CCXXXV. THE SPLITTING OF HAEMOGLOBIN BY ACIDS

By NILS GRALÉN

From the Institute of Physical Chemistry, University of Upsala, Sweden

(*Received 14 September 1939*)

It is well known that haemoglobin is split by acids into its protein constituent, globin, and the prosthetic group haem. That these two dissociation products can be recombined has long been known, and the resynthesis has been extensively studied. The purpose of the present work was to investigate the products of fission and reconstitution with the aid of the ultracentrifuge. Globin has been previously examined in the ultracentrifuge [Svedberg, 1930] and found to be heterogeneous as regards molecular weight. Svedberg suggested that this heterogeneity was caused by severe chemical conditions during preparation of the globin, and, further, that milder methods would probably yield a monodisperse product. Some of the results of the present investigation have already been quoted by Svedberg [1937; 1938].

Experimental

The method of Anson & Mirsky [1930] was used for the splitting and reconstitution of haemoglobin. Horse COHb was treated with 0·1 *N* HCl, and the globin thus split off was precipitated by acetone, separated, air-dried and dissolved in water. The solution obtained was slowly neutralized with 0·1 *N* NaOH, which precipitated denatured protein, and the "native" globin was further freed from denatured globin (paraglobin, according to Roche [1930]) by 40 % saturation with ammonium sulphate. The native globin was then precipitated by adding solid ammonium sulphate (16 g./100 ml. solution), redissolved and dialysed against distilled water in the cold. The solutions obtained were never very stable. (Cf. Roche *et al.* [1932], who report a similar instability of native horse globin.) During dialysis part of the globin was always precipitated; moreover, a clear solution of globin soon became turbid if kept at room temperature. On electrodialysis the protein was completely precipitated.

Alkaline haem solutions were prepared by adding to the acetone solution 1 % of its volume of 2 *N* sodium acetate, and dissolving the precipitate so obtained in a buffer consisting of equal parts of Na_2CO_3 and $NaHCO_3$ [cf. Anson & Mirsky, 1930]. By adding this solution to a solution of neutral globin, methaemoglobin was obtained; this was reduced by the addition of a small amount of $Na_2S_2O_4$, then immediately saturated with CO and dialysed against distilled water. Some precipitate always formed during the coupling and subsequent dialysis, but the residual solution was very stable and could be kept for months at 4°. Electrodialysis, however, precipitated the protein. In order to obtain a successful coupling of haem and globin, it was necessary to use a freshly prepared solution of haem [cf. Herzog, 1933].

Sedimentation experiments. Sedimentation experiments were carried out in the ultracentrifuge at a speed of 59,000 r.p.m., corresponding to a force of gravity ×250,000. The temperature varied from 25 to 30°. Observations were

made with the light absorption method and with the scale method of Lamm [1937], both of which have been described by Svedberg [1937; 1938] and by Svedberg & Pedersen [1939]. The acid acetone globin, namely, an aqueous solution of the globin precipitated by acetone from the HCl solution, examined before neutralization and dialysis, appeared to be heterogeneous with regard to molecular weight. The size of molecules present varied within a wide range around a maximum corresponding to a sedimentation constant (reduced to water at 20°, s_{20}) of about 2·5.[1] In order to decide whether this heterogeneity was caused by the acetone precipitation, experiments were also made with haemoglobin in acid solution (*p*H about 2), where fission into haem and globin will have taken place. The haem was observed by light absorption at 546 mμ and was found to sediment at a rather rapid rate ($s_{20}=7{\cdot}5$), showing that it forms aggregates in the solution. The globin could be observed by light absorption in the ultra-violet (chlorine and bromine filters) and also by the scale method. It sedimented more slowly than the haem and was appreciably heterogeneous. The sedimentation constant by either method was found to be 2·2, which is in satisfactory agreement with that obtained for the acetone-precipitated globin, if allowance is made for the unsatisfactory sedimentation diagrams obtained with heterogeneous substances.

The neutralized globin solutions, from which paraglobin had been removed, were dialysed against phosphate buffers at *p*H 7·0 (in general 0·2 *M* NaCl + 0·03 *M* Na_2HPO_4 + 0·02 *M* KH_2PO_4) before ultracentrifuging. This material gave much better sedimentation curves than the acid acetone globin, but no preparation was found to be entirely homogeneous. The best criterion of homogeneity is that the diffusion constant, calculated from a sedimentation experiment, should agree with the results from a static diffusion experiment. The diffusion constant of globin is 6·5 (see below). In the best sedimentation experiment the values 7·8, 8·1 and 9·8 were obtained for the diffusion constant at different times. This is a fair agreement, but it still indicates a slight inhomogeneity. The preparation used for this experiment was carried out entirely at 4°, which proved to be more suitable than room temperature. The sedimentation constants were readily reproducible. The results obtained from four different preparations are recorded in Table I.

