Chem. Soc. 92, 3824.
Shibahara, T. (1973), *J. Chem. Soc., Chem. Commun.,* 864.
Siiman, O., Young, N. M., and Carey, P. R. (1974), *J. Am. Chem. Soc. 96,* 5583.
Spiro, T. G., and Strekas, T. C. (1974), *J. Am. Chem. Soc. 96,* 338.
Tomimatsu, Y., Kint, S., and Scherer, J. R. (1973), *Biochem. Biophys. Res. Commun. 54,* 1067.
Vaska, L. (1963), *Science 140,* 809.
White, D. A., Solodar, A. J., and Baizer, M. M. (1972), *Inorg. Chem. 11,* 2160.

The Binding of Azide to Human Methemoglobin A_0. Error Analysis for the Interpolative and Noninterpolative Methods†

A. D. Barksdale, B. E. Hedlund, B. E. Hallaway,* E. S. Benson, and A. Rosenberg

ABSTRACT: The binding of azide to human methemoglobin A_0 has been studied at 6°, pH 7, and $I = 0.2$ by three spectroscopic methods: (1) the conventional interpolative method, (2) an interpolative dialysis technique, and (3) a noninterpolative method. The interpolative methods assume that the fractional spectral change equals the fraction of heme sites bound by ligand, while the noninterpolative method measures the extent of binding directly, i.e., without the interpolative assumption. Both experiment and error analysis show that method 1 has low precision, and consequently, gives an inherently unreliable binding isotherm. Method 2 achieves high experimental and intrinsic precision. However, method 3, which also has high precision, clearly proves that the interpolative assumption of method 2 is incorrect. That is, the true fractional extent of binding becomes equal to the fractional spectral change only after about 97% of heme sites have been bound with ligands.

Recently, studies of the interactions of ferrihemoglobin A (Hb^+) with anionic ligands, none of which bind to ferrohemoglobin itself, have become especially germane, in that an understanding of these processes should increase our understanding of the in vivo function of ferrohemoglobin, especially in light of the reported crystallographic near-isomorphism of oxyferrohemoglobin and ferrihemoglobin (Perutz et al., 1974).

A review of the literature concerning the binding of azide to Hb^+ is particularly disconcerting. That is, several laboratories have reported substantially dissimilar estimates of the cooperativity of the binding process, although the experiments were conducted under essentially similar conditions. To wit: Banerjee et al. (1973) have reported a Hill coefficient ("n" value) of about 1.5 at pH 6, which indicates that the binding process may be somewhat cooperative (i.e., $n > 1$). In sharp contrast, Epstein and Stryer (1968) find $n = 1$ independent of temperature and pH (from 5.5 to 9.5). These results suggest that the binding of azide to Hb^+ might be noncooperative (four independent heme groups). All of these sets of Hill coefficients were obtained at moderate ionic strengths (0.05–0.1) and temperatures (10–25°) and should, therefore, have been more nearly in agreement, especially in the cases where the pH was the same.

In view of the discrepancies in Hill coefficients in the literature and in our own laboratory (vide infra) for the binding of azide to Hb^+, we have undertaken an investigation of the usual interpolative tube method for determining the Hb^+-N_3^- isotherm.

The interpolative tube method consists of this series of steps: a set of tubes containing a constant amount of Hb^+, buffer, and total azide concentrations of sufficient span to assure total azide/heme ratios of 0 to about 50 is incubated until equilibrium is reached. After incubation, the absorbance of these solutions is measured at an appropriate wavelength at the temperature of incubation. The ligand bound per heme is equated to the fractional spectral change, Y, which, in turn, is interpolated from the absorbance changes by the relationship

$$Y = (A_s - A)/(A_s - A_0) \quad (1)$$

where A_s, A_0, and A represent the absorbances at saturating, zero, and intermediate concentrations. The free azide concentration is subsequently calculated by

$$[L]_f = [L]_t - Y[Hb^+] \quad (2)$$

with $[L]_f$, $[L]_t$ = concentrations of free and total ligand, respectively, $[Hb^+]$ = concentration of protein in moles heme per liter, and with the product $Y[Hb^+]$ being taken as the concentration of bound ligand.

