SOME PROPERTIES OF CRYSTALLINE GUINEA PIG HEMOGLOBIN

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Several lines of evidence lead to the conception of wet protein crystals as made up of protein molecules with interstices between them that are large and filled with loosely held solvent molecules. Some of the evidence leading to this conclusion may be briefly summarized: (a) the high water content of protein crystals, early noted by Sørensen (1), who estimated that over 20 per cent by weight of egg albumen crystals is water; (b) the ease of water loss under slightly diminished aqueous tension as observed by Katz (2); (c) the change in density when suspended in solutions of different osmotic pressures as studied by Adair and Adair (3); (d) the staining of protein crystals with dyes; (e) x-ray data on wet pepsin crystals, from which Bernal and Crowfoot (4) infer that the protein molecules are relatively dense globular bodies separated by relatively large spaces which contain water.

We wish to present further evidence in support of this idea, and also to describe several other properties of crystalline guinea pig hemoglobin.

I

Action of $K_3Fe(CN)_6$ on Crystalline Oxyhemoglobin.—When a suspension of crystals of guinea pig oxyhemoglobin is treated under the microscope with $K_3Fe(CN)_6$ solution, the red crystals are observed to become yellow-brown while the crystal shape remains intact. (Other oxidizing agents of high potential such as quinone or $KMnO_4$ also bring about this change in color.) With the Abbe microspectroscope the yellow-brown color of the crystals was identified as Mth.¹ This experiment suggested that ferricyanide was penetrating into the crystal to react with the iron in HbO_2 .

In order to determine whether all of the HbO_2 in the crystals could be converted into Mth, a quantitative study with Warburg manometers was made of the O_2 released when HbO_2 is acted upon by ferricyanide. Freshly prepared

¹ Abbreviations used.

Hb = ferrous hemoglobin

HbO_2 = ferrous oxyhemoglobin

Mth = ferri hemoglobin or methemoglobin

washed crystals of guinea pig HbO_2 were suspended in $\text{M}/30$ phosphate buffer pH 7.0 and carefully pipetted into Warburg vessels that had two side arms containing the ferricyanide. The ferricyanide was added in two successive portions. The first portion, 0.05 cc., was sufficient to theoretically react with only

TABLE I
Recovery of O_2 Released by Ferricyanide from Crystals of HbO_2

Each vessel contained 2.0 cc. of HbO_2 crystals* suspended in $\text{M}/30$ Sørensen's PO_4 buffer, pH 7.0 at 29.8°C . (1). The dry weight of HbO_2 per cc. of suspension is 0.0493 gm. $\approx$ 66 c. mm. O_2 when quantitatively converted into methemoglobin with ferricyanide. If recovery of O_2 is 100 per cent then 0.100 cc. of 0.05 M $\text{K}_3\text{Fe}(\text{CN})_6$ should release 112 c. mm. O_2 from HbO_2 .

Vessel No.	0.05 M $\text{K}_3\text{Fe}(\text{CN})_6$ added		O_2 produced		Recovery of O_2
	cc.		c. mm.		per cent
1	0.05		54.6		97.3
	0.10	0.15	72.6	127.2	96.3
1A	0.05		51.8		92.5
	0.05	0.10	53.0	104.8	94.0
3A	0.30			133	100
5A	0.05		54.6		97.5
	0.20	0.25	82.6	137.2	105
7	0.05		53.0		94.6
	0.20	0.25	81.4	134.4	102
8	0.05		57.4		102.5
	0.20	0.25	78.0	135.4	102
1A'	0.20		135.0		102
	+ Veronal sodium†		135.0		102

* The uniformity of pipetting the crystal suspension into Warburg vessels gives rise to a maximum error of 2 per cent, as shown by the dry weight of three samples of HbO_2 suspension pipetted under the same conditions: 0.1190 gm.; 0.1220 gm.; 0.1181 gm.

† 0.20 cc. of a saturated sodium veronal solution was added from the side arm to make the solution sufficiently alkaline to dissolve the crystals. It is seen that no additional oxygen is recovered when the hemoglobin is in solution.

42 per cent of the total oxyhemoglobin. It is seen from Table I that this reaction was quantitative and corresponded stoichiometrically to the amount of ferricyanide added. From Table II it is seen that 85 per cent of the resulting Mth was in the crystalline state at the end of the experiment. Therefore the ferricyanide molecules must have penetrated the crystals and reacted with all the prosthetic groups of oxyhemoglobin. Considering the size and shape

of our crystals, this would mean that some molecules of ferricyanide in order to get to the center of the crystals would have had to penetrate a distance equivalent to the diameter of 1,500 hemoglobin molecules. The conclusion is obvious that there must be interstices in the crystals large enough to permit the movement of ferricyanide into them.

