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The Determination of the Redox States and Phosphorylation Potential in Living Tissues and Their Relationship to Metabolic Control of Disease Phenotypes

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This paper reviews the development in the 1950s of methods to determine the redox states of the free [NAD^+]/[NADH] in cytoplasm of yeast by Helmut Holzer and Feodore Lynen and in rat liver by Theodore Bucher and Martin Klingenberg. This work was extended in the 1960s in the laboratory of Hans Krebs, where the use of basic thermodynamic and kinetic principles allowed the extension of this approach to the determination of the free mitochondrial [NAD^+]/NADH] in mitochondria and the redox state of the free NADP system in cytoplasm and mitochondria. This work also outlined the linkage between the redox states in the various couples to the phosphorylation state or the free [ATP]/[ADP][P_i] ratio, the central energy parameter of living cells. This work has since been extended to include other energy-linked systems including the gradients of inorganic ions between extra and intracellular phases of the cell and the redox state of the co-enzyme Q couple of mitochondria. This system of linked near-equilibrium redox and phosphorylation potentials constitutes a framework of primitive metabolic control that is altered in a number of disease phenotypes. The alteration of such disease phenotypes by substrate availability is discussed, as well as the importance of a thorough grounding in basic kinetics and thermodynamics in designing new therapies to normalize the metabolic abnormalities that are the proximate cause of many common and some rare diseases states.

Keywords: Free cytoplasmic and mitochondrial [NAD^+]/ [NADH], [$NADP^+$]/ [NADPH], [ATP]/ [ADP] [P_i], energetics, metabolism, redox states, phosphorylation potential, $\Delta G'$ of ATP, linked metabolic networks.

EARLY ATTEMPTS TO DETERMINE REDOX STATES

The importance of proton equilibria in biochemistry was recognized early in the 20th century as exemplified by work of Van Slyke, Henderson, and Hasselbalch and codified in the famous Henderson-Hasselbalch equation defining the pH of the blood and its relationship to the primary mammalian [HCO_3^-]/[CO_2] buffer system [1]. A quantitative study of redox potentials was the life work of W. Mansfield Clark, professor of biochemistry at The Johns Hopkins University, whose review on potentiometric and other determinations of redox potentials remains [2] a classic in the field. Until the 1960s, studies of redox potentials of cells largely made use of redox dyes to estimate the redox potential. As Clark pointed out, however, there are no enzymes present to ensure that the redox potential of a dye will equilibrate with the redox couple intended for study. A shift of emphasis to the use of enzymes to determine the redox states of various tissue components soon bore fruit. This approach required a cataloging of the thermodynamic characteristics of the reactions involved, as exemplified by the tables of the free energies involved in reactions of biological interest complied by Hans Krebs, Sir Hans Kornberg, and Kenneth Burton [3]. This approach required a thorough understanding of the kinetics of the enzymes bringing about these reactions.

Measurement of total NAD and NADH gives no information on the extent and direction of oxido-reduction in cells because such measurements fail to differentiate between free and bound nucleotides and because the distribution of these nucleotides between cytoplasm and mitochondria was known to be uneven [4]. Additionally, the pyridine nucleotides did not move freely between the cytoplasm and mitochondria. These difficulties in compartmentation and binding were overcome in 1956 when it was shown in yeast by Helmut Holzer and Feodore Lynen ([5]) that the ratio of free [NAD^+]/[NADH] could be estimated from the measured ratio of acetaldehyde/ethanol on the assumption of near-equilibrium in the alcohol dehydrogenase reaction. (Fig. 1). It was critical that the enzyme being measured have a high activity and be in a state of "near-equilibrium" and that the enzyme be located in the cell compartment of interest. Using that approach, the free cytoplasmic [NAD^+]/[NADH] was estimated to be about 500. The same principle was soon applied to liver by Theodore Bucher and Martin Klingenberg (Fig. 2([6]), where they measured ratios of [lactate]/[pyruvate] and [α-glycerophosphate]/[dihydroxyacetone-P], both giving

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FIG. 1. **Feodor Lynen.**

the same calculated free cytoplasmic [NAD^+]/[NADH] ratio.

THE LABORATORY OF HANS KREBS AND THE REDOX STATE OF FREE MITOCHONDRIAL [NAD^+]/[NADH]

This was the state of knowledge of redox states when I arrived at the laboratory of Hans Krebs in July of 1966. I had received the mandatory 1 year of biochemistry at Harvard University School of Medicine, where I had been a research fellow for 1 year. I later spent 2 years as a research fellow at the NIH.[1] As most will understand, such training is totally inadequate to do much meaningful research. Luckily, my preceptor at the NIH was Han Weil-Malherbe, a physician and biochemist, who had served as a house officer with Krebs at Frieberg, where he elucidated the urea cycle. As a result of Weil-Malherbe's intercession, I was accepted as a worker in Krebs' laboratory. Krebs only had 20 Ph. D. students in his entire 50-year career in biochemistry. By this stage in his career, his "post-doctoral" positions were largely filled by distinguished U. S. professors on sabbatical. For those who decry "old boy" networks, they sometimes work. Krebs, a young student with no meaningful research training or special distinction, obtained his position in Otto Warburg's laboratory through the intercession of his friend Bruno Mendel, who recommended Krebs as an assistant in Warburg's laboratory while attending a dinner party with Warburg and Albert Einstein at Warburg's father's house [7]. He considered this training with Warburg crucial. In his autobiography, the proofs of which he received on the day he died in 1981, Krebs wrote [8], "Among all my teachers, he had by far the greatest influence on my development and I owe him an immense debt." Many who worked with Hans Krebs, including myself, would say the same of him.

I arrived in Oxford after the overnight flight from the United States and went to Krebs office at 9 a.m., still carrying my bags. He had just returned from a symposium in Indianapolis organized by George Weber, where he presented his ongoing work on the redox state of the mitochondrial pyridine nucleotides. In the course of discussion, he was asked by George Cahill, "What is the redox state of the NADP system?" He announced to me at that first meeting that working out the redox potential of the NADP system was to be my project. He introduced me to Pat Lund (Fig. 3), who had the onerous duty of teaching me to do everything. Krebs was visibly disappointed that I would not start work that day, before I had arranged my accommodations. After being taught by Pat to measure metabolite concentrations enzymatically, my first project was to determine the equilibrium constant of the malate dehydrogenase reaction under intracellular conditions of ionic strength 0.25 and temperature of 38 °C. The result of this determination was acknowledged in a line in Table XII of Krebs' report on the determination of the free mitochondrial [NAD^+]/[NADH] ratio by measurement of the substrates of the β-hydroxybutyrate and glutamate dehydrogenase reactions [9]. One of Krebs' co-authors had severe doubts that the measurements of the three substrates of the glutamate dehydrogenase reaction would ever indicate the redox state of the mitochondrial NAD couple. Krebs response was Warburgian, "just do it." The result was, in my opinion, the most remarkable paper in the entire series

FIG. 2. **Martin Klingenberg (*top*) and Theodore Bucher.**

[1] The abbreviations used are: NIH, National Institutes of Health; PDH, pyruvate dehydrogenase.