We have addressed ourselves to the following possible weaknesses in the interpolative tube method. First, does the increase in absorbance truly represent ligand binding alone, or can that increase also have contributions from other sources? That is, may the fractional spectral change, Y, be correctly equated to the fraction of heme ligated? Second, is the concentration of free ligand correctly and precisely described by eq 2?

† From the Stone Memorial Laboratories and the Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota 55455. *Received January 20, 1975.* Research supported by National Institutes of Health Grant HL-16883 and by the Minnesota Medical Foundation.

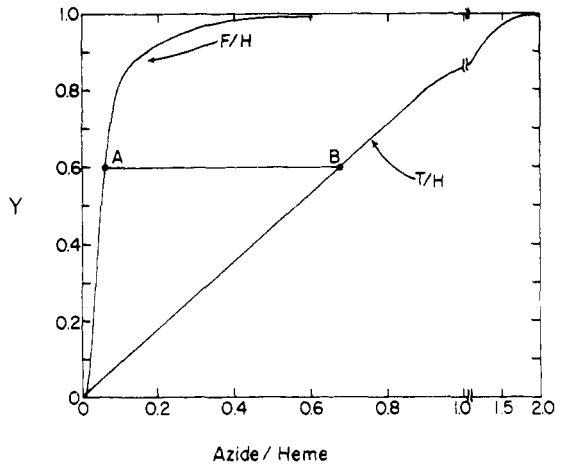

FIGURE 1: Schematic representation of the noninterpolative method. Fractional spectral change, Y, plotted as a function of both free azide per heme (F/H) and total azide per heme (T/H). Azide bound per heme = horizontal distance between the F/H and T/H curves, e.g., between points A and B.

To find answers to these questions, we have investigated the binding of azide to ferrihemoglobin in additional ways, two experimental and one mathematical. The first experimental method consists of dialyzing Hb^+ against large volumes of an azide solution whose concentration, by virtue of the exhaustive nature of the dialysis, exactly equals the concentration of free azide in equilibrium with the Hb^+-N_3^- complex. Therefore, in this, the interpolative dialysis method (interpolative because the fractional ligand binding continues to be equated to the fractional spectral change) the errors and assumptions of eq 2 are avoided.

A second experimental technique, the noninterpolative method, gives a direct measure of the azide bound per heme without assuming any proportionality between spectral change and extent of binding. As Figure 1 indicates, the method is, in principle, straightforward: two experimental curves are generated, one of total azide per heme, another of free azide per heme, both plotted against Y. Then, at one value of Y, i.e., at one point in the titration, the azide bound per heme is simply the distance between the two curves (e.g., between points A and B in Figure 1). The isotherm is completed by calculating the concentration of free azide at the same Y from the known experimental relationship between Y and $[N_3^-]_f$ established in the dialysis experiments.

Finally, whatever method used to establish the isotherm must withstand rigorous error analysis which will reveal the limits of precision inherent in that method. These limits may not always be apparent, and may frequently be masked by excellent reproducibility.

Experimental Methods

Hemoglobin Preparation. Hemoglobin was prepared from 21 day old red cells by either the ammonium sulfate method (Rossi-Fanelli et al., 1961) or by the method of Hedlund et al. (1972) in which xylene was substituted for ethyl ether. Salt and organic solvent were removed by passage through a Sephadex column and organic phosphates were removed by means of a mixed bed ion exchange column. The hemoglobin contained less than 0.05 mol of inorganic phosphates/mol of hemoglobin tetramer at this stage. The minor components of human hemoglobin were removed by fractionation of DEAE-Sephadex A-50 (Williams and Tsay, 1973). The final preparation of human hemoglobin A_0 contained only traces of other components as determined by isoelectric focusing. The oxyhemoglobin contained less than 2% methemoglobin at this stage.

Methemoglobin A_0 was prepared by oxidation with 4 equiv/heme of potassium ferricyanide for 1 hr at room temperature. The ferricyanide was removed by passing the solution through a Sephadex G-25 column. This preparation showed the normal spectral characteristics discussed by Cameron and George (1969).

