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Hydroxide binding equilibrium in hemerythrin

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The measurement of the equilibrium constant and the thermodynamic parameters of the binding of hydroxide ions to the active site of hemerythrin, an invertebrate iron containing protein, was done by a new difference spectroscopic method. The method enables accurate measurements of equilibrium constants without accurate knowledge of molar absorptivities or protein concentrations and is novel in that it takes

the difference between the two equilibrium mixtures, thus enabling the measurement of equilibrium constants when it is not convenient or possible to produce either substance as a pure reference material.

Hemerythrin from the sipunculid Themiste dyscritum was shown to bind hydroxide ion to its binuclear iron chromophore in a 1:1 stoichiometry with the logarithm of the equilibrium constant being 5.71±0.11 at 25°C. The temperature dependence of the equilibrium constant showed that the enthalpy change upon hydroxide binding was -10.01±1.45 kcal/mole and the entropy change was found to be -7.68±2.41 cal/mole-K.

Future applications of this difference spectroscopic method are suggested.

HYDROXIDE BINDING EQUILIBRIUM IN HEMERYTHRIN

by

JOHN DAVID MCCALLUM

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TO THE OFFICE OF GRADUATE STUDIES AND RESEARCH:

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DEDICATION

I would like to dedicate this thesis to Dr. Joann Loehr, without whose academic and personal advice it never would have been completed, and to Regina, without whose loving encouragement it would not have been as much fun.

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INTRODUCTION

The presence of molecular oxygen in the earth's atmosphere provides an abundant oxidant for nearly any fuel that nature could provide. Although oxygen is found in combination with almost every element, molecular oxygen is relatively stable and will not enter chemical reactions at biologically acceptable rates and temperatures unless it has been activated. One of the means used by biological organisms to activate molecular oxygen is by coordination to a transition metal ion. The understanding of metal containing proteins, which are responsible for sequestering energy from the environment, is a requisite beginning to the understanding of life.

HEMERYTHRIN

Hemerythrin is the oxygen transport protein of three phyla of marine invertebrates. It composes about 95% of the protein found in the blood cells of these organisms and is therefore readily available for study. The hemerythrin molecule has a molecular weight of 108,000 daltons which is distributed among eight identical subunits (Klotz and Keresztes-Nagy, 1963). Each subunit contains two iron atoms and has the capacity to bind one molecule of oxygen (Boeri and Ghiretti-Magaldi, 1957).

Although there has never been a cooperative effect attributed to the interactions between subunits, as is found in hemoglobin, the octameric structure may influence the active site since the oxygen storage protein found in muscle, myohemerythrin, which is a monomeric unit similar to hemerythrin, has a greater oxygen affinity (Kubo, 1953). This difference in oxygen affinity facilitates the transport of oxygen from the coelomic fluid to the muscle tissue.

There are two physiological forms of hemerythrin. The oxygen free protein, called deoxyhemerythrin, has both iron atoms in the reduced or ferrous state while the oxygenated form, known as oxyhemerythrin, has both iron atoms oxidized to the ferric state (Klotz and Klotz, 1955) while the bound oxygen molecule has been reduced to peroxide (Dunn, et al, 1973). It has been shown that the iron dimer can be oxidized with chemical reagents, such as ferricyanide, forming a species now known as methemerythrin (Marrian, 1927) which is no longer capable of reversibly binding oxygen but which tends to bind various anions (Keresztes-Nagy and Klotz, 1965). By studying the interactions of these anion-containing forms of the protein it is possible to learn more about the nature of metal ion sites in proteins than if the study were limited to the two physiological forms.

There are similarities between the oxidized forms of hemerythrin and inorganic μ-oxo bridged iron dimers. The electronic spectra are similar (Garbett, et al, 1969;

Schugar, et al, 1972), they have nearly identical Mössbauer spectra (York and Bearden, 1970; Garbett, et al, 1971), and the magnitude of antiferromagnetic coupling is very close (Okamura, et al, 1969).

The various anion adducts of methemerythrin tend to fall into three classes of bound ligand. Aquomethemerythrin is characterized by monophasic formation from oxyhemerythrin (Bradić, et al, 1980) and a biphasic reduction by dithionite (Harrington, et al, 1978). Another class, which includes azide, cyanide, and cyanate, can be characterized by biphasic formation from oxyhemerythrin (Bradić, et al, 1980) and no or very slow reduction by dithionite (Olivas, et al, 1979). This second class also shows a shift of the 508 cm^{-1} Raman peak assigned to the iron-oxygen vibration of the μ-oxo bridge upon transfer to solvent with labelled oxygen (Freier, et al, 1980). A third class, which includes thiocyanate, fluoride, and nitrite, can be characterized by monophasic formation from oxyhemerythrin (Bradić, et al, 1980), monophasic reduction by dithionite ion (Olivas, et al, 1979), and also shows no interchange of the μ-oxo bridge with solvent (Freier, et al, 1980).

The interconversions of these forms of hemerythrin are shown in Figure 1 with thiocyanate being shown as representative of the third class of ligands and azide being representative of the second class of ligands.

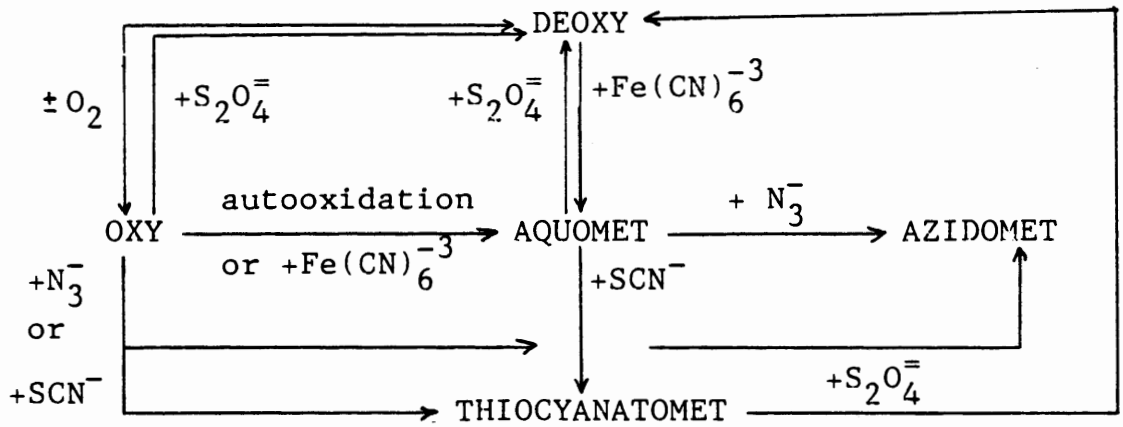

Figure 1. The Reactions of Hemerythrin. Azide has been included as being characteristic of all ligands in its class and thiocyanate has been included as being characteristic of all ligands in its class.

THE CRYSTAL STRUCTURE OF HEMERYTHRIN

An X-ray diffraction crystal structure has been done on aquomethemerythrin from Themiste dyscritum (Stenkamp, et al, 1976). The first model at low (2.8 Å) resolution had the iron atoms as the centers of octahedra which shared a common face. The vertices of the common face were two protein carboxylates and the water ligand. The other vertices were occupied by five histidine residues and one tyrosine residue from the protein. The revised higher (2.2 Å) resolution map (Stenkamp, et al, 1981) shows that the tyrosine is not close enough to be an iron ligand and the azidomethemerythrin difference electron density map (Stenkamp, et al, 1981) shows that the shared oxygen bridging group is conserved and therefore is probably the μ-oxo bridge and not a water ligand. The structure of the active site is shown in Figure 2 below.

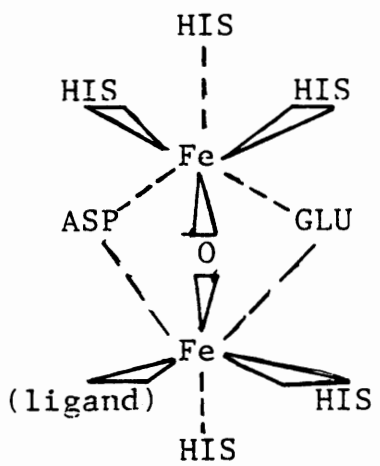

Figure 2. The Active Site Structure of Hemerythrin. Dotted lines recede, triangles protrude; the site marked (ligand) is vacant in aquomethemerythrin and occupied by azide ion in azidomethemerythrin.

One aim of this study is to help in the determination of the active site structure of both of the physiological forms of hemerythrin. Is the oxygen binding site similar to the azide binding site and what are the structural changes upon oxygen binding to deoxyhemerythrin? The active site structure of the methemerythrins has been fairly well established but the structure of the oxygen containing protein and, especially, the structure of the reduced protein must be determined before any mechanism can be implied.

The active site structure of deoxyhemerythrin can best be determined by X-ray diffraction methods, but until recently crystals of the reduced protein have not been available. In conjunction with this thesis deoxyhemerythrin has been crystallized for X-ray analysis which is currently being done by Ron Stenkamp, Larry Sieker, and Lyle Jensen at the University of Washington.

Another structural technique which can be used with deoxyhemerythrin is extended X-ray absorption fine structure spectroscopy (EXAFS) in which the probability of absorption of X-rays with subsequent emission of a K-shell electron depends on the final state of that electron, which is dependent on the surrounding electronic matrix. This technique is relatively new and not as well refined as X-ray diffraction but has the advantage of being able to be used on samples in solution. EXAFS on deoxyhemerythrin as well as the oxy and met adducts has been done on samples prepared in

conjunction with this thesis by Tim Elam and Ed Stern at the University of Washington (Elam, et al, 1982a; 1982b).

INTERCONVERSION OF AQUO AND HYDROXY METHEMERYTHRINS

Methemerythrin obtained from the oxidation of oxyhemerythrin with no binding ligands present has been referred to as aquomethemerythrin; the implication in the name being that a water molecule occupied the small anion binding site. If the pH of a methemerythrin solution is raised another species, known as hydroxymethemerythrin, is formed; the implication in the name being that a hydroxide ion is bound to the same site.

The X-ray crystallographic data were collected on methemerythrin from Themiste dyscritum and show an empty site in the azide binding location. These crystals were believed to have been aquomethemerythrin but the ultraviolet and visible spectra of the mother liquor suggested that there may have been some hydroxymethemerythrin present in the crystals. Knowledge of an equilibrium constant for the conversion of aquomethemerythrin to hydroxymethemerythrin would give a clearer understanding of the composition of the crystals used in the crystallographic model.

The primary aim of this thesis is the determination of the equilibrium constant and its temperature dependence for the aquomethemerythrin-hydroxymethemerythrin conversion. Although the temperature dependence of the equilibrium

constant yields the thermodynamic parameters ΔG, ΔH, and ΔS their significance at the active site is obscured in a large protein molecule where conformational changes of the protein matrix as well as secondary protonations can contribute as much as the active site reaction. The thermodynamics can be used as a comparison in future studies.

There recently appeared a difference spectroscopic method for the determination of equilibrium constants which does not require knowledge of the molar extinction coefficients (Gorman and Darnall, 1981) and is therefore convenient to use for proteins where an accurate extinction coefficient is difficult to obtain. Gorman and Darnall demonstrated this technique on hemerythrin from Phascolopsis gouldii. This method was to be used for the equilibrium constant determination of teh aquomet-hydroxymet equilibrium in Themiste dyscritum in this thesis but some objections were raised that led to the development of a new technique which is outlined in the following chapter and is the major focus of this thesis.

The main criticism of the Gorman and Darnall method is that the assumption was made that the molar extinction coefficient of aquomethemerythrin was exactly the same as methemerythrin with perchlorate. It was shown (Darnall, et al, 1968) that the addition of nitrate, carbonate, phosphate, and especially perchlorate raised the pK of the aquomet to hydroxymet equilibrium and it has now become common practice

to stabilize the aquomet form with perchlorate. Since aquomethemerythrin is not believed to be obtainable in pure solution without raising the pK with perchlorate it can not be shown that the spectral similarities that the perchlorate form has towards aquomethemerythrin are an accurate mimic. Furthermore, crystallographic studies (Stenkamp, et al, 1978) have shown that although perchlorate binds at a site 15 angstroms away from the iron center it also induces some negative electron density near, but not at, the ligand binding site. In light of this observation there must be serious doubts as to whether the addition of perchlorate preserves the electronic spectrum and since the aquo form cannot be obtained in pure form without raising the pK by perchlorate addition this assumption cannot be rigorously tested.

Another criticism of the Gorman and Darnall method is that it is necessary to test ΔA, the difference in absorbance, as a function of hemerythrin concentration at constant ionic strength. Due to the indeterminant nature of the myriad charged sites on the hemerythrin molecule it is difficult to keep ionic strength constant as hemerytrhin concentration is varied but still concentrated enough to give reasonable spectral differences.

THEORY

A NEW METHOD FOR DETERMINING EQUILIBRIUM CONSTANTS FROM DIFFERENCE SPECTROSCOPY

Difference spectroscopy has been used in many investigations of protein-ligand binding constants. One of the most recent applications was made in a study of the hydroxide and thiocyanate binding equilibria with hemerythrin by Gorman and Darnall (1981). In the Gorman and Darnall method it is not necessary to know the molar extinction coefficients of either the reactants or the products as long as the ligand is transparent in the region of interest. This is a boon to the measurement of protein equilibrium constants where molar absorptivities are often difficult to obtain. In this method, however, it is necessary to have a sample of unligated or fully ligated protein in the reference beam. This is not always practical if the investigation is of a secondary binding site or if the ligand is a ubiquitous agent such as a water molecule or a hydroxide ion or if the protein is sensitive to wide ranges of ligand concentration (e.g. denaturation due to high concentrations of acid, base, or salt).

Herein is presented an extension of the Gorman and Darnall method which is especially suited to these exceptions. It is a convenient method to use for determin-

ing the equilibrium constants of samples which have relatively large spectral changes upon ligand binding, cannot be subjected to wide ranges of ligand concentration, and are not easily obtainable in pure form.

Assume a protein, P, binds a ligand, L, resulting in an equilibrium given by the expression:

$$P + nL \rightarrow PL_n \qquad (II\text{-}1)$$

with an equilibrium constant defined by:

$$K_{eq} = \gamma_{PL_n}[PL_n] \; / \; \gamma_P[P](\gamma_L[L])^n \qquad (II\text{-}2)$$

where the bracketed terms are molar concentrations, γ denotes the activity coefficient of the subscripted species, and n is the number of ligand moieties bound at the binding site.

The absorbance, A, for an equilibrium mixture of free and bound protein is given, provided that both species obey the Beer-Lambert law and the law of additive absorbances, by the expression:

$$A = (\epsilon_P[P] + \epsilon_{PL_n}[PL_n])\ell . \qquad (II\text{-}3)$$

The path length is given by ℓ and ϵ indicates the respective molar absorptivities. This relation is valid at any wavelength.

If a different equilibrium mixture is put in the reference beam the measured absorbance, ΔA, through a path length of unity is given by:

$$\Delta A = \epsilon_P[P]_s + \epsilon_{PL_n}[PL_n]_s - \epsilon_P[P]_r - \epsilon_{PL_n}[PL_n]_r \qquad (II\text{-}4)$$

where the subscripts s and r refer to the sample and reference, respectively.

