in assessing the veracity of a macroscopic energy coupling scheme such as that of Scheme 3, the proper question to ask (Kell, 1986) is whether or not the pmf generated by electron transport exceeds this threshold.

This is a particularly thorny problem, reviewed in extenso elsewhere (e.g. Ferguson and Sorgato, 1982; Ferguson, 1985; Kell and Westerhoff, 1985; Westerhoff et al., 1984; Kell, 1986), but our view is that the most self-consistent explanation of the available data is that the actual pmf generated by electron transport does not in fact exceed this threshold value (Guffanti et al., 1981; Kell and Hitchens, 1982; Hitchens and Kell, 1984; Kell, 1986). The circularity arises from the fact that many signals purporting to measure the pmf can be obtained in a form that apparently corresponds to values of the pmf above the threshold. However, if one can reliably obtain other data which show that energy coupling is localized (in the sense of Scheme 2) then, evidently, Scheme 3 is inappropriate (with Δp as X) and values of the pmf purporting to lie above the threshold must, logically, be in error. The most direct measurements of the membrane potential component of the pmf in mitochondria (Tedeschi, 1980), for instance, do indeed suggest that it is energetically insignificant.

In any event, and whilst much of the present concern (Kell, 1979; Ferguson and Sorgato, 1982; Westerhoff et al., 1984; Ferguson, 1985) about the veracity of the chemiosmotic coupling hypothesis has arisen from the lack of correlation between the reactions of ETP and the apparent pmf, what one is trying to convey is that the avoidance of the circularity referred to above requires that one's arguments and experiments designed to assess the veracity of a macroscopic coupling scheme, such as Scheme 3, should not require the measurement of the concentration of X. One approach, which fulfills this criterion, which can serve in principle to distinguish microscopic from macroscopic coupling schemes (and may therefore be of general interest), and with which we have recently been concerned, is known as the double-inhibitor titration method. Two general types are of interest with reference to Scheme 3.

DOUBLE-INHIBITOR TITRATIONS OF ELECTRON TRANSPORT-LINKED PHOSPHORYLATION

Many species of Rhodospirillaceae may be grown aerobically in the dark or phototrophically under anaerobic (or semi-anaerobic) conditions. Under the latter regime, the bacterial cytoplasmic membrane differentiates to form intracytoplasmic membrane (ICM) invaginations; upon cell disruption these vesiculate to form so-called chromatophores, which possess a cyclic electron transport system and provide a well-coupled and convenient system for the study of ETP. Parenthetically, it

may be mentioned that this membrane differentiation is hard to reconcile with the widespread view that membrane protein complexes normally possess lateral diffusion coefficients exceeding 10^{-10} cm^2/s (Kell, 1984), but we will not pursue this issue here (see Harris and Kell, 1985; Kell and Harris, 1985).

In recent work (Hitchens and Kell, 1982a,b, 1983a,b) we have carried out double-inhibitor titrations of photophosphorylation by bacterial chromatophores. With reference to Scheme 3, it is possible to inhibit ATP production using at least three types of inhibitor: electron transport inhibitors (I_1) which inhibit E_1, ATP synthase inhibitors (I_2) which inhibit E_2, or uncouplers. We consider first I_1/I_2-type titrations (Fig. 1). In this case, we first titrate the rate of photophosphorylation, J_p (at saturating light intensity) using a tight-binding and specific I_1-type inhibitor (Fig. 1a). Then, using a fresh batch of chromatophores, we first inhibit photophosphorylation, by say 50%, using a tight-binding and specific I_2-type inhibitor (Fig. 1b, c). The simple view is that, in a delocalized coupling scheme, there is now spare capacity in E_1, so that the residual rate of phosphorylation will be proportionally less sensitive to I_1 than in the control chromatophores (Fig. 1b). Conversely, in a fully localized system, the relative titre will be unchanged (Fig. 1c), for one molecule of I_1 will still block one molecule of E_1, whether it is capable of driving phosphorylation in "its" E_2 or not. In practice, the same (or no increase in) titre is obtained in the partially inhibited case (Hitchens and Kell, 1982 a,b; Kell and Hitchens, 1983, and see Venturoli and Melandri, 1982), and similar results are obtained with energy-linked reactions in submitochondrial particles (Baum et al., 1971; Baum, 1978; Westerhoff et al., 1983a,b; Ferguson, 1985) and chloroplast thylakoids (Davenport, 1985), a finding strongly suggestive of a localized coupling system. However, there is a clever and interesting counterargument, which (if true) might still allow this type of finding to be accommodated in a delocalized scheme, and we must needs rehearse it here now.

