PROCESSING AND ANALYSIS OF POTENTIOMETRIC DATA

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ABSTRACT Potentiometric titration curves have traditionally been collected as the difference in absorbance at two wavelengths, and analyzed by plotting voltage vs. log (oxidized/reduced). The collection method, designed to monitor changes in local peak height, is effective for that purpose only when spectral backgrounds do not change slope as voltage changes, and the analysis method is valid only for a single isolated component (one whose midpoint potential is far from that of anything else in the mixture). Yet these methods are commonly used where such restrictions do not pertain, e.g. the study of cytochromes in mitonchondria. In this paper, we present more appropriate methods of collection and analysis, and suggest that, even with the best available methods, any conclusion should be confirmed in several ways. Experimental results are presented in accompanying papers.

INTRODUCTION

Our current understanding of the structure and thermodynamic characteristics of electron transport chains is based to a large extent on controlled spectral potentiometric titrations. Data relating to cytochromes are collected as a difference in absorbance (ΔA) between a peak wavelength and a reference wavelength. These data are analyzed by a graphic procedure that relates voltage to the log of the supposed ratio of oxidized to reduced species. The purpose of this paper is to demonstrate that these methods of data collection and analysis are not able to extract the kind of information that is sought. Consequently, the conclusions reached in all of these studies must be questioned and reexamined.

With the realization of these problems, we have devised procedures that allow us to collect entire spectra at each controlled voltage (Reddy and Hendler, 1983) and to analyze this increased amount of data by two newer mathematical procedures (Shrager and Hendler, 1982, and Reddy and Hendler, 1983). Using this new approach, we have examined the *b* cytochromes (Reddy and Hendler, 1983), the c_1 cytochromes (Reddy and Hendler, 1986), and the aa_3 cytochromes (Reddy et. al., 1986), all in situ in beef heart mitochondria. We have also studied isolated purified cytochromes aa_3 (Hendler et al., 1986). These studies suggest an electron transport mechanism fundamentally different from that which is currently accepted.

MODELS OF ELECTROCHEMICAL TITRATION CURVES

When voltage E is varied, and optical absorbance $A_\lambda(E)$ is observed at some fixed wavelength λ, the set of observations is called a potentiometric titration curve, with electrons as the titrant being added or removed. If a single transition (oxidized to reduced state or the reverse) is occurring, its form, called Nernstian, is defined as:

$$A(E) = A_{\text{Base}} + h/(1 + 10^{[n(E - E_m)/60]}). \quad (1)$$

The parameter h is the total absorbance change induced by the transition, E_m is the midpoint potential of the transition (representing equal amounts of oxidized and reduced state), and n represents the number of electrons passed when a molecule is converted from one state to the other. The value 60, derived from 2.303 RT/F at 30°C, is a typical constant for this class of experiments. The curve $A(E)$, as described by Eq. 1 and as shown in Fig. 1, is a sigmoidal curve with level asymptotes, A_{Base} for $E \gg E_m$, and $A_{\text{Base}} + h$ for $E \ll E_m$. A brief table of values for $A(E)$ will give an indication of its behavior. Let $A_{\text{Base}} = 0$, $h = 1$, and $d = 60/n$:

$E - E_m$	$-\infty$	$-3d$	$-2d$	$-d$	0	d	2d	3d	$+\infty$
$A(E)$	1	0.999	0.99	0.91	0.5	0.09	0.01	0.001	0

$A_{\text{Base}} + h$ and A_{Base} are sometimes referred to as A_{Red} and A_{Ox}, because those absorbances represent the all-reduced and all-oxidized states of some chemical component.

When A_λ is influenced by more than one transition, the curve $A(E)$ becomes a sum of Nernstians from Eq. 1:

$$A(E) = A_{\text{Base}} + \sum_{j=1}^{k} h_j/(1 + 10^{[n_j(E - E_{mj})/60]}), \quad (2)$$

where the parameters h, E_m, and n are now subscripted by j. Because the Nernstian terms are being added in some proportion given by the h's, Eq. 2 is called a linear

