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A Preliminary X-ray Study of Crystals of the Complex Ribonuclease-Parachloromercuribenzoate

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It is shown that crystals of the complex ribonuclease-parachloromercuribenzoate are not strictly isomorphous with the metal-free crystalline enzyme. The difference is probably due to a movement of the molecules, relative to those in the free crystals, to allow for incorporation of the mercury derivative in the crystals of the complex.

1. Introduction

In 1954 H. Dix succeeded in crystallizing ribonuclease with sodium parachloromercuribenzoate (Na-*pcmb*) from aqueous alcohol containing one mercury atom per protein molecule; these crystals were isomorphous with the metal-free ones originally grown by Kunitz (1943). Following the work of Green, Ingram & Perutz (1954) and now of Bluhm *et al.* (1958) we have been attempting to apply the isomorphous replacement technique of phase determination to the X-ray reflections of ribonuclease, but unfortunately we have had some difficulty in locating the mercury atoms in the crystals of the complex. In the course of this work, however, we found that (*a*) it is possible to diffuse the Hg derivative into the metal-free crystals, where they very likely occupy approximately the same sites as they do when crystallized with the protein itself, and that (*b*) the crystals of the complex are not truly isomorphous with the metal-free ones, for there is some evidence that the protein molecules move relative to each other to accommodate the Hg derivative.

We began soaking crystals in solutions of the mercury derivative, after we discovered that crystals of the mercury complex (Dix's crystals) when kept in metal-free aqueous alcoholic solutions gradually lost their mercury and gave X-ray photographs almost identical with the metal-free ones. X-ray pictures showed the monoclinic symmetry of Dix's crystals to be preserved after immersion in the mercury solution, while similar treatment made the metal-free crystals triclinic.

2. Preliminary X-ray and optical studies

The cell dimensions and space group of the three crystals are given below.

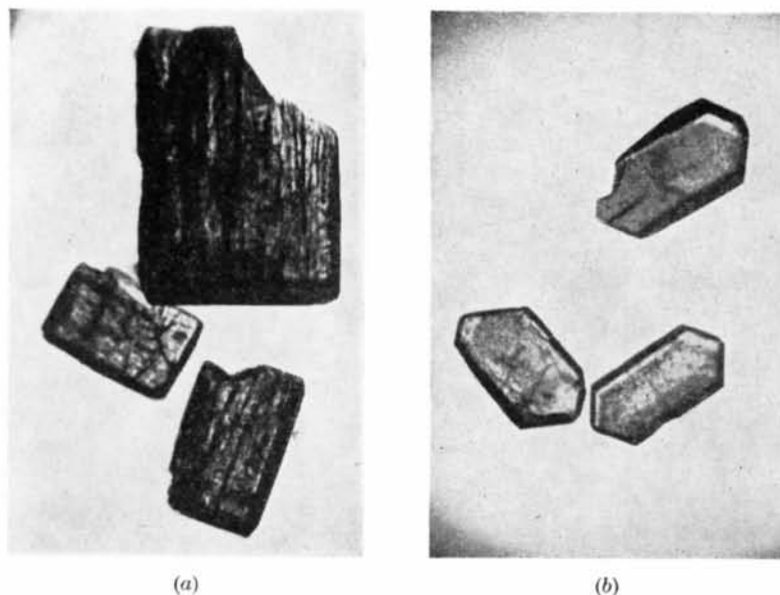
	a (Å)	b (Å)	c (Å)	β	Z	Space group
Ribonuclease	30·90	38·8	54·06	106°	2	$P2_1$
Ribonuclease-*pcmb* (Dix crystal containing 1 Hg : 1 protein molecule)	30·76	38·9	53·9	106°	2	$P2_1$
Ribonuclease-*pcmb* soaked in 50% Na-*pcmb* 60% aq. alcohol (Dix soaked crystal 2 Hg : 1 protein molecules)	30·45	38·9	53·65	105·7°	2	$P2_1$

Fig. 1 shows the habits of the two crystals; while the normal crystals show a marked tabular development with a dominant (001) face, the crystals with the mercury derivative show a dominant (100) face. The optical properties of both forms were the same, both showing negative birefringence with γ parallel to b and β lying about 40–45° to the c axis in the obtuse monoclinic angle. The density of the wet ribonuclease-*pcmb* or Dix's crystals has not been measured, but it can be assumed that its unit cell contains 2 molecules of ribonuclease like that observed for the normal form (Fankuchen, 1941; Carlisle & Scouloudi, 1951; King *et al.*, 1956).

