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Quantitative analysis of absorption spectra and application to the characterization of ligand binding curves

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Summary. The spectrum of a chromophore may change as a result of perturbations in its environment. The spectral changes resulting from the perturbation are often followed by measurements at just one or two wavelengths but it is usually no more difficult to collect entire spectra. The problem comes in analysing the data from such a series of spectra. In this paper we will suggest a simple procedure in which the spectrum observed under any particular set of conditions may be considered to consist of the sum of two distinct spectral forms. The method, which is free of any assumptions regarding the quantitative relationship between the perturbation and the extent of spectral change, defines any given spectrum in terms of an apparent molar fraction of the contributing spectral forms. The variation of this apparent molar fraction provides information from which a quantitative relationship can be developed to describe the dependence of the spectral change on the perturbant. The method is illustrated using the model system of phenol red protonation and is applied to the characterization of the binding of azide ions to cobalt-substituted carbonic anhydrase.
Key words. Spectral titrations; absorption spectra; fluorescence spectra; binding curves; carbonic anhydrase.

Many compounds contain a chromophore with spectral properties which depend on its environment. Perturbations of the environment result in spectral changes which can be used to obtain a quantitative description of the effect of the perturbant. For example, the chromophore might be a group in a protein and the perturbant a ligand which binds to the protein; by following the spectral change resulting from ligand addition, the binding curve may be determined.

In these types of titration, it is a relatively simple matter to collect many complete spectra, each representing a different amount of added perturbant. The resulting spectra contain a bewildering amount of information, so much so that it is common practice to ignore most of it.

Often, the absorbance or fluorescence at a single wavelength is selected and used as a measure of the spectral change.
A number of authors [1-7] have proposed analyses of spectra obtained from the titration of small molecules with metal or hydrogen ions. All of these procedures have a common feature in that they perform an overall fit to an entire collection of spectra, on the assumption that the observed spectral changes result from one or more simple dissociation reactions. In cases where this assumption is inappropriate these fitting procedures will be of no value. For example, a spectral change might be observed as a function to time rather than of the concentration of a ligand; Martorana et al.[8] report a study of this genre.
In this paper we will suggest a method by which entire spectra can be used to characterize the effect of a perturbing agent. The method is free of any assumptions about the quantitative relationship between the extent of spectral change and the perturbing agent. The only assumption made is that the spectrum may be described as the sum of two spectral forms whose relative concentrations change as a result of the perturbation. The method gives an estimate of the relative amounts of the spectral forms and this information can then be used to deduce the qualitative and quantitative relationship governing the spectral perturbation.

Materials and equipment

Bovine erythrocyte carbonic anhydrase was obtained from Sigma and the native zinc atoms replaced with cobalt using the dipicolinate method[9]; the product was dialyzed against water, lyophilized and stored at −20 °C until required.
The protein (approx. 13.5 mg) was dissolved in 2.0 ml of 30 mM Na_2SO_4 in 10 mM potassium phosphate buffer (pH 7.10) and passed through a 0.45-μ Millipore filter. Spectra between 680 nm and 480 nm at 2.5-nm intervals were obtained in 10-mm path length cuvettes using a Cary 118 spectrophotometer. The raw data, consisting of an absorbance measurement at each wavelength, were collected using a modification of the OLIS software package in conjunction with a Northstar computer which was interfaced to the spectrophotometer. Titration by successive additions of small volumes of 40 mM sodium azide yielded a series of spectra which were corrected for light scattering[10] before further analysis.
Spectra of 11.5 μM phenol red (phenolsulphophthalein) between 620 nm and 380 nm at 3-nm intervals were obtained in mixtures of 0.1 M Tris and 0.1 M citric acid of the required pH; the data were collected using the same equipment as described above for carbonic anhydrase.
Nonlinear regression analysis of binding curves was performed using the DNRP53 computer program[11].