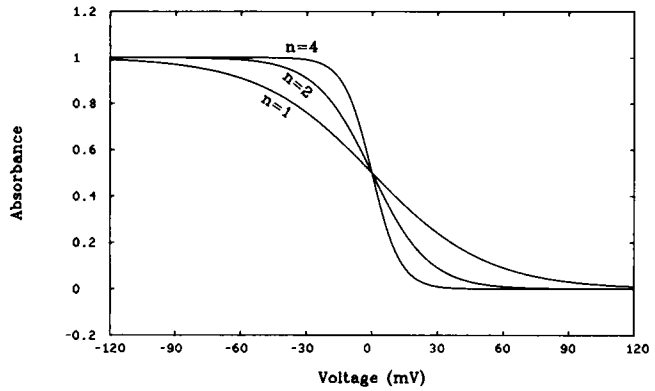

FIGURE 1 Nernstian curves with $h = 1$, $E_m = 0$, $A_{Base} = 0$, and n values of 1, 2, and 4. For the study of cytochromes, the Nernstian is defined as decreasing with voltage.

combination of Nernstians. The h's can be positive or negative, the n's can be any positive integers, and the E_m's can be widely separated or arbitrarily close. Degrees of separation are illustrated in Fig. 2. Overlapping Nernstians present a difficult analytical problem. In the next section we will discuss the extraction of titration curves of the form (Eq. 2) from spectroscopic data, and in the succeeding sections, we will describe some analyses of these curves that attempt to resolve overlapping Nernstians.

GENERATION OF TITRATION CURVES

The simplest approach to the detection of spectral change is a measurement of the absorbance at a single wavelength, which we will denote by 1W. The 1W method is frequently rejected as unsuitable, because a spectrum is viewed as one or more narrow absorbance peaks, whose heights monitor the interesting chemical states, superimposed on a broad background whose voltage-induced changes are not pertinent. The 1W method produces a titration curve that exhibits both narrow and broad effects. To isolate the peak behavior from the background, the 2W method is used: measure the difference in absorbance (ΔA) between a wavelength near the center of the peak and a wavelength subject only to background. The reasoning is that any rise or fall in base level with respect to voltage will be canceled out by subtraction. The flaws in the 2W method become evident when spectral change is observed at more than two wavelengths (*a*) Background change is not restricted to changes in base level, but is often a complex movement with tilts and bends. Such changes are not suppressed by the 2W method. (*b*) It may be impossible to find two wavelengths whose ΔA will be influenced by only one interesting peak or transition. (*c*) The notion of "background" is questionable. In many instances, we have observed that the broad features of a spectrum exhibit Nernstian behavior just as the narrow peaks do. Suppression of "background" is suppression of information which may, in fact, be pertinent.

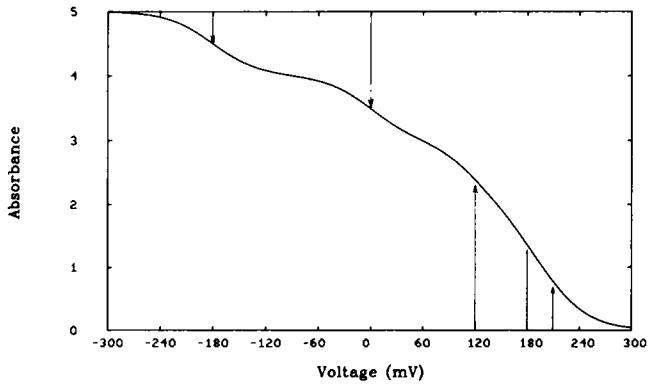

FIGURE 2. A sum of five Nernstian components showing varying degrees of overlap. For all components, $h = 1$ and $n = 1$. The E_m's are −180, 0, 120, 180, and 210. Notice that visual separation is very difficult for E_m separation ≤60 mV.

Whether background is significant or not, one may wish to isolate the behavior of local absorbance peaks, and the issue of tilts and bends in the background is one that cannot be completely resolved by any numerical technique. But in some cases, the isolation of local behavior can be improved. Consider the case where changes in voltage induce significant changes in background slope, but negligible changes in curvature. Then a local measure of the second derivative (e.g. the leading coefficient of a least-squares parabola through three or more points on the peak) will ignore changes in base level and slope, such information being absorbed entirely by the linear and constant terms of the parabola. We observe that this method, called 2D for second derivative, often yields cleaner isolation than the 2W method in the sense that titration curves measured by the 2D method will level off at the tails, as they should, where curves measured by the 2W method continue to slope.