This argument was first given by Parsonage and Ferguson (1982) (and see Parsonage, 1984; Ferguson, 1985), and is reiterated by Davenport (1985); we may discuss it with reference to Fig. 2, for the case of

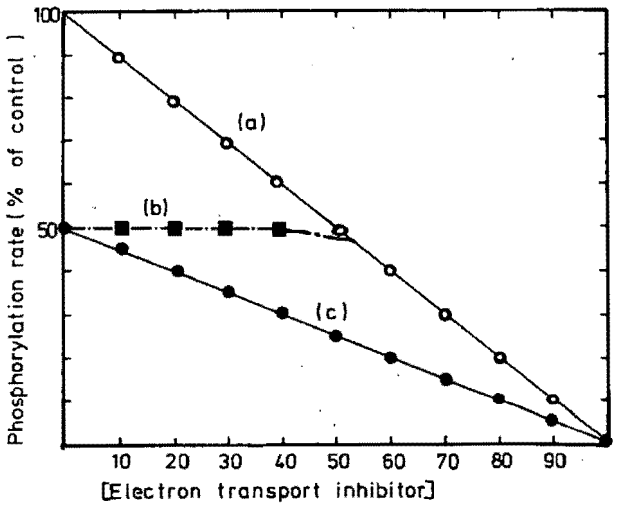


Fig. 1. The principle of an I_1/I_2-type double inhibitor titration of electron transport phosphorylation. For explanation, see text.

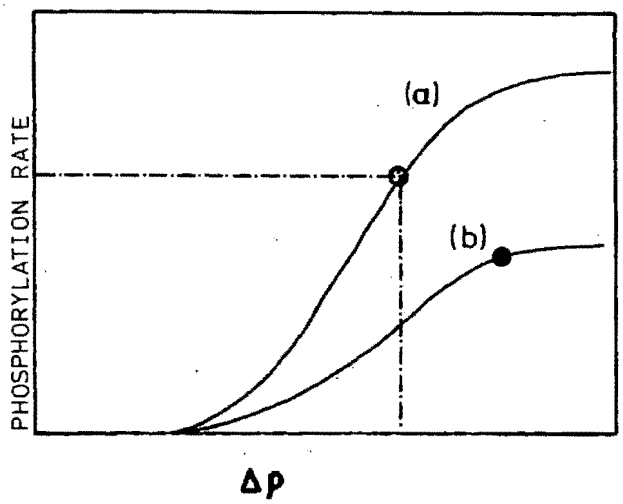


Fig. 2. Possible relationships between the protonmotive force and the rate of phosphorylation in a double inhibitor titration of electron transport-linked phosphorylation according to a delocalized, chemiosmotic coupling model.

