Discontinuous Lattice Changes in Haemoglobin Crystals

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A technique has been developed for observing the X-ray pattern of a protein crystal while its water content is altered by known and controlled changes in the vapour pressure of the atmosphere surrounding it. The method adopted is to equilibrate the crystal with the vapour above calcium chloride solutions of known concentration in an evacuated, thermostatically-controlled enclosure. When crystals of horse met-haemoglobin are shrunk by this technique the lattice constants always change in a discontinuous manner, at a series of well-defined vapour pressures. During this work five new types of haemoglobin lattice were observed.

1. Introduction

When crystals of globular proteins are removed from their mother-liquor, they shrink. This shrinkage, as seen under the microscope, often takes place without any major disruption of the crystal. It is accompanied by changes in the X-ray diffraction pattern. For example Perutz (1946) has observed that the pattern of shrunk haemoglobin crystals differs, both in cell dimensions and in distribution of intensities, from that of the normal wet crystals. The shrinkage of these monoclinic crystals (space group $C2$) was characterized by a change in β, and hence in $c \sin \beta$, while the other cell dimensions remained nearly constant (Fig. 1).

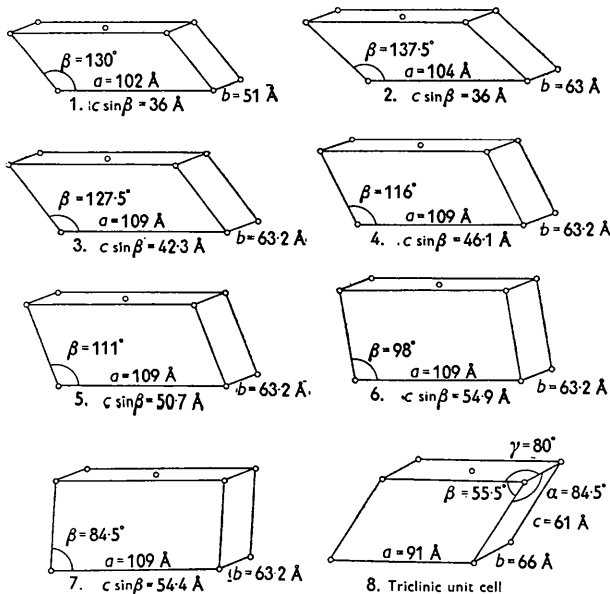Perutz's interpretation is that the structure consists

Fig. 1. Unit-cell dimensions of horse met-haemoglobin at various stages of swelling and shrinkage (after Perutz, 1946). 1, rapidly dried; 2, slowly dried; 3, 4, intermediate shrinkage stages; 5, normal cell; 6, expended in dilute $(NH_4)_2SO_4$; 7, expanded in acid; 8, in saturated $(NH_4)_2SO_4$.

of layers of protein molecules in the $a\,b$ plane which, on shrinkage, move closer together and at the same time slide over one another in the a direction, the molecules always remaining in a regular crystalline lattice. He observed that the shrinkage apparently took place in a series of definite steps; lattices with intermediate values of $c \sin \beta$ were never found. Similar changes can be produced by altering the ionic composition of the mother-liquor; in one case (see Fig. 1) there was a change in the cell symmetry to a triclinic form. The behaviour of other types of protein crystal suggests that they shrink in a similar way, though the details are different in each case. In the earlier stages of shrinkage the crystal generally remains as highly ordered as it was when fully wet, i.e. X-ray reflexions are observed out to spacings of 2–3 Å; in the last stage the order usually deteriorates considerably, reflexions of short spacing no longer being observed.

Perutz's experiments were carried out by allowing crystals, mounted in a capillary tube in the usual way, to lose water slowly to the atmosphere through a small hole pierced in the seal of the tube. The X-ray pattern was observed until a change in the lattice dimensions occurred, and the capillary was then sealed off. This method does not permit accurate control or measurement of the vapour pressure of the crystal.

Kendrew (1946) improved this technique by mounting the crystals, in capillary tubes, over a calcium chloride solution of known concentration, so that the vapour pressure was known if the temperature was known. In this way he was able to plot the approximate relation between vapour pressure and lattice dimensions for horse haemoglobin. However, in these experiments the temperature (that of the room) was not controlled, and furthermore the system took a long time to reach equilibrium.

The purpose of the present investigation was four-fold:

(1) To develop a convenient technique for observing

the X-ray pattern of a protein crystal while changes in its water content were produced by known and accurately controlled changes in vapour pressure. The immediate object was to apply this technique to the study of horse met-haemoglobin, but it was felt that it might find general application.

