SOLUBILITY OF ADULT AND FETAL CARBONYLHEMOGLOBIN OF THE COW

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It is well known that the hemoglobins obtained from members of different species are different, and that the differences originate in the globin portion of the molecules. It is not entirely clear, however, whether the hemoglobins obtained from different members of the same species are the same, or whether the blood of a given individual contains more than one kind of hemoglobin molecule, and, if so, whether there are changes in the amounts of the different kinds with the age and condition of the individual.

Various experiments have been reported which deal with these questions. In 1927 Valer (1) made analytical measurements of the percentage of iron and sulfur in purified hemoglobin from different sources. In all cases the amount of iron was the same (0.33 per cent), but the amount of sulfur varied from one species to another. In the cat and cow, samples from different individuals were always alike, but in the horse and dog the sulfur content varied from one sample to another in a way which was interpreted to mean that dog hemoglobin is a mixture of several different kinds of molecules and that different individuals differ in respect to their hemoglobin. These results received confirmation in later studies of Kaiser (2), Timár (3), and Simonovits and Balassa (4). Schenck (5), in 1930, reported differences in the amino acid content of hemoglobin from different human individuals but it is questionable whether the differences do not lie within the limits of error of his analyses. Schenck has also reported a difference in the rate of digestion by pepsin of adult and fetal hemoglobin of man. On the basis of studies of the rate of denaturation of hemoglobin by strong alkali, von Kruger (6) and subsequently von Kruger and Bischoff (7) and Bischoff and Schulte (8) concluded that fetal hemoglobin in man is different from that of the adult, being more resistant to denaturation. Similar conclusions have been reached by Haurowitz (9, 10) and by Brinkman and his associates (11), who state that the blood of new born infants contains, in addition to the fetal type of hemoglobin, 20 per cent of a less resistant form which they identify with that of the adult. Brinkman and his associates also state that adult human blood contains 8 to 20 per cent of a resistant hemoglobin which may or may not be identical with fetal hemoglobin. These conclusions are supported by later studies of Brinkman and Jonxis.
SOLUBILITY OF CARBONYLHEMOGLOBIN

(12) on the rate of spreading of hemoglobin in monolayers. In these studies of Brinkman it was found that the resistant form of hemoglobin in fetal blood disappears 7 months after birth and is replaced by the resistant form in adult blood at $2\frac{1}{4}$ years.

There are numerous other studies directed to the problem of establishing a difference between adult and embryonic hemoglobins. Perrier and Jannelli (13) made optical measurements of crystals of hemoglobin from adult and new born human beings. They reported that the crystals from adults were biaxial; those from the new-born, uniaxial. These results were later confirmed by Haurowitz (10). On the other hand, careful spectroscopic studies of Jongbloed (14) in the ultraviolet region showed no difference between hemoglobin from adults and new-born. The greatest amount of work, however, has to do with the oxygen equilibrium. Haselhorst and Stromberger (15), in connection with the problem of oxygen secretion by the placenta, made measurements of the oxygen dissociation curves of maternal and fetal whole blood of man. They determined the oxygen affinity of the two kinds of blood in relation to pH and showed that at a given pH the affinity was higher in the case of fetal blood than in the adult. They believed that the difference is the result of an adaptation on the part of maternal blood, resulting in a decrease of oxygen affinity. In 1933, McCarthy (16) made gasometric measurements of the oxygen dissociation curves of purified goat hemoglobin dissolved in $M/15$ phosphate at pH 6.8. All samples of maternal hemoglobin gave the same curve, with $n = 2.2 \pm 0.3$. In fetal hemoglobin the value of $n$ was found to be $2.0 \pm 0.4$ and the oxygen affinity was approximately twice that of maternal hemoglobin. The two kinds of hemoglobin gave rise to different membrane potentials, but the osmotic pressure measurements showed no evidence of a difference of molecular weight. In a very recent communication (17) McCarthy reported similar results on solutions of fetal and adult hemoglobin of the sheep. The hemoglobin of the adult has a lower oxygen affinity and a higher value of $n$ than fetal hemoglobin, but there is no difference between the osmotic properties of the two. In 1934 Barcroft (18) and others made an extensive study of fetal and maternal whole blood from goats. In agreement with the work of McCarthy, they found that the oxygen affinity of maternal blood was less than that of the embryo. The difference was attributed in part to a difference of pH and in part to a

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1 The term oxygen affinity used in this discussion is the reciprocal of the value of the oxygen pressure corresponding to 50 per cent saturation of the protein with oxygen.

