THE COMPOSITION AND SWELLING PROPERTIES OF HAEMOGLOBIN CRYSTALS

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It has long been known that protein crystals can undergo swelling and shrinkage, that a large proportion of their volume normally consists of water, and that they are permeable to smaller ions. In recent years their composition has been studied by Adair and Adair,[1] and by McMeekin and Warner,[2] principally with a view to measuring the hydration of proteins.

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[1] Adair and Adair, *Proc. Roy. Soc. B.*, 1936, **120,** 422.

[2] McMeekin and Warner, *J. Amer. Chem. Soc.*, 1942; **64,** 2393.

The present study started as an attempt to determine the exact amounts of liquid in wet hæmoglobin crystals and the electron density in the liquid regions, data which were needed as part of a crystal structure analysis of horse methæmoglobin. It was mainly the difficulties encountered there which led to the study of the ionic equilibria between the interior of protein crystals and their suspension medium; this in turn drew our attention to the interesting swelling properties of the hæmoglobin crystals and raised the problem of the lattice forces which hold them together. The question of protein hydration, though outside the scope of the present discussion, has a direct bearing on the mechanism of swelling in protein crystals and gels, and is therefore treated as part of this investigation.

The lattice forces in protein crystals might be of several kinds, but for the present purpose it will be convenient to distinguish two main types: long range forces such as appear to exist in certain gels, and short range forces of the type more commonly found in crystal structures. Evidence of the former comes principally from studies of tobacco mosaic virus and bentonite gels and, more recently, from experiments with soap and phospholipoid micelles. The interparticle distances in those systems range from tens to several thousands of Ångstrom units, and vary as a function of both hydrogen ion and neutral electrolyte concentration. The nature of the forces which keep particles parallel and equidistant across such great thicknesses of water is not yet clear. If the lattice forces in protein crystals were of this long-range type, the unit cell dimensions should vary with hydrogen ion and electrolyte concentration. On the other hand, if short-range electrostatic forces between hydrated ions predominate, and hydrogen bonds act either directly between protein molecules or through intermediary water molecules, as in certain hydrated crystals, the unit cell dimensions should be independent of the composition of the suspension medium, except when a change in *p*H actually alters the distribution of fixed ionic changes on the surface of the protein molecule.

Before proceeding further, it will be useful to summarise our present knowledge of the structure of horse methæmoglobin crystals.[3, 4] These crystals are monoclinic, belong to the space-group $C2$, and have two molecules in the unit cell. They possess a layer structure, with rigid layers of hæmoglobin molecules extending throughout the crystal parallel to the crystallographic c plane, and alternating with layers of liquid of crystallisation whose thickness in the normal wet crystal is 15 A. If the crystals are gradually dried, the thickness of the liquid layers is reduced in steps first to 10 A., then to 6 A., and finally to zero. The layers of hæmoglobin molecules retain a thickness of 36 A. at all degrees of wetness of the crystal; they are rigid and apparently impenetrable to water. Evidence concerning their internal structure need not be described here.

Experimental.

Crystals of horse methæmoglobin were grown either by dialysis of hæmoglobin solutions against concentrated salt solutions or by electrodialysis of highly concentrated hæmoglobin solutions against tap water. In either case well developed crystals of up to 1 mm. diameter were obtained. The effect of different suspension media was studied by first washing them with the new medium on a slide and then leaving them for several days to reach equilibrium in the new medium before taking any measurements. The densities of the wet crystals were determined by flotation in mixtures of xylene and bromobenzene, according to the method of McMeekin and Warner.[2] Adair and Adair's method [1] of flotation in concentrated salt solutions was also employed. The different results given by these two methods can best be visualised by comparing a protein crystal to a wet sponge: McMeekin and Warner float the sponge in a

[3] Perutz, *Nature*, 1942, **149**, 491.
[4] Boyes-Watson and Perutz, *ibid.*, 1943, **151**, 714.

liquid which does not mix with water and thus measure the density of the wet sponge, while Adair and Adair float the sponge in salt solutions; as the salt diffuses freely into the sponge, the property one would expect to be measured by this method is the density of the dry sponge skeleton. Actually, a certain proportion of the water inside protein crystals does not seem to be available to the diffusing salt, so that the observed density is considerably lower than that of the dry protein. In calculating the crystal composition, we also had to use the partial specific volume of the anhydrous protein or its reciprocal, the apparent density. This quantity is the same [1] whether hæmoglobin is present in solution or in the form of a crystalline suspension; it is also the same in the absence of salt or in the presence of M/NaCl. The results obtained with a salt-free methæmoglobin crystal are given in Table I.