Theory

Our basic proposition will be that there is a substance which can exist in two forms (X and Y) which have different spectral properties. The relative amounts of X and Y depend upon a perturbant, which will normally be referred to as if it is a compound or ion which is added to the solution. It might equally well represent a change in temperature, polarity, dielectric constant and so on; it could also be time, when there is a slow spectral change as a result of a sudden shift in experimental conditions. The theory to be presented applies equally to both fluorescence and absorbance spectra, but will be phrased in terms of the latter. In principle, the procedure could be extended to cases where there are three or more spectral forms but we will limit the present discussion to two spectral forms only. The only assumption that will be made regarding the quantitative relationship between the amount of perturbant and the relative amounts of X and Y is that the perturbant promotes the conversion of X to Y.
First we note that it may not be possible to prepare a solution consisting of only Y since this may require a quantity of perturbant which exceeds solubility, economic or some other practical limit. In some cases, it may not be possible to obtain a solution containing only X either. Where, for example, X is a protein and Y is its protonated form, instability of the protein may preclude raising the pH sufficiently far above the pK to effectively convert all Y to X.
We shall consider the worst possible case where neither extreme is experimentally accessible, so that we can only measure the spectral properties of mixtures containing unknown proportions of X and Y. For any such mixture, where the mole fraction of Y is f_m, the absorbance ($A_{m,\lambda}$) at any wavelength (λ) is given by:

$$A_{m,\lambda} = f_m A_{y,\lambda} + (1 - f_m) A_{x,\lambda} \quad (1)$$

where $A_{x,\lambda}$ is the absorbance if only X was present and $A_{y,\lambda}$ is the absorbance if only Y was present. Since the absorbances of X and Y are precisely those which we are taking to be unknown, equation 1 is of little immediate value in determining f_m. Let us denote the experimental conditions which produce the lowest fraction of Y as 'zero' conditions and the corresponding spectrum, the 'zero' spectrum. Similarly, the conditions in which the mole fraction of Y is highest will be referred to as 'unit' conditions and as producing the 'unit' spectrum. We may now write two new expressions, precisely analogous to equation 1, for the absorbance ($A_{0,\lambda}$ and $A_{1,\lambda}$) in terms of the mole fraction of Y (f_0 and f_1) for the zero and unit spectrum, respectively. Using these two equations, we may eliminate $A_{x,\lambda}$ and $A_{y,\lambda}$ from equation 1 to obtain an expression for any intermediate spectrum in terms of the zero and unit spectra, and an apparent mole fraction (f'_m):

$$A_{m,\lambda} = f'_m A_{1,\lambda} + (1 - f'_m) A_{0,\lambda} \quad (2)$$

where:

$$f'_m = (f_m - f_0)/(f_1 - f_0) \quad (3)$$

Since all three absorbances in equation 2 may be measured, f'_m may be calculated at any wavelength but, due to experimental variations in the measurements, the value of f'_m will fluctuate from one wavelength to another. Our aim is to represent the entire intermediate spectrum with a single value of f'_m and we have selected this value using a least squares criterion; f'_m is chosen such that the square of the difference between the actual absorbance and that calculated from equation 2, summed over all wavelengths, is minimized. The decision to use a least squares method was based on its algebraic convenience, and the fact that it worked well in practice. However, if experimental errors happen to follow a Gaussian distribution, minimizing the sum of squares has the additional advantage of yielding the most likely value of f'_m. The least squares criterion is met when f'_m is calculated from equation 4.

$$f'_m = \sum dr / \sum r^2 \quad (4a)$$

$$d = A_{m,\lambda} - A_{0,\lambda} \quad (4b)$$

$$r = A_{1,\lambda} - A_{0,\lambda} \quad (4c)$$

It should be noted here that a spectrum is a continuous curve which we are approximating with a series of discrete points. The inaccuracies introduced in this manner will depend on the spacing of the points in relation to the size of the features seen in the spectrum. Obviously, complex infra-red spectra will require more points to define them than spectra obtained at visible or ultra-violet wavelengths which usually have a small number of quite broad peaks. Common sense should prevail here; the points should be spaced at sufficiently small intervals that connecting adjacent points with straight lines would give a visually acceptable representation of the spectrum.

