THE FUNCTION OF ENERGY-DEPENDENT REDOX REACTIONS IN CELL METABOLISM

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1. Introduction

A major interest of H. A. Krebs has been the mechanisms that regulate the steady-state concentrations of metabolic intermediates within the various cell compartments. Almost 15 years ago, studies by Williamson et al. [1] confirmed earlier findings [2,3] which suggested that the components of the lactate dehydrogenase system in liver are maintained in a constant state apparently close to thermodynamic equilibrium. The ratio, [lactate]/[pyruvate] was thus considered to reflect the 'redox state', the ratio of free [NAD]/[NADH], within the cytoplasmic compartment of the hepatic cell [1,4]. Estimates of cytoplasmic [NAD]/NADH] ratios of about 400–2000 under various experimental circumstances were calculated from the values found for [lactate]/[pyruvate] [5–7]. These calculations assumed that there is only one functional pool of NAD(H) in the cytoplasm and that the oxidized and reduced forms of the dinucleotide act like coenzymes, not prosthetic groups, and are able to diffuse freely throughout the cytoplasmic compartment of the hepatic cell.

Similar studies were carried out in relation to the hepatic mitochondrion where the situation is more complex, owing to its multicompartmental structure. The measurements of Williamson et al. [1] of the components of the reactions catalysed by the matrix enzyme, glutamate dehydrogenase, and the membrane-bound 3-hydroxybutyrate dehydrogenase suggested that the redox state of the matrix and inner membrane NAD(H) pools are identical, and it was concluded from this that reducing equivalents could exchange freely between these two compartments. From this work arose the idea that there is only one functional pool of NAD(H) within the mitochondrion and no shuttle is required for reducing-equivalent transfer between mitochondrial matrix and inner membrane. The redox state of this putative pool could be determined by measurement of the substrates either for the 3-hydroxybutyrate dehydrogenase or the glutamate dehydrogenase reactions, and proved to be some 100-times more reduced than the cytoplasmic compartment [7].

This work was subsequently expanded to include the NADP-linked couples of the cytoplasm and mitochondria, and the relationship of the redox state for cytoplasmic NAD(H) to the phosphorylation state of the cytoplasmic and mitochondrial compartments [8]. From these studies developed the concept that a network of near-equilibria exists within the living cell, ultimately regulated by the phosphorylation state of the respiratory chain, poising the redox states of cytoplasmic and mitochondrial NAD(H) and NADP(H) pools [7,9–11]. On the other hand, Sies argued that in an open system, such as a living cell, the maintenance of steady-state redox potential differences between different compartments, and between NAD-linked and NADP-linked systems in the same compartment, must be dependent on energy flow [12]. The question of whether energy-dependent mechanisms are involved in maintaining intracellular redox states still remains unresolved. The studies reported here address this problem.

2. Experimental

Hooded Wistar rats (250–300 g body wt) were fed, or starved for 24 h to deplete liver glycogen. Isolated liver cells were prepared from them by a modification [13] of the method of Berry and Friend [14]. For some experiments, rats were injected daily with clofibrate (ethyl-2-(4-chlorophenoxy)-2-methylpropionate) at a dose of 100 mg/kg body wt for

Dedicated to Professor Sir Hans Krebs, FRS, on his eightieth birthday

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14–21 days. For studies on hyperthyroid animals, rats were injected with L-thyroxine (0.6 mg/kg body wt), daily for at least 7 days. No weight loss occurred over this period. After preparation, the cells (80–100 mg wet wt) were washed 3 times and incubated in 2 ml balanced bicarbonate–saline medium with a gas phase of 95% O_2, 5% CO_2 [15]. Incubations were terminated by addition of 2 M perchloric acid and the extracts neutralized with KOH. Metabolites were measured in the neutralized extracts by standard enzymic techniques as in [15,16]. When [1-^{14}C]palmitate was a substrate, $^{14}CO_2$ was trapped in phenethylamine and measured by liquid scintillation counting. The uptake of O_2 was measured in the presence of CO_2 by a manometric method [17].

Enzymes for metabolite determination were from Boehringer Mannheim (Sydney, NSW), collagenase from Worthington Biochemical (Freehold, NJ), [1-^{14}C]palmitate from the Radiochemical Centre (Amersham) and rotenone from Sigma, (St Louis, MO).

3. Results and discussion

3.1. *Interactions of cytoplasmic dehydrogenase systems*

If the assumption is correct that the cytoplasmic compartment contains a single NAD(H) pool, for which the [lactate]/[pyruvate] ratio acts as an indicator, it should be possible to find other redox couples that behave similarly [3]. Metabolite distribution and assay problems [3,5] limit the choice of enzyme systems, so that we considered only the components of the sorbitol dehydrogenase and 1-glycerophosphate dehydrogenase reactions as suitable for analysis. Although there is considerably less sorbitol dehydrogenase activity than lactate dehydrogenase activity in rat liver [18,19], it can still catalyze a rate of sorbitol removal of >3 μmol . g^{-1} . min^{-1} [16], almost as fast as the maximal rate of lactate utilization. Fructose can also be reduced to sorbitol at substantial rates (about 1 μmol . g^{-1} . min^{-1}) [20]. Hence, it seemed valid to use the ratio [sorbitol]/[fructose] as an independent indicator of cytoplasmic [NAD]/[NADH], particularly over 40 min incubation, during which the [lactate]/[pyruvate] is known to reach steady-state (fig.1). The use of the [glycerol 1-phosphate (GP)]/[dihydroxyacetone phosphate (DHAP)] ratio as a measure of cytoplasmic [NAD]/[NADH] is 20 years old [3]. It is generally considered to give valid

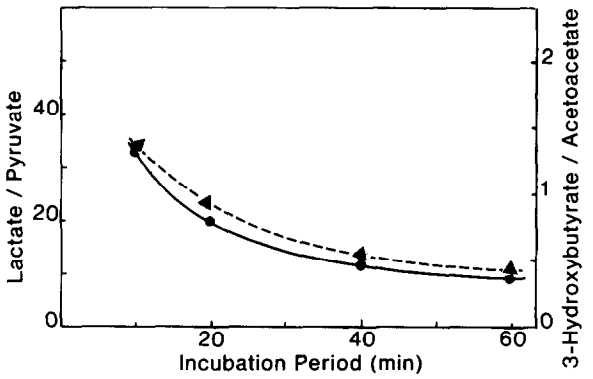

Fig.1. Isolated liver cells from starved rats were incubated in bicarbonate–saline medium which initially contained 10 mM lactate and 1 mM 3-hydroxybutyrate. Values are for a representative experiment: [lactate]/[pyruvate] (●——●); [3-hydroxybutyrate]/[acetoacetate] (▲– – –▲).

results because of the high activity of cytoplasmic GP-dehydrogenase.