TABLE I.

Method of density determination.	Crystal density at 18°C.
1. Flotation of wet crystal in xylene-bromobenzene mixture .	1·160
2. Flotation of wet crystal in concentrated salt solution . .	1·242
3. Flotation of air-dried crystal in xylene-bromobenzene mixture	1·270
4. Apparent density of crystal suspension in water . . .	1·33

The unit cell dimensions of the crystals were determined with the help of oscillation photographs, with an accuracy varying between 1 % and 0·3 %, according to the angle to which the X-ray reflections extend.

Evaluation of Results.

The molecular weight of horse hæmoglobin is known from analysis of its iron content, combined with osmotic pressure measurements, to be 66,700; its molecular volume is obtained by multiplying the weight with the partial specific volume of the anhydrous protein in solution (the justification for this procedure is shown in the previous section). The specific volume of the liquid crystallisation (i.e. the total liquid in the crystal), can now be derived from the equation:

$$v = \frac{V - nMv_p}{VD - nM} \quad . \quad . \quad . \quad . \quad . \quad (1)$$

where v = specific volume of the liquid crystallisation, V = unit cell volume in A^3., n = number of hæmoglobin molecules per unit cell M = molecular weight of hæmoglobin in 10^{-24} g., D = crystal density, and v_p = partial specific volume of anhydrous protein in solution.

We now assume that the liquid of crystallisation consists of two distinct components, viz., water which is bound to the protein and therefore not available as solvent to mobile ions (" bound water ") and a solution through which mobile ions diffuse freely (" free liquid ") and which has the same composition as the suspension medium outside the crystal. It follows that

$$v = xv_{H_2O} + (1 - x)v_m$$

where x is the weight fraction of bound water, v_{H_2O} is the specific volume of water, and v_m that of the suspension medium. The weight fraction of bound water is therefore given by

$$x = \frac{v - v_m}{v_{H_2O} - v_m} \quad . \quad . \quad . \quad . \quad . \quad (2)$$

whence, protein hydration (expressed as grams water per gram protein, w), is

$$w = x\left(\frac{VD - nM}{nM}\right) = \frac{v - v_m}{v_{H_2O} - v_m}\left(\frac{VD}{nM} - 1\right) \quad . \quad . \quad (3)$$

Of the four possible sources of error only those in the crystal density would seriously affect the value of w which should be accurate to ± 0·03, at least.

The Effect of Neutral Electrolyte.

The unit cell dimensions of horse methæmoglobin crystals were found to be the same whether the crystals had been grown by dialysis against neutralised $(NH_4)_2SO_4$ solution or neutralised K_2HPO_4 solution, or by electrodialysis against tap water. The different methods of growth also had little influence on the distribution of intensities in the X-ray diffraction pattern. In order to test whether the concentration of neutral electrolyte in the suspension medium has any effect on the crystal structure, a crop of crystals was grown by dialysis against $(NH_4)_2SO_4$ solution, and the crystals were then immersed in a series of eight $(NH_4)_2SO_4$ solutions varying in strength between 1·25 and 4·05 molar; this series was prepared by successive dilution of a saturated solution which had been brought to p_H7 by the addition of 5 ml. N NaOH per litre. The use of buffers was avoided because they are ineffective at very high salt concentrations.

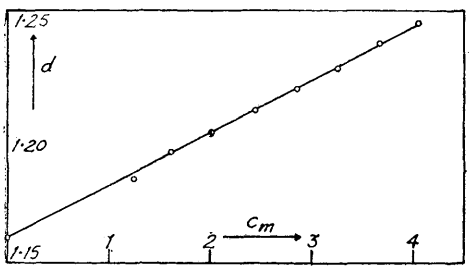

FIG. 1.—Density of wet horse methæmoglobin crystals plotted against ammonium sulphate concentration in the suspension medium c_m = M $(NH_4)_2SO_4/l$.