If the total protein concentration, C, is equal in both the sample and reference cells such that:

$$C = [P]_s + [PL_n]_s = [P]_r + [PL_n]_r \qquad (II\text{-}5)$$

then ΔA can be rewritten as (Eq. II-6):

$$\Delta A = \epsilon_P[P]_s + \epsilon_{PL_n} C - \epsilon_{PL_n}[P]_s - \epsilon_P[P]_r - \epsilon_{PL_n} C + \epsilon_{PL_n}[P]_r$$

which can be rearranged to:

$$\Delta A = \Delta\epsilon([P]_s - [P]_r). \qquad (II\text{-}7)$$

One can then make another independent measurement on a different equilibrium mixture against the same reference mixture and have another measured absorbance, $\Delta A'$, which is equal to $\Delta\epsilon([P]_t - [P]_r)$ where the subscript t refers to the second sample. The ratio of ΔA to $\Delta A'$ which I will call α will eliminate the need to know $\Delta\epsilon$ and will be given by:

$$\alpha = \Delta A/\Delta A' = ([P]_s - [P]_r) \,/\, ([P]_t - [P]_r). \qquad (II\text{-}8)$$

This equation contains three unknown quantities: $[P]_s$, $[P]_t$, and $[P]_r$ but our desire is to know the equilibrium constant which can be expressed by three independent equations:

$$K_{eq} = \gamma_{PL_n}[PL_n]_s \,/\, \gamma_P[P]_s(\gamma_L[L]_s)^n \qquad (II\text{-}9)$$

$$K_{eq} = \gamma_{PL_n}[PL_n]_t \,/\, \gamma_P[P]_t(\gamma_L[L]_t)^n \qquad (II\text{-}10)$$

$$K_{eq} = \gamma_{PL_n}[PL_n]_r \,/\, \gamma_P[P]_r(\gamma_L[L]_r)^n. \qquad (II\text{-}11)$$

These equations introduce the unknown activity coefficients and the unknown quantity n. We can simplify them by assuming that $\gamma_{PL_n} = \gamma_P$. Since ligand binding does not appreciably alter the large protein molecule the activity coefficients should be similar at similar concentrations and because this technique is designed for small changes in concentrations this assumption is reasonable. The activity coefficient of the ligand is often included in the measurement of ligand activity. If the ligand binding constant is high and the ligand concentration low then the activity of the ligand will be what is measured by most techniques. Only when the binding constant is low and a large reservoir of ligand is added in known concentration will a correction for ligand activity coefficient be necessary.

To find n it would be possible to add another sample yielding a sixth equation but these equations would not be linear and it is possible that they would generate an extraneous solution. It is more convenient to determine n by another method (e.g. Benesi and Hildebrand, 1949; Scatchard, 1949) or to implicitly determine it by finding the most consistent set of equilibrium constants obtained from several samples and different integer values of n.

Solving Equations II-8, II-9, II-10, and II-11 by the method of Gauss keeping n as an arbitrary parameter (see Appendix A) gives the equilibrium constant as a function of the ligand activities (or concentrations) and the measured

spectral differences (expressed as α) such that:

$$K_{eq} = \frac{\alpha[L]_r^n - \alpha[L]_t^n + [L]_s^n - [L]_r^n}{[L]_r^n[L]_t^n + \alpha[L]_s^n[L]_t^n - [L]_s^n[L]_t^n - \alpha[L]_s^n[L]_r^n} \quad . \qquad (II\text{-}12)$$

CONSIDERATIONS ON THE ERRORS IN K_{eq} CALCULATION

There are two measured quantities which contribute to error in the calculation of equilibrium constants by this technique: the measurement of ligand activities and the spectral differences. The measurement of spectral difference is affected by two sources of error: the mismatch of sample concentration and the performance of the spectrophotometer (including errors due to electronic noise, stray light, and bandpass errors).

The performance of the spectrophotometer can be estimated by doing repeated experiments and noting the deviations. If the spectral changes upon ligand binding are small these errors can be very significant.

The error in sample concentration can be reduced by following certain techniques. The preparation of samples of identical protein concentration with different concentrations of ligand is achieved by adding different amounts of ligand to aliquots from the same protein solution. The addition of ligand changes the concentration by the factor $V_p/(V_p + V_l)$, where V_p is the volume of protein solution and V_l is the volume increase due to the added ligand. This gives a final concentration, C, which can be expressed as a function of

the initial concentration, C_o, as follows:

$$C = C_o(V_p \pm E_p)/(V_p + V_l \pm E_p \pm E_l), \qquad (II\text{-}13)$$

when there is a measurement error, E_p and E_l, associated with the volume delivered.

The relative error in concentration, $(C' - C)/C'$, where C', the calculated concentration, is given by:

$$C' = C\,[V_p/(V_p + V_l)] \qquad (II\text{-}14)$$

can be expressed as:

$$(C'-C)/C' = \frac{V_p/(V_p+V_l) - (V_p \pm E_p)/(V_p+V_l \pm E_p \pm E_l)}{V_p/(V_p+V_l)}. \qquad (II\text{-}15)$$

This can be simplified to:

$$(C' - C)/C' = \frac{E_p V_l + V_p E_l}{V_p(V_p + V_l + E_p - E_l)} \qquad (II\text{-}16)$$

when the error in the protein and ligand volumes have opposite signs (this is when the error is the greatest).

Most commercially available volume measuring devices have an inherent error of about 0.1% which is usually independent of volume for most of the volume range of the apparatus. So if $E_i = kV_i$, where k is the error constant of the device (approximately 0.001 for most good quality calibrated glassware) then the relative error given in Equation II-16 can be further simplified to:

$$(C' - C)/C' = \frac{\pm 2kV_l}{V_p + V_l + E_p - E_l}. \qquad (II\text{-}17)$$

From Equation II-17 it is clear that the relative error is

minimized by making V_l, the volume increase due to added ligand, as small as practical with respect to the volume of protein solution used.

The error in the measurement of ligand activities will probably be the largest error in the equilibrium constant. Ligands can be measured by ion-selective electrodes where the accuracy is often limited by interfering ions but even under optimum conditions will rarely achieve greater than ±3% accuracy. This spectroscopic method increases this error since the calculation involves the difference of two measured ligand activities. For example, a glass membrane electrode which is capable of measuring hydrogen ion activities to within 0.02 pH units will give an error of about 4% in the hydrogen ion concentration when measuring a pH of 7 but when measuring a difference of 0.3 pH units this error is increased to almost 10%.

One of the difficulties with difference spectroscopy in general is that the total absorbance is often quite high in order to get an appreciable signal for the difference. The signal is often increased by increasing the bandpass. This contributes an error given by the function:

$$\frac{\int e^{f(y)} dy}{e^{f(y)} \int dy}, \qquad (II\text{-}18)$$

where y is the wavelength being observed and f(y) is a function given by: $-1/2\sigma^2 y^2 + \mu/2\sigma^2 y - \mu^2/2\sigma^2$, where μ is the center of the band which is broadened by a Gaussian

function and σ^2 is the variance of that Gaussian function. The integrals are evaluated over the bandpass. The wider the bandpass the greater the spectral flattening but in this method this error source is corrected by the use of the ratio of $\Delta A/\Delta A'$ which would have the same percentage of reduction in both the numerator and the denominator.

BEST ESTIMATE OF K_{eq}

In an ordinary experiment where the desired value is obtained directly from a single measurement the best estimate of that value is given by the mean of the measurements with the 95% confidence interval given by $\pm t\sigma/\sqrt{n}$, where σ is the population standard deviation (estimated from the sample standard deviation), n is the number of samples, and t is a constant which approaches the limiting value of 1.96 at infinite degrees of freedom. However, in an experiment where the desired value is obtained from a calculation involving two or more separate and independent measurements the best estimate is given by the mean only when the calculation does not involve multiplication, division, or exponentiation by another error-containing measurement.

In this technique a normal distribution of errors in ΔA will not give a normal distribution to the calculated equilibrium constants but will skew the mean toward lower values when the chosen ligand concentrations produce less than 50% bound ligand and towards higher values when the

chosen ligand concentrations produce the opposite effect. If there are a large enough number of chosen ligand concentrations these effects will cancel one another according to the Central Limit Theorem but in cases of small data sets a mean of the calculated equilibrium constants will be skewed in an indeterminate direction.

In this technique several ΔA values at different ligand concentrations can be measured and all possible combinations of three used to represent samples r, s, and t in Equation II-12. The generated equilibrium constants are not randomly distributed but are a function of the samples used in the calculation. If the magnitude of error in one ΔA measurement is greater than that of another then that error will show up as a non-random pattern in the calculations involving that measurement. The existence of these patterns can indicate the direction and approximate magnitude of error (relative to the other samples) in a given measurement. A better estimate than the mean can be obtained by doing a series of experiments and generating equilibrium constants for all possible combinations of the data and then adjusting (within a limit defined by the standard error of an individual ΔA) the data in order to find the one equilibrium constant which requires the least adjustment to be generated by every possible combination.

This section has given a general outline for the treatment of data generated by this difference spectrophoto-

metric technique. The final section of the chapter entitled "Results" shows how these general considerations can be applied.

EXPERIMENTAL PROCEDURES

Aquomethemerythrin undergoes a reaction with aqueous hydroxide ions to form a new species called hydroxymethemerythrin. This reaction can be observed spectroscopically (Figure 3) by the increased absorbance in the 300-400 nanometer region of the ultraviolet spectrum of hydroxymethemerythrin from Themiste dyscritum. Since the structure of aquomethemerythrin has not been shown to contain bound water molecules this reaction can be written in two ways. Either as an acid dissociation reaction:

$$Hr(H_2O)_n \rightarrow Hr(OH)_n + nH^+, \text{ where } K_a = \frac{[Hr(OH)_n][H^+]^n}{[Hr(H_2O)_n]} \qquad (III\text{-}1)$$

or as a hydroxide binding reaction:

$$Hr + nOH^- \rightarrow Hr(OH)_n, \text{ where } K_f = \frac{[Hr(OH)_n]}{[Hr][OH^-]^n} . \qquad (III\text{-}2)$$

Equation III-2 has been chosen as the reaction expression in this thesis because it can be applied equally well to the binding of other small molecules which hemerytrhin is known to do. Equations III-1 and III-2 are related in that $K_a = K_f K_w$ where K_w is the equilibrium constant for the ionization of water.

The equilibrium constant for hydroxide binding to methemerythrin can be determined by the method previously described where:

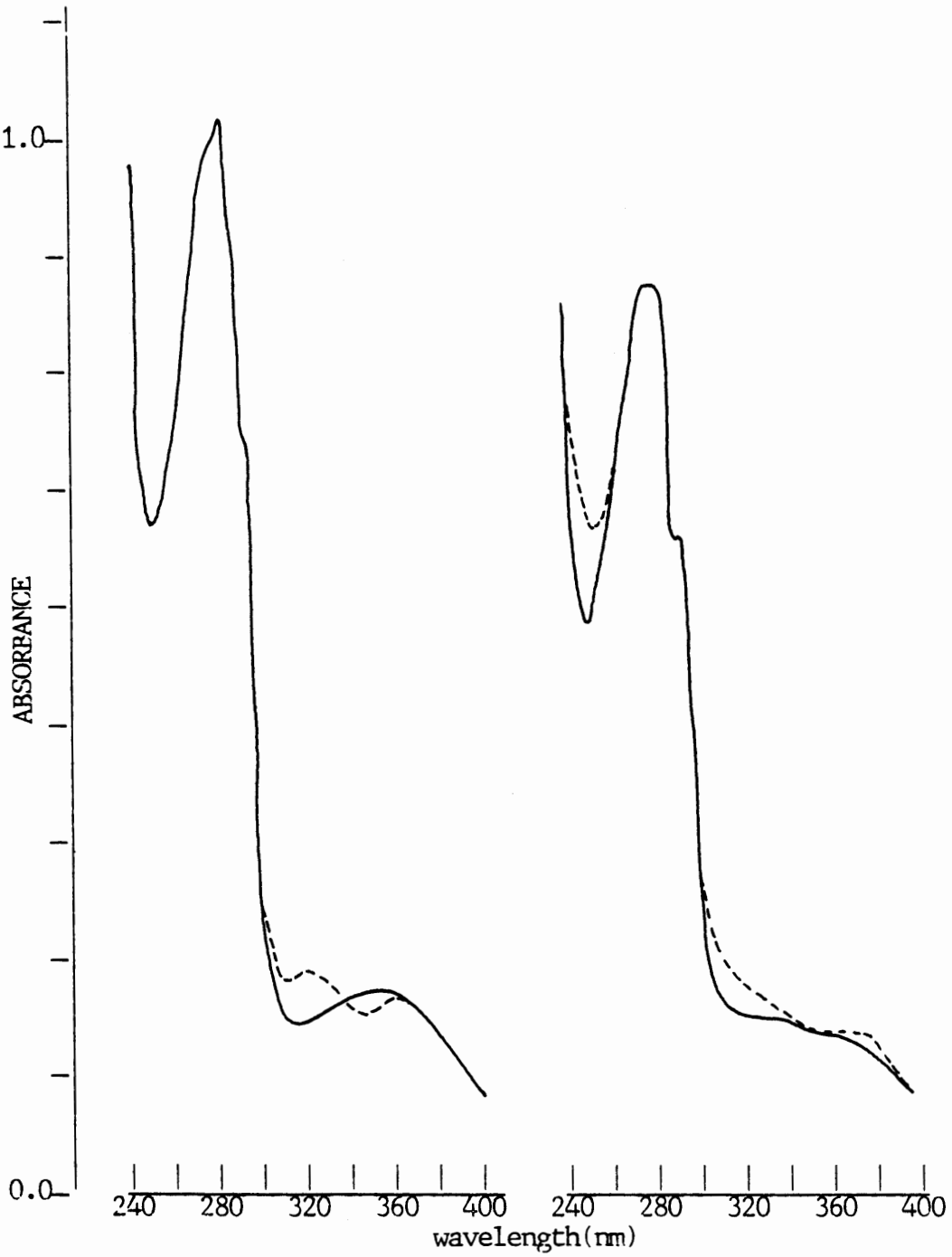

Figure 3. Absorption Spectra of Methemerythrins From Two Species. The 240–400 nm region of the spectrum from P. gouldii (left) and T. dyscritum (right). Solid lines indicate aquomethemerythrin and broken lines are hydroxymethemerythrin. Concentrations are .0288 mM for P. gouldii and .0263 mM for T. dyscritum (in iron).

$$K_{eq} = \frac{\alpha[L]_r^n - \alpha[L]_t^n + [L]_s^n - [L]_r^n}{[L]_r^n[L]_t^n + \alpha[L]_s^n[L]_t^n - [L]_s^n[L]_t^n - \alpha[L]_s^n[L]_r^n} \cdot \qquad (III-3)$$

For this system (using n = 1):

$$K_f = \frac{\alpha([OH]_r - [OH]_t) + ([OH]_s - [OH]_r)}{\alpha[OH]_s([OH]_t - [OH]_r) + [OH]_t([OH]_r - [OH]_s)} \cdot \qquad (III-4)$$

where [OH] is the activity of hydroxide ions determined from a measured pH value and a calculated K_w and α is $\Delta A/\Delta A'$, ΔA being the spectral difference between sample s and reference r and $\Delta A'$ being the spectral difference between sample t and reference r.

