Relationship between Concentration and Sedimentation in Different Preparations of Bovine CO-Hemoglobin

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It has been customary to regard different preparations of the same protein as being generally identical, provided that the preparation methods are more or less the same. It appears probable, however, that due to difficulties in repeating exactly the same preparative procedure from time to time, as well as possible variations in the biological starting material, one may obtain certain variations from preparation to preparation.

Two years ago Jacobsen¹ published an extensive investigation of different preparations of β -lactoglobulin, in which comparisons were made of different preparative methods, as well as of different preparations made at different times but according to the same method. Jacobsen showed that there are appreciable variations among preparations made at different times and from different materials, but was not able to prove any definite differences for preparations from the same material but obtained by different methods.

The present paper concerns itself with comparable ultracentrifugations of six preparations of bovine CO-hemoglobin, all obtained by the same method. Unlike β -lactoglobulin the results here reported seem to indicate, that hemoglobin is affected by handling during the preparative work. This can be explained however by the fact that Jacobsen studied certain chemical properties, whereas the present investigation is concerned only with the relationship between concentration (c) and rate of sedimentation (s), *i.e.* with a function solely dependent on size and shape of the molecules.

It is generally accepted that there is a linear relationship between s and c , providing that one deals with a well defined single component system with approximately spherical molecules. Under these conditions it is then permissible to use the method of least squares to calculate this relationship, which was done here in every case.

The linear equations were set up according to the form

$$s_{20} = s_{20}^0 - K \cdot c$$

or according to the formulation used by Enoksson ²,

$$s_{20} = s_{20}^0 (1 - c \cdot \Phi)$$

where s_{20}^0 is the s_{20} value extrapolated to zero concentration, c is the concentration, K the slope of the line and Φ the specific sedimentation volume, or a factor directly proportional to this.

As the differences here obtained are comparatively small, a great difficulty in the present investigation is the estimation of the magnitude of the experimental error. In the appendix there is given an approximate calculation of the accidental error for each run, as well as the method of calculation of the mean error given in the table. As can be seen from the respective formulae, the mean error is a good measure as to how well the points fit a certain line, and will therefore together with the values of $\text{Max } \Delta$ (see Table I), give a relative measure of the accuracy.

EXPERIMENTAL

For the preparation of CO-hemoglobin a slightly modified method by Adair and Adair ³ (Method 1) was used. The only difference consisted in increasing the number of washings of the blood corpuscle with 1.5 % sodium chloride from 4 to 6 (seven washings were used for preparation IX). All preparations were made in the cold room at a temperature of about 5° C.

For preparation VI, three different determinations, designated a, b and c, were made at different times and different pH. (See Fig. 1 b, c and d.)

In all subsequent experiments the pH was kept around the isoelectric point in order to avoid too large variations of pH with different hemoglobin concentrations. The buffer then used contained 0.0588-M Na_2HPO_4 + 0.0236-M NaH_2PO_4 .

For the last two preparations the blood was immediately divided into two parts, designated A and B, and worked up separately but simultaneously, in order to insure the greatest possible uniformity of conditions.

Part of preparation IX B; designated as IX B (F) was freeze-dried and the concentration dependence determined.

The experimental solutions, prepared at most two days before the run, were prepared by diluting a concentrated mother liquid with CO-saturated buffer. The concentration of the mother liquid was obtained by dry substance determinations. For prep. V and VI this was done by drying at 40° C to constant weight. For the other preparations the dry weight was obtained from an average of one freeze-drying to constant weight and one drying in vacuo over phosphorus pentoxide at 20° C.

All runs were performed with the same centrifuge (A) and with the same rotor, which was not removed between September 15, 1949 and April 14, 1950. The optical system used has been Lamm's scale method. Hydrogen pressure was 15 mm Hg. The number of revolutions has been determined by means of a stroboscope and a vibrating-reed frequency meter for preps. V and VI and with a LKB-frequency meter for the other preparations.

RESULTS AND DISCUSSION

The results obtained are shown in Table 1. Two different calculations (1) and (2) were made for preparation V. In the first instance, all points were taken into consideration whereas in the second only those between concentra-

