AN ULTRACENTRIFUGAL STUDY OF THE DISSOCIATION OF HUMAN CARBOXYHEMOGLOBIN

Thesis by

William Day Hutchinson

In Partial Fulfillment of the Requirements

For the Degree of

Master of Science in Chemistry

California Institute of Technology

Pasadena, California

1957

Abstract

The dissociation of human carboxyhemoglobin has been investigated by means of sedimentation velocity measurements. Sedimentation studies on this protein have been extended to concentrations as low as 0.002% by the use of absorption optics. Hemoglobin was observed to dissociate increasingly as the pH was changed from 7 to 5. Apparent dissociation constants at each pH have been calculated. Values of the apparent specific volume of human carboxyhemoglobin have been found.

Acknowledgments

I would like to express my sincere appreciation and gratitude to Doctor Jerome Vinograd, who not only suggested this research but showed great interest in its progress and outcome.

I also wish to express my thanks to those people who have contributed the blood used in the hemoglobin preparations.

For financial support during the course of this research I am indebted to the California Institute of Technology for granting me the Paul E. Lloyd Fellowship.

TABLE OF CONTENTS

INTRODUCTION

The hemoglobins of several species of mammals have been reported to dissociate into smaller molecules under the influence of various conditions such as high urea concentration, low pH, or low protein concentration. Burk and Greenburg reported that between pH 7.3 and 9, in 6.6 M urea, the molecular weight of horse carboxyhemoglobin, as determined by osmotic pressure measurements, is half that found in the absence of urea (1). This finding was confirmed by Wu and Yang (2), who found, however, that the hemoglobins of the dog and sheep did not exhibit this behavior. Steinhardt, using the sedimentation and diffusion method, confirmed the earlier work on horse hemoglobin and effected recombination to a species having the original molecular weight by removing the urea by dialysis (3). A reported increase (4) in the diffusion coefficient of this protein, horse carboxyhemoglobin, with dilution has been interpreted as a dissociation (5). Dissociation of horse carboxyhemoglobin by high salt concentrations (0.5-1.0 M) has been observed though no such behavior was found for human carboxyhemoglobin (6).

More recently, Field and O'Brien have reported a decrease in the sedimentation coefficient with a concomitant rise in the diffusion coefficient of human carboxyhemoglobin in solutions of pH below 5 (7). Based upon comparisons of the observed spreading of the boundary in the

ultracentrifuge, Ogston and Field concluded that the equilibrium in the reaction was rapidly established (8).

There have been no clear demonstrations of the adherence of any of these alleged dissociations to the law of mass action. A knowledge of the mass action expression, if any, would yield useful information about the group, or groups, involved in the reaction. A study of the effects of temperature on the equilibrium, if it is found to be a true equilibrium, would give useful information about the energy changes taking place. It has therefore been the purpose of this investigation to demonstrate whether or not one of these proteins, human carboxyhemoglobin, dissociates in accord with the law of mass action.

PROCEDURE

It may be shown* that the measured sedimentation coefficient, $\bar{S}$, in a system in dissociation equilibrium is given by $\bar{S} = \sum_i f_i S_i \alpha + (1-\alpha) S_o$ where S_o and S_i are the sedimentation coefficients of the original species and the products of its dissociation respectively and α is the degree of dissociation. The quantity f_i is the ratio of the molecular weight of the i^{th} species to that of the original species. Since α should be a function of the concentration, a study of the sedimentation rate as a function

---

* See appendix I.

of protein concentration should reveal the effects of mass action. A judicious choice of analytical optics and cell depths makes it possible to study sedimentation behavior over a ten thousandfold range of concentration.

The possibility of a change in the partial specific volume of the protein in conditions favoring dissociation must be considered.

The data of Field and O'Brien indicate that the apparent extent of dissociation is increased by the presence of urea in the solutions, and, contrary to the behavior of horse hemoglobin as was found earlier, the dissociation is unaffected by the presence of high salt concentrations. Since the presence of urea in protein solutions has been shown to increase the rate of protein denaturation (9) its effect in this instance is ambiguous. In this investigation the extent of dissociation has been studied, therefore, as a function of the hydrogen ion and protein concentrations in solutions of single buffer salts.

EXPERIMENTAL METHODS

All sedimentation experiments were performed in a Spinco Model E analytical ultracentrifuge. Most sedimentation coefficient determinations were made at the full speed of the instrument. This speed provided a conveniently large movement of the boundary during a running time of about three hours. All the determinations using

the cells of 30 mm. depth were performed at 50,740 RPM, the maximum speed of the rotor used with these cells. The sedimentation rate was followed in the concentration range of 2.00% to approximately 0.20% with the schlieren optical system of the instrument. This method records the refractive index gradient between the solution and the solvent. At lower concentrations a more sensitive optical system based on light absorption was used. It was found that light of a wavelength of 546 mu was convenient in the concentration range from 0.30% to 0.05%; ultraviolet light of a wavelength of 254 mu could be used from 0.12% to 0.02%. The absorption of the protein at 436 mu was such that concentrations below 0.03% down to 0.002% could be studied.

In the measurement of the sedimentation coefficients by absorption optics it was necessary to establish the relation between protein concentration and the blackening of the film. This relation is given by O.D. (film) = $\gamma\{\Phi - \text{O.D.(sol'n)}\}$ where the constant Φ is a function of the length of the light path and the extinction coefficient of the protein, and γ is the slope of the D-log E curve of the film for the conditions of development.* This equation is strictly valid only for systems in which Beer's law is obeyed and under conditions where the optical densities of the film fall on the linear part of the D-log E curve.

---

* See appendix II.

The sedimentation coefficients were determined from the schlieren diagrams by measurements made on 20X enlargements such that 1 cm. on the enlargement corresponded to an actual 0.05 cm. in the cell, thus simplifying the calculations. In view of the spreading of the boundary in solutions buffered at pH 5, the position of the boundary was determined in some instances from the second moment of the curve (10). The result differed from that obtained from the more conventional but less accurate zeroth moment by .02 cm. in the last picture of a run. It was estimated that this difference leads to errors no greater than the experimental error with normal spreading.