The concentration of methemoglobin was determined spectrophotometrically as either the cyanide derivative at 419 nm or as the azide derivative at 540 nm. Extinction coefficients for both derivatives are given by Antonini and Brunori (1971, p 45).

Reagents and Solutions. Azide solutions (1 M) were prepared by weight (±0.001 g) from one sample of the Fisher "Purified" reagent, and all other solutions were made by serial dilution of aliquots of stock. In no case were more than two dilutions used to obtain any desired azide concentration. Dilutions were made with phosphate buffer solutions of pH 7.00, $I = 0.2$ at 6°.

Conventional Interpolative "Tube" Method. A number of tubes containing solutions of methemoglobin (concentration *ca.* 0.1 mM in heme) in sodium phosphate buffer (pH 7.0, $I = 0.2$) were incubated with varying amounts of azide (10^{-7}–10^{-2} M) for a period of about 24 hr at 6°. At this time, no further change in the spectrum was observed when the solutions were scanned at 6° on a Cary 15 recording spectrophotometer. Isosbestic points were observed at 517 and 604 nm. The fractional spectral change, Y, was calculated by eq 1 from the increase in absorbance at 572 nm (Banerjee et al., 1973) where the maximum spectral change occurs.

Interpolative Dialysis Method. Aliquots of methemoglobin were dialyzed at 6° for ca. 48 hr against buffer containing known concentrations of azide. The concentration of protein was about 0.1 mM in heme. The buffer solutions were changed several times to ensure that the free concentration of ligand inside the tubing was the same as the known concentration of ligand outside the tubing.

After the dialysis, the dialysates were scanned at 6° from 700 to 480 nm. The observed isosbestic points were slightly less sharp in these scans than in those for the tube method, presumably because of light scattering in some samples. The contribution of light scattering to the observed absorbance could be quantitated and corrected for, and the corrections amounted to less than 2% of the total absorbance. As in the tube method, Y was calculated from the absorbance increase at 572 nm. The highest concentration of free azide was 1 mM.

Methods of Data Analysis

Propagation of Errors. Whenever one quantity is calculated from others, it is necessary to propagate the error in that quantity, knowing the errors in the quantities from which the calculation was made. Thus, in general, if

$$x = f(p,q,\ldots) \tag{3}$$

then the error in x is propagated as (Bevington, 1969, p 59),

$$S_x^2 = (\partial f/\partial p)^2 S_p^2 + (\partial f/\partial q)^2 S_q^2 + \ldots \tag{4}$$

where an S represents a standard deviation.

Weighting of Data Points. In all of the data fitting procedures described below, each data point was assigned a weight (Bevington, 1969, p 188)

Table I: The Interpolative Tube Method: Log ($[N_3^-]_f$) and Its Standard Deviation.

$[N_3^-]_t$ (μM)[a]	Y[a]	Log ($[N_3^-]_f$)[b]	$S_{\log([N_3^-]_f)}$[c]
1	0.01	−6.83	5.90
10	0.10	−6.00	0.85
30	0.30	−5.50	0.32
50	0.50	−5.27	0.27
75	0.74	−5.07	0.21
100	0.90	−4.68	0.09
200	0.99	−3.95	0.02

[a] Representative data obtained at pH 7.0, I = 0.2, 6°. [b] $[N_3^-]_f$ calculated (in molarity) from eq (2) with $[Hb^+] = 1 \times 10^{-4} M$ in heme. [c] Calculated from eq 4–6, with $S_{[Hb^+]} = 1 \times 10^{-6} M$ in heme, S_Y = 0.005, and with a 1% standard deviation in $[N_3^-]_t$.

$$W_i = (1/S_i^2)/Q \quad (5)$$

where W_i and S_i are the weight and standard deviation of the ith point, and Q, the average weight, is given by

$$Q = (1/N)\Sigma(1/S_i^2) \quad (6)$$

with N = number of data points.

