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The Thermochemical Characterization of Sodium Dithionite, Flavin Mononucleotide, Flavin–Adenine Dinucleotide and Methyl and Benzyl Viologens as Low-Potential Reductants for Biological Systems

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The heat of reaction (ΔH) of $Fe(CN)_6^{3-}$, Methyl Viologen, FMN and FAD with $S_2O_4^{2-}$ in aqueous buffer solutions was measured calorimetrically. In addition ΔH values for reduction of $Fe(CN)_6^{3-}$, FMN and FAD by reduced Methyl Viologen were determined. The resulting calorimetric data and corresponding E_0' values were combined to yield thermodynamic data for these simple reducing agents in a form useful for applications to biological reactions. Thermodynamic data for the reduction of spinach ferredoxin are also presented.

As part of a programme to determine the energetics of biologically important redox reactions occurring near the potential of the pH7 hydrogen electrode, a thermodynamic study involving simple reductants capable of supplying electrons to biochemical systems at this potential was begun. The almost universal use (Postgate, 1971) of $Na_2S_2O_4$ and, to a smaller extent, various Viologen dyes (Peck & Gest, 1956; Bulen *et al.*, 1965) as reductants for low-potential redox-active proteins (ferredoxins, nitrogenases and hydrogenases) and studies implicating FMN-containing proteins (Benemann & Valentine, 1971) in biological nitrogen fixation prompted us to study these simple reductants before undertaking thermochemical measurements involving them in more complex biological reactions. We report here thermochemical studies for the redox reactions of $S_2O_4^{2-}$,Methyl Viologen and Benzyl Viologen, FMN, FAD and $Fe(CN)_6^{3-}$.

Experimental

Chemicals

$Na_2S_2O_4$ (98% pure) was a special order from Associated Chemical Co., Harrogate, Yorks., U.K. The sealed metal container was opened and subsequently stored in a Vacuum Atmosphere Corp. Dry Box under argon. Samples of $Na_2S_2O_4$ for laboratory use were removed from the stock supply and stored in argon-filled desiccators over anhydrous $CaCl_2$. These precautions maintained $Na_2S_2O_4$ at greater than 95% purity (measured by amperometric or calorimetric titrations) for periods greater than a year. Reagent-grade $K_3Fe(CN)_6$ was purchased from Fisher Scientific, Cincinnati, Ohio, U.S.A. FMN, FAD, Methyl Viologen (MV), Benzyl Viologen (BV), Tes buffer [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid] and Tris were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. and were used without further purification. All gases were passed through a quartz tube containing fine copper turnings at 600°C to remove O_2.

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Calorimetry

The calorimetric measurements were performed by using a Beckman model 190 Micro-Flow calorimeter (Lyons & Sturtevant, 1969) having the Teflon inlet lines coaxially jacketed with larger Teflon tubing through which purified N_2 or argon was passed. The calorimeter and flow system were flushed with O_2-free N_2 or argon for 30min before the air-sensitive solutions were introduced.

Degassed solutions of FMN, FAD, $Fe(CN)_6^{3-}$ and Methyl Viologen were prepared under argon and were 1–10mM in 0.05 or 0.10M buffers. The pH values of these solutions were adjusted to 7.00, 7.25 or 8.00 with a Radiometer expanded-scale pH-meter. The solutions were standardized spectrophotometrically by using the following extinction coefficients at the indicated wavelengths: FMN, $12200\,cm^{-1}\cdot M^{-1}$ at 450nm (Sober, 1968); FAD, $11300\,cm^{-1}\cdot M^{-1}$ at 450nm (Sober, 1968); $Fe(CN)_6^{3-}$, $1000\,cm^{-1}\cdot M^{-1}$ at 420nm (Sober, 1968); $S_2O_4^{2-}$-reduced Methyl Viologen, $8250\,cm^{-1}\cdot M^{-1}$ at 600nm (Yu & Wolin, 1968). Methyl Viologen and Benzyl Viologen were also standardized coulometrically by using a Wenking potentiostat equipped with a saturated calomel reference electrode.

Solutions of $Na_2S_2O_4$ in 0.05 or 0.10M buffers were prepared at the desired pH by adding samples of the solid to degassed buffer solutions. The concentration of the $Na_2S_2O_4$ solution was 1.5–2.0 times that required to fully reduce the oxidant. A larger than

twofold excess of $Na_2S_2O_4$ caused a slight endothermic reaction which increased in magnitude as the $Na_2S_2O_4$ concentration increased. This effect is probably due to slow $S_2O_4^{2-}$ decomposition (Lem & Wayman, 1970) because oxidation by air is highly exothermic.