Chemical analysis of Dix's crystals shows them to contain 1 Hg atom per protein molecule, whilst the chemical analysis of the soaked crystals, for periods of a month or so, shows that they rarely contain more than 2 Hg atoms per protein molecule. In fact we can now deduce the mercury content of a crystal from the intensity changes of certain groups of reflections.

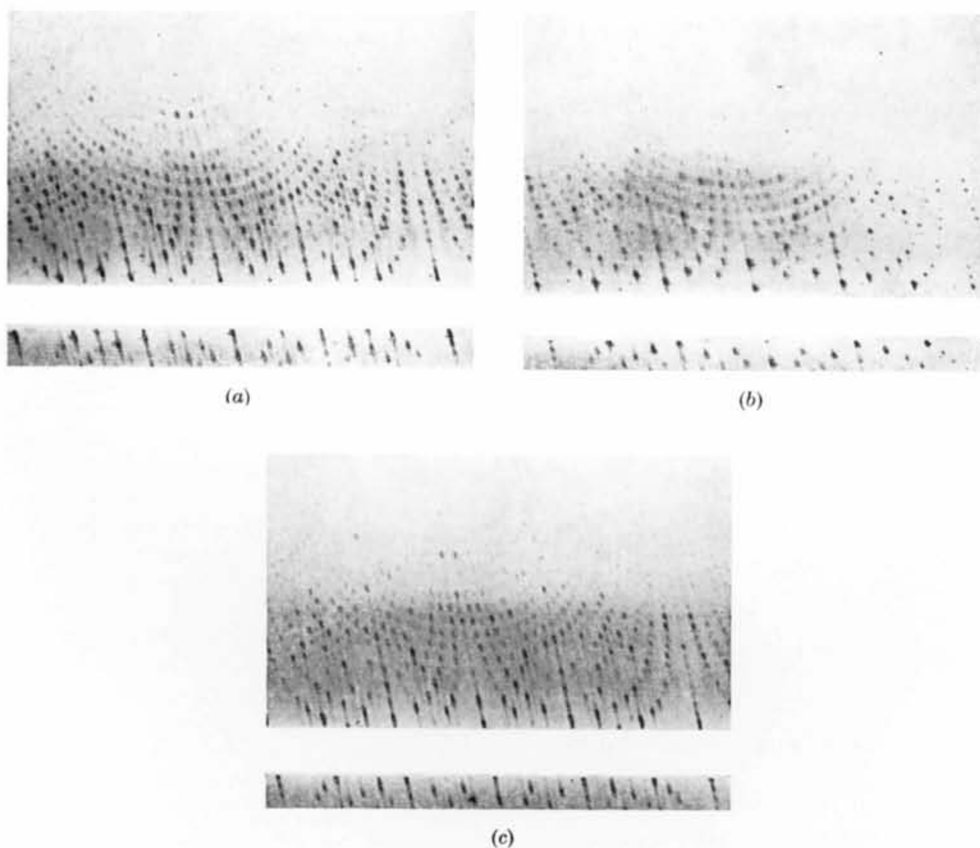
X-ray photographs were taken of normal crystals and Dix's crystals soaked in 0·25–0·5% sodium parachloromercuribenzoate solution in 60% aqueous alcohol. Fig. 2 shows the $F^2(0kl)$ intensities for the three crystals. It is seen that while the X-ray pattern for the normal soaked crystals, Fig. 2(*b*), has become triclinic, that of Fig. 2(*c*) showing the X-ray pattern for Dix's soaked crystal exhibits large intensity changes, but has retained its monoclinic symmetry; we therefore decided to work on them.