EXTRACTION AND PURIFICATION OF HEMERYTHRIN

Hemerythrin for these studies was extracted from two species of sipunculid, Phascolopsis (syn. Golfingia) gouldii, obtained live from Marine Biological Laboratories in Woods Hole, Massachusetts and Themiste dyscritum, obtained live from the Oregon Institute of Marine Biology in Charleston, Oregon.

The worms were cut open with dissecting scissors. The coelomic fluid was filtered through glass wool and collected in an Erlenmeyer flask on ice. The body cavity was rinsed with 0.54 molar sodium chloride and this rinse was pooled with the coelomic fluid. The coelomocytes were collected by centrifugation for ten minutes at 1000 g. The cells were then resuspended in 0.54 molar sodium chloride and collected again by centrifugation. This washing process was repeated

until the pale amber color of the coelomic proteins was no longer visible. The cells were then lysed by addition of an equal volume of distilled water. This suspension was then centrifuged for thirty minutes at 10,000 g to remove cellular debris. This resulted in a translucent dark red solution of hemerythrin. If the pellet had any visible red color it was resuspended in distilled water and recentrifuged. The hemerythrin, being 95% of the cellular protein (Clarke, 1977) was then almost pure.

The final step was the purification of the protein by crystallization. Hemerythrin from Phascolopsis gouldii was dialysed against a solution of 15% ethanol in water while the protein from Themiste dyscritum was crystallized by dialysis against an aqueous solution of 0.02 molar tris-(hydroxymethyl)aminomethane (obtained from Sigma Chemical Co. under the trademark Trizma) which was adjusted to a pH of 8.0 with sulfuric acid. If crystals failed to appear within 24 hours the dialysate was diluted by the addition of distilled water. This buffer was selected because of the optical transparency of both Trizma (also known as Tris) and sulfate between 230 and 1350 nanometers and because Tris and sulfate were believed to bind only weakly to hemerythrin (Keresztes-Nagy and Klotz, 1965). These buffers will be referred to as Tris-sulfate with the concentration of Tris preceding and the pH following parenthetically.

The large crystals of hemerytrhin were allowed to

settle while the smaller ones were collected by a slow centrifugation (less than 1000 g) for two to three minutes. The crystals were rinsed with distilled water and recollected by settling and centrifugation. The crysatls were then dissolved in 0.2 M Tris-sulfate (8.0). All hemerythrin solutions obtained in this manner were mostly oxyhemerythrin but had some residual methemerythrin and will be referred to as cellular hemerythrin. This method of preparation is essentially that of Klotz, Klotz, and Fiess (1957) with only minor changes.

Dissolution of hemerythrin crystals in 0.2 M Tris-sulfate made it possible to obtain stock solutions of 2-5 mM in iron dimer. The concentration of the stock solution was determined by measuring the ultraviolet absorbance at 280 nm. The molar absorptivities at 280 nm are 35,400 and 33,300 per iron dimer for P. gouldii and T. dyscritum protein, respectively (Dunn, et al, 1971). The purity of oxyhemerythrin was determined by measuring the absorbances at 280, 360, and 500 nm and comparing the ratios of these values with those of the molar absorptivities reported by Dunn, et al.

PREPARATION OF METHEMERYTHRIN FOR DIFFERENCE SPECTROSCOPY

A stock solution of methemerytrhin was prepared by diluting cellular hemerythrin to obtain a solution that had a total absorbance of between 1 and 2 at 360 nm (about 0.2 mM in iron dimer). This solution was then oxidized by the

addition of a four-fold molar excess (per iron dimer) of solid potassium ferricyanide. This solution was then dialysed three times against a fifty-fold volume excess of 0.02 M potassium hydrogen sulfate that had been adjusted to a pH of between 7.2 and 7.8 by addition of solid Trizma base. This gave a Tris-sulfate buffer with an ionic strength of 0.06. [see Appendix C for a discussion of the calculation of ionic strength]. This dialysis removed any possible chromophores due to an excess of ferricyanide or its reaction product ferrocyanide but also caused swelling which could reduce the hemerythrin concentration by as much as 50%.

For spectral measurements 3.00 ml of this stock solution were added to a test tube with 0.30 ml of a Tris-sulfate buffer with an ionic strength of 0.90 and variable pH with a syringe buret.[1] The test tubes were then covered with Para-

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[1]The buret was a product of Micrometric, Inc. of Cleveland, Ohio and had divisions of 3 microliters. By repeatedly delivering volumes of water and weighing them it was found that the buret was capable of reproducing volumes within 0.2% (See Appendix A) but only when the procedure was rigorously consistent. For these experiments all volumes were delivered at approximately the same rate from a reading of 0.00 to 3.00 for the protein solution and from 1.00 to 1.30 for the buffer solution. The initial setting was established from the negative side and the buret tip was wiped with tissue before delivery. The final delivery was always capped off by touching the buret tip to the side of the glass test tube. Occasionally this buret would "run on" after delivery. This was a hazard that was constantly watched for. For reloading the syringe the setting was brought back beyond the initial delivery point and the syringe plunger gradually pulled back to be flush with the machine plunger. It was held in this position for several seconds before adjusting the buret to its initial delivery position and wiping the tip clean.

film (Marathon Products) and mixed by several inversions. This gave a final ionic strength of 0.14 (excluding the contribution of the protein). For each temperature six of these solutions were made up. The lowest pH buffer was added to two samples one to be used in the reference beam and one to be used as the zero line while four other buffers of progressively higher pH were added to the remaining four samples.

The solutions of differing pH were placed in a water bath and allowed to equilibrate for one-half to one hour. It was found that 95% of the change in spectrum occurred in the first ten minutes as measured by the magnitude of the difference peak at 310 nm.

Samples were transferred directly from the water bath to the spectrophotometer cell and allowed to equilibrate for an additional ten minutes before measurements were taken. There were occasionally slight differences in temperature between the spectrophotometer and the water bath and there was the possibility of warming of the samples during transfer so this additional ten minute equilibration was added to minimize errors due to thermal differences.

Measurements were made on a recording Cary-14 spectrophotometer with a 0-0.1 slidewire. It had been fitted with a circulating water/antifreeze system (from Neslab, Inc.) through jacketed cells available from Varian Associates. These cells were further insulated with styrofoam to help minmize heat loss and maintain constant temperature between

the sample and reference compartments. At low temperature the cell compartments were continually flushed with dry nitrogen or argon to prevent moisture condensation from refracting the light beam.

Five samples of differing pH were run against a single reference sample at each temperature. Repeat scans were run to determine the stability of the samples and the instrumental reproducibilty (see Appendix A). The self-adjusting slit width of the Cary-14 remained below 0.15 mm between 305 and 400 nm for all experiments.

Protein samples were reclaimed by dialysis against a large excess of 0.02 M $KHSO_4$-Tris (7.2-7.8). There was slight dilution caused by this process but since the absolute concentration was not critical these samples were reused. However, upon repeated use of the protein there was a loss of spectral response to changes in pH. The absolute spectrum of the protein remained unchanged but this loss of activity towards hydroxide was paralleled by a loss of ability of the protein to bind azide. The protein solution was reused in these experiments as long as the addition of azide to a small aliquot showed at least 80% azidomethemerythrin $[(A_{azide,446}/\epsilon_{azide,446})/(A_{azide,280}/\epsilon_{azide,280}) \geq 0.8]$.

Although curious this phenomenon is not considered to interfere with the measurement of the difference spectrum since both the sample and the reference cells would have

equal concentrations of this non-reactive species. It was thought that this phenomenon might be a photochemical reaction occurring in the spectrophotometer but prolonged exposure to ultraviolet light did not produce this non-reactive species so it was considered to be an artifact of the high salt additions followed by dialysis. Since the absolute spectrum did not change it is assumed that the access to the active site has simply been blocked. It was found that after several days in sodium azide solution this non-reactive species did show a slight increase in the azide to iron charge transfer at 446 nm.

After the spectra were taken the temperature of each sample was measured with a thermometer. The thermometer gave sufficiently accurate temperatures since there were a few tenths of a degree difference between the reference and sample compartments and the equilibrium constant is not very sensitive to variations of one or two degrees.

While the sample was still in the spectrophotometer cell the pH was measured with a combination glass membrane/calomel electrode and a Radiometer pH meter (from the London Company, Westlake, Ohio). The meter was calibrated using a phosphate buffer (pH = 7.00 at 25°C; pHydrion trademark from Micro Essential Laboratories of Brooklyn, N.Y.) and a borate/carbonate buffer (pH = 10.01 at 25°C from the same manufacturer). These standard buffers were equilibrated in the same water bath as the hemerytrhin samples and they were

used to set the meter at 7.00 and 10.00 at all temperatures after the temperature correction dial on the pH meter had been set to the experimental temperature. Since phosphate, borate, and carbonate all have significant changes of their pK values with changing temperature a correction for pH had to be made after making the reading on the sample.

The temperature dependence of the pH values of the phosphate and borate/carbonate buffers were obtained from the manufacturer. These values are plotted as change in pH from 25°C as a function of temperature in Figure 4.

The pH of each sample was determined by adding the correction taken from Figure 4 to the meter reading. The error at a pH of 10.00 would be the error of a borate/carbonate buffer at experimental temperature, while the error at a pH of 7.00 would be the error of a phosphate buffer at that temperature. The correction at each temperature was a linear interpolation involving the measured pH and the errors at these bounds by the following equation:

$$pH = pH' + c_7 + \frac{pH' - 7}{10 - 7}(c_{10} - c_7),$$

where pH′ is the meter reading, c_7 is the correction at a pH of 7 and c_{10} is the correction at a pH of 10.

The hydroxide ion activity was calculated by subtracting the pH from the pK_w (the ionization constant of water) and using that value as the negative exponent of 10. The temperature dependence of the pK_w was obtained from data in the Chemical Rubber Company Handbook of Chemistry and Physics

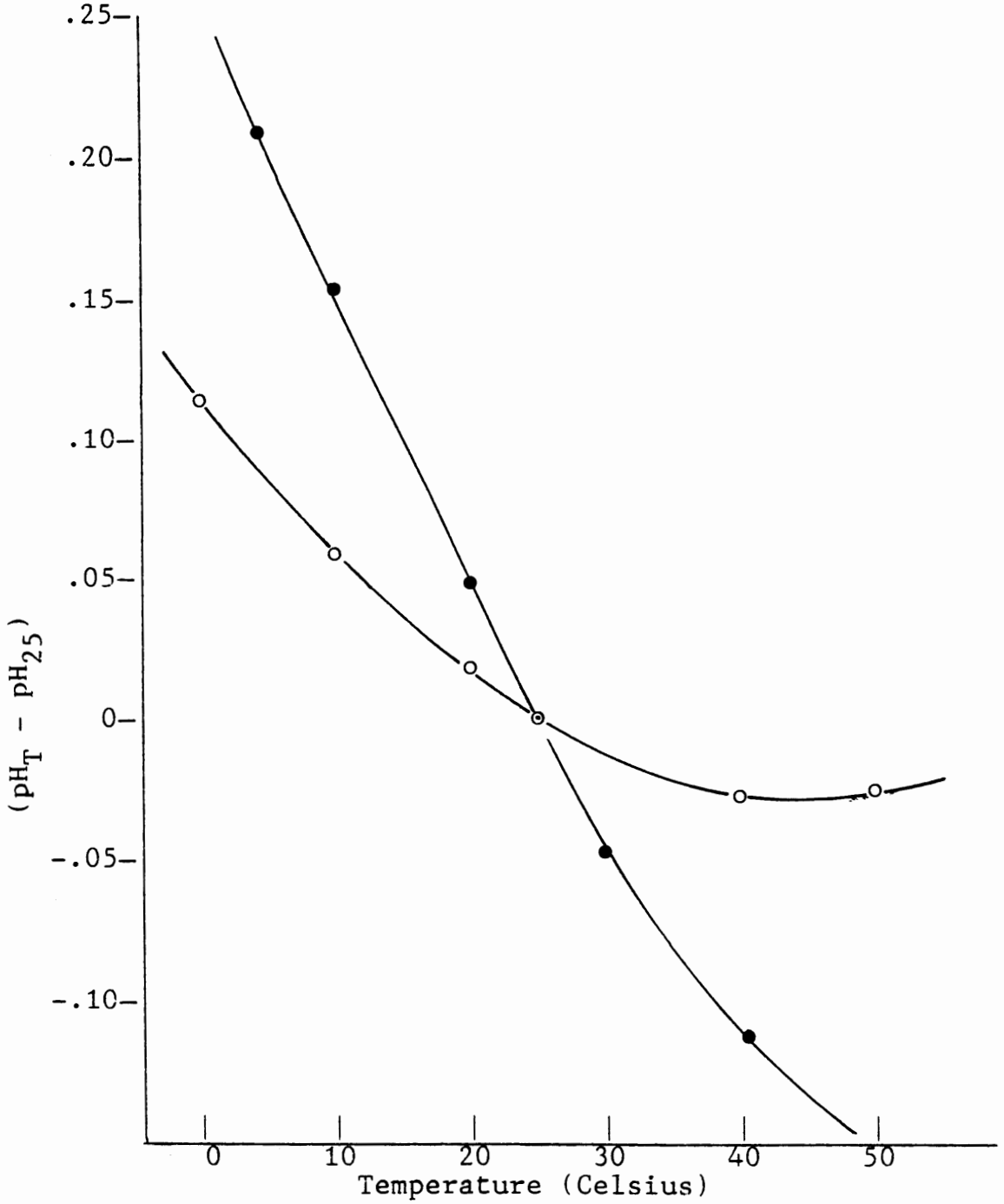


Figure 4. The Temperature Dependence of the Standard Buffers. The difference in pH of phosphate and borate/carbonate buffers from their values at 25°C as a function of temperature. Data supplied by Micro Essential Laboratories. Phosphate (o) Borate/Carbonate (●)

56th Edition (page D-152) and the ionic strength correction taken from Harned and Copson (1933). The data between 0 and 40 degrees and a least squares quadratic fit of that data are shown in Figure 5. The quadratic equation, which has numerical parameters such that $pK_w = 1.823 \times 10^{-4} T^2 - 4.267 \times 10^{-2} T + 14.946$, was used to calculate the pK_w at the experimental temperatures.

