CCXVII. ON THE SPREADING OF DIFFERENT HAEMOGLOBINS, MUSCLE HAEMOGLOBINS AND CYTOCHROME *c*

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STUDIES of Gorter & Grendel [1929] and Hughes & Rideal [1932] have shown that proteins, although mostly soluble in water, are usually almost completely absorbed in a homogeneous layer on the surface, provided that this is large enough. The thickness of this protein layer is, if the layer is under a small pressure (2 dynes per cm.), of the order of the length of one amino-acid, i.e. c. 7 Å. This is far less than the diameter of a protein molecule. Therefore the molecule must be unfolded so that every amino-acid can have its place on the water surface. At a pressure of more than 2 dynes the hydrocarbon chains of the amino-acids are nearly vertical to the surface. At a lower pressure they are flat on the water. The time necessary for complete unfolding to occur on dilute buffer solutions is short when the *pH* of the solution is the same as the isoelectric point of the protein. On diluted buffer solutions of other *pH* values the unfolding of the protein molecules proceeds far more slowly; some of the protein dissolves in the fluid of the trough and in most cases homogeneous monolayers are formed extremely slowly [Gorter & Philippi, 1934]. On more strongly acid and alkaline solutions (*pH* 1 and 13) monolayers are immediately formed.

The time taken by a protein molecule to unfold at its isoelectric point varies greatly, even in closely related proteins [Jonxis, 1935; Brinkman & Jonxis, 1935; 1937]. The results to be presented deal with "unfolding time" and area measurements of different haemoglobins, muscle haemoglobin and cytochrome *c*, and the influence of concentrated salt solutions, urea and reducing substances on the spreading of these proteins.

MATERIALS AND METHODS

A Langmuir trough with Gorter's modifications [Gorter & Seeder, 1935] was used to measure the surface areas. The *pH* of the trough solutions was determined with indicators. The protein solutions were applied on the surface by means of a micro-pipette as used by Gorter & Seeder [1935]. Whilst blowing out the protein solution the pipette is slowly moved over the surface of the liquid in the trough, so that the protein solution comes into better contact with the surface. Mitchell [1937] has found that with this method gliadin only forms its maximum surface when the concentration of the gliadin solution is less than 0.1 mg. per ml. I therefore tested the influence of the concentration of the protein solution on the maximum area and the time after which this area is reached. The protein I used was horse haemoglobin; it was placed on 0.1 *N* HCl and on a 0.003 *M* phosphate buffer solution (*pH* 6.8).

Between concentrations of 25 and 0.05 mg. per ml. there was no influence of the concentration of the protein on the maximum area or on the time in which this area was reached.

In order to have sufficient protein on the surface it was necessary to use greater quantities of the more diluted solutions (50 μ l. for a concentration of 0.5 mg. per ml., 500 μ l. for a concentration of 0.05 mg. per ml.).

The concentrated solutions (10–25 mg. per ml.) must be blown out of the pipette very slowly because otherwise a part of the protein does not remain on the surface, but sinks to the bottom of the trough. All area measurements were done at room temperature.

Preparation of the proteins

Horse haemoglobin. (a) Horse blood was washed three times with a 0.9 % NaCl solution. To 100 ml. red blood corpuscles there were added 30 ml. distilled water and 30 ml. washed ether. After shaking for 5 min. 13 g. NaCl were added. After centrifuging, the clear haemoglobin solution was dialysed against distilled water in the ice chest.

Alcohol was added at a low temperature till the concentration was 20 %. The solution was left in the ice chest and the next day the crystals could be removed and washed with 20 % alcohol. Then they were dissolved in water by adding a few drops of *N*/10 NaOH at 39° till the fluid became dark and clear. After addition of the same amount of *N*/10 HCl the haemoglobin solution was placed in the ice chest and the next day a mass of crystals could be centrifuged off. This was repeated three times. Finally the haemoglobin crystals were dissolved in a diluted alkaline phosphate buffer solution and dialysed against a diluted phosphate buffer solution of pH 6.8. The spreading properties of this haemoglobin solution were just the same as those obtained with a solution prepared in the following way.

(b) The horse blood was washed three times with 0.9 % NaCl. The red blood corpuscles were haemolysed with the same volume of distilled water. After adding 0.25 volume *M*/10 phosphate buffer solution the solution was centrifuged to remove the precipitate of stroma proteins. The clear haemoglobin solution was diluted with distilled water to a concentration of 5 mg. per ml. The latter method has the advantage of taking less time. This is very important because the spreading properties of a haemoglobin solution change when the haemoglobin solution is some days old, although it has been stored in the ice chest. Small amounts of stroma proteins and lipins seem to have no influence on the spreading properties of haemoglobin, so it is not necessary to remove them completely.

