Analytical Ultracentrifugation with Absorption Optics and a Scanner–Computer System. Applications to Molecular Weight Measurements on Interacting Systems†

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ABSTRACT: Experiments on the measurement of the tetramer–dimer equilibrium for hemoglobin are described using an on-line computer system in conjunction with an analytical ultracentrifuge and absorption optics. The computer system includes the elements described earlier (R. H. Crepeau, S. J. Edelstein, and M. J. Rehmar (1972), *Anal. Biochem. 50,* 213) with the addition of a disk unit for on-line data analysis, an *x–y* plotter for data display, and a modified triggering system using pulses from the rotor collar. Values of optical density *vs.* position in the rotor are measured at sedimentation equilibrium, with the computer collecting one transmittance point per revolution per cell. In a normal experiment 5000 transmittance points are collected from each sample–reference pair. The data are averaged in groups of 50 pairs of points and the logarithm of the ratio of reference to sample transmittance is computed to leave a set of 50 final values of radius, optical density, and standard deviation. Values of optical density in the range of 0.01–2.0 can be recorded with standard deviations, usually about 0.01 or less. Under conditions examined the data for carboxyhemoglobin can be described in terms of two exponentials, corresponding to tetramers and dimers; the coefficients of the exponential terms yield a dissociation constant, $K_{4,2} = 1.1 \times 10^{-6}$ M with a standard deviation of 0.05×10^{-6} M. Differences in the dissociation constant are observed in the presence and absence of inositol hexaphosphate; addition of the organic phosphate effector results in a 50-fold reduction of the dissociation constant for carboxyhemoglobin. Studies on hemoglobin S, the mutant form of hemoglobin obtained from individuals with sickle cell disease, indicate a dissociation constant in the carboxy form that is identical with that of normal hemoglobin within the limits of the accuracy of the measurements.

The range of experimental measurements that can be performed on interacting systems by analytical ultracentrifugation has been substantially enhanced by using an on-line computer system in conjunction with the absorption optical system (Spragg and Goodman, 1969; R. Cohen, private communication; Pekar *et al.*, 1971; Crepeau *et al.*, 1972). In the first paper of this series (Crepeau *et al.*, 1972), an on-line system was described which collects a point on each revolution of the rotor from each cell that is present and applications of the system were described for molecular weight measurements on simple, noninteracting systems. The system had a number of advantages in convenience of data handling and analysis, as well as improved performance, based on (1) replacement of mathematical operations performed with analog electronics by digital processing; (2) availability of precise statistical indices obtained from evaluating a large number of data points; (3) extension of the range of OD over which reliable data could be collected. More recent work involving use of the scanner–computer system for molecular weight measurements on interacting systems has revealed that the system is especially advantageous for such studies. The ability to examine up to 15 solution–reference pairs (in a six-hole rotor with five multichannel cells and one counterbalance) simultaneously and process the data rapidly to obtain M_w *vs.* concentration has permitted experiments of a scope and precision previously unavailable. The advantages are particularly striking when compared to the laborious processes previously required: reading data from recorder traces or fringe diagrams, converting the data to a form for entry into a computer, and awaiting analyses from a computer facility. In this paper studies on the tetramer–dimer equilibrium reaction of hemoglobin are described with emphasis on the techniques involved. The aspects described here will include additions to the original systems (Crepeau *et al.*, 1972) to improve operation and the use on-line of a fitting program based on sums of exponentials (Edelstein *et al.*, 1970) to analyze sedimentation equilibrium data for dissociation of hemoglobin and directly yield tetramer–dimer dissociation constants.

Methods

Design Considerations. Since the first description of the computerized ultracentrifugation system developed in this laboratory (Crepeau *et al.*, 1972), some modifications in the basic system have been introduced. Figure 1 shows a block diagram of the equipment currently in use. The output of the ultracentrifuge scanner and multiplexer must be passed to the computer in a useful and convenient form. The scanner produces the photomultiplier pulses which contain all the information concerning the absorption of the solutions within the spinning rotor. The multiplexer provides complicated signals which can be used to identify and separate the output from the scanner into its components from each cell in a multi-hole rotor. The commercial equipment described in Figure 1 consists of a Beckman Model E ultracentrifuge with scanner, Data General Nova computer with 20K memory, 10 bit analog-to-digital converter (A/D), Honeywell *x–y* plotter, Centronics high speed printer, Diablo disk system, Tektronics oscilloscope for display pur-

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poses, and ASR 35 teletype. The complete system is available from Comptek Research Inc., Buffalo, N.Y.