(2) To use the technique to test the supposition that the lattice changes in haemoglobin always took place in discrete steps.

(3) To search for new intermediate shrinkage stages which might have been missed in the earlier experiments: such new forms of the haemoglobin lattice would provide additional information for structure determination by making possible a more complete survey of the Fourier transform of the unit cell (see Bragg & Perutz, 1952a).

(4) To measure accurately the vapour pressure at which each lattice transition occurred and to investigate its variation with temperature, with a view to studying the thermodynamics of the process.

When these experiments were begun, it was thought (Boyes-Watson, Davidson & Perutz, 1947) that sheets of protein molecules in the haemoglobin crystal were completely separated by aqueous regions three or four molecules thick, and that the stepwise shrinkage was due to an ordered layering of water molecules in these regions, layers being lost in succession as shrinkage proceeded; and it was hoped that a study of the thermodynamics of shrinkage might give some information about the nature of the forces maintaining this structure in an ordered arrangement. Since that time it has become probable (Dornberger-Schiff, 1950; Bragg & Perutz, 1952b) that at any rate most of the data can be better explained on the basis of hydrated molecules in contact, and that shrinkage occurs, not by removal of an ordered layer of water, but by the sliding past one another of molecules which are always in contact and which, to explain the discontinuities, must be assumed to be irregular in shape. Pending further clarification of this structure, part (4) of the investigation has not yet been completed.

2. Technique

It was found in preliminary experiments that the whole shrinkage process took place (at 18° C.) between 9·0 and 15·3 mm. Hg pressure of water vapour. In order to obtain vapour pressures in this range solutions of calcium chloride were employed, the crystal being allowed to come into equilibrium with the vapour above this solution in a closed vessel. The concentration of $CaCl_2$ solutions was determined by measuring their densities, and hence the vapour pressure at a given temperature could be found from published tables with an error less than 0·01 mm. Hg.

Vapour pressure isotherms were normally explored at intervals of 0·1 mm. Hg; in $CaCl_2$ solutions a variation in vapour pressure of this order is produced by a temperature change of 0·1° C. Accordingly,

accurate thermostatic control was necessary to keep the vapour pressure constant within the required limits. Instead of arranging a small thermostatically controlled enclosure round the crystal itself, it was found more convenient to construct a large enclosure holding the whole X-ray camera; in this way cameras of various types could be accommodated, including the Buerger precession camera normally used, whose precessing layer-line screen and goniometer head severly restrict the free space round the crystal. The constant-temperature enclosure was double-walled, with internal baffles to ensure uniform water flow over all its surface; the jacketing was continuous, with the exception of a small area round the X-ray tube and of the lid, which was itself double with a cork liner. The jacket was supplied with continuously circulated water from a large double-jacketed reservoir, insulated with glass wool, stirred and thermostatically controlled to within ±0·05° C. The temperature near the crystal was measured with a thermocouple, and was found to vary less than 0·05° C.

It is desirable to arrange that the crystal reaches equilibrium with the $CaCl_2$ solution as rapidly as possible. The crystal was mounted in the conventional manner in a thin-walled glass capillary tube of about ½–1 mm. bore; the lower end of the capillary was sealed into, and communicated with, a small vessel which contained the $CaCl_2$ solution, and was mounted directly on the goniometer head of the camera, as in the earlier experiments previously referred to (Kendrew, 1946). However, in this arrangement as it stands, even though the distance between crystal and solution is only of the order 2 cm., equilibrium takes an inconveniently long time to establish itself: thus, for a 0·1 mm. difference in vapour pressure between the crystal and the $CaCl_2$, the time of equilibration is of the order of days for normal dimensions of capillary and crystal. To reduce this time the apparatus was designed so that it could be evacuated (with a water pump). Under these conditions the factor limiting the rate of equilibration is the rate at which latent heat of vaporization can be lost or gained by the crystal, rather than the rate of diffusion of water vapour; however, it can be shown that the time of equilibration
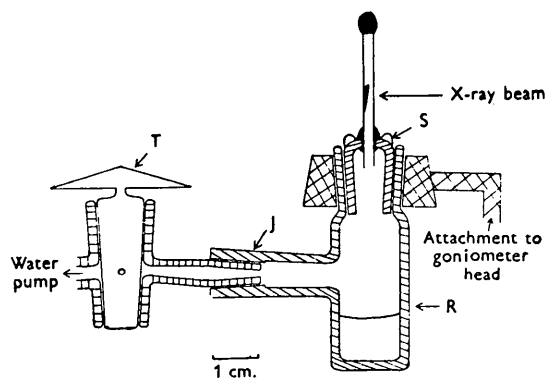

Fig. 2. Apparatus for controlled shrinkage of protein crystals.