The 2W and 2D methods are both attempts to isolate local spectral changes from global changes, and in the context of optical spectra with peaks, 2D is more robust because it suppresses changes in background slope as well as background level. There is a contrasting view of spectral analysis: rather than try to isolate the narrow features (e.g. peaks), regard the entire spectrum as significant. Analytical methods deriving from this viewpoint use all the wavelengths (in a judiciously selected region) to extract the significant titration curves. The extraction process should not suppress any spectral changes. The SVD method of Shrager and Hendler (Shrager and Hendler, 1982; Shrager, 1984; Frans and Harris, 1985) is one such method. Formulas 1, 2, and 9 in the 1982 paper served as the basis of two other approaches, the first by Frans and Harris (1984), and the second by Koland et al. (1984). All three methods produce resolved Nernstians with corresponding difference spectra as a by-product, but neither of these last two methods uses the SVD operator.

The SVD method is unique in the special assistance it offers in determining a minimum number of transitions required to explain the data. Since it was not stressed in

previous papers, we will point it out here. The "titration" curves produced by SVD (assuming that all data trends are Nernstian) are linear combinations of the Nernstians. These linear combinations are stored in the columns of the matrix V, to be plotted against voltage. The columns of V are orthogonal, which means that they must have unique patterns of numerical sign, which usually involves unique curve shape as well. It can be proven that every Nernstian in the data must show itself in at least one column of V. In many cases, determining the number of Nernstians present is simply a matter of counting the upward and downward slopes in the most oscillatory columns of V, although more rigorous analysis described in the reference should be used to draw final conclusions.

All of the above methods, 1W, 2W, 2D, and SVD, are valid ways of generating titration curves, and the choice will depend upon which aspects of the data one wishes to emphasize or isolate. In subsequent sections, we will refer to the ordinate of any titration curve as A(E), regardless of the method by which it was generated.

REPLOTTING METHOD FOR RESOLVING NERNSTIANS

In 1970, Wilson and Dutton introduced a replotting method for resolving overlapping Nernstians (Wilson and Dutton, 1970; Dutton et al., 1970). We will refer to this method simply as "replotting." At that time, computers were considerably less convenient than they are today, and graphical methods held a prominent position in laboratory computing. The convenience of replotting, as with several other graphical methods, led to its established use in problems beyond its capabilities. Because replotting is the basis of most analyses of potentiometric data, and because our own more rigorous methods have produced results in sharp contrast to established results, we are obliged to discuss this matter in some detail.

It is our position that replotting should no longer be used to resolve overlapping Nernstians because it is subject to a variety of errors, some human, but most inherent in the method itself. This should by no means be construed as a criticism of those who have used the method. We discovered these discrepancies by setting out to use replotting in the same way as everyone else, and we were fortunate that some convenient computer programs were at our disposal for cross-checking, particularly the MLAB mathematical modeling system (Knott, 1979).

Graphical methods and transformations of data have well-recognized pitfalls, as explained in Johansen and Lumry (1961), Wilkinson (1961), Cleland (1963), Dowd and Riggs (1965), Colquhoun (1971), Cornish-Bowden and Eisenthal (1974), and Ackerman and Gatewood (1979). None of these works addresses itself specifically to replotting, so we single out several pitfalls that apply to replotting as well as to other methods in the literature. For completeness, the authors also offer an unpublished report, Shrager and Hendler (1985; A comparism of methods for the processing and analysis of potentimetric data.), explaining some of these pitfalls in greater detail than is appropriate here.

(*a*) The log transformation, particularly as used in replotting, routinely distorts the data beyond all bounds of utility.

Example 1: there is a high probability that some arguments to the log function will be small-positive, zero, or negative in certain regions. Such logs are either nonexistent or impossible to fit on a conveniently-scaled plot. They are, of course, omitted from the log plots, yet the data from which they came may be just as informative as the data that is shown. Theoretical means and variances of the logs, in such cases, do not exist. The apparent (i.e. edited) means, variances, and visual trends are functions of the range chosen for plotting.

Example 2: when the arguments of the log are comfortably positive and the error distribution of those arguments is symmetric about zero with constant variance, the mean of the log curve is biased toward minus infinity, and approaches that limit too rapidly as the argument curve approaches zero.

Example 3: the variances of small arguments are magnified more than the variances of large arguments. Least-squares procedures would compensate for disparate variances with weights, to prevent the worst points from dominating the results. But replotting, being a graphical method, fails to address the problem.