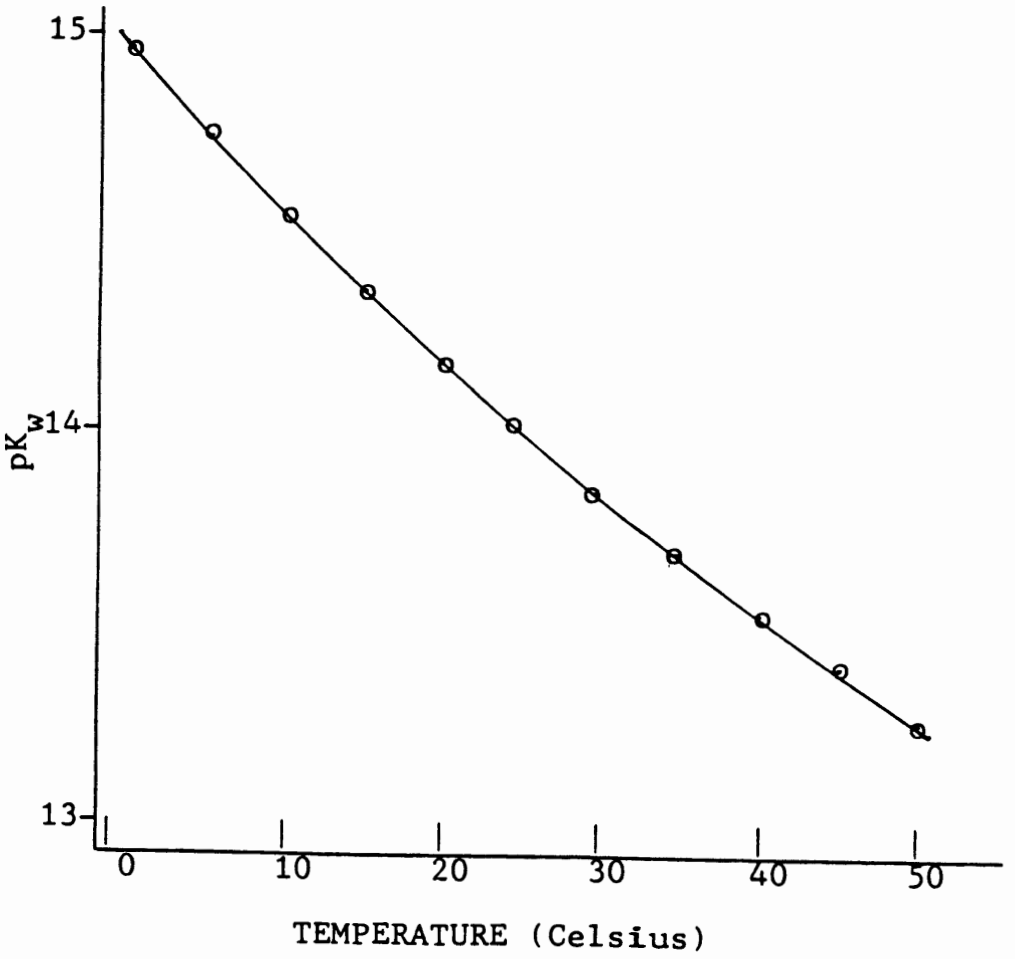


Figure 5. The Temperature Dependence of the pK_w. The solid line is the quadratic least squares fit of the equation $pK_w = 1.823 \times 10^{-4} - 4.267 \times 10^{-2} + 14.946$.

RESULTS

The equilibrium constant for the binding of hydroxide to methemerythrin from Themiste dyscritum was determined at a series of temperatures between 4.2°C and 34.5°C using Equation II-12. One other experiment was done at 38°C in which the protein precipitated in the ultraviolet beam. The difference spectra taken at 9.2°C are shown in Figures 6a-6e to illustrate the sensitivity of the measurements and also the range of their interpretation. Figure 6a shows difference spectra for five samples ranging in pH from 7.47 to 8.82. The reference cell contained an aliquot of the pH 7.47 sample. Figure 6b shows the variation of repeated measurements on the same sample. The extreme values of the samples at pH values of 7.87 and 8.82 are shown. Figure 6c is the result of two independently prepared samples both at a pH of 7.87. While Figure 6b shows the reproducibility of the instrument, Figure 6c is more the result of sample preparation.

There are theoretically an infinite number of wavelengths where measurements could be taken since α is independent of wavelength. However, the peak positions give the smallest errors ($dA/d\lambda = 0$) in measuring absorbance from chart paper, so the difference spectral peaks at 380 nm and approximately 310 nm were chosen for ΔA determinations.

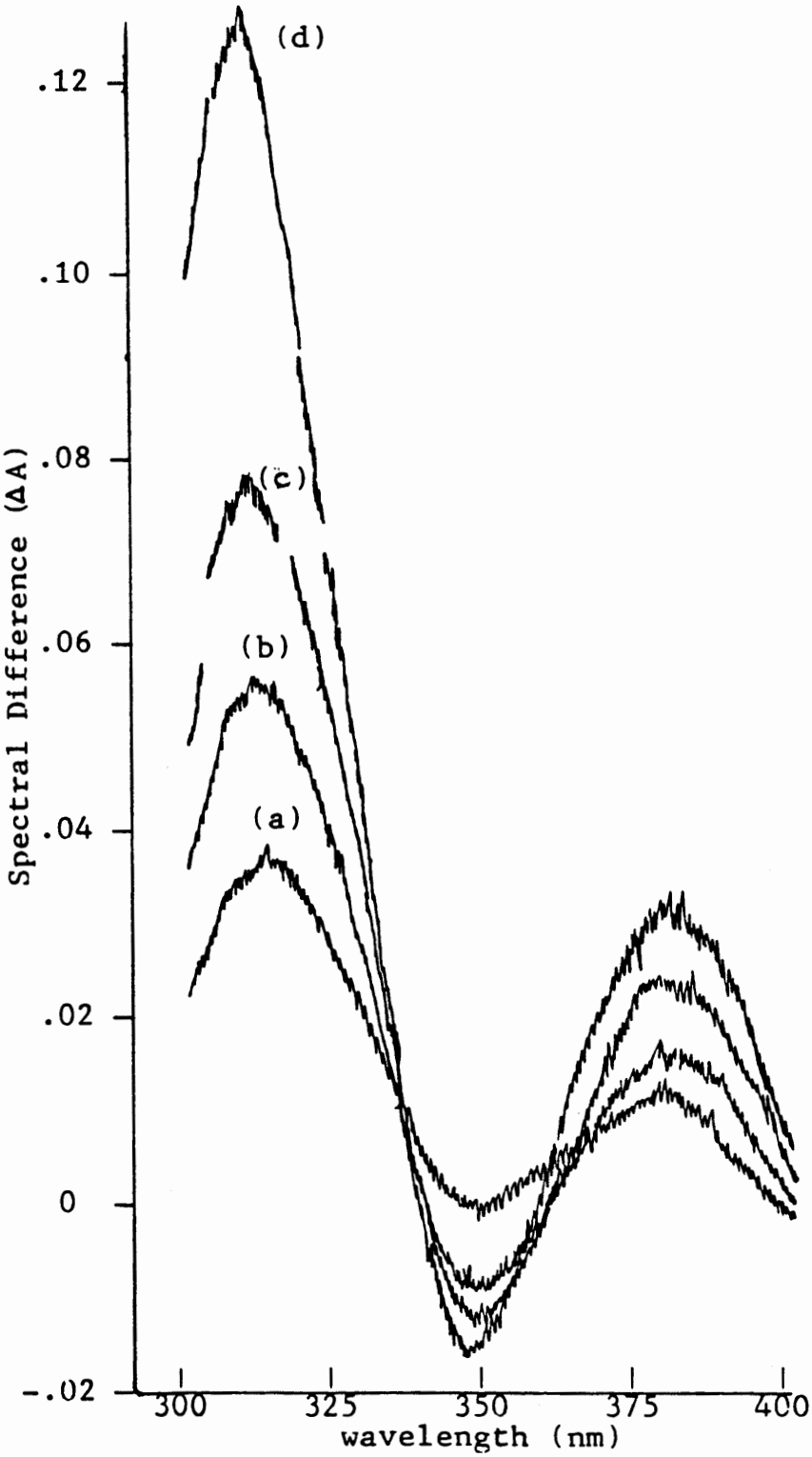


Figure 6a. Methemerythrin Difference Spectra at 9.2 C. The reference cell pH is 7.47 and the samples have pH values of (a) 7.87, (b) 8.16, (c) 8.38, (d) 8.82.

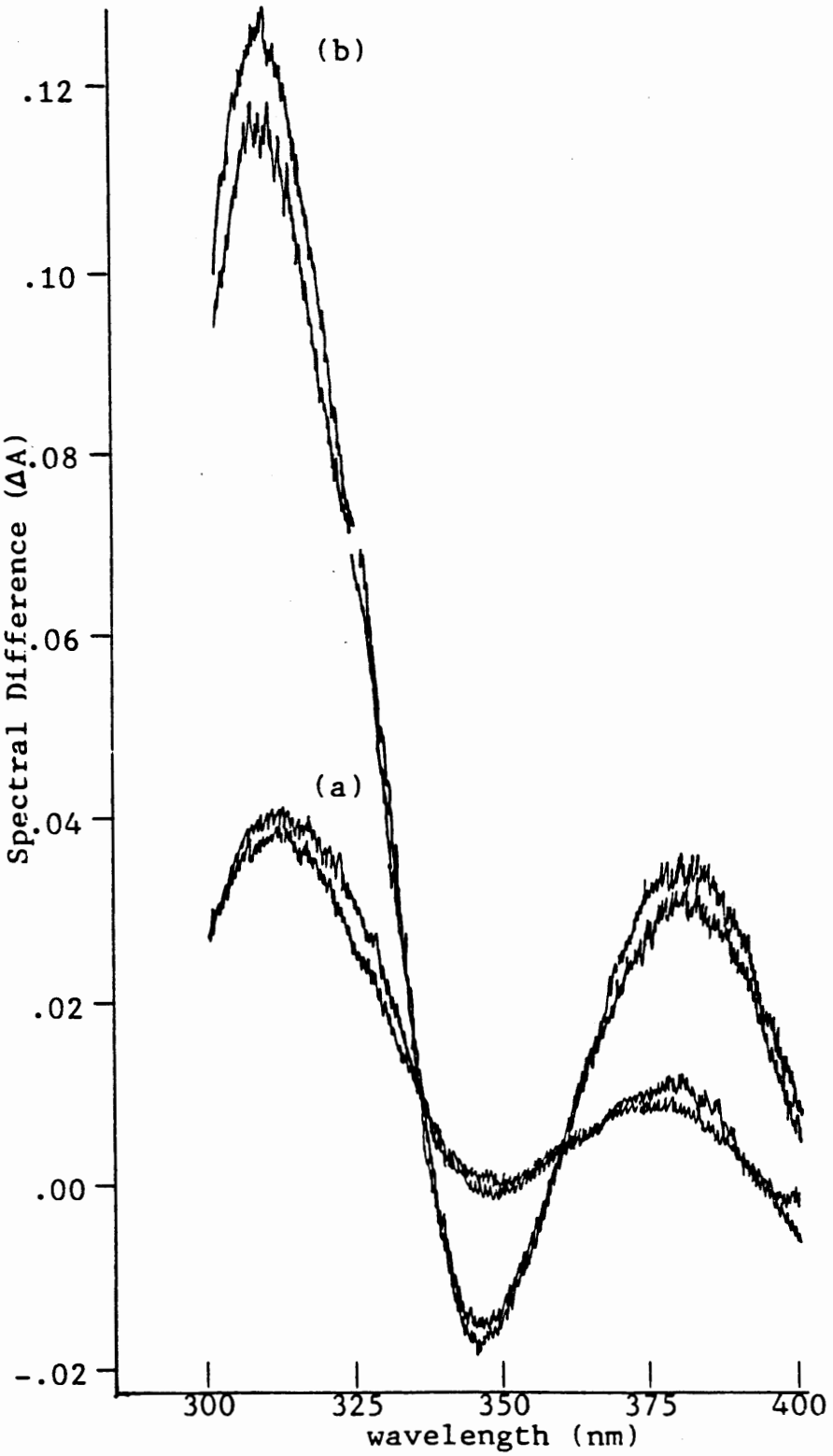


Figure 6b. Difference Spectra of Methemerythrin. Two independent measurements on the same sample at pH values of (a) 7.87 and (b) 8.82 against a reference at pH=7.47

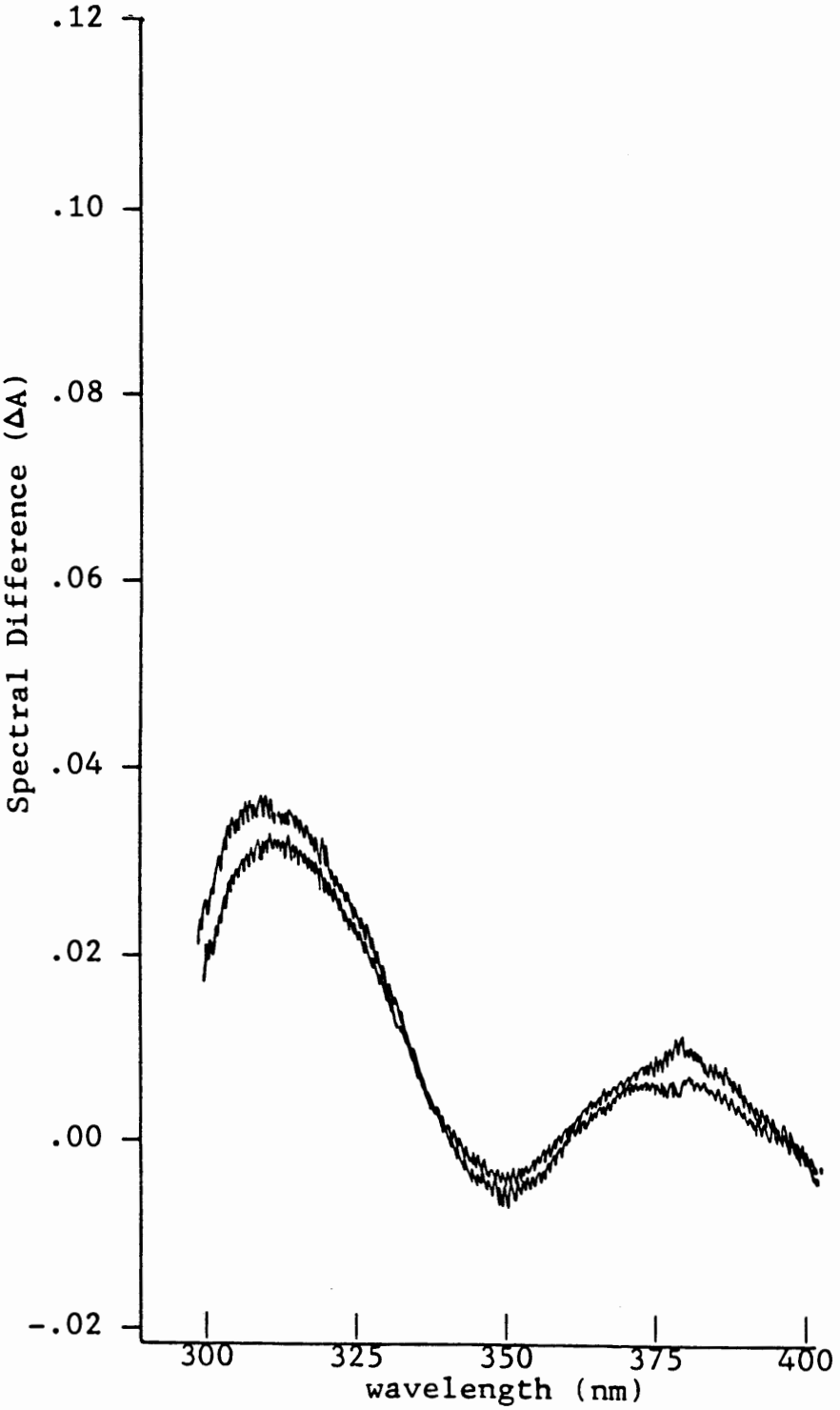


Figure 6c. Difference Spectra of Methemerythrin. The difference spectra of two independently prepared samples both at a pH of 7.87 against a single reference sample at a pH of 7.47.

