LETTER TO THE EDITOR

Artifacts in the Measurement of Diffusion Coefficients for Hemoglobin by Means of Intensity Fluctuation Spectroscopy

Dear Sir:

In a recent paper, LaGattuta et al. (1) presented results and conclusions concerning the concentration dependence of mutual diffusion coefficients for the liganded hemoglobins oxy-HbA and oxy-HbS, which are greatly at variance with previous findings. In particular these authors claim that (*a*) the dependence of the diffusion coefficient (D) on the concentration of hemoglobin (CHb) is nonlinear at pH = p*I* and at both higher and lower values of pH. The structure of the concentration dependence is interesting and significant. (*b*) The intercepts of the plots of D vs. CHb at the low CHb limit exhibit strong pH dependence with D ranging from 7.6×10^{-7} cm^2/s at pH 6.3 to 6.5×10^{-7} cm^2/s at pH 7.4 for oxy-HbA. This corresponds to a change of effective radius from 30.5 Å at pH 6.3 to 35.7 Å at pH 7.4. (*c*) The variance shows a strong dependence on CHb. It ranges from 0.05 to 0.4, and at pH 7.4 is found to increase as CHb approaches zero.

These are striking conclusions, and in view of the importance of Hb as a model system in the study of protein interactions and especially in connection with the sickle cell problem it is imperative that the major differences with earlier studies be understood.

For point (*a*) direct comparisons can be made for the case where pH = p*I*. Previous investigations using quasielastic light scattering including those in this laboratory have found a linear dependence of D on CHb, and as the errors have decreased in more recent work the magnitude of the correlation coefficient in the linear fit has increased (2–5). For example Jones and Johnson (4) found for oxy-HbA and oxy-HbS a linear fit in the concentration range from 0.1 to 37 g/dl with a correlation coefficient of -0.94. The errors in individual points were 3–4% and the variance was ~0.02 at concentrations up to 20 g/dl. In the more extensive studies on unliganded hemoglobin by Hall et al. (5), it was found that $D = D_o[1 - (0.00568 \pm 0.00028)\,\mathrm{CHb}]$ where $D_o = (6.75 \pm 0.05) \times 10^{-7}$ cm^2/s and CHb is measured in units of g/dl. The correlation coefficient was -0.988 and the variance was always <0.04.

The data on which LaGattuta et al.(1) base their conclusions are shown in Figs. 1–5 for D and 6–10 for variance in reference 1. In the plots of D vs. CHb the errors are as large as 15% and there is a lot of scatter. In fact it is difficult to understand how linear fits were ruled out on the basis of these data. These plots should be compared with Figs. 2 and 13 in references 4 and 5, respectively. The plots of variance vs. CHb in reference 1 also show exceptionally large errors, in some cases from 50–100%. Since the variance is an indicator of sample purity at low concentrations, these results suggest that experimental conditions should be reviewed.

LaGattuta et al. (1) report that the Hb samples were prepared by filtering the hemolyzate and then dialyzing. The samples were centrifuged before taking data. Aliquots were drawn from the top of the culture tubes and then sealed in 100λ glass capillary tubes using wax. It has been our experience, which we have reported (4), that filtration is not sufficient to remove membrane fragments from Hb samples, and that chromatographic procedures are necessary. It is important to note that the effectiveness of centrifugation depends on the bulk density of the sample which varies with salt concentration. Centrifugation is also necessary to remove denatured material, but it is almost impossible to transfer centrifuged samples to scattering cells without introducing some dust. After the transfer, the samples must be circulated in a closed loop system through a filter at least as fine as 0.22 μm. The circulating solution can then be inspected through a microscope while under laser irradiation to make sure that contaminants are not being introduced through leaks. Even with this procedure we have found it extremely difficult to remove all dust particles from solutions at very low ionic strengths.

Three additional comments concerning the experimental conditions are in order. An optical path length of 0.3 mm was used to minimize absorption. With such a small path length and the resulting curvature of the cell wall, it is exceedingly difficult to avoid mixing stray light with the light scattered from the hemoglobin. The expected result is heterodyne detection with unknown efficiency. The authors do not indicate how this problem was avoided, though they assume homodyne detection in the analysis. The use of strongly scattering latex spheres for calibration and testing of the instrumentation is not sufficient when dealing with scatterers as small as Hb. Even with the short cell and small sample volume, we are concerned about heating by the laser beam unless the cell wall was held at constant temperature. It is best to extrapolate the results to zero power when strongly absorbing samples must be used. Finally, the met-Hb concentration is likely to be much larger than reported since up to four days elapsed between the met-Hb determination and the light scattering measurements. It should be noted that the conversion to met-Hb is accelerated by concentrating the HbA under nitrogen.

In view of the large errors in D, the large magnitudes of the variance especially at low cencentrations, and the experimental conditions reported by LaGattuta et al., I conclude that the samples used in reference 1 were contaminated with membrane fragments, dust, or other particulate matter. This would explain the increase of the variance in some cases as CHb approached zero. For whatever reasons, the data presented do not have sufficient accuracy to establish that the dependence of D on CHb is nonlinear. In all cases straight lines appear to fit the data as well as the curves calculated by LaGattuta, et al., using Phillies' theory (6, 7). As far as the magnitude of D is concerned, it is not clear in reference 1 how the results were corrected to standard conditions. Since the viscosity depends on the concentration of NaCl, this correction could be quite important.

It is argued in reference 1 that the dependence of D on both pH and ionic strength should be investigated. This is an important point since intermolecular interactions may be quite different away from the isoelectric point, and the range of these interactions is expected to depend on the ionic strength. However, for such studies to have validity the errors must be reduced to the level commonly expected for intensity fluctuation spectroscopy, i.e., 3% or less. Unfortunately, the level of errors shown in reference 1 is so large that the data do not provide suppport for the conclusions in points (b) and (c) concerning the pH dependence of D and the variance.

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