No alteration in unit cell dimensions was observed over the entire range, although any lattice change exceeding 0·5 % could easily have been detected. The density of the wet crystals was found to be a linear function of salt concentration (Fig. 1). The unit cell volume of the normal wet crystals is 350,000 A.^{3}; by subtracting the volume of two hæmoglobin molecules it is found that 52·4 % of the cell volume are taken up by liquid of crystallisation. Fig. 2 shows the relationship between the salt concentration in this liquid, and that in the suspension medium, calculated from formula (1). Hydration of the protein is indicated by the fact that c_l is always lower than c_m. Fig. 3 shows the composition of the protein crystal as a whole, plotted against salt concentration in the suspension medium; it is seen that any salt which penetrates the crystal

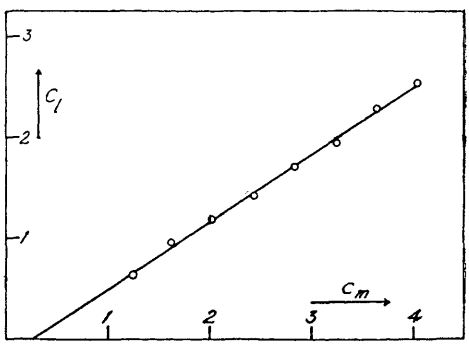

FIG. 2.—Ammonium sulphate concentration in the liquid of crystallisation (c_l) plotted against that in the suspension medium (c_m). No significance need be attached to the fact that the line does not pass through the origin, as the amount of deviation is within the experimental error.

displaces an equivalent volume of water. In consequence the *total water content* of the crystals varies between 0·82 g. per g. protein in the absence of salt and 0·69 g. in saturated ammonium sulphate solution, while the *total volume of liquid* in the crystal remains the same. Fig. 4 shows the protein hydration as derived from equation (3). The hydration is seen to be of the order of 0·3 g. H_2O/g. protein throughout the whole concentration range studied. Salt concentrations near to the saturation point seem to have a slight dehydrating effect, otherwise the hydration appears to remain constant. (The unusually high value of w at $c_m = 1{\cdot}25$ may be due to the presence of liquid-filled cracks in the crystals which showed signs of dissolving at this low concentration.)

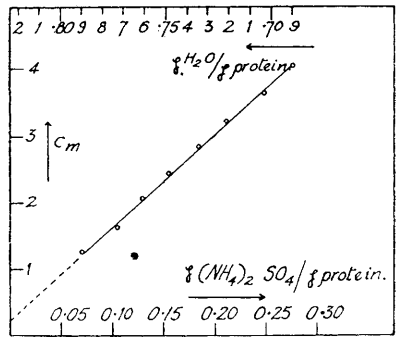

FIG. 3.—Composition of a horse methæmoglobin crystal as a function of c_m.

The Effect of Hydrogen Ion Concentration.

The effect of *p*H was studied at a constant salt concentration of 2·5 M to prevent dissolution of the crystals. Ammonium sulphate solution was again used as the basic medium whose *p*H was varied between 3·6 and 10·0 by replacing $(NH_4)_2SO_4$ with different quantities of mono- and di-ammonium phosphate, and, for *p*H values above 8, by adding concentrated ammonia. The *p*H determinations were made with the help of a glass electrode by kindness of Dr. E. F. Gale at the Department of Biochemistry.

The unit cell dimensions of the methæmoglobin crystals were not affected by immersion in media between *p*H 6 and 10. At *p*H 5·4 the lattice expanded by 7·3 %, and this expanded form in turn remained

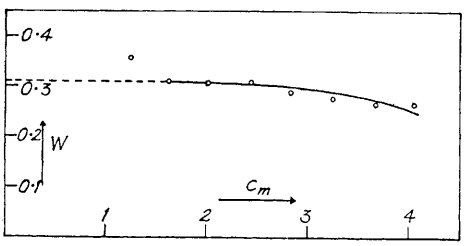

FIG. 4.—Hydration of protein in the crystal in *g.* H_2O/*g.* protein (w) as a function of c_m.

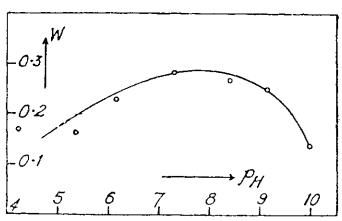

FIG. 5.—Hydration of protein in the crystal as a function of *p*H.

stable between *p*H 5·4 and 3·9. The expansion could be reversed by returning the crystals to a more alkaline medium. At *p*H 3·6 the crystals broke up into an amorphous coagulate. Their density changed little with *p*H; Fig. 5 shows protein hydration to be at a maximum near the isoelectric point and to diminish on either side.