Table 1 contains the results of measurements of [lactate]/[pyruvate], [sorbitol]/[fructose] and [GP]/[DHAP] ratios, after lactate and sorbitol were added separately and in combination, to isolated liver cells from fed rats. The cells were incubated without other additions or in the presence of various inhibitors. The results were unexpected in that they showed that the [lactate]/[pyruvate] ratio was far more sensitive to the action of inhibitors than were the other two couples. Hence, treatments which caused large shifts in the [lactate]/[pyruvate] ratio had little or no effect on [sorbitol]/[fructose] or [GP]/[DHAP] (table 1). In consequence, no constant relationship could be demonstrated between the three substrate pairs [4]. These observations could be taken to mean that neither sorbitol dehydrogenase nor GP-dehydrogenase are suitable indicator enzymes for measuring the redox state of cytoplasmic NAD(H). Alternatively, they could indicate that these two enzymes do not equilibrate with the same pool of cytoplasmic NAD(H) as does lactate dehydrogenase.

This was tested by examining rates at which lactate and sorbitol were removed from the medium under control conditions or in the presence of inhibitors. It was found that sorbitol inhibited lactate uptake, whereas lactate slightly stimulated sorbitol removal (table 2). Antimycin was a powerful inhibitor of both lactate and sorbitol utilization, but rotenone was much more effective an inhibitor of lactate

Table 1
Cytoplasmic redox ratios in liver cells from fed rats

Additions	[Lactate] / [Pyruvate]	[Sorbitol] / [Fructose]	[GP] / [DHAP]
Lactate	10.8 ± 0.6		3.4 ± 1.0
Lactate, rotenone	25 ± 3		6.2 ± 2.4
Lactate, antimycin	164 ± 23		3.5 ± 0.6
Lactate, DNP	78 ± 5		4.2 ± 1.7
Lactate, DNP, rotenone	43 ± 4		3.3 ± 2.0
Sorbitol	160 ± 14	14.9 ± 2.2	14.4 ± 1.0
Sorbitol, rotenone	225 ± 15	8.2 ± 1.4	10.8 ± 0.9
Sorbitol, antimycin	261 ± 27	15.6 ± 1.0	14.1 ± 2.1
Sorbitol, DNP	242 ± 25	7.2 ± 1.6	8.9 ± 1.7
Sorbitol, DNP, rotenone	258 ± 39	4.8 ± 0.6	5.5 ± 1.0
Lactate, sorbitol	108 ± 11	16.1 ± 2.5	10.7 ± 0.7
Lactate, sorbitol, rotenone	237 ± 27	11.0 ± 0.6	16.3 ± 2.2
Lactate, sorbitol, antimycin	641 ± 106	7.7 ± 0.7	24.7 ± 2.5
Lactate, sorbitol, DNP	332 ± 66	7.6 ± 0.9	8.5 ± 0.6
Lactate, sorbitol, DNP, rotenone	256 ± 28	4.9 ± 0.5	9.7 ± 0.8

Isolated liver cells from fed rats were incubated for 40 min. Metabolite levels were measured on total vessel contents. Substrates were added where indicated at 10 mM initial conc. Inhibitors were present at (μM): rotenone, 16; antimycin, 2.3; 2,4-dinitrophenol (DNP), 80. Values are the mean ± SEM of at least 5 expt. GP, glycerol-1-phosphate; DHAP, dihydroxyacetone phosphate

Table 2
Metabolism of lactate and sorbitol by liver cells from starved normal and hyperthyroid rats

Additions	Normal (μmol . g^{-1} . min^{-1})		Hyperthyroid (μmol . g^{-1} . min^{-1})	
	Lactate	Sorbitol	Lactate	Sorbitol
Lactate	−1.89 ± 0.17		−2.89 ± 0.17	
Lactate, rotenone	−0.31 ± 0.03		−0.46 ± 0.10	
Lactate, antimycin	−0.29 ± 0.02		−0.34 ± 0.18	
Lactate, DNP	−0.29 ± 0.05			
Sorbitol	+0.50 ± 0.06	−1.70 ± 0.16	+0.92 ± 0.07	−2.72 ± 0.14
Sorbitol, rotenone	+0.85 ± 0.13	−0.76 ± 0.12	+2.00 ± 0.27	−1.72 ± 0.06
Sorbitol, antimycin	+0.27 ± 0.04	−0.09 ± 0.02	+0.38 ± 0.13	−0.15 ± 0.03
Sorbitol, DNP	+0.89 ± 0.18	−1.57 ± 0.28		
Lactate, sorbitol	−0.24 ± 0.04	−1.99 ± 0.09	−1.12 ± 0.17	−2.62 ± 0.13
Lactate, sorbitol, rotenone	+0.50 ± 0.12	−0.85 ± 0.17	+1.66 ± 0.23	−1.54 ± 0.17
Lactate, sorbitol, antimycin	−0.25 ± 0.07	−0.28 ± 0.11	−0.43 ± 0.23	−0.24 ± 0.09
Lactate, sorbitol, DNP	+0.32 ± 0.06	−2.18 ± 0.18		

Isolated liver cells from starved normal or hyperthyroid rats were incubated for 40 min. Substrate and inhibitor concentrations were as for table 1. The rate of substrate removal was linear with time. Values are the mean ± SEM of at least 5 expt.

uptake than of sorbitol consumption. These findings suggest that the hydrogen arising during the oxidation of these two substrates was channelled through separate pools of cytoplasmic NAD(H) and that sorbitol utilization was much less sensitive to inhibition by rotenone.

A logical inference from this is that reducing equivalents arising from sorbitol metabolism enter the flavin-linked GP-shuttle [21,22], whereas hydrogen generated in lactate oxidation is transferred to the mitochondria by a rotenone-sensitive malate-aspartate shuttle [23]. Support for this idea came from the observation that fluoromalate, which impaired lactate uptake [24], had almost no effect on sorbitol removal [16]. Additional support for these assumptions was obtained with cells from hyperthyroid animals. In rats, hyperthyroidism is known to increase the activity of the GP-shuttle [25,26]. As expected, cells from thyroxine-treated animals removed sorbitol at an increased rate that was largely rotenone-insensitive, but lactate uptake, though also stimulated, remained sensitive to rotenone (table 2).

Thus, the assumption that the cytoplasm can be regarded as one compartment, containing a single pool of NAD(H), seems invalid. Rather, several NAD(H) pools may exist, interacting with different dehydrogenases and supplying reducing equivalents to the mitochondria through distinct shuttles. Thus, the cytoplasmic NAD(H) pools associated with specific shuttles may not interact, implying that the dinucleotides may not diffuse rapidly through the whole of the cytoplasmic compartment of the cell. For example, the NAD(H) pool interacting with lactate dehydrogenase cannot interact with that associated with sorbitol dehydrogenase. Substrate-specific shuttles could not function if all cytoplasmic dehydrogenases were to interact with a single pool of NAD(H). Thus, the original logical assumption that the dinucleotides act like co-enzymes may not be valid; rather they may function as prosthetic groups for their respective dehydrogenases.

