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Linear relationships between mitochondrial forces and cytoplasmic flows argue for the organized energy-coupled nature of cellular metabolism

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We have studied rates of formation of glucose, urea and lactate by isolated hepatocytes incubated with a variety of inhibitors of energy transduction. Linear relationships have been found between these metabolic rates and mitochondrial forces (membrane, redox and phosphorylation potentials). The findings are suggestive of extensive enzyme organization within these metabolic pathways.

Electrochemical potential; Irreversible thermodynamics; Linear force-flow relationship; Gluconeogenesis; Urcogenesis; Metabolic regulation; (Isolated hepatocyte)

1. INTRODUCTION

A number of theoretical constructs have been developed for the quantitative description of flow through metabolic pathways [1–3]. While these differ in many important details, they are consistent in favouring a kinetic modality of regulation. It is assumed that control of flux depends on 'regulatory enzymes' that catalyse far-from-equilibrium reactions and follow non-linear kinetics. It is to be expected, therefore, that pathways containing regulatory enzymes would inevitably show non-linear flows in response to alteration in driving forces. Nevertheless in various studies on biological energy conversion, linear relationships between flows and forces have been observed [4–10]. These linear relationships are suggestive of thermodynamic rather than kinetic control of metabolism [10]. Analysis using the formalism of (near-equilibrium) irreversible thermodynamics indicates that such linearity does not reflect local processes actually operating near true thermodynamic equilibrium, but rather a balanced interplay of energy-coupled far-from-equilibrium forces [10]. It has been argued that in mitochondria such interactions must reflect a high degree of organization within these organelles [9].

We have previously reported linear flow-force relationships in isolated liver cells [11], but a comprehensive study has not been carried out. Since such linear flow-force relationships would have important implications in regard to our understanding of metabolic control within the cytoplasmic compartment, we have examined in greater detail the correlation between various cytoplasmic flows and mitochondrial forces. We found that linear flow-force dependencies in hepatocytes are far more extensive than has been recognized previously, and consider that these rela-

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tionships are suggestive of a substantial degree of enzymic organization in the cytoplasmic phase.

2. MATERIALS AND METHODS

Collagenase and enzymes for metabolite determination were from Boehringer-Mannheim (FRG) as was bovine serum albumin (fraction V); defatted according to [12]. Palmitate, rotenone, antimycin, oligomycin, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and gramicidin were obtained from Sigma (USA). [^{14}C]Methyltriphenylphosphonium iodide was purchased from Amersham (England). Palmitate (8 mM) was prepared in isotonic saline containing 9% (w/v) bovine serum albumin. Other water-insoluble compounds were dissolved in dimethylsulphoxide.

Isolated liver cells from male Hooded Wistar rats (250–280 g body wt), starved for 24 h to deplete liver glycogen, were prepared and incubated at 37°C in a bicarbonate-buffered medium as described [13,14]. Metabolites were measured by standard enzymic techniques as in [15] on neutralized perchloric acid extracts of the incubated cells and medium [14]. The metabolite assays were performed in a Cobas FARA centrifugal analyser (Roche Diagnostics, Switzerland) and the data transferred to a PDP 11/73 computer (DEC, USA) for subsequent processing. All fitted, straight line relationships were calculated by least-squares linear regression analysis of untransformed data. Most of the measurements of cytoplasmic flows (i.e. rates of metabolite production) were made during incubation periods of 40 min, but similar results were also obtained using shorter incubation periods. Each plot is a representative example from at least three related experiments.

Mitochondrial membrane potential ($\Delta\psi$) was measured with the [^{14}C]methyltriphenylphosphonium cation according to [16], after correction for non-specific binding and plasma membrane potential. Phosphorylation potential (ΔG_p) was calculated as $\Delta G_p^{o'} + (RT/nF)\ln([ATP]/[ADP][P_i])$, taking a value for $\Delta G_p^{o'}$ of 29 kJ·mol^{-1} [17], and for [P_i] of 9 mM [18], since [P_i] did not change significantly under our experimental conditions (unpublished). Values for mitochondrial redox potential (E_h), defined as the half-cell reaction potential of free [NAD^+]/free [NADH] ($E_h = E^{o'} + (RT/nF)\ln$[acetoacetate]/[3-hydroxybutyrate]) were obtained by measurement of the concentrations of acetoacetate and 3-hydroxybutyrate, on the assumption of an $E^{o'}$ at 37°C of −0.297 V [19]