2 This is the $n$ which occurs in Hill’s equation describing the combination of hemoglobin with oxygen. The equation is $y/100 = kp^n/(1 + kp^n)$ where $y$ denotes percentage saturation of hemoglobin with oxygen, $k$ and $n$ are constants, and $p$ is the partial pressure of oxygen.
real difference in the hemoglobin molecules. In the same year, Hall (19),
using a spectroscopic method, obtained oxygen dissociation curves of
solutions of goat hemoglobin, maternal and fetal, at a concentration one-
one-hundredth that in blood. At pH 6.8 the oxygen affinity of fetal
hemoglobin was seven-fourths that of maternal blood. Similar results
were obtained with hemoglobin of incubating chicks (20), for which it was
shown that there was a gradual decrease in oxygen affinity during develop-
ment. In 1935 Haurowitz (10) showed that with human hemoglobin in
dilute solution (0.15 per cent) the curve obtained for the adult lies to the
left of that for the embryo, but that the relationship is reversed when the
hemoglobin is present in corpuscles. In 1936 Hill and Wolvekamp (21)
showed that the dilution of adult human hemoglobin might lead to a 2-fold
increase in oxygen affinity, without any considerable change in the shape
of the curve. Fetal hemoglobin was found to be much less affected by
dilution. Similar effects were observed with other animals. This effect
dilution on oxygen affinity has been quite recently confirmed by Gine-
zinsky (22). Still more recently McCarthy (23) has shown by gasometric
measurements that the affinity of embryonic human hemoglobin in con-
centrated solution is slightly less than that of maternal hemoglobin, but
that when the hemoglobin is in the corpuscles the relationship is reversed.

In view of this rather complicated picture and of the importance of the
question of the existence of different kinds of hemoglobin, we have under-
taken a study of the solubility of adult and embryonic cow hemoglobin.
Solubility represents perhaps the most significant criterion of chemical
identity, and is a property which can be studied fairly easily. The present
paper presents results which, though somewhat incomplete, are believed
to be decisive.

Cow hemoglobin was employed in all the experiments. The crystalliza-
tions of the protein and the subsequent equilibrations were all made in
strong phosphate solutions of pH ≈ 6.8, in the salting-out region of the
solubility curve. Approximately 1000 cc. of blood from adults and 500
cc. of fetal blood were used for each experiment. This was collected from
the slaughter-house in bottles containing 4 per cent trisodium citrate in
amounts equal to approximately 10 per cent of the volume of blood. In
addition to citrate a very small amount of toluene was added to minimize
bacterial growth. The blood was cooled as soon as possible after it was
received, and the cold blood was then saturated with carbon monoxide.
After this the cells were washed three or four times with cold 1.85 per cent
sodium chloride, being centrifuged each time in the cold. The washed
cells were then laked by being shaken after addition of approximately
one-fifth of their volume of toluene and one-fifth of their volume of water.
After the laked cells were centrifuged to remove the stroma, the subnatant
liquor was syphoned off and filtered in a Buchner funnel through No. 5 filter paper covered with Celite, or sometimes simply through paper. The clear filtrate was then saturated with carbon monoxide and stored in the cold as a stock solution.

When an aliquot of cold stock solution was added to concentrated ice-cold phosphate buffer, pH 6.8, the amounts being such as to give a final concentration of phosphate equal to about 2.15 moles per liter, an amorphous precipitate resulted at once, which, on standing and being shaken, became crystalline. Sometimes, after the precipitate had stood overnight in the cold, crystals could be seen attached to the side of the flask, which were an appreciable fraction of a mm. in length. It was apparent that the amount of precipitate obtained with solutions prepared from blood of adults was always greater than that obtained with solutions from embryonic blood and that the crystals from the latter were more soluble than those from the adult.