The speed of free diffusion of ferricyanide as calculated from the Stokes-Einstein equation under the conditions of our experiment is approximately 4×10^{-8} cm. per second. It was calculated that if the crystal offered no appreciable resistance to diffusion of ferricyanide, the whole journey through liquid and crystal would take the maximum time of 2 to 3 seconds. The maximum time experimentally determined in the manometer for complete

TABLE II
Solubility of the Crystals Used, at 29.8°C.

To each of two centrifuge tubes with conical ends, add 2.5 cc. HbO₂ crystal suspension in M/30 PO₄ buffer pH 7.0. To tube 1 add 0.2 cc. H₂O; to tube 2 add 0.2 cc. of 0.05 M K₃Fe(CN)₆. These are kept at 29.8° for 2 hours. They are then centrifuged sharply, the supernatant liquid decanted, and adhering liquid wiped away. Tube 1 now contains crystals of HbO₂ and tube 2 contains crystals of Mth. The crystals were weighed wet, and then dried at 110°C. to constant weight.

Tube No.	Dry weight of Hb in 2.5 cc.	Weight of crystals after decanting		Final amount of Hb suspension in crystal- line state <i>per cent</i>
		Wet	Dry	
1	0.1227 g.	0.2874	0.1097	89
2	0.1227	0.2790	0.1053	85

release of O₂ was 2 to 3 minutes. Undoubtedly a considerable portion of this time is due to the slowness of equilibration between gas and liquid phase in the manometer vessels. If the ferricyanide ions are to diffuse somewhat readily into the crystals of oxyhemoglobin the interstices must be at least 1.5 to 2.0 times the diameter of the ion, or from 6 to 10 Å.

Let us assume that the hemoglobin molecules are spheres and that they are arranged in closest hexagonal packing. It may be readily calculated that the volume of space between the spherical molecules occupies about 25 per cent of the total volume of the cube, or if filled with water this would mean that 25 per cent by volume or 20 per cent by weight of the wet crystal is water. These are, of course, minimum figures based on the assumption of closest packing and spherical molecules. The distance between the spheres would be just sufficient to permit ferricyanide movement into the spaces. It may be predicted that optically isotropic protein crystals will be found to contain at least this amount of loosely held water between the molecules.

Preparations Used

The tetrahedral crystals of HbO_2 were prepared from guinea pigs, bled by heart puncture into warm sodium citrate solution. The blood was washed twice with warm isotonic saline by centrifugation. To luke the cells three volumes of water were added to one of packed cells. Crystals started to form at once. The laked blood was kept at 37°C . for 2 hours in a 2-inch thick layer in a beaker and then centrifuged slowly for a short time. In this way only the large crystals are deposited, the supernatant solution being discarded. The crystals are suspended in water and centrifuged slowly, the process being repeated two more times. The crystals thus prepared were kept under a thin layer of water at 1°C . overnight. The next morning 10 cc. of the packed crystals were added to 45 cc. of Sørensen buffer ($\text{M}/15$ in phosphate) pH 7.0 and the suspension finally made up to 90 cc. with water. These guinea pig HbO_2 crystals were found to be practically free of catalase activity.

Mth was prepared from HbO_2 crystals by treatment with a slight excess of ferricyanide and subsequently washing the crystals three times by means of centrifuging, and then dialyzing them against water for 24 hours. A solution of Mth was then prepared by making the cold suspension slightly acid with dilute acetate buffer and bringing the solution to neutrality with $\text{M}/15$ disodium phosphate.

II

Location of the Prosthetic Groups.—A corollary of the quantitative oxidation of the hemes by ferricyanide appears to be that the heme groups are on the surface of the globin molecules, otherwise one would have to assume penetration of ferricyanide into the molecules. The latter is made unlikely because of the closeness with which the peptide chains are packed within the molecule. Mirsky (5) has offered this juxtaposition of peptide chains as an explanation for the non-detection of $-\text{SH}$ groups in some native proteins by means of ferricyanide. The data presented in Table I show that the hemes react quantitatively with the ferricyanide added. If some ferricyanide had oxidized $-\text{SH}$ groups before it oxidized the heme groups then a lower O_2 production per ferricyanide molecule would have been expected. For example, assuming only one free $-\text{SH}$ group reacting per hemoglobin molecule, one would expect to recover only 75 per cent of the O_2 .