We have attempted to scale the intensities from



(a) (b)

Fig. 1. (a) Crystalline ribonuclease from aqueous alcohol. (b) Crystalline ribonuclease-paramercuribenzoate (Ri-*pcmb*) from aqueous alcohol.



(a) (b)

(c)

Fig. 2. Photographs showing the (0*kl*) reflections for (a) normal crystal, (b) normal crystal soaked in 0·5% Na-*pcmb* solution, (c) Dix crystal already containing 1 Hg:1 protein molecule soaked in 0·5% Na-*pcmb* solution.

crystals with and without the metal atom by an empirical procedure. Fig. 3 shows a plot of $|F(h0l)|$

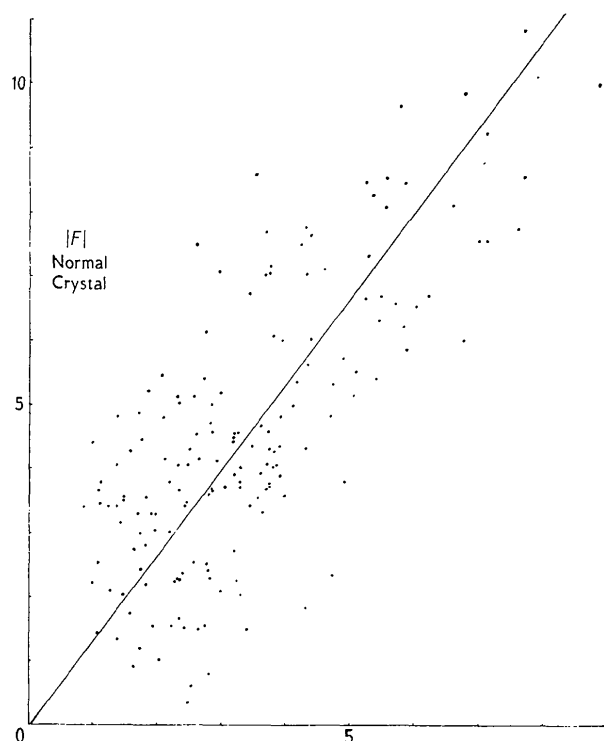


Fig. 3. Plot of $|F(h0l)|$ protein against $|F(h0l)|$ for Dix's soaked crystal for each reflection.

for the normal crystal against $|F(h0l)|$ for Dix's soaked crystal, out to spacings of about 2·5 Å, using crystals of approximately the same size and shape. Rough calculations showed that the maximum variation of intensities for X-ray reflections of the normal ones was of the order of that due to 2 mercury atoms. A line of best fit ought then to pass through the origin of Fig. 3 or near to it and the tangent of the angle between the line and the abscissa gives a scaling

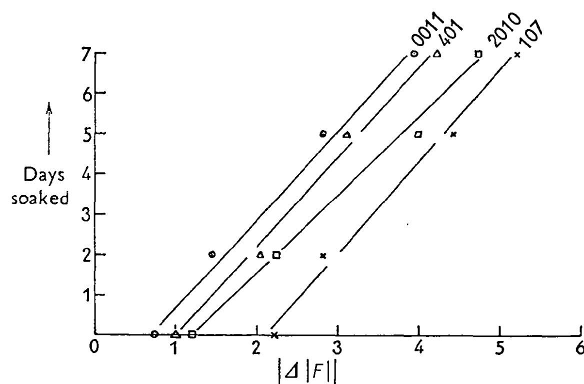


Fig. 4. Plot of time of soaking of Dix's crystal against change of intensity compared with normal crystal, for four reflections 0011, 401, $\bar{2}$010, $\bar{1}$07.

factor. This is a very crude method of scaling and it will be subsequently replaced by a statistical procedure embodying all *hkl* reflections, when collected, to spacings of 1·5 Å or less.