We give the following description of an orienting experiment which illustrates this. Two stock solutions of adult and embryonic hemoglobin were prepared by the method described above. A phosphate buffer was made up from $K_2HPO_4$ and $KH_2PO_4$, containing 4.346 moles per liter. The ratio $K_2HPO_4:KH_2PO_4$ was 0.577:0.423 and the density at 28° was 1.458. The calculated pH was very close to 6.8. This buffer was saturated with carbon monoxide. To each of two 100 cc. volumetric flasks, labeled Flasks A and E, for adult and embryonic hemoglobin respectively, were added exactly 50 cc. of this buffer. The flasks were then chilled to −4° and diluted to the mark with the cold stock hemoglobin solutions. An amorphous precipitate at once developed, which was much greater in Flask A than in Flask E. Both flasks were left in the cold overnight. On the next morning some crystals were apparent in each flask, but the bulk of the material appeared to be amorphous. After the flasks were shaken and allowed to stand for an hour or two, the precipitate in each had become completely crystalline so far as could be observed. When samples from Flasks A and E were centrifuged for half an hour at 3000 R.P.M., the crystals, being lighter than the mother liquor, collected at the top. The clear liquid underlying the crystals from the blood of the adult was colorless; that underlying the crystals from embryonic blood was a deep pink. After these liquids were syphoned off, the crystals were washed three times with a solvent made by diluting the stock phosphate buffer with water to twice its volume. The molar concentration of this solvent in total phosphate was 2.173 and the density 1.245 at 25°. During the second washing the crystals were left in contact with the solvent overnight. Throughout the process it was evident after each centrifugation that the clear liquid underlying the crystals of embryonic carbonylhemoglobin was always much
darker in color than that underlying the crystals of carbonylhemoglobin from the adult, and that the former crystals disappeared much faster than the latter. At the end of the third washing the crystals of embryonic carbonylhemoglobin were nearly gone.

In view of these results, further and more careful experiments were done in which the concentrations of the solutions were determined spectrophotometrically. For this purpose either of two instruments was employed. One of these was the spectrophotometer made by the Central Scientific Company but modified by the substitution of a much more sensitive photometric device. In the measurements made with this instrument the nominal slit width was 37 A. The other was the Beckman photometric quartz spectrophotometer. In the measurements made with this instrument the nominal slit width was 8 A. All the measurements were made at a wave-length of 5000 A and concentrations were calculated by taking the value of the absorption coefficient of cow carbonylhemoglobin as $5.50 \times 10^6$ sq. cm. per equivalent, where the weight of the equivalent is 16,700 (24). This means that if the depth of the absorption cell is 1 cm., as in all our measurements, the concentration of hemoglobin in gm. per 100 cc. may be obtained by multiplying log $I_0/I$ by 0.303. It may be noted that at a wave-length of 5000 A the absorption coefficients of oxygen and carbonylhemoglobin are almost identical.

The first experiment was carried out as follows: Two stock solutions, Solutions A and E, containing adult and fetal hemoglobin respectively, were prepared in the usual way except that, before filtration through Celite, the solutions obtained from the laked cells were dialyzed against water to remove traces of salt. After saturation with carbon monoxide, the concentrations of hemoglobin in these two stock solutions were measured with the spectrophotometer. That in Solution A was 22.0 gm. per 100 cc., and that in Solution E was 19.1 gm. per 100 cc. The density of Solution A was 1.0493, which, on the basis of a partial specific volume of hemoglobin of 0.749, would mean a concentration of 20.6 gm. per 100 cc. The concentrations of these two stock solutions were equalized by diluting Solution A with water to $22.0/19.1 = 1.15$ times the original volume. After this the concentrations of the two solutions were checked with the Beckman instrument and found to be, Solution A, 1705 gm. per 100 cc.; Solution E, 17.10 gm. per 100 cc. These two figures may be taken as equal within the limits of experimental error. The difference between the concentrations of Solution E as measured with the two photometers must be ascribed to factors involving the instruments themselves, but it is not relevant to the present experiments to inquire into it further. A phosphate buffer like that described in the preliminary experiment was made up and saturated with carbon monoxide. Exactly 50 cc. of this were added to each of two
425 cc. centrifuge cups. These were then cooled in an ice-salt mixture and to one were added exactly 50 cc. of the cold stock Solution A. To the other, an equal amount of cold Solution E was added. The cups were then stoppered, put in the ice chest, and left overnight. On the next morning they were placed in a rotator in a water bath at 7° and left rotating 4 days. At the end of this time the cups were centrifuged and the subnatant liquids were syphoned off and filtered in the cold. The concentrations of hemoglobin in the subnatant liquids were then determined with the spectrophotometer and found to be as follows: Solution A, 0.228 gm. per 100 cc.; Solution E, 2.59 gm. per 100 cc. While these equilibrations were being carried out, two additional precipitations were made in which 18 cc. of adult or embryonic hemoglobin were added to 20 cc. of phosphate buffer in smaller centrifuge cups. The solutions were then equilibrated by rotating the cups in the 7° bath for 3 days, and were removed and measured at the same time as the solutions in the large cups. The concentrations of hemoglobin in this second pair of solutions were as follows: Solution A, 0.0636 gm. per 100 cc.; Solution E, 0.616 gm. per 100 cc.