Table 1.*

Prep. No.	Date of prep.	s_{20}^0	$K \cdot 10^{-2}$	$\Phi \cdot 10^{-2}$	Max. Δ	Notes
V (1)	30.10.48	4.60 ± 0.05	17.1 ± 1.6	3.72	0.23	All points taken
V (2)	»	4.78 ± 0.05	21.0 ± 1.2	4.39	0.07	Conc. from 2.0 to 7.0 % Hb
VI, a	24. 1.49	4.59 ± 0.05	18.0 ± 1.4	3.92	0.17 **	Run 31.1—16.2 pH ca. 7.0
VI, b	»	4.60 ± 0.06	17.7 ± 1.4	3.85	0.08	Run 17—19.2 pH ca. 5.4—6.2
VI, c	»	4.54 ± 0.02	16.9 ± 0.5	3.72	0.03	Run 21—24.3 pH ca. 7.1
VIII A	6.10.49	4.48 ± 0.02	16.4 ± 0.5	3.66	0.04	Run 20—29.10
VIII B	»	4.50 ± 0.01	15.8 ± 0.3	3.51	0.02	Run 3—5.11
IX A	16. 2.50	4.53 ± 0.02	15.4 ± 0.6	3.40	0.05	Run 8—13.3
IX B	»	4.57 ± 0.03	17.8 ± 0.7	3.89	0.06	Run 16—20.3
IX B (F)	»	4.57 ± 0.03	16.5 ± 0.8	3.61	0.06	Run 1.—12.4

s_{20}^0 and Max. Δ are given in Svedberg units.

Max. Δ is the greatest deviation in s_{20}^0 from the corresponding line, shown by any point (see also appendix).

* Note added in proof (9. 12. 51): Recent meltingpoint experiments by Dr Pedersen indicate that the real temperature of the cell was higher than the one recorded by the thermo-element. That means that the absolute values of s_{20}^0 given here may be of the order of 5 % too high. It does not however affect the relative temperature nor the discussion here.

** The next highest value of Δ is 0.09.

Fig. 1. Relationship between concentration and sedimentation for preparations V and VI. For prep. V two different calculations have been made, in (1) all points are taken into consideration, in (2) only those between concentrations of 2.0 and 7.0 % hemoglobin.

tions of 2.0 and 7.0 % hemoglobin were taken. The reason for this is a noticeable tendency of the points corresponding to the lower concentrations to deviate from the line, as can be seen from Fig. 1a. This may be due either to accidental experimental errors, or to some alteration in form or size of the molecules in the more deluted solutions, as for example, incipient dissociation.

Corresponding deviations could not, however, be observed with the other preparations. Since there is no cause to assume that the conditions for the runs of the preparations V and VI were different from one another the only possible explanation for the unusually large error for V (1) (see appendix) is that there is no linearity when the low hemoglobin concentrations are taken into account.

With the buffer system used in V and IV b, ($0.0666 \text{ } M \text{ KH}_2\text{PO}_4 + 0.200 \text{ } M \text{ NaCl}$) the pH of the solutions varies approximately linearly with the concentration of hemoglobin (Fig. 2). If one then assumes that the deviation discussed above is due to incipient dissociation, one could explain it by the fact, that at low protein concentrations, the pH is at the limit of the stability range for the hemoglobin.

On the other hand, however, a comparison between VI a and VI b (see Fig. 1 b and c) indicates that variations in pH such as the ones that comes into

Fig. 2. The variations of the pH with the concentration of hemoglobin for the buffer-system 0.0666-M KH_2PO_4 and 0.200-M NaCl .

Fig. 3. The specific sedimentation volume Φ plotted against the time elapsed between the preparation and the respective runs.

consideration here do not affect the sedimentation. Further, since V and VI b were run with the same buffer concentrations, the large deviations of V from the rest of the material could be explained only by pH influence if it is assumed that the pH stability varies somewhat from one preparation to the next.

By comparing VI a, b and c, one finds that the value for K has a noticeable tendency to fall. Since the error areas partly overlap, this observation is naturally somewhat uncertain. By plotting, the specific sedimentation volume Φ against the time elapsed between the preparation and the respective runs, one obtains three points on a somewhat curved line (Fig. 3).

Although these observations are also subject to some uncertainty, they were thought to be of some interest, due to the comparatively short time (2 months) in which the alterations took place. During this time the preparation was kept at about 3°C in the presence of toluol.

But even if there should not be any changes according to time, these three determinations for prep. VI will give an idea of the reproducibility of the relationship between concentration and sedimentation for one preparation.

The duplicate preparations (VIII A — B and IX A — B) were made in order to ascertain whether the preparation work as such had any influence on the sedimentation. By comparing the values for s_{20}^0 one sees that they agree rather well two by two. There is, however, a difference between the K values of IX A and IX B and possibly also between those for VIII A and VIII B. Since variations in K are due to variations in Φ , they indicate that the preparation procedure can affect the specific sedimentation volume.

Finally, considering all comparable determinations, *i.e.* preparations V (2), VI a, VIII A and B and IX A and B, the following can be concluded. Even after excluding prep. V which evidently is different from the others, the values of the other determinations of s_{20}^0 and K are spread over so wide an area, that it seems hardly possible to attribute these variations exclusively to experimental error, especially in view of the relatively small mean errors.