The value of the sedimentation coefficients from absorption runs were found from densitometer tracings. The optical densities of the film were measured on the Spinco Model R Analytrol using the microanalyzer attachment supplied with the instrument. The method used for determining the position of the boundary was that described by Shooter and Butler (11).

The concentrations of the solutions were determined either refractometrically or spectrophotometrically. The value used for the specific refractive index increment for the 623 mu line of the mercury arc was 0.00181 (12) and the value of the specific extinction coefficient, $E_{1\ cm.}^{1\%}$, 540 mu, was 8.60 (13). The instruments used for the respective measurements were a differential refractometer made

by the Phoenix Precision Instruments Company and a Beckman Model B spectrophotometer.

For absorption runs with 254 mu light the chlorine-bromine filter supplied with the Spinco Model E ultracentrifuge was used. At other wavelengths this filter was removed. An interference filter was substituted to pass light of 436 mu wavelength, and a Wratten #77A filter was used for 546 mu light. The lightsource used was the AH-4 low pressure mercury arc lamp.

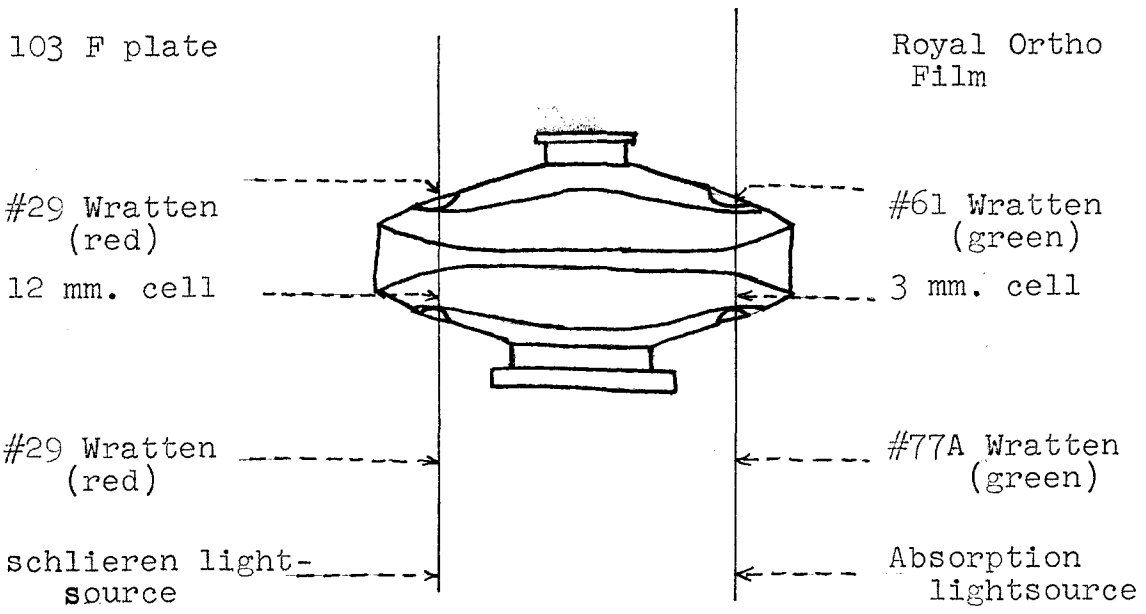
Except for the first few runs which were performed at 25°C., all runs were performed at 20.0°C.; the sedimentation coefficients, $S_{w,20}$, thus correspond to velocities and equilibria at 20°. The density of the solution was used in the $(1-\bar{v}\rho)$ (9) term which reduces the buoyancy factor to standard conditions.

Since two analytical methods were used in this work the problem arose as to the equivalence of the results obtained by the two methods. In order to check this unequivocally the sedimentation coefficients were measured by the two methods simultaneously in the same rotor employing two separate cells, thus avoiding the possibility of different effects of temperature, denaturation, and methemoglobin formation in the comparison. To achieve reasonable sensitivity it was necessary to vary the cell thickness and the corresponding liquid layer thickness since the regions of equal sensitivity for the two methods do not overlap

for equal cell depths. For example a 12 mm. cell was used for the schlieren optical system, and a 3 mm. cell was used for the absorption system with 546 mu light. In order that one system would not conflict with the other, the cells were equipped with individual gelatin filters, and suitably sensitive photographic emulsions were employed. An example of the method is given in figure 1.

Figure 1

An Experimental Arrangement for Simultaneous Schlieren and Absorption Runs



While it was not possible in all cases to carry out such simultaneous experiments because of the requirements of reasonable sensitivity, consecutive experiments, using cells of the appropriate thicknesses were carried out to achieve an overlapping in the ranges of the different methods. For instance, the same solution was studied in a 30 mm. cell by schlieren optics, a 12 mm. cell by UV light, and a 3 mm. cell by light of a wavelength of 436 mm.

MATERIALS

The samples of carboxyhemoglobin used in this investigation were prepared by the method of Drabkin (14) as it has been modified by Rhinesmith, Schroeder, and Pauling (15).

The buffers used were prepared from reagent grade chemicals. The buffer of pH 7.09 was that of Kegeles and Gutter (16). The phosphate buffer of pH 6 was prepared by dissolving per liter of distilled water, 2.66 gms. of $Na_2HPO_4 \cdot 7H_2O$, 9.71 gms. of $NaH_2PO_4 \cdot H_2O$, and 8.77 gms. of NaCl. The acetate buffer of pH 5.06 was prepared by dissolving in a liter of distilled water 8.19 gms. of NaAc, 2.4 cc. glacial acetic acid, and 9.71 gm. of NaCl. The phosphate-acetate buffer was prepared by adding 12.30 gm. NaAc per liter to the buffer of Kegeles and Gutter (16).