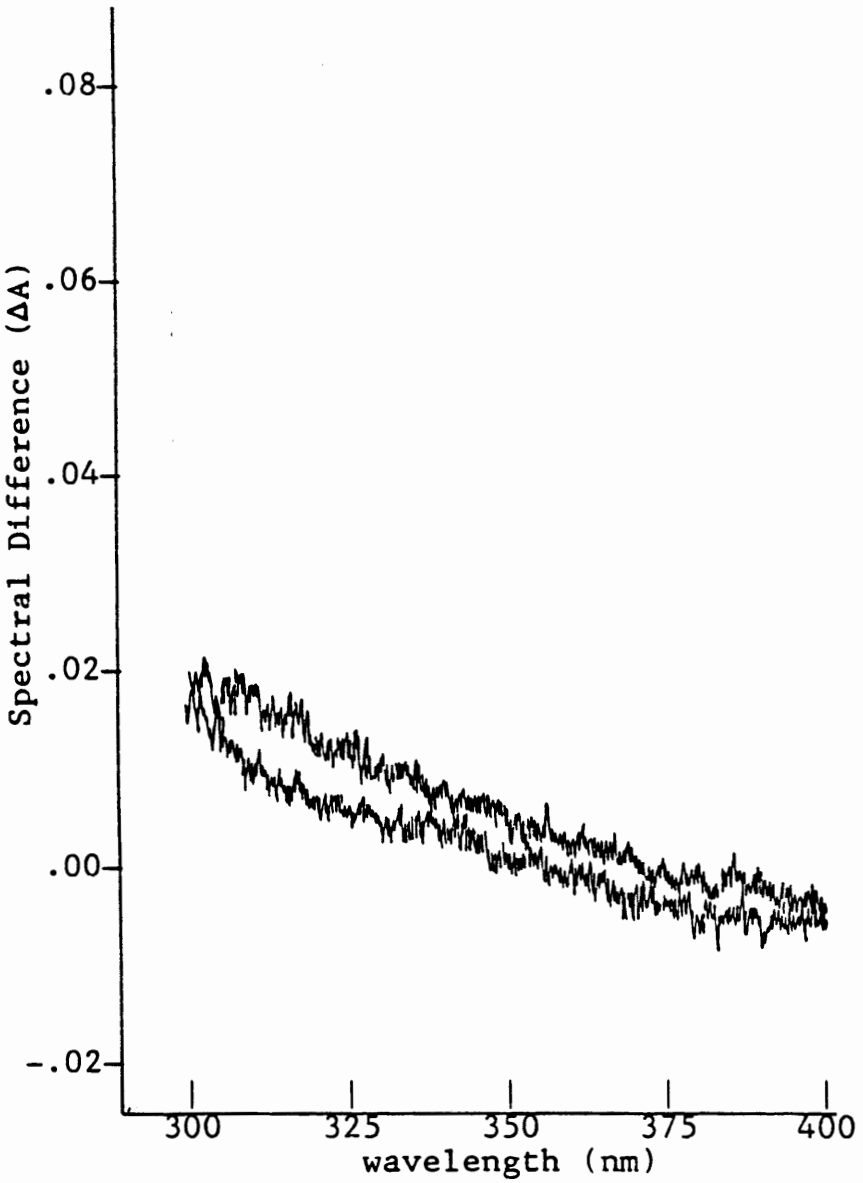


Figure 6d. Baseline Spectra of Methemerythrin. Two spectra of the same sample before and after measurement of other samples. Both sample and reference are at a pH of 7.47. This fluctuation of the baseline was common with this instrument. Although slope was rarely affected the position changed with time. This was thought to be due to electronic noise.

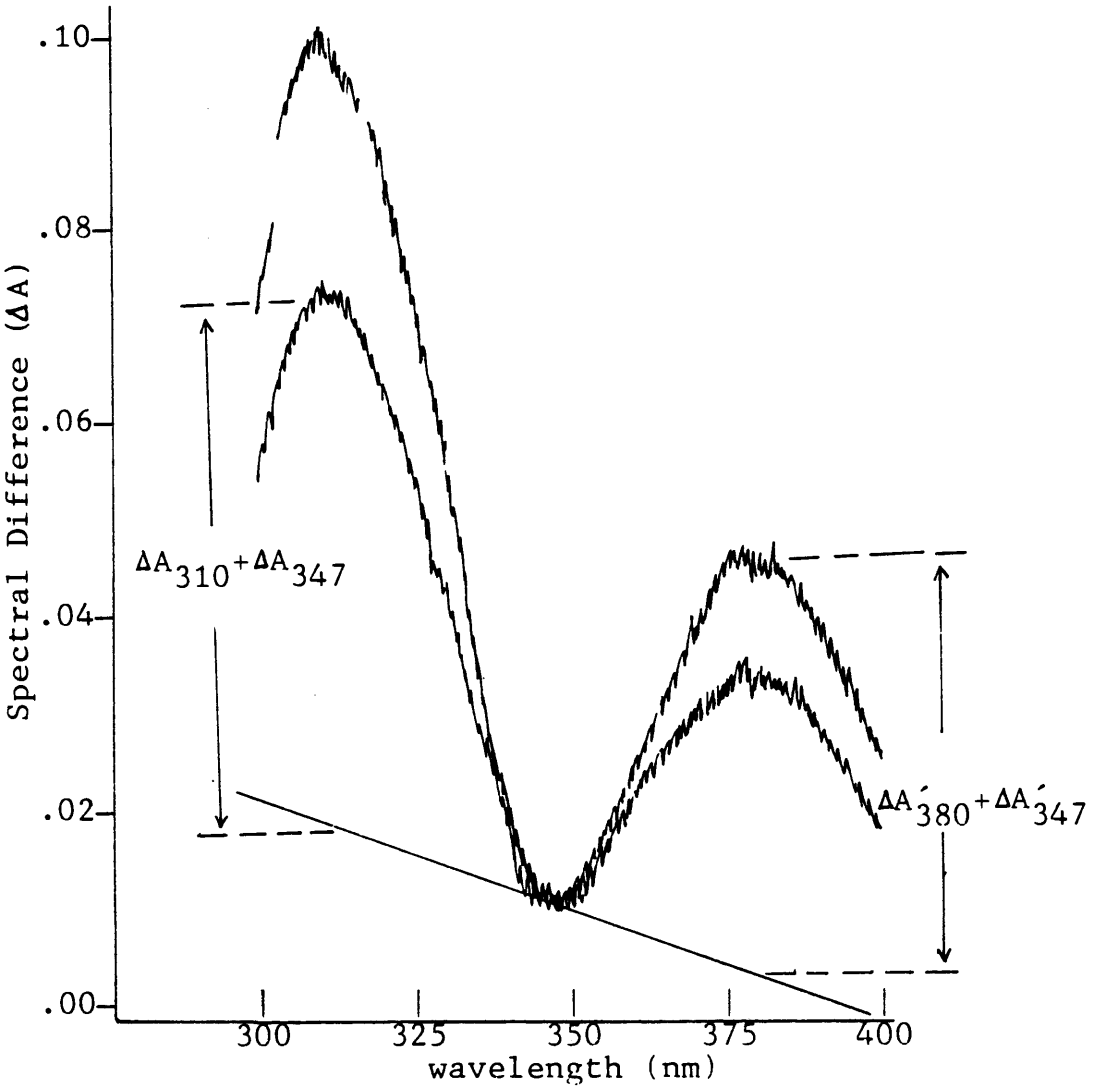


Figure 6e. The difference spectrum of Methemerythrin at 9.2°C. Samples at pH 8.38 and 8.16 against a reference sample of 7.47. Solid line shows slope of the 7.47 vs. 7.47 spectrum which was used as a zero point for the spectral minimum. Absorbances were measured from dotted line to dotted line.

The magnitude of the negative peak at 347 nm was considered too small to give meaningful α values in and of itself.

There appeared to be two isosbestic points at 336 nm and 360 nm but because of small fluctuations (<0.01 absorb.) in the baseline of the spectrophotometer (see Figure 6d) these were difficult to pinpoint precisely. As a correction to these fluctuations it was decided to choose the spectral minimum at 347 nm as an arbitrary zero point. The effect of this procedure was to add additional absorbance to each peak. The procedure for measuring absorbance values is illustrated in Figure 6e.

Due to the selection of this arbitrary zero point the absorbance values reported in Table I represent sums of spectral features. Assuming that all spectral features are due to the same equilibrium the α values calculated from these sums should not be affected by this addition since:

$$\text{if } \alpha = \frac{\Delta A_{310}}{\Delta A'_{310}} = \frac{\Delta A_{380}}{\Delta A'_{380}} = \frac{\Delta A_{347}}{\Delta A'_{347}}\ , \qquad \text{(IV-1)}$$

$$\text{then } \alpha = \frac{(\Delta A_{310} + \Delta A_{347})}{(\Delta A'_{310} + \Delta A'_{347})} = \frac{(\Delta A_{380} + \Delta A_{347})}{(\Delta A'_{380} + \Delta A'_{347})}\ . \qquad \text{(IV-2)}$$

Unless the spectral features at the different wavelengths are the result of separate equilibria, errors in α are reduced by using the larger peaks. Equilibrium constants were determined individually from the peaks at 380 nm and 310 nm, instead of averaging them, because the peak position at 310 nm was pH dependent (see Figure 7) while the peak at

TABLE I

MEASURED DIFFERENCE ABSORBANCE VALUES[a]

TEMP	REF pH	pH	310 nm[b]	380 nm[b]
4.2	7.87	8.14	.0240	.0088
		8.33	.0452	.0184
		8.52	.0672	.0280
		8.95	.1340	.0448
5.5	8.19	8.34	.0124	.0128
		8.57	.0246	.0176
		8.80	.0546	.0264
		9.10	.1044	.0368
9.2	7.47	7.87	.0316	.0168
		8.16	.0596	.0304
		8.38	.0864	.0420
		8.82	.1356	.0560
12.0	7.74	8.01	.0128	.0052
		8.25	.0276	.0120
		8.59	.0516	.0236
		9.06	.0900	.0380
16.5	7.50	7.78	.0104	.0048
		8.07	.0256	.0128
		8.34	.0452	.0228
		8.83	.0832	.0356
21.0	7.58	7.80	.0168	.0116
		7.99	.0380	.0236
		8.19	.0596	.0372
		8.58	.1108	.0620
21.4	7.62	7.81	.0296	.0112
		8.00	.0440	.0196
		8.20	.0640	.0324
		8.58	.1120	.0536
30.0	7.32	7.52	.0100	.0052
		7.67	.0220	.0128
		7.89	.0392	.0200
		8.23	.0720	.0304
34.5	7.33	7.55	.0064	.0048
		7.70	.0208	.0112
		7.96	.0420	.0184
		8.31	.0814	.0248
38.0	protein denatured in UV beam			

[a] the error in all measurements was estimated to be .005.

[b] these peaks also include a contribution from the negative peak at 347 nm.

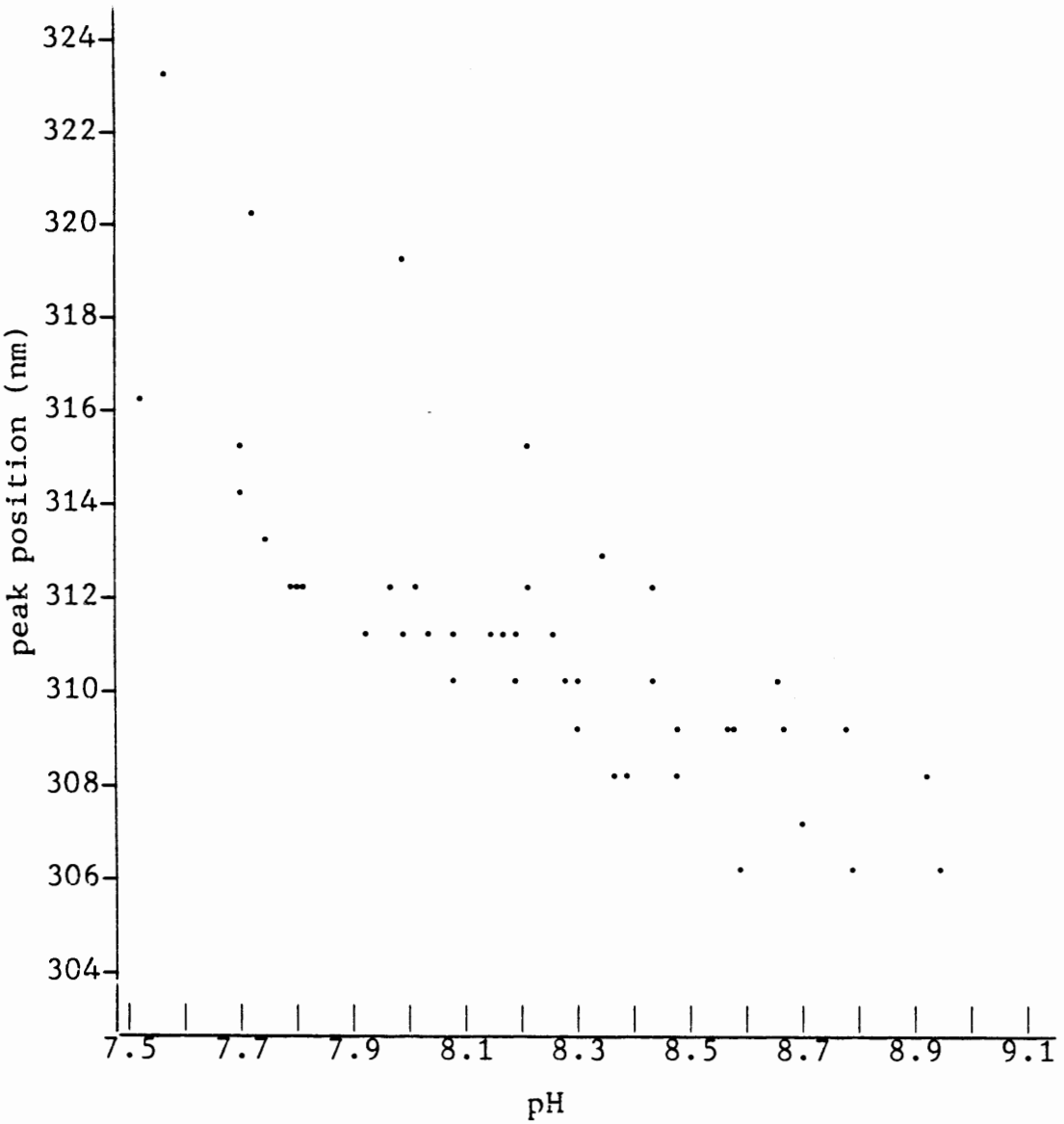


Figure 7. The Position of the c. 310 nm Difference Spectral Peak in Methemerythrin as a Function of pH.

380 nm did not appear to be. This suggested that these two peaks might be caused by different phenomena so they were treated separately to see if different equilibrium constants would be generated by treating them independently. Although for the most part the equilibrium constant derived from the 310 nm peak was within experimental error of that derived from the 380 nm peak there was an exception for the high temperature experiments where a separation into two constants was noted.

To determine the equilibrium constant at a specific temperature the ΔA of five samples, each at a different pH, were measured. This enabled the generation of ten equilibrium constants as combinations of three were used for each calculation (see Table II). Each combination can represent samples r, s, and t of Equation II-12.