3. RESULTS

Fig.1 shows the effects of alterations in energy flow on the forces, ΔG_p, $\Delta\psi$ or E_h, and on each of two cytoplasmic flows, namely production of lactate from pyruvate ($J_{lactate}$), and formation of glucose from lactate ($J_{glucose}$). The forces and flows have been altered by addition to the cell suspensions of graded concentrations of a variety of inhibitors of energy transduction, affecting different facets of the bioenergetic apparatus. Palmitate has been included in the incubation medium in order to achieve a wider range of $J_{glucose}$ and $J_{lactate}$ [20]. In the presence of added lactate, $J_{glucose}$ decreases with increasing concentrations of each metabolic inhibitor (fig.1a–c). In the presence of added pyruvate, $J_{lactate}$, likewise, is found to decrease as the concentration of each inhibitor is raised (fig.1d–f). ΔG_p invariably falls whichever inhibitor is present as does $\Delta\psi$, except in the case of titrations with oligomycin where $\Delta\psi$ is unchanged or decreases only slightly. E_h becomes more positive as the amount of gramicidin or FCCP is increased, and more negative when hepatocytes are titrated with rotenone or oligomycin. It is also apparent that as the concentrations of rotenone, antimycin and oligomycin are raised, $J_{lactate}$ declines even though E_h is becoming more reduced (fig.1f). Since the reducing equivalents for pyruvate reduction are generated in the mitochondria, this observation might appear paradoxical. It has, however, been reported previously and taken to indicate that the transfer of reducing equivalents from mitochondria to cytoplasm is an energy-dependent process [21,22].

The observation that inhibitors of energy transduction can bring about a decrease in $J_{glucose}$ and $J_{lactate}$ are unremarkable in themselves. The significant feature of these data is the linearity of the relationships between flows and forces which is observed over a wide range of inhibitor concentrations. The slope of each relationship, and indeed the sign of the slope, is dependent on the inhibitor used. At high inhibitor concentrations, where flow rates are greatly reduced, linearity is lost.