chromatophores that have had 0% or 50% of their ATP synthase inhibited. This illustrates a possible relationship between the pmf and the rate of phosphorylation (i.e. J_p = [ATP synthases] x f(Δp)). The argument is that if, in the absence of I_1, the pmf is not saturating for the ATP synthases, then each titration with I_1 will, simply by decreasing Δp, take one down the two curves in a similar fashion, so that there will be no difference in the titre of I_1 whether I_2 is present or not, despite the real existence of delocalized behavior. Thus the argument requires (1) that the pmf is <u>not</u> saturating for the ATP synthases and (2) that there is a decrease in Δp upon inhibition with an I_1-type inhibitor. Thesis (1) is necessary because if Δp <u>is</u> saturating, and is increased slightly upon partial inhibition of J_p with I_2 (Parsonage, 1984), then the initial stages of the titration with I_1 will indeed be less inhibitory when a partial titre of I_2 is present, as expected for a simple delocalized system (Fig. 2, open circle and closed circle). However, if, as is found (Venturoli and Melandri, 1982; Berden et al., 1984; Ferguson, 1985), the decrease in J_p occasioned by the partially inhibitory titre of I_2 is mirrored by a comparable decrease in the number of active (or potentially active) ATP synthases, then one has to assume, in a delocalized framework, that Δp in the absence of I_1 or I_2 <u>is</u> saturating for the ATP synthases. In other words (Kell and Hitchens, 1983; Kell, 1986), as is found (Hitchens and Kell, 1982b), these titrations are symmetrical, in that partial inhibition of J_p with an appropriate titre of I_1 does not increase the titre of I_2 (Hitchens and Kell, 1982b). Thus part (1) of the counterargument fails.

Since part (2) of the counterargument should require measurements of the pmf, it is subject to the circularity described above. However, it is worth mentioning that in the initial part of a titration curve of J_p with an I_1-type inhibitor (Kell et al., 1978; Sorgato et al., 1980), or upon decreasing the flash frequency in photosynthetic systems (Venturoli and Melandri, 1982), the usual methods claimed to measure the pmf do not in fact suggest that it decreases from its value in the absence of

inhibitor. More importantly, in well-coupled systems as isolated (e.g. Ferguson et al., 1976), including bacterial chromatophores (Jackson et al., 1981; Venturoli and Melandri, 1982), the P/2e ratio is unchanged upon decreasing the rate of electron transport. Not only does this finding eliminate arguments based upon "energy leaks" (Hitchens and Kell, 1982a), but, we should like to stress, it also eliminates the argument that one can explain these titrations in terms of a decrease in Δp. This is because, with a chemiosmotic scheme, the P/2e ratio cannot be a less sensitive function of Δp than is J_p, for if one attempts to uncouple (or reduce Δp), the respiratory chain should attempt to act faster to try and maintain Δp (and thus J_p) at its initial value. Thus since the P/2e ratio is not decreased by an I_1-type inhibitor, then neither should the pmf be; and this also eliminates part (2) of the counterargument.

It is worth mentioning that one might artefactually obtain a delocalized result of the type shown in Fig. 1 for trivial reasons, such as if the system is heterogeneous, or contains inhibitor-binding sites which are not involved in coupled electron-transport phosphorylation, etc. However, we confine our considerations here to the well-defined systems in which a localized coupling mechanism does seem to be the only defensible explanation of the data obtained.

It is appropriate to mention at this point the metabolic control theory developed and described by Kacser and Burns (1973) and by Heinrich and Rapoport (1974) (and see Kacser, this volume), and which has been reviewed by Groen et al. (1982a) and by Westerhoff et al. (1984a). The theorems contained therein provide a rigorous mathematical treatment with which one may describe the extent to which each enzyme in a system such as that of Schemes 1 to 3 controls the pathway flux, and of the importance of different effectors in this control. In particular, the "flux-control coefficient" of an enzyme is given by the fractional change in the pathway flux divided by the fractional change in the amount of that enzyme (as these changes tend to an infinitesimal amount), and these coefficients may obviously be determined experimentally by the use of specific inhibitors (e.g. Groen et al., 1982a, b). Further, according to the Summation Theorem, the sum of the flux-control coefficients of each of the enzymes in the pathway will equal identically 1, bearing in mind that "leaks", or enzymes removing substrates from the pathway, will tend to have negative flux control coefficients. However, what we wish to concentrate upon is the implicit assumption of pool behavior for each substrate that is built into these metabolic control theories.