Table I

Sedimentation Coefficients and Calculated Equilibrium Constants for Carboxyhemoglobin in Acetate-NaCl Buffer, pH 5.06, μ = 0.25

Run#	conc.%	Method	λ mμ	$S_{w,20}$	$S^{*}_{w,20}$	K x M
372*	1.79	schl.		3.42	3.97	.88
337*	1.55	"		3.32	3.69	2.28
353*	1.5	"		3.52	3.89	1.03
371*	1.16	"		3.61	3.90	.76
786	1.00	"		3.65	3.90	.65
355*	.75	"		3.70	3.88	.51
786	.75	"		3.74	3.93	.44
338*	.72	"		3.71	3.89	.50
379*	.60	"		3.56	3.71	.81
767	.535	"		3.74	3.87	.40
767	.400	"		3.67	3.77	.44
768	.32	"		3.52	3.60	.48
768	.27	"		3.44	3.50	.74
748	.26	"		3.32	3.38	1.06
865	.22	"		3.60	3.65	.38
342*	.19	"		3.37	3.42	1.18
870	.13	abs.	546	3.37	3.34	.56
871	.066	"	"	3.34	3.35	.30
659	.027	"	436	2.99	2.99	.48
611	.026	"	"	3.03	3.03	.39
614	.026	"	"	3.00	3.00	.44
575	.025	"	"	2.57		
577	.020	"	"	2.93	2.93	.49
689	.0043	"	"	2.91	2.81	.25

* These runs were made at 25.0°C., all others at 20.0°C.

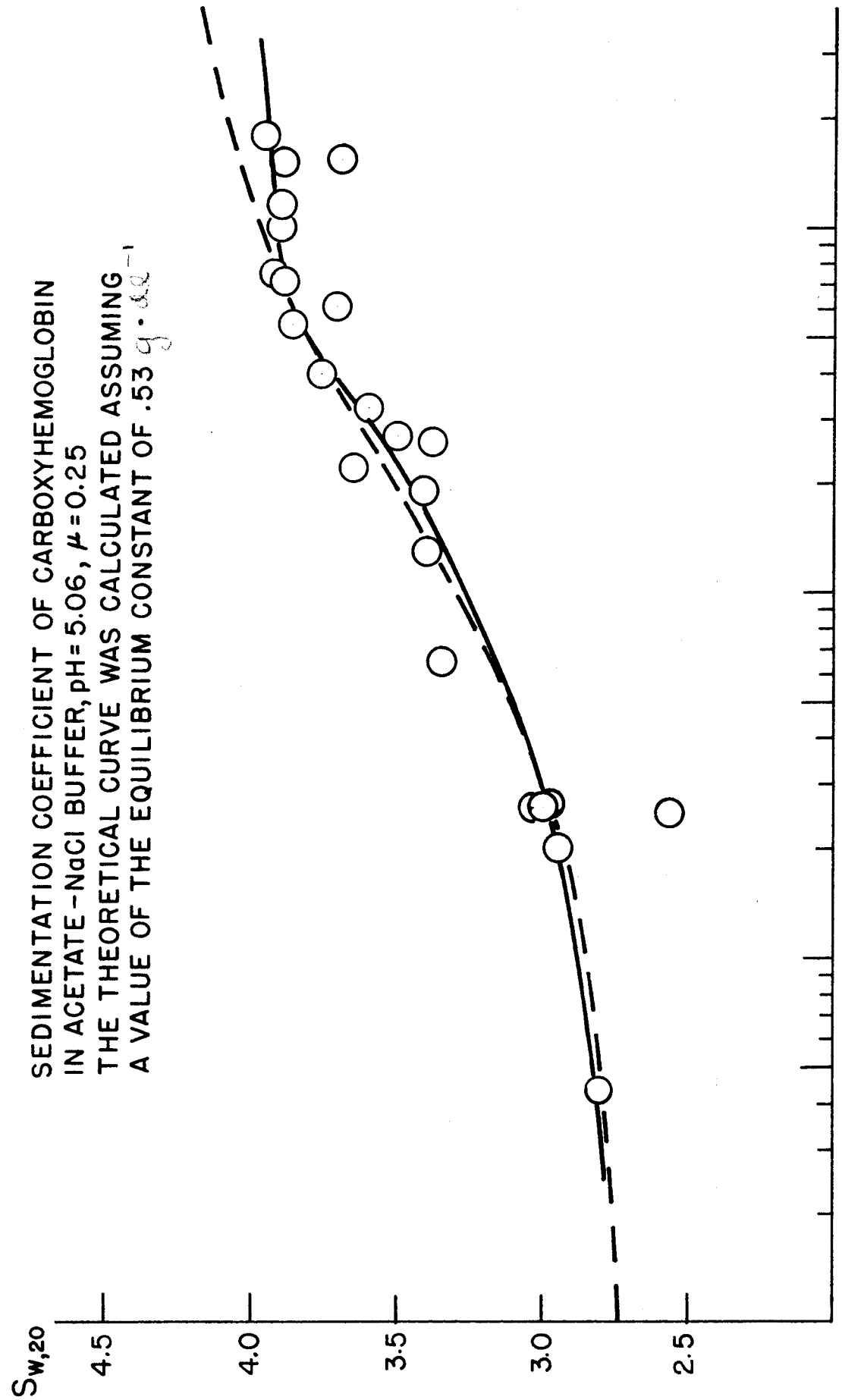

SEDIMENTATION COEFFICIENT OF CARBOXYHEMOGLOBIN
IN ACETATE-NaCl BUFFER, pH= 5.06, μ=0.25
THE THEORETICAL CURVE WAS CALCULATED ASSUMING
A VALUE OF THE EQUILIBRIUM CONSTANT OF .53 g·dl⁻¹
$S_{w,20}$
4.5
4.0
3.5
3.0
2.5
.001
.005
.010
.050
.100
.500
1.00
CONCENTRATION, %

FIGURE II

Table II

Sedimentation Coefficients and Calculated Equilibrium Constants for Carboxyhemoglobin in Phosphate-NaCl Buffer, pH 6.00, μ = 0.25, T 20.0°C.