FIG. 3. **Members of the Krebs laboratory in 1968 who contributed significantly to our understanding of cellular redox states.** 1, Han Krebs; 2, Dereck Willilamson; 3, Pat Lund; 4, Richard Veech; 5, Marion Stubbs; 6, Sean Brosnan; 7, Dulce Veloso. For specific contributions of each, see the references.

of attempts to determine redox states of the pyridine nucleotides. In this paper, he showed that one enzyme in the mitochondrial cristae, β-hydroxybutyrate dehydrogenase, shared the same pyridine nucleotide co-factor pool with the glutamate dehydrogenase residing in the mitochondrial matrix. He also showed that during alloxan diabetes, not only is the free cytoplasmic NAD couple reduced, but the redox state of the free mitochondrial NAD couple moved in the opposite direction, becoming oxidized. He correctly interpreted this finding as the result of a deficiency of citric acid cycle intermediates, caused by the rapid conversion of oxaloacetate to phosphoenolpyruvate due to the exuberant gluconeogenesis characteristic of the diabetic state. It would be over a decade before the implications of this paradoxical reduction of the free cytosolic NAD couple and the oxidation of the free mitochondrial NAD couple on the free energy of ATP hydrolysis would be fully appreciated.

THE DETERMINATION OF THE FREE CYTOSOLIC [$NADP^+$]/[NADPH]

Having been assigned the topic of determining the redox state of the free cytoplasmic [$NADP^+$]/[NADPH], and following the lead outlined earlier by Holzer, Lynen, Klingenberg, Bucher, and Krebs, I determined, with the help of Leonard Eggleston, a technician with 30 years experience in Krebs' laboratory, the activity of the NADP-linked dehydrogenases in liver. The activity of the NADP-linked dehydrogenases was 1–2 orders of magnitude lower than those of the cytosolic NAD-linked dehydrogenases, whose activity was 250 μmoles/min/g of wet weight at 25 °C for lactate dehydrogenase and 400 for malate dehydrogenase or double that at 38 °C, as compared with glycolytic flux rates of the order of 0.5–1 μmol/min/g. The activity of the NADP-linked dehydrogenases is listed in Table I.

Next I determined the equilibrium constants of each of these reactions. Many of these enzymes were commercially available, and their constants were determined, in the forward and backward direction, under the appropriate intracellular conditions of I = 0.25, T = 38 °C. Malic enzyme was not commercially available, but Krebs suggested that it could be obtained free of the more active malate dehydrogenase by fractionating wheat germ, which lacks malate dehydrogenase. Fresh wheat germ was provided from the flour mill in Wantage, and a crude fraction was made that allowed the determination of this equilibrium constant. As an interesting aside, isocitrate of the proper L_s isomeric configuration was not commercially available in 1966. I therefore used isocitrate, which had been extracted from *Sedum praeltum* grown in Krebs' own garden and presented to me in a hand-cut glass vial that Krebs received from Otto Warburg's laboratory. I used this sample to determine the K_{eq} of the isocitrate dehydrogenase reaction, which I found to be 0.91 M. Later, more careful determinations of this constant were performed in the laboratory of Keith Dalziel and were found to be 1.17 M. The determination of the constant for glutathione reductase was a special problem because of the near impossibility of obtaining glutathione standards without small contaminating amounts of oxidized glutathione. The value I obtained for the following reaction,

$$K_{eq} = \frac{[GSSG][NADPH][H^+]}{[GSH][NADP^+]}$$

REACTION 1

TABLE I
Activity of NADP-linked dehydrogenases in rat liver (10)

Cytosolic NADP-linked dehydrogenase	Activity
	μmol/min/g of wet weight
Isocitrate dehydrogenase	22
Glutathione reductase	7
6-Phosphogluconate dehydrogenase	2.8
Glucose-6-phosphate dehydrogenase	1.4
Malic enzyme	1.3

was 2×10^{-9} M. A better value is likely to be about 1×10^{-9} M obtained at the Arctic Research Laboratory by E. M. Scott *et al* [64]. Lest one think that determinations of equilibrium constants are not without problems, it is worth reporting that T. W. Rall and Albert Lehninger [65] reported values for this constant of 9×10^{-16} M, and the great physical chemist Isherwood reported a value of 5.6×10^{-13} M. Because equilibrium constants are just that, constants, it is with some trepidation that one reports them because if the value is incorrect, there is little one can do but admit error. As we will see, I would have to do that later in my first determination of the equilibrium constants for glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase, where I ignored the very large effects of free $[Mg^{2+}]$ on the value of the later constant.

In the case of measuring the ratio of oxidized to reduced glutathione in tissue, the issue was moot. Preventing small amounts of oxidation during perchloric acid treatment of frozen tissue inevitably leads to the oxidation of small amounts of reduced glutathione. We even resorted to preparing frozen extracts under liquid carbon monoxide made specially for my use in the low temperature physics laboratory at Oxford, to remove all active heme, which produces oxygen free radicals when subjected to acid treatment in a Lemberg-Legg type reaction. This was all to no avail despite 4–5 months of work.

The equilibrium constants of the relevant dehydrogenases linked to the cytoplasmic NAD and NADP systems and the free mitochondrial $[NAD^+]/[NADH]$ systems were either determined in the Krebs laboratory or obtained from the literature and were as reported (Table II).

The determination of the other NADP dehydrogenase-linked substrates enzymatically was quite accurate. We soon had our answer about the ratio of free cytosolic $[NADP^+]/[NADPH]$. The values of the free NAD and NADP couples were calculated from the equilibrium constants given in Table I, using the concentrations of substrates measured enzymatically in freeze-clamped liver, the $[CO_2]$ measured in hepatic venous blood, and assuming intracellular pH of 7.0 (Table III).

The agreement between the free cytosolic $[NADP^+]/[NADPH]$ calculated from the substrates of the malic enzyme and isocitrate dehydrogenase, the parallel changes with diet and the much lower redox potential of the free cytosolic NADPH couple at -0.41 V as compared with the potential of the free cytosolic NAD couple at -0.19 V, made it almost certain that these estimates were correct. This difference explained why the very negative NADP system was used for reductive synthesis of fatty acid and cholesterol synthesis, whereas the more oxidized potential of the free cytosolic NAD system was poised to accept the reducing equivalents produced in glycolysis. On the other hand, the potential of the free mitochondrial NAD couple at -0.28 V was too oxidized to effect reductive synthesis of fats, whereas the potential of the free cytosolic NAD system was too oxidized to produce sufficient redox potential to effect the synthesis of 3 ATP in the mitochondria. It was also clear that the nucleotide specificity of the cytosolic pyridine nucleotide dehydrogenases for NAD or NADP was determined by the equilibrium constants of the reactions themselves [19]. Later, the relationship of the NADP system to the reactions of the hexose monophosphate pathway was demonstrated, as was the linkage of the non-equilibrium portion of the hexose monophosphate pathway to the glycolytic pathway [20]. Only recently, the work of Kosaku Uyeda has shown that this cross-talk between the cytosolic NAD and NADP systems, which determine the concentrations of metabolites of the non-oxidative portion of the pentose phosphate shunt, in turn control the activity of the glucose responsive transcription factor, ChREBP [21]. This transcription factor regulates the co-coordinated transcription of many of the enzymes of the fatty acid synthesis pathway, along with certain glycolytic enzymes and enzymes producing NADPH, both required for fatty acid synthesis.