Using the $|F(h0l)|$ from the metal-free crystal as a standard we examined the intensities of reflections for Dix's crystals soaked for various periods. Fig. 4 shows a plot of the time of soaking against $|\Delta|F||$, for four reflections. We are now extending these experiments to longer soaking times and different concentrations, but these preliminary observations suggest a regular relationship between the time of soaking of the crystals and the difference in intensity

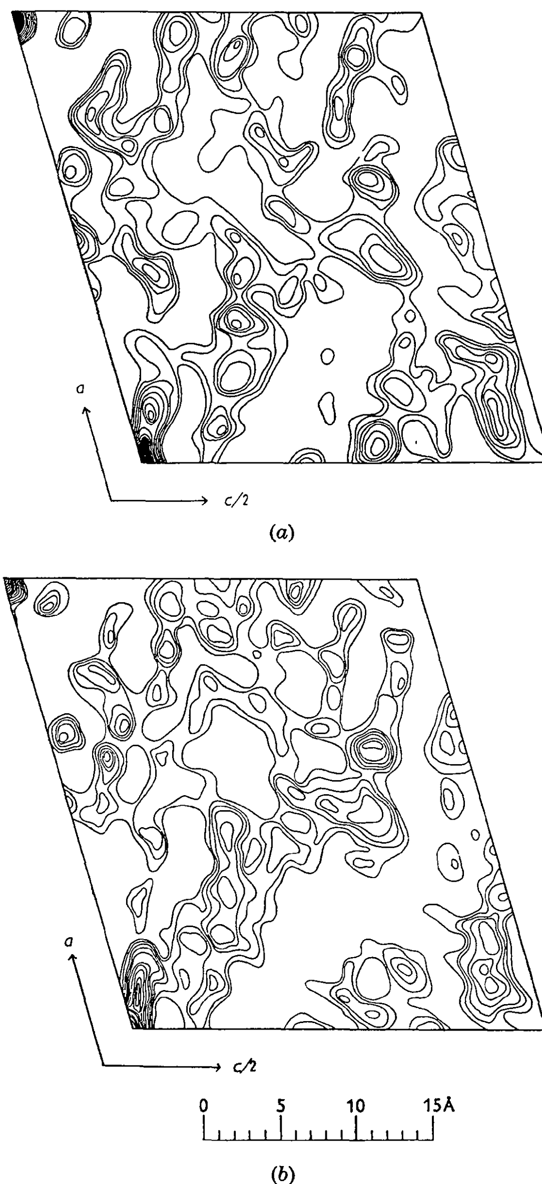


Fig. 5. Patterson difference maps projected on (010) for: (*a*) Normal soaked crystal—normal crystal. (*b*) Dix soaked crystal—normal crystal.