Table 1. *Shrinkage stages of horse met-haemoglobin*

(Includes only lattices observed in the current series of experiments)

| Lattice no. | | Dimensions | | | | |
Salt-containing	Description	a (Å)	b (Å)	c (Å)	β (°)	$c \sin \beta$ (Å)
5	Normal	109	63·2	54·4	111	50·7
4	First shrinkage	109	63·2	51·4	116	46·1
4d*	First shrinkage, c doubled	109	63·2	102·8	116	92·2
9	Fully dry	102	56	49	134	36
7	Acute-angled expanded	109	63·2	54·6	84·5	54·4
13*	Shrinkage stage of 7	109	61·6	50·5	84	50·3
6	Obtuse-angled expanded	109	63·2	55·4	98	54·9
Salt-free						
5	Normal	109	63·2	54·4	111	50·7
5d*	Normal, b doubled	109	126·4	54·4	111	50·7
8*	Shrinkage stage	108	63·2	65·8	138	43·9
9*	Fully dry	?	?	?	?	36

* Lattices not previously observed.

for 0·1 mm. pressure differences is now of the order of a few hours, which is quite acceptable.

The final arrangement is shown in Fig. 2. The capillary is sealed to the ground-glass stopper S with picene wax; the stopper can be removed and replaced in the same position with the aid of a fiduciary mark. The reservoir R containing the solution is fixed to the goniometer head; the whole apparatus is evacuated through the side-arm J and then sealed off by closing the tap T.

The mounting procedure consists in first sealing a glass capillary to the stopper and then mounting the crystal in the capillary, this order being adopted to avoid heating the crystal (which must be near the lower end of the capillary to hasten diffusion); the upper end of the capillary is sealed last, and the stopper replaced in the reservoir bottle, which contains mother-liquor while the preliminary setting of the crystal is carried out. $CaCl_2$ solution of the required vapour pressure is then substituted for the mother-liquor, the stopper being replaced in the same position, and the system is pumped out, replaced in the thermostat, and left for two or three hours to equilibrate.

Re-setting is generally unnecessary, so the new cell dimensions can be rapidly determined. The procedure is repeated at as many different vapour pressures as are required; for a search over the whole range, steps of 0·1 mm. Hg were convenient, 'bracketing' being used for the accurate determination of the vapour pressure of a stage once it had been found.

3. Results

(a) *The discontinuous nature of the shrinkage process*

Experiments were carried out with both salt-free and salt-containing crystals of monoclinic horse met-haemoglobin. Table 1 lists all the shrinkage stages observed throughout the series of experiments; it in-

cludes five not previously reported (indicated by an asterisk). It should be noted that the salt-free crystals examined (all from the same batch of crystals) showed a doubling of the b dimension, but were otherwise normal; this phenomenon is discussed in c(i) below.

We consider that the following evidence is conclusive in establishing the discontinuous nature of the shrinkage:

(i) No lattices intermediate between those listed were ever found in any experiment; the discontinuities are evident in the curves of Figs. 3 and 4. In all 37 crystals were examined.

(ii) Occasionally shrinkage occurred during the progress of a single X-ray exposure. In such cases the photographs showed two sets of reflexions, corresponding to the lattice spacings before and after shrinkage; these sets of reflexions were always quite distinct and sharp, and were not joined by streaks.

In several instances it was found possible to reverse the process, i.e. by raising the vapour pressure to cause the crystal to expand. Salt-free crystals could even be re-expanded from the 'fully-dry' stage; when dry, these crystals gave hardly any reflexions, but on re-wetting strong reflexions of the normal wet lattice reappeared out to spacings of 3–4 Å.

(b) *Relation of cell dimensions to vapour pressure*

(i) *Salt-free crystals.*—Fig. 3 shows the relation between vapour pressure and cell dimensions for salt-free crystals; only the value of $c \sin \beta$ is plotted since the other dimensions vary but slightly.

Lattice change occurred in all the crystals examined at a sharply defined and reproducible vapour pressure (± 0.1 mm. Hg), the whole crystal changing over to the new form at the same time.