The concentration was determined first gasometrically by the Van Slyke method, later colorimetrically.

Native globin was made and recombined with haem to haemoglobin according to the method of Anson & Mirsky [1930].

Muscle haemoglobin was prepared by the method of Theorell [1934] and was twice recrystallized.

Cytochrome c was prepared by the method of Keilin & Hartree [1937].

RESULTS

The relation between area and pressure for a monolayer of horse haemoglobin on 0.003 *M* phosphate buffer solution (pH 6.8) after the maximum area is reached is given in Fig. 1.

In its compressed state 1 mg. of horse haemoglobin covers (extrapolated to zero pressure) an area of 9400 cm.² This gives for one molecule an area of 10,560 Å.² A small part of this area (4 × 70 × sq. Å.) will be occupied by the four

haem groups of the molecule [Adam, 1938]. One molecule of horse haemoglobin is built up from 576 amino-acids [Bergmann & Niemann, 1937], therefore the area per amino-acid on the water surface will be 17.8 sq. Å. This value agrees very well with the results of Hughes & Rideal [1932] and Philippi [1934], who found for gliadin and ovalbumin under the same circumstances an area of 16.5 and 17 sq. Å. respectively per amino-acid. The side-chains under these conditions are up in the air or down in the water and only the polypeptide chains will be on the surface.

Fig. 1. Surface pressure curve obtained with monolayers of different haemoglobins.
 • Horse Hb at pH 6.8. ○ Cow Hb at pH 6.8. + Horse Hb at pH 1. □ Muscle Hb at pH 1.

I investigated haemoglobins of man, cow, dog, sheep, rabbit, cat and rat (both adult and foetal forms), and found that after the moment of reaching the maximum area the same area-pressure curve was obtained in the compressed state; when extrapolated to zero pressure the area was about 9400 sq. cm. per mg. The same curve was also found for the native globins prepared from these different haemoglobins. This shows that the total numbers of amino-acids of all these haemoglobins are the same per g., and if the mol. wt. is the same in the surface as in the interior of the solution, there will be the same number of amino-acids per per mol., i.e. 576.

At a lower pressure, below 3 dynes per cm., there are small differences between the surface pressure curves of the haemoglobins of different species (see Fig. 1). At these lower pressures the hydrocarbon chains of the amino-acids are mostly horizontal on the water surface and in this position the area is determined not only by the number, but also by the nature of the amino-acids.

On 0.1N HCl and on 0.1N NaOH monolayers are somewhat more compressible. The surface-pressure curves for pressures higher than 3 dynes per cm. are the same for all haemoglobins.

In Fig. 2 the relation between area and time at pH 6.8 is shown. The "unfolding time", after which the maximum area is reached, is, for human haemoglobin 5 min., for horse haemoglobin 18 min., for cow haemoglobin 35 min.

The time is independent of the way in which the haemoglobin has been prepared. Small amounts of impurities, such as stroma proteins and lipins, have no influence on this "unfolding time".

Fig. 2. Influence of the time on the area of monolayers of different haemoglobins.
• Human Hb. ○ Horse Hb. × Cow Hb. + Horse Hb on 25% urea at pH 6.8.

Higher salt concentrations of the liquid in the trough greatly increase the spreading tendency of proteins on the acid and alkaline sides of their isoelectric points [Gorter *et al.* 1932], but at the isoelectric point the concentration and the nature of the ions in the solution have no influence on the unfolding time; except on very concentrated solutions, where the unfolding time is somewhat smaller (see Table I).

Table I

Phosphate concentration	pH	Unfolding time in minutes	
		Horse Hb	Cow Hb
0.001 <i>M</i>	6.8	16	35
0.010 <i>M</i>	6.8	18	35
0.100 <i>M</i>	6.8	16	33
0.200 <i>M</i>	6.8	12	30
0.010 <i>M</i> + 0.001 <i>M</i> glutathione	6.8	17	35

Probably these differences in unfolding time are the results of different intramolecular linkages (especially S—S linkages) between the cysteine groups. No correlation could, however, be found between the amount of sulphur [Roche, 1936] and the unfolding times of haemoglobin molecules of different species. Other proteins, although containing S—S linkages, are immediately unfolded at their isoelectric points. This suggests that the S—S linkages do not seriously hinder the unfolding of a protein molecule. After keeping the haemoglobin solution a few days, the unfolding time decreases; it is therefore necessary to work only with fresh haemoglobin.