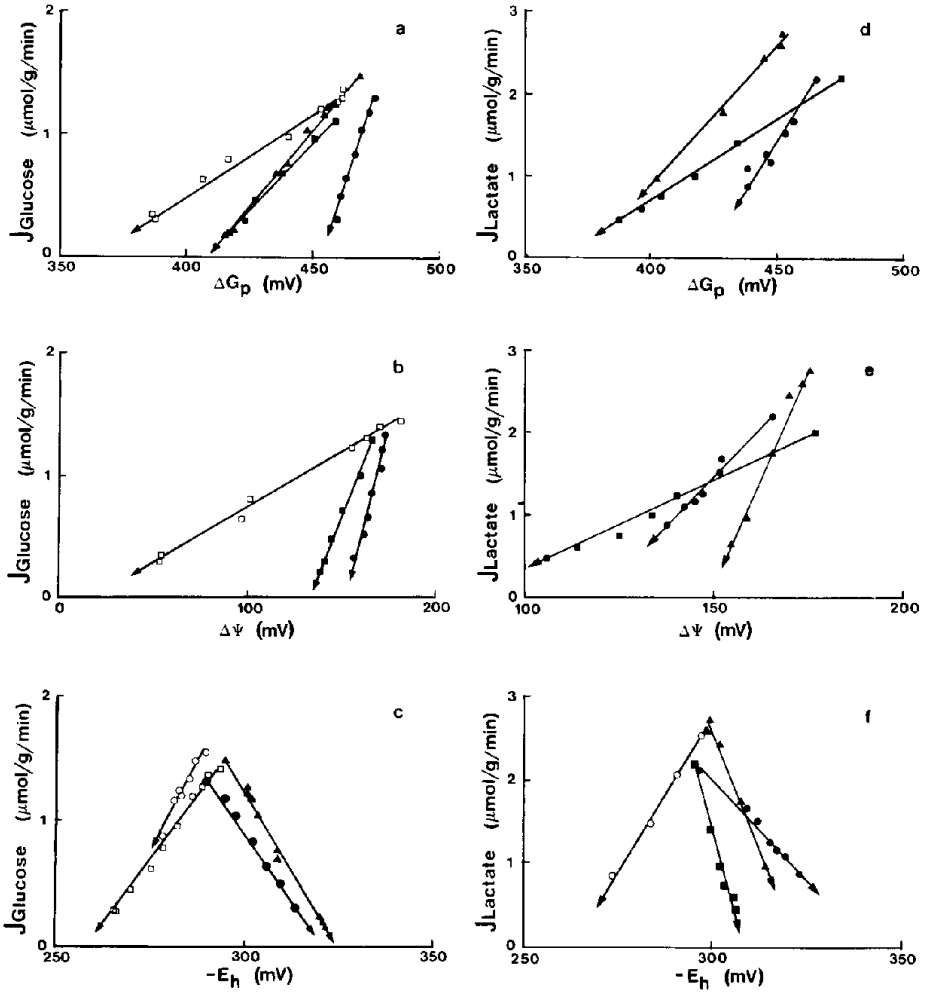

Fig.1. Relationships between metabolic rates and electrochemical potentials. Isolated liver cells were incubated as described in section 2 with either 2 mM palmitate, 10 mM lactate and 1 mM pyruvate (a–c) or 2 mM palmitate and 15 mM pyruvate (d–f). Other additions included: 0–3.5 μM antimycin (■), 0–0.57 μM oligomycin (▲), 0–8 μM rotenone (●), 0–40 μM FCCP (○) or 0–100 μM gramicidin (□). The arrowhead denotes the direction of increasing inhibitor concentration. For the measurement of $\Delta\psi$, 2.4 μM [^{14}C]methyltriphenylphosphonium iodide was included. Each set of data is from a single representative experiment.

We have found that a linear relationship between a cytoplasmic flow and a mitochondrial force can be observed even when there is no apparent direct participation of mitochondrial metabolism in the cytoplasmic process. For example, fig.2 illustrates the relationship between $J_{glucose}$ and E_h when fructose is the gluconeogenic substrate. In the presence of increasing concentrations of inhibitors of mitochondrial energy transduction, $J_{glucose}$ declines (fig.2a) whereas $J_{lactate}$ rises (fig.2b). This decrease in gluconeogenesis and increase in glycolysis as mitochondrial energy metabolism is impaired is to be expected, even though under these conditions the cells are capable of generating sufficient ATP for their gluconeogenic needs from fructolysis to lactate. Although glucose formation from fructose appears to have no stoichiometric requirement for reducing equivalents, an excellent linear correlation between $J_{glucose}$ and E_h is observed. Moreover,

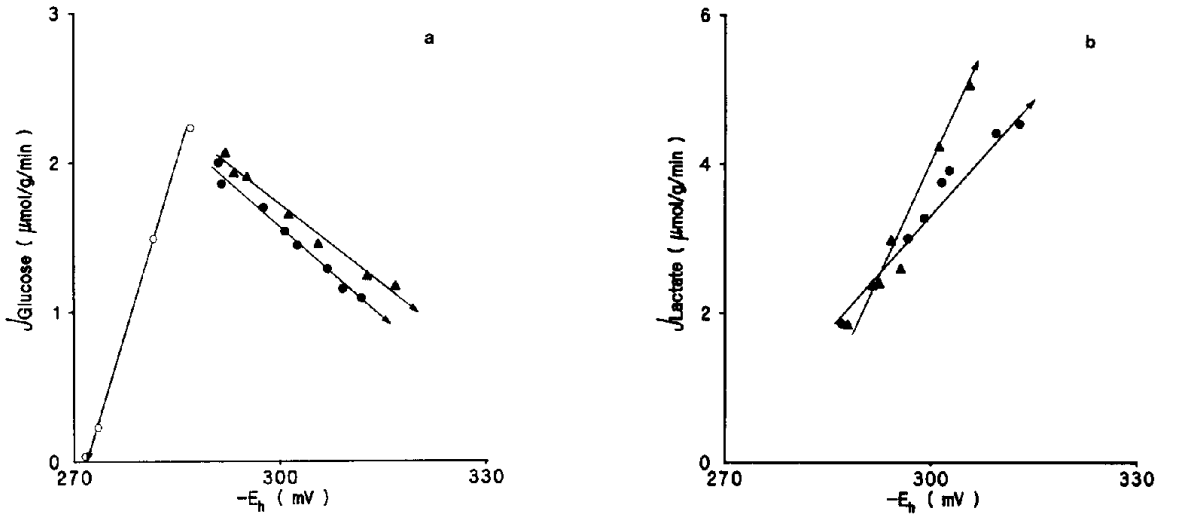

Fig.2. Relationship between mitochondrial redox potential and rates of formation of glucose and lactate from fructose. Isolated liver cells were incubated as described in section 2 with added fructose (10 mM). Redox potential and metabolic rates were varied by the addition of metabolic inhibitors as follows: 0–0.75 mM oligomycin (▲), 0–4 mM rotenone (●) and 0–20 µM FCCP (○). The arrowhead denotes the direction of increasing inhibitor concentration. Each set of data is from a single representative experiment.