The shape of the curve is formally similar to that which would, from application of the Phase Rule, be expected if the shrinkage stages could be regarded as

distinct phases (cf. vapour-pressure isotherms of salt hydrates).

(ii) *Salt-containing crystals.*—The case of salt-containing crystals is more complicated, because
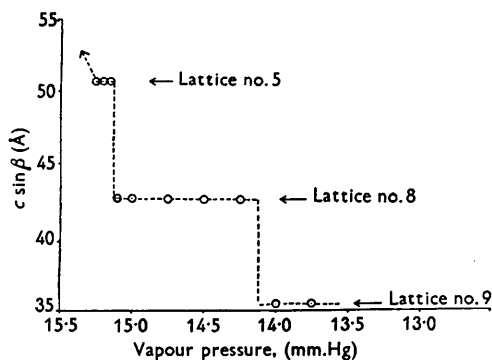

Fig. 3. Vapour-pressure isotherm of salt-free horse met-haemoglobin crystals at 18° C.

although experiment shows that, for any given crystal, shrinkage takes place at sharply-defined vapour pressures, different crystals are found to shrink at slightly differing vapour pressures, and sometimes through a different set of intermediate lattices (e.g. the acid-expanded lattice, or the 'doubled' first shrinkage stage—see c(iii)). The cause of this variation is an uncertainty in either the pH or the salt concentration within the crystal during shrinkage; this uncertainty arises as follows.

It is known that the over-all salt concentration inside a protein crystal is always less than, but bears a fixed relation to, that outside (Perutz, 1946). If the external liquid is concentrated by evaporation, salt must enter the crystal to preserve this relation, which will be maintained right up to the stage when the external liquid is saturated if, but only if, an adequate amount of external solution is present; if—as may easily happen—there was very little external liquid at the beginning of the experiment, so that the total salt present in the whole system is less than would be present inside the crystal if it were equilibrated with saturated solution, then at a certain point the internal salt concentration becomes dependent on the initial conditions, which are impossible to measure accurately. This variable salt concentration affects the vapour pressure at which shrinkage takes place.

If on the other hand the amount of external salt solution initially present is enough to maintain the equilibrium internal salt concentration until the external solution becomes saturated, then the salt content of the crystal when dried is determinate; but now a new uncertainty arises, since under these conditions the external salt, once saturated, will deposit solid salts, the buffer salts present will in general crystallize at different rates, and the pH will change. The effects of pH on crystal habit and lattice dimensions are well-known (e.g. Fig. 1, no. 7). In these

experiments, indeed, it often happened that the 'acid-expanded' stage (no. 7) was obtained when attempts were made to shrink the crystal. On drying, these acid-expanded crystals sometimes gave a new shrinkage stage not previously observed; further reductions in vapour pressure did not however cause $c \sin \beta$ to decrease further still, but merely disrupted the whole pattern.

Thus in these experiments, as we performed them, one variable was inevitably uncontrolled—either the internal salt concentration or the pH, according to the initial conditions. Further refinement of technique would be required if reproducible isotherms were to be
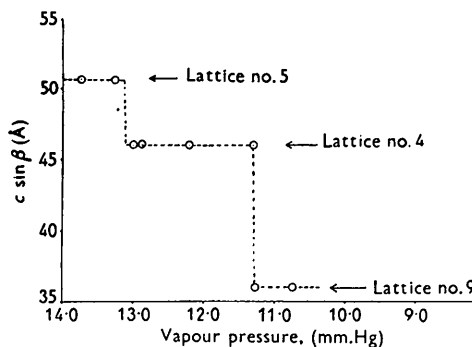

Fig. 4. Typical vapour pressure isotherm of salt-containing horse met-haemoglobin crystal at 18° C.

obtained from salt-containing crystals. For the sake of example an isotherm obtained from one particular crystal is shown in Fig. 4.

(c) *Doubled and disordered lattices*

(i) *Unshrunk salt-free crystals (lattice no. 5), b doubled.*

As already mentioned, all crystals from the batch of salt-free crystals used exhibited doubling of b; this effect disappeared when the crystals shrank. The 'extra' reflexions were very weak or absent in the low orders, and more pronounced in higher orders, suggesting that the 'doubling' is concerned with a displacement small compared with the cell dimensions.

This lattice is being studied in detail by Mr F. H. C. Crick.