As was stated in Chapter 2:Theory, the generation of more than five equilibrium constants from only five independent measurements means that these generated equilibrium constants can not be independent and will appear as a function of the combination of samples used in the calculation. The non-random patterns appearing in the set of generated equilibrium constants enable a quick and accurate estimation of the actual equilibrium constant. This is illustrated in Table II which shows the generated equilibrium constants from the measured ΔA values of the 310 nm peak at 4.2°C and in Table III which shows a few more

examples of the patterns these interrelated constants yield. Table III shows the generated equilibrium constants from the 310 nm peak and the 380 nm peak for samples at 9.2, 12.0, 16.5, 21.0, 30.0, and 34.5 degrees.

TABLE II

THE CALCULATED pK VALUES AT 4.2°C FROM THE MEASURED ABSORBANCES AT 310 NM

Sample #s	Calculated pK
1 2 3	6.32
1 2 4	6.42
1 2 5	6.23
1 3 4	6.51
1 3 5	6.21
1 4 5	6.07
2 3 4	6.61
2 3 5	6.19
2 4 5	5.99
3 4 5	5.79
Sample 1	pH=7.75
Sample 2	pH=8.01
Sample 3	pH=8.19
Sample 4	pH=8.38
Sample 5	pH=8.78

One can see from Table II the consistency of the pK values generated from combinations which do not include sample 4. Further analysis shows that whenever sample 4 was used as the lower pH sample (contributing to ΔA) the pK was lower than the mean and that when sample 4 was used as the higher pH sample (contributing to $\Delta A'$) the pK was higher than the mean. This trend throughout the generated pK values indicates that the error in the measurement of the peak height of sample 4 was greater than the errors in the other peak heights and that the true value was actually higher

TABLE III

CALCULATED EQUILIBRIUM CONSTANTS AT VARIOUS TEMPERATURES FROM THE VALUES IN TABLE I[a]

Calculated from the peak at 310 nm

Sample #'s	9.2°C	12.0°C	16.5°C	21.0°C	30.0°C	34.5°C
1 2 3	6.50	6.03	5.99	5.36	0.*	0.*
1 2 4	6.38	6.01	5.89	5.77	5.51	0.*
1 2 5	6.31	5.91	5.85	5.76	5.61	3.75
1 3 4	6.23	6.00	5.79	6.01	5.89	5.51
1 3 5	6.22	5.88	5.80	5.84	5.77	5.40
1 4 5	6.22	5.78	5.81	5.76	5.69	5.33
2 3 4	6.06	5.99	5.73	6.20	6.18	6.28
2 3 5	6.14	5.85	5.77	5.91	5.90	5.80
2 4 5	6.18	5.74	5.79	5.75	5.72	5.47
3 4 5	6.22	5.68	5.81	5.61	5.60	5.26
mean:	6.25	5.89	5.82	5.80	5.76	5.35
std. dev:	.13	.13	.06	.22	.22	.81

Calculated from the peak at 380 nm

Sample #'s	9.2°C	12.0°C	16.5°C	21.0°C	30.0°C	34.5°C
1 2 3	6.61	5.76	5.69	5.98	0.*	0.*
1 2 4	6.52	5.84	5.73	5.99	5.57	5.50
1 2 5	6.54	5.88	5.91	5.98	5.86	5.81
1 3 4	6.39	5.89	5.77	5.99	6.36	6.14
1 3 5	6.50	5.91	5.97	5.98	6.23	6.18
1 4 5	6.58	5.92	6.09	5.98	6.14	6.23
2 3 4	6.25	5.92	5.79	5.99	0.*	7.44
2 3 5	6.46	5.93	6.00	5.98	6.68	6.83
2 4 5	6.61	5.93	6.14	5.97	6.26	6.50
3 4 5	6.87	5.94	6.28	5.97	6.00	6.32
mean:	6.53	5.89	5.94	5.98	6.14	6.33
std. dev.	.16	.06	.19	.01	.38	.60

* if the selected pH values are not well spaced about the pK small errors in α can results in negative equilibrium constants. The program in Appendix B sets all negative equilibrium constants to zero and drops them from the calculation of the mean value and deviation.

[a] The equilibrium constants in this table are expressed as the logarithm.

than was measured.

The data in Table II was calculated before the pH corrections were made and so is not actually used in this thesis but it was this set of data which showed a wide scatter of equilibrium constants and led to the realization that these generated equilibrium constants were not independent. In this specific example inspection of the spectrum of sample 4 showed that the peak occurred during an instrumental scale change and that a poor reading of the absorbance had been made. An adjustment of 0.004 units of absorbance was made to this sample before calculating the equilibrium constant with the corrected pH values.

The procedure used to calculate the best estimate of the equilibrium constant at each temperature was to use the estimated peak heights and measured pH values to generate an initial set of pK values, like those in Table II and Table III, and then to search those values for the one measurement that had the greatest error, relative to the others, and adjust its value, within a given estimate of the noise, until the variance of the entire set was minimized. Then, using the noise level as the adjustment of the previous sample, this procedure was repeated for other samples. Appendix B lists a FORTRAN program to execute this iterative process. Table IV shows the calculated equilibrium constants (expressed as the logarithm) determined by this method as a function of temperature and Figure 8 shows this data

graphically as $-\log K_{eq}$ as a function of the reciprocal temperature. This is the familiar van't Hoff plot. The van't Hoff equation reads: $-\ln K_{eq} = \Delta H/RT - \Delta S/R$, so with the appropriate constants (natural logarithm of 10 and R, the gas constant) the slope will yield the enthalpy change, ΔH, and the intercept will yield the entropy change, ΔS.

The van't Hoff plots shown in Figures 8 and 9 have used the equilibrium constants derived from data at 310 nm. There was a marked deviation from linearity at the higher temperatures when the 380 nm data was included. The separation of the data into two apparent equilibrium constants at high temperature is not explained but in view of the fact that at temperatures above 38°C the protein precipitated this phenomenon could be the result of partial thermal degradation. For this reason the 380 nm data was not used in further calculations.

The splitting of the derived equilibrium constants into two values at high temperatures suggests that there is a possibility that n in Equation II-12 is greater than one. Although n can not be calculated directly from this equation when the values of two and three were used to calculate the equilibrium constant the equation failed to give meaningful numbers for the equilibrium constants. [An interesting note on this equation is that small errors in α produce negative numbers for the equilibrium constants. When n was assumed to be 2 and 3 all equilibrium constants generated were less

than zero. It is on this basis that the value of n is determined to be unity and the 380 nm peak is probably more affected by the degradation of the protein than the transition at 310 nm.

SUMMARY OF VAN'T HOFF EQUATION

Depending on the way the equilibrium is expressed, as an acid dissociation or a hydroxide binding, the thermodynamic parameters obtained from the van't Hoff equation are different. Since the exact structure of the active site of aquomethemerythrin is still unknown both possiblities are presented here. From Figure 8 which treats the equilibrium as a hydroxide binding the slope is 2190 and the intercept is -1.68 with a linear regression coefficient, $r^2 = 0.92$. Multiplying the slope and intercept by Rln10 gives values of 10.01 kcal/mole and 7.68 cal/mole-K for the enthalpy change and entropy change, respectively. Assuming an error of 0.10 in the pK_f values the error in the enthalpy change is ±1.45 kcal/mole and the error in the entropy change is ±2.41 cal/mole-K from the standard error of the intercept of the van't Hoff plot. The error in the entropy measurement is probably higher than reported here since the standard error in the slope of the van't Hoff plot would only give an error of 0.70 kcal/mole, which is better than I have estimated that this method can do (see following section).

From Figure 9 the values of ΔH and ΔS when treated as an acid dissociation are 3.88 kcal/mole and -25.13 cal/mole-K.

TABLE IV

BEST ESTIMATE EQUILIBRIUM CONSTANTS
(expressed as log)

TEMP (C)	1/T(Kelvins) ($x10^6$)	log K_f (310 nm peak)	log K_f (380 nm peak)
4.2	3606	6.15	6.30
5.5	3589	[5.59][a]	[6.34]
9.2	3542	6.23	6.57
12.0	3507	5.94	5.89
15.2	3468	[5.95]	[5.74]
16.5	3452	5.80	5.81
21.0	3400	5.75	5.98
21.4	3395	[5.88]	[5.93]
30.0	3299	5.61	6.26
34.5	3250	5.39	6.18
38.0			

[a]bracketed numbers were not used in the van't Hoff plots because of large standard deviations or insufficent data

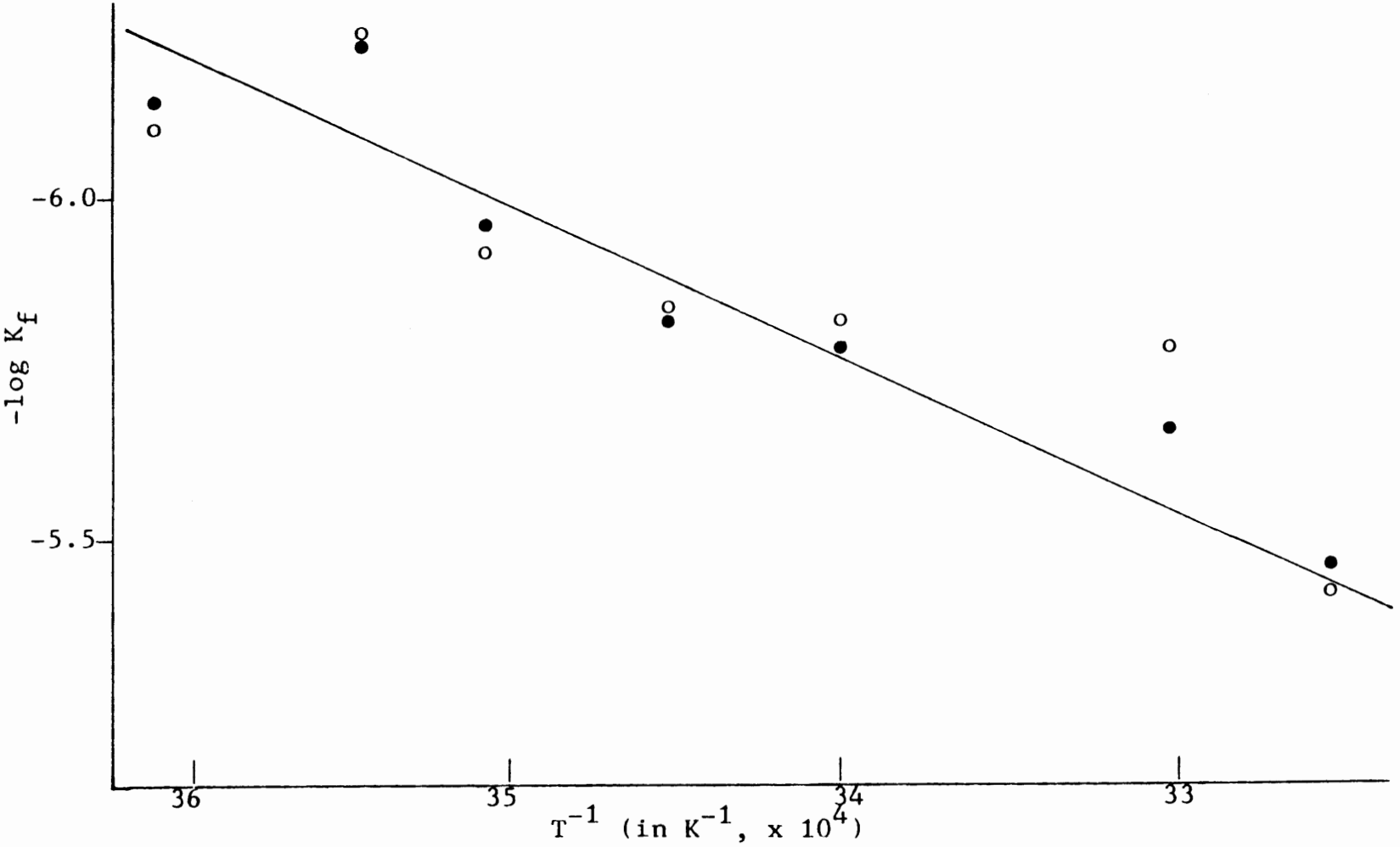


Figure 8. van't Hoff Plot of the Equilibrium Constant of Methemerythrin. Data is from Table IV, column 3: the log K derived from the difference peak at 310 nm. Dark circles are the best estimate values, open circles are the mean values. Solid line is the least squares linear fit of the data.

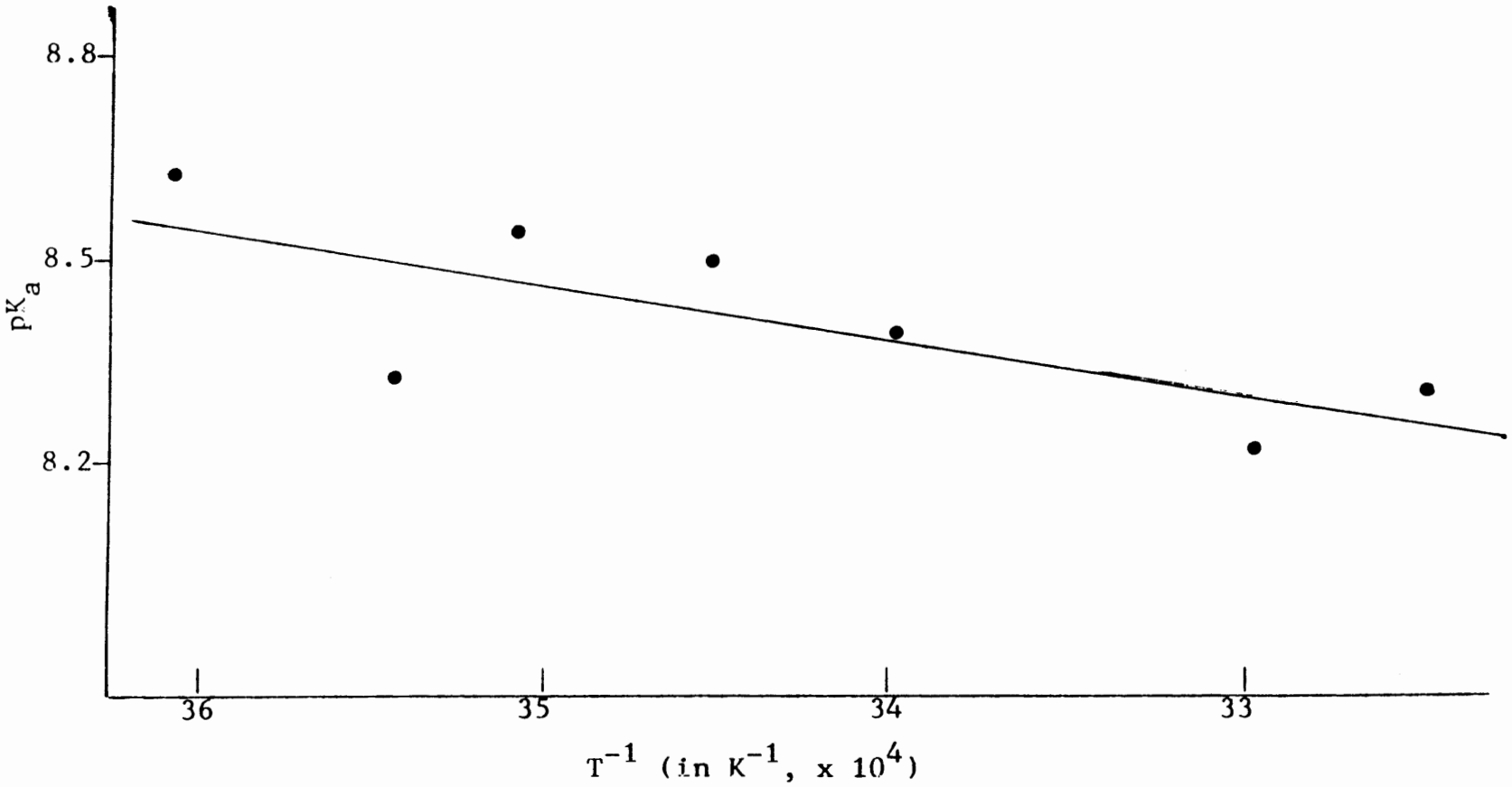


Figure 9. Van't Hoff Plot of the Equilibrium Constant of Methemerythrin written as an acid dissociation reaction. Dark circles are best estimate values. The solid line is the linear least squares fit.

with approximately the same error limits as the values generated from the assumption that the reaction was a hydroxide binding. The square of the linear regression coefficient of the van't Hoff plot for the treatment as an acid dissociation is only 0.62 but this is due to the deviant point at 9.2°C affecting the flatter curve to a greater extent. When the data from 9.2°C is excluded from the calculation the square of the linear regression coefficients increase to 0.97 and 0.91.