These results reveal the considerable difference in solubility between adult and fetal carbonylhemoglobin. However, it should be pointed out that the nature of the experiment accentuates this difference; as the protein precipitates, taking with it a certain amount of water of crystallization, it gives rise to an increase in the phosphate concentration in solution which tends to lower the solubility of the protein further. The effect is greater in the case of the less soluble protein.

In a second experiment the same stock solutions, Solutions A and E, and the same phosphate buffer were employed to make up a set of solutions in test-tubes. To each tube was added a certain volume of phosphate buffer, a certain volume of stock hemoglobin solution, Solution A or E, and a certain volume of water, the water, like the phosphate buffer, having been saturated with carbon monoxide. The sum of the volumes of water and hemoglobin solution was in each case equal to the volume of the phosphate buffer. These solutions were then equilibrated by rotating the test-tubes for several days in the bath at 7°. At the end of this time, the contents of all the tubes were filtered to remove crystals and the concentrations of the hemoglobin in the filtrates were measured with the Beckman spectrophotometer. In each case the measured concentration of hemoglobin was tabulated against the hypothetical concentration calculated from the way in which the corresponding test-tube was made up, on the assumption that all the protein remained in solution. In these calculations the concentration of hemoglobin in both stock solutions was taken as 17.07 gm. per 100 cc., the value obtained with the Beckman instrument, since the concentrations of dissolved hemoglobin were measured with this in-
instrument. No account was taken of the slight volume contraction resulting from mixing the liquids, since this is of the order of only 1 to 2 per cent and is very nearly constant for all the tubes. Its only effect would be to alter slightly the slope of the theoretical straight line shown in Fig. 1. This experiment was carried out twice, although lack of stock Solution E limited the number of points obtained the second time. The results are shown graphically in Fig. 1.

This type of solubility experiment is different from that used by Northrop. So long as the solution is unsaturated, the concentration of dissolved protein remains equal to the calculated concentration of added protein, subject only to the slight effect of volume changes discussed above. As soon as the saturation point is reached and crystals begin to form, the volume of the liquid phase is reduced, and, owing to the water of crystallization of the hemoglobin, the proportion of phosphate to water in this phase is increased. This leads to the phenomenon shown in Fig. 1, involving a sharp drop in the concentration of dissolved protein as more stock solution is added. The effect provides a sharp "end-point" corresponding to saturation. There is also another factor in the situation to be considered. As more stock solution and correspondingly less water are added, the ratio of total water in the system to total phosphate decreases whether or not there is any precipitate. Thus at the point of saturation the solvent is more concentrated in phosphate in the case of the more soluble protein.

This experiment makes it possible to calculate with fair accuracy the solubility of each of the two hemoglobins in a solvent of known ionic strength, different for each. It may be estimated from Fig. 1 that saturation occurs at a concentration of 4 gm. of protein in 100 cc. in the case of the hemoglobin of the adult and of 7.7 gm. of protein in 100 cc. in the case of
embryonic hemoglobin. These concentrations would result from combining the following quantities of water, stock hemoglobin solution, and phosphate buffer.

<table>
<thead>
<tr>
<th></th>
<th>Adult cc.</th>
<th>Fetus cc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock solution</td>
<td>23.4</td>
<td>45.1</td>
</tr>
<tr>
<td>Water</td>
<td>26.6</td>
<td>4.0</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

From these figures, taking account of densities, we calculate the following quantities of materials to be present in the two systems.

