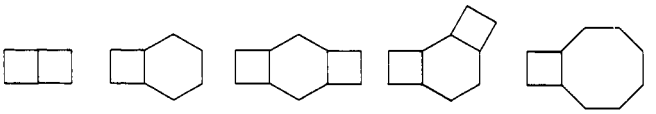
exactly one negative value of x^2, i.e., the values of x are imaginary numbers. $P^{ref}(G/R_4)$ (eq 8) also has imaginary roots, a result that can be demonstrated by direct numerical solution for the two real positive values of x^2 and factorization to give a quadratic equation in x^2. The discriminant of the quadratic equation is negative, which shows that there are four complex roots for the original eq 7.[26] In either case, the obtaining of imaginary values obscures the meaning and the chemical interpretation of the graph-theoretical resonance energy.

Published works[7,8,10] on graph-theoretical individual ring resonance energies contain real number entries for the π molecular graphs shown in **1–5**, each of which can be demonstrated to possess

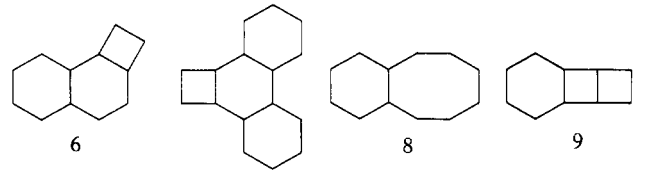


2 1 3 4 5

two or more complex or pure imaginary roots for particular G/R reference polynomials. The reason for these errors may be the use of an approximate formula and numerical integration to obtain the total ring resonance energy, which bypasses finding the actual exact solutions to the polynomials.

The recent discussion[12] of a "unified theory of aromaticity and London diamagnetism" uses bicyclo[6.2.0]decapentaene molecular graph **5** as one of its two examples, also reporting the total sum of the ring resonance energies as a real number.[27] Here the explanation is that roots are obtained by an expression based on Newton's method to approximate roots that is not capable of finding complex solutions.

In general, annelated alternant polycyclic π molecular graphs with corrected structure count (CSC)[28] of unity or zero will have complex roots for ring reference polynomials defined as in eq 3. This class includes many benzannelated derivatives of the compounds **1–5** and many other types of related systems, e.g., **6–9**,



6 7 8 9

which comprise a group of compounds that are of particular interest to theoreticians concerned with definitions of aromaticity and calculations of resonance energies. The use of the reference polynomial, eq 3, is interdicted for this group, and its successful use in other systems including benzenoid alternants and nonalternants *should be considered problematic.*[29]

A less explored way to define GTRE has been suggested in earlier work by Aihara.[5] The reference polynomial is constructed by subtracting all ring terms, eq 1, and reintroducing the algebraic terms only for the ring under consideration. The roots of such ring polynomials have all been found to be real numbers in several benzenoid systems[5] and in the π graphs considered in the present paper. Whether or not one wishes to employ this alternate definition where no proof exists that difficulties of the type outlined above are absent is a matter of individual predilection.

Acknowledgment. I thank Professor Edgar Heilbronner for a useful exchange of unpublished information. The financial support of the Robert A. Welch Foundation of Houston, TX, is also gratefully acknowledged.

(25) Uspensky, J. V. "Theory of Equations"; McGraw-Hill: New York, 1948; pp 121–124.

(26) The numerical values of the roots for eq 8 are $x^2 = 0.163\,901$, 5.143 381, and $(1.846\,359 \pm 0.386\,849)(-1)^{1/2}$.

(27) The reference polynomial for the 10-membered ring component of **5** has one negative value of x^2, showing that the corresponding roots are $\pm x\text{-}(-1)^{1/2}$. Columns 3 and 4 of Table II, ref 12, should therefore contain some entries with imaginary numbers.

(28) Herndon, W. C. *Tetrahedron* **1973**, *29*, 3–12.

(29) The significance of the necessity for real roots of reference polynomials is pointed to by the number of papers where this question is discussed. For leading references see: Graovac, A. *Chem. Phys. Lett.* **1981**, *82*, 248–251. Godsil, C. D.; Gutman, I. *Croat. Chem. Acta* **1981**, *54*, 53–59. The real root requirement is considered to be inconsequential in the following: Aihara, J. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 2010–2012. Gutman, I. *Z. Naturforsch.* **1978**, *33a*, 840–841. The use of the so-called "sextet" polynomial is described here.

