of small molecules which are lost during the transition from 'wet' to 'damp', assuming that the density of the various components remains unchanged and that no holes are formed in the crystal. The change in volume of the unit cell, assuming b_0 changes in about the same ratio as a_0 and c_0, is about 3%, that is 1800 Å^3. The volume of a water molecule is 30 Å^3, and that of an ethyl alcohol molecule about 96 Å^3. Thus the number of small molecules lost per protein molecule (twice this for the unit cell) is either 30 water molecules, 10 alcohol molecules, or more likely, an intermediate number made up of both types. This enables one to form some picture of the effect.

The apparently continuous nature of the shrinkage suggests, as does all other evidence, that at least a portion of the solvent in the crystal is in a fairly liquid state. It seems that a well-ordered state of the crystal occurs only over a small part of the range from the wet stage to the first shrinkage stage. It is probably very difficult, if not impossible, to maintain the crystal in a state, say, half way between the normal wet stage and the first shrinkage stage.

The most likely explanation, then, is that most of the solvent in the crystal is in a liquid state, and that the structure of the crystal is maintained by the protein molecules being 'in contact' in some ill-defined sense. In the shrinkage we have been describing we surmise that the points of contact remain the same, but that the system is strained as the solvent molecules evaporate. In the *discrete* shrinkage, on the other hand, the molecules are envisaged as moving so that at least one of the 'points of contact' is changed.

The apparently continuous shrinkage of β-lactoglobulin reported by McMeekin, Rose & Hipp (1954) might be due, on this picture, to the closeness of the discrete shrinkage stages, because of the large size of the unit cell, since the molecules could rather easily move their points of contact by a few Ångströms without causing more than a very small percentage change in the volume of the unit cell. This, together with the small continuous shrinkage due to straining the configurations, might give the appearance of continuous shrinkage. If this view is correct such an appearance would be more common with large protein molecules than with small ones.

We should like to thank Dr Murray Vernon King, who grew and mounted all the crystals for us; Mrs Dalia Rojansky David, who did all the computation so cheerfully; Dr Benjamin Post, who suggested the use of large jets; and all our colleagues at the Protein Structure Project for general assistance. The calculations were carried out on IBM machines at the Watson Laboratories, for which facilities we are very grateful.

One of us (F. H. C. C.) would like to express thanks to Dr David Harker for the hospitality shown to him during his year's stay at the Project.

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A New Crystal Form of Ribonuclease*

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Crystals of the protein ribonuclease grown in the presence of iodophenol blue have a new unit cell, space group $C2$. The Patterson projection shows that it is closely related to the common monoclinic form of ribonuclease, space group $P2_1$. From a study of the low-order reflections the relative positions of the molecules can be found within certain limits.

Introduction

In order to attach a heavy atom to the ribonuclease molecule, attempts have been made to grow crystals of the protein in the presence of certain dyes. In most cases some dye was incorporated in the crystal (usually ribonuclease II) without any change in the space group, and with only very slight changes in the

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cell dimensions (King, Magdoff, Adelman & Harker, 1955). In one case, however, the dyed crystals were found to be of a new type, which we have called ribonuclease VI.

Although the space group ($C2$) was different from that of ribonuclease II ($P2_1$) it soon became clear that the two forms were closely related. This note describes briefly our preliminary studies of ribonuclease VI, and discusses the nature of its relationship to ribonuclease II.

Method of growth

The crystals were grown by Dr Murray Vernon King by methods described elsewhere (King *et al.*, 1955) as part of his general program of attaching heavy atoms to ribonuclease. The protein was Armour bovine ribonuclease. The dye was tetraiodophenolsulfonphthalein (iodophenol blue) (Fig. 1). The sample

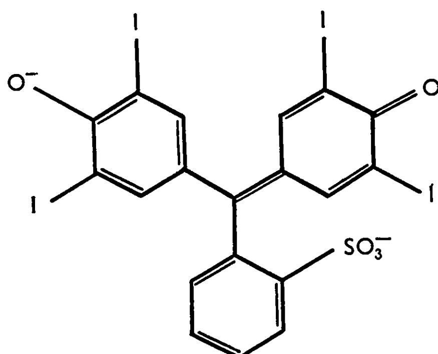

Fig. 1. Iodo-phenol blue.

was prepared with $2\frac{1}{2}$% protein, and the dye was present in a 1:1 molar ratio. The crystals were grown at a pH of about 5 in a solvent having 50% by volume of tertiary butyl alcohol. Under these conditions, but without the dye, they would have grown as ribonuclease II.