The standard error of f'_m is given by:

$$S.E.(f'_m) = \{[\sum d^2/\sum r^2 - (f'_m)^2]/[N - 1]\}^{1/2} \quad (5)$$

where N is the number of wavelengths at which the absorbance is measured.

We will refer to the process in which the apparent molar fraction is calculated using equation 4 as spectral 'fractionation'. The resulting value of f'_m can be substituted into equation 2 to give a calculated spectrum which we will call a 'conformed' spectrum.

Methodology

The method that is being proposed here consists of two essential stages, a desirable third stage and an optional fourth stage. These are:

1) measurement of the spectrum of a chromophore in the presence of several different amounts of a perturbing agent; 2) calculation of an apparent mole fraction (f'_m, see equation 4) which best describes any given spectrum in terms of two designated reference spectra; 3) qualitative and quantitative characterization of the effect of the perturbant from the observed variation of f'_m; and 4) interpolation or extrapolation of f'_m under perturbant conditions which were not examined experimentally and prediction of the corresponding spectrum.

Results and discussion

The procedure we are proposing was devised during a series of experiments on cobalt-substituted carbonic anhydrase which exhibits a spectral change when azide binds. These results will be shown later; first we will demonstrate the validity of our procedure using a model system. The one we have chosen is a pH indicator in which the perturbant is the hydrogen ion and the chromophore is an ionizable dye (phenol red). The spectrum of this compound was determined over a pH range from 7.00 to 8.90 and the results are shown in figure 1a.

Each of these spectra is composed of a mixture of the spectra of the protonated (or more correctly, the monoanionic[12]) and unprotonated (bianionic) forms of phenol red. That obtained at pH 8.90 contains the lowest proportion of the protonated form and was selected as the zero spectrum as defined in the theory section. The spectrum obtained at pH 7.00 contains the highest proportion of the protonated form and was used as the unit spectrum. These two spectra are shown again in figure 1b, together with an intermediate spectrum at pH 8.00. Fractionation of this intermediate spectrum using equation 4 gave $f'_m = 0.4525 \pm 0.0004$. Substitution of this value into equation 2 gave the conformed spectrum shown as the solid circles in figure 1b. There is excellent agreement between the observed spectrum and the conformed spectrum which results from adding together 45.25% of the unit spectrum (pH 7.00) and 54.75% of the zero spectrum (pH 8.90).

This analysis of the spectrum of phenol red at pH 8.00 does not assume any particular quantitative relationship between the pH of the solution and the relative proportions of the two spectral forms. All we have done is to describe one spectrum in terms of a mixture of two others. The reason for using this model system is that we know that f'_m should depend on the pH in a predictable manner and a confirmation of this dependence provides evidence for the validity of our procedure. To this end, each of the spectra depicted in figure 1a was fractionated as described above and the resulting values of f'_m plotted against pH (fig. 1c). The results follow a simple titration curve with a pK of 7.86 ± 0.02.

It was implied earlier that neither zero nor unit conditions produce a pure ionic species of phenol red. This is