The buffer solutions used with their ΔH values in kJ/mol (4.184kJ/kcal) for proton ionization are: Tris–HCl (+47.40), acetate (+0.85), Tes (+33.47), phosphate (+4.73), bisulphite (+26.23) and cacodylate (−2.43). Except for Tris–HCl, all buffers were made from their sodium salts. The wide range of ΔH values for proton ionization and the production of a buffering component (HSO_3^- pK6.9) when $S_2O_4^{2-}$ is oxidized made necessary the precise pH control of both the oxidant and $S_2O_4^{2-}$ solutions.

The ΔH values for oxidizing $S_2O_4^{2-}$ to SO_3^{2-} by the oxidants used in this study were determined by mixing a slight excess of $S_2O_4^{2-}$ with a known limiting amount of oxidant and then correcting the measured heat for heats of dilution and buffer ionization.

Electromotive-force measurements

A Radiometer pH-stat was used for routine pH measurement and to determine the protons released when $S_2O_4^{2-}$ reacted with the various oxidants at pH8.0. The procedure consisted of adding either solid $Na_2S_2O_4$ or 0.10ml of a $S_2O_4^{2-}$ solution at pH8 to 2ml of oxidant (also at pH8) in the titration cup of the pH-stat. The protons released per molecule of oxidant were calculated by measuring the volume of 0.1M-NaOH required to restore the initial pH conditions.

Mid-point potentials and concentrations of $S_2O_4^{2-}$ were determined with a Sargeant model XXI Polarograph equipped with dropping mercury and saturated calomel electrodes. Mid-point potentials for Benzyl Viologen and Methyl Viologen were also determined by a potentiostatic procedure described by Watt & Bulen (1975).

Results and Discussion

The stoicheiometries (1)–(3) were confirmed from proton-release measurements during oxidation or reduction and from variation or lack of variation of mid-point potentials with pH.

$$S_2O_4^{2-} + H_2O = 2SO_3^{2-} + 2e^- + 4H^+ \quad (1)$$

$$MV_o(BV_o) + e^- = MV_r(BV_r) \quad (2)$$

$$FMNH_2(FADH_2) = FMN(FAD) + 2e^- + 2H^+ \quad (3)$$

The Viologens (Michaelis & Hill, 1933) are able to transfer electrons with no accompanying proton involvement whereas electron transfer by both $S_2O_4^{2-}$ and FMN(FAD) involves the indicated proton change. The pK of HSO_3^- was 6.9 under the conditions of this study and consequently reaction (1) represents the reaction only at pH8.0 or greater. Below pH8, reaction (1) must include the protonated form of SO_3^{2-} and a corresponding decrease in total released protons.

Table 1 contains calorimetric data for the indicated chemical reactions. The calorimetric measurements were made in different buffers at several pH values and involved various combinations of the redox reagents. This procedure insured that proper buffer-ionization corrections were applied and eliminated the possibility of unusual buffer–redox reactant or redox reactant–redox reactant interactions. The consistency of the corrected ΔH values in Table 1 indicates that proper buffer-ionization corrections have been applied and that the ΔH values are independent of buffer components. An anomaly is observed for reaction (*h*) in Table 1 in phosphate buffer at pH7.25 where the ΔH value is in poor agreement with the other ΔH values for this reaction. Although this result has been obtained consistently, the reason for it is unknown. However, normal behaviour is observed at pH8.0 under otherwise identical conditions.

Significant product interaction was observed only with the $Fe(CN)_6^{3-}$ oxidation of the reduced Viologens. It amounted to −13.0 and −18.8kJ/mol of $Fe(CN)_6^{4-}$ for the interaction of $Fe(CN)_6^{4-}$ with oxidized Methyl Viologen and Benzyl Viologen respectively. This effect undoubtedly results from charge interaction between the dipositive Viologens and the tetranegative $Fe(CN)_6^{4-}$ ion. These results may be important when interpreting thermodynamic quantities involving Viologens and acid proteins because Viologen-binding to negatively charged proteins could be significant. The other combinations of redox species produced insignificant product interactions.