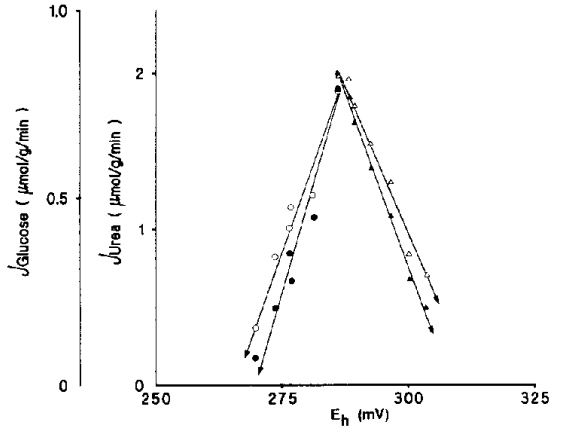

Fig.3. Relationships between mitochondrial redox potential and rates of formation of glucose from lactate and urea from ammonium chloride. Isolated liver cells were incubated as described in section 2 with 2 mM palmitate, 10 mM lactate, 1 mM pyruvate, 12 mM ammonium chloride and 2 mM ornithine. Redox potential and metabolic rates ($J_{glucose}$, solid symbols; J_{urea}, open symbols) were varied by the addition of metabolic inhibitors as follows: 0–0.6 µM oligomycin (▲,△) or 0–25 µM FCCP (●,○). The arrowhead denotes the direction of increasing inhibitor concentration. Each set of data is from the same representative experiment.

in the same experiment an equally good correlation occurs between $J_{lactate}$ (from fructose) and E_h (fig.2).

We have also examined the perturbation of two unrelated flows in association with variation in a single force. Variations in $J_{glucose}$ from lactate, and urea synthesis (J_{urea}) from ammonium chloride have been studied as functions of change in E_h.

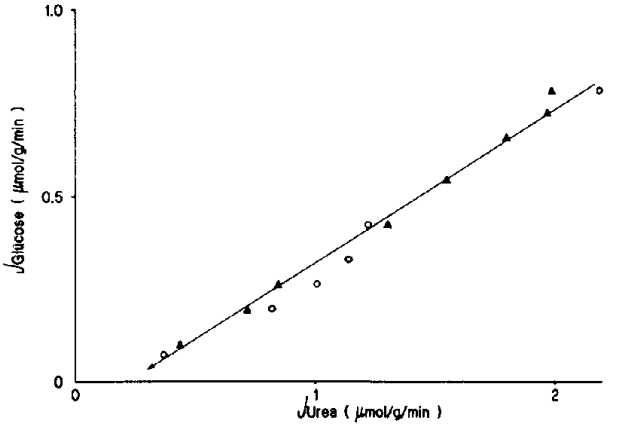

Fig.4. Relationship between rates of formation of glucose from lactate and urea from ammonium chloride. The data are derived from fig.3.

Each of the inhibitors tested yields a linear correlation for both flows. Fig.3 shows two representative examples. Moreover, the plot of $J_{glucose}$ against J_{urea} (fig.4) demonstrates that a given change in gluconeogenesis is accompanied by a corresponding alteration in the rate of urea synthesis, i.e., a change in the magnitude of the mitochondrial force is associated with an identical degree of change in two unrelated flows, that would normally be expected to be regulated by separate and different mechanisms.

4. DISCUSSION

In the glycogen-depleted state the hepatocyte is entirely dependent on mitochondrial ATP for cytoplasmic syntheses. Hence, it is to be expected that impairment of mitochondrial energy metabolism in cells from fasted rats will lead to a decline in ΔG_p and a corresponding fall in $J_{glucose}$, J_{urea} and $J_{lactate}$. Not to be expected, however, is the frequent finding of linear relationships between the alterations in these metabolic flow rates and the changes in mitochondrial forces (i.e. ΔG_p, E_h or $\Delta\psi$), that accompany the inhibition of energy transduction.

We infer from our data that these apparent linkages of individually measured mitochondrial forces to the various cytoplasmic flows are not direct. If a direct causal relationship existed between each mitochondrial force and a particular cytoplasmic flow, the same slope for a given flow-force relationship would be expected, regardless of the nature of the inhibitor of energy transduction present. Yet in our experiments not only the slopes but even the sign of the slopes differed, depending on the inhibitor added. Moreover, under some experimental conditions, straight-line correlations were not observed (not shown). Also in some instances cytoplasmic flow changed substantially in response to exposure of the cells to a particular inhibitor, whereas the measured mitochondrial force remained unaltered. An example of this is the relative stability of $\Delta\psi$ when hepatocytes are exposed to oligomycin [23]. All these findings led us to conclude, on the basis of the chemiosmotic theory [24], that the observed changes in mitochondrial forces, associated with exposure of hepatocytes to inhibitors of electron transport or ATP synthesis, uncoupling agents or ionophores, are secondary to alteration in a more fundamental element of biological energy transduction, namely, mitochondrial protonmotive force (Δp). Hence, the linear relationships between ΔG_p, E_h or $\Delta\psi$ and $J_{glucose}$, J_{urea} or $J_{lactate}$ can be taken to reflect the coupling of Δp to mitochondrial and cytoplasmic energy-utilizing processes.