(ii) *Shrinkage stage of salt-free crystals (lattice no. 8).*— Some of these crystals gave a normal diffraction pattern; in others, however, there was evidence of anisotropic disordering. The appearance of the X-ray photographs from the latter suggests that the reciprocal lattice consists of a set of rods parallel to c^*, only the basal $b^* c^*$ reciprocal lattice plane still consisting of a set of spots. This effect may be interpreted as meaning that sheets of molecules (in the $a \, b$ plane) maintain their integrity, but slide over one another in the a direction, their separation ($c \sin \beta$) remaining constant. A very similar phenomenon has been observed in horse myoglobin (Kendrew, 1950).

(iii) *First shrinkage stage of salt-containing crystals (lattice no. 4) c doubled.*—This lattice is closely related
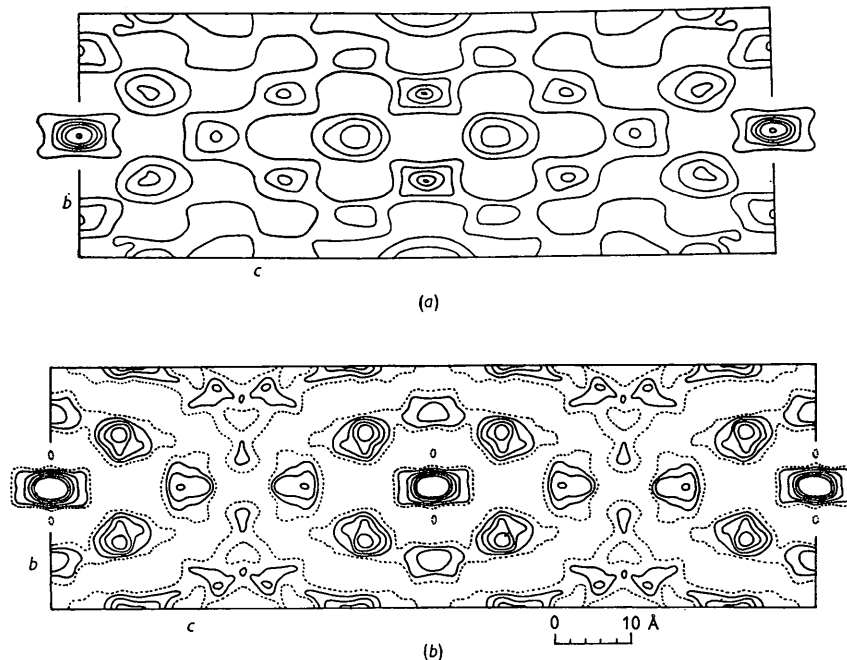
Fig. 5. (a) a Patterson projection of doubled first shrinkage stage of salt-containing horse met-haemoglobin crystals (lattice 4d). (b) a Patterson projection of normal wet crystals (lattice 5).

to the normal first shrinkage stage, sometimes passing over to it spontaneously on standing, and having about the same vapour-pressure transition points.

The *hkl* (general) reflexions exhibited the doubling effect, but the *h0l*'s were identical with those of the normal first shrinkage stage, showing no additional reflexions or abnormal intensities. It is concluded that the doubling arises from a displacement of alternate (*a b*) layers of molecules in the *b* direction.

This interpretation is supported by the appearance of the *a* Patterson projection (Fig. 5(*a*)) of this lattice. This projection may be compared with two unit repeats of the *a* projection of the normal unshrunk lattice (Fig. 5(*b*)); the prominent peaks at $y = \frac{1}{2}b \pm$ 5·5 Å, $z = \frac{1}{2}c$ are clearly due to intermolecular vectors and might be called 'pseudo-origins'. The sideways displacement parallel to *b* is clearly 5·5 Å.

It should be noted that the displacements in (ii) and (iii) are such that the molecules retain their original relative orientation, and that the direction of slip is perpendicular to the direction of the polypeptide chains (*a*).

4. Summary of conclusions

(*a*) When crystals of horse methaemoglobin shrink the lattice constants always change in a discontinuous manner.

(*b*) In salt-free crystals, each successive change in lattice dimensions takes place when the external vapour pressure is reduced below a characteristic and sharply-defined value, which is the same for all crystals at a given temperature.

(*c*) In salt-containing crystals shrinkage also takes place at a series of sharply defined vapour pressures; these values, however, differ somewhat in different crystals. Furthermore, different crystals may shrink by different paths. These effects arise from an uncertainty in either the salt concentration or the pH inside the crystal.

(*d*) Five new types of lattice were observed.

(*e*) Shrinkage is reversible, even, in favourable cases, at the 'fully dry' stage.

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