The ΔH data in Table 1 together with corresponding mid-point potentials are combined to yield the thermochemical data in Table 2 in a form more useful for biological applications. These reactions are all referred to the pH7 hydrogen electrode [$P_{H_2} = 10^2$kPa (1atm), activity $H^+ = 10^{-7}$M] for which E_0', $\Delta G^{0'}$, $\Delta H^{0'}$ and $\Delta S^{0'}$ for the reaction $\frac{1}{2}H_2 = H^+ + e^-$ are all defined to be equal to zero. This procedure has two important advantages. First, the reactions in Table 2 either consume or produce molecular H_2, which puts them in the form desired for studies of biological redox systems producing or consuming molecular H_2 or otherwise operating near the voltage of the pH7 hydrogen electrode [$E_0' = -425$mV for $P_{H_2} = 10^2$kPa (1atm) and activity $H^+ = 10^{-7}$M]. For example, reduced Methyl Viologen or $S_2O_4^{2-}$ can serve as electron donors to the enzymes, hydrogenase (Peck & Gest, 1956) and nitrogenase (Bulen *et al.*, 1965), which then catalytically produce hydrogen according to reactions (4) and (5). Nitrogenase also requires

Table 1. *Enthalpy measurements at 25°C for redox reactions in various buffers*

The ΔH observed values have been corrected for heats of dilution and any thermal effects due to product interaction but not for proton interaction with buffer components. To convert into kcal/mol, the values below should be divided by 4.184 kJ/kcal.

Designation	Reaction	Buffer (pH)	ΔH observed (kJ/mol)	ΔH corrected (kJ/mol)
(*a*)	$Fe(CN)_6^{3-} + MV_r = Fe(CN)_6^{4-} + MV_o$	Tris (8.00)	−127.9 ± 1.1	−127.9 ± 1.1
		Phosphate (8.00)	−130.5 ± 0.8	−130.5 ± 0.8
		Phosphate (8.00)	−134.7 ± 0.9	−134.7 ± 0.9
(*b*)	$Fe(CN)_6^{3-} + BV_r = Fe(CN)_6^{4-} + BV_o$	Tes (8.00)	−134.3 ± 0.5	−134.3 ± 0.5
(*c*)	$2MV_r + 2H^+ + FMN = 2MV_o + FMNH_2$	Phosphate (8.00)	−79.1 ± 1.0	−88.0 ± 1.0
		Tes (8.00)	−27.5 ± 0.7	−94.4 ± 0.7
		Tes (7.00)	−26.2 ± 0.5	−93.2 ± 0.5
	$2MV_r + 2H^+ + FAD = 2MV_o + FADH_2$	Phosphate (8.00)	−92.1 ± 1.6	−101.2 ± 1.6
(*d*)	$MV_o + \frac{1}{2}S_2O_4^{2-} + H_2O = MV_r + SO_3^{2-} + 2H^+$	Tris (8.50)	−65.1 ± 2.1	+29.8 ± 2.1
(*e*)	$Fe(CN)_6^{3-} + \frac{1}{2}H_2 = Fe(CN)_6^{4-} + H^+$	Phosphate (8.00)	−115.5 ± 2.2	−110.9 ± 2.2 (−111.7)*
(*f*)	$Fe(CN)_6^{3-} + FMNH_2 = Fe(CN)_6^{4-} + \frac{1}{2}(\cdot FMNH)_2 + H^+$	Phosphate (8.00)	−103.8 ± 1.5	−98.7 ± 1.5
(*g*)	$FMN + S_2O_4^{2-} + 2H_2O = FMNH_2 + 2SO_3^{2-} + 2H^+$	Tris (8.00)	−116.6 ± 3.1	−21.8 ± 3.1
		Tes (7.25, 8.00)	−83.2 ± 0.7, −85.9 ± 0.9	−22.9 ± 0.7, −22.3 ± 0.9
		Phosphate (7.25, 8.00)	−40.6 ± 0.7, −28.2 ± 0.9	−23.5 ± 0.7, −12.9 ± 0.9
		Cacodylate (7.25)	−18.0 ± 0.5	−23.0 ± 0.5
	$FAD + S_2O_4^{2-} + 2H_2O = FADH_2 + 2SO_3^{2-} + 2H^+$	Phosphate (8.00)	−31.4 ± 1.5	−23.0 ± 1.5
		Tes (8.00)	−90.2 ± 1.5	−23.2 ± 1.5
(*h*)	$Fe(CN)_6^{3-} + \frac{1}{2}S_2O_4^{2-} + H_2O = Fe(CN)_6^{4-} + 2H^+ + SO_3^{2-}$	Tris (7.25, 8.50)	−173.8 ± 3.0, −192.5 ± 3.0	−96.0 ± 3.0, −97.5 ± 3.0
		Tes (7.25, 8.00)	−155.4 ± 3.1, −161.5 ± 2.9	−99.8 ± 3.1, −97.1 ± 2.9
		Phosphate (7.25, 8.00)	−134.0 ± 2.3, −104.2 ± 1.8	−126.2 ± 2.3, −95.2 ± 1.8
		Cacodylate (7.25)	−106.3 ± 2.1	−99.1 ± 2.1
		Acetate (7.00)	−107.7 ± 2.5	−96.2 ± 2.5

* Value taken from Hanania *et al.* (1967).