The block diagram of the computer centrifuge system in Figure 1 illustrates how this equipment is configured to produce a useful system. Amplified photomultiplier signals are converted into digital form by the A/D converter and transmitted to the computer. These pulses consist of the amount of light transmitted through the sample and reference sides of up to five double-sector cells and measurements of the dark voltage or base line. All information needed to identify and separate these photomultiplier pulses is obtained from the Schmitt pulses (S1 and S2) present in the multiplexer of the Model E. This information consists of cell and sector identification, start pulses for the A/D converter, and a revolution count pulse for the computer.

The cell count information is coded in the form of an interrupt request pulse passed to the computer for each hole in the rotor. This information is derived from the rotor collar pulses and, due to the orientation of this collar, is generated before the photomultiplier pulses for the corresponding cell. Additional information, required to identify each rotor hole, is a pulse once per revolution to identify rotor hole number one. This pulse is also used as a revolution counter which, in connection with the known position of the counterbalance holes and a constant scan speed, allows calculation of the radius position of the scanner throughout the scan. A real time clock is currently being implemented for the purpose of timing the scan and later calculating the radius of each point collected. This device will also make it possible to easily determine the accurate revolutions per minute of the rotor.

The A/D converter requires an external signal, the start pulse, to start the conversion process at a time when the desired signal from the photomultiplier is at the input of this device. In our present apparatus, the start pulse is generated by the interface equipment from the rotor collar pulses as indicated in the block diagram. In our original design of this system the start pulses were obtained from the Schmitt pulses present at the scanner test point. This design required the photomultiplier signals to be above the discrimination level of the scanner before they could be seen by the computer. This restriction, while not severe if there is adequate light, made observation of high optical densities under conditions of low light intensity difficult. The present design ensures that the A/D converter is able to provide unambiguous sample and reference pulses at all optical densities since the collar pulses are always available. This development is also necessary to eliminate the scanner entirely when alternate optical systems are used such as the laser scanner system (Crepeau *et al.*, 1973).

The sector identification information is passed to the computer by an additional two bits of information accompanying the 10 bit result of the A/D conversion. One bit identifies either the sample or reference pulse and the other provides discrimination between the signal or the dark response of the photomultiplier. As an example, a bit configuration of 11 would identify the sample dark signal. The origin of this sector information is also the rotor collar making it independent of the presence of transmitted light.

Computer Programs. The collection program in the Nova computer correlates the incoming information during a scan and produces a permanent record of optical density, radius, and standard deviation of each point for later reference on the tape cassette and on the disk system. One scan can give sufficient data to allow analysis of up to 15 solu-

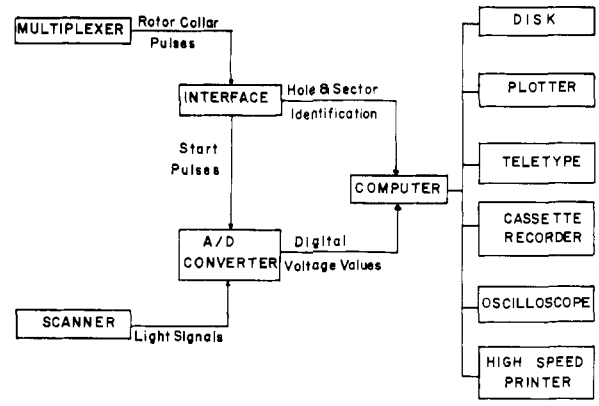

FIGURE 1: A block diagram of the apparatus used to collect, analyze, store, and output data from a Beckman Model E ultracentrifuge.

tion–reference pairs when using five Yphantis cells in a six-hole rotor. The computer is programmed to output these data to the teletype, high speed printer, plotter, or oscilloscope as desired by the operator.