Fig. 3 shows the influence of the pH on the formation of monolayers of haemoglobin. Between pH 3–6 and 7.5–10 monolayers on 0.003*M* buffer solu-

tions are very imperfectly formed, most of the protein dissolving in the liquid of the trough. On concentrated buffer solutions (0.1M) the maximum spreading is almost immediately reached on solutions of pH 3-6 and 7.5-10, the unfolding

Fig. 3. The effect of pH on the spreading of horse haemoglobin and cytochrome. Time 5 min. • Horse Hb on diluted solutions. + Horse Hb on 0.2M phosphate buffer solutions. × Globin on diluted solutions. ○ Cytochrome on diluted solutions. □ Cytochrome on concentrated solutions.

time at the isoelectric point being the same as that on diluted solutions. Thus on concentrated solutions we find a minimum spreading tendency at the isoelectric point [Philippi, 1936; Danielli, 1938]. Native globin forms monomolecular films very easily. At pH 6.8 the maximum area is almost immediately reached (see Fig. 4). Haemoglobin synthesized from native globin and haem has the spreading properties of globin. By splitting off the haem the structure of the molecule must so be changed that the unfolding of the molecule proceeds more easily. In the synthesized haemoglobin the original structure is not restored. That synthesized haemoglobin is not identical with the normal haemoglobin is known from the work of Hill [1939], who found that the dissociation curve of synthesized haemoglobin is not of S-shape, but is a simple hyperbola.

The influence of urea on the spreading of haemoglobin

Burk [1932], Burk & Greenberg [1930] and Steinhardt [1938] have shown that in concentrated solutions of urea (25%) the molecular weight of horse haemoglobin is halved or even further diminished. The change in molecular weight was confirmed by Wu & Yang [1932] who showed that horse and ox haemoglobins are dissociated in concentrated urea solutions, but that the haemoglobins of sheep and dog are not. What, now, is the influence of urea on the spreading properties of haemoglobin?

Horse haemoglobin was dissolved in a 2% urea solution and brought on to the surface of a 0.003*M* buffer solution, which contained 2% urea. This concentration of urea had only a slight influence on the spreading. The surface pressure curve remained unchanged and the unfolding time was decreased from 18 to 15 min. The influence of more concentrated urea solutions, in which the haemoglobin dissociates, is greater. The surface pressure curve is flatter (Fig. 4). At greater areas, more than 1 sq. m. per mg., the pressure is higher and the layer more compressible. At pressures higher than 15 dynes/cm. the surface pressure curve is the same as on normal buffer solutions. This seems to indicate that on the concentrated urea solutions the side-chains are attracted by the surface. For horse haemoglobin the unfolding time is reduced from 18 to 9 min. For sheep haemoglobin, which is not dissociated by 25% urea, the surface pressure curve is the same as that of horse haemoglobin and the unfolding time is diminished from 30 to 17 min. This indicates that the dissociation unto molecules of half the normal size is not the cause of the diminution in the infolding time.

Fig. 4. Area pressure curve of horse haemoglobin and cytochrome and the influence of urea.
 • Horse Hb at pH 6.8. ○ Horse Hb on 25% urea at pH 6.8. + Cytochrome at pH 9.7.

The spreading of reduced haemoglobin

When a small amount of sodium hydrosulphite is added to the fluid in the trough, the maximum spreading is reached after 3 min. It does not make any difference whether reduced or oxyhaemoglobin is used. In both cases the maximum spreading is reached after 3 min. It is therefore improbable that the shorter unfolding time of haemoglobin on a reduced liquid is to be accounted for by the heat developed when the oxyhaemoglobin is reduced by the hydrosulphite.

The surface pressure curve is the same as that of oxyhaemoglobin on non-reducing solutions. If more haemoglobin solution is brought on to the surface some minutes later, when the sodium hydrosulphite in the trough has been oxidized by the air, it again takes 15 min. to reach the maximum spreading.

This shows that the unfolding time for reduced haemoglobin is far shorter than that for oxyhaemoglobin.

I have tried to repeat this experiment with other reducing substances (Stokes' solution), but the reducing power was already exhausted before the haemoglobin could be brought on to the surface. Haurowitz [1938] has found that there are differences in the forms of the crystals of reduced and oxyhaemoglobin probably due to differences in positions of the haem groups in the molecule. The differences in unfolding time between reduced and oxyhaemoglobin also suggest that there are differences in structure between reduced and oxyhaemoglobin. The resistance of the oxyhaemoglobin molecule to unfolding must be the result of special properties of the oxygenated haem groups, because reduced haemoglobin and globin are almost immediately unfolded.