TABLE II

The equilibrium constants of NAD and NADP-linked dehydrogenases

Values were obtained at 38 °C, I = 0.25. The substrates are listed as the predominant ionic species at physiological pH of 7.0 but indicate the concentration of the sum of all the ionic species present. While not the case in most dehydrogenase reactions, in reactions such as ATP hydrolysis or the aconitase reaction where the Mg^{2+} binding constants of substrates vary significantly, account must be taken of the effects of changes in free $[Mg^{2+}]$ upon the apparent equilibrium constant (11–13).

Enzyme	Definition of K_{eq}	Value	Reference
NADP Linked Dehydrogenases			
Isocitrate Dehydrogenase EC 1.1.1.42	$\frac{[\alpha\text{-ketoglutarate}^{2-}][CO_2][NADPH]}{[\text{isocitrate}^{3-}][NADP^+]} = K'$	1.17 M	(14)
Glutathione Reductase EC 1.4.6.2	$\frac{[GSSG][NADPH][H^+]}{[GSH]^2[NADP]} = K'$	1.98×10^{-9}	(10)
6-Phospho-gluconate Dehydrogenase EC 1.1.1.44	$\frac{[\text{Ru-5-P}^{2-}][NADPH][CO_2]}{[\text{6-P-gluconate}^{3-}][NADP^+]} = K'$	0.17 M	(15)
The Malic Enzyme EC 1.1.1.40	$\frac{[\text{pyruvate}^-][CO_2][NADPH]}{[\text{malate}^{2-}][NADP^+]} = K'$	3.44×10^{-2} M	(10)
Cytoplasmic NAD Linked Dehydrogenases			
Lactate Dehydrogenase EC 1.1.1.28	$\frac{[\text{pyruvate}^-][NADH][H^+]}{[\text{L-lactate}^-][NAD^+]} = K'$	1.11×10^{-11} M	(9)
Malate Dehydrogenase EC 1.1.1.37	$\frac{[\text{malate}^{2-}][NADH][H^+]}{[\text{oxaloacetate}^{2-}][NAD^+]} = K'$	2.86×10^{-12} M	(16)
α-Glycerophosphate Dehydrogenase EC 1.1.1.8	$\frac{[DHAP^{2-}][NADH][H^+]}{[\alpha\text{-GP}^{2-}][NAD^+]} = K'$	1.35×10^{-11} M	(17)
Glyceraldehyde phosphate Dehydrogenase EC 1.2.1.12	$\frac{[\text{1,3 diP-glycerate}^{4-}][NADH][H^+]}{[\text{glyceraldehyde 3-P}^{2-}][Pi^{2-}][NAD^+]} = K$	5.1×10^{-13}	(13)
Mitochondrial NAD Linked Dehydrogenases			
β-Hydroxybutyrate Dehydrogenase EC 1.1.1.30	$\frac{[\text{acetoacetate}^-][NADH][H^+]}{[\beta\text{-hydroxybutyrate}^-][NAD^+]} = K'_{\text{bHBDH}}$	4.93×10^{-9} M	(9)
Glutamate dehydrogenase EC 1.4.1.2	$\frac{[\alpha\text{-ketoglutarate}^{2-}][NH_4^+][NADH][H^+]}{[\text{glutamate}^-][NAD^+]} =$	3.87×10^{-13} M^2	(18)

THE LINKAGE OF REDOX STATES

To me however, there were even more exciting implications than the simple determination of the redox state of the NADP system. The redox states of the various pyridine nucleotide couples, despite their large differences in poten-

TABLE III

Free cytosolic [NADP$^+$]/[NADPH] and free cytosolic and mitochondrial [NAD$^+$]/[NADH] ratios in free clamped rat livers fed different diets (10)

Diet	Free cytosolic [NAD$^+$]/[NADH] from lactate dehydrogenase	Free cytosolic [NADP$^+$]/[NADPH] malic enzyme	Free cytosolic [NADP$^+$]/[NADPH] isocitrate dehydrogenase	Free mitochondrial [NAD$^+$]/[NADH] β-hydroxybutyrate dehydrogenase
Fed Chow (n = 12)	1164 ± 218	0.0118 ± 0.0019	0.0101 ± 0.0008	7.74 ± 1.10
48-h fasted (n = 8)	564 ± 81	0.00186 ± 0.00031	0.00442 ± 0.0003	5.48 ± 0.53
3-day 70% sucrose (n = 8)	1820 ± 237	0.0146 ± 0.0024	0.0105 ± 0.0012	5.14 ± 1.13
3-day low carbohydrate (n = 12)	526 ± 126	0.00134 ± 0.0003	0.00264 ± 0.00035	4.09 ± 0.97

tial, were not independent of one another but rather were algebraically linked by shared metabolite concentrations. Pyruvate concentrations were common to both the NADP couple through the malic enzyme reaction and the free cytosolic NAD couple through the lactate dehydrogenase reaction, containing a ratio of metabolites and the ratio of free nucleotide ratios in equations of the following form,

$$\frac{[NADH]_{cyto}[NADP^+]_{cyto}}{[NAD^+]_{cyto}[NADPH]_{cyto}} = \frac{[CO_2][lactate^-]}{[H^+][malate^{2-}]} \times \frac{K_{LDH}}{K_{ME}}$$

REACTION 2

Similar statements could be written linking the free mitochondrial NAD system and the free cytosolic NADP system through the common substrate α-ketoglutarate. However, when I tried to calculate this relationship, the answer was off by a factor of 10^3. It should work, but it did not. I showed my calculations to Pat Lund, who had been my instructor in enzymatic analysis, and she pointed out that the glutamate dehydrogenase reaction had unequal numbers of metabolites and therefore had units of mM because of the substitution of metabolite measurements in μmol/g of wet weight. Although physical chemists insist on writing equilibrium constants without units, as a working biochemist, I have since always insisted on balancing units and charges in any equation. Although this irritates the trained physical chemists, it has saved me from many errors over subsequent years.

One last thing had to be added in this linkage of redox states, and that was that the free cytosolic NAD system had to be related to the free cytosolic [ATP]/[ADP] [P_i] ratio or phosphorylation potential due to the high activity of over 100 μmol/min/g of the glyceraldehyde-3-phosphate dehydrogenase and 3-phosphoglycerate kinase reactions and the known reversibility of glycolysis and gluconeogenesis in liver in a reaction equation of the following form,

$$\frac{[ATP^{4-}]_{cyto}[NADH]_{cyto}}{[ADP^{3-}]_{cyto}[P_i^{2-}]_{cyto}[NAD^+]_{cyto}} = K_{combined}\frac{[GAP^{2-}]}{[3PG^{3-}][H^+]}$$

REACTION 3

It took about 3 days to write up the results of my determination of the redox state, the free cytosolic NADP system and its difference from the redox potentials of the free cytosolic and mitochondrial NAD systems, plus their linkage to one another and to the phosphorylation potential. I presented my results to Krebs in the laboratory. He looked at my five written pages and said, "This can't be right." I responded with, "Well damn it, it is." Neither of us were angry, we were just expressing our opinions freely, which was both of our habits. Krebs never became out of sorts when discussing experiments, even if they were critical, only when people, often visiting professors, wanted to talk, and waste his time. I have often remembered his dictum, "time is the only irreplaceable commodity," particularly when attending obligatory meetings by one or another self-important, but largely irrelevant, administrator. For the next month, Krebs offered no further opinions on the subject, nor did I. He continued to talk to me every day, but not on this subject. He was thinking things over.