<table>
<thead>
<tr>
<th></th>
<th>Adult</th>
<th>Fetus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin, gm</td>
<td>4</td>
<td>7.7</td>
</tr>
<tr>
<td>Water, gm</td>
<td>83.65</td>
<td>83.10</td>
</tr>
<tr>
<td>Phosphate, mole</td>
<td>0.2173</td>
<td>0.2173</td>
</tr>
</tbody>
</table>

These figures mean that, at 7°, 4 gm. of the hemoglobin of the adult will dissolve in a solvent consisting of 85.65 gm. of water and 0.2173 mole of phosphate in which $\text{K}_2\text{HPO}_4$:$\text{KH}_2\text{PO}_4 = 577:423$; and that at the same temperature 7.7 gm. of fetal hemoglobin will dissolve in a solvent consisting of 83.10 gm. of water and the same amount of phosphate. It is a simple matter to calculate the ionic strengths of these two solvents. All that is needed for this is a knowledge of the densities. These were estimated on the basis of density measurements on other phosphate solutions of known composition as 1.257 and 1.265. These two figures give for the ionic strengths of the two solvents the following values: carbonylhemoglobin solution for the adult, 4.90; for the embryo, 5.05.

A final experiment remains to be considered. Stock Solutions A and E of adult and embryonic hemoglobin were prepared as already described, without the step involving dialysis. The same phosphate buffer was employed as before, and this was saturated as before with carbon monoxide. Stock Solutions A and E were added cold to equal volumes of phosphate buffer. After the precipitations were complete, samples of the mother liquor were withdrawn and centrifuged. The densities were then measured and found to be, Solution A, 1.2926; Solution E, 1.2806. A phosphate buffer was prepared by diluting the original buffer so that the concentration was 2.57 moles per liter, the ionic strength 5.54, and the density 1.2880. This was used as a solvent to wash the two sets of crystals. Six washings were carried out with this solvent over a period of 10 days at 7°. During each washing the crystals were equilibrated with the solvent by rotating the container in the constant temperature bath. At the end of each equilibration the material was centrifuged, the solvent syphoned off, put aside for measurement, and replaced with fresh solvent. At the end of the process the last four washings were filtered, and, after being suitably diluted,
measured with the Beckman spectrophotometer. The results are given in Table I.

These experiments show that embryonic cow hemoglobin is more soluble than adult and would seem to furnish decisive proof that the two proteins are distinct. It is possible to make use of the collected data to obtain crude salting-out curves for the two hemoglobins by plotting log, solubility against ionic strength. For this purpose we assemble the following data for the solubility, $S$, in gm. per 100 cc., of the two kinds of hemoglobin in relation to $\Gamma$, the ionic strength of the solvent per liter:

<table>
<thead>
<tr>
<th>Washing No.</th>
<th>Adult</th>
<th>Embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>gm. per 100 cc.</td>
<td>gm. per 100 cc.</td>
</tr>
<tr>
<td>3</td>
<td>0.012</td>
<td>0.080</td>
</tr>
<tr>
<td>4</td>
<td>0.006</td>
<td>0.062</td>
</tr>
<tr>
<td>5</td>
<td>0.016</td>
<td>0.070</td>
</tr>
<tr>
<td>6</td>
<td>0.012</td>
<td>0.076</td>
</tr>
<tr>
<td>Average</td>
<td>0.0115</td>
<td>0.072</td>
</tr>
</tbody>
</table>

The result of the plot is shown in Fig. 2. The curves may be interpreted in terms of the equation

$$\log S = \beta - K' \frac{\Gamma}{2}$$

It should be mentioned that a preliminary and somewhat less reliable experiment on the solubility of the hemoglobin of the adult at an ionic strength of 4.68 would indicate a considerably lower solubility than corresponds to that in Fig. 2.
in which \( \beta \) and \( K' \) are constants, by assuming the following values of the constants: adult hemoglobin, \( \beta = 20.237, K' = 4 \); fetal hemoglobin, \( \beta = 21.033, K' = 4 \). The values of \( \beta \) would indicate that, in the range of ionic strengths studied, the solubility of fetal hemoglobin is about 6.3 times that of the adult. However, since the curves drawn in Fig. 2 are each based on only two points, too much weight cannot be attached to the exact values of the constants, although there would seem to be no question of the greater solubility of fetal hemoglobin. Whether or not the adult and fetal hemoglobin preparations both represent a single kind of molecule or a mixture of two or more different kinds is a question which cannot be settled on the basis of these experiments. In the latter alternative it would seem likely that the crystals are solid solutions.

**SUMMARY**

Solubility measurements have been made on carbonylhemoglobin of adult and fetal cow blood. In strong phosphate buffers, of ionic strength 4.9 to 5.5 and pH 6.8, the hemoglobin from embryonic blood is more than 6 times as soluble as that from the adult. This would indicate that the two hemoglobins are distinct.

**BIBLIOGRAPHY**

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