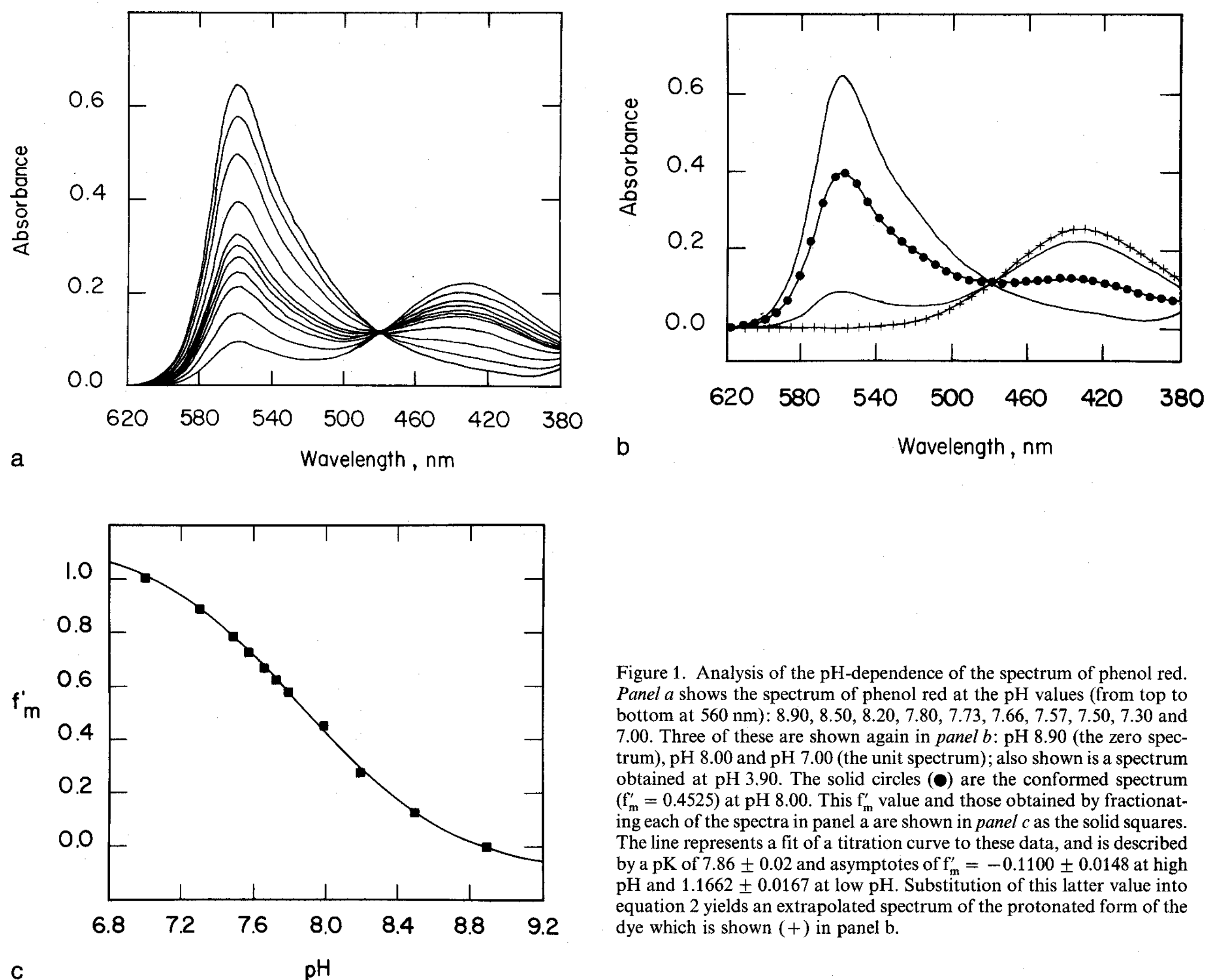

Figure 1. Analysis of the pH-dependence of the spectrum of phenol red. *Panel a* shows the spectrum of phenol red at the pH values (from top to bottom at 560 nm): 8.90, 8.50, 8.20, 7.80, 7.73, 7.66, 7.57, 7.50, 7.30 and 7.00. Three of these are shown again in *panel b*: pH 8.90 (the zero spectrum), pH 8.00 and pH 7.00 (the unit spectrum); also shown is a spectrum obtained at pH 3.90. The solid circles (●) are the conformed spectrum ($f'_m = 0.4525$) at pH 8.00. This f'_m value and those obtained by fractionating each of the spectra in panel a are shown in *panel c* as the solid squares. The line represents a fit of a titration curve to these data, and is described by a pK of 7.86 ± 0.02 and asymptotes of $f'_m = -0.1100 \pm 0.0148$ at high pH and 1.1662 ± 0.0167 at low pH. Substitution of this latter value into equation 2 yields an extrapolated spectrum of the protonated form of the dye which is shown (+) in panel b.