After about 1 month or more, he came to me and announced that we would start writing a paper. He had thought about it in his careful way and decided. The writing of this paper took at least an hour a day for several months. Although these findings were reported in a number of different forms, the first version turned out to be the longest paper Krebs ever wrote [19]. In his autobiography, Krebs listed his 356 papers written over 50 years in biochemistry. He cited 23 of these as "more important", including the report of the urea cycle [22] and the citric acid cycle [23]; the list also included a shorter version of this research entitled "Interrelations between diphospho- and triphospho-pyridine nucleotides" [24]. The network of near-equilibrium reactions linking the redox states of the free pyridine nucleotide couples continued to grow. Immediately following the work on the free NADP couple, several reactions were worked on in the Krebs laboratory. These were the active transaminases and the isomerases in the initial portion of glycolysis. Their equilibrium constants were determined (Table IV).

The equilibrium constants for aspartate aminotransferase were performed by Marion Stubbs and those for alanine aminotransferase were performed by Sean Brosnan, but these were never published. The constants for aldolase and triose phosphate isomerase were performed by Louisa Raijman and me, with the consultation of Keith Dalziel and Krebs. Although conditions were carefully set at 38 °C and I = 0.25 to match the physiological situation, no consideration was given at the time to the fact that the concentration of free [Mg^{2+}] must be considered in reactions in which the substrates have differing Mg^{2+} binding constants.

Over a Christmas holiday in the Krebs laboratory in 1967, I determined the equilibrium constant of the combined glyceraldehyde-3-phosphate dehydrogenase plus 3-phosphoglycerate kinase reactions, which in the absence of free Mg^{2+} I found to be 59. At the time, I was unaware of the effects that variations of free [Mg^{2+}] would have in reactions in which the Mg binding constants of the substrates and products differed significantly, a fact that was clearly explained by Robert Alberty [26] soon after these results were obtained. My value was incorrect be-

TABLE IV

Near equilibrium transaminases and glycolytic isomerases

These determinations were performed at I = 0.25, T = 38 °C, pH 7.0, and at 0 free [Mg^{2+}]. The substrates are listed as the predominant ionic species at physiological pH but indicate the concentration of the sum of all the ionic species present.

Linking reactions	Definition of K_{eq}	Value	Reference
Aspartate amino transferase EC 2.6.1.1	$\frac{[\alpha\text{-ketoglutarate}^{2-}][\text{aspartate}^{-}]}{[\text{glutamate}^{-}][\text{oxaloacetate}^{2-}]} = K'$	6.61	(10)
Alanine amino transferase EC 2.6.1.2	$\frac{[\alpha\text{-ketoglutarate}^{2-}][\text{alanine}]}{[\text{glutamate}^{-}][\text{pyruvate}^{-}]} = K'$	1.52	(10)
Triose phosphate isomerase EC 5.3.1.1	$\frac{[\text{dihydroxyacetone-P}^{2-}]}{[\text{glyceraldehyde 3-P}^{2-}]} = K'$	22	(25)
Aldolase EC 4.1.2.13	$\frac{[\text{DHAP}^{2-}][\text{GAP}^{2-}]}{[\text{Fru-1,6 bisP}^{4-}]} = K_{\text{Aldolase}}$	0.99 x 10^{-4} M	(25)

cause the conditions under which it was determined, that is, without Mg^{2+}, failed to reflect intracellular conditions. The value for this constant in the absence of free Mg^{2+} obscured many important physiological features present in animal tissues, such as the mitochondrial sequestration of ADP in mitochondrial-containing tissues, and led to the widespread measurement of total ATP, ADP, and AMP as meaningful estimates of cellular energy status, an error that persists in many publications to this day. My failure to consider the effects of free [Mg^{2+}], the predominant intracellular divalent cation, on the value of this constant would take 10 years of very painstaking work to overcome.

THE LINKAGES OF THE REDOX STATES TO THE ENERGY OF ATP HYDROLYSIS AND THE PROBLEM OF FREE [MG^{2+}]

One problem concerning the effects of free [Mg^{2+}] was that, although total intracellular Mg content was known, no one knew the value of free intracellular [Mg^{2+}]. The free [Mg^{2+}] of plasma had been calculated to be about 0.5 mM [27]. With the help of Dulca Veloso, who joined me after I left the Krebs laboratory in 1969, Robert Guynn, and Marianne Oskarson, we embarked on a year-long project aimed at determining the free cytosolic [Mg^{2+}], comparing the values of the aconitase equilibrium constant with free [Mg^{2+}] with Scatchard plots of Mg^{2+} binding to cellular components. Our study estimated that the free cytosolic [Mg^{2+}] varied between 0.6 and 1 mM in all tissues studied [12].

Many reactions of central metabolic importance vary with changes in free [Mg^{2+}], some of which are listed in Table V. The effects of variations of free [Mg^{2+}] and pH on the equilibrium constants of the 3-phosphoglycerate kinase reaction, the ATP hydrolysis reaction, and the creatine kinase reaction can be illustrated graphically (Fig. 4) and are quite large [11]. The proof that near-equilibrium existed in the creatine kinase reaction and in the combined glyceraldehyde-3-phosphate dehydrogenase 3-phosphoglycerate kinase reactions [32] became the basis for estimating the $\Delta G'$ of ATP hydrolysis in tissues non-invasively using ^{31}P magnetic resonance spectroscopy and has been confirmed by this technique many times [33, 34].

These errors that we made in the initial determination of these important equilibrium constants should not detract from the importance of thermodynamics to biochemistry, a fact that Krebs emphasized throughout his career, along with his student, Kenneth Burton [3], Sadly, this emphasis

TABLE V

Equilibrium constants of reactions varying with free [Mg^{2+}]

Values were measured at I = 0.25, T = 38 °C, and reported for free [Mg^{2+}] = 1 mM.