The No. VI crystals which grew were colored a deep blue, which seems to the eye rather a different color from a dilute solution of the dye. It was found that both the old form (No. II) and the new form (No. VI) would grow from the solution, sometimes in the same tube.

So far it has not proved possible to obtain ribonuclease VI without dye, or with a different dye.

Habit and optical properties

The typical habit of the new crystals is shown in Fig. 2(*a*). That of the more usual No. II is shown in Fig. 2(*b*) for comparison. At one time it was thought that the two types could be distinguished by their habit and general appearance, but this has turned out on occasions to be misleading. Frequently, the only distinctive and easily observable difference is the high pleochroism of the No. VI form. The No. VI crystals are usually elongated blades, the dimensions in the *a*, *b* and *c* directions being typically in the ratio

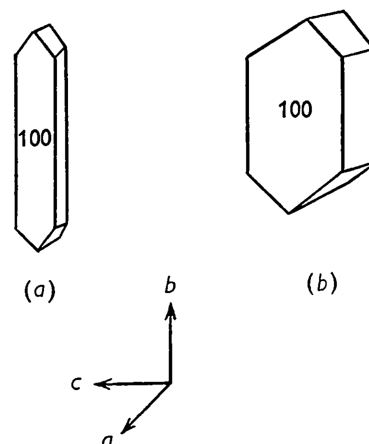

Fig. 2. Typical habit of (*a*) ribonuclease VI, (*b*) ribonuclease II. The interaxial angles are given in Table 1.

$1:10:2\frac{1}{2}$. Viewed in polarized light traveling approximately perpendicular to the *b*–*c* plane, they show a high degree of pleochroism, the strong absorption being when the electric vector is parallel to *b*. The maximum pleochroic ratio was judged visually to be about 10:1. The normal to the plane of the dye molecule is therefore not far from the *c* direction.

The strong pleochroism shows that all the dye molecules in the crystal are parallel, or very nearly so. The most likely reason for this is that the dye occupies a single site on the protein molecule.

Space group and unit-cell dimensions

The space group was found by taking precession photographs. They showed unambiguously that it was $C2$. No forbidden reflections were found in the three zones covered ($1/d$ to 0·44 $Å^{-1}$) so that the space group is probably a true one and not a pseudo one.

The cell dimensions were measured roughly from the precession photographs and accurately on the spectrometer. They are given in Table 1, with those for ribonuclease II for comparison.

Table 1

		a_0 (Å)	b_0 (Å)	c_0 (Å)	β (°)	Volume ($Å^3$)
Ribonuclease VI	$C2$	70·60	38·99	51·65	103·96	138,000
Ribonuclease II	$P2_1$	30·28	38·39	53·16	105·83	59,400

The volume of the *primitive* cell of No. VI is thus 16% greater than that of No. II. As there are two molecules of ribonuclease per unit cell of No. II there are almost certainly two for the primitive cell of No. VI, and thus four for the side-centered cell; that is, one molecule in the asymmetric unit.