In addition to being localized on the surface of the globin, the four hemes per globin must be facing the spaces between the molecules, or in other words, the hemes are not the points of closest contact between the molecules.

III

Further Evidence of Permeability of HbO_2 Crystals.—Hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$) may also be shown to penetrate the crystal of HbO_2 removing the oxygen and producing Hb. This becomes evident by diminishing the solubility of Hb with ammonium or sodium sulfate. When HbO_2 crystals were suspended in 50 per cent saturated ammonium sulfate and hydrosulfite added, the crystals could be seen to turn violet under the microscope and the Hb band became

visible at the same time that the HbO_2 bands vanished in the micro spectro-scope. These violet crystals go into solution within a few minutes. If the concentration of ammonium sulfate is increased to near saturation many of the purple crystals do not dissolve.

The staining of protein crystals with dyes may also represent penetration of the dye molecules into the crystal spaces. Several dyes in aqueous solution were added to HbO_2 crystals and examined microscopically. Due to the intense color of the crystals it is difficult to judge their penetration. Crystal violet, however, stained the crystals intensely but not methyl green. Catalase prisms and plates were better objects because of their negligible color (6). Methylene blue and toluidine blue stained them faintly, crystal violet very strongly, and methyl green very faintly. If the dye concentration, is properly chosen the crystals will become deeply colored and the solution colorless. The staining is not merely a surface adsorption since thicker crystals take up a very much deeper stain than thinner ones.

IV

The Relative Solubilities of Hb, HbO_2 , and Mth.— HbO_2 , as isolated from guinea pig blood is rather insoluble at room temperature. A suspension kept in $\text{M}/30 \text{ PO}_4$ buffer, pH 7.0, for 1 day at 1°C . and then for 3 hours at 30° was 89 per cent in the crystalline state. Mth is slightly more soluble, 85 per cent remaining in the crystalline state under these conditions. Hb, however, is very soluble at this pH.

When crystals of HbO_2 are watched under the microscope, as hydrosulfite diffuses to them, they are seen to melt away rapidly while still retaining their tetrahedral shape. At the same time the Hb band becomes visible. A thick suspension of HbO_2 crystals is a muddy red-brown. When treated with hydrosulfite it becomes a clear deep purple; on shaking with air it gradually becomes red, then cloudy, and begins to deposit crystals all within 1 to 2 minutes. Here is an example, then, of an aggregation and dissolution brought about by the mere addition or removal of oxygen.

V

The O_2 - HbO_2 Equilibrium in Crystalline HbO_2 .—The removal of O_2 from a solution of HbO_2 may be readily accomplished by lowering the pressure of O_2 . Under our conditions of evacuation from a Thunberg tube with an oil pump, it required less than 2 minutes to completely convert the dilute HbO_2 solution to Hb. However, if a suspension of HbO_2 crystals is treated in the same manner as the HbO_2 solution, then no appreciable O_2 can be removed even after 15 minutes of constant pumping, although the pressure was 15 mm. to 5 mm. of mercury (decreasing as the temperature of the suspension decreased due to evaporation).

The following experiments show that the oxygen of HbO_2 crystals can be

removed only by considerably decreasing the oxygen tension: (a) Bubbling either H_2 or N_2 gases (free from O_2) through a suspension of HbO_2 crystals at 30° for 20 to 30 minutes completely converted them to Hb solution. (b) Removal of O_2 by hydrosulfite: HbO_2 crystals in $M/15$ PO_4 buffer pH 7 were placed in a Thunberg tube and evacuated. A minute amount of hydrosulfite was then added from a side arm. Within 1 to 2 minutes the suspension became a solution of Hb. The pH after the experiment was 6.9. (c) Removal of O_2 by activated hydrogen: A suspension of HbO_2 crystals in the presence of traces of methylene blue or catalase was treated with H_2 + colloidal Pd. Within 1 to 2 minutes this suspension had been completely converted to a solution of Hb. (d) Replacement of O_2 by CO: A suspension of HbO_2 crystals was treated with pure CO gas for 5 minutes. The CO replaced the O_2 in the crystals without changing their shape, since on the addition of a small amount of hydrosulfite no Hb results and the crystals remained intact. On the addition of a large excess of hydrosulfite, the crystals began to dissolve very slowly and unevenly, this being due to a decrease in pH.