Run#	Conc. %	Method	λ mμ	$S_{w,20}$	K x M
414	2.11	schl.		4.21	
415	1.05	"		4.31	
796	.77	"		4.28	
796	.75	"		4.18	
798	.58	"		4.40	
798	.55	"		4.35	
416	.53	"		4.34	
837	.28	"		4.44	
709	.24	abs.		4.48	
976	.119	abs.	254	4.23	
977	.117	schl.		4.45	
982	.043	abs.		3.88	0.0315
618	.031	abs.	436	3.91	0.0194
578	.024	abs.	436	3.38	0.098
984	.0209	abs.	254	3.11	0.222
988	.0208	abs.	436	3.21	0.154

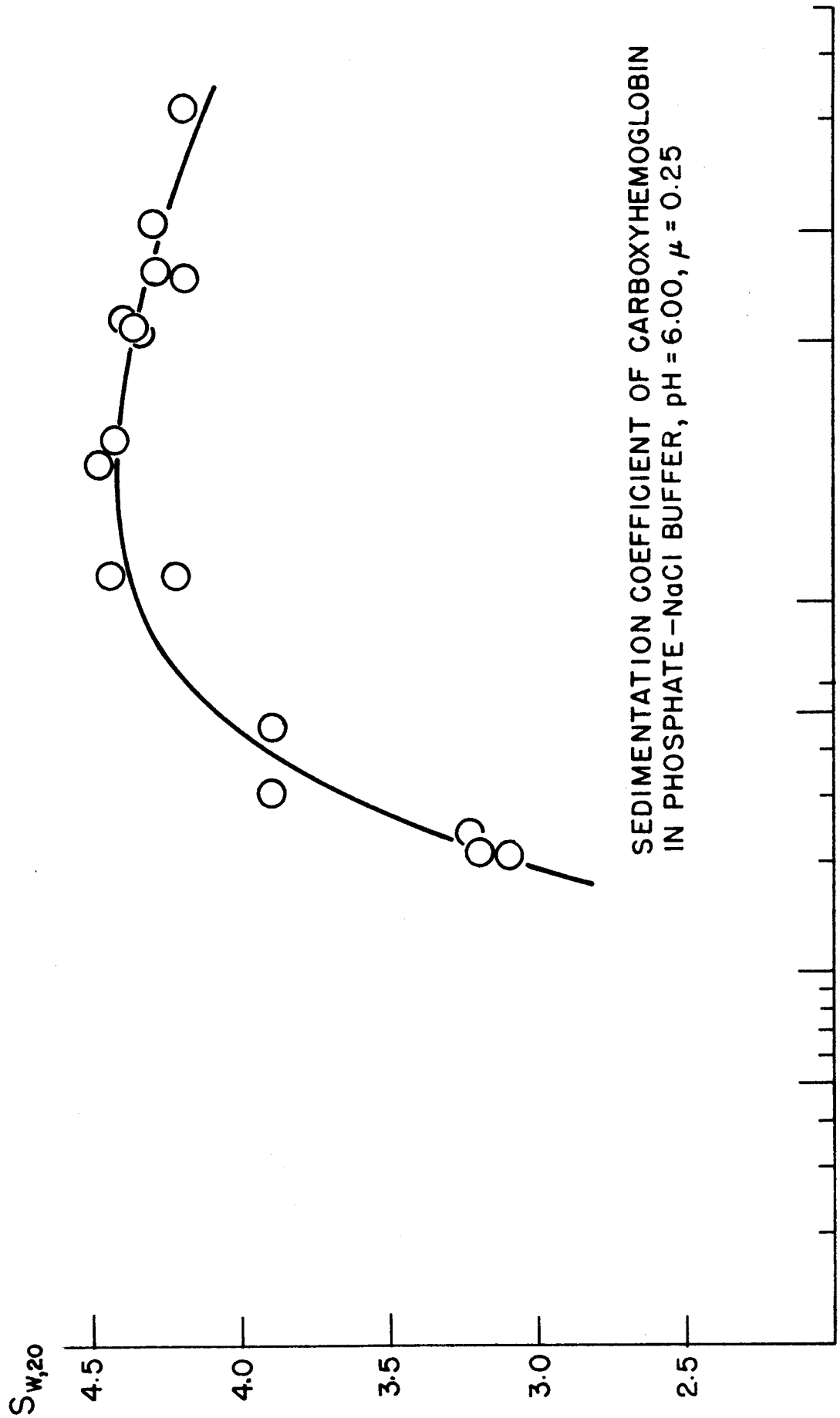

SEDIMENTATION COEFFICIENT OF CARBOXYHEMOGLOBIN
IN PHOSPHATE–NaCl BUFFER, pH = 6.00, μ = 0.25
$S_{w,20}$
4.5
4.0
3.5
3.0
2.5
.001
.005
.010
.050
0.1
0.5
1.0
CONCENTRATION, %

FIGURE III

Table III

Sedimentation Coefficients and Calculated Equilibrium Constants for Carboxyhemoglobin in Phosphate Buffer, pH 7.09, $\mu = 0.10$

Run #	Conc. %	Method	λ mμ	$S_{w,20}$	K x M
385*	1.52	schl.		4.16	
396*	1.52	"		4.09	
397*	1.14	"		4.20	
386*	.75	"		4.31	
387*	.38	"		4.20	
734a	.239	schl.		4.46	
734b	.239	abs.	546	4.43	
726	.229	"	"	4.37	
956	.0985	schl.		4.47	
957	.0985	abs.	436	4.47	
961	.0985	"	"	4.40	
571	.0413	"	"	4.04	.0145
674	.0324	"	"	4.01	.0133
669	.028	"	"	4.00	.0119
673	.016	"	"	3.55	.0373
966	.0105	"	"	3.04	.15
951**	.0087	"	"	3.14	.083
675	.0065	"	"	3.35	.0295
686	.00605	"	"	3.27	.036
968	.0043	"	"	2.56	
676	.0032	"	"	2.94	.0442

*Runs 385-397 were performed at 25.0°C., all others at 20.0°C.
**The actual pH of the solution in this case was 6.95.