3.2. *Role of energy in the maintenance of the steady-state [lactate]/[pyruvate] ratio in liver*

These observations do not invalidate the original concepts of Bücher, Krebs and others concerning the significance of the hepatic [lactate]/pyruvate] ratio. However, they draw attention to an area of some contention. The observations in [1] led to the surprising conclusion that the mitochondrial NAD couple was about 100-times more reduced than that of the cytoplasm. From this it was inferred [27,28] that the transfer of reducing equivalents from cytoplasmic to mitochondrial NAD(H) pools would be against the electrochemical potential gradient and was therefore likely to be energy dependent. Hence, the re-adjustment of the [lactate]/[pyruvate] ratio after a load of lactate would require energy input. On the other hand, energy would not be needed for sorbitol uptake, which involves the transfer of reducing equivalents into the mitochondria via the flavin-linked GP shuttle.

To test this, the utilisation of lactate and sorbitol was examined in the presence of 2,4-dinitrophenol (DNP; table 2). Although sorbitol uptake was less sensitive to high concentrations of uncoupling agent than was lactate removal, the results were somewhat equivocal. This was because a substantial change in the [lactate]/[pyruvate] ratio was observed only at the highest concentration of DNP added even though cellular [ATP]/[ADP] ratios and O_2-uptake were affected at relatively low levels of uncoupler (table 3). This could be taken to mean that there is only a minimal requirement for energy in the establishment of the normal steady-state [lactate]/[pyruvate] ratio. However, rates of gluconeogenesis were also influenced only to a small extent by uncoupler at <0.1 mM. Since gluconeogenesis from lactate is an energy-dependent process, these results show that energy-dependent activities may proceed even in the presence of relatively low [ATP]/[ADP] ratios.

Because of the difficulties of obtaining definitive results by the use of uncouplers alone, an alternative approach was adopted. Isolated liver cells from starved rats were incubated with rotenone which, by inhibiting NADH dehydrogenase [29], strongly depressed oxidation of [1-^{14}C]palmitate to $^{14}CO_2$ and ketone bodies, and decreased respiration by $>50\%$. The addition of 10 mM acetoacetate completely restored ketone body formation and brought about a partial recovery of O_2-uptake and $^{14}CO_2$ production. These effects were associated with a substantial reduction of the added acetoacetate to 3-hydroxybutyrate (table 4). The explanation of this phenomenon is straightforward. The NAD-linked hydroxyacyl-CoA oxidation step of each loop of the fatty acid spiral is blocked by NADH accumulating as a consequence of the inhibition of NADH dehydrogenase by rotenone. Acetoacetate acts as a hydrogen sink for this NADH, allowing the oxidation of hydroxyl-CoA to proceed.

Table 3
Effects of 2,4-dinitrophenol on lactate metabolism and adenine nucleotide ratios in isolated liver cells

DNP (μM)	O_2 (μmol . g^{-1} . min^{-1})	Glucose	[Lactate]/[Pyruvate]	[ATP]/[ADP]
0	−3.65	0.65	6.7	3.3
10	−3.98	0.53	7.4	2.5
20	−5.03	0.53	7.9	1.5
50	−5.80	0.52	12.6	1.2
100	−1.90	0.04	33.7	0.4

Isolated liver cells from normal starved rats were incubated for 40 min. Lactate was added at 10 mM initial conc. ATP in cells incubated without DNP was 1.87 mM. Values are from a representative experiment

Because the flavin-linked dehydrogenation of acyl-CoA circumvents the rotenone block, palmitate oxidation is largely restored. The flow of reducing equivalents from flavin to O_2 is coupled to phosphorylation so that levels of ATP, depressed by rotenone-treatment, are raised to about 60% of control values. Since some oxidation of acetyl-CoA derived from palmitate occurs, as evidenced by generation of $^{14}CO_2$, it appears that NAD-linked dehydrogenases of the tricarboxylic acid cycle can also couple with 3-hydroxybutyrate dehydrogenase to a limited extent, thereby circumventing the rotenone-induced block of cycle activity.

Table 4 also shows that the impairment of lactate uptake, induced by rotenone, was accompanied by a marked rise in the [lactate]/[pyruvate] ratio. This rise was not prevented by added acetoacetate; indeed, the [lactate]/[pyruvate] ratio increased still further in its presence. Palmitate was also unable to prevent the rise in the [lactate]/[pyruvate] ratio induced by rotenone. However, the combination of acetoacetate and palmitate promoted a gradual accumulation of substantial amounts of pyruvate, and reduced the [lactate]/[pyruvate] ratio to a value about twice that of cells incubated with lactate and palmitate in the absence of rotenone. Concomitantly a >3-fold increase in production of $^{14}CO_2$ from [1-^{14}C]palmitate was observed, whereas ketone body production was slightly depressed. Hence the lowering of the [lactate]/[pyruvate] ratio towards normal appears to have been associated with an enhancement of tricarboxylic acid cycle activity.

Although many of these features can be attributed only to mitochondrial metabolism, the question arose as to whether some oxidation of lactate to pyruvate might have been an extramitochondrial function mediated by a peroxisomal flavin-linked L-2-hydroxy-acid dehydrogenase [30]. To test this, experiments were conducted with cells from starved clofibrate-treated rats in which total hepatic L-2-hydroxyacid oxidase activity is <50% normal [31], even though peroxisomal proliferation occurs [32]. In the presence of either palmitate or acetoacetate, the [lactate]/[pyruvate] ratio in rotenone-treated cells remained >10-times the control value (table 4). However, with palmitate and acetoacetate present in combination, the cells were able to decrease the [lactate]/[pyruvate] ratio drastically. The ability to synthesize glucose was also partially restored. Since it is to be expected that palmitate would compete with, rather than facilitate, lactate oxidation in the peroxisomes [33], these results reinforce the view that the effects of palmitate were mediated by mitochondrial mechanisms, in which the fatty acid acted as an energy source. This conclusion is also supported by the finding that substitution of antimycin for rotenone prevented both reduction of acetoacetate by palmitate and pyruvate accumulation (not shown).