The unit cells of the normal and expanded crystals are seen in Fig. 6 (No. 5 and 7) as part of a comprehensive diagram of the unit cells of methæmoglobin crystals at all stages of swelling and shrinkage. Swelling at *p*H 5·4 is accompanied by a change of β from 111° to 84·5°; * the a

* The surprising change from the obtuse to the acute angle is genuine, and was confirmed by recording the X-ray pattern from a crystal as it changed from the normal to the expanded form. Comparison of the vector projections of the two forms also supports this observation.

and b axes remain constant in length and c hardly alters, while the layer spacing $c \sin \beta$ expands from 50·7 to 54·4 A. The perfection of the X-ray pattern is not affected by these lattice changes.

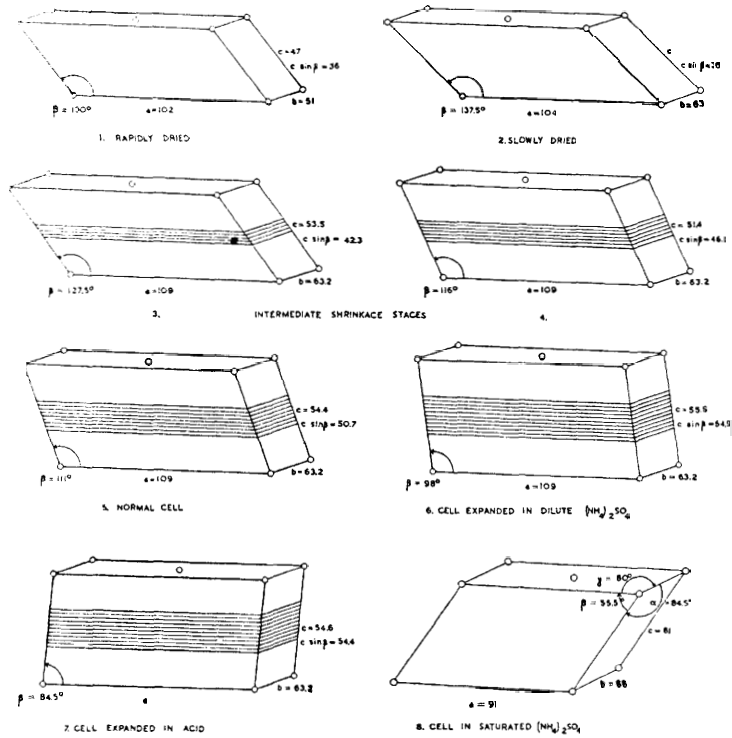

FIG. 6.—The diagrams show the unit cell dimensions of horse methæmoglobin crystals at all stages of swelling and shrinkage. The shaded parts in the centre of some of the parallelopiped indicate the thickness of the liquid layer. 1 mm. = 4 A.

The Effect of Pure Ammonium Sulphate Solution.

The pH of pure ammonium sulphate solutions decreases with increasing concentration. As salt concentration and pH vary simultaneously, the behaviour of methæmoglobin crystals immersed in a series of un-neutralised $(NH_4)_2SO_4$ solutions is more complex than in the experiments just described. The unit cell dimensions were normal only in the neighbourhood of a salt concentration of 2·8 M. Above this concentration the crystals changed into the acid-expanded form (Fig. 6, No. 7) already mentioned; this in turn became unstable in saturated $(NH_4)_2SO_4$ solution where the crystals assumed a triclinic form accompanied by a deterioration in the diffraction pattern and a marked increase in density (Fig. 6, No. 8). Below 2·8 M salt the crystals expanded by 8 % to a new and different unit cell, which remained stable up to 1·6 M. Dissolution of the crystals prevented observations at lower concentrations.

The triclinic cell is seen in Fig. 6, No. 8; its density is 1·28, indicating a hydration of only 0·04. Diagram No. 6 shows the unit cell which is stable between 2·8 and 1·6 M; it is similar to the acid-expanded one, except that β is 98° instead of 84·5°. This expansion, too, can be reversed by returning the crystals to a 2·8 M $(NH_4)_2SO_4$ solution; the whole process of swelling and reshrinking can be carried out without any adverse effect on the X-ray diffraction pattern.

Shrinkage.

Methæmoglobin crystals were made to shrink in stages (cf. [3]), by drying them extremely slowly. The two intermediary stages observed between the normal wet and the air-dried state are shown in Fig. 6, No. 3 and 4. The lengths of a and b remain constant while β changes first to 116° and later to 127·5°, involving a shrinkage of $c \sin \beta$ by 4·6 and 3·8 A. respectively. The cell dimensions of the air-dried crystals vary according to the method of drying, slowly dried ones having a larger unit cell and more perfect X-ray pattern than rapidly dried ones (Fig. 6, No. 1 and 2). Slow drying evidently preserves the alignment of the hæmoglobin molecules in a comparatively loose structure, while rapid drying produces closer packing, but destroys the phase relationship between corresponding groups of atoms in neighbouring molecules. The first shrinkage stage can be reversed by rewetting the crystals; no attempt at reversing the second stage was made, as it was only obtained once. The air-dried crystals tend to break up on rewetting, and a reversal of the shrinkage is therefore not feasible. The dried crystals can, of course, be dissolved and recrystallised.