(*b*) As a graphical method, replotting is subject to the visual bias of the user. Finding asymptotes and inflection points in noisy data, as required by replotting, are particularly subjective tasks.

(*c*) Replotting is a multi-phase method, involving at least four plots: plot 1 is the original absorbance vs. voltage data, plot 2 is a replot using logs and estimated asymptotes from plot 1, and plots 3, 4, etc. are like plot 2, using subsets of the data determined by the inflection points from plot 2. Errors in the asymptotes and inflection points of plots 1 and 2 bias all the data in the succeeding plots, from which the final E_m and n values are derived.

(*d*) Replotting is inherently biased. Despite perfectly executed procedures carried out on noiseless data, the answers may be substantially wrong. This point is discussed more fully in the above-mentioned report of Shrager and Hendler, 1985.

(*e*) There is no goodness criterion. Replotting does not employ any statistical check of the E_m and n values against the original data, to determine how well the problem has been solved.

In summary, replotting is a series of biases loosely connected by data: visual bias, log bias, multi-phase bias, and inherent bias, all with no redeeming corrections or measures of merit. This method is not a compelling basis for the resolution of overlapping Nernstians. In the next section, we present a method more appropriate to the problem.

A DIRECT METHOD FOR RESOLVING NERNSTIANS

Nonlinear curve-fitting, available in most scientific computing systems, including microcomputers, offers a means of comparing the data to various linear combinations of Nernstians. This approach does not require any transformation of the titration curves, and while it does require initial estimates of the E_m's (e.g.), these estimates are refined by the curve-fitting procedure in order to achieve a best fit in the least-squares sense. In contrast to graphical estimation, such a fit has some established statistical merit as explained, for example, in Draper and Smith (1966).

Curve-fitting programs vary somewhat, but their general use in this context can be described as follows: (*a*) Provide a titration curve. (*b*) Specify that Eq. 2 is the general model you wish to use. (*c*) Provide k and estimates of A_{Base}, h_j, E_{mj}, and n_j. (*d*) Invoke the curve-fitter. (*e*) Plot the residuals (data minus computed curve) to see if the fit is reasonable. Optionally, use a goodness-of-fit test, but beware that the data may not meet the assumptions of the test. Do not settle for a plot of the data with the computed curve superimposed, because visual bias can make a bad fit look good. (*f*) If the fit is inadequate, change some quantities in step *c* and try again.

Some curve-fitting procedures provide additional amenities. For example, our current curve-fitter (Shrager, 1970; Shrager, 1972) provides the ability to constrain the parameters to predetermined regions, and it solves linear problems (where the parameters are all first power multipliers of distinct terms) in one step. This last feature enables us to use zero as the first estimates of A_{Base} and all h_j's if we wish, because in one step, the curve-fitter will set them to their best least-squares values given the initial E_{mj}'s and n_j's.

The user can help his cause by using data generated in a variety of ways, some of which are described in the section titled Generation of Titration Curves. For example, in the problem of Fig. 3, are there really two components, or only one? SVD further reduces this ambiguity by producing either one or two titration curves. The first curve looks essentially like Fig. 3, but the second curve, if any, rises in the low voltage region, then falls (or vice-versa). The fact that two titration curves are generated, and the fact that the second curve needs at least two transitions to explain it (one Nernstian cannot produce both upward and downward slopes on the same curve) proves that at least two components are required here. Exploitation of this technique is graphically illustrated in Shrager and Hendler (1982) using laboratory data, and in Shrager (1984) using artificial data to illustrate the limits of the procedure. Further work on the method by Frans and Harris (1985) indicates that these limits have already been improved upon.

The user can further help his cause by using prior knowledge. For example, on those occasions when we have allowed the curve-fitter to control the values of the n_j's,