Table I

Exp. no.	Temperature of neutralization of globin °C.	Method of observation	Sedimentation constant	Protein concentration %
H 10	+20	Light absorption	2·64	—
G 6	+20	Scale	2·59	0·2
G 10	+ 4	,,	2·53	0·2
G 12	+ 4	,,	2·51	1·0

The protein concentrations are calculated by integration of the sedimentation curves, assuming a refractive index increment of $1{\cdot}96 \times 10^{-3}$ for globin at a wave-length of 436 mμ. Owing to the instability of the solutions, it was difficult to determine the concentration by any other method. The figures given are to be regarded as approximate. In concentrations below 1%, the sedimentation constant is independent of concentration.

The synthetic COHb proved to be homogeneous in the ultracentrifuge. Observations were made by the light absorption method at a wave-length of 546 mμ, and the photometer curves obtained indicate monodispersity as for

[1] Sedimentation constants are given in units of 10^{-13}.

native haemoglobin. The sedimentation constant was about 4·5, in a *p*H range from about 6 to 9·8, for moderate protein concentrations (0·5–1 %) and in buffer solutions of a molarity of about 0·2. Exposures at 546 mμ and with ultra-violet light made during the same experiment gave the same sedimentation constant, and the ultra-violet photographs show that no protein is left behind the sedimenting boundary. This provides good evidence that all the globin present after dialysis has recombined with haem, and that denatured globin has been precipitated. At a low concentration (0·1 %) of the synthetic haemoglobin, s_{20} was 4·0, and the same value was obtained from an experiment at a relatively high concentration (2·8 %), the *p*H being 6·9 in each case. The *p*H stability diagram of the synthetic COHb is shown in Fig. 1; it is almost identical with that of native haemoglobin [Andersson & Pederson, unpublished].

As mentioned above, it was observed that the haem in acid solution sediments rather rapidly. In alkaline solutions the same effect was shown though to a lesser degree (in different experiments s_{20} varied from 2·2 to 3·6). In all instances the aggregates formed were very polydisperse. Aggregation in alkaline solutions was noted by Anson & Mirsky [1930] who observed that the haem did not pass through collodion membranes made to retain haemoglobin.

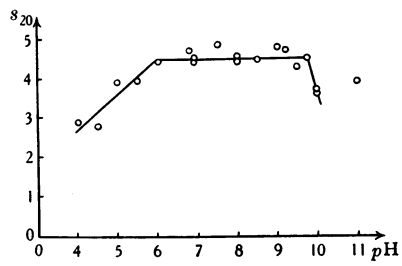

Fig. 1.

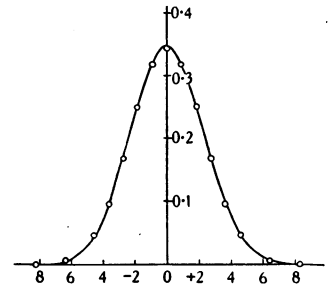

Fig. 2.

Fig. 1. *p*H-stability diagram of synthetic carbon monoxide haemoglobin.

Fig. 2. Diffusion curve of native globin. Abscissa: distance from original boundary position (mm.). Ordinate: scale line displacement (mm.). Time after start: 36,000 sec. The experimental curve is continuous, the circles indicate the theoretical curve for a homogeneous substance.

Diffusion experiments. The best globin preparation was used for a diffusion experiment at 20°, using Lamm's method [1937]. The value $D_{20} = 6{\cdot}5$[1] was obtained (concentration 0·54 %). The diffusion curves obtained showed a very slight indication of heterogeneity (see Fig. 2, which gives the globin diffusion curve, compared with a normal distribution curve that should be given by a homogeneous substance).

A 0·8 % solution of the synthetic haemoglobin gave a diffusion constant of 6·3, which is in good agreement with the value (6·27 in a 0·8 % solution) found by Tiselius & Gross [1934] for native horse haemoglobin. Polson [1939, 1] has reported a higher value for the diffusion constant of horse haemoglobin (7·09 in a 1 % solution).