The energy requirement for the maintenance of a normal [lactate]/[pyruvate] ratio was even more apparent when cells were incubated with an uncoupling agent. Cells from clofibrate-treated rats tolerated the combination of DNP and rotenone better than did cells from normal animals, possibly due to the greater rate of fatty acid oxidation in the drug-exposed animals [34]. DNP, at a concentration which stimulated respiration in the absence of rotenone, did not affect O_2-uptake and ketone body formation

Table 4
Effects of rotenone, palmitate and acetoacetate on lactate metabolism in liver cells from starved normal and clofibrate-treated rats

Additions	Rotenone	O_2-uptake	$^{14}CO_2$	Acetoacetate	3-Hydroxybutyrate	Lactate	Glucose	Pyruvate	*L/P*
		(μmol · g^{-1} · min^{-1})						(μmol)	
Normal									
None	–	2.25 ± 0.08		0.70 ± 0.04	0.09 ± 0.01				
Palmitate	–	3.46 ± 0.28	0.228 ± 0.026	0.66 ± 0.05	1.23 ± 0.09				
Lactate	–	3.60 ± 0.24				−1.88 ± 0.13	0.65 ± 0.05	1.14 ± 0.16	10.8
Lactate, palmitate	–	5.03 ± 0.43	0.738 ± 0.069	0.73 ± 0.05	0.63 ± 0.07	−2.39 ± 0.25	1.04 ± 0.10	0.54 ± 0.20	17.4
Palmitate	+	1.57 ± 0.07	0.024 ± 0.008	0.05 ± 0.01	0.47 ± 0.06				
Lactate	+	1.59 ± 0.07				−0.31 ± 0.03	0.03 ± 0.01	0.18 ± 0.04	109
Lactate, palmitate	+	1.51 ± 0.15	0.032 ± 0.001	0.06 ± 0.01	0.76 ± 0.11	−0.16 ± 0.05	0.02 ± 0.01	0.21 ± 0.05	68
Acetoacetate	+	1.27 ± 0.10		−0.65 ± 0.18	1.20 ± 0.09				
Palmitate, acetoacetate	+	2.59 ± 0.30	0.110 ± 0.011	−1.41 ± 0.11	3.37 ± 0.29				
Lactate, acetoacetate	+	1.19 ± 0.11		−1.25 ± 0.18	1.91 ± 0.22	−0.33 ± 0.01	0.03 ± 0.01	0.08 ± 0.02	270
Lactate, palmitate acetoacetate	+	2.92 ± 0.30	0.336 ± 0.076	−1.75 ± 0.07	3.36 ± 0.26	−0.48 ± 0.08	0.04 ± 0.01	0.54 ± 0.03	34
Clofibrate									
Lactate	–	3.78 ± 0.09				−1.77 ± 0.15	0.59 ± 0.04	1.39 ± 0.08	8.2
Lactate, palmitate	+	1.42 ± 0.06	0.037 ± 0.005	0.08 ± 0.04	0.97 ± 0.06	−0.67 ± 0.13	0.03 ± 0.01	0.26 ± 0.04	78
Lactate, acetoacetate	+	0.96 ± 0.14		−1.19 ± 0.15	2.23 ± 0.18	−0.87 ± 0.13	0.05 ± 0.01	0.08 ± 0.01	252
Lactate, palmitate, acetoacetate	+	3.13 ± 0.22	0.298 ± 0.029	−1.96 ± 0.21	4.10 ± 0.22	−0.59 ± 0.09	0.15 ± 0.02	0.69 ± 0.10	22
Lactate, palmitate, acetoacetate, DNP	+	2.85 ± 0.21	0.123 ± 0.005	−1.87 ± 0.17	4.12 ± 0.19	−0.42 ± 0.06	0.04 ± 0.01	0.16 ± 0.02	125

Isolated liver cells from starved normal or clofibrate-treated rats were incubated for 40 min. Rotenone was present, where indicated, at 16 μM. Initial substrate levels were (mM): lactate, 10; acetoacetate, 8; [1-^{14}C]palmitate, 2. Values for pyruvate represent the amount accumulated in the incubation vessel after 40 min, at which time [lactate]/[pyruvate] (*L/P*) was measured. Values for rates of metabolite uptake or formation are the mean ± SEM of 5–20 expt.

when rotenone-treated cells were incubated in the presence of lactate, palmitate and acetoacetate (table 4). In the presence of uncoupler, ATP levels were decreased to 20% of control values and the [lactate]/[pyruvate] ratio remained very high.

Gluconeogenesis and $^{14}CO_2$ formation were also considerably depressed, but reduction of acetoacetate to 3-hydroxybutyrate was not affected.

3.3. *Relationship between energy demand and respiration; the 'redox cycle'*

The data in table 4 demonstrate another intriguing feature of lactate metabolism. The stimulation of O_2-uptake by addition of lactate is substantially in excess of the energy required for glucose synthesis. This conclusion is based on the assumption that the formation of 1 mol glucose from 2 mol lactate requires 6 mol ATP, derived from the consumption of 1 mol O_2. An O_2-uptake in excess of that required for gluconeogenesis has been observed in cells incubated with lactate or other glucogenic substrates, but the reason remains unclear [35–37]. In view of the energy-dependent nature of the reactions involved in transfer of lactate hydrogen to the mitochondria, it seems reasonable to assume that this process accounts for at least some of the O_2-consumption in excess of that required by the ATP demand of gluconeogenesis. Nevertheless, it is noteworthy that the eventual attainment of a normal steady-state [lactate]/[pyruvate] ratio of 8/1 is not associated with a substantial diminution in their rate of respiration. Moreover, the initial [lactate]/[pyruvate] ratio may be varied from >500/1 to <8/1 by addition of appropriate amounts of lactate and pyruvate to the cell suspension, with little effect on the respiratory rate (unpublished).

An explanation emerges when reduction of pyruvate is also considered. Pyruvate is rapidly converted to glucose and lactate as well as being oxidized in the tricarboxylic acid cycle [38]. Again, O_2-uptake is substantially in excess of that anticipated for the energy needs of gluconeogenesis [37]. This finding is in keeping with studies, using inhibitors of oxidative phosphorylation, which showed that the reduction of pyruvate to lactate, by means of reducing-equivalent transfer from mitochondria to cytoplasm, is also energy dependent [38]. These studies were supplemented by experiments with cells from starved rats treated with clofibrate to enhance fatty acid oxidizing activity [34] (table 5). Exposure of these cells to rotenone inhibited the uptake of pyruvate by 75%, and its reduction to lactate by 63%. O_2-consumption was depressed >50%. Addition of acetoacetate, as in [38], partially restored pyruvate uptake, but acetoacetate did not overcome the inhibition of respiration or of pyruvate reduction to lactate. Palmitate, added in the absence of acetoacetate, likewise had little effect on pyruvate metabolism in rotenone-treated cells, but when pyruvate, palmitate and acetoacetate were present together, O_2-uptake was stimulated, pyruvate reduction to lactate was restored to a considerable extent and significant amounts of glucose were formed (table 5). The respiration of rotenone-inhibited cells in the presence of all three substrates reached 72% of that of the corresponding control and was substantially less when pyruvate was omitted. As was the case for uninhibited cells, this stimulation of O_2-uptake by pyruvate was considerably in excess of that necessary for the quantity of carbohydrate synthesized.