In contrast, none of the examples of (electro)chemical phenomena, reported by others as giving rise to linear flow-force relationships, have involved cytoplasmic components. Rather, they have entailed organized membrane complexes wherein the separate forces and flows are coupled [4–10]. In these membrane complexes it is believed that metabolic reactions (or transport steps) can be under thermodynamic control, being poised apparently near equilibrium by the application of a vectorial far-from-equilibrium force, which modulates the individual kinetic rate constants of the relevant enzymes or transport proteins. Important examples of this principle are the action of Δp on the kinetic constants of the ATP synthase [25] and energy-linked transhydrogenase [26] of the mitochondrial inner membrane. On the other hand, an enzyme dissolved in a bulk aqueous phase could not respond to such a force, since the Haldane relationship decrees that any force altering the properties of an enzyme in solution would affect forward and back reactions equally, leaving the equilibrium constant unchanged.

In view of this we infer that the apparently 'soluble' regulatory enzymes of glycolysis, gluconeogenesis and ureogenesis must be embedded in an organized matrix that in some manner enables coupling to Δp. Although the exact form of this organization is uncertain, it is now well-established that many enzymes of intermediary metabolism are membrane-associated [27–32] and that enzymes in pathways such as glycolysis can bind reversibly to cytoplasmic substructures with concomitant modulation of their kinetic properties [27,28,33–37]. It is feasible that enzymes bound in this manner could come within the domain of electric fields generated as a consequence of Δp [38]. Under these circumstances, ΔG for reactions catalysed, for example, by dehydrogenases and kinases (involving uptake or release of protons) would be expected to be considerably different from ΔG for the same reactions taking place in the bulk aqueous phase. Stucki [9] has suggested that

modulation of enzyme-kinetic parameters by local electric fields may lie at the heart of the force-flow linearity of some mitochondrial processes (cf. [39]).

These proposals are compatible with the suggested roles of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase as mediators of communication between mitochondrial and cytoplasmic compartments [19,40] provided that these enzymes are membrane-associated [28,29]. Furthermore, they accommodate well the numerous observations of apparent near-equilibrium between cytoplasmic and mitochondrial redox and phosphorylation states [19,41]. However, it would seem that such relationships should be interpreted in terms of irreversible thermodynamics [10] rather than on the basis of the classical equilibrium model proposed [19,41,42].

We have examined whether our results can be explained in more conventional terms, without postulating an essential requirement for enzyme organization. Under contrived conditions the kinetics of enzymes in solution can give rise to linear relations between flows (reaction rates) and forces (ΔG) [7,10,43–45]. However, the maintenance of even a single enzyme reaction in the linear regime places some rather specific (and in some cases, physiologically unrealizable) constraints on the steady-state concentrations of substrate and/or product [10]. It seems even more unlikely therefore that linear flow-force relationships could be sustained over the length of a metabolic pathway catalysed by a sequence of enzymes in solution. In our studies individual concentrations of metabolic intermediates varied over a ten-fold range (not shown), thereby greatly exceeding the limits required for kinetic control of linearity [43]. Although it is possible that a multi-enzyme complex could be more readily constrained kinetically to function in the linear regime [45], most of the metabolic pathways under consideration involve both the mitochondrial matrix and the cytoplasm, and no enzyme complexes are known that extend between these two compartments. Thus it seems improbable that the observed linear flow-force relationships can be attributable to the existence of static multi-enzyme complexes.

The physiological question, why intermediary metabolism should be maintained within the linear thermodynamic regime over a wide range of metabolic rates, remains to be answered. Most discussion has focused on considerations of efficiency and free-energy transduction, optimization of energy conversion and power output, etc. [4–10,44,45]. The main thrust of the present paper is to demonstrate that linear flow-force relationships are far more extensive than previously appreciated and may indicate a widespread network of macromolecular connections between energy-generation sites in the mitochondria and energy-utilization processes in the cytoplasm.

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