Now, it should be obvious that for a strictly localized system, each of the main pathway enzymes will have a flux-control coefficient of 1, since inhibiting x% of the enzymes of a given type will reduce the pathway flux by x%, whichever enzyme is chosen, so that the sum of the flux-control coefficients will exceed 1. Thus the violation of the summation theorem also constitutes a powerful criterion for a localized system (Kell and Hitchens, 1983; Kell and Westerhoff, 1985). A full description of the application of this approach to the analysis, in more mathematical terms, of the I_1/I_2 type of double-inhibitor titration described above, is given by Westerhoff and Kell (1985), and is not repeated here. One point is, however, worth making. The flux-control coefficients may be obtained, in principle, either by increasing or decreasing the amount of the enzyme of interest. However, the addition of molecules of a given exogenous enzyme will have no effect (i.e. flux-control coefficient = 0) on the pathway flux if the coupling is "perfectly" localized, whilst inhibiting the enzymes of the same type will cause a proportional inhibition of pathway flux (flux-control

coefficient = 1); this difference in behavior also constitutes a useful criterion of "channelling".

The other main type of double-inhibitor titration of ETP (see Kell and Hitchens, 1983; Ferguson, 1985; Westerhoff and Kell, 1985; Kell and Westerhoff, 1985; Kell, 1986) concerns the titration of J_p with an uncoupler, in the presence and absence of a partially inhibitory titre of I_2 (Hitchens and Kell, 1982b, 1983a,b). In terms of the chemiosmotic analysis ("counterargument") given above (see Fig. 2), the ATP synthase inhibitor probably raises, and certainly cannot lower, the pmf in the absence of uncoupler, so that one should expect that the uncoupler is equally or less potent when a partially inhibitory titre of I_2 is present. In practice, and provided that the experiments are not done under non-stationary conditions, when almost any behavior is possible (Cotton and Jackson, 1983), the uncouplers act more potently when a fraction of the ATP synthases is inhibited. Similar data are obtained in thylakoid photophosphorylation (Davenport, 1985) and in ATP-driven reversed electron transport in submitochondrial particles (Westerhoff et al., 1983; Berden et al., 1984), and may be explained, within a localized framework, in terms of the (most) rate-limiting step for uncoupling being not the diffusion of uncoupler molecules to their sites of action but the uncoupling step itself (e.g. Hitchens and Kell, 1983a,b).

A point worth stressing, and apparently not appreciated by some workers (O'Shea and Thelen, 1984; Davenport, 1985), is that the starting points for each of these two types of titration (with I_1 or uncoupler) are the same, so that whatever I_2 does in one case (if one wishes to defend a delocalized model) it must do in the other: if I_1 stays equally potent when a partially inhibitory titre of I_2 is present then so should the uncoupler, if the only means by which I_1 and uncouplers affect J_p is by decreasing the pmf. That I_2 can affect the titration behavior differently constitutes perhaps the simplest argument necessary to illustrate that this type of approach shows that the energy coupling systems of ETP do not interact via a macroscopic, delocalized high-energy intermediate.

In outlining the double-inhibitor titration approach, we have concentrated on bioenergetic systems, for most of the work has been done on them. However, we believe that the approach has a general utility for discerning whether or not a metabolic pathway is organized macroscopically or microscopically. In this regard, a nice example approximating this type of approach, involving the demonstration of a "replitase supercomplex" in DNA synthesis, is given by the work of Pardee and Reddy (1983).

We now wish to turn to another approach to understanding the organization of energy metabolism, in this case of glycolysis in the clostridia.