clearly so as each condition is only one pH unit from the pK. Even though the spectrum of neither pure ionic species is included in the data, it is possible to extrapolate the appearance of either. The fitted titration curve shown in figure 1c has limits of -0.1100 ± 0.0148 at high pH and 1.1662 ± 0.0167 at low pH. Substitution of either of these limiting values of f'_m into equation 2 will yield a calculated spectrum corresponding to that of either the protonated or unprotonated form of phenol red. The former is shown (plus symbols) in figure 1b, together with an experimental spectrum obtained at pH 3.90. At this pH, where protonation should be 99.99% complete, there is excellent agreement between the predicted spectrum and that observed experimentally.

This extrapolation obviously requires that a quantitative dependence of f'_m upon the perturbing agent can be established. However, it should be stressed that the existence and nature of such a relationship is only required for the extrapolation. The determination of f'_m itself does not require any assumptions about this relationship. Indeed, the main function of our procedure is to establish the relationship, as was done for phenol red; the only difference here is that we had a reasonably good idea of the likely dependence of f'_m on pH for this model system.

Having established the validity of the procedure, we applied it to the analysis of azide binding by cobalt-substituted carbonic anhydrase. Figure 2a shows the spectrum of this protein and the effect of addition of sodium azide. In the absence of azide (zero conditions) the protein exhibits several distinct absorbance bands but addition of 999 μM sodium azide (unit conditions) results in a broad and relatively featureless spectrum. A lower concentration of azide (174 μM) produces an intermediate spectrum which was fractionated to give $f'_m = 0.4737 \pm 0.0016$, corresponding to a conformed spectrum (figure 2a, solid circles) which closely resembles that measured experimentally.

Again we emphasize that the calculation of f'_m and the conformed spectrum is unrelated to any quantitative dependence of the spectral change upon the azide concentration. Nevertheless, such a dependence clearly exists as is illustrated in figure 2b; values of f'_m calculated from a