Table 2. *Thermodynamic data at 25°C from Table 1 converted into redox reactions relative to the pH 7.0 hydrogen electrode*

The thermochemical data ($\Delta H'$, $\Delta G'$ and $\Delta S'$) can be converted into kcal/mol by dividing by 4.184 kJ/kcal.

Reaction	E_0' (V)	$\Delta H'$ (kJ/mol)	Derivation	$\Delta G'$ (kJ/mol)	$\Delta S'$ ($J \cdot mol^{-1} \cdot °K^{-1}$)
(i) $Fe(CN)_6^{3-} + \frac{1}{2}H_2 = Fe(CN)_6^{4-} + H^+$	0.444*	−111.7 ± 3.0‖		−42.7	−230
		−110.9 ± 1.7	(*e*)		
(ii) $MV_o + \frac{1}{2}H_2 = MV_r + H^+$	−0.441†‡	17.2 ± 0.8	(*a*) − (*e*)	+42.7	−88
			(*d*) − h + e		
(iii) $BV_o + \frac{1}{2}H_2 = BV_r + H^+$	−0.360†‡	23.0 ± 0.8	(*a*) − (*b*)	+34.7	−39
(iv) $FMN + H_2 = FMNH_2$	−0.215†	−54.8 ± 2.9	2(*e*) − 2(*f*) − 6.6	+41.4	−322
$FAD + H_2 = FADH_2$	−0.219†	−56.5 ± 2.9	(*g*) − 2(*h*) + 2(*e*)	+42.3	−330
			(*c*) − 2(*a*) + 2(*e*)		
(v) $2SO_3^{2-} + H_2 + 2H^+ = S_2O_4^{2-} + 2H_2O$	0.511†‡	−29.3 ± 0.8	2(*h*) − 2(*e*)	−98.3	−427
(vi) $FMN + FMNH_2 = (FMNH)_2$	—	−27.6 ± 0.8	—	−25.5	−8.4
(vii) $NAD^+ + H_2 = NADH + H^+$	−0.320†§	−29.2§, −27.5§	—	+61.9	−301
$NADP^+ + H_2 = NADPH + H^+$	−0.324†§	−25.3§	—	+62.5	−293

* Beaudette & Langerman (1974) and references therein.
† Sober (1968).
‡ Present study.
§ Burton (1974) and references therein.
‖ Hanania *et al.* (1967).

concomitant ATP hydrolysis (Bulen & LeComte, 1966).

$$S_2O_4^{2-} + 2H_2O \xrightarrow{\text{Hydrogenase or nitrogenase+ATP}} 2SO_3^{2-} + H_2 + 2H^+ \quad (4)$$

$$2MV_r + 2H^+ \xrightarrow{\text{Hydrogenase or nitrogenase+ATP}} 2MV_o + H_2 \quad (5)$$

The data for reactions (ii) and (v) in Table 2 provide the necessary thermodynamic information for these enzyme-catalysed reactions.

The second advantage of this procedure is that the thermodynamic changes occurring for redox reactions where H_2 production or consumption occurs can be attributed to the other partner of the redox reaction. This is an important feature when comparing the thermodynamic quantities for reaction of a series of redox species being reduced by or oxidizing molecular H_2. An interesting biological comparison can be made to illustrate this point by comparing the thermodynamics of reduction by H_2 of the two important iron-containing electron-transfer proteins, horse heart cytochrome *c* (Fe-cyt. *c*) and spinach ferredoxin (Fd). The $\Delta G'$, $\Delta H'$ and $\Delta S'$ values are −25.0kJ/mol, −59.4kJ/mol (−14.2kcal/mol) and $-114.2\,J\cdot mol^{-1}\cdot{}^\circ K^{-1}$ for reaction (6) (Watt & Sturtevant, 1969) and +42.7kJ/mol (Benemann & Valentine, 1971), −21.3kJ/mol (−5.1kcal/mol) and $-216.3\,J\cdot mol^{-1}\cdot{}^\circ K^{-1}$ for reaction (7) respectively.