ORGANIZATION OF GLYCOLYSIS IN CLOSTRIDIUM PASTEURIANUM

Clostridium pasteurianum is a fermentative obligate anaerobe, which derives the free energy necessary for growth by glycolysis (using the EMP pathway) to produce acetate and butyrate in approximately equimolar amounts (Thauer et al., 1977). Glucose is taken up via a phosphotransferase system (PTS) (Booth and Morris, 1982; Mitchell and Booth, 1984); and in intact cells of strain 6013-ES1, which lacks granulose phosphorylase (Mackey and Morris, 1974), continuing glycolytic acid

production is strictly dependent upon an exogenous carbon source. As may be seen in Fig. 3, the addition of glucose to a washed cell suspension causes, after a lag of approximately 20-30s, a steady rate of acid production. The lag might be ascribed to the build-up of pools of glycolytic intermediates, but, for reasons which will become apparent, is more likely associated with the optimal poising of the adenine (and perhaps pyridine) nucleotide pools. Permeabilization of the cell membrane by treatment with an appropriate concentration of toluene:ethanol (1:10), a very common method of cell permeabilization (Felix, 1982), releases intracellular cofactors such as ATP (data not shown) and leads to an immediate inhibition of glycolysis (Fig. 3). (Under these conditions, without toluene and with periodic adjustment of the pH, a steady rate of acid production may be maintained for as long as 30 minutes; and the major acidic products may be shown by gas chromatography to be acetate and butyrate, with a small proportion (up to 10%) of D- and L-lactate.

The product of the PEP-dependent PTS system is glucose-6-phosphate (G6P) (which does not penetrate the membrane of intact cells of this organism (Booth and Morris, 1982)), and the question arises concerning the extent to which glycolysis from G6P is inhibited in toluenized cells, according to the following reasoning. If all glycolytic intermediates (together with cofactors such as NAD(H) and ATP) exhibit "pool" behavior, then the ability to restore flux through the glycolytic pathway using G6P, NAD and ATP alone should be severely compromised, since most enzymes, including those of this pathway (e.g. Kotze, 1968; Uyeda and Kurooka, 1970) have $S_{0.5}$ values in the mM range. Thus, in a reaction mixture containing 1.25 mg dry weight cells/ml, with a specific enclosed volume of 2.12 ml/l (Clarke et al., 1982), the dilution of cytoplasmic "pool" constituents upon toluenization will be 450-fold, so that their concentration (if their *in vivo* concentration corresponds to the $S_{0.5}$

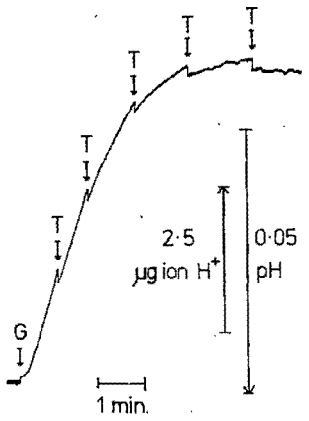


Fig. 3. Glycolytic acid production by intact cells of *Clostridium pasteurianum* 6013-ES1. *Clostridium pasteurianum* 6013-ES1 was grown in a glucose minimal medium (Clarke et al., 1982) and washed and resuspended in 25 mM triethanolamine phosphate pH 6.5/50 mM KCl/5 mM $MgSO_4$/0.05% cysteine-HCl. All procedures were carried out anaerobically, and pH changes were recorded as described (Hitchens and Kell, 1982a) at a temperature of 37°C. The reaction volume was 8 ml, and 10 mg dry weight cells were present. At the arrows indicated, 20 mM glucose (G) and 0.02 ml aliquots of toluene:ethanol (1:10) (T) were added.

values of the enzymes for which they are substrates) will be lowered by a factor of at least 100, giving an initial glycolytic rate which would (Fig. 3) be immeasurably small. Alternatively, and even if the lag observed in the initiation of steady-state glycolytic acid production by intact cells (transient time) upon addition of glucose were to be caused by the build-up of pools of glycolytic intermediates, the lag would be increased by a similar amount (Keleti, 1984), and would also escape detection.