Interpolative Binding Isotherm: Tube Method. The fraction spectral change at 572 nm was equated to the fraction of heme groups ligated (the interpolative assumption). Errors in Y were propagated from the errors in absorbance readings (typically, about 0.002 absorbance unit including the observational error in the reading itself and in the base line measurement). The concentration of free azide was calculated from eq 2 and the error in free azide concentration from eq 4. Errors in total azide concentration and in heme concentration were arrived at by propagation of errors in weight and dilution, and for the protein concentration, estimates of the error in extinction coefficients and absorbance readings. Because the binding isotherm consists of Y vs. log ($[N_3^-]_f$), errors in log ($[N_3^-]_f$) were propagated from the errors in $[N_3^-]_f$.

Interpolative Binding Isotherm: Dialysis Method. As discussed earlier, the free azide concentration at each Y was set equal to the azide concentration in the dialysis solution. Consequently, the only error in the free azide concentrations in the dialysis experiments is that due to dilution.

Noninterpolative Binding Isotherm. Total azide per heme (T/H), Y pairs were calculated from the input and output of the tube method. Similarly, free azide per heme or (F/H), Y pairs were determined from the input and output of the dialysis experiments. Errors in T/H or F/H were propagated by the appropriate form of eq 4.

Since it was nearly impossible to obtain both T/H and F/H points at exactly the same value of Y, the following curve fitting procedure was used to generate the noninterpolative isotherm. First, the T/H points were transformed to log (T/H) with error propagation, and weighting of each log(T/H) point by eq 5. Then the weighted log (T/H), Y pairs were fitted to an orthogonal least-squares polynomial of the form[1]

$$\log(T/H) = a_0 + a_1 Y + a_2 Y^2 + \ldots . \quad (7)$$

for Y = 0.1–0.9. It was necessary to restrain Y to this range because the fitting procedure became very imprecise at Y greater than 0.9 due to the sudden and sharp curvature of the log (T/H), Y plot (Figure 3). The analogous fit was obtained for log (F/H). In both cases, a fourth degree polynomial gave the best fit.[2] We then calculated the two log quantities at the same value of Y over the range Y = 0.1–0.9, such that the azide bound per heme, V, at a given Y could be calculated as

$$V = \exp(2.303 \log'(T/H)) - \exp(2.303 \log'(F/H)) \quad (8)$$

where log′ (T/H) and log′ (F/H) indicate the calculated rather than observed quantities. The standard deviations of log′ (T/H) and log′ (F/H) were calculated from a fit to Y of the residuals in the original fittings. These standard deviations were subsequently propagated by eq 4 and 8 to yield the standard deviation of V at the given Y.

Finally, the information available from the dialysis method (log ($[N_3^-]_f$) as a function of Y) was fitted as above for log (T/H) or log (F/H) and the resultant polynomial was used to calculate log ($[N_3^-]_f$) at each Y for which a value of V, the azide bound per heme, was calculated.

Hill Plots. Each of the three isotherms was subjected to the Hill plot (Hill, 1910; Antonini and Brunori, 1971, p 164) to determine the apparent degree of cooperativity of the binding process. Thus, each X (X = a calculated V or an observed Y), log ($[N_3^-]_f$) pair was transformed to a log ($X/(1 - X)$), log ($[N_3^-]_f$) pair with error propagation. The weighted log ($X/(1 - X)$), log ($[N_3^-]_f$) pairs were fitted to a first degree least-squares program. The slope of the fit and its standard deviation then became the Hill coefficient n, and its standard deviation.

Results and Discussion

Application of error analysis to the tube method (viz., Table I) reveals inherent errors in the free azide concentration (calculated by eq 2) which become, as expected, very large at low $[N_3^-]_f$. In our own experience, the magnitude of the inherent error may not always manifest itself as scatter in the experimental data because a particular worker may be extremely consistent in his or her experimental methods and procedures over a long period of time and thereby produce a seemingly smooth and precise isotherm. For example, two of us, using the experimental techniques described above, measured Hb^+-N_3^- interpolative tube isotherms which, in one case, gave an n of 1.6 but in the second, gave an n of 0.97, i.e., essentially duplicating all the literature estimates of the degree of cooperativity for this system. The error analysis (Table I) clearly demonstrates that our own two seemingly disparate isotherms are in fact identical within the error of the method.