The firmer binding of O_2 in HbO_2 crystals, as contrasted to HbO_2 in solution, may indicate that the coordination of the molecule in the crystal has diminished the competition of other groups than O_2 for the iron. The HbO_2 crystals can be readily dissolved, away from the isoelectric point, and in the dissolved state the HbO_2 molecules readily give up their oxygen on diminishing the O_2 tension. The firm binding of O_2 in HbO_2 crystals would obviously prevent hemoglobin in the crystalline state from functioning as a readily reversible oxygen carrier.

VI

Action of H_2O_2 on HbO_2 .—The crystals of HbO_2 of the guinea pig, crystallized once, are practically free from catalase. On addition of a few drops of 0.5 per cent H_2O_2 to a suspension of HbO_2 crystals, the two prominent bands at 578 and 540 become faint, the 578 band appears to broaden, spread out to 590, and is relatively stronger than the 540 band, while the original crystalline shape remains unchanged. This compound has the same absorption bands as that of H_2O_2 -methemoglobin solution first described by Keilin and Hartree (7).

VII

Action of Activated Hydrogen on Mth.—Methemoglobin either in solution or in crystalline suspension at pH 7.0 in PO_4 buffer is very slowly reduced by activated hydrogen. Mth plus a trace of catalase and caprylic alcohol was bubbled with hydrogen for 3 minutes and then 2 drops of colloidal Pd added. The bubbling was continued for 15 minutes during which time only traces may have been reduced as observed spectroscopically in comparison with the control.

This slow interaction may be partially explained by the fact that we are dealing with two colloids. If a trace of methylene blue or rosindulin GG is

added to the Pd-H₂ solution containing Mth plus a trace of catalase, then reduction to ferrohemoglobin is complete in 1 minute in the case of the solution, and reduction and solution of Mth crystals is complete in 2 to 3 minutes. The methylene blue molecules being small and reversibly oxidized act as intermediary between the two colloids.

This is in agreement with the fact that also the electron exchange between hemoglobin and the bright Pt electrode is so sluggish that potentials are only poorly established.

VIII

The Action of Activated Hydrogen on HbO₂.—One of the intermediates produced in the reduction of O₂ either as such or in the form of HbO₂, by activated Pd-H₂ is H₂O₂. This may be shown by the following experiment. HbO₂ either in solution or as crystal suspension in PO₄ buffer pH 7, was treated with Pd-H₂. Within 45 seconds a band at 630 mμ had arisen and also the H₂O₂-Mth bands had appeared. The 630 band was identified as Mth by forming the Mth-NaF compound. If a trace of catalase is added to the HbO₂ solution, then Pd-H₂ added, the Hb is formed within 1 minute and in the case of the crystalline HbO₂, removal of O₂ and solution of crystals occurred in 1 to 3 minutes. No Mth band was observed.

Instead of using catalase to destroy the H₂O₂, a trace of methylene blue was used with identical results. Within 3 seconds after PdH₂ was added, the dye was decolorized and within 20 to 40 seconds the HbO₂ bands had vanished. Methylene blue may act here in three ways. Its reduced form may be oxidized by H₂O₂ thus destroying some peroxide; it may act on any Mth that might have arisen from the action of peroxide on Hb; and it may react with the minute amount of O₂ in solution.

SUMMARY

1. Guinea pig hemoglobin crystals are shown to be readily permeable to ferricyanide and hydrosulfite, indicating the presence of interstices between the protein molecules of the crystal.
2. The assumptions of closest hexagonal packing and of spherical molecules of HbO₂ lead to a crystal lattice having interstices between the molecules which represent 25 per cent by volume of the crystal. These spaces would be just large enough at their narrowest junctures to permit ferricyanide ions to diffuse through them. If these spaces were filled with water, then 20 per cent by weight of the crystals would be water.
3. The hemes are on the surface of the globin and are arranged facing the interstices between the molecules of the lattice.
4. The binding of O₂ in HbO₂ is stronger in the crystal lattice than in solution.

5. Hydrogen, activated with colloidal palladium, will not reduce ferrihemoglobin except in the presence of a redox dye.

6. In the reduction of O_2 by activated hydrogen, H_2O_2 can be demonstrated by the formation of the H_2O_2 -ferrihemoglobin spectrum.

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