The plot program allows the user many options for presenting that data in graphical form. After reading in previously collected data from cassette, the program requests a range of points to be plotted and then requests an option number. The currently available options are as follow:

1 Fetch new data
2 Select a new fit range
4 Take the log of the optical density and square the radius
8 Plot the x and y axes
16 Print the axes calibration on the high-speed printer
32 Allow the user to scale the axes
64 Retain the previous axes scale
128 Direct the plot to the oscilloscope display
256 Force the y axis to have the value $y = 0$
512 Smooth random noise from the data

These options may be combined by adding the corresponding numbers of the desired options since each number corresponds to changing one additional binary bit from 0 to 1. The convenience of this program will be illustrated by an example. The first option requested would be 1 to recall new data from cassette and select a fit range. (This is automatic for new data.) The next option could be a $392 = 8 + 128 + 256$. This would give a quick display of optical density *vs.* radius on the oscilloscope display screen. Since 256 is contained in that sum, the origin has the value OD = 0 and any base line present in the data is seen immediately. At this time if we wish to modify the fit range to omit points beyond the meniscus or too close to the bottom of the cell to give meaningful data, a mode 2 is appropriate. Next a mode $140 = 4 + 8 + 128$ will produce a plot of log OD *vs.* radius squared on the oscilloscope to allow an estimate of a dissociation constant or a measure of the purity of the sample. A pure single molecular weight species (ideal) would produce a straight line on this plot. Finally, if a permanent copy is desired, these two plots would be directed to the x–y plotter and a copy of the coordinates directed to the high speed printer. This plotting program has proven very useful in evaluating the quality of the collected data and the suitable range of points to be directed to the fitting program.

Calculation of Dissociation Constants. Data for sedimentation equilibrium experiments are collected and screened as described above and then fit to an equation of the form

TABLE I: Sample Data from Computer High-Speed Printer Output for HbA.

Point No.	Radius	OD	σ
1	6.122	1.853	0.0581
3	6.113	1.504	0.0161
5	6.104	1.227	0.0120
7	6.095	0.972	0.0105
9	6.087	0.769	0.0090
11	6.078	0.622	0.0080
15	6.061	0.409	0.0067
20	6.039	0.244	0.0053
25	6.017	0.151	0.0046
30	5.995	0.099	0.0043
35	5.973	0.062	0.0044
40	5.951	0.041	0.0046
45	5.930	0.029	0.0046

$$\text{OD} = \text{base line} + A\exp(\alpha M_i r^2) + B\exp(2\alpha M_i r^2)$$

where $\alpha = 2RT\omega^2/(1 - \bar{v}\rho)$ and M_i = molecular weight of monomer in a monomer-dimer equilibrium or M_i = dimer weight in a dimer-tetramer equilibrium. The constants A, B, and the base line were calculated by a least-squares fit or, if the base line had been measured by overspeeding, then it was subtracted from the data and only A and B were calculated. The base line for the experiments reported here was measured to be zero and hence only A and B are calculated. The dissociation constant is then found from

$$K_{4,2} = [\text{dimer}]^2/[\text{tetramer}]$$

where [dimer] = $A/1000\epsilon$ and [tetramer] = $B/1000\epsilon$. Here ϵ is the heme millimolar extinction coefficient for the path length of the cell (usually 1.2 cm) and the wavelength used (405 nm in all the experiments reported here). Solving for $K_{4,2}$ the following relationship is obtained: $K_{4,2} = A^2/1000\epsilon B$. The program for this least-squares fitting procedure was written in Fortran 4 and was based on a general multiple linear-regression fit to a function which is linear in its coefficients (Bevington, 1969). The program also provides three statistical parameters: the coefficient of determination, the chi-square parameter, and the F-test. The coefficient of determination is a measure of how well the data can be fit by the given equation. The chi-square test determines if the scatter in the data can be explained by the known standard deviation of the points, and the F-test is a measure of the need for all the fitting parameters. A standard deviation of the $K_{4,2}$ is also calculated by this program from the input standard deviations of the initial points. The program was written using the Data General disk operating system which makes Fortran programming relatively simple and fast. In addition to calculating the dissociation constant, the program also outputs to the high-speed printer the calculated values for OD and the apparent molecular weight as a function of concentration.