Enzyme	Definition of K_{eq}	Value	Reference
Kinase Reactions			
Aconitase EC 4.2.1.3 at 1 mm free [Mg^{2+}]	$\frac{[\text{citrate}^{3-}]}{[\text{L}_s\text{-isocitrate}^{3-}]} = K'_{\text{Acon}}$	18	(12)
Phosphoglycerate kinase EC 2.7.2.3	$\frac{[\text{3-P-glycerate}^{3-}][\text{ATP}^{4-}]}{[\text{1,3 diP-glycerate}^{4-}][\text{ADP}^{3-}]} = K'$	3.8 x 10^{-3}	(13)
Creatine kinase EC 2.7.3.2	$\frac{[\text{creatine}^{+}][\text{ATP}^{4-}]}{[\text{creatine-P}^{2-}][\text{ADP}^{3-}][\text{H}^{+}]} = K'$	1.66 x 10^{+5} M^{-1}	(28)
Myokinase EC 2.7.4.3	$\frac{[\text{ATP}^{4-}][\text{AMP}^{2-}]}{[\text{ADP}^{3-}]^2} = K'_{\text{MYO}}$	1.05	(29)
Hexokinase EC 2.7.1.1	$\frac{[\text{Glu 6-P}^{2-}][\text{ADP}^{3-}][\text{H}^{+}]}{[\text{Glucose}][\text{ATP}^{4-}]} = K'_{\text{HEX}}$	1.99 x 10^{-4} M	(29)
Adenosine triphosphatase EC 3.6.1.3	$\frac{[\text{ADP}^{3-}][\text{HPO}_4^{2-}][\text{H}^{+}]}{[\text{ATP}^{4-}][\text{H}_2\text{O}]} = K'_{\text{ATP}}$	2.19 x 10^{-2} M	(30)
ATP pyrophosphatase EC 3.6.1.8	$\frac{[\text{AMP}^{2-}][\text{PPi}^{3-}][\text{H}^{+}]}{[\text{ATP}^{4-}][\text{H}_2\text{O}]} = K'_{\text{ATP-PPiase}}$	9.98 x 10^{-1} M	(31)

has occupied a much diminished place in the current teaching and practice of biochemistry. Re-emphasis of the fundamentals of kinetics and thermodynamics in biochemical teaching, such as was done so succinctly in the text for beginning biochemistry students by Edwin A. Dawes, professor of biochemistry at Hull [35], particularly in an era of "big science" initiatives such as genomics, proteomics, and metabolomics, could only be salutary.

FURTHER LINKS BETWEEN THE $\Delta G'$ OF ATP HYDROLYSIS AND INORGANIC ION GRADIENTS AND REDOX COUPLES IN THE MITOCHONDRIA AND THEIR RELATIONSHIP TO ATP SYNTHESIS AND FREE RADICAL FORMATION

During the late 1980s, we became interested in the linkage of the variations in the redox states of cells and the energy of ATP hydrolysis [32] powering the inorganic ion gradients between the intra- and extracellular phases of cells. We therefore undertook, with the encouragement but not total agreement of the great physical biochemist from Duke, Charles Tanford, a study on the relationship of the $\Delta G'$ of ATP hydrolysis to the extent and direction of the Na^+, K^+, and Ca^{2+} gradients in perfused working rat heart [36]. We presented evidence that the Na^+ and K^+ gradients in heart resulted from the action of the $3Na^+/2K^+$ ATPase and an open K^+ channel in the electroneutral reaction shown below.

$$\Delta G^{\circ}_{\text{ATPhydrol}} - RT \ln \frac{[\Sigma\text{ATP}]}{[\Sigma\text{ADP}][\Sigma\text{P}_i]} + RT \ln \frac{[\text{Na}^+]_i^3[\text{K}^+]_i^3}{[\text{Na}^+]_i^3[\text{K}^+]_o^3} \approx 0$$

REACTION 4

This statement views the extent of two inorganic ion gradients and the electrical potential between the extra and intracellular phases of the cell as critically dependent upon the energy of ATP hydrolysis, which in turn could vary, due to the high activity of the cytoplasmic glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase system, with the redox state of the free cytoplasmic NAD couple.

The question naturally arose as to what relationship existed between the energy of the gradients of inorganic ions and the $\Delta G'$ of ATP hydrolysis in those tissues where the

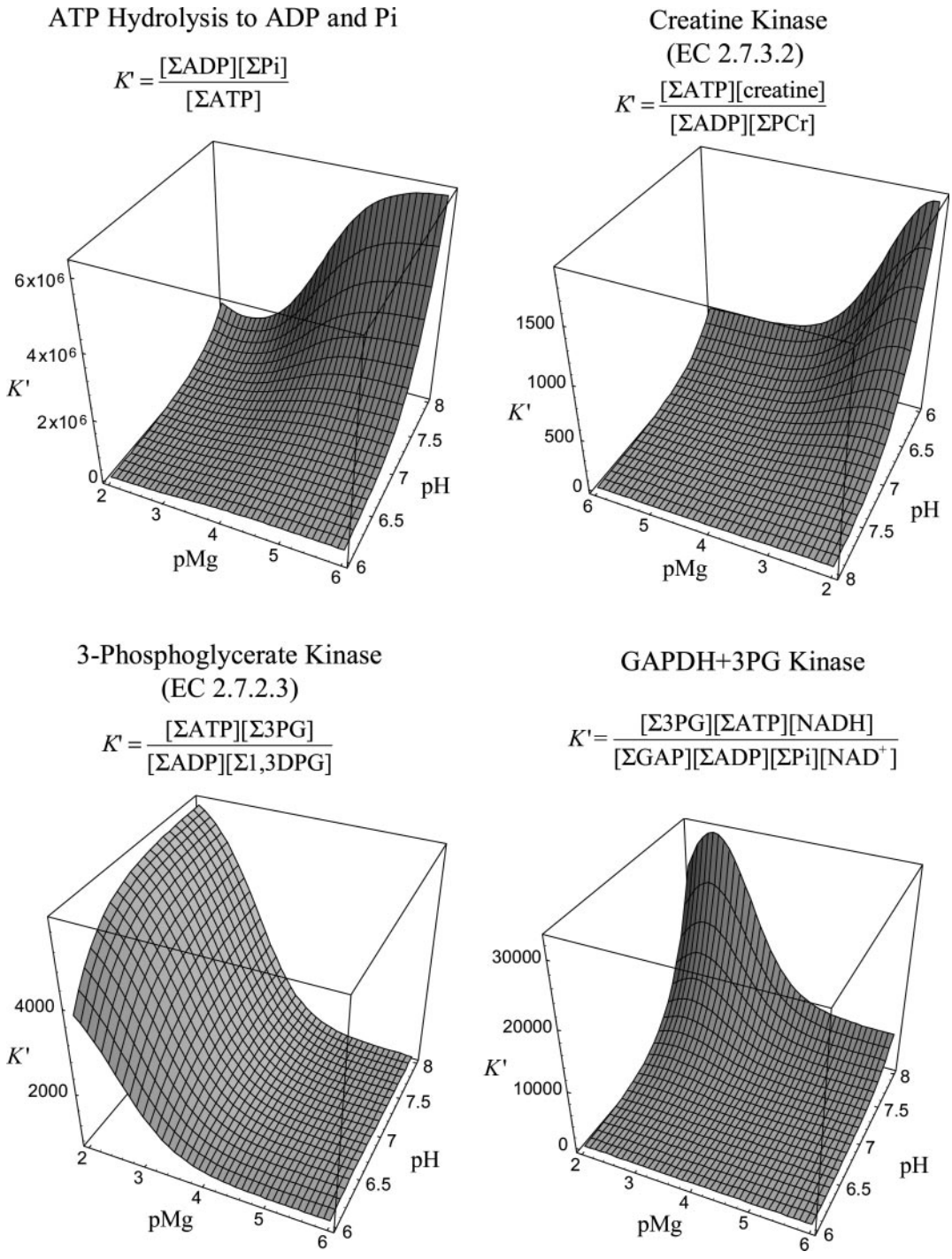

FIG. 4. **The Effects of pMg2 = and pH on the K_{eq}.**

electric potential between extracellular and intracellular phases was not set by an open K^+ channel, giving a neat electrically balanced equation. Rather, most cells, from liver to red blood cells, have electric potential in the range of −50 to −7 mV rather than the −83 mV characteristic of excitable tissues such as muscle and nerve. We therefore surveyed heart, liver, and red blood cells under a variety of altered voltages and were able, 10 years later, to empirically write the statement relating the linked transport reactions of all nine common inorganic ions to the energy of ATP hydrolysis in cells where Cl^-, not K^+, is the permeant ion [37].