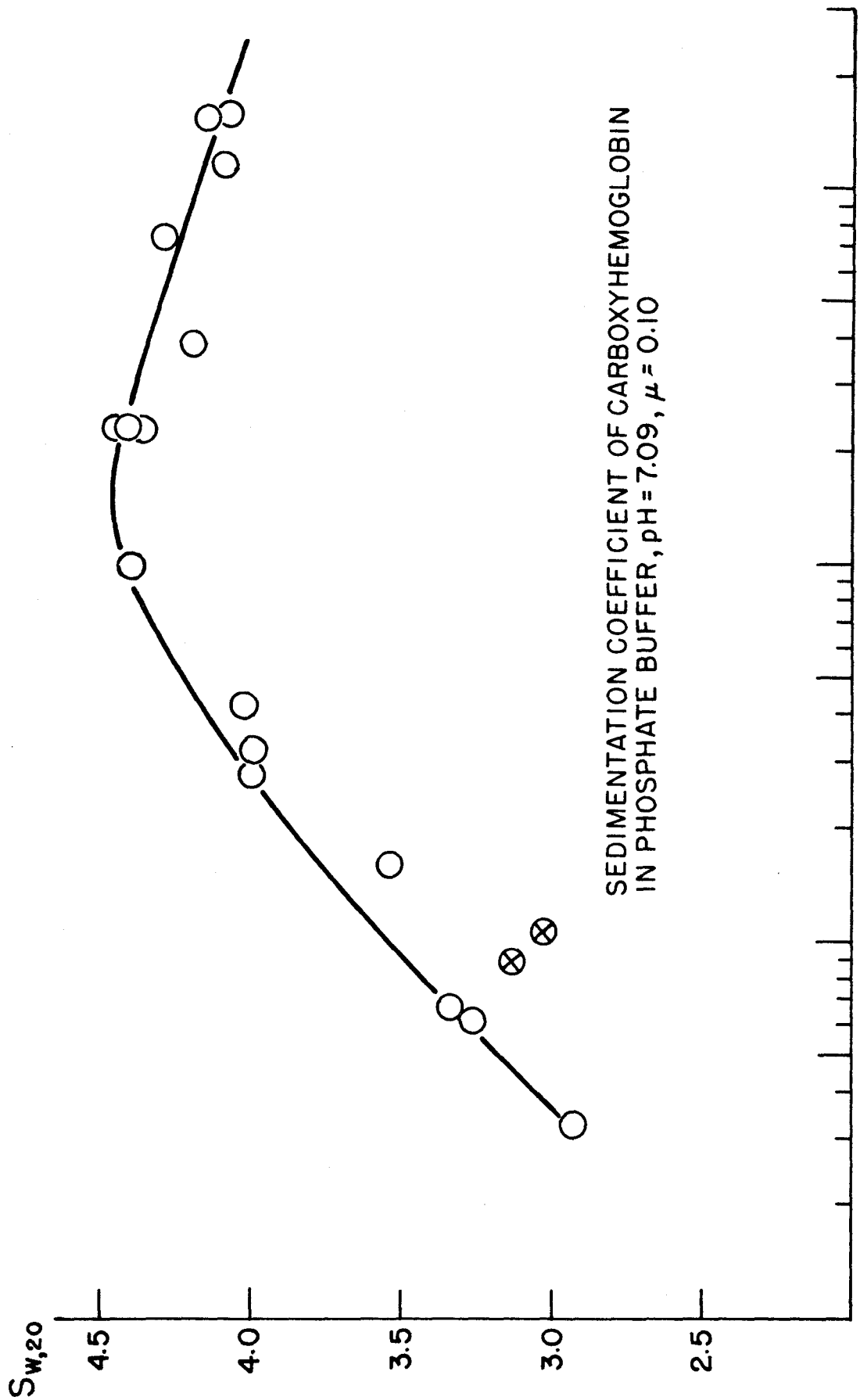
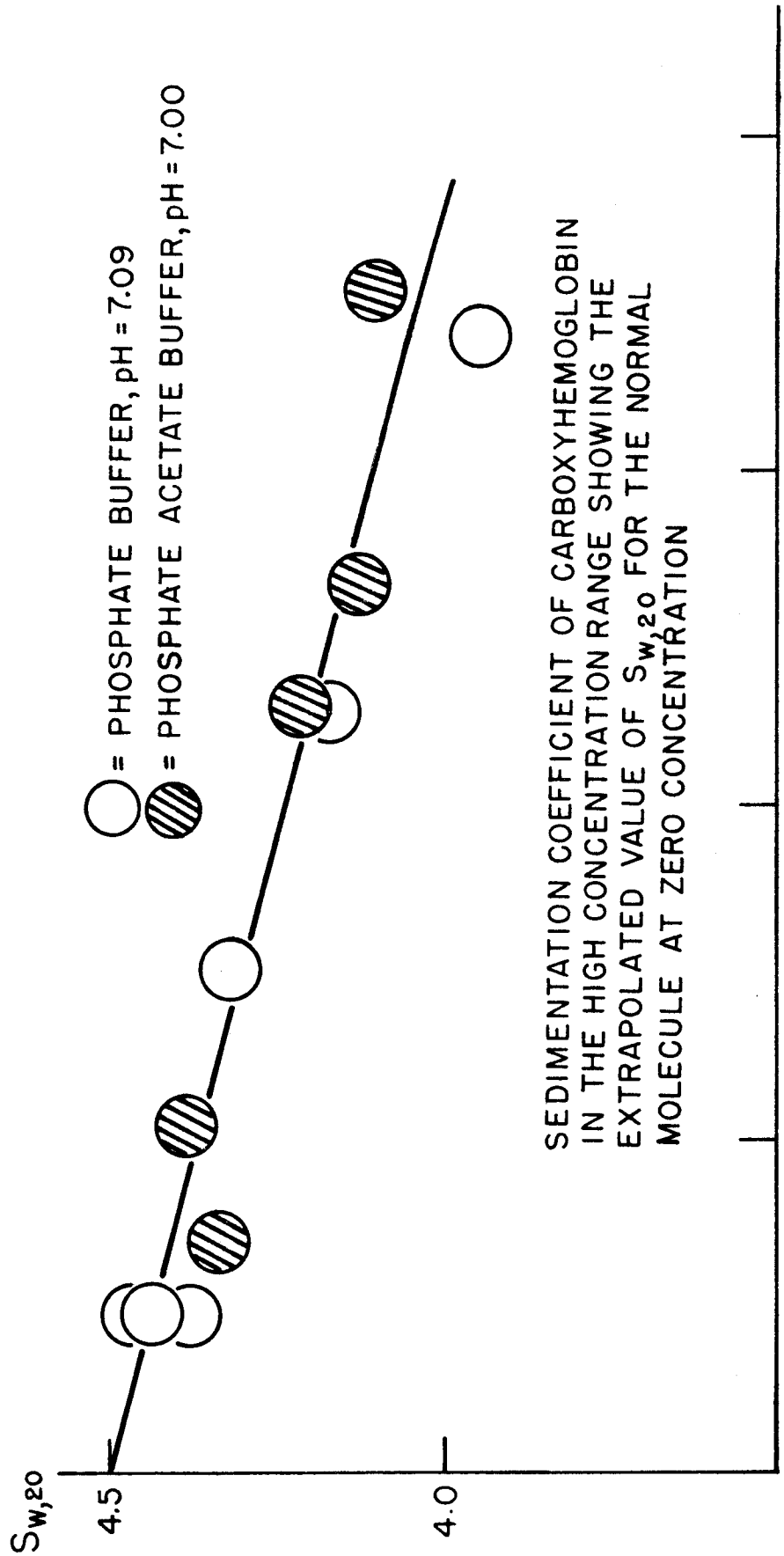