It can be seen that the two unit cells are dimen-

Table 2

l \ $h \rightarrow$	$\overline{22}$	$\overline{20}$	$\overline{18}$	$\overline{16}$	$\overline{14}$	$\overline{12}$	$\overline{10}$	$\overline{8}$	$\overline{6}$	$\overline{4}$	$\overline{2}$	0	2	4	6	8	10	12	14	16	18	20	22
0	—	—	—	—	—	—	—	—	—	—	—	—	1060	29	1	16	18	25	246	341	300	157	3
1	28	167	102	5	4	3	113	73	83	100	112	363	1	63	17	68	3	79	1	5	21	7	10
2	—	3	30	288	311	44	173	15	79	510	77	266	133	40	3	3	23	15	6	3	6	17	3
3	3	4	2	152	8	16	13	13	269	—	22	36	53	10	14	130	34	10	15	—	26	17	—
4	2	206	49	60	13	16	1	118	35	91	32	19	11	78	43	3	3	18	—	51	63	24	—
5	2	—	3	31	69	4	32	—	399	543	94	2	18	4	42	33	67	2	9	60	—	10	—
6	33	3	7	—	163	48	4	1	12	71	3	2	2	1	—	2	60	1	22	44	8	—	—
7	1	13	15	57	9	5	11	105	96	9	3	23	18	6	31	1	121	12	88	68	1	—	—
8	6	91	234	187	6	7	56	5	2	1	28	107	17	16	13	187	—	5	34	44	—	—	—
9	2	3	25	120	5	29	1	—	98	61	9	6	1	9	106	6	42	5	5	15	—	—	—
10	15	105	27	8	379	348	86	2	48	9	26	60	274	110	36	2	21	1	1	—	—	—	—
11	—	89	4	157	18	58	288	67	13	83	25	56	95	79	47	8	46	20	5	—	—	—	—
12	—	11	14	3	42	41	10	9	30	6	1	21	213	2	1	61	8	2	—	—	—	—	—
13	—	—	11	—	93	54	41	1	40	13	4	—	—	—	3	1	41	—	—	—	—	—	—
14	—	—	12	3	8	2	25	3	10	92	39	19	8	1	9	90	—	—	—	—	—	—	—
15	—	—	—	1	3	2	19	55	122	6	7	69	109	5	3	—	—	—	—	—	—	—	—
16	—	—	—	—	16	31	2	28	16	2	64	40	1	—	—	—	—	—	—	—	—	—	—
17	—	—	—	—	—	—	5	—	19	17	—	—	—	—	—	—	—	—	—	—	—	—	—

sionally rather similar. The b axes are almost identical, the c axes not very different, and $\frac{1}{2}a$ for No. VI is only 16% greater than a for No. II. It was therefore decided to make some preliminary measurements of the intensities of No. VI in the hope of throwing additional light on this relationship.

We have tried to obtain dry cell dimensions from No. VI crystals, but so far the dry crystals we have obtained have been too disordered to yield a unit cell. The disordering occurs, through evaporation of solvent, not later than ten minutes after the capillary has been cracked.

Experimental

The values of the $h0l$ and the $0k0$ intensities were measured on the Geiger-counter spectrometer. The instrument and the technique have already been described (Furnas & Harker, 1955). Adequate precautions were taken to ensure that the crystal was in a stable condition (Magdoff & Crick, 1955) and the usual correction was made for the absorption of the capillary (Furnas, unpublished: described in Magdoff & Crick, 1955). The Lorentz factor and the absorption correction were made using IBM machines. The relative $|F|^2$ are listed in Table 2. Approximate absolute values in (electrons)^2 for the *primitive cell* can be obtained by multiplying these figures by 1900 (Furnas, unpublished).

Relative values of the $0k0$ intensities are given in Table 3, together with *relative* values of those for ribonuclease II.

A two-dimensional Patterson projection was calculated using IBM machines from the relative $|F|^2$ in Table 2, and is shown in Fig. 3(a). Each contour is about 1000 $e^2.\text{Å}^{-2}$ for the primitive cell. The corresponding Patterson projection of ribonuclease II is given in Fig. 3(b). The data for this crystal, for which the solvent was 50% by volume tertiary butyl alcohol, have not been published, but the intensities are very similar to those for a crystal grown from 55% by volume ethyl alcohol listed in Magdoff & Crick (1955). Visually estimated values have also been published earlier by Carlisle & Scouloudi (1951) who also show a Patterson projection which is similar to ours. The intensities making up our data extend to $2\theta = 30°$, as they do for the No. VI crystal, and the contours of the two Pattersons (in Fig. 3(a) and (b)) are approximately on the same scale.