Enzyme-Catalyzed Transhydrogenation between Nicotinamide Cofactors and Its Application in Organic Synthesis[1]

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Several of the most convenient systems that have been proposed for the regeneration of the nicotinamide cofactors are specific for NAD(H).[2,3] Although NADP(H) is less common in synthetically interesting biochemical pathways than NAD(H), on those occassions when NADP(H) is required, one is restricted for its regeneration to two procedures.[4,5] Here we describe three practical systems that accomplish transhydrogenation between NAD(H) and NADP(H) cofactors (eq 1) and demonstrate their

$$\text{NADH} + \text{NADP}^+ \rightleftharpoons \text{NAD}^+ + \text{NADPH} \qquad (1)$$

application to problems in practical synthesis (Scheme I).[6] These systems permit regeneration procedures that are specific for one cofactor (NAD(H) *or* NADP(H)) to be coupled to the other.

The first of these schemes utilizes the flavoenzyme diaphorase (EC 1.6.4.3) from *Clostridium klyveri*; the second uses alcohol dehydrogenase from *Leuconostic mesenteroides* (ADH, EC 1.1.1.1) in a system containing both ethanol and acetaldehyde; the third involves glutamic dehydrogenase (GluDH, EC 1.4.1.3) in solutions containing glutamate (Glu), 2-ketoglutarate (2-KG), and ammonium ion. Added redox-active substrates are required for transhydrogenation activity with ADH and GluDH but not for diaphorase,[7] which contains FAD and which catalyzes hydride transfer between oxidized and reduced nicotinamide coenzymes directly. We used concentrations of the added substrates for ADH and GluDH that were $5\text{–}10K_m$. Relevant kinetic parameters for these enzymes are summarized in Table I.

Demonstration of the coupling of a $NADP^+$-specific enzymatic oxidation to a regeneration system specific for NADH → NAD^+ was accomplished by conversion of pyruvate to L-lactate (**1**) and

(1) Supported by the National Institutes of Health (GM 26543 and GM 30367).

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(6) The flavoenzyme pyridine nucleotide transhydrogenase (EC 1.6.1.1) from *pseudomonas* or spinach catalyzes direct transhydrogenation (Kaplan, N. O.; Colowick, S. P.; Neufeld, E. F. *J. Biol. Chem.* **1953**, *205*, 1–15). Since $NADP^+$ is a potent dead-end inhibitor for this enzyme, the forward reaction ($NADP^+$ + NADH → NAD^+ + NADPH) is very slow (Cohen, P.T.; Kaplan, N. O. *J. Biol. Chem.* **1970**, *245*, 4666–4672). Moreover, the enzyme isolated from either source (ca. 20 units from 1 kg of spinach or *pseudomonas* cells) has low specific activity (0.1–0.3 unit mg^{-1}). We did not attempt to use this enzyme for large-scale synthesis.

(7) Pig heart lipoamide dehydrogenase is essentially specific for NAD^+ ($NADP^+$ is only 5% as active a substrate as NAD^+, in either the presence or the absence of added lipoic acid). Addition of lipoic acid to the transhydrogenation system containing diaphorase from *C. klyveri* did not improve its catalytic activity, because lipoic acid (or lipoamide) is a poor substrate for this enzyme.

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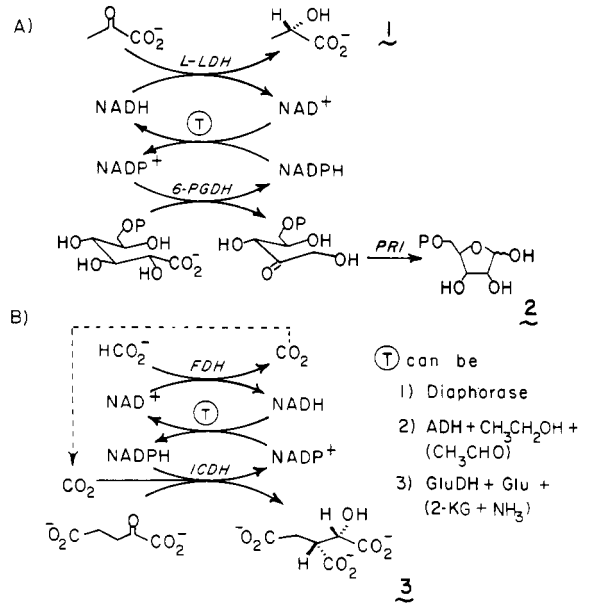
Table I. Kinetic Parameters for Diaphorase, ADH, and GluDH