SEDIMENTATION COEFFICIENT OF CARBOXYHEMOGLOBIN
IN PHOSPHATE BUFFER, pH = 7.09, μ = 0.10
$S_{w,20}$
4.5
4.0
3.5
3.0
2.5
.001
.005
.010
.050
.100
.500
1.0
CONCENTRATION, %

FIGURE IV



SEDIMENTATION COEFFICIENT OF CARBOXYHEMOGLOBIN IN THE HIGH CONCENTRATION RANGE SHOWING THE EXTRAPOLATED VALUE OF $S_{w,20}$ FOR THE NORMAL MOLECULE AT ZERO CONCENTRATION

FIGURE V

Table IV

Comparison of Results Obtained by Different Methods

Run #	Date	Conc.%	$S_{w,20}$	pH	Temp. °C.	Method	mμ
schlieren with schlieren,							
385	5/12/56	1.52	4.16	7.09	25.0	schl.	
396	5/18/56	1.52	4.09	7.09	25.0	"	
* 798a	2/6/57	.58	4.40	6.00	20.0	"	
798b	2/6/57	.55	4.35	6.00	20.0	"	
416	5/30/56	.53	4.34	6.00	20.0	"	
* 796a	2/6/57	.77	4.28	6.00	20.0	"	
796b	2/6/57	.75	4.18	6.00	20.0	"	
355	4/30/56	.75	3.70	5.06	25.0	"	
786	1/29/57	.75	3.74	5.06	20.0	"	
768b		.27	3.44	5.06	20.0	"	
748	1/7/57	.26	3.32	5.06	20.0	"	
436 with 436,							
659	11/7/56	.026	2.99	5.06	20.0	abs.	436
611	10/1/56	.027	3.03	5.06	20.0	abs.	436
614	10/2/56	.026	3.00	5.06	20.0	abs.	436
675	11/19/56	.0065	3.35	7.09	20.0	abs.	436
686	11/28/56	.00605	3.27	7.09	20.0	abs.	436
546 with 546,							
734b	12/28/56	.239	4.43	7.09	20.0	abs.	546
726	12/27/56	.229	4.47	7.09	20.0	abs.	546
schlieren with absorption,							
976	5/2/57	.119	4.23	6.00	20.0	abs.	254
977	5/2/57	.117	4.45	6.00	20.0	schl.	
* 734a	12/28/56	.239	4.46	7.09	20.0	schl.	
734b	12/28/56	.239	4.43	7.09	20.0	abs.	546
956	4/25/57	.0985	4.47	7.09	20.0	schl.	
957	4/25/57	.0985	4.47	7.09	20.0	abs.	436
961	4/28/57	.0985	4.40	7.09	20.0	abs.	436

* These runs were done simultaneously in the same rotor.

RESULTS AND DISCUSSION

The results of the sedimentation coefficient determinations are compiled in tables I, II, and III, and the data of these tables are plotted in figures 2, 3, 4, and 5.

In table IV are shown the reproducibility of results obtained by the different methods. It was found that sedimentation coefficient determinations by schlieren optics agreed within $\pm$ 2% as is seen from the results of runs 385 and 396, 798a and b, 355 and 786, 768b and 748. With absorption optics using light of a wavelength of 436 mu the results agreed within $\pm$ 2% as is seen in runs 659, 611, and 614, and 675 and 686. The agreement among the results using light of a wavelength of 546 mu is given in runs 734b and 726. The agreement between schlieren optics and absorption optics may be seen from the runs 734a and b, 976 and 977, and 956, 957, and 961.