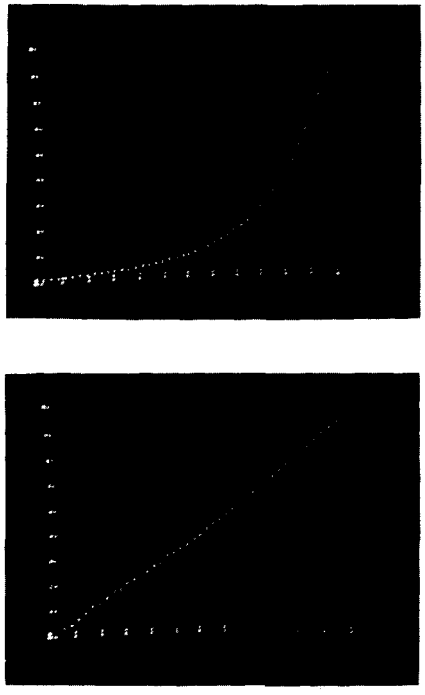

FIGURE 2: Photograph of oscilloscope plot of CO-HbA data displayed by the plot program: (upper plot) OD plotted as a function of radius; (lower plot) log OD *vs.* radius squared.

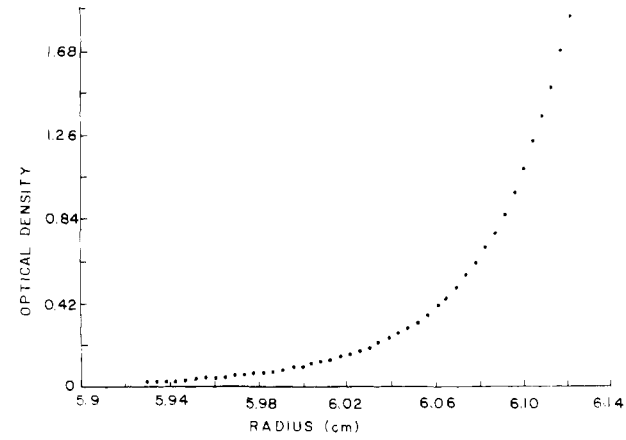

FIGURE 3: Reproduced computer plot on x–y plotter of CO-HbA data. OD plotted as a function of radius.

Results

Measurement of Tetramer–Dimer Equilibrium Constant for Hemoglobin. To illustrate the on-line data collection and analysis the steps required to obtain a $K_{4,2}$ value from

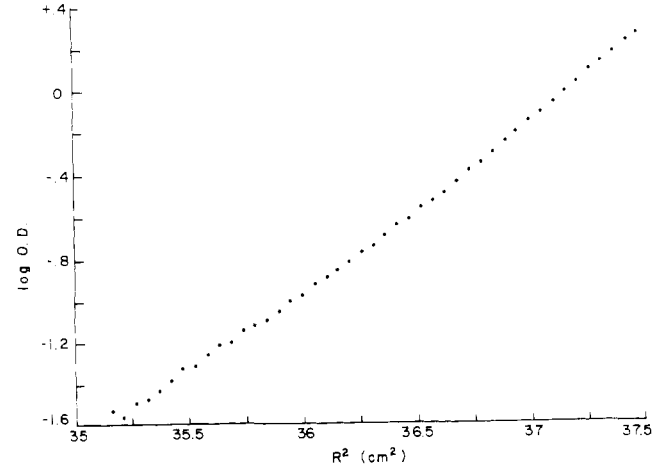

FIGURE 4: Reproduced computer plot of same data as in Figure 3 with log OD plotted as a function of radius squared.

TABLE II: Computer Output to Printer from Program Fitting for Dissociation Constant.