The spreading of methaemoglobin

When some drops of ferricyanide are added to the solution in the trough the Fe^{++} of haemoglobin is oxidized to Fe^{+++} and methaemoglobin is formed. This change has no influence on the spreading. The surface pressure curve and the unfolding time are the same as those of oxyhaemoglobin.

The spreading of muscle haemoglobin

Muscle haemoglobin differs from the blood haemoglobin of the same species in its lower molecular weight (17,000) [Svedberg, 1939], its hyperbolic dissociation curve and a small difference in absorption spectrum. It is far more soluble and is precipitated only by saturated ammonium sulphate. Its spreading properties are also different from those of the blood haemoglobin. Between pH 4 and 11 monolayers are hardly formed at all, even on concentrated buffer solutions; the solubility seems to be too great. On more acid and alkaline solutions monolayers are formed, but under these circumstances the muscle haemoglobin is denatured. No differences between the spreading properties of horse and cow muscle haemoglobins could be detected. The globin prepared from muscle haemoglobin has, on buffer solutions with a pH between 3 and 11, the same spreading properties as the globin of blood haemoglobin. This indicates that the total number of amino-acids per Svedberg unit is the same as that of blood haemoglobin: viz. for a molecular weight of 17,000, 144 amino-acids. On solutions of pH 1 and 13 the surface pressure curve is flatter than that of blood haemoglobin, so there must be some differences between the two globins.

The methaemoglobin formed from muscle haemoglobin has the spreading properties of muscle oxyhaemoglobin, but reduced muscle haemoglobin immediately forms monolayers.

When a small amount of sodium hydrosulphite is added to a phosphate buffer solution (pH 6.8) a monolayer of reduced muscle haemoglobin is formed and the normal maximum area (9400 cm^2 per mg.) is reached in 5 min. When the same experiment is repeated after some time the hydrosulphite is oxidized and the muscle oxyhaemoglobin will not form monolayers. This demonstrates again that the condition of the haem groups has a great influence on the spreading properties of haemoglobin.

Do these changes in the spreading properties only occur when oxygen is added or removed, or can the oxidation of Fe^{++} to Fe^{+++} , as in oxidation of reduced cytochrome, also influence the spreading properties?

Cytochrome forms monolayers on solutions of a wide range of pH [Harkins & Anderson, 1938] (see Fig. 3). At pH 9.6 (close to the isoelectric point of cytochrome) a monolayer is easily formed and the maximum spreading is

reached after 5 min. At lower pressure the surface pressure curve is remarkably flat. When hydrosulphite is added, the unfolding time becomes greater; after 5 min. the area is only 25 % of the maximum area and the maximum is not reached, probably because a certain amount of the cytochrome is dissolved in the solution in the trough.

When the experiment is repeated, after the hydrosulphite has been oxidized by the air, the normal spreading of oxidized cytochrome is found again. Here also a difference in spreading properties between the oxidized and reduced forms can be found. The differences are just the opposite of those between oxygenated and reduced haemoglobin: oxyhaemoglobin forms monolayers with greater difficulty than reduced haemoglobin, oxidized cytochrome more easily than reduced cytochrome.

SUMMARY

The maximum areas of all haemoglobins in the compressed state are the same, 9400 sq. cm. per mg. This indicates that they are built up from the same number of amino-acids per Svedberg unit.

The times after which the maximum area is reached at the isoelectric point are different for the haemoglobins of different species.

This time is independent of the total ion concentration of the solution on which the films are spread, provided that the pH is that of the isoelectric point of the protein.

Small concentrations of urea have hardly any influence on the spreading properties of haemoglobin.

On concentrated solutions of urea the unfolding time is shorter, and the surface pressure curve is flatter, but there are no differences in the behaviours of horse and sheep haemoglobins, although horse haemoglobin is dissociated into molecules of half the normal molecular weight and sheep haemoglobin is not.

There are differences in unfolding time between oxygenated and reduced haemoglobin. Reduced haemoglobin spreads more rapidly than oxyhaemoglobin, but oxidized cytochrome spreads more rapidly than reduced.

Muscle haemoglobin does not form monolayers on solutions of a pH between 4 and 11, but reduced muscle haemoglobin forms monolayers at pH 6.8. The maximum area is reached after 5 min.

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