Preparation IX B and the freeze-dried prep. IX B (F) give also the same s_{20}^0 but show slightly deviating K -values.

If one assumes that the specific volume of hemoglobin is constant and that the shape of the molecules is approximately the same, the variations in Φ could be explained by assuming that the layer of hydration around the hemoglobin molecules is not constant. Whether these variations depend only on the preparative procedure or if there are also some biological variations to be taken into consideration cannot be answered at the present time. A much larger number of preparations would be necessary to determine the magnitude of the preparative variations.

APPENDIX

Accidental errors in the calculation of s_{20}^0 values are due to the following three factors:

1. The number of revolutions as calculated by means of the stroboscope and vibrating-reed frequency meter can be given with an accuracy of ± 1.7 r.p.s. With a frequency meter the error depends only on the calibration, which in the LKB instrument is done with a tuning fork with 150.0 ± 0.1 vibrations/sec. Consequently the error in the speed of the ultracentrifuge will be less than ± 0.5 r.p.s. The corresponding error in s_{20} will then be ± 0.33 % for the stroboscope and ± 0.1 % with the frequency meter.

2. The *relative* temperature in the cell. It is difficult to determine how accurately this is recorded by the thermo-element but if no sudden changes in the temperature are registered it is assumed that the value recorded by the instrument and the real temperature value correspond within $\pm 0.3^\circ\text{C}$. To this has to be added an error of about $\pm 0.1^\circ\text{C}$ in reading of the instrument. The total error in the determination of the temperature is therefore $\pm 0.4^\circ\text{C}$ which corresponds to ± 1.0 % error in s_{20} .

3. Determination of the x -values. Taking into consideration that the great majority of all calculated exposures — with exception of a small minority — give narrow and symmetrical peaks, it can be assumed that the x -values in a diagram can be given to ± 0.5 mm. Since the scale is 1 : 20 the position in the cell should be determined to within ± 0.0025 cm, which agrees with the accuracy given by Cecil and Ogston⁴ for their diagonal schlieren system according to Philpot. The corresponding error in s_{20} should then be less than ± 0.5 %. The total error in s_{20} thus should not exceed ± 1.9 % if the speed is determined by means of the stroboscope or ± 1.6 % if it is determined by means of a frequency meter.

The error in the determination of the concentration is due partly to the error in the determination of the concentration of the mother liquid and partly to an error of dilution. Estimating each separately with ± 0.5 %, the total error is ± 1.0 %. Especially for the later determinations, however, with more experience in the technique, this error is probably too high.

As can be seen from the column "Max Δ " in Table 1, all values from runs performed with a frequency meter lie within the limits of error. For prep. VI a two runs are outside the limits. If for prep. V the concentrations between 2.0 and 7.0 % only are taken into consideration (alternative 2) all runs lie within the limits of error (Max Δ 0.07). If all points from prep. V are taken into the calculation (alternative 1) as many as four points deviate from the limite (Max Δ 0.23).

Assuming that the only errors are the deviations from a linear relationship which the separate points show, one obtains the following expression for the mean errors ⁵:

$$E_{s_{20}} = \pm \sqrt{\frac{\bar{s} \cdot \Sigma \Delta^2}{n - 2}}$$

$$E_K = \pm \sqrt{\frac{\bar{K} \cdot \Sigma \Delta^2}{n - 2}}$$

$$\text{where } \bar{s} = \frac{\Sigma c^2}{n \cdot \Sigma c^2 - (\Sigma c)^2}; \bar{K} = \frac{n}{n \cdot \Sigma c^2 - (\Sigma c)^2}$$

$\Delta_i = s_{20}^0 - K \cdot \gamma_i - \xi_i$; (γ_i ; ξ_i coordinates for a point) and n the number of points. The errors shown in Table 1 have all been calculated according to the above formulas.

SUMMARY

The linear relationship between concentration and sedimentation has been studied for six preparations of bovine CO-hemoglobin, all obtained by the same method. The differences between the slopes of the lines seem to be greater than could be expected from the experimental errors. This indicates that hemoglobin is affected by handling during the preparative work. Further it is shown that there is a small alteration upon storage during the time of two months. A possible explanation in both cases is that the layer of hydration is not constant.

The author wishes to express his thanks to Dr Kai O. Pedersen for suggesting the present problem and for his kind interest and valuable advise. Further thanks are given to Prof. Stig Claesson for his interest and helpful criticism, and to Mr. Evald Hellman and Miss Annalisa Norling for assistance with the calculations.

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Received March 21, 1951.