Such a view would explain why injury to cells by almost any means results in the stereotypic reaction in which cells gain Na^+, loose K^+, and swell. Surprising as it may seem, during World War II, it was not recognized that troops suffering burn or hypoxic injuries died from an increase in

$$\Delta G'_{ATP} + RT \ln \frac{[Ca^{2+}]_o[Na^+]_o^2[Mg^{2+}]_i[H^+]_i[K^+]_i^2[Cl^-]_o[HCO_3^-]_i[H_2PO_4^-]_o[HPO_4^{2-}]_i}{[Ca^{2+}]_i[Na^+]_i^2[Mg^{2+}]_o[H^+]_o[K^+]_o^2[Cl^-]_i[HCO_3^-]_o[H_2PO_4^-]_i[HPO_4^{2-}]_i} \approx 0$$

REACTION 5

extracellular K^+. This fact was demonstrated by Herbert Tabor [38], the scholarly long time Editor of the *Journal of Biological Chemistry*, working in the laboratory of Sanford Rosenthal at NIH. His paper was thought so out of the mainstream thinking at the time that it was published in the *Public Health Reports*, a publication with less than stellar impact scores, but one sponsored by an organization of which NIH was a part. It is fair to say that what I viewed as an important paper that extended the scope of redox state linkages was met with an underwhelming response in scientific circles but with more interest in clinical circles in which treatment of electrolyte imbalance is a major concern.

Krebs died in 1981 in the Radcliffe Infirmary less than 50 yards from the laboratory where he worked for 20 years until a week before his death. In the years between my leaving his laboratory at Oxford in 1969, until 1981, he visited my laboratory every year. We took this opportunity to convene a meeting of, depending upon the major topic, five of the best metabolic biochemists to review past studies and future projects. Included in these review meetings at various times, depending upon the subjects being discussed, were: George Cahill, Britton Chance, Ronald Estabrook, Edwin Krebs, Henry Lardy, Albert Lehninger, J. E. Rall, Paul Srere, Merton Utter, Stewart Wolf, and Harland Wood, to mention a few. These were the most enjoyable and stimulating meetings I have ever been privileged to attend. The reviewers received the grand sum of $75 per day, over a 2-day weekend, and a free dinner at a cheap restaurant funded by the laboratory chief since government rules prevent such amenities as free food. Nonetheless, they appeared to enjoy these meetings as much as the laboratory staff and students.

By the mid 1980s, review processes at NIH became more bureaucratized and centralized by NIH administrators along the lines of the "peer-review" process in which "experts", usually grantees of the institute under review, reviewed the intramural NIH laboratories. The work of our laboratory went from being a "national treasure" to a "waste of money," depending upon the eye of the beholder. By 1991, the work of the laboratory at NIH was judged to be inadequate, and I was notified that the laboratory would be shut down in 2 years time. I decided that I would spend the last available 2 years studying a subject that I thought to be important and to take no notice whatsoever of programmatic goals set by administrators without actual laboratory experience. To their great credit, the laboratory members, who were all to be terminated at the end of this period, accepted my explanation that this was a "Birkenhead drill" and stayed at their posts, completing the work outlined during the remaining 2 years.

The problem chosen was to determine the effects of changes caused by the ketone bodies (D-β-hydroxybutyrate and acetoacetate), insulin, and their combination on the working perfused rat heart. Although ketone bodies had earlier been shown to be a metabolic fuel replacing glucose in brain by Owen and Cahill [39], the effects of ketone body metabolism by the brain, although more interesting, was judged to be too technically difficult for the techniques we had in hand. The working, perfused heart was chosen because the heart performs little synthetic or transport work and uses its metabolic energy simply to pump. In addition, unlike the brain, it is largely homogeneous in cell type and is amenable to a determination of the relevant parameters needed for a complete metabolic control analysis.

The parameters required to complete the study chosen were:

1) determination of the concentrations of the substrates and products of each reaction in a metabolic pathway,
2) the K_m and V_{max} of each enzyme in the pathway,
3) the K_{eq} of each enzymatic step, and
4) a measure of flux through the pathway being studied.

The two pathways chosen for study were the glycolytic pathway of cytoplasm and the combined citric acid cycle and the oxidative phosphorylation pathways of mitochondria. The aim was a complete metabolic control analysis of these two most fundamental and ancient pathways of the great metabolic chart. Completion of these studies required five people working for 2 years using the techniques of enzymatic analysis. Our metabolic control analysis of the glycolytic pathway was published in the *Journal of Biological Chemistry* in 1994 [40]. Because of the multiple branch points of the citric acid cycle, no complete metabolic control analysis could be performed for mathematical reasons; however, informative results were nonetheless obtained [41]. We found that the metabolism of ketones as compared with the metabolism of glucose alone:

1) bypasses the block of PDH induced by insulin insufficiency,
2) increases the substrates of the first one-third of the citric acid cycle from 4- to 20-fold,
3) reduces free mitochondrial $[NAD^+]/[NADH]$,
4) oxidizes the free mitochondrial coenzyme Q/coenzyme QH_2,
5) decreases the E between cytoplasmic and mitochondrial phases from -140 to -120 mV,
6) increases the $\Delta G'$ $[H^+]$ across mitochondrial inner membrane,
7) increases the $\Delta G'$ of ATP hydrolysis, and
8) increases the hydraulic efficiency of cardiac work by 28%.

The potential medical applications of mild ketosis follow directly from its basic biochemical effects, and in particular, its effects on mitochondrial redox states in heart. This work on mitochondrial energetics made clear other links between the mitochondrial redox states and the $\Delta G'$ of ATP hydrolysis, in addition to the already known links between the cytoplasmic NAD couple and the phosphorylation potential brought about by the combined glyceraldehyde-3-phosphate dehydrogenase 3-phosphoglycerate kinase reactions of the glycolytic pathway.