enzyme	K_m, mM NAD	NADH	NADP	NADPH	2nd substrate	specific activity,[a] units mg^{-1} NADPH + NAD ⇄ NADP + NAD forward	reverse
diaphorase[b] (*C. kluyveri*)		0.1		0.1		2	3
ADH[c] (*L. mesenteroides*)	0.5	0.25	0.085	0.2	EtOH, 50 (NAD^+)		
					17 ($NADP^+$)	7	10
					CH_3CHO, 0.25 (NADH)		
					0.20 (NADPH)		
GluDH[d]	0.02	0.03	0.047	0.026	Glu, 1.8 ($NADP^+$)		
					2-KG, 0.7 ($NADP^+$)	2	2
					NH_4^+, 3.2 (NADPH)		

[a] The reaction mixture was quenched by adding an equal volume of 1 N $NHClO_4$ to destroy NAD(P)H and enzymes. The concentration of NADP was then determined by G-6-P/G-6-PDH, and NAD was determined by ethanol and ADH. [b] Wren, A.; Massey, V. *Biochim. Biophys. Acta* 1966, *122*, 436–449. [c] Hatanaka, A.; Adachi, O.; Chiyonobu, T.; Ameyama, M. *Agr. Biol. Chem.* 1971, *35*, 1304–1306. [d] Fahien, L. A.; Wiggert, B. O.; Cohen, P. P. *J. Biol. Chem.* 1965, *240*, 1083–1090.

Scheme I. Regeneration of Nicotinamide Cofactors for Use in Enzyme-Catalyzed Synthesis Using Enzyme-Catalyzed Transhydrogenation: (A) Regeneration of NADH and NADP; (B) Regeneration of NAD and NADPH[a]



[a] Abbreviations: L-LDH, L-lactic dehydrogenase; 6-PGDH, 6-phosphogluconic dehydrogenase; PRI, phosphoriboisomerase; ADH, alcohol dehydrogenase; GluDH, glutamate dehydrogenase; ICDH, isocitrate dehydrogenase; FDH, formate dehydrogenase; Glu, glutamate; 2-KG, 2-ketoglutarate. The components in parentheses were not added but generated in the reaction.

6-phosphogluconic acid to ribose-5-phosphate (**2**). A 600-mL solution containing 6-phosphogluconate (0.2 M), pyruvate (0.2 M), NAD^+ (0.2 mM), $NADP^+$ (0.2 mM), mercaptoethanol (4 mM), and 200 units each of immobilized phosphoriboisomerase (PRI, in 6 mL of PAN[8] gel), 6-phosphogluconate dehydrogenase (6-PGDH, in 8 mL of gel), diaphorase (in 20 mL of gel), and L-lactate dehydrogenase (L-LDH, in 0.5 mL of gel) was deoxygenated with Ar and stirred at 25 °C with the pH automatically controlled in the range 8.0–8.2 by adding 1 N KOH through a peristaltic pump. The reaction was complete in 8 days, and **1** and **2** were isolated as described previously:[4,9] zinc lactate (15.3 g, 94% pure calculated as $Zn(L\text{-lactate})_2 \cdot 2H_2O$, 81% yield, 94% enantiomeric excess); barium ribose-5-phosphate (39.5 g, 92% purity determined enzymatically, 83% yield). The turnover numbers and residual activities for each component at the conclusion of the reaction were as follows: NAD(H), 1000 (82%); NADP(H), 1000 (70%); 6-PGDH, 2×10^6 (79%); PRI, 3×10^6 (86%); diaphorase, 6×10^5 (82%); L-LDH, 6×10^7 (88%).

Replacement of diaphorase with ethanol (1.2% v/v) and immobilized ADH (from *L. mesenteroides*, 200 units in 5 mL of PAN gel) or glutamic acid (18 mM) and GluDH (200 units in 5 mL of PAN gel) resulted in very similar results with approximately the same TN for $NAD(P)^+$.