Point No.	OD	OD Fit	OD–OD Fit	Molecular Weight
1	1.853	1.910	−0.0572	58,523
3	1.504	1.519	−0.0150	57,879
5	1.227	1.210	0.0176	57,182
7	0.972	0.966	0.0054	56,435
9	0.769	0.774	−0.0052	55,640
11	0.622	0.623	−0.0013	54,799
15	0.409	0.408	0.0016	52,994
20	0.244	0.245	−0.0012	50,559
25	0.151	0 152	−0.0011	48,033
30	0.099	0.096	0.0022	45,520
35	0.062	0.063	−0.0008	43,150
40	0.041	0.042	−0.0004	40,999
45	0.029	0.028	0.0009	39,130

an equilibrium run with carboxyhemoglobin A will be described. A sample of 5 μM HbA prepared in the usual manner (Edelstein *et al.*, 1970) in pH 7 ammonium phosphate buffer (0.05 M) and saturated with CO is loaded into a Yphantis cell to give a column height of about 3 mm. This sample is spun overnight in the centrifuge at a speed of 24,000 rpm (rotor temperature 20°). The following morning, data are collected with the computer and printed on the high-speed printer. A data sample is given in Table I. Each final data point represents an average value from 100 revolutions of the rotor. It is from this average that the standard deviation σ is calculated. After each scan the data are stored on a cassette for permanent storage. When all the data are safely stored, the plot program is loaded from cassette and the plots of Figures 2–4 are produced as previously described. Figure 2 shows an oscilloscope plot of the optical density as a function of radius (upper plot) and the log of the optical density as a function of radius squared (lower plot). Figures 3 and 4 are repeats of these data taken from the x–y plotter output and with the axis information added. The log plot shows the characteristic curvature of an associating system.

The data are now transferred from the cassette to the disk where fitting programs written with the disk operating system can most conveniently be applied. Tables II and III and Figure 5 show the results of the $K_{4,2}$ calculation. The parameters in Table II can be used as an indicator of the quality of the data. The column OD-OD FIT should show a random variation of the data points from the calculated line. This difference is plotted in Figure 5 as a function of

TABLE III: Output from Dissociation Constant Fitting Program with a Sample of HbA.

Fit range, 1–45
Base line, 0.0
Dimer coefficient, 1.28×10^{-20}; SD, 2.64×10^{-22}
Tetramer coefficient, 2.03×10^{-39}; SD, 1.93×10^{-41}
Coefficient of determination, 0.99987
Chi-Square statistic, 0.349
F statistic, 1.72×10^{5}
$K_{4,2} = 1.09 \times 10^{-6}$, SD, 5.57×10^{-8}

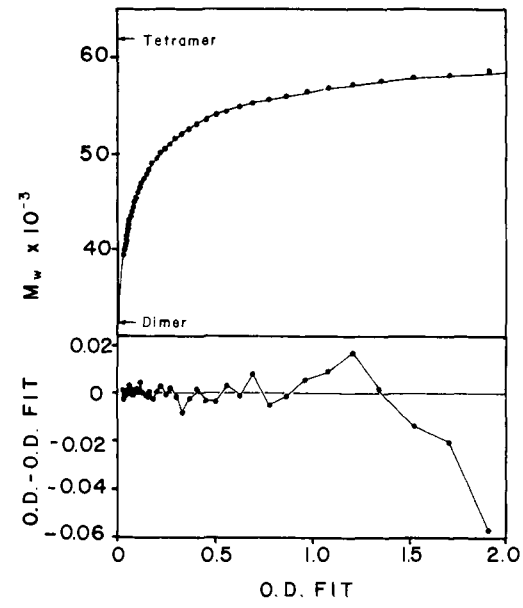

FIGURE 5: Graphical representation of the output of the $K_{4,2}$ fitting program given in Table II. Upper plot: apparent molecular weight *vs.* OD for CO-HbA data. Lower plot: deviation of measured OD from calculated OD *vs.* calculated OD.

OD. The apparent molecular weight was used to plot molecular weight *vs.* concentration in Figure 5. Extrapolation to zero concentration gives the dimer molecular weight indicating that there was no monomer present in our solutions. This particular set of data corresponded to a value of $K_{4,2} = 1.09 \times 10^{-6}$, with standard deviation = 5.6×10^{-8} (Table III). The coefficient of determination is nearly one, indicating the correct number of variables was used to fit the data. Chi square is less than one, indicating that the data were better than indicated by the standard deviation given in Table I. The F statistic is a large number, which indicates that two variables (dimer coefficient and tetramer coefficient) were sufficient to fit the data.