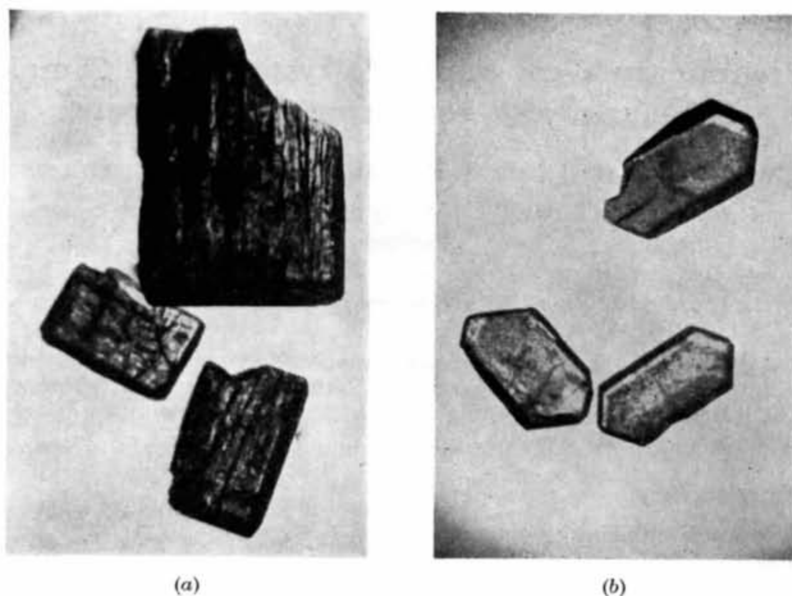


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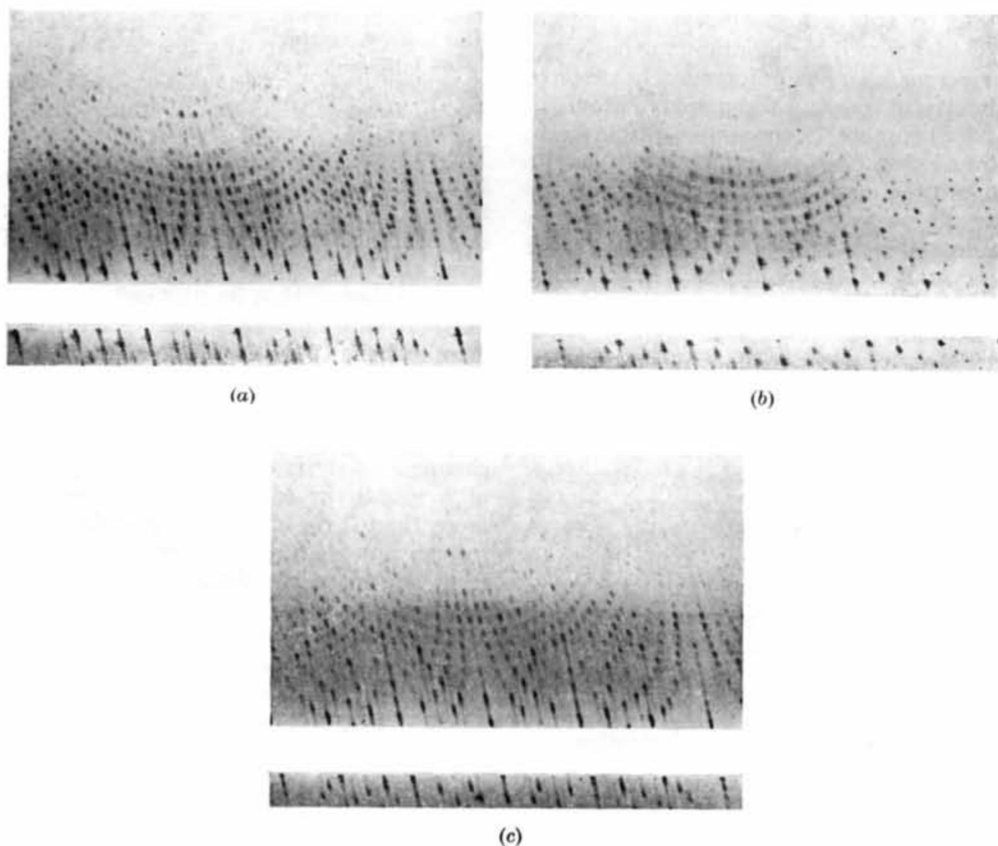
Fig. 1. (a) Crystalline ribonuclease from aqueous alcohol. (b) Crystalline ribonuclease-paramercuribenzoate (Ri-*pcmb*) from aqueous alcohol.



(a) (b) (c)

Fig. 2. Photographs showing the (0*kl*) reflections for (a) normal crystal, (b) normal crystal soaked in 0·5% Na-*pcmb* solution, (c) Dix crystal already containing 1 Hg:1 protein molecule soaked in 0·5% Na-*pcmb* solution.