The inhibition of [1-^{14}C]palmitate oxidation to $^{14}CO_2$ by rotenone was, again in part, overcome by acetoacetate. However, a much greater stimulation of $^{14}CO_2$ formation was induced by acetoacetate when pyruvate was also present (table 5). Thus, as for lactate oxidation in rotenone-treated cells, pyruvate reduction was accompanied by enhanced tricarboxylic acid cycle activity. As observed previously, palmitate and acetoacetate in combination raised cellular ATP levels in cells poisoned with rotenone to about 60% of control values, but in the presence of DNP levels only 20% of those in control cells were attained. The uncoupling agent had little effect on the interactions of pyruvate and palmitate with acetoacetate, leading to the formation of 3-hydroxybutyrate, but reduction of pyruvate to lactate and glucose synthesis were largely prevented. Formation of $^{14}CO_2$ was also depressed.

These results support the view [38,39] that the reduction of pyruvate to lactate in liver is an energy-dependent function. It is known that the shuttle system for hydrogen transfer from mitochondria to cytoplasm involves malate [40,41], but is distinct from that responsible for passage of reducing equivalents into the mitochondria [23,24]. It therefore seems feasible that, within the inner membrane, more than one NAD(H) pool may be involved in poising the [lactate]/[pyruvate] ratio. A simple model, formulated on this basis, postulates two pools, each linked to the respiratory chain, but maintained at

Table 5
Effects of rotenone, palmitate and acetoacetate on pyruvate metabolism in cells from starved clofibrate-treated rats

Additions	Rotenone	Pyruvate	O_2-uptake	$^{14}CO_2$ from [1-^{14}C]palmitate	Acetoacetate	3-Hydroxy-butyrate	Lactate	Glucose
Pyruvate	–	−3.49 ± 0.09	2.99 ± 0.12		0.80 ± 0.08	0.38 ± 0.06	1.14 ± 0.03	0.33 ± 0.02
Palmitate	–		3.85 ± 0.20	0.163 ± 0.016	0.66 ± 0.10	1.70 ± 0.18		
Pyruvate, palmitate	–	−3.48 ± 0.09	5.00 ± 0.15	0.640 ± 0.048	1.03 ± 0.06	1.38 ± 0.13	1.58 ± 0.11	0.35 ± 0.04
Pyruvate	+	−0.94 ± 0.09	1.41 ± 0.11		0.13 ± 0.02	1.14 ± 0.10	0.42 ± 0.02	<0.01
Pyruvate, palmitate	+	−1.04 ± 0.11	1.65 ± 0.09	0.133 ± 0.005	0.10 ± 0.01	1.10 ± 0.11	0.30 ± 0.03	<0.01
Pyruvate, acetoacetate	+	−2.24 ± 0.09	1.19 ± 0.13		−1.11 ± 0.12	2.60 ± 0.11	0.44 ± 0.03	<0.01
Palmitate, acetoacetate	+		2.86 ± 0.12	0.213 ± 0.037	−1.45 ± 0.15	3.44 ± 0.13		
Pyruvate, palmitate, acetoacetate	+	−2.26 ± 0.15	3.58 ± 0.11	0.496 ± 0.042	−1.11 ± 0.08	3.30 ± 0.13	1.16 ± 0.08	0.14 ± 0.02
Pyruvate, palmitate, acetoacetate, DNP	+	−1.33 ± 0.12	2.56 ± 0.11	0.219 ± 0.046	−0.93 ± 0.14	2.87 ± 0.28	0.55 ± 0.08	0.03 ± 0.01

Isolated liver cells from starved clofibrate-treated rats were incubated as described in table 4. Pyruvate was added at 10 mM initial conc. Values for lactate and glucose formation have been corrected for metabolite production in the absence of added pyruvate and are expressed in $\mu mol \cdot g^{-1} \cdot min^{-1}$ as the mean ± SEM for 5 expt.

different redox states by energy-dependent mechanisms such as 'reversed electron transfer' [42,43] (fig.2). The pool (NAD_{OX}) participating in lactate oxidation would be kept in a more oxidized state by the energy-driven flow of reducing equivalents from it, whereas the pool (NAD_{RED}), responsible for pyruvate reduction, would be held more reduced, again by energy-dependent mechanisms. It follows from this that the shuttle responsible for bringing lactate hydrogen into the mitochondria (malate–aspartate shuttle [23]) would equilibrate only with (NAD_{OX}), whereas its counterpart exporting hydrogen from the mitochondria (malate shuttle [40,41]) would equilibrate only with (NAD_{RED}).

The extensive studies of Krebs et al. [1,5–11] led to the development of the concept of a single cyto-