Of most significance in the plots of $S_{w,20}$ versus c is the fact that all curves are similar in their general appearance; the sedimentation rate appears to decrease rapidly at low concentrations at all pH values studied. Above 0.20% concentration at pH 6 and 7 the results of this study are slightly higher than the results of previous workers; this is caused by the employing of solution densities instead of solvent densities in reducing the measured sedimentation coefficients to standard conditions, $S_{w,20}$. The value of the sedimentation coefficient of the undissociated hemoglobin

molecule at zero concentration obtained by extrapolation of the data found at pH 6 and 7 is 4.5 as opposed to the values 4.3-4.4 found by others (16). The reason for this is not understood; the effect described above should have no effect on the value of the sedimentation coefficient at zero concentration.

Apart from the effect described above, the sedimentation rate appears to be normal at pH 6 and 7 above a concentration of 0.20%, showing only the concentration dependence of the sedimentation coefficient of the unchanged hemoglobin molecule. For all protein concentrations studied at pH 5, the values are low, indicating that at these concentrations in this buffer there is an appreciable degree of dissociation. This was also evident from the spreading and shapes of the schlieren peaks.

Schumaker and Schachman (17) have recently reported that the sedimentation coefficient of human carboxyhemoglobin at pH 7 is normal in concentrations as low as 0.005% in contradiction to the present work. The reason for this difference is not understood. It is unlikely that errors in the concentration determinations can account for the difference since a factor of approximately 10 in the concentration would be required. It is not unreasonable for dissociation to occur at pH 7 if it occurs to the extents observed at pH 5 and 6. It may be that the explanation lies in the experimental method especially in view of the statement made by these authors that different values of the sedimentation

coefficient for a cytochrome preparation were found when light of different wavelengths was employed. It should be pointed out that the method used by these authors was different from the method described in this paper in the respect that they modified the schlieren optical system and used it as an absorption system.

The critical test of the adherence of the dissociation of carboxyhemoglobin to the law of mass action would be the calculation of an equilibrium constant for the reaction from the degrees of dissociation found from the sedimentation studies. In the calculation of degrees of dissociation it has been assumed that the dissociation reaction produces two new species having half the molecular weight of the normal hemoglobin molecule. The assumption of dissociation into equal fragments is in accord with earlier findings that the hemoglobins of various species dissociate into equal fragments and with the discovery that human carboxyhemoglobin molecule has symmetry about a diad axis (18).

For a system undergoing dissociation into half molecules, the sedimentation coefficient is given by $S=\alpha S_p+(1-\alpha)S_o$, where S_o and S_p are the sedimentation coefficients of the original molecule and the reaction product respectively. The value of the sedimentation coefficient of the intact hemoglobin molecule has been taken as 4.5 which is the extrapolated value of the sedimentation coefficient of the normal molecule at zero concentration. The value of the sedimentation coefficient of the half molecule has been taken as 2.7 which

is the value of the asymptote at zero concentration on the log plot of $S_{w,20}$ against c for acetate buffer of pH 5.

Since the experimental curve of the sedimentation coefficient is the sum of two curves, the regular curve of the concentration dependence of S, through its dependence on the frictional coefficient f, and the mass action dependence of $S_{w,20}$, the experimental data must be corrected to yield only the mass action dependence of $S_{w,20}$. Since dissociation occurs at high concentrations at pH 5 this correction has been applied to the data for this buffer; no correction was applied to the $S_{w,20}$ values in buffers of pH 6 and 7 since dissociation is appreciable only at very low concentrations where the effects of concentration are negligible. The corrections applied in the case of the acetate buffered solutions were made on the basis that the concentration dependence of the sedimentation coefficient of the half molecule was the same as for the whole molecule. The correction made was 0.25 svedbergs per 1.00% protein, as derived from a plot of S against c for the high concentration range in pH 7 buffer.

From tables II, III, and IV the values for K x M are seen to be reasonably constant for the solutions of pH 5 and 7. The corrected experimental curve for pH 5 (figure II) is fitted by the constant 0.53. The best average value of the constant at pH 7.09 is 0.024, deleting the high values 0.15 and 0.083 from the calculation of the average. The points corresponding to these high K values are designated by the

crosses in figure IV. The data at pH 6 seem to be ambiguous for a calculation of the constant at this pH.

If the values of K are taken as 0.53 at pH 5.06 and 0.024 at pH 7.09, the hydrogen ion concentration can enter the mass action expression only to some power less than the first power. To pinpoint the hydrogen ion dependence more accurately than this is beyond the scope of the data reported here. It is suggested that more work be done at pH values 6 and 7 and 4.5 to determine more closely the hydrogen ion dependence.

The reactive group involved in the dissociation reaction is probably the histidine, for of all the acidic groups in the protein, this would be expected to undergo most change in the pH range from 5-7.

It has been observed that hemoglobin upon dissociation is converted more rapidly into methemoglobin. This qualitative observation is subject to a number of interpretations. It may be considered to be evidence for the position of the hemes in the hemoglobin molecule. If the hemes are located close to newly exposed surfaces of the two halves (and deeply submerged within the whole molecule), they would, upon dissociation of the molecule, be exposed to oxidation. Another explanation of this behavior may be that the groups which become acidic are the hemes themselves or other ligands attached to the iron atom. Under these circumstances the iron atom may be perturbed upon acidification and more easily oxidized.

Measurement of Partial Specific Volume. To detect any change in the specific volume of the protein upon dissociation, measurements of the specific volume were made. The quantity measured in this study was the apparent specific volume; it was assumed that it approximates the thermodynamically significant partial specific volume of the protein within the accuracy needed. The value of the apparent specific of hemoglobin in conditions favoring dissociation was found to be unchanged, within the accuracy of the determinations, from the value found in conditions where dissociation was negligible. The values found are given in table V.