Measurement of Variations in Tetramer–Dimer Equilibrium Constants. The opportunity of performing multiple measurements of dissociation constants on several samples simultaneously permits small but functionally significant changes in dissociation with different conditions to be monitored with confidence. For example, the tetramer-dimer equilibrium of carboxyhemoglobin may be perturbed in the presence of inositol hexaphosphate (IHP),[1] since it is believed to bind at an axis of symmetry to the tetramer with a stoichiometry of one per tetramer (Arnone and Perutz, 1974). The effects of IHP binding to deoxyhemoglobin are well known, although the interactions with liganded forms of hemoglobin are less well characterized. Results of experiments on the dissociation of carboxyhemoglobin with and without IHP in bis-tris buffer are summarized in Figure 6. A definite shift in molecular weight *vs.* concentration is observed in the presence of IHP, with the organic phosphate producing higher molecular weights. Average values from several experiments indicate a dissociation constant, $K_{4,2} = 2 \times 10^{-5}$ M, for carboxyhemoglobin under these conditions, with a decrease to $K_{4,2} = 4 \times 10^{-7}$ M in the presence of IHP. The implications of these results for the general questions concerning the conformational changes accompanying IHP binding are considered in a separate paper which

[1] Abbreviation used is: IHP, inositol hexaphosphate.

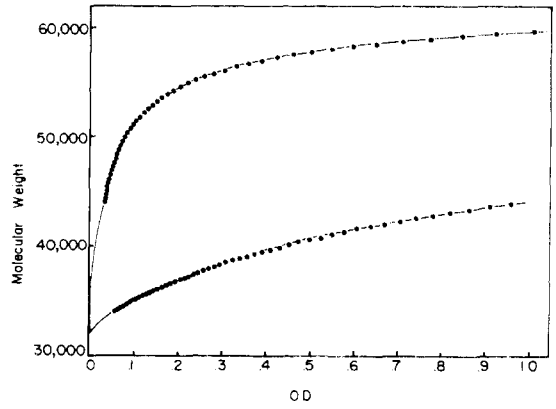

FIGURE 6: Plot of molecular weight as a function of OD for carboxyhemoglobin in 0.01 M bis-tris buffer (pH 6.0)–0.1 M NaCl (lower curve) and upon addition of 50 μM IHP (upper curve).

includes experiments on methemoglobin (Hensley *et al.*, 1974).

Comparison of Dissociation Constants for Different Samples under Identical Conditions. The ability to detect relatively small differences in dissociation constants enables experiments to be performed to determine if structural differences are reflected by differences in physical properties, such as subunit dissociation. For example, normal and sickle cell hemoglobin (hemoglobin S: β6 Glu → Val) have much different behavior at the high concentrations found in erythrocytes. The sickle cell hemoglobin, or hemoglobin S, aggregates with itself to form long rods which distend the cells and are responsible for a wide variety of clinical symptoms. However, in dilute solutions hemoglobin A and hemoglobin S are generally thought to have very similar properties, although HbS has recently been found to be markedly more sensitive to denaturation (Jones *et al.*, 1973). In order to test whether this observation is a reflection of differences in subunit dissociation, simultaneous experiments on hemoglobins A and S have been performed. In addition, mixtures of hemoglobins A and S have been examined to test whether hybrid tetramers, which are formed under these conditions, might also vary in their stability as has been suggested on the basis of cross-linking experiments (Macleod and Hill, 1973). The results, summarized in Table IV and Figure 7, indicate no consistant differences in any of the samples, with dissociation constants of $K_{4,2} = 1.0 \pm 0.2 \times 10^{-6}$ M obtained throughout. Therefore, differences in stability between hemoglobins A and S must have their bases in structural properties other than subunit dissociation.