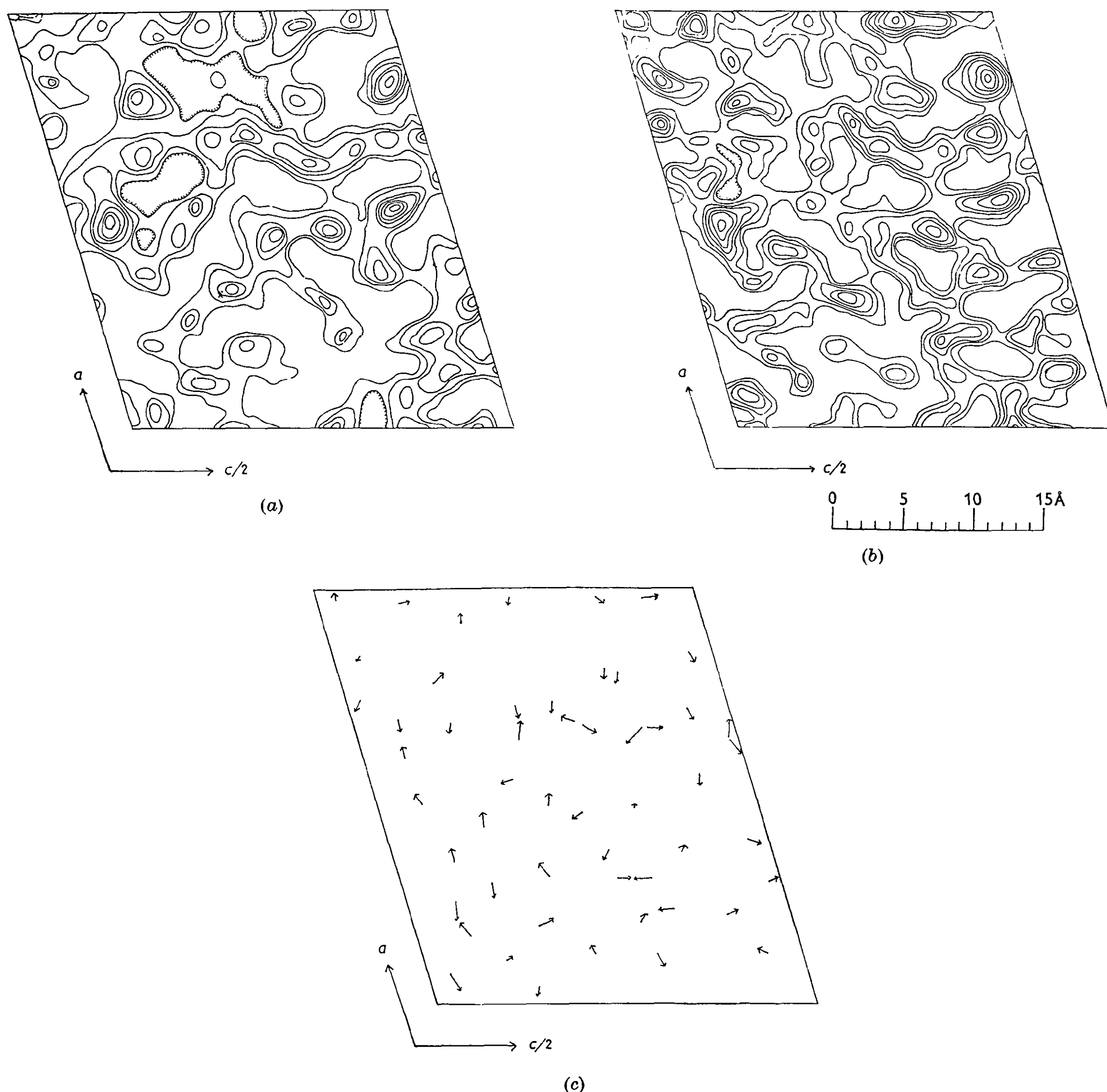


Fig. 6. (*a*) Patterson section at $V = \frac{1}{2}$ for Dix's soaked crystal. (*b*) Patterson section at $V = \frac{1}{2}$ for the normal crystal. (*c*) Schematic representation of peak movements between (*a*) and (*b*) indicating a movement of ribonuclease molecules to incorporate mercury derivative.

between corresponding reflections of the soaked and normal ones. The absence of perceptible intensity changes of the reflections of the crystals on prolonged soaking suggests that diffusion of the metal derivatives into the crystals of the complex almost ceases when there are about 2 Hg atoms per protein molecule.