The reverse (reductive) transhydrogenation was demonstrated by using the synthesis of *threo*-D_s(+)-isocitric acid (**3**). A 800-mL solution containing 2-KG (0.2 M), formate (0.2 M), $NaHCO_3$ (0.2 M), NAD^+ (0.2 mM), $NADP^+$ (0.2 mM), mercaptoethanol (4 mM), and 80 units each of immobilized isocitric dehydrogenase (ICDH, in 20 mL of gel), diaphorase (in 10 mL of gel), and formate dehydrogenase (FDH, in 35 mL of gel) was deoxygenated and stirred at room temperature with pH controlled at 7.6. The reaction was 62% complete in 8 days, and no further reaction was observed.[10] GluDH (100 units, in 3 mL of gel) and ammonium sulfate (60 mmol) were added to the reaction mixture to consume the unreacted 2-KG, and the reaction was allowed to proceed for 3 additional days. Compound **3** was isolated as its barium salt (82 g),[4] which contained 88% (91 mmol) of $Ba_3(isocitrate)_2$ (57% yield). The turnover number and residual activity of each component was as follows: NAD(H), 620 (90%); NADP(H), 1000 (66%); ICDH, 1.6×10^6 (88%); diaphorase, 4×10^5 (78%); FDH, 1.5×10^5 (80%).

A very similar result was obtained in this conversion of 2-KG to **3** when the diaphorase was replaced with ethanol (1.2% v/v) and ADH (200 units). The turnover number and residual activity of ADH was 8×10^6 (80%); TN and residual activity for other components were similar to those observed in the system using diaphorase. The Glu/GluDH system could not be used in this instance, because the high concentration of 2-KG (present as a substrate for ICDH) inhibits the dehydrogenation of Glu.[11]

This work establishes simple experimental protocols, using commercially available enzymes, that catalyze transhydrogenation between NAD(H) and NADP(H). Of the three reactions, that based on ADH has the highest rate and is the most convenient to use. It has, however, the disadvantage that the system must contain organic components (ethanol and acetaldehyde), which might deactivate some enzymes used in synthesis. The diaphorase-catalyzed transhydrogenation requires no added components but is kinetically slower (and more expensive) than that based on ADH.

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(10) The extent of reaction was limited by the equilibrium constant of the ICDH-catalyzed reaction (K = [2KG][CO_2][NADPH]/[isocitrate][$NADP^+$] = 0.77 M; pH 7.0, 22 °C: Ochea, S. *Method. Enzymol.* **1955**, *1*, 699–704) and by product inhibition (K_m(isocitrate) = 2.6 M: Rose, Z. B. *J. Biol. Chem.* **1960**, *235*, 928–933).
(11) Fahien, L. A.; Wiggert, B. O.; Cohen, P. P. *J. Biol. Chem.* **1965**, *240*, 1083–1090.

These procedures for catalyzing transhydrogenation introduce an element of flexibility into nicotinamide cofactor regeneration and provide another method of controlling the redox behavior of systems of enzymes used for organic synthesis.

Registry No. NAD, 53-84-9; NADPH, 53-57-6; NADH, 58-68-4; NADP, 53-59-8; diaphorase, 37340-89-9; alcohol dehydrogenase, 9031-72-5; glutamic dehydrogenase, 9001-46-1; ribose-5-phosphate, 4300-28-1; L-lactate, 79-33-4; *threo*-D_s(+)-isocitrate, 6061-97-8; 6-pheophogluconic acid, 2464-13-3; pyruvate, 127-17-3; 2-ketoglutarate, 328-50-7.

Reduction of Ferricenium Ion by Horse Heart Ferrocytochrome *c*

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The electron-transfer reactions that can occur between metalloproteins and small transition-metal complexes have been the subject of considerable recent study,[1-4] with horse heart cytochrome *c* being the most extensively investigated protein. In these studies Gray and co-workers[3,5,6] have used the Marcus relationship[7] and Debye–Hückel theory to interpret the second-order cross reaction rate constants for these systems. A major conclusion of these interpretations is that the hydrophobicity and π-bonding character of the ligands of the transition-metal complex are very important in determining the facility with which the protein can transfer electrons to or accept electrons from the complex.

In an effort to test these ideas with a new class of small complexes with hydrophobic π-bonding ligands, we have begun a study of the electron-transfer reactions of ferrocene, ferricenium ion, and their derivatives with metalloproteins. We report here the results of the reduction of ferricenium ion with horse heart ferrocytochrome *c*. The products of this reaction are ferrocene and ferricytochrome *c*.

The rate of the one-electron reduction of ferricenium ion by ferrocytochrome *c* from horse heart (type VI ferricytochrome *c* obtained from Sigma) has been measured with a Durrum Model D-110 stopped-flow spectrophotometer interfaced with a Nicolet Model 1090 digital oscilloscope and an Apple II computer. The reaction, monitored at 550 nm, was run with ferricenium ion and is pseudo-first-order excess over ferrocytochrome *c* in all experiments. Ferricenium hexafluorophosphate was prepared by the oxidation of ferrocene (Alfa Chemical Co.) by using the method of Yang, Chan, and Wahl.[8] Solutions of ferricenium hexafluorophosphate were prepared by dissolving the salt in argon-purged buffer and analyzed by measuring the solution's absorbance at 617 nm with a Cary 14 recording spectrophotometer.