The free energy change, ΔG, can be determined at any temperature by the equation $\Delta G = \Delta H - T\Delta S$. At 25°C the value is 7.72 kcal/mole when treated as a hydroxide binding and 11.37 kcal/mole when treated as an acid dissociation. These two values are related by the free energy change for the dissociation of water.

ANALYSIS OF ERRORS

The question arises as to how reliable the equilibrium constants calculated by this method are. The random errors are found in the ΔA values and the logarithms of the hydroxide ion activities. Because there is not a normal distribution about the equilibrium constants and because the average equilibrium constants are calculated from a non-independent set of equilibrium constants their standard deviations do not accurately reflect the error involved in the measurement.

To calculate the probable reliability in the equilib-

rium constant due to random sources of error we can estimate the errors of the various constituents by their standard deviations and see how those errors propagate in the calculation. We can estimate the errors in pH and ΔA measurements by the standard deviation of repeated samples. The reproducibilty of pH measurement was found to be ±.02 when a single sample was measured several times with temperature changes and reequilibration between measurements (see Appendix A).

The contribution of error to ΔA comes from two sources. First the reproducibility of sample preparation and second the reproducibility of spectral measurements. Sample preparation involved the mixing of two unequal volumes, 3.0 ml of protein solution and 0.3 ml of buffer solution (±.003 and ±.001, respectively; see Appendix A). The relative error in the final concentration using these values is:

$$\frac{E_p V_l + V_p E_l}{V_p(V_p + V_l + E_l - E_p)} = .0004 \qquad (II\text{-}15)$$

Since the concentration error contribute to error in A, the total absorbance, and not ΔA, the concentration error is magnified by the ratio of A to ΔA. For these experiments ΔA lies between about .02 and .10 while A is between 1.0 and 2.0. The error in ΔA is the relative error in concentration (.0004) times A/ΔA or between .02 and .05. The peak at 310 nm having the smaller errors due to larger ΔA values.

The reproducibilty of the spectrophotometer was not

rigorously calculated but was estimated by the range of several measurements to be about 5%. This adds to the error in ΔA due to concentration differences to give a range of relative errors between .055 for the typical peak at 310 nm and .069 for the average size peak at 380 nm. Since α is a function two ΔA values the relative errors in α range from .078 to .098.

Combining the probable errors in α with the pH measurement error of .02 (.02 pH units is about a 5% change in hydroxide ion activity) the estimated errors in the equilibrium constants range from 24% to 28% or errors in the pK values of .09 to .11. This is the best that can be expected from this technique and these are only the random errors.

Note: All calculations in this section were based on relative errors. For any multiplicative function, f, of several variables, x, y, z, ... the relative error, e, will be given by:

$$e^2(f) = e^2(x) + e^2(y) + e^2(z) + \ldots$$

For additive functions e represents absolute error and not relative error, but by assuming that the variables are approximately the same magnitude and dividing by the square of the function value we find that the relative error is given by the same expression as in multiplicative functions if we divide by the number of measurements being summed. This approximation was used here because it simplified the calculations due to the lack of any constants in the equation.

DISCUSSION

The most obvious result of these experiments is the magnitude of the pK for the aquomet/hydroxymet equilibrium for the protein from Themiste dyscritum. Values have been reported for protein from Phascolopsis gouldii ranging from 7.33 (Gorman and Darnall, 1981) to 7.83 (Garbett, et al, 1971). These values are acid dissociation values, whereas this thesis treats the equilibrium as a hydroxide binding. To avoid confusion the notation, pK_a and pK_f will be used to designate acid dissociation and hydroxide binding, respectively.

At room temperature the pK_a for T. dyscritum hemerythrin is 8.4 (see Figure 9) which is much larger than the value seen in P. gouldii. The major significance of this value now is in its assistance in interpreting previous work. When the crystal structure was done on T. dyscritum hemerythrin (Stenkamp, et al, 1976) the crystals were believed to have been aquomethemerythrin because the pH of the mother liquor was 6.5 (Loehr, personal communication) but later the assignment was changed to hydroxymethemerythrin on the basis of the absorption spectrum of the mother liquor which, having two maxima in the 300-400 nm region, appeared more like hydroxymet than aquomet. The existence of hydroxymet in a solution of pH 6.5 seemed to imply that the pK_a was

much lower for the Themiste dyscritum protein than that from Phascolopsis gouldii, but Themiste hemerythrin was observed to undergo a color change and drastic solubility change at pH values closer to 8. Since the different anion binding forms were initially described by their spectral properties (Garbett, et al, 1969) I wanted to spectrally define the equilibrium constant for this transition to aid in the interpretation of the crystallographic data.

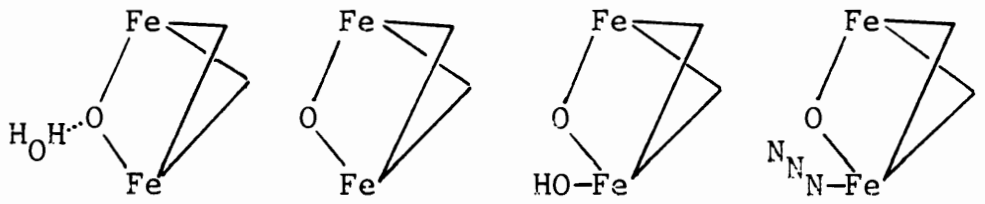
Single crystal visible spectroscopy has also been done on crystals from the batch used in the diffraction studies (Marvin Mackinen, personal communication) and it was found that no significant spectral changes occurred on raising the pH of the medium from 5.0 to 8.7. Above a pH of 8.7 the crystals dissolved. From this data and the knowledge that the pK_a is 8.4 it appears that the crystals were purely aquomethemerythrin and that raising the pH did not produce hydroxymet until the crystals began to dissolve. This is consistent with the observation of increased solubility of hydroxymet.

It is difficult to obtain much useful information regarding the structures and mechanism of conversion of methemerythrin from the temperature dependence of the equilibrium constant. When the reaction is written as an acid dissociation the enthalpy change is calculated to be 3.88 kcal/mole and when written as a hydroxide binding the value is calculated to be -10.01 kcal/mole, but without additional

corresponding kinetic data, the thermodynamics are meaningless in trying to deduce which mechanism is correct.

When aquomethemerthrin crystals were exposed to perchlorate two things were observed by the crystallographers (Stenkamp, et al, 1978). There is a binding of perchlorate 15 Å away from the active site on the surface of the molecule and there is also a loss of electron density near the μ-oxo bridge. It is possible that there is a water molecule at this site and that addition of perchlorate somehow induces its loss. Preliminary low resolution difference maps indicate that hydroxymethemerythrin has also lost electron density at this site, though bound hydroxide has not been observed (Stenkamp, personal communication).

If we propose that this is indeed a water molecule and that it is involved in the conversion reaction of aquomethemerythrin to hydroxymethemerythrin then we can propose the following structures seen in Figure 10.



met(aquo) met(ClO_4^-) met(OH^-) met(N_3^-)

Figure 10. Proposed Structure of Four Methemrythrins.

These structures are proposed from the crystallographic work

of Stenkamp, et al, (1976, 1978, 1981). Knowing the pK for the aquomet to hydroxymet conversion has given a clearer picture. Although a hydroxide ion has not been seen in the early results of the X-ray diffraction analysis of hydroxymethemerythrin there is evidence for its existence. The inclusion of a bound hydroxide at the active site was based on the resonance Raman spectrum reported by Freier, et al, (1981). There is, in addition to the 508 cm^{-1} enhanced vibration of the μ-oxo bridge another vibration at 492 cm^{-1} in hydroxymethemerythrin. This vibration is not seen in any other form of the protein, and is most likely the Fe-OH vibration.

These structures are supported by the EXAFS spectrum of the two methemerythrins. Elam, (personal communication) showed that although there was no discernable difference in the X-ray absorption adge of aquomet and hydroxymet the fine structure showed a great difference in their Fourier transforms. While crystallography supports a vacant site and a five coordinate iron atom in aquomet the EXAFS points to uniform octahedral fields around the iron atoms but vast structural differences between their forms.

How do these results relate to the physiological forms? Evidence for a five coordinate iron atom in aquomethemerythrin immediately draws analogy to the five coordinate iron in deoxymethemoglobin, another oxygen carrier. Although a five coordinate ferric ion is unusual the aquomethemeryth-

rin structure might be similar to that of deoxyhemerythrin which is in the ferrous state. There are two known classes of oxygen carriers. The synthetic carriers, such as Vaska's complex, are low valent d^8 ions which are square planar and bind oxygen in an axial position (Vaska and DiLuzio, 1962). The other class contains the iron porphyrins and cobalt in a variety of ring systems. These carriers all have four ring ligands and an axial base which regulates oxygen affinity. On binding oxygen there is a formal charge transfer from the metal to the oxygen molecule which binds at the base of the square pyramid resulting in an octahedral iron(III) or cobalt(III) complex (Lapidot and Irving, 1974). Hemerythrin could bind oxygen in a very similar manner with the μ-oxo bridge and other iron atom constituting the regulatory base. Aquomethemerythrin could be an oxidized form of deoxy with the active site geometry fairly well preserved. The rapid binding of anions to aquomethemerythrin to produce the other forms of methemerythrin would yield the more stable six coordinate ferric ion.

A structural model for deoxyhemerythrin and oxyhemerythrin has come out of a recent EXAFS study (Elam, et al, 1982b) and there is evidence that one iron atom in deoxyhemerythrin is five coordinate, while oxyhemerythrin has two six coordinate iron atoms. X-ray diffraction results also show that oxyhemerythrin is structurally the same as the azidomethemerythrin with an oxygen molecule

situated at the azide binding site (Ron Stenkamp, personal communication). The proposed structures for the physiological forms of hemerythrin are given in Figure 11.

deoxyhemerythrin oxyhemerythrin

Figure 11. Proposed Structure of Oxy and Deoxy Hemerythrin.

The majority of the work done on hemerythrin has been done on protein from P. gouldii but all the crystallographic work has been done on protein from Themiste. It is assumed that the active sites are the same across specie lines. Although it has been shown in this thesis that the pK values of the aquo to hydroxy methemerythrin conversion is quite a bit different as are the absolute spectra Table V compares the thermodynamic parameters which are quite similar.

TABLE V

THERMODYNAMICS OF TRANSITION BETWEEN AQUO AND HYDROXY METHEMERYTHRIN

Species	ΔG^a	ΔH	ΔS
	kcal/mol	kcal/mol	cal/mol-K
T. dyscritum	11.4	3.88	-25.1
P. gouldii[b]	10.2	1.79	-28.3

a at 25 C
b from Gorman and Darnall (1981)

CONCLUSIONS

There is a difficulty in drawing conclusions to any scientific endeavor for nature has a way of answering our questions with more questions. What has been done in this thesis has added little to the understanding of hemerythrin in particular and iron proteins in general but it is hoped that the methods developed here will find uses elsewhere. The basic idea of determining equilibrium constants without knowledge of concentrations could be extended to other analytically methods than just electronic spectroscopy. The idea of not needing a pure substance as a reference could extend the range of possible measurements up to in vivo studies.

As a conclusion to this thesis I would like to add that Equations II-7, II-9, and II-10 can be combined into the linear function:

$$\frac{[L]_r - [L]_s}{\Delta A_s} = \frac{K_{eq}}{C\Delta\epsilon}[L]_s + \frac{1}{C\Delta\epsilon} \qquad (VI-1)$$

which when the left hand function is plotted as a function of ligand concentration in the sample gives the equilibrium constant such that: K_{eq} = slope/intercept. This function gives similar results to the iterative smoothing process in this thesis but is much more convenient to use. A FORTRAN program for its use is appended to Appendix B.

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APPENDIX A

DERIVATION OF EQUATION II-12

The expression for the equilibrium constant (Equation II-12) can be derived from Equations II-8, II-9, II-10, and II-11 by the method of Gauss. Most solutions for K_{eq} using this substitution technique yield a quadratic with two real roots. Although it can be shown that one of the roots is never valid the following solution for K_{eq} does not involve a quadratic equation.

To make these equations less cumbersome the symbols $[P]_r$, $[P]_s$, $[P]_t$, $[L]_r^n$, $[L]_s^n$, and $[L]_t^n$ will be replaced by r,s,t,i,j, and 1, respectively; K_{eq} will be denoted by k; $[PL]_r$, $[PL]_s$, and $[PL]_t$ will be replaced by C-r, C-s, and C-t, where C signifies the total protein concentration; γ_L and γ_{PL}/γ_P will be assumed to be unity; the use of these new symbols transforms Equations II-8, II-9, II-10, and II-11 to:

$$\alpha = (s - r)/(t - r) \qquad \text{(A-1)}$$

$$k = (C - s)/sj \qquad \text{(A-2)}$$

$$k = (C - t)/tl \qquad \text{(A-3)}$$

$$k = (C - r)/ri. \qquad \text{(A-4)}$$

Equations A-2, A-3, and A-4 can be rearranged as explicit functions of free protein concentration as follows:

$$r = C/(ki + 1) \qquad \text{(A-5)}$$

$$s = C/(kj + 1) \qquad \text{(A-6)}$$

$$t = C/(kl + 1). \qquad \text{(A-7)}$$

Isolation of s from Equation A-1 gives:

$$s = \alpha t - \alpha r + r \qquad \text{(A-8)}$$

and substituting into Equation A-6 gives:

$$C/(kj + 1) = \alpha t - \alpha r + r. \qquad \text{(A-9)}$$

Isolating r in Equation A-9 gives:

$$r = [C/(kj + 1) - \alpha t]/[1 - \alpha] \qquad \text{(A-10)}$$

and substituting the value of r from Equation A-7 and the value of t from Equation A-6 eliminates all the unknown free protein concentrations yielding:

$$C/(ki + 1) = [C/(kj + 1) - C/(kl + 1)]/[1 - \alpha]. \qquad \text{(A-11)}$$

Multiplication by $1 - \alpha$ and division by C yields:

$$(1 - \alpha)/(ki + 1) = [1/(kj + 1) - \alpha/(kl + 1)]. \qquad \text{(A-12)}$$

A combination of terms with subsequent multiplication of cross terms and factoring out k gives:

$$k(il - \alpha ji - jl + \alpha jl) = (j - \alpha l + \alpha i - i) \qquad \text{(A-13)}$$

Substituting the old labels back in and dividing by the parenthetical term on the left side of the equation gives Equation II-12:

$$K_{eq} = \frac{\alpha[L]_r^n - \alpha[L]_t^n + [L]_s^n - [L]_r^n}{[L]_r^n[L]_t^n + \alpha[L]_s^n[L]_t^n - [L]_s^n[L]_t^n - \alpha[L]_s^n[L]_r^n}.$$

ERROR IN SYRINGE BURET

Addition of 3.00 ml aliquots		Addition of 0.30 ml aliquots	
weight	increment	weight	increment
27.6657		25.3420	
30.6006	2.9349	25.6359	0.2939
33.5657	2.9651	25.9296	0.2937
36.5300	2.9643	26.2230	0.2934
39.4849	2.9549	26.5187	0.2957
42.4429	2.9580	26.8137	0.2950
45.4107	2.9678	27.1079	0.2942
48.3718	2.9611	27.4017	0.2938
51.3379	2.9661	27.6947	0.2930
54.2950	2.9571	27.9905	0.2958
57.2536	2.9586	28.2844	0.2939
60.2139	2.9603	28.5801	0.2957
	$\bar{x}$ = 2.9613		$\bar{x}$ = 0.2944
	s = .0043		s = 0.0010
3.00 ml ± 0.003		0.30 ml ± .001	

Error limits are $t_{.95}\ s/\sqrt{n}$. Statistical calculations were done on a Hewlett-Packard programmable 33-E calculator.