$$Fe^{III}\text{-cyt. } c + \tfrac{1}{2}H_2 = Fe^{II}\text{-cyt. } c + H^+ \quad (6)$$

$$Fd_{ox.} + \tfrac{1}{2}H_2 = Fd_{red.} + H^+ \quad (7)$$

where $Fd_{ox.}$ and $Fd_{red.}$ are oxidized and reduced ferredoxin respectively. The ΔH value for reaction (7) was calculated from the measured ΔH value of -6.69 ± 2.1kJ/mol (1.6 ± 0.5kcal/mol) for the reduction of spinach ferredoxin by $S_2O_4^{2-}$ measured in our laboratory and reaction (v) from Table 2. Reactions (8), (9) and (10) summarize the thermochemical cycle used for this calculation

$$Fd_{ox.} + \tfrac{1}{2}S_2O_4^{2-} + H_2O = Fd_{red.} + SO_3^{2-} + 2H^+;\ \Delta H = -6.69\,kJ/mol \quad (8)$$

$$SO_3^{2-} + H^+ + \tfrac{1}{2}H_2 = \tfrac{1}{2}S_2O_4^{2-} + H_2O;\ \Delta H = -14.6\,kJ/mol \quad (9)$$

$$Fd_{ox.} + \tfrac{1}{2}H_2 = Fd_{red.} + H^+;\ \Delta H = -21.3\,kJ/mol \quad (10)$$

and illustrate the general procedure for using the data in Table 2 to convert reactions so that they either consume or produce H_2 gas. The favourable free-energy change for reaction (6) is the result of a favourable enthalpy change overcoming an unfavourable entropy effect. The decrease in entropy is mainly a result of the known (Margoliash & Schejter, 1966) minor conformation change which occurs on reduction of cytochrome *c* making the reduced form more compact relative to the oxidized form. Spinach ferredoxin undergoes an even larger unfavourable entropy change on reduction, overwhelming the modest but favourable enthalpy change to make the overall free-energy change positive for reduction. By analogy with cytochrome *c* reduction, this large negative entropy effect may indicate conformational changes on reduction. The large negative (positive) entropy change and the small negative (positive) enthalpy change accompanying reduction (oxidation) of ferredoxin are noteworthy and may suggest an entropy-driven energy-transfer mechanism by this low-potential redox protein.

No significant difference between the ΔH values for reduction of FAD and FMN was observed. This result is expected because the AMP group, which, when attached to FMN forms FAD, is remote from the site of reduction and should have negligible influence on reduction enthalpies. The $\Delta G^{0\prime}$ and $\Delta S'$ values were found to be likewise unaffected.

The reaction of excess of $FMNH_2$ with $Fe(CN)_6^{3-}$ produced a ΔH value slightly larger than expected for the simple oxidation of $FMNH_2$ to FMN. This same observation was reported by Beaudette & Langerman (1974) during the course of this study from calorimetric measurements of FMN redox reactions. A detailed analysis of the various FMN-containing species led these authors to conclude that the observed enthalpy excess resulted from reaction (11). By following their treatment, we find a ΔH of −6.6kcal/mol (−27.6kJ/mol) for reaction (11) in agreement with their reported value of −7kcal/mol. Our measured ΔH of -13.1 ± 0.7kcal/mol (−54.8kJ/mol) for FMN reduction by H_2 is also in agreement with the previously reported value (Beaudette & Langerman, 1974) of -14.2 ± 0.7kcal/mol of FMN.

$$FMN + FMNH_2 = (\cdot FMNH)_2 \quad (11)$$

The thermodynamic data in Table 2 reported (Burton, 1974) for NAD^+ and $NADP^+$ reduction by H_2 add to the list of redox species, both natural and artificial, that can operate near the pH7 hydrogen electrode. The flavin and nicotinamide nucleotide coenzymes usually operate in conjunction with protein carriers and the thermodynamic data in Table 2 for these free coenzymes can be shifted to be either more positive or more negative by this protein–coenzyme interaction. The degree to which the thermodynamic data in Table 2 for the free coenzymes are shifted in the presence of their protein carriers, gives a good indication of the magnitude of the protein–coenzyme interaction.

The ΔH values in Table 2 and the Gibbs–Helmholtz equation can be used to calculate E_0' ($\Delta G^{0\prime}$) values at any other temperature for the redox couples listed. For example, E_0' values for Methyl Viologen are

calculated to be 0.462 and 0.442V respectively at 40° and 15°C compared with measured values of 0.460 and 0.445V. In this manner the use of the E_0' (ΔG^0) values found in Table 2 can be greatly extended.

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