Table 3. *Comparison of intensities*

Reflection	No. II	No. VI
020	1970	2600
040	500	490
060	46	27
080	16	21
0,10,0	269	220
0,12,0	81	27

Interpretation

(a) *The molecular positions in No. VI*

Turn first to Fig. 3(a). It can be seen that the vectors are concentrated in a comparatively narrow region running through the cell parallel to c. This effect is produced by the very large $|F|^2$ of 200, which is the largest intensity among the $h0l$'s. It can only mean that the molecules form layers in the b–c plane. The reason this shows up here is that the solvent has a much lower electron density than that of the hydrated protein (0·3 e.Å^{-3} compared to 0·4 e.Å^{-3}).

The comparatively strong 001 suggests that the 'centers' of the molecules related by a twofold axis are not $\frac{1}{2}c$ apart. Thus although we cannot locate the relative positions of the two molecules unambiguously we can narrow down the possibilities considerably. This is shown in Fig. 6 by the regions marked A.

(b) *Comparison between No. VI and No. II*

Now compare Fig. 3(a) with Fig. 3(b). To facilitate this comparison Fig. 4 has been constructed. This consists of the Patterson of ribonuclease II—a little

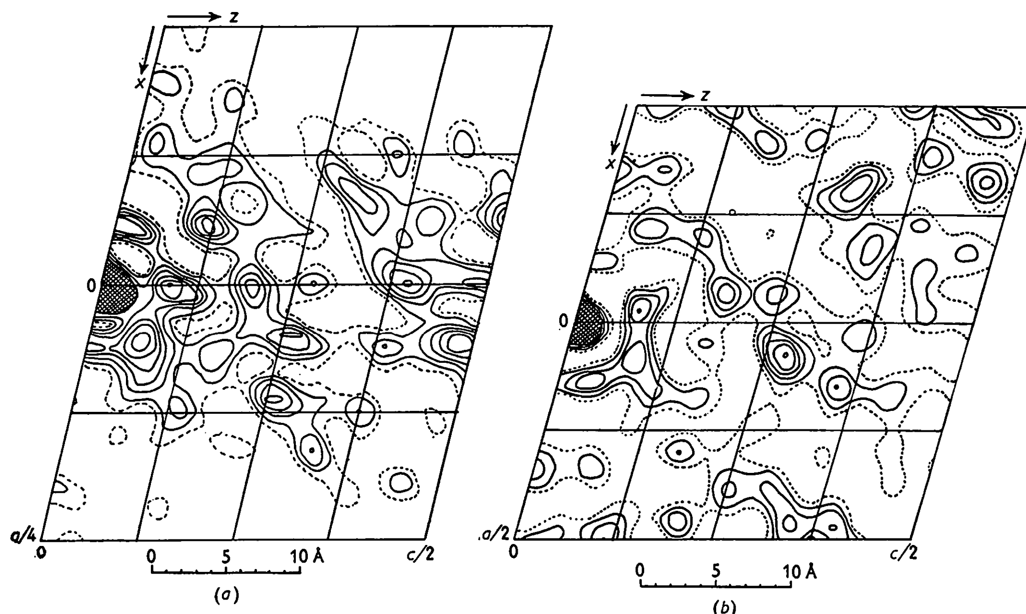

Fig. 3. Patterson projection along the b axis of (a) ribonuclease VI, (b) ribonuclease II. Zero contours broken; negative contours omitted.

more of it than is shown in Fig. 3(b)—to which has been added, as a pattern of dots, all the positive regions of ribonuclease VI (Fig. 3(a)) above the first contour. However, the two cells have not been superimposed with their a or c axes parallel, but twisted so that their respective c axes are some 25° apart.