Mechanism of Swelling and Shrinkage.

Neglecting such extreme treatments as rapid drying in air or immersion in saturated ammonium sulphate solution, a study of Fig. 6 shows that all swelling and shrinkage is practically confined to changes in the c spacing, brought about by a change in β from 84·5° in the acid-expanded crystals to 137·5° in the slowly dried ones. Of the total change of 53°, 32° at least can be produced reversibly. Detailed analysis of the X-ray pattern shows that the hæmoglobin molecules form coherent sheets which remain parallel to the c plane throughout the entire range of swelling and shrinkage. The sheets of hæmoglobin molecules are sheared parallel to each other as the angle β changes, but no alteration either in the thickness of the hæmoglobin molecules or in their internal structure seems to occur; it is only the thickness of the liquid layers between them that alters. Swelling and shrinkage in these crystals, therefore, is an inter- and not an intra-molecular process.

All swelling and shrinkage proceeds in discrete and reproducible steps. The lattice seems to click from one equilibrium position to another, while all intermediary positions are too unstable to be observed, at any rate on diffraction photographs requiring not less than one hour's exposure. In terms of the spacing between two neighbouring layers of hæmoglobin molecules (equivalent to $c \sin \beta$), the magnitude of the steps between the different equilibrium positions varies between 3·7 and 4·6 A., with the exception of the last step of drying which amounts to 6 A. These values were all obtained in ammonium sulphate solutions; other suspension media may well give rise to different equilibrium positions.

Discussion.

The lattice dimensions of wet methæmoglobin crystals are independent of the concentration of neutral electrolyte present in the suspension medium. They are, moreover, independent of p_H over wide ranges, and only at one specific p_H does a definite and reversible lattice change occur. It must be concluded, therefore, that the intermolecular forces which operate in protein crystals are of a totally different character from the long range forces present in gels of tobacco mosaic virus or bentonite. The lattice change which takes place around p_H 5·5 provides at least a pointer to the type of force which may predominate. A survey of the dissociation constants of the amino-acid residues with polar side chains shows that only the imidazole group of histidine has a p_K value in the region between 5 and 7; in pure histidine solutions its amino group

carries a positive charge below *p*H 6·0 and is uncharged above it [5]; in peptides or proteins and in the presence of salts the *p*K value may be shifted considerably. Since there are 33 histidine residues in one hæmoglobin molecule, it may reasonably be suggested that the charging of their NH groups so alters the balance of electrostatic forces on the surface of the protein molecules that the lattice energy "troughs" undergo a substantial shift. The swelling could be explained as a result of the repulsive forces introduced by the appearance of the positive charges. Since no *p*K of any other ionised group is within the region investigated (3·9-10·0), it may be suggested that the absence of any lattice change other than that around *p*H 5·5 is due to the comparative constancy of the charge distribution on the hæmoglobin molecules in the *p*H ranges 3·9-5·4 and 5·6-10·0, say.

It is interesting to note that the transition from acid to alkaline methæmoglobin which takes place around *p*H 8·2 and is accompanied by a transformation of the absorption spectrum produces no lattice change. It was also found by allowing NaN_3 to diffuse into the crystals that the methæmoglobin could be transformed into azide-methæmoglobin, involving the attachment of an azide group 4 A. long to each of the four iron atoms, without change in cell dimensions. Since it is improbable that four such large groups could be tucked away in the interior of the protein molecule without causing structural changes affecting the X-ray pattern, it seems likely that the hæms lie on the surface; nevertheless the iron atoms, at any rate, can play no part in intermolecular binding, since otherwise the attachment of the azide groups would be expected to cause a change in cell dimensions.

The discontinuous character of all swelling and shrinkage may have two alternative explanations. Either the surface structure of the protein molecules is such that only certain distances between successive layers of protein molecules are structurally stable, or the liquid between those layers has itself a laminated structure, such that only one layer of salt solution can be added or removed at a time. The latter hypothesis is perhaps the more plausible one, though at the moment there is no evidence to distinguish between them. Hendricks *et al.*[6] found montmorillonite particles to incorporate several distinct layers of water of 3 A. thickness. The thickness of the layers in hæmoglobin crystals would have to be about 4 A., since this is the order of magnitude of the steps which separate successive states of swelling. This increased thickness compared to the pure water layers found in montmorillonite may be due to the presence of the sulphate ions; it remains to be seen whether ions of different size would alter the magnitude of the steps.