Molecular weight calculations. The molecular weights of the proteins investigated have been calculated by the formula

$$M = \frac{RTs}{D(1 - V\rho)}$$

[1] Diffusion constants are given in units of 10^{-7}.

[Svedberg, 1937; 1938; 1939]. The partial specific volume, V, for COHb was shown by Svedberg & Fåhraeus [1926], to be 0·749. The same value has been used here for the synthetic haemoglobin and for the native globin, which presumably cannot introduce any large error. By this means a molecular weight of 37,000 has been obtained for globin and 69,000 for synthetic haemoglobin. The frictional ratio, f/f_0, namely, the ratio between the observed frictional constant and that of a compact spherical molecule of the same weight [Svedberg, 1939] was 1·47 for globin and 1·23 for synthetic haemoglobin.

Electrophoretic experiments. The synthetic COHb was investigated in the electrophoresis apparatus with quartz tubes, described by Tiselius [1930, cf. Pedersen, 1933]. All the protein solutions used for electrophoresis were dialysed overnight at room temperature against buffers of ionic strength 0·02. The electrophoresis experiments were carried out at 20°. Control experiments were made with native COHb which had been treated with a few crystals of $Na_2S_2O_4$ and then dialysed. The controls gave results which are in good agreement with those obtained by Pedersen, who found an isoelectric point of 6·92 for horse haemoglobin, using the same apparatus (unpublished results, quoted by Svedberg [1939]). It is therefore evident that the hydrosulphite has not affected the protein.

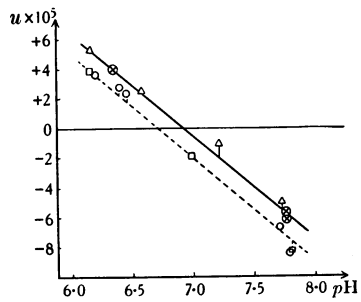

Fig. 3. Electrophoretic mobility (u), of native and synthetic carbon monoxide haemoglobin. The continuous curve is the mobility curve of native Hb and synthetic Hb III; the dotted curve refers to synthetic Hb I and II. ⊗ Native Hb. □ Synthetic Hb, I. o Synthetic Hb, II. △ Synthetic Hb, III.

Three different preparations of synthetic haemoglobin were examined. The results are given in Fig. 3. All the preparations were electrophoretically homogeneous, and all showed the same picture in the ultracentrifuge. On electrophoresis, one of them (III) gave results that agree with those obtained from the native protein (isoelectric point 6·92), but the two others (I and II) had a slightly more acid isoelectric point (6·72), and the whole mobility curve was moved about 0·2 pH unit to the acid side. The difference is definitely outside the range of experimental error.

Light absorption experiments. The light absorption of native and recoupled COHb was measured in a König-Martens spectrum photometer. The maxima of the characteristic absorption bands were found to be at 539 and 568 mμ for the native protein, and 537 and 567 mμ for the recoupled haemoglobin. The differences are not necessarily significant, as the limits of error of the instrument are rather wide. The extinction coefficients, relative to the nitrogen contents of the solutions (Kjeldahl), agree well for the two preparations. This fact shows that the molecules contain the same number of haem groups per equivalent of globin. These results are in agreement with those reported by Herzog [1933].

Discussion

Roche & Combette [1937] have measured the molecular weight of synthetic haemoglobin by the osmotic pressure method. They obtained a value of 66,000 for the methaemoglobin formed, in good agreement with their values for methaemoglobin prepared directly from oxyhaemoglobin. During the present study methaemoglobin formed by coupling haem and globin was found to have a

sedimentation constant of about 4·5 (three different preparations gave values of 4·55, 4·42, and 4·45), which corresponds closely with the results obtained by Roche & Combette. In this work, COHb was used in most experiments, because it appears to be more homogeneous than methaemoglobin, as shown by Pedersen for native haemoglobin (personal communication), and indicated also by the experiments on recombined haemoglobin. The experiments on synthetic COHb show, moreover, that the molecular weight of the recombined protein is the same as that of the native protein (69,000 from latest ultracentrifugal measurements, [cf. Svedberg & Fåhraeus, 1926; Svedberg, 1939]. The frictional ratios before fission and after recombination are also the same, which would indicate that the shape and the hydration of the molecule are unchanged.