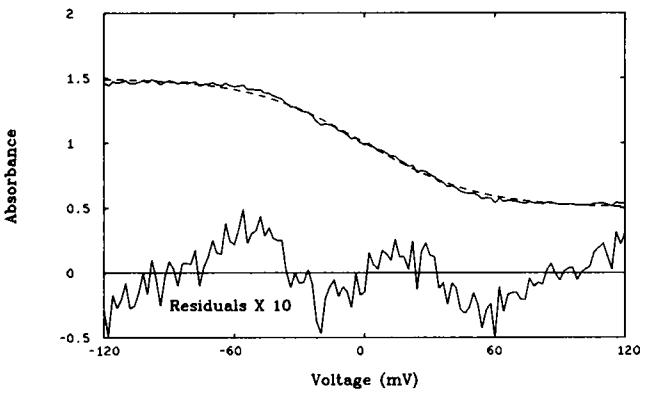

FIGURE 3 A sum of two Nernstians having $h = 0.47$ and $n = 2$, with E_m's of -26.5 mV and 26.5 mV. $A_{Base} = 0.53$. Noise level = 0.01. Data points are joined by solid lines. The data is fitted by a single Nernstian ($A_{Base} = 0.5$, $h = 1$, $E_m = 0$, n = 1), the dashed curve. Residuals (data minus fitted) are shown plotted about $A = 0$, and joined by solid lines. The residuals exhibit only 24 runs of sign where the mean is 61 runs ±6.5.

those values came out close to integers, with fractional deviations of one-tenth or less. Consequently, we have chosen various fixed integer values for the n's to save curve fitting time, and to improve resolution by eliminating parameter dependencies. (Dependency is the ability of changes in the model curve, induced by one parameter, to be negated by changes induced by other parameters. This negation, if complete enough, can cause severe increases in the variances of the parameters involved.) We anticipate that if a markedly noninteger n is required, the merits of two curve-fits using the neighboring integers will be difficult to distinguish, thus indicating an ambiguity in the data.

Since our curve-fitter relieves us of the burden of estimating A_{Base} and the h's, and, by prior assumption, we are guessing and fixing the n's at various integer values (the number of reasonable combinations to be tried is usually small), the only critical parameters the curve-fitter requires from us are the number of Nernstians k, and an estimated E_m for each. If the n values are truly in doubt, the option is always open to let the curve-fitter control them also.

Judging from our own experience, nonlinear curve-fitting will be an exercise in man-computer interaction for the foreseeable future. Computerized parameter refinement is a very convenient tool, but it is not totally reliable. The results must be monitored, preferably at a terminal that can produce graphs rapidly. For each set of data, we generate titration curves in at least two ways (2D and SVD are currently favored). For each titration curve, we try several different models (choices of k and the n's). For each model, we try several first estimates of the E_m's. For an example of why all this effort is necessary, see Figs. 3 and 4. In some cases, we can extract a consensus model from this effort, and in other cases, we must settle for describing the ambiguities. But if a consensus is reached, it is not likely due to biased treatment of the data, nor to a

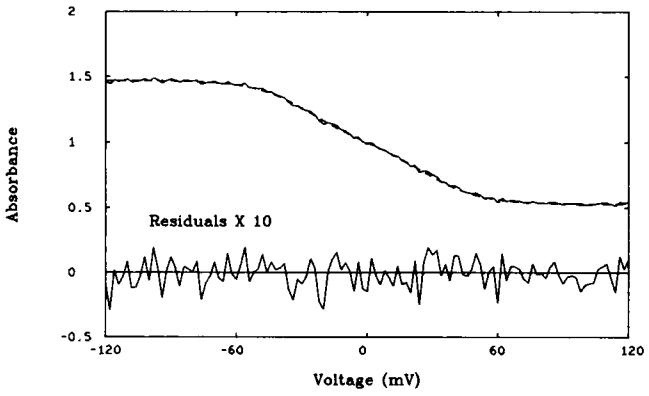

FIGURE 4 The data of Fig. 3 compared with the proper model, with the same format as Fig. 3. The residuals exhibit 56 runs of sign, within one standard deviation of the mean.

single-minded choice of model, nor to a spurious local solution of the least-squares problem.

CONCLUSION

The resolution of sums of similar components, whether they be Nernstians, Gaussians, Lorentzians, or exponentials, is difficult. Not only is there the danger that two similar shapes may be too close to resolve. There is also the danger that two such components may combine to resemble a third, and so on. Graphical methods are inadequate to handle the questions of discrimination that arise. Replotting is additionally burdened by the various idiosyncracies described in Generation of Titration Curves. Curve-fitting, combined with variety in data treatment and a thorough selection of models, holds the best hope of resolving overlapping components. SVD is a particularly helpful data treatment because of its ability, through the unique shapes of the titration curves it extracts from the data, to establish a minimum number of components necessary to explain the total titration.

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