Since swelling and shrinkage is a purely intermolecular process, and does not affect the internal structure of the hæmoglobin molecules, hydration should be confined to the surface. X-ray data indicate that the hæmoglobin molecules correspond roughly to cylinders with an ellipitical base having a total surface area of 11,990 A^2. Supposing this area were covered with a layer of water one molecule thick and a density of 1·0, the weight of that water layer would be 31,000 g. per mole or 0·28 g. per g. protein, which agrees with the measured hydration of 0·3 g. per g. protein. There is no conclusive evidence as yet whether the additional intracrystalline liquid which has the same composition as the suspension medium is of a purely interstitial character. The fact that its composition can vary within such wide limits suggests that it is, and that its presence cannot easily be ascribed to protein hydration, as McMeekin and Warner have done in the case of salt-free lactoglobulin crystals.[2]

It should perhaps be pointed out that our results differ from McMeekin and Warner's in one important respect. They state that the total amount

[5] Cohn and Edsall, *Proteins, Amino-Acids and Peptides* (New York, 1943).
[6] Hendricks, Nelson and Alexander, *J. Amer. Chem. Soc.*, 1940, **62**, 1456; Hendricks and Teller, *J. Chem. Physics*, 1942, **10**, 147.

of water in β-lactoglobulin crystals is the same in the absence of salt and in the presence of high concentrations of ammonium sulphate. Since salt diffuses freely into the crystals, they have to assume that the space for the salt is provided by expansion of the lattice, an assumption which is not supported by any of their observations. Our experiments, on the other hand, prove that the lattice is rigid, and that any neutral electrolyte penetrating the crystal therefore displaces an equivalent volume of water; thus it is the total volume of liquid in the crystal and not the total water content which remains constant.

For reasons of space, the details of the X-ray evidence are not discussed in this paper, and will be described in a later publication elsewhere. I have to thank Dr. Edna Davidson, Miss Joy Boyes-Watson and Miss Olga Weisz, who have helped me with certain of the experiments, Dr. H. Carlisle, who measured the unit cell of azide methæmoglobin and Dr. J. C. Boursnall, who kindly analysed some of the suspension media. I am also grateful to Dr. Gale for carrying out the *p*H determinations. The research was financed by a grant from the Rockefeller Foundation.

Conclusions and Summary.

The composition and swelling properties of horse methæmoglobin crystals were investigated by combining density measurements with X-ray diffraction studies. The following conclusions were reached.

(1) 52·4 % of the volume of normal wet methæmoglobin crystals consists of liquid whose composition can be varied within wide limits.

(2) It was shown by immersing crystals in a series of ammonium sulphate solutions at *p*H 7 that the unit cell dimensions and the general intensity distribution in the X-ray diffraction pattern are independent of the concentration of neutral electrolyte in the suspension medium. Similarly, they are independent of *p*H if the salt concentration is kept constant, except at *p*H 5·4, when the unit cell swells by a definite amount on acidification. In pure ammonium sulphate solutions any change in concentration involves a change in *p*H; more complex swelling effects were therefore observed.

(3) In methæmoglobin crystals layers of hæmoglobin molecules alternate with layers of liquid. Swelling and shrinkage produce variations in the layer spacing and shearing of the layers. All lattice changes proceed in definite, reproducible steps, involving changes in the layer spacing of the order of 4 A. at a time. The thickness and structure of the protein layers remain unaltered during swelling and shrinkage.

(4) At *p*H 7 the salt concentration in the liquid of crystallisation is a linear function of the salt concentration in the suspension medium; in ammonium sulphate solution the former is about two-thirds of the latter. If it is assumed that the liquid of crystallisation consists of two components, namely, "bound water" which is not available as solvent to mobile ions and "free liquid" through which ions can diffuse and which has the same composition as the suspension medium, it can be shown that protein hydration ' ("bound water") amounts to 0·3 g. H_2O per g. protein. At the isolectric point hydration is largely independent of electrolyte concentration; it is, however, a function of *p*H and decreases on both sides of the iso-electric point. It is shown that this hydration is of the right order of magnitude for a monomolecular layer of water molecules covering the surface of the hæmoglobin molecule.

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