Table V

Apparent Specific Volume of Human Hemoglobin 25.3°C.

Solvent	ml./gm.
0.1 μ K phosphate pH 7.0	0.747 ± .001
0.1 μ Na Acetate 0.1 M Urea pH 4.8	0.750 ± .002

These values referred to 20.0°C. become 0.744 and 0.747 ml./gm. Thus, it is seen that no appreciable change takes place in the specific volume on dissociation.

During the course of the specific volume determinations it was found that there had been no other determinations of

the partial specific volume of human carboxyhemoglobin; the value 0.749 ml./gm. quoted in the literature is based on the conjecture that the values for horse hemoglobin and human hemoglobin are the same.

APPENDICES

I. In the ultracentrifuge, the flux due to sedimentation is:

$$\frac{\Delta m}{\Delta t} = qc_t\omega^2 S_t r = \sum_i qc_i\omega^2 S_i r$$

$$c_t\bar{S}_t = \sum_i c_i S_i$$

where q is a function of the cell dimensions, r is the distance from the center of rotation of the plane through which sedimentation is occurring, and the c's are expressed in grams of solute per ml. of solution. For a dissociation of the type $A \rightleftharpoons B+C+\ldots$, if we start with c_o grams of A per ml., when equilibrium is established we will have in the solution $c_o(1-\alpha)$ grams of A and $f_i c_o \alpha$ grams of the i^{th} product of dissociation. The quantity f_i is the ratio of the molecular weight of the i^{th} species to the molecular weight of the substance A. The total flux across a plane in a cell containing such a system would be:

$$\lim_{\Delta t \to 0} \frac{\Delta m}{\Delta t} = qc_o(1-\alpha)\omega^2 S_o + qc_o\omega^2 \sum_i f_i S_i \alpha = c_o q \omega^2 \bar{S}$$

$$c_o(1-\alpha)S_o + \sum_i S_i c_o f_i \alpha = \bar{S} c_o$$

$$(1-\alpha)S_o + \sum_i f_i S_i \alpha = \bar{S}$$

In the case of a dissociation into fractional parts having molecular weights half that of the original species this expression becomes:

$$(1-\alpha)S_o + \alpha S_1 = \bar{S}$$

II. On the straight line portion of the D log E curve of the film, the condition

$$\frac{d\{O.D.(film)\}}{d\{\log t\}} = \gamma \qquad (1)$$

a constant, is satisfied. For a solute obeing Beer's law then

$$\log \frac{I_i}{I_t} = Kc = O.D.\ (sol'n)$$

$$\log I_t = \log I_i - O.D.\ (sol'n) \qquad (2)$$

From (1), $O.D.(film) = (\log t - \log t^*)$ (3)

where $\log t^*$ is the extrapolated value of log t when O.D.(film) = 0. For a plane surface the amount of light reaching the surface is

$$I = \bar{K}t, \text{ or } \log t = \log I - \log \bar{K} \qquad (4)$$

Substituting (4) into (3),

$$O.D.(film) = \gamma(-\log K + \log I - \log t^*)$$

$$O.D.(film) = \gamma(\log K + \log \alpha I_t - \log t^*), \text{ if}$$

$I = I_t$, where α is a factor introduced to take account of the attenuation of the beam of light transmitted through the solution as it passes through the optical system of the ultracentrifuge.

$$\text{O.D.(film)} = \gamma \log \bar{K} + \gamma(\log \alpha - \log I_t - \log t^*)$$

Substituting in this equation the value of I_t from equation 2,

$$\text{O.D.(film)} = \gamma \log \bar{K} + \gamma \log \alpha - \gamma \log t^* + \gamma \log I_i - \gamma[\text{O.D.(sol'n)}].$$

Combining terms so as to include all constants in Φ, we find

$$\text{O.D.(film)} = \gamma\Phi - \gamma[\text{O.D.(sol'n)}]$$

REFERENCES

1. Burk, N. F., and Greenberg, D. M., J. Biol. Chem., 87, 197-238 (1930).

2. Wu, H., and Yang, E. F., Chinese J. Physiol., 6, 51 (1932).

3. Steinhardt, J., J. Biol. Chem., 123, 543-575 (1938).

4. Tiselius, A., and Gross, D., Kolloid Z., 66, 12 (1934).

5. Lamm, O., and Polson, A., Biochem. J., 30, 528-541 (1936).

6. Svedberg, T., and Pedersen, K., The Ultracentrifuge. Oxford Univ. Press, London, 1940. p. 407.

7. Field, E. O., and O'Brien, J. R., Biochem. J., 60, 656-661 (1955).

8. Field, E. O., and Ogston, A. G., Biochem. J., 60, 661-665 (1955).

9. Neurath, H., Cooper, G. R., and Erickson, J. O., J. Biol. Chem., 142, 249-263, 265-276 (1942).

10. Goldberg, R. J., J. Phys. Chem., 57, 194-202 (1953).

11. Shooter, K. V., and Butler, J. A. V., Trans. Faraday Soc., 52, 734-742 (1956).

12. Howard, F. H., J. Biol. Chem., 41, 537-547 (1920).

13. Winegarden, H., and Borsook, H., J. Cell. Comp. Physiol., 3, 437-448 (1933).

14. Drabkin, D. L., J. Biol. Chem., 164, 703-723 (1946); Arch. Biochem. Biophys., 21, 224-232 (1949).

15. Rhinesmith, H., Schroeder, W., and Pauling, L., J. Am. Chem. Soc., 79, 609-615 (1957).

16. Kegeles, G., and Gutter, F., J. Am. Chem. Soc., 73, 3770-3777 (1951).

17. Schumaker, V. N., and Schachman, H. K., Biochem. Biophys. Acta, 23, 628-639 (1957).

18. Perutz, M. F., as quoted in Lemberg, R., and Legge, J., Hematin Compounds and Bile Pigments., Interscience, New York, 1949. p. 248.