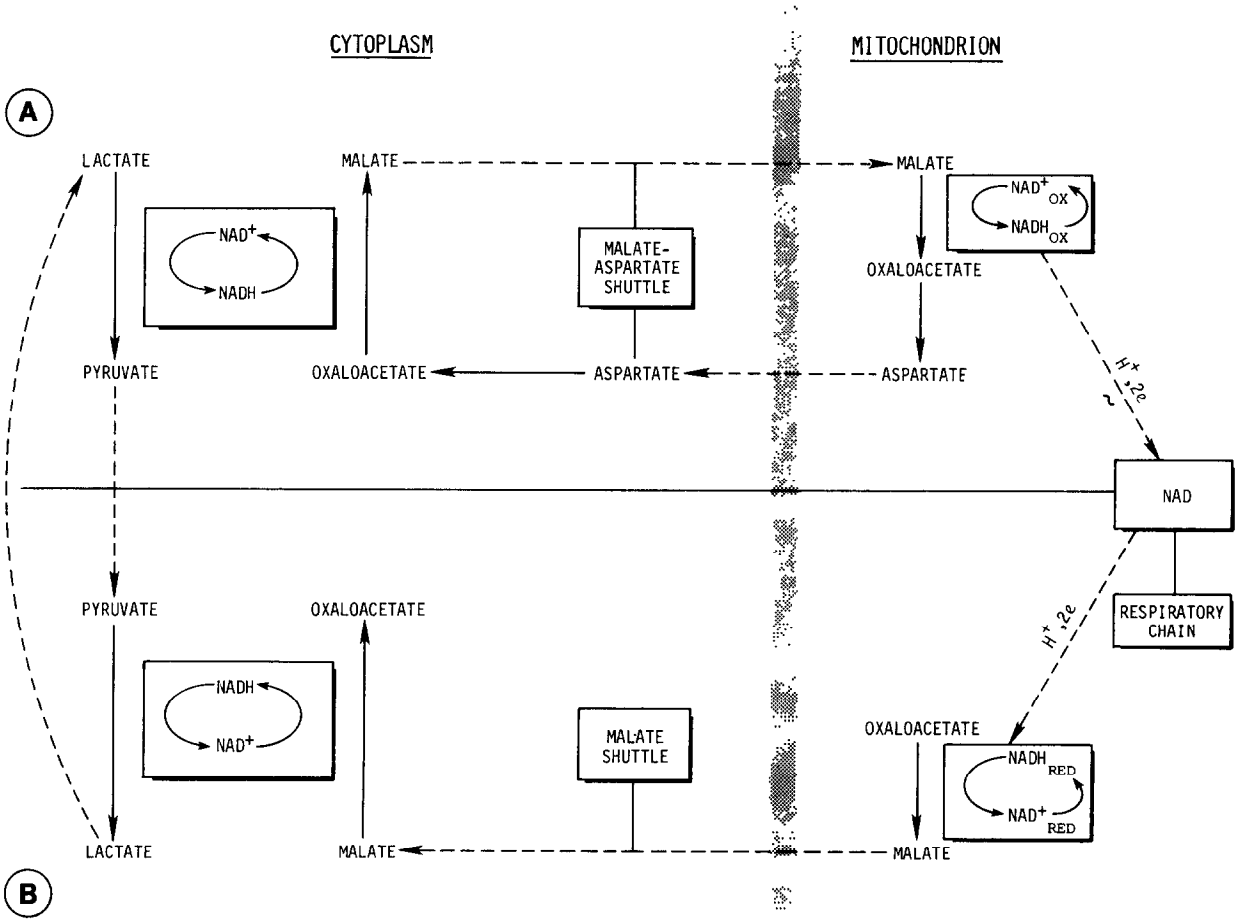

Fig.2. Schematic diagram of putative NAD pools within mitochondrial and cytoplasmic compartments. For simplicity, not all components of the transaminase reactions involved in the malate–aspartate shuttle are shown, and the pathway of gluconeogenesis from oxaloacetate is omitted. (– –) Metabolite diffusion; (——) metabolic reactions. Lactate and pyruvate are assumed to diffuse freely between compartments. Bulk flow of pyruvate is from (A) to (B) while lactate flows from (B) to (A). The carrier of reducing equivalents between NAD_{OX}, NAD_{RED} and NAD of respiratory chain is not specified, but this may be a function of the 3-hydroxybutyrate:acetoacetate couple (see text). The diagram is not intended to imply that macroscopic compartmentation occurs within cellular structures. Segregation is likely to be effected at the molecular level. Assuming the reactants in each compartment are poised sufficiently close to equilibrium, a physiological lactate load will displace lactate + NAD ⇄ pyruvate + NADH + H^+ in compartment (A) further from equilibrium, thereby increasing the rate of oxidation of lactate to pyruvate. Conversely in compartment (B), pyruvate + NADH + H^+ ⇄ lactate + NAD^+ will be brought closer to equilibrium, slowing the rate of pyruvate reduction to lactate. A similar pyruvate load will have equal but opposite effects. Thus, any perturbation of the steady-state will to be self-correcting. For further details, see text.

plasmic pool of NAD(H) in thermodynamic equilibrium with the components of the lactate dehydrogenase reaction and forming part of a network of equilibria throughout the cell. However, their experimental data are also compatible with the possibility that two compartments exist within the cytoplasm (Ⓐ and Ⓑ, fig.2), each of which contains both lactate dehydrogenase and a separate pool of NAD(H), the individual pools being held at different redox states by equilibration with two distinct mitochondrial NAD(H) pools (NAD_{OX} and NAD_{RED}, fig.2). Under such circumstances it would not be feasible for lactate and pyruvate to be in thermodynamic equilibrium with both cytoplasmic pools. Instead these metabolites would shuttle reducing equivalents between each pool, lactate being oxidized at one site while pyruvate was reduced at the other. The inevitable consequence of this would be the establishment of an energy-dependent redox cycle, so that a stationary [lactate]/[pyruvate] ratio would reflect not thermodynamic equilibrium but a dynamic steady-state in which the rate of pyruvate reduction was exactly balanced by the rate of lactate oxidation. This balance point would necessarily be displaced from the equilibrium position in relation to each individual cytoplasmic NAD(H) pool. Accordingly, the [lactate]/[pyruvate] ratio would be a composite value and would not indicate the true ratio within either cytoplasmic compartment.

3.4. *The function, distribution and regulation of redox cycles*

In recent years the notion of 'futile' or 'substrate cycles' has been debated vigorously [44–46]. It has been suggested that such cycles could play an important role in the regulation of metabolism and attempts have been made to quantify them. Indeed it has been proposed that an ATP-splitting 'futile cycle' might be involved in pyruvate reduction to lactate [47], but the contribution appears to be small under physiological conditions [48]. Since such cycles are regarded as energetically wasteful it has been assumed that their operation must necessarily be limited. Thus, Katz and Rognstad [46] have calculated that the most active cycles of this kind in liver would together utilize only 5–10% of hepatic cell ATP production. The redox cycle activity discussed here is substantially greater in magnitude, accounting for perhaps up to 50% of the energy expenditure involved in the utilisation of lactate or pyruvate in the fasting state, based on measurement of O_2-uptake.

The consumption of energy by the cyclical interconversion of lactate and pyruvate might seem to be purposeless. For this reason, acceptance of the reality of 'redox cycling' may require a substantial revision of present attitudes. For many years it has been recognized that ATP is the mediator of energy transfer for the great majority of energy utilising reactions of the living cell [49]. Further, it is generally assumed that the direction of a sequence of reactions, such as the conversion of glucose to lactate, can readily be reversed if additional steps in which ATP is consumed are incorporated into the backward pathway [50]. This implies that the metabolic pathways such as glycolysis or fatty acid oxidation can be reversed in vivo without energy input other than in the form of ATP, the reducing power for gluconeogenesis and lipogenesis coming from concomitant oxidations. A major difficulty with this concept is that the great majority of these oxidations have too positive a redox potential to allow direct coupling to reductive syntheses under physiological conditions [51]. In any case, these reductive syntheses occur in an oxidizing environment where the reduction of O_2 is by far the most favourable reaction thermodynamically [50]. Nor does ATP participate directly as a chemical component of redox reactions, even though its utilisation in other portions of the synthetic pathway may be of paramount importance. The conclusion seems inescapable that an additional driving force is necessary. In vivo, an injection of energy is required to shift the apparent redox potentials of the involved couples so that flux can take place in what would be, in the absence of energy input, a thermodynamically unfavourable direction.

The utilisation of energy to drive electrons against an electrochemical potential gradient has been named 'reversed electron transfer', and has been the subject of considerable debate for 25 years. The concept was clearly expounded by Krebs and Kornberg in 1957 [50].

> 'The fact that the synthesis of, and the release of energy from, ATP is independent of the redox scale allows ATP to be used as an 'energy currency' or 'energy transmitter' in a wide range of situations. It can in particular act as an energy link between two oxido–reduction systems. This means that one oxido–reduction (e.g., the oxidation of ferrocytochrome by O_2), by producing ATP, can theoretically drive another similarly ATP-producing oxido–reduction backwards. Thus the system NADH + Fp = NAD^+ + FpH_2 could be driven from right to left to pro-

duce NADH, providing that the coupling of oxido–reduction and ATP synthesis in the system is reversible.'