3. Comparison of Patterson maps

Figs. 5(*a*), (*b*) show Patterson difference maps using $(\Delta|F|)^2$ as coefficients for the *b* plane of the normal soaked crystal and for Dix's soaked crystal respectively. In the calculation of these functions we discarded all coefficients that were less than the arithmetic average of $\Delta|F|$ for all observed reflections. Neither of the maps depict the clear-cut appearance of Hg–Hg vectors which is such a convincing feature of the corresponding Patterson difference maps obtained by Green *et al.* (1954) in their location of the mercury atoms in haemoglobin. Figs. 5(*a*), (*b*) bear a superficial resemblance to one another but there are differences in detail. It is not unlikely that the mercury atoms occupy approximately the same sites in the two crystals.

The three-dimensional Patterson vector sections at $V = \frac{1}{2}$ for the normal and Dix's soaked crystal are

shown in Figs. 6(*a*), (*b*), and Fig. 6(*c*) shows, by means of arrows, the displacement of corresponding peaks between the maps. The arrows in the neighbourhood of the *a* axis lie parallel to it, but at increasing distances from it they lie in a random manner. This indicates a rotation of the molecules about an axis nearly parallel to the *c* axis to accommodate the Hg derivative. The rather large number of peaks seen on Figs. 5(*a*), (*b*) and the differences in detail between them are not inconsistent with such a movement and would serve to explain the difficulty we experienced in locating the Hg atoms.

This suggests that the diffusion of the mercury derivative into the normal crystal may result in a movement of the protein molecules which lowers the symmetry of the crystal. The corresponding process in the Dix soaked crystal merely results in more equivalent sites being filled by heavy atoms with little or no resultant movement of the molecules, because the latter are already in their new positions.

Discussion

We conclude from these observations that isomorphism, as commonly understood in crystals of smaller molecules, need not strictly apply to protein crystals owing to the relative movement of the molecules needed to incorporate the heavy atom derivative. Provided the distortions are small, this should not prejudice the appearance of the heavy atoms by the use of *low order* reflections as carried out by Green, Ingram & Perutz (1954) and more recently by Kendrew (1958). Such non-isomorphism can be recognized by the appearance of multiple peaks on a difference Patterson projection.

The soaking experiments indicate that the mercury atoms cannot be strongly attached to the protein molecule, as would be expected since there are no free *SH* groups in this enzyme (Hirs, Moore & Stein, 1956; Rabinovitch & Barron, 1955; Gowron *et al.*, 1956; Redfield, Anfinsen & Cooke, 1956). There must, however, be weak directive forces holding them to specific sites on the protein molecule, because of the reproducibility of X-ray photographs. It may thus be possible to find other heavy metal derivatives specific in their attraction to certain residues on the molecule such as those discovered by Kendrew and his collaborators in myoglobin—another protein without *SH* groups.

The large number of peaks on the Patterson difference map (Fig. 5) may be due either to molecular rotation or to multiple mercury sites. In view of the movement of vector peaks on the Patterson sections at $V = \frac{1}{2}$ (Fig. 6) we believe the former explanation to be the more likely, but at this stage the latter cannot be rigorously excluded. There is, however, a degree of isomorphism between the crystals as shown by the low order reflections whose Patterson difference maps have already yielded clues to the possible location of the Hg atoms. This later work will be published in connection with the more detailed analysis now being undertaken of the complete sharpened three-dimensional Patterson maps for the two crystals.

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