It can be seen that there is a very considerable degree of coincidence. Practically every peak of the No. II Patterson is covered by the dots. The peaks which are not so covered—for example, those near the bottom of the figure—will be found to be covered when they occur elsewhere (as they do here at the top of the figure, which is simply a repetition of the bottom due to the repeat of the Patterson). Moreover, the dots cover hardly any negative regions of the No. II Patterson, and when they do it can usually be explained as due to the symmetry inherent in the Patterson, which gives the same peak in more than one place.

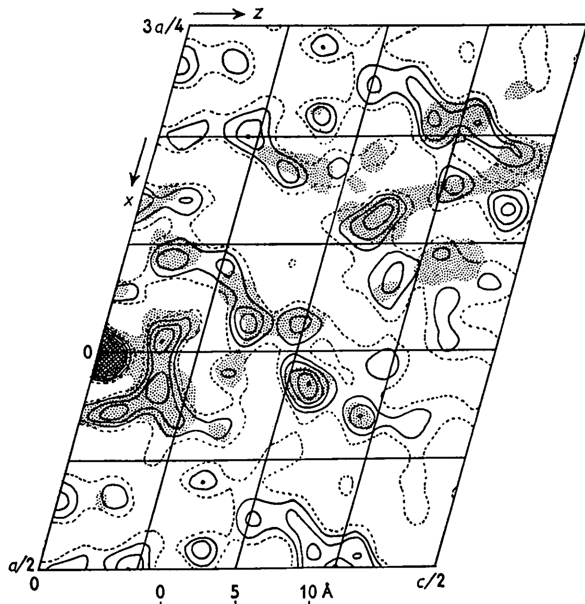

Fig. 4. Patterson projection along the b axis of ribonuclease II. Dotted regions are those above 1000 e.Å^{-2} for ribonuclease VI, appropriately rotated. Zero contours broken; negative contours omitted.

The only exception to this seems to be near the origin. In the Patterson of No. II there is a continuous negative ring at 3 Å from the origin; in that of No. VI there is a conspicuous bridge. However, closer inspection of the former shows that it has a submerged bridge. This can be seen from Fig. 5(a), which shows the region in question for ribonuclease II (50% ter-

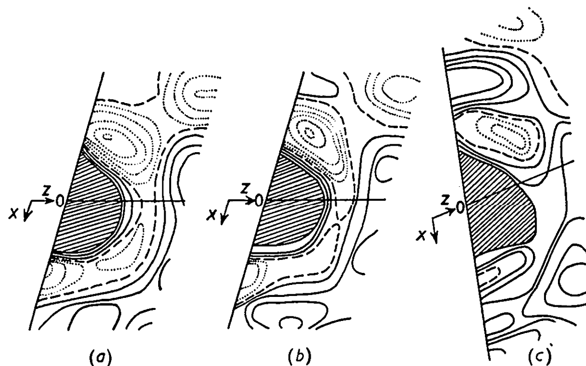

Fig. 5. Patterson projections along the b axis of (a) ribonuclease II, grown from 50% aqueous tertiary butyl alcohol, (b) ribonuclease II, grown from 55% aqueous ethyl alcohol, (c) ribonuclease VI, appropriately rotated. Zero contours broken; negative contours dotted.

tiary butyl alcohol) with the negative contours as well as the positive ones. The corresponding region for ribonuclease II, but from 55% ethyl alcohol as solvent, is shown in Fig. 5(*b*), and that for ribonuclease VI in Fig. 5(*c*). It can be seen that the submerged bridge in Fig. 5(*a*) has broken surface in Fig. 5(*b*) and is high and dry in Fig. 5(*c*). We believe that this effect is caused by the increased intensities of the low-order reflections, produced in Fig. 5(*b*) by the lower electron density of the solvent and in Fig. 5(*c*) by the increased separation of the molecules.