The analogy of a series of oxido–reduction cells was used, whereby a high electromotive force in one cell could drive a second cell backwards. The situation regarding the action of ATP was seen as exactly analogous to the action of electrons in the oxido–reduction cell system [50,52], in that ATP acted as an 'energy currency', energetically linking systems of widely different potential.

These conjectures were soon supported by experimental evidence obtained with mitochondrial preparations, first by Chance and Hollunger [42] and later by many others [43,53–56], who demonstrated that reversed electron transfer could involve not only flavin-coupled systems but also NAD-, NADP- and cytochrome-linked reactions. It was subsequently realised that ATP itself might not be directly involved in the energy transfer [57], and the notion of a 'high-energy intermediate' of oxidative phosphorylation was invoked instead [58]. With the development of the 'chemiosmotic hypothesis' by Mitchell [59], and suggestive evidence for the 'reversibility' of oxidative phosphorylation [10,60], there seems no difficulty in postulating that a proton gradient may be dissipated in moving electrons towards a more electronegative potential [59]. Even so, it does not follow that the process of energy-dependent reversed electron flow represents merely a reversal of oxidative phosphorylation [61].

Although it was recognized early that the light-induced energy-dependent movement of electrons against the electrochemical potential gradient is an essential element of photosynthesis in plant cells [62], general acceptance of reversed electron transfer as a process of primary physiological importance in animals has developed rather slowly. Possible reasons for this have been the limited demonstration of the phenomenon in whole cell or tissue preparations and a lack of any satisfactory explanation of how such electron flow might be regulated. Moreover, the problem of energy 'wastage' due to the interaction of energy-linked and non-energy-linked redox reactions was seen as a major obstacle to accepting the validity of reversed electron transfer as an important physiological mechanism in animals [6]. On the other hand, its function in reductive syntheses in certain autotrophic bacteria is generally acknowledged [63,64].

The results presented here (tables 4 and 5) provide evidence that energy-dependent reversed electron flow occurs in whole cell preparations. The extra energy-demand is manifest only when substrate is added, so that no energy 'wastage' occurs in the absence of an ample supply of fuel. Nevertheless, some queries will doubtless be raised concerning the likelihood that, during lactate or pyruvate metabolism, up to 20% of the O_2-consumption of the hepatocyte is utilized for the operation of a redox cycle, shuttling reducing equivalents between pyruvate and lactate. It is well-recognized that the liver converts lactate or pyruvate to glucose with equal facility, and that rapid changes of flux in the direction of glycolysis or gluconeogenesis can occur under appropriate conditions. It seems doubtful that such metabolic flexibility could be achieved by poising the reactants of the lactate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase systems close to chemical equilibrium, where flux would be minimal. Instead, it seems more likely that the extra O_2-consumption expended over that required to meet the immediate ATP demands of gluconeogenesis reflects the energy cost of poising the redox states of the relevant NAD(H) pools of the mitochondrial and cytoplasmic compartments away from chemical equilibrium. These redox states could be maintained steady, by constant energy input, at values which allow substrate pressures, that is the free energy of the reactions involved at physiological substrate concentrations [45,65,66], to become a determining factor governing the direction and net rate of metabolic flux.

A relatively simple, albeit mechanical, analogy can be made by considering a motor vehicle, with automatic transmission but without brakes, held stationary though facing up a steep hill, by the expedient of running the engine at an appropriate speed with the selector in 'drive', Energy is consumed and appears within the transmission as heat in this process, though no work is performed on the immobile car. Nevertheless, the consequence of the energy expenditure is immediately recognized by the relative ease with which the vehicle can be pushed uphill. In fact the same effort is now required to push the car uphill or down. Thus, functionally, the energy input counteracts the effects of the slope so that the car behaves as if on level ground. That this steady-state is maintained by energy expenditure is proved by turning off the engine whereupon the vehicle inevitably rolls backwards down the hill.

The redox cycle described here must likewise be

regarded as an open system which adjusts the [lactate]/[pyruvate] ratio to a steady-state. In terms of non-equilibrium thermodynamics this situation is termed 'static head' [67], reflecting the continuing expenditure of energy in the apparent absence of work. This energy input is required to maintain the steady-state against the entropic forces imposed on all natural phenomena under the second law of thermodynamics. By holding this steady-state, entropy is produced at a minimum rate [68]. These considerations are of course not compatible with assumptions made for completely coupled systems within the framework of equilibrium thermodynamics [10]. However, it is generally recognized that, whereas classical thermodynamic treatments can be of great value in describing closed systems at equilibrium, they may not be appropriate for application to open systems associated with the living cell.

There is independent evidence that other redox cycles, besides that involving the lactate:pyruvate couple, exist within cells [45,69]. Moreover, it can be predicted that any redox reaction for which bi-directional fluxes can be demonstrated readily in vivo, is likely to require energy input, at least in one direction. An example of this is the redox cycle embracing the 3-hydroxybutyrate:acetoacetate couple. Under appropriate experimental conditions the fall of the [3-hydroxybutyrate]/[acetoacetate] ratio towards steady-state parallels exactly the decline in the ratio of [lactate]/[pyruvate] (fig.1). This close relationship between the two ratios is underlined by the experiments of table 4 where, in the presence of rotenone, the energy-dependent correction of the [lactate]/[pyruvate] ratio is achieved in association with acetoacetate reduction. Moreover, the reduction of acetoacetate in an energy-dependent manner, by oxidations of the fatty acid spiral and tricarboxylic acid cycle can also be observed (tables 4 and 5). Since 3-hydroxybutyrate oxidation is coupled to phosphorylation [70], the circumstance exists for the establishment of an energy-dependent redox cycle. It appears that reducing equivalents from both cytoplasmic and mitochondrial matrix oxidations, pass to the inner membrane, probably via malate shuttles, and there bring about acetoacetate reduction in an energy-driven reaction, possibly involving succinate dehydrogenase [42,53]. The 3-hydroxybutyrate formed can then be oxidized in the respiratory chain, so that the 3-hydroxybutyrate dehydrogenase system acts as an intramitochondrial reducing-equivalent shuttle [71]. It follows that the [3-hydroxybutyrate]/[acetoacetate] ratio is a composite value, steady-state representing a balance where the rate of acetoacetate reduction exactly equals the rate of 3-hydroxybutyrate oxidation.

The operation of such a cycle can explain the elevated O_2-consumption associated with the oxidation of short or long chain fatty acids by liver cells ([37]; tables 4 and 5). Since triglyceride and phospholipid synthesis can be ruled out as significant sources of ATP depletion (unpublished), the only apparent requirement for energy is in activation of the added fatty acid. It is thus difficult to identify the source of the energy demand that the enhancement of hepatic O_2-uptake by fatty acid implies. However, if addition of fatty acid to liver cells induces the operation of energy-dependent redox cycles, involving interactions of 3-hydroxybutyrate, acetoacetate and tricarboxylic acid cycle intermediates, the requirement for additional O_2-uptake can be readily understood. Such a mechanism would allow the liver to handle rapidly a large load of lipid and convert it to ketone bodies for export to the periphery, independently of the organ's own need for ATP.