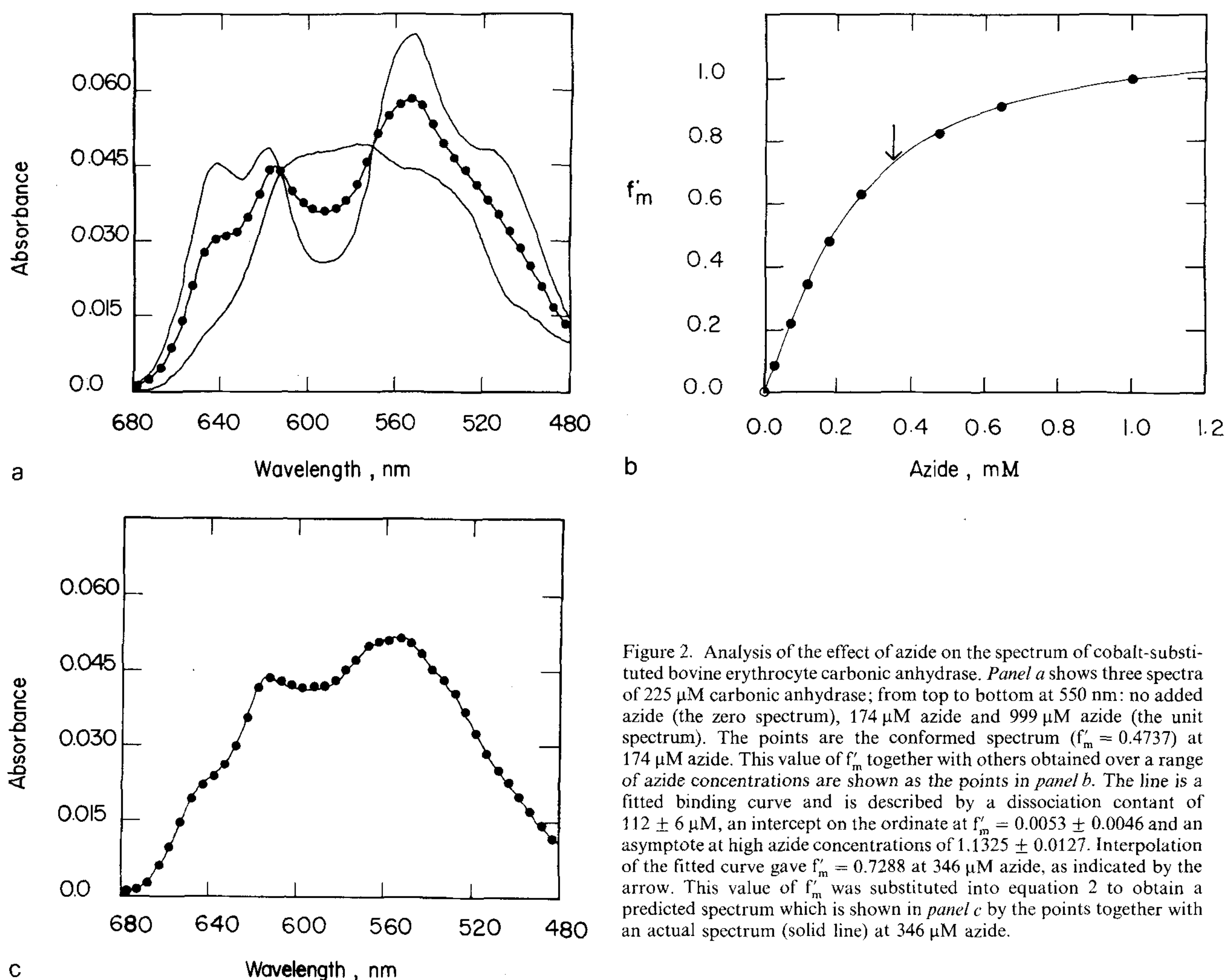

Figure 2. Analysis of the effect of azide on the spectrum of cobalt-substituted bovine erythrocyte carbonic anhydrase. *Panel a* shows three spectra of 225 μM carbonic anhydrase; from top to bottom at 550 nm: no added azide (the zero spectrum), 174 μM azide and 999 μM azide (the unit spectrum). The points are the conformed spectrum ($f'_m = 0.4737$) at 174 μM azide. This value of f'_m together with others obtained over a range of azide concentrations are shown as the points in *panel b*. The line is a fitted binding curve and is described by a dissociation contant of 112 ± 6 μM, an intercept on the ordinate at $f'_m = 0.0053 \pm 0.0046$ and an asymptote at high azide concentrations of 1.1325 ± 0.0127. Interpolation of the fitted curve gave $f'_m = 0.7288$ at 346 μM azide, as indicated by the arrow. This value of f'_m was substituted into equation 2 to obtain a predicted spectrum which is shown in *panel c* by the points together with an actual spectrum (solid line) at 346 μM azide.

series of spectra (not shown) increase smoothly as the azide concentration increases. Using these values of f'_m, it is now possible to determine the relationship between f'_m and the concentration of azide.

In some instances [13], the spectral change might indicate that a sigmoidal binding function is appropriate in which case a cooperative model could be fitted to the data. Since there was no indication of sigmoidicity in figure 2b, the data were fitted to the equation for simple binding of an univalent ligand to an univalent acceptor, allowing for the fact that there is significant depletion of the ligand as a result of binding. A dissociation constant for the azide-carbonic anhydrase complex of 112 ± 6 μM gave an extremely good fit to the data (figure 2b, solid line). By comparison, the conventional way of following azide binding to carbonic anhydrase is to measure the absorbance at a single wavelength, 640 nm. Fitting the same binding equation to the single-wavelength data gave a similar dissociation constant (106 μM) but a much larger standard error (18 μM).