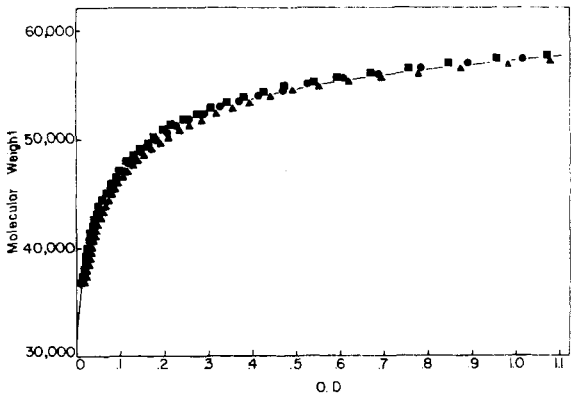

FIGURE 7: Apparent molecular weight as a function of OD for HbA (■), HbS (●), and a 1:1 HbA–HbS mixture (▲) under identical conditions. Other details described in the text.

TABLE IV: Observed Dissociation Constants for Hemoglobins A, S, and AS Mixture in 0.05 M Ammonium Phosphate Buffer at pH 7.0.

HbA $\times 10^{-6}$	HbS $\times 10^{-6}$	Mixed $\times 10^{-6}$
1.23	1.17	1.03
0.867	0.884	1.10
1.09	0.826	0.982
Av 1.06	0.96	1.04

Discussion

The studies described in this paper indicate that it is now possible to measure dissociation constants for the tetramer–dimer equilibrium of hemoglobin conveniently and with high precision using an on-line computer system. The system is sufficiently precise that the dissociation constant of carboxyhemoglobin, $K_{4,2} = 1.1 \times 10^{-6}$ M, can be measured with a standard deviation of the analysis for a single determination of 0.05×10^{-6} M. Average values from multiple determinations are in general agreement with these estimates as judged by the studies on normal and sickle cell hemoglobin (Table IV, Figure 7). Thus the dissociation constant can now be used as a convenient probe of conformational change and the studies with inositol hexaphosphate reveal some conformational alteration in carboxyhemoglobin caused by this molecule as reflected by a 50-fold decrease in dissociation constant. Such changes are also found for methemoglobin (Hensley *et al.*, 1974). Similarly, relatively small pH-dependent changes in dissociation constant for cat hemoglobin can be detected (Hamilton and Edelstein, 1974).

The development of the on-line computer system for the absorption optical system represents an out-growth of earlier work with the electronic scanner (Schachman and Edelstein, 1966, 1973). The conversion of voltage pulses from the photomultiplier directly to digital values with an analog/digital converter (Crepeau *et al.*, 1972) provides the data in a form where statistical indices can be tabulated and analysis conducted directly to yield association–dissociation equilibrium constants, as reported here. Although the system is particularly well suited to studies on hemoglobin (which dissociates in a convenient range of concentrations), applications to many other proteins should be possible, including proteins which absorb only in the uv. Successful measurements on association–dissociation equilibria have been performed with the computer system in this laboratory on the acetylcholine receptor of the electric fish and the electric eel, the cold-labile adenosine triphosphatase, and malic dehydrogenase (unpublished results). The system is under continuous development and substantial progress has been realized on studies to be described in detail in a future publication. A new collection program has recently been completed which makes use of disk storage and permits collection of a greater number of data points and more convenient output and analysis of the data. In addition, pulse integration has been achieved using a triggering system based on rotor collar pulses and has been incorporated into circuits which permit a "fine-tuning" of the pulses to bring them into coincidence and compensate for any unevenness in the spacing of light and dark spots on the rotor collar. The adjusted collar pulses can then be used to trigger the

initiation and termination of a voltage ramp-type integrating circuit which makes use of all the information contained in the scanner pulses, rather than the partial sample employed in the system used for the experiments described in this paper. Experiments with the integration circuit indicate a substantial reduction in the standard deviation of each final OD value, particularly at lower rotor speeds where pulse duration is appreciable. When the integrator is used in conjunction with the scanning laser optical system (Crepeau *et al.*, 1973) in place of the current optical system, even greater precision in measurements is obtained and measurements on proteins based on uv absorbance are possible at concentrations substantially lower than previously accessible. Such measurements may reveal functionally significant subunit dissociation in other oligomeric proteins, similar to the linkage of the allosteric constant to dissociation in hemoglobin (Thoman and Edelstein, 1972).

Acknowledgments

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