Not all redox reactions take part in redox cycles. Those that in living cells are apparently irreversible are unlikely to be energy dependent (e.g., the oxidation of pyruvate to acetyl-CoA). In keeping with this view is the fact that ethanol, added to liver cells, suppresses the oxidation of other substrates, but does not itself stimulate O_2-consumption during its conversion to acetate [72]. The failure of an added substrate to stimulate respiration of a tissue would seem to imply that its oxidation is not energy-dependent.

The proposal that cell metabolism involves numerous energy-dependent cycles embracing many of the redox reactions of the tricarboxylic acid cycle, fatty acid oxidation (and presumably synthesis), glycolysis and gluconeogenesis, can be accommodated within biochemical principles. I suggest that for many redox reactions energy input is necessary in order to overcome thermodynamic barriers; a consequence of this is the establishment of energy-dependent cycles, the energy-driven reverse process being a separate entity segregated from the thermodynamically favourable forward reaction. A few years ago, the operation of such cycles would not have been considered possible. However, in the light of new knowledge of cytoplasmic ultrastructure [73], the flow of reducing equivalents between and within localized areas of the

cytoplasm and the mitochondria now seems quite feasible, since the physical framework exists for the separation of NAD(H) pools and enzyme sequences. It is argued that only by such segregation can the cell achieve the energy-dependent gradients required to maintain the steady-state against the forces of entropy.

The question arises as to how the rate of reducing-equivalent flux through redox cycles is controlled. In considering this it is necessary to distinguish between the rate of re-adjustment of a redox pair to steady-state after perturbation, and the overall rate of flux of reducing equivalents through the corresponding redox cycle, which continues even though steady-state has been reached. Studies to be reported in detail elsewhere indicate that, for the former, the degree of displacement of the redox reaction from steady-state is the determining factor ([65]; fig.1,2). As far as regulation of total flux is concerned, it seems improbable that the process of reversed electron transfer is rate-limiting, for if this were the case it is difficult to envisage how the constancy of the redox states of cellular dinucleotide pools could be maintained. A more likely constraint may be the capacity of the shuttle mechanisms for reducing-equivalent flux between cell compartments. However, at present it must be acknowledged that the factors controlling the overall rate of metabolism in cells and animals are poorly understood.

Nevertheless, even in the present state of our knowledge, it is an attractive speculation that the hormone, thyroxine, which is known to increase the basal metabolic rate, may act to stimulate the rate of redox cycle activity. It has been shown that in the hyperthyroid state the O_2-consumption by liver cells, in excess of that expected on the basis of the energy demand of gluconeogenesis, is much greater than under control conditions and reducing-equivalent transfer between cell compartments is greatly enhanced [37,74]. A stimulation of redox cycle activity by thyroxine would be in keeping with the hormone's calorigenic effect, since in view of their dissipative nature, redox cycles involving reversed electron transfer (tables 4 and 5), are likely to have a major function in heat production within the animal. What controls the rate of cycling in individual species and whether there is a relationship to body mass must be a matter for further study.

It is concluded that energy-dependent reversed electron transfer is a fundamental feature of the living state. It provides a mechanism for the reversal of thermodynamically unfavourable redox reactions by energy coupled steps, distinct from the forward reactions. These processes are dependent on the high degree of organization of cell fine structure which allows energy transfer, not only within the cellular membranes, but also within other microcompartments throughout the cell. The constant energy input, mediated by reversed electron transfer, thus maintains the cellular dinucleotide pools at redox states removed from chemical equilibria and helps bring about the establishment of steady-states for cell metabolites and a dynamic balance between degradative and synthetic processes.

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Professor Krebs' work has had a major impact on everyone who studies the regulation of metabolism. The experiments and ideas presented here have been inspired in large measure by the example that he has set during the twenty years I have been privileged to know him. The authorship of much of the more recent work, carried out at Flinders University School of Medicine has been shared with Dr A. R. Grivell and Dr P. G. Wallace, and owes much to the efforts and diligence of a group of dedicated assistants, ably led by Ms D. C. Fanning. The work conducted in Australia has been supported in part by the National Health and Medical Research Council.

Note added in proof

Recent studies on the energy-linked transhydrogenase [75] tend to confirm that the process of reversed electron transfer can be understood in the light of Mitchell's hypothesis that an electrical field can drive a chemical reaction in an otherwise thermodynamically unfavourable direction. A simple mechanism for this in relation to intramitochondrial redox reactions can be developed on the basis that NAD(P)- or flavin-linked redox reactions generate or consume protons. It seems possible that these protons are not released immediately into the bulk aqueous medium of the cell [76], but flow along hydrogen-bonded channels within proteins [77]. This flow will be influenced by the electric field created by the inner mitochondrial membrane potential and will be

enhanced or impaired depending on the orientation of the dehydrogenase within the membrane. In this way the energy of the electric field may be used to shift the equilibrium of redox reactions and thereby bring about the phenomenon described as reversed electron transfer. These considerations can also apply to extramitochondrial dehydrogenases, provided that the protons generated can be conducted by means of hydrogen-bonded chains ('proton wires' [77]) through an appropriately oriented electric field.

These concepts emphasize the over-riding importance of structure in relation to cellular function. It must be assumed that the interactions described here take place in a structured cellular phase, segregated from the bulk aqueous medium [78]. Such an arrangement would permit enzymes to be organized in relation to intracellular electric fields in such a way that exergonic reactions were impaired by the fields whereas endergonic reactions were promoted. In this way electrical energy could be used to poise metabolic pathways very close to equilibrium so that flux along anabolic or catabolic pathways was entirely a function of substrate or product pressure.

These ideas provide a means of reconciling the findings of H. A. Krebs, concerning the existence within cells of networks of near-equilibrium reactions traversing intracellular boundaries, with the view that differences in redox states between cell compartments must reflect systems functioning far from equilibrium. It would appear that living systems are able to conserve energy derived from degradation of foodstuffs, not only by synthesizing new chemical bonds, but also by storing separated charge. The energy conserved in the electric field so created can be used to drive oxidative phosphorylation or reversed electron transfer [59]. Conversely ATP can be utilized to generate proton currents which can be harnessed for cellular work [79]. This interaction of chemical and electrical energy within the cell makes living systems highly efficient units operating close to equilibrium. This conclusion is in marked contrast to current concepts which, despite the influence of Mitchell, discount the role of electrical energy in metabolism and assume on the basis of chemical analyses that living systems function far from equilibrium.

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