It was noted in the theory section that f'_m is chosen such that it minimizes the sum of the squares of the differences between the actual spectrum and that predicted by equation 2. The validity of this procedure could be questioned as it seems to imply that the zero and unit spectra are known exactly. Obviously, these two spectra are obtained from experimental measurements and will carry as much uncertainty as any of the fractionated spectra. There are two lines of evidence to suggest that this does not really matter.

Which two spectra are chosen as reference spectra is somewhat arbitrary although it is obviously desirable that they are near the extremes of the series. However, we can choose various pairs of reference spectra to see whether these different choices make any real difference. In the analysis reported earlier for the three spectra in figure 2, for which the zero and unit spectra were those obtained at 0 and 999 μM azide respectively, fractionation of the 174 μM azide spectrum gave $f'_m = 0.4737$. However, we could solve equation 2 for $A_{1,\lambda}$ and calculate a new quantity (f''_m) which should equal the reciprocal of f'_m. In effect, we are treating the 0 and 174 μM azide spectra as fixed references and fractionating the 999 μM spectrum. This analysis gave $f''_m = 2.1084$, corresponding

to a value for f'_m of 0.4743. Similarly, treating the 174 and 999 μM azide spectra as references gave $f'_m = 0.4732$. Evidently, it does not make very much difference which of the spectra are treated as fixed references and which as the unknown.

The second point concerns any further analysis to which the f'_m values are subjected. A necessary feature of the procedure we are proposing is that there will be one datum where f'_m is exactly zero and one where it is exactly unity. However, if some binding or other function is then fitted to the entire set of data, these two reference points should not be assigned any special significance. They are treated as if they are no more or less precise than any other datum. This ensures that if one of the reference spectra was faulty resulting in a systematic displacement of all of the other f'_m values, it is the faulty reference which would stand out as a single aberrant point and any further analysis could be adjusted accordingly.

The analysis we are proposing takes an entire series of spectra and describes each of them in terms of two limiting spectra and a single parameter (f'_m) defining the relative amounts of these two limiting spectra. If the dependence of f'_m on the perturbing agent can be accurately described by a theoretical or empirical equation, it is then possible to predict the spectrum at levels of the perturbant which were not examined experimentally. This type of prediction was illustrated by extrapolating the expected spectrum for the protonated form of phenol red (fig. 1b). A similar type of prediction was carried out for carbonic anhydrase, in this case interpolating a spectrum for 346 μM azide which is shown in figure 2c (solid circles) together with an experimental spectrum at this concentration of azide, which is shown by the line in figure 2c. This predicted spectrum imitates the actual spectrum quite well and it must be stressed that this particular experimental spectrum was not used at all in the calculations. The points in figure 2a are a *conformed* spectrum, deliberately chosen so as to give the best overall match to the experimental observations. By contrast, the points in figure 2c are a *predicted* spectrum which was calculated independent of the experimental spectrum.

We regard this ability to predict a spectrum as a useful benefit of the procedure described in this report. However, the primary purpose is to characterize the quantitative effect of perturbing agent which promotes a spectral change and our procedure has two important features. First, it uses all the spectral data whereas common practice has been to follow the absorbance at just one or two selected wavelengths. Second, unlike analyses which have been proposed before[1–7], the method described here makes no assumptions about the mathematical form of the relationship between the amount of perturbant and the extent of the spectral change; rather it is this relationship which may be deduced from the analysis.

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Short Communications

Short axon ganglion cells in the chick retina

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Summary. Using Golgi's staining technique, we investigated some ganglion cells whose axons do not project out of the retina area. These axons, after following a short trajectory through the optic nerve fiber layer or through the 5th stratum of the inner plexiform layer (IPL), change their direction and end in the inner stratum of the IPL.
Key words. Retina; ganglion cells; axon; chick.

The cytological organization of the vertebrate retina is basically considered to consist of five major classes of neurons with different locations and function[1–4]. However, the classic neuronal disposition pattern sometimes presents morphological variations, shown by the displaced location of some of the neurons.

The displacement of retinal cells has been the object of several descriptive studies; for example, of ganglion cells in the