Therefore, the large inherent error in the tube interpolative method probably accounts for the discrepancies in Hill coefficients in the literature for this system. Although our binding experiments and their results were confined to one wavelength (572 nm), and, as Banerjee et al. (1973) suggest, n may vary with wavelength, nevertheless, it is highly probable that the interpolative tube method brings to

[1] The orthogonal least-squares polynomial fitting program was obtained from the University of Minnesota Computer Center program library. The computer center had in turn received the program, "LSFITW", from NBS. "LSFITW" seems to use Legendre polynomials in a manner essentially similar to that described by Bevington (1969, p 155) but in Basic rather than Fortran language.

[2] Not only did a fourth-order polynomial give a reduced χ^2 of about 1, indicating that our fitting function was a reasonably accurate representation of the parent data population (Bevington, 1969, p 188) but also a fifth or higher order fit gave a incremental F ratio of less than 1, indicating that terms beyond degree of 4 are unwarranted (Bevington, 1969, p 201). In fact, higher order fits began to follow the data points, rather than passing smoothly through them.

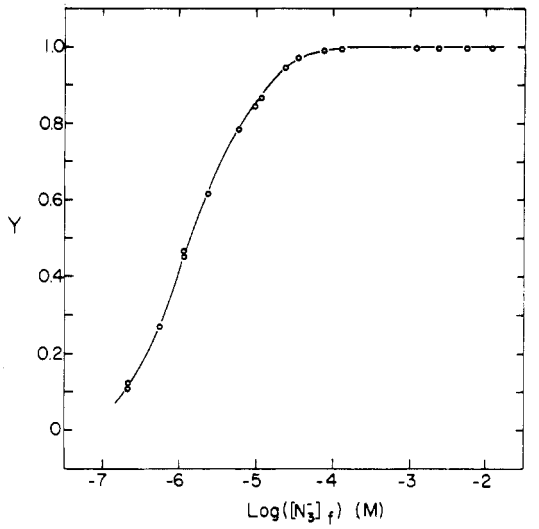

FIGURE 2: Hb^+-N_3^- binding isotherm measured by the interpolative dialysis method. Experimental conditions: pH 7.00, I = 0.2 (sodium phosphate buffer), $[Hb^+] \sim 10^{-4}$ M, 6°.

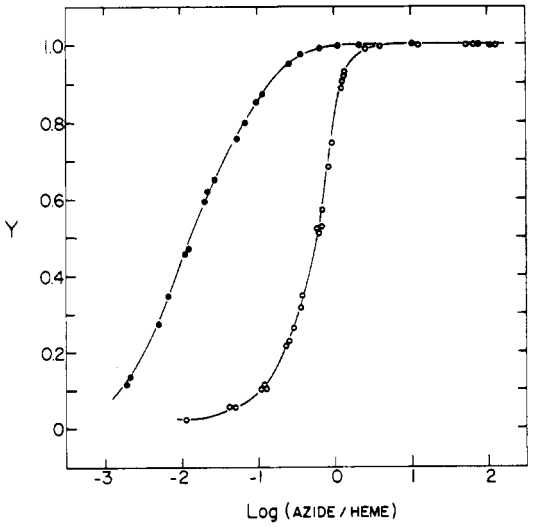

FIGURE 3: Raw data for the noninterpolative method. (●) Log (F/H); (○) log (T/H). Experimental conditions as in Figure 2.

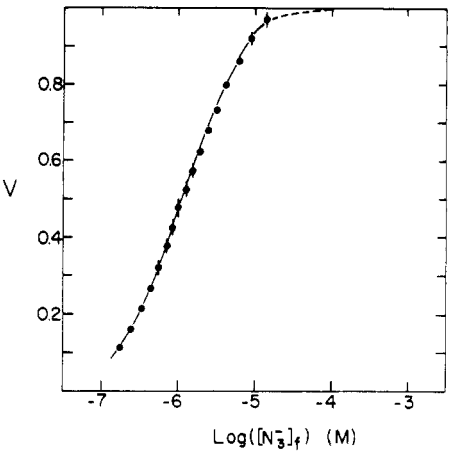

FIGURE 4: Hb^+-N_3^- binding isotherm from the noninterpolative method, calculated as described in text from data in Figure 3. (●) Values of V calculated for Y = 0.1-0.9 in 0.05 increments. Error bars indicate ±1 SD of V. Points having no error bars have standard deviations masked by the closed circle. (- - -) Suspected approach of V to 1.0.