In contrast, if glycolysis, say from G6P to pyruvate, were to be organized as a 'supercomplex' (e.g. Mowbray and Moses, 1976; Ottaway and Mowbray, 1977; Gorringe and Moses, 1980; Masters, 1981; Friedrich, 1984; Keleti, 1984), whether physically or just functionally, such that glycolytic intermediates did not exhibit "pool" behavior, then the rapid and extensive restoration of glycolytic flux from G6P might require the addition only of adenine and pyridine nucleotide cofactors. Fig. 4 illustrates an experiment designed to obtain information on this point.

Fig. 4(a) illustrates acid production in toluenized cells of Clostridium pasteurianum 6013-ES1, indicating a contribution to acid production by ATP hydrolysis (cf. Hitchens and Kell, 1982a), whilst Fig. 4(b) shows that a maximal rate of glycolytic acid production is strictly dependent upon the addition of adenine and pyridine nucleotides. There is some variability in the lag phase, and the traces have been chosen to illustrate the range that we have so far experienced (from virtually no lag to ca. 4 min); this variability is ascribed predominantly to the requirement for an optimum poise of the ATP/ADP ratio (since both nucleotides are required at reasonable concentrations for a maximum rate of glycolysis). Since exogenous CoA is not present in this assay, glycolysis to acetate and butyrate is not observed, but acid production continues until the G6P is exhausted and recommences immediately upon injection of another aliquot of 5 mM G6P (not shown).

The steady-state rate of acid production in this case (Fig. 4), when corrected for the ATP hydrolase activity, is approx. 0.08 mol/min/mg dry-weight cells, corresponding to approx. 51% of the rate of acid production of intact cells in this run. Whilst a restoration of glycolytic acid production to 100% of the rate exhibited in intact cells has not so far

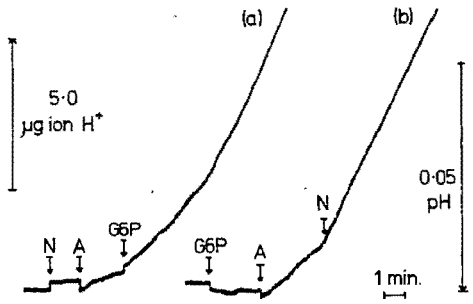


Fig. 4. Glycolytic acid production by toluenized cells of Clostridium pasteurianum 6013-ES1. Acid production was measured with the system, and in the medium, described in the legend to Fig. 3. 20 mg cells were present, and were toluenized with 0.1 ml toluene:ethanol (1:10), and 1 mM NAD^+ (N), 2 mM ATP (A) and 5 mM glucose-6-phosphate (G6P) were added at the arrows indicated.

been obtained, we would mention that it is known that organic solvents *per se* can noncompetitively inhibit glycolysis in intact cells under conditions in which the cell membrane is not permeabilized (Nogodiwathana et al., 1977; Millat et al., 1982). Also, toluenization does release a certain amount of intracellular protein (not shown). In any event, the relatively small lags and large rates of acid production observed in toluenized cells would seem to argue in favor of an organization of glycolysis in this organism, that does not allow the intermediates to exhibit pool behavior. The dependence of acid production upon catalytic concentrations of both ATP and NAD serves as a control to show (i) that *multi-step* reactions are being observed and (ii) that cell permeabilization has indeed been effected by the toluene treatment.

Although toluenized cells have often been used to assay the activities of single enzymes (Felix, 1982), including those of glycolysis (Serrano et al., 1973), we are not aware of any report in which a significant segment of the glycolytic pathway of a prokaryote has been shown to function *en bloc*, without added intermediates, in this way, although Clegg (1984) has obtained and discussed similar data in dextran sulphate-permeabilized mouse L-cells. Further work will be aimed at assessing the ability or otherwise of exogenous glycolytic intermediates to decrease the specific radioactivity of the products derived from radioisotopic G6P, and at assessing the extent to which glycolytic intermediates are released to the extracellular space during G6P dissimilation, together with other comparable approaches discussed by Friedrich (1984) and by Keleti (1984). However, we should like to mention that the type of metabolic organization evidenced by the present type of observation has the interesting and biotechnologically significant corollary, that *multistep and complex* biotransformations of membrane-impermeant xenobiotics of commercial importance might be effected by this type of permeabilized-cell system.