As discussed earlier, metabolism of ketone bodies reduces the free mitochondrial [NAD+]/NADH] couple while

oxidizing the mitochondria Q couple, as judged by measurement of the [fumarate^{2-}]/[succinate^{2-}] ratio, the metabolite couple succinic dehydrogenase, or complex II of the respiratory chain [41]. The co-enzyme Q pool is in near-equilibrium with complex II and III, where it is the common intermediate. Sites I, III, and IV use the redox energy of electrons moving up the electron transport system to extrude protons from mitochondria, creating an electrical potential between cytoplasmic and mitochondrial phases of the cell [42]. The free energy of this electrical gradient is converted to free energy contained in ATP when protons are returned from cytoplasm to mitochondria through the F_0F_1 ATPase. We found that the changes in the $\Delta G'$ between the redox potential (the half-reaction relative to that of the hydrogen electrode, which is taken to be zero, E_h) of the mitochondrial NAD and coenzyme Q couples, written formally as

$$\Delta G'_{QH_2/NAD^+} = -nF(Eh_{Q/QH_2} - Eh_{NAD^+/NADH}) = \Delta G'_{ATP}$$

REACTION 6

paralleled changes in the measured $\Delta G'$ of ATP hydrolysis, Also, the $\Delta G'_{[H+]}$ (the $\Delta G'$ of the proton gradient between mitochondrial and cytosolic phases) determines, through the reversible F_0F_1 ATPase [43, 44], the $\Delta G'$ of the ATP synthesized. These values indicate a stoichiometry of four protons ejected per site, agreeing with the estimates of others [45], which results in the statement

$$\frac{\Delta G'_{ATP}}{4} = RT \ln \frac{[H^+]_{mito}}{[H^+]_{cyto}} + FE_{m/c} \quad (41)$$

REACTION 7

Thus the redox state of the NAD couple is linked to the $\Delta G'$ of ATP through the central enzymes of the glycolytic pathway, as well as to the differences in the redox potential between the free mitochondrial NAD and co-enzyme Q pool in the mitochondria. The network of near-equilibria that was first clearly defined in the Krebs laboratory in the late 1960s continues to expand after almost half a century.

WHY DO REDOX STATES CHANGE?

During fasting, the redox state of the free cytoplasmic [NAD$^+$]/[NADH] couple falls to around 500; when animals are fed normal chow, the ratio is around 1000; whereas when animals are fed a high carbohydrate diet, the ratio becomes 1800 (Table III). The changes in the mitochondrial NAD couple are generally in the same direction. The question obviously arises as to why do the redox states change. The answer would appear to reside in the fundamental thermodynamic properties of the substrate selected. During fasting, the liver is metabolizing free fatty acids and using amino acids in the gluconeogenic pathway. During high carbohydrate feeding, liver is metabolizing glucose to form pyruvate, which in turn is metabolized by the mitochondrial citric acid cycle. The citric acid cycle is simply the pathway whereby nearly all substrates are converted to CO_2 and H_2O [3]. It is a very old pathway, probably over 3.5 billion years old [46]. Originally, before the endosymbiosis of chloroplasts and their generation of O_2, the atmosphere was reducing, and the citric acid cycle ran backward. It was the major anaplerotic pathway responsible for amino acid synthesis. With the emergence of O_2 into the atmosphere, the change in redox state induced the cycle to run forward, and it became the central metabolic pathway for the combustion of food stuffs.

TABLE VI
Heats of combustion of substrates in $\Delta H°$ (kJ/C_2 units)

	Formula	C_2 unit	kJ/C_2 unit
Palmitic	$C_{16}H_{32}O_2$	$C_2H_4O_{1/4}$	−1247
Butyric	$C_4H_8O_2$	C_2H_4O	−1088
D-β-Hydroxybutyric	$C_4H_8O_3$	$C_2H_4O_{3/2}$	−1021
Glucose	$C_6H_{12}O_6$	$C_2H_4O_2$	−933
Acetic	$C_2H_4O_2$	$C_2H_4O_2$	−874
Pyruvic	$C_3H_4O_3$	$C_2H_{8/3}O_2$	−778

The stoichiometry of the citric acid cycle has been fixed for a very long time. To enter the cycle, most foodstuffs are converted to a C_2 unit, after which they are converted to CO_2, and their reducing power, or electrons, become substrates for the electron transport chain that generates the mitochondrial proton gradient required for ATP synthesis by the F_0F_1 ATPase. However the inherent energy of the various C_2 units traversing the cycle varies widely, as illustrated by a comparison of the heats of combustion of the non-nitrogen-containing nutrients metabolized by the cycle (Table VI).

The C_2 units entering the citric acid cycle vary in their heats of combustion by almost a factor of 2. When pyruvate enters the cycle, 15 high energy phosphates are formed by the reducing equivalents produced. The $\Delta G'$ of ATP hydrolysis is held in very narrow limits between −53 to −60 kJ/mole [37], a variation of less than 10%, not a factor of 2. To produce these 15 ATP requires −795 to −900 kJ/15 moles. If pyruvate is being metabolized, there are only −778 kJ available, meaning that the ATP produced must be of low energy. If glucose is being metabolized to produce the pyruvate, 18 ATP are produced because of the extra NADH produced in the cytoplasm. These 18 ATP would require −954 to −1080 kJ, leading to the same conclusion since C_2 units from glucose contain only −933 kJ. On the other hand, if C_2 units from fats are being metabolized in the cycle, there is too much energy contained to be accommodated in the 15 ATP produced, making uncoupling a thermodynamic imperative. It turns out that the metabolism of D-β-hydroxybutyrate, containing −1021 kJ/mole C_2 unit, has only about 10% excess energy required, some of which must be used to accommodate the obligatory energy loss between cytochrome oxidase and O_2. The optimum amount of energy contained inherently in this metabolite seems an adequate explanation for the increased metabolic efficiency observed during ketone metabolism.

The data in Table VI also account for the reduction of the cytoplasmic NAD couples during fasting. In this state, the liver is metabolizing fatty acids, which are themselves more reduced than pyruvate and account for the reduced state of the NAD couples. In contrast, the NAD couples become more oxidized during the metabolism of glucose, a partially oxidized substrate. In general then, the redox states of the NAD couple reflect the redox state of the principle substrate being metabolized, and they usually

change in parallel with one another.

Under unusual situations, the redox state of the NAD couples in the cytoplasm and mitochondria can change in opposite directions. Examples of the unusual situation in which reduction of the cytoplasmic NAD couple is associated with oxidation of the mitochondrial NAD couple are: during excess NH_4^+ administration to liver [9], during administration of uncoupling agent, ([63]), or during certain rare disease states such as pyruvate carboxylase deficiency [47]. In the cases of NH_4^+ administration and pyruvate carboxylase deficiency, there is a deficiency of citric acid cycle intermediates because of either excessive loss into glutamate or insufficient anaplerotic production. In the case of uncoupling agents, the demand of the electron transport system for reducing equivalents exceeds the rate at which they can be produced, leading to oxidation of the mitochondrial NAD couple and insufficient redox energy in the chain to produce high energy ATP, leading to reduction of the cytoplasmic NAD couple, which is sensitive to the level of ATP energy.