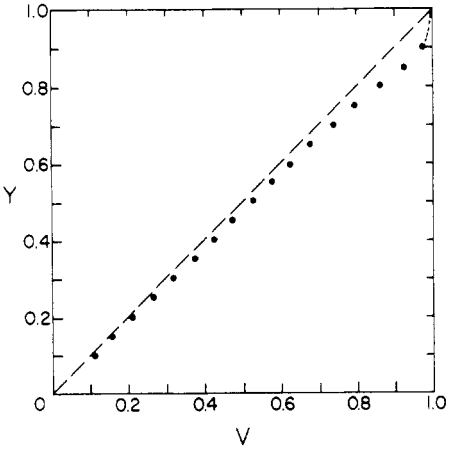

FIGURE 5: Comparison of the interpolative and noninterpolative isotherms. (●) Y = fractional spectral change vs. V = fractional ligand binding at the same log ($[N_3^-]_f$). (– –) Expected relationship between V and Y if the interpolative assumption was valid. (- - -) Inferred relationship between V and Y as both approach 1.0 (see footnote 3).

any wavelength the poor precision it possesses in our experiments. Clearly this method gives an intrinsically unfocused picture of the binding of azide to methemoglobin.

Interpolative Isotherm by the Dialysis Method. Figure 2 displays the isotherm obtained from the interpolative dialysis method. Error analysis reveals any inherent standard deviation of 0.005 for Y and of about 1% for each $[N_3^-]_f$, which translates into an almost constant error of 0.03 in log ($[N_3^-]_f$). Therefore, the dialysis method has a high and essentially constant precision for all Y, log ($[N_3^-]_f$) pairs, as reflected in the smoothness and reproducibility of the isotherm.

The weighted Hill plot for the isotherm in Figure 2 yields an n of 0.96 ± 0.03. Because the isotherm is precisely known, the Hill coefficient derived from that isotherm is, consequently, an accurate estimate of the cooperativity in the binding of azide to ferrihemoglobin, given the interpolative assumption, and the limits of the Hill model.

To summarize: if the interpolative assumption (that the increase in absorbance behaves linearly with the extent of heme ligation) is correct, then the dialysis method gives unequivocal results of high inherent reliability from which a clear picture of the ligand course of the binding process may be developed. Without doubt, this method, though it requires more labor and materials, is strikingly better than the conventional tube method.

The Noninterpolative Isotherm. The raw data from which the noninterpolative isotherm was derived appear in Figure 3. These data were treated as described in Experimental Methods.[3] The resultant isotherm is shown in Figure 4.

From the weighted Hill analysis, we find a Hill factor of 1.100 ± 0.008, a value which is substantially higher than

[3] As discussed in Experimental Methods, the calculation of V was limited to values of Y no greater than 0.9. At Y = 0.9, V = 0.973, which is the highest point in Figure 4. Because this upper limit in Y is imposed by the exigencies of obtaining a good fit at lesser values of Y, and because at Y greater than 0.9, the error in V becomes large (i.e., the difference between free ligand and bound ligand becomes small), we can only infer the relationship between Y and V as they both approach 1. (We know with certainty (Figure 2), that Y = 1 at 10^{-4} M free azide): hence the dotted lines in Figures 4 and 5.