Leaving this possible difficulty on one side, it seems virtually certain that the aspect of the pair of molecules, looking along *b*, making up the unit cell of No. II is very similar to that of the pair of molecules forming the (primitive) unit cell of No. VI, and that the two aspects can be made to coincide by twisting one pair of molecules about the *b* axis through 25°.

(*c*) *The molecular positions in No. II.*

Now the symmetry elements of No. II consist only of twofold screw axes; those of No. VI of both twofold screw axes and ordinary twofold axes. It is therefore a natural hypothesis to *assume that one of the screw axes is the same in both cells*. That is, the relationship between adjacent molecules, within an infinite line of molecules in the *b* direction, which leads to one of the screw axes in No. II, is exactly the same as that expressed by one of the screw axes in No. VI.

If this hypothesis were correct we should expect the *b* axis of the two cells to be identical, and the 0*k*0 reflections to remain unchanged in going from one cell to the other. Reference to Tables 1 and 3 shows that these expectations are obeyed approximately. The *b* axes differ by only 0·60 Å, and the 0*k*0 intensities differ by no more than might be expected from this change and from the effect of the dye molecules.

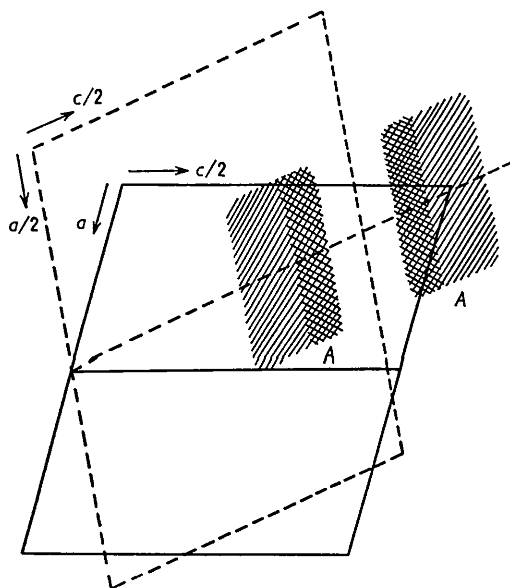

Fig. 6. Relative positions of the ribonuclease molecules in the two cells $P2_1$ and $C2$; first molecule at the origin, second molecule in regions *A*. The broken cell is ribonuclease VI and the solid is ribonuclease II.

We are now in a position to use this information to narrow down the position of the two molecules in ribonuclease II. This is shown diagrammatically in Fig. 6. The shaded areas labeled *A* show, in an approximate manner only, the location of the 'center' of the second molecule when the first one is placed with its center on the origin. The size and shape of these areas have been estimated by using the $|F|$ values for 001 and 002. The fact that 002 is also rather strong suggests that the 'center' of the second molecule is more likely to lie in the parts of *A* which are shown with *double* cross-hatching. It should be realized that in any case the 'center' of a protein molecule is not a clear-cut idea, and depends on how it is defined.

(*d*) *Concluding remarks*

Notice that it would be difficult from a simple study of ribonuclease II alone to find where the molecules are, since they are closer together than in No. VI, and thus the contrast between them and the solvent is less marked. Similarly, from No. VI alone it would be difficult to decide if the molecules in the same layer were related by rotation or by screw axes, while the argument given above suggests that the latter alternative is correct and that the rotation axes relate one layer to the next.

Finally it should be remembered that the dye molecule (Fig. 1) has a pseudo mirror plane and a pseudo twofold axis. It is thus possible that the dye molecules lie on one or more of the rotation axes.

We feel it is unwise to go beyond these rather limited deductions for the time being, since speculations from simple packing considerations have been known to give misleading results in the past. The first successful isomorphous replacement should in any case establish the main features of the molecule and it seems better to wait till this is achieved.

We should like to thank Dr Murray Vernon King for both growing and mounting all the crystals; Mrs Dalia Rojansky David who did all the computing and drew many of the figures; and all our colleagues at the Protein Structure Project for general assistance and discussion. The calculations were carried out on IBM machines at the Watson Laboratories, for which facilities we are very grateful.

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