IMPLICATIONS FOR CURRENT WORK, THE EXTENSION OF BASIC BIOCHEMISTRY TO MEDICINE

With a body of work defining measurement of the redox states in living tissues stretching back over half a century, and involving some of the most distinguished biochemists of the 20th century, one would have thought that these principles would be part of standard biochemical practice. This is particularly so since a number of emerging transcription factors such as the clock gene [48], the NAD-linked histone deacetylase Sirt1 [49], thought to be implicated in the life-extending properties of caloric restriction, are regulated by changes in the redox states of NAD. With only two exceptions, attempts to understand the redox control of transcription factors have involved measurement of total NAD and NADH, which give no information about the redox state in the compartment of interest. One of those exceptions, exploring the effects of changes in the free cytosolic $[NAD^+]$/[NADH] ratio on the activity of Sirt1 in muscle [50], hardly testifies to the general acceptance of this body of knowledge within the biochemical community. Richard H. Goodman and colleagues [51], studying the regulation of the transcription co-activator, carboxyl terminal binding protein, CtBP, elegantly demonstrated, using two-photon microscopy, that the ratio of free $[NAD^+]$/[NADH] within the nucleus corresponded to that of the free cytosolic $[NAD^+]$/[NADH] ratio.

Although the failure of the biochemical community to widely accept the simple application of thermodynamics to living systems is puzzling, on writing this article, it became clear that this has generally been the case with thermodynamics. Josiah Willard Gibbs, who defined the concept of chemical potential and presented the first comprehensive formulation of thermodynamics, was not widely accepted by his colleagues at the time. He sent copies of his work [52] to the leading scientists in Europe and America, where it was met with silence [53]. Rudolf Clausius, whom Gibbs quoted in the opening of his work, never made any acknowledgment. Sir William Thompson, later Lord Kelvin, and his colleague, P. G. Tait, withheld approval and quibbled about the concept of entropy. Only James Clerk Maxwell recognized and championed Gibbs' contribution, which led, after his being awarded the Rumford Medal by the Royal Society, to his finally being awarded a salary of $2000 per year from Yale, where he had worked all his life in an unpaid position. M. M. P Muir's 1885 textbook on thermal chemistry [66] commented on Gibbs' work and concluded that there was no new physical principle involved and that in any case, the mathematical treatment is beyond the ability of chemistry students to understand. I think the failure to grasp a firm understanding of Gibbs' work and its wide applicability of thermodynamics and its handmaiden kinetics could be extended to most contemporary courses in biochemistry and particularly to the students produced by these courses. For me, the twin pillars of thermodynamics and kinetics, the relationship of which was succinctly formulated by J. B. S. Haldane in the "Haldane relationship," are essential to an understanding of metabolism and particularly for the metabolic defects that are the proximate cause, providing a therapeutic window into disease phenotypes. They constitute the basis for what has been later called metabolic control analysis. Such a detailed biochemical analysis of metabolite patterns depends not upon some computer-driven, and usually futile, search for patterns but rather upon firm thermodynamic, kinetic, and analytical data.

By the time the work defining the metabolic effects of ketones and insulin on glycolytic [40] and mitochondrial [41] metabolism was published, my laboratory had been closed, and its workers and techniques had been dispersed. As an overage civil servant, I could not be "fired" but had no other visible means of support. I used this sabbatical in the closet to reflect on the implications of our findings on the remarkable effects of ketone bodies on mitochondrial redox potentials. Ketosis, resulting from either prolonged fasting or feeding a high fat, low carbohydrate diet, has been used to treat refractory epilepsy for over 100 years [54]. An in-depth understanding of the biochemical details of the mechanism of action of ketone bodies led to other and more widespread potential applications. What emerged from a detailed biochemical analysis of the effects of ketone metabolism was a surprising array of disease phenotypes, including specific rare monogenetic diseases and common polygenic diseases that might be benefited by mild ketosis [55]. The major disease phenotypes encompassing these groups of diverse disease states fall into four major groups [56], as shown below.

1) Diseases of Substrate Insufficiency or Insulin Resistance—The metabolism of ketones leads to a remarkable increase of up to 20-fold in citric acid cycle intermediates. Therefore disease states characterized by a deficiency of such substrates might be amenable to therapy with these agents. Such disease phenotypes include rare diseases such as Friedreich ataxia, acyl CoA dehydrogenase deficiency, and Duchene muscular dystrophy and common diseases such as heart failure. In addition, ketones enter the citric acid cycle without requiring PDH activity. Many disease states, including extreme stress or infection, are associated with inhibition of PDH activity. In this group are: Leprechaunism, type I and II diabetes [57], Alzheimer disease [58], and burns and traumatic injuries.

2) Diseases of Free Radical Toxicity—The major source of free radicals comes from the non-enzymatic reaction of Q semiquinone with O_2 [59]. The metabolism of ketones reduces the co-enzyme Q couple, thus decreasing the amounts of Q semiquinone. Free radicals can also be produced when mitochondrial electrical potential becomes elevated over about −150 mV [60]. The metabolism of ketones decreases the electrical potential from −140 to −120 mV, whereas insulin, which also increases efficiency, leaves the potential unaffected. A lowering of potential between mitochondrial and cytosolic phases tends to lessen mitochondrial proton leakage and free radical production. Diseases associated with excessive free radical toxicity are: Parkinson disease, amyotrophic lateral sclerosis, and reperfusion injury.

3) Diseases of Hypoxia—These disease phenotypes include myocardial infarction, stroke, multi-infarct dementia, angina, chronic obstructive pulmonary disease, and peripheral vascular disease.

Ketone bodies not only have the basic metabolic effects we have described but have also been found recently to have "pharmacological" effects. The natural substrate for the nicotinic acid receptor on the adipocyte is, in fact, D-β-hydroxybutyrate [61, 62]. Nicotinic acid and D-β-hydroxybutyrate, both acting on a G protein-coupled receptor, block the action of the cyclic AMP-dependent protein kinase and thus the release of free fatty acids from adipocytes. Elevated serum free fatty acids contribute to the pathophysiology of a number of conditions, including both heart failure, and in general to states of insulin resistance. High doses of nicotinic acid have long been used to decrease elevated serum triglycerides, so that there will emerge another group of disease phenotypes in which ketone bodies may be able to play a therapeutic role by a mechanism that differs from their effects upon the redox and phosphorylation state of the cell.

Krebs once remarked that it usually takes about a generation for advances in basic science to be applied in medicine. This has certainly been the case in applying our understanding of the linked network of equilibria involving the great nucleotide systems and ion gradients to one another. As is so often the case in the history of science, luck plays an important role. I am well aware that the biomedical research community considers metabolism to "be dead" and that funding for this type of research would be impossible to obtain from any conventional NIH study section. In the late sixties, Krebs foresaw the decline of metabolic biochemistry, which had been a major part of the curriculum in biochemical departments. Despite valiant efforts to re-emphasize this area in the biochemical community, notably by people such as Richard Hanson of Case Western Reserve University, I think it likely that Krebs' prediction was correct. He thought at the time that metabolism would "Not return to biochemistry departments, but would be reborn in medical departments." For those few remaining biochemists with an interest in metabolism, I would remind them of the old Norman motto, "*Famam extendimus facti.*" (We spread our fame by our deeds.)

Fortunately for my research, other sources of non-traditional funding became available that allowed us the chance to determine whether our hypotheses about the therapeutic benefit of alteration in metabolic substrates in a number of disease phenotypes were true. If these hypotheses prove to be correct, it will be ironic that the funding was provided by the Department of Defense, not the Department of Health and Human Services. Little did I know in 1966, when Krebs assigned me the task of determining the redox state of the NADP system, that I would still be working on the problem 40 years later. What a ride!

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