Logarithmic plots of absorbance change vs. time were linear for more than 3 half-lives, establishing a first-order dependence on ferrocytochrome *c*. The reaction was also observed to be first order in ferricenium ion as indicated by data displayed in Figure 1. The second-order rate constant derived from a linear least-squares analysis of the data displayed in Figure 1 is (6.20 ± 0.18) × 10^6 M^{-1} s^{-1}. All measurements were made at 25.0 °C and μ = 0.50 (phosphate) at pH 7.0 under strictly anaerobic conditions. No hint of rate saturation, as observed[4] for some other protein complex reactions, was observed over the range of concentrations employed here. This is not surprising considering the high ionic strength and limited ferricenium ion concentration range possible in this study. The range of ferricenium ion concentrations was limited by the short reaction half-times at high concentrations and vanishingly small absorbance changes at lower concentrations. It should also be noted that ferrocene, the product of ferricenium ion reduction, is not appreciably soluble in water. However, in our experiments its concentration never exceeded 2.5 × 10^{-6} M, and at this concentration no precipitation of ferrocene results.

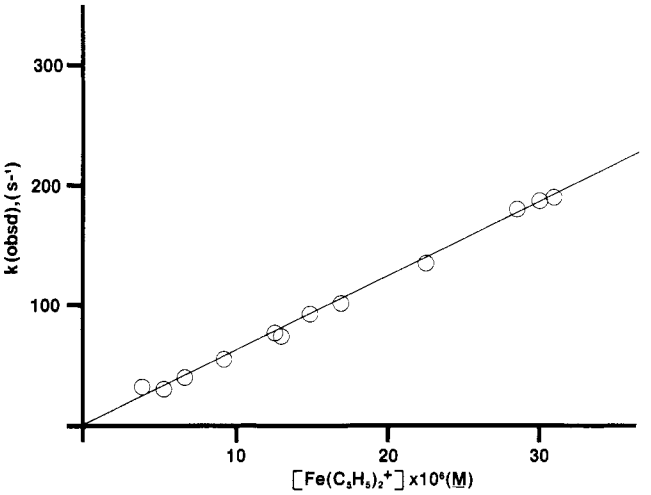

Figure 1. Plot of observed first-order rate constant, k_{obsd}, vs. initial ferricenium ion concentration, $[Fe(C_5H_5)_2^+]$, at 25.0 °C, pH 7.0, and μ = 0.50 (phosphate).

Electron-transfer rates and mechanisms of ferrocene and its derivatives have been of interest, and some information is available regarding their reactivity. Wahl[8] and co-workers have measured the ferrocene–ferricenium ion electron-exchange rate constant directly in NMR studies (k = (5.7 ± 1.0) × 10^6 M^{-1} s^{-1} in acetonitrile) and Pladziewicz and Espenson[9] have measured rate constants for cross reactions between a number of derivatives of ferrocene and ferricenium ion. Using the Marcus relationship,[7] Pladziewicz and Espenson derive a value of 5.7 × 10^6 M^{-1} s^{-1} for the ferrocene–ferricenium ion electron-exchange rate constant in 1:1 v/v 1-propanol–water. This rate constant shows little dependence on solvent and ionic strength for the conditions employed in these two studies.

Gray and co-workers[3,5,6] have evaluated protein–metal complex electron-transfer reactions by correcting the second-order cross reaction rate constants and the small complex exchange reaction rate constants for nonspecific electrostatic effects by using Debye–Hückel theory to adjust these rate constants to infinite ionic strength. They then use the Marcus relationship[7] (eq 1) to

$$k_{12} = (k_{11}k_{22}K_{12}f)^{1/2} \quad (1)$$

$$\ln f = (\ln K_{12})^2/(4 \ln (k_{11}k_{22}/Z^2))$$

calculate an exchange rate constant (k_{11}) for the protein at infinite ionic strength. Algebraic rearrangement of eq 2 gives[11] which

$$\ln k_{11} = (\ln k_{12} - \tfrac{1}{2} \ln K_{12} + \ln Z) - \ln k_{22} - [(\ln Z - \ln k_{12})^2 + \ln K_{12}(\ln Z - \ln k_{12})]^{1/2} \quad (2)$$

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