The electrophoretic measurements are possibly more sensitive to changes in the linkage of haem and globin, and would seem, at least in some instances, to give reason for doubt as to the identity of the native and the resynthesized haemoglobins. This view is further supported by the fact that electrodialysis causes precipitation of the recombined protein and not of the native protein.

Roche *et al.* [1932] measured the osmotic pressure of solutions of native ox globin, and found that the molecular weight depended on the protein concentration. Their experiments extended over a wide concentration range, from 0·8 to 10·18 g./100 ml. They also investigated native horse globin at *p*H 5·6, but only at low concentrations (< 1·11 %). In solutions of about 1 % they report a molecular weight for native globin of about one-half that of haemoglobin. The present results support this finding. The molecular weight of native horse globin is independent of concentration below 1 %, which is not contradictory to the results of Roche *et al.* for higher concentrations. It is therefore probable that the splitting of haemoglobin into haem and globin by acid is accompanied by a splitting of the protein molecule into halves. Steinhardt [1938] has shown that the haemoglobin molecule can be split into halves by urea. Pedersen & Andersson found that haemoglobin is split into halves in very dilute solutions (unpublished work, quoted by Svedberg [1939]). It thus seems evident that the linkages between the two halves of the haemoglobin molecule are rather weak.

It was not possible to obtain globin in as homogeneous a state as native or recoupled haemoglobin. This would indicate that the globin solutions always contained some denatured protein still in solution, or else that part of the globin was present as complexes which broke up when the haem was combined with the globin. The fact that some precipitate was always formed on recoupling gives support to the former view, and the precipitate given was probably a combination product of haem and denatured globin.

The frictional ratio of haemoglobin is 1·23 and that of globin 1·47. This would indicate that the smaller molecule has a more extended shape. As pointed out by Polson [1939, 2], the products of fission of protein molecules have often higher frictional ratios than the original molecule. Polson found that the *p*H dissociation of protein takes place along the long axis of the molecule. Even if his numerical calculations are not strictly accurate (as he does not take into account the hydration of the molecules) it is probable that his finding is applicable to the splitting of haemoglobin by acid.

Summary

1. Globin and haem, obtained by splitting horse haemoglobin with acid, could be recoupled to form a haemoglobin that has the same molecular weight (69,000) as native haemoglobin, the same *p*H-stability diagram and the same molecular shape.

2. Electrophoretic experiments have, in some instances, given reason to doubt the identity of native and recoupled haemoglobins.

3. Native globin has a molecular weight about half that of haemoglobin. It is probable from the frictional ratios of the molecules that the splitting has taken place along the long axis of the haemoglobin molecule.

The author wishes to thank Prof. T. Svedberg for suggesting this research, for the facilities provided at his laboratory and for his kind interest in the work. Thanks are due also to Dr K. O. Pedersen for valuable advice and to Dr G. S. Adair and Mrs M. E. Adair for useful criticism.

The expenses of the work were defrayed by grants from the Rockefeller and the Wallenberg Foundations.

REFERENCES

Anson & Mirsky (1930). *J. gen. Physiol.* **13**, 469.
Herzog (1933). *Biochem. Z.* **264**, 412.
Lamm (1937). *Nova Acta Soc. Sci. upsal.* IV, **10**, No. 6.
Pedersen (1933). *Kolloidzschr.* **63**, 268.
Polson (1939, 1). *Kolloidzschr.* **87**, 149.
—— (1939, 2). *Kolloidzschr.* **88**, 51.
Roche (1930). *C.R. Lab. Carlsberg*, **18**, No. 4.
—— & Combette (1937). *Bull. Soc. Chim. biol., Paris*, **19**, 627.
—— Roche, Adair & Adair (1932). *Biochem. J.* **26**, 1811.
Steinhardt (1938). *J. biol. Chem.* **123**, 543.
Svedberg (1930). *Kolloidzschr.* **51**, 10.
—— (1937). *Nature, Lond.*, **139**, 1051.
—— (1938). *Industr. Engng Chem.*, Anal. Ed. **10**, 113.
—— (1939). *Proc. roy. Soc.* B, **127**, 1.
—— & Fåhraeus (1926). *J. Amer. chem. Soc.* **48**, 430.
—— & Pedersen (1939). The Ultracentrifuge. Oxford (in the Press).
Tiselius (1930). *Nova Acta Soc. Sci. upsal.* IV, **7**, No. 4.
—— & Gross (1934). *Kolloidzschr.* **66**, 11.