In our final section, we wish to draw attention to yet another type of system in which we believe that one should also invoke a more microscopic organization of cellular metabolism than is commonly construed, illustrated with reference to two recently discovered anaerobic bacteria.

ENERGY TRANSDUCTION BY THE ATP SYSTEM AND THE "BIOLOGICAL QUANTUM"

When discussing the organization of cellular metabolism from an energetic standpoint, it is usual to consider that the free energy-generating reactions of microbial catabolism (or photosynthetic electron transport) are stoichiometrically coupled to the anabolic, free energy-requiring reactions of biosynthesis by means of the adenine nucleotide pool, as illustrated in Fig. 5 (Westerhoff et al., 1982) for microbial growth on glucose. In this figure, ΔG_p, the phosphorylation potential, is generally poised at a value of approx. -44 kJ/mol (-10.5 kcal/mol) (Thauer et al., 1977), such that it should be more negative than the free energy of anabolism (ΔG_a) is positive and, more importantly, if ATP molecules are formed stoichiometrically, should be less negative than is the free-energy change of catabolism (ΔG_c). In other words, it is considered that the free energy of catabolism is conserved as a "biological quantum" of "packets" corresponding to a (free) energy of ca. 44 kJ/mol, any excess catabolic energy being dissipated as heat, presumably as variable frictional losses in "molecular machines" (Welch and Kell, 1986), with such free-energy conservation being generally

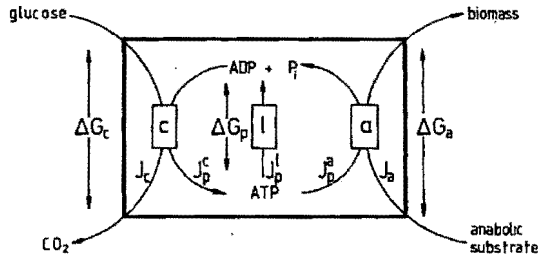


Fig. 5. Energy coupling in microbial growth, in which the catabolic reactions (c) are stoichiometrically coupled to the anabolic reactions (a) by means of the adenine nucleotide pool. "l" represents "leak" reactions which result in a loss of free energy and an apparent non-stoichiometry between the catabolic synthesis and the anabolic utilization of ATP. For further explanation see text, where we discuss in particular the possibility of a sub-integral stoichiometry between catabolism and ATP synthesis itself.

effected by substrate-level or electron transport-linked phosphorylation.

However, there are at least two anaerobic bacteria which cannot conform simply to the above, macroscopic, continuum picture, viz. Thermoproteus neutrophilus (Fischer et al., 1983) and Syntrophomonas wolfei (McInerey et al., 1981); for their ΔG_c values are significantly less negative than -44 kJ/mol. As discussed by Thauer and Morris (1984), the only mechanistic escape from this paradox is to posit a non-integral stoichiometric coupling between some reversed electron-transfer reaction and an ATP hydrolase reaction, so that even macroscopic schemes, such as the chemiosmotic scheme outlined (Thauer and Morris, 1984), render meaningless the concept of "the biological quantum". However, the existence of threshold behavior in membrane energy-coupling systems (see Kell, 1986), a characteristic of quantal free energy-coupling devices (Welch and Kell, 1986), indicates that coupling systems of this type cannot be arbitrarily flexible, and both provides an illustration of a system in which the concept of a macroscopic "concentration" fails and which, in this case, from a macroscopic viewpoint, would appear to violate the Second Law. Further work, to determine exactly whether the coupling in these systems is microscopic or macroscopic, perhaps using the double-inhibitor titration method, seems warranted.

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