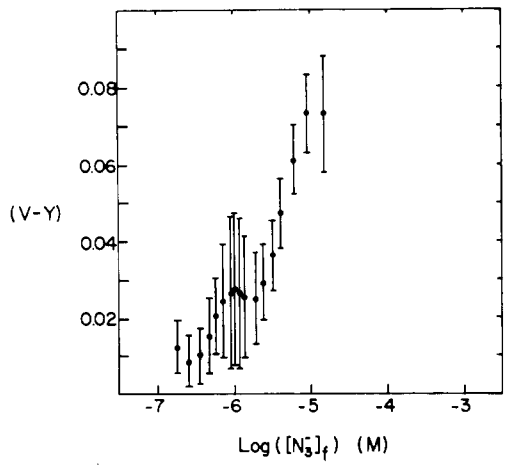

FIGURE 6: The difference between fractional binding and fractional spectral change as a function of log ($[N_3^-]_f$). Error bars represent ± 1 SD in the difference ($V - Y$).

the n factor from the Hill plot of the interpolative dialysis isotherm. That the Hill coefficients differ, and that the difference (1.100 ± 0.008 for the noninterpolative isotherm *vs.* 0.96 ± 0.03 for the interpolative dialysis isotherm) lies well outside error limits, gives good indication that the two isotherms differ in shape.

Since the noninterpolative method affords a direct measure of the extent of heme ligation by azide, while the interpolative method assumes a linearity between absorbance and degree of binding, it follows that, given the high inherent reliability and reproducibility of the results which go into the construction of both isotherms, any differences between the information given by the two isotherms must be caused by or related to the interpolative assumption.

For purposes of comparing the interpolative and noninterpolative isotherms, we have, in Figure 5, plotted Y, V pairs at the same log ($[N_3^-]_f$).[3] That the differences between Y and V lie beyond experimental error is demonstrated in Figure 6, in which we have plotted the difference ($V - Y$) with its error, as a function of log ($[N_3^-]_f$).

Figures 5 and 6 provide convincing evidence that Y, the fractional spectral change, equals V, the fraction of sites bound, only at some free ligand concentration greater than that at which essentially all (i.e., 97%) of all the heme sites have been ligated. In fact, Y lags behind V in a nonlinear fashion over much of the course of binding.

In summary, our findings clearly show that any isotherm in which the degree of binding is equated to the degree of spectral change may ab initio be incorrect and may, with even the most reproducible and inherently precise measurements, lead to a false picture of the binding process, simply because the interpolative assumption may be incorrect.

In closing we offer these brief comments about the cause of the V,Y differences in Figures 5 and 6. It appears that the spectrally observed shift in heme state from met to the met-azide complex may be somewhat out of phase with the fractional azide binding. The reason for this nonlinearity is not clear, but one possibility is the presence of nonheme binding sites, which upon ligand binding shift the equilibrium between the two spectrally observable states. Another possibility is tetramer-dimer dissociation (White, 1975). Further experiments relating to this system are in progress.

References

Antonini, E., and Brunori, M. (1971), Hemoglobin and Myoglobin in Their Reactions with Ligands, Amsterdam, North-Holland Publishing Co.

Anusiem, A. C., Bettlestone, J. G., and Irvine, D. H. (1966), *J. Chem. Soc. A*, 357.

Banerjee, R., Henry, Y., and Cassoly, R. (1973), *Eur. J. Biochem. 32*, 173.

Bevington, P. R. (1969), N.Y., Data Reduction and Error Analysis for the Physical Sciences, New York, N.Y., McGraw-Hill.

Cameron, B., and George, P. (1969), *Biochim. Biophys. Acta 194*, 16.

Epstein, H. F., and Stryer, L. (1968), *J. Mol. Biol. 32*, 113.

Hedlund, B. E., Danielson, C., and Lovrien, R. (1972), *Biochemistry 11*, 4660.

Hill, A. V. (1910), *J. Physiol. 40*, IV–VII.

Perutz, M. F., Fersht, A. R., Simon, S. R., and Roberts, G. C. K. (1974), *Biochemistry 13*, 2174.

Rossi-Fanelli, A., Antonini, E., and Caputo, A. (1961), *J. Biol. Chem. 236*, 397.

White, S. L. (1975), *J. Biol. Chem. 250*, 1263.

Williams, R. C., Jr., and Tsay, K. Y. (1973), *Anal. Biochem. 54*, 137.