ERROR IN pH MEASUREMENT

Temp	pH	pH	
21	7.56	8.19	
12	7.74	8.38	
21	7.58	8.18	7.588±0.020
12	7.68	8.37	
21	7.62	8.20	7.723±0.020
12	7.71	8.35	
21	7.61	8.23	8.377±0.014
12	7.75	8.36	
21	7.56	8.17	8.181±0.018
12	7.72	8.39	
21	7.57	8.20	
12	7.77	8.42	
21	7.55	8.16	
12	7.72	8.36	
21	7.61	8.18	
12	7.74	8.37	
21	7.63	8.16	
12	7.69	8.38	
21	7.59	8.14	
12	7.71	8.39	

APPENDIX B

```
0010 DIMENSION ALPH(20)
0020 DIMENSION PK(20)
0030 DIMENSION PH(6)
0040 DIMENSION HT(5)
0044 DIMENSION ZHT(5)
0045 DIMENSION THT(5)
0046 DIMENSION TPH(6)
0047 DIMENSION STD(5)
0050 5 WRITE(6,21)
0060 21 FORMAT('TITLE: BEGINNING WITH TEMPERATURE')
0080 READ(5,11)TEMP,T1,T2,T3,T4
0085 IF(TEMP.EQ.0)GO TO 102
0090 11 FORMAT(F4.1,4A4)
0100 WRITE(6,32)                                          *0100
0101 READ(5,31)N                                          *0101
0102 31 FORMAT(I1)                                        *0102 N=5
0103 WRITE(6,23)                                          *0104
0104 32 FORMAT('HOW MANY SAMPLES WERE RUN?')              *0190
0110 23 FORMAT('LIST PH VALUES IN UNCREASING ORDER')      *0200 A=15.0
0120 READ(5,13)(PH(I),I=1,N)                              *0210
0130 13 FORMAT(6F5.2)                                     *0220
0140 WRITE(6,24)                                          *0225
0150 24 FORMAT('WHAT WERE THE MEASURED PEAK HEIGHTS?')    *0227 T=0.03
0160 READ(5,14)(HT(I),I=1,N-1)                            *0228
0170 14 FORMAT(5F5.1)                                     *0229
0180 FLAG=0
0190 WRITE(6,27)
0200 27 FORMAT('WHAT IS THE NOISE LEVEL?')
0210 READ(5,16)A
0220 16 FORMAT(F4.1)
0225 WRITE(6,34)
0227 19 FORMAT(F4.2)
0228 34 FORMAT('WHAT IS THE ERROR TOLERANCE?')
0229 READ(5,19)T
0230 FHC=A
0231 JM=7
0232 44 TA=FHC/1.2
0233 TS=1.0
0235 DO 440 I=1,N-1
0236 ZHT(I)=HT(I)
0237 440 CONTINUE
0238 INDEX=0
0239 HC=0.0
0240 TSTD=1.0
0241 55 DO 330 L=1,N-1
0242 THT(L)=ZHT(L)
0243 330 CONTINUE
0250 L=1
0260 DO 100 M=1,N-2
0270 DO 10 J=M,N-2
0280 DO 20 I=J,N-2
0290 ALPH(L)=THT(J)/THT(I+1)
0300 L=L+1
0310 20 CONTINUE
0320 10 CONTINUE
0330 DO 30 K=M+1,N-1
0340 THT(K)=THT(K)-THT(M)
0350 30 CONTINUE
0360 100 CONTINUE
0365 AKW=0.0001823*TEMP**2.0-0.04267*TEMP+14.946
0370 DO 40 I=1,N
0380 TPH(I)=10**(PH(I)-AKW)
0390 40 CONTINUE
0400 SUM=0.0
0410 SSQ=0.0
0420 M=0
```

```
0430 IO=0
0440 DO 50 I=1,N-2
0450 DO 50 J=I+1,N-1
0460 DO 50 L=J+1,N
0470 M=M+1
0480 U1=TPH(L)-TPH(I)
0490 U2=TPH(J)-TPH(I)
0500 EQK=(ALPH(M)*U1-U2)/(U2*TPH(L)-ALPH(M)*TPH(J)*U1)
0510 IF(EQK.LE.0)GO TO 500
0520 PK(M)=ALOG10(EQK)
0530 150 SUM=SUM+PK(M)
0550 SQ=PK(M)**2
0560 SSQ=SSQ+SQ
0570 50 CONTINUE
0580 M2=M-IO
0590 AV=SUM/M2
0600 V=(SSQ-M2*AV**2)/(M2-1)
0610 S=V**0.5
0611 SE=2*S/M2**0.5
0612 IF(SE.LE.TS)TS=SE
0613 IF(SE.LE.TS)TAV=AV
0614 IF(SE.LE.TS)FHC=HC
0619 IF(INDEX.EQ.1)GO TO 1
0620 GO TO 300
0630 500 PK(M)=0.0
0640 IO=IO+1
0650 GO TO 150
0660 300 IF(FLAG.EQ.1.0)GO TO 99
0670 L=0
0680 DO 70 M=1,N-2
0690 DO 70 J=M+1,N-1
0700 DO 70 I=J+1,N
0710 L=L+1
0720 WRITE(6,25)M,J,I,PK(L)
0730 25 FORMAT('SAMPLES: ',3I1,' PK= ',F5.2)
0740 70 CONTINUE
0750 FLAG=1.0
0760 88 WRITE(6,26)AV,SE
0770 26 FORMAT('THE MEAN PK IS: ',4X,F5.2,' PLUS OR MINUS: ',F4.2)
0772 IF(N.NE.5)GO TO 77
0780 99 IF(SE.LE.T)GO TO 101
0790 SUM=0.0
0791 SSQ=0.0
0792 IM=0
0793 DO 200 I=7,10,1
0794 PKI=PK(I)
0795 CALL SUB(PKI,SSQ,SUM)
0796 200 CONTINUE
0797 GO TO 201
0798 212 SUM=PK(10)
0799 SSQ=PK(10)**2
0800 DO 600 I=4,6,1
0801 PKI=PK(I)
0802 CALL SUB(PKI,SSQ,SUM)
0803 600 CONTINUE
0808 GO TO 201
0810 213 SUM=PK(2)
0812 SSQ=PK(2)**2
0816 DO 700 I=3,9,3
0817 PKI=PK(I)
0818 CALL SUB(PKI,SSQ,SUM)
0819 700 CONTINUE
0820 GO TO 201
0822 214 SUM=PK(8)
0824 SSQ=PK(8)**2
0826 DO 800 I=1,5,2
0827 PKI=PK(I)
0828 CALL SUB(PKI,SSQ,SUM)
```

```
0830 800 CONTINUE
0832 GO TO 201
0834 215 SUM=PK(2)
0836 SSQ=PK(2)**2
0838 DO 900 I=1,7,3
0839 PKI=PK(I)
0840 CALL SUB(PKI,SSQ,SUM)
0845 900 CONTINUE
0850 201 AVE=SUM/4.0
0851 IM=IM+1
0852 VAR=(SSQ-4.0*AVE**2)/3.0
0854 STD(IM)=VAR**0.5
0855 IF(STD(IM).LE.TSTD)TSTD=STD(IM)
0856 IF(STD(IM).LE.TSTD)KM=IM
0865 IF(IM.EQ.1)GO TO 212
0870 IF(IM.EQ.2)GO TO 213
0875 IF(IM.EQ.3)GO TO 214
0880 IF(IM.EQ.4)GO TO 215
0890 INDEX=1
0895 1 IF(SE.LE.T)GO TO 101
0898 IF(JM.EQ.KM)GO TO 78
0900 MODE=1
0902 KS=KM-1
0904 IF(KS.NE.0)GO TO 250
0920 IF(PK(8).GT.PK(6))MODE=0-MODE
0930 DO 140 I=1,4
0934 ZHT(I)=ZHT(I)+MODE*TA/2.0
0936 140 CONTINUE
0938 GO TO 260
0940 250 IF(PK(7).GT.PK(10))MODE=0-MODE
0942 IF(KS.EQ.1.OR.KS.EQ.3)MODE=0-MODE
0944 ZHT(KS)=ZHT(KS)+MODE*TA/2.0
0950 260 HC=HC+MODE*TA/2.0
0960 TA=TA/2.0
0970 IF(TA.GT.0.2)GO TO 55
0980 JM=KM
0986 IF(KS.EQ.0)GO TO 22
0990 HT(KS)=HT(KS)+FHC
0995 GO TO 44
1000 22 DO 460 I=1,4
1002 HT(I)=HT(I)+FHC
1004 460 CONTINUE
1010 GO TO 44
1020 101 HT(KS)=HT(KS)+FHC
1030 WRITE(6,28)AV,SE,(HT(I),I=1,4)
1040 28 FORMAT('THE ADJUSTED PK IS: ',F5.2,' PLUS OR MINUS: ',F4.2,
1041$/,'WITH NEW PEAK HTS OF: ',4F6.1)
1060 GO TO 33
1062 78 WRITE(6,36)TAV,TS
1064 36 FORMAT('THE BEST FIT PK IS: ',F5.2,' PLUS OR MINUS: ',F4.2)
1065 WRITE(6,37)(HT(I),I=1,4)
1066 37 FORMAT('WITH NEW PEAK HTS OF: ',4F6.1)
1068 GO TO 33
1070 77 WRITE(6,29)
1080 29 FORMAT('TO USE AUTO ADJUSTMENT USE 5 PHS PER RUN')
1085 33 GO TO 5
1090 102 STOP
1100 END
1101 SUBROUTINE SUB(P,SX2,SX)
1102 SX=SX+P
1103 S2=P**2
1104 SX2=SX2+S2
1105 RETURN
1106 END
```

ALERNATE PROGRAM USING EQ. VI-1

```
0010 DIMENSION PH(5)
0030 DIMENSION AB(5)
0040 DIMENSION OH(5)
0050 1 WRITE(6,16)
0060 READ(5,11)T1,T2,T3,T4
0070 IF(T1.LE.0.0)GO TO 2
0080 W=0.0001823*T1**2.0-0.04267*T1+14.946
0090 WRITE(6,17)
0100 READ(5,12)(PH(I),I=1,5)
0110 WRITE(6,18)
0120 READ(5,13)(AB(I),I=2,5)
0130 DO 100 I=1,5
0140 OH(I)=10**(PH(I)-W)
0150 100 CONTINUE
0160 SX=0.0
0170 SY=0.0
0180 SX2=0.0
0190 SXY=0.0
0200 DO 200 N=2,5
0210 X=OH(N)
0220 Y=(OH(1)-OH(N))/AB(N)
0230 XY=X*Y
0240 X2=X**2.0
0250 SX=SX+X*2.0
0260 SY=SY+Y*2.0
0270 SX2=SX2+X2*2.0
0280 SXY=SXY+XY*2.0
0290 200 CONTINUE
0295 M=(N-1)*2
0300 D=(SX2*M-SX**2.0)
0310 A=(SXY*M-SX*SY)/D
0320 B=(SX2*SY-SX*SXY)/D
0330 E=A/B
0340 PK=0.0-ALOG10(E)
0350 WRITE(6,26)PK
0360 WRITE(6,27)T1,T2,T3,T4
0370 DO 300 I=2,5
0380 UY=A*OH(I)+B
0385 ABS=(OH(1)-OH(I))/UY
0390 WRITE(6,28)I,ABS
0400 300 CONTINUE
0410 GO TO 1
0420 11 FORMAT(F4.1,3A4)
0430 12 FORMAT(5F5.2)
0440 13 FORMAT(4F7.4)
0450 16 FORMAT('TEMPERATURE: ')
0460 17 FORMAT('PH VALUES: ')
0470 18 FORMAT('ABSORBANCES: ')
0480 26 FORMAT('THE PK IS: ',3X,F5.2)
0490 27 FORMAT('CURVE FIT FOR: ',F4.1,3A4,/,'SAMPLE NO. ABSORBANCE')
0500 28 FORMAT(5X,I1,7X,F7.4)
0510 2 STOP
0520 END
```

APPENDIX C

CALCULATION OF IONIC STRENGTH IN BUFFER SOLUTIONS

There are several ways of computing the ionic strength of various buffer solutions. The most common is to use prepared nomographs. The construction of nomographs is described by Davis (1945). However, in a weak acid or base solution the ionic strength depends on the dissociation constant of the acid or base, but if this solution is buffered with a highly ionizable component then the ionic strength will depend almost entirely on the concentration of that component. This is illustrated quite vividly by Boyd's nomogram for acetate buffers (1945). It is clear from inspection that except in extremely concentrated solutions the ionic strength of acetate buffers is simply the concentration of sodium acetate used to make the buffer. It would be much easier to make acetate buffers by adding glacial acetic acid to solutions of sodium acetate while monitoring the pH with a glass electrode. I have found that the error involved in this approximation is no greater than involved in reading the scales of a carefully constructed nomograph.

For a Tris-sulfate buffer of relatively low ionic strength it can be assumed that the sulfate is totally

ionized and that every proton has ionized a Tris molecule and that no other Tris has been ionized. The ionoc strength is given by:

$$I = \tfrac{1}{2}\Sigma c_i z_i^2 = \tfrac{1}{2}([Tris^+] + 4[SO_4^{-2}]) = \tfrac{1}{2}(2M + 4M) = 3M$$

where M is the molarity of sulfuric acid used to make the buffer. If potassium hydrogen sulfate was used to make the buffer the ionic stength is given by:

$$I = \tfrac{1}{2}([K^+] + [Tris^+] + 4[SO_4^{-2}]) = \tfrac{1}{2}(M + M + 4M) = 3M$$

also. The error introduced by the assumption that the ionized Tris is equal to the protons added is less than 0.1% in a solutino of ionic strength of 0.1 when the pH is raised to 9.0, since raising the pH to 9.0 only removes 10^{-5} molar protons.

All ionic strength calculations in this thesis have used this approximation.