

Structure of ribulose-1,5-bisphosphate carboxylase-oxygenase: Form III crystals

(protein structure/x-ray diffraction/molecular symmetry/chloroplast crystals)

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ABSTRACT A new crystal form (III) of tobacco leaf ribulosebisphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39] has been grown by dialysis procedures, and is suitable for structural studies at near atomic resolution. The crystals exhibit birefringence, grow as pseudo-regular rhombic dodecahedrons, and belong to the tetragonal space group $P4_22_12$ with $a = b = 149 \text{ \AA}$, $c = 138 \text{ \AA}$, and $V = 3.04 \times 10^6 \text{ \AA}^3$. Each unit cell contains two molecules, with two large and two small subunits per asymmetric unit. At low resolution ($>10 \text{ \AA}$) the crystal structure is body centered belonging to space group I422 with one large/small pair in the asymmetric unit. Thus, at low resolution the molecular symmetry is D_4 , the highest possible symmetry for an oligomer of stoichiometry large₂small₂. Form III crystals may be identical to crystalline inclusions found in chloroplasts.

In 1947, Wildman and Bonner (1) isolated a protein in large quantities from spinach leaves. Later it was realized that this protein comprises nearly 50% of the soluble protein of leaf extracts, and that it catalyzes the initial dark reaction of the photosynthetic Calvin cycle (2, 3)

D-ribulose 1,5-bisphosphate + CO₂ + H₂O



Further work has established that this enzyme, ribulosebisphosphate carboxylase [3-phospho-D-glycerate carboxy-lyase (dimerizing), EC 4.1.1.39], is oligomeric, with eight copies of each of two types of subunits [L (large) $\approx 55,000$ molecular weight; S (small) $\approx 15,000$ molecular weight]. An octomer of the large subunit is capable of catalysis, with or without the presence of small subunits (4), whose function is unknown.

Because of the abundance of this enzyme in chloroplasts, investigators have suggested that it composes the crystalline inclusions that are observed in chloroplast stroma. These microcrystals have been observed in micrographs of sectioned and freeze-etched chloroplasts of both spinach and tobacco (5-12). In some cases, these crystallites may be induced by chemical treatments but in other cases they are believed to exist *in situ*. Both the extent (up to 3 μm in one dimension) and number of these crystallites, and their lattice spacings (65-105 \AA) have led investigators to conclude that they are ribulosebisphosphate carboxylase. The dimensions of the enzyme are approximately $115 \times 115 \times 100 \text{ \AA}$ (13). The function of the inclusions remains unknown; they may be stored protein.

We report here a new crystal form (III) of the enzyme, which is more stable than Forms I and II, and is suitable for structural studies of near atomic resolution. Form III crystals may be identical to the crystalline inclusions found in chloroplasts.

Abbreviation: L, large subunit; S, small subunit.

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EXPERIMENTAL

Protein. The carboxylase was a gift from Kevin Chen of the Department of Biology, University of California, Los Angeles. It is received and stored in salt-free buffer at 4° as Form I crystals, and is isolated from *Nicotiana tabacum* by published methods (14).

Crystallization. Form I crystals of the enzyme are dissolved in 0.025 M Tris-HCl buffer at pH 7.4 containing 0.1 M NaCl and dialyzed overnight against 0.05 M K phosphate buffer at pH 7.2. The protein is diluted to 3.0-5.0 mg/ml in this buffer, and centrifuged. Then aliquots of 0.05 ml are introduced into glass capillaries (7 mm outer diameter, 2 mm inner diameter) which are sealed with dialysis membrane at one end, and are submerged in a reservoir of the precipitant solution. The precipitant solution is 0.2 M K phosphate with 0.1-0.75 M (NH₄)₂SO₄, adjusted to pH 4.8-5.2.

Density Measurements. The wet density of 10 Form III crystals was measured by the density gradient technique of Low and Richards (15). The gradient is made in a 25 ml graduated cylinder with xylene and bromobenzene (each of which is presaturated with mother liquor), and calibrated with 5-10 μl droplets of 0.5-4.0 M KBr solutions of known densities. Crystals settle to equilibrium positions in 60-90 sec.

RESULTS

Form III crystals $0.4 \times 0.4 \times 0.2 \text{ mm}$ in extent (Fig. 1) grow in 1-2 weeks at room temperature in 0.2 M K phosphate at pH 5.0 and 0.3 M (NH₄)₂SO₄. These crystals are mechanically more stable than either Forms I or II, and unlike these other forms can be transferred from a crystallization capillary to an x-ray capillary without suffering damage.

Precession photographs (Fig. 2) reveal that Form III is tetragonal, with diffraction symmetry $4/mmm$. At low resolution ($>10 \text{ \AA}$), the structure is body centered because reflections of the form $h + k + l = \text{odd}$ are absent. The unit cell dimensions ($a = b = 148.7 \pm 0.2 \text{ \AA}$; $c = 137.5 \pm 0.2 \text{ \AA}$) are such that the cell volume ($V = 3.04 \pm 0.01 \times 10^6 \text{ \AA}^3$) is only 2.3 times the envelope of a single molecule ($1.3 \times 10^6 \text{ \AA}^3$), and indicate that there cannot be more than two molecules per unit cell. Space group I422 is the only space group compatible with this symmetry that can contain fewer than four protein molecules per cell. Because the body centering requires some multiple of two molecules per cell, there must be exactly two molecules in the unit cell.

At higher resolution (about 4.4 \AA), reflections with indices $h + k + l = \text{odd}$ begin to appear, and show that the unit cell is actually primitive with the dimensions given above. There are eight primitive space groups of point group 422, but only two are compatible with the basic data: four of the eight require four or more molecules per cell, and two others cannot accommodate a pseudobody-centered packing arrangement. The remaining two are $P4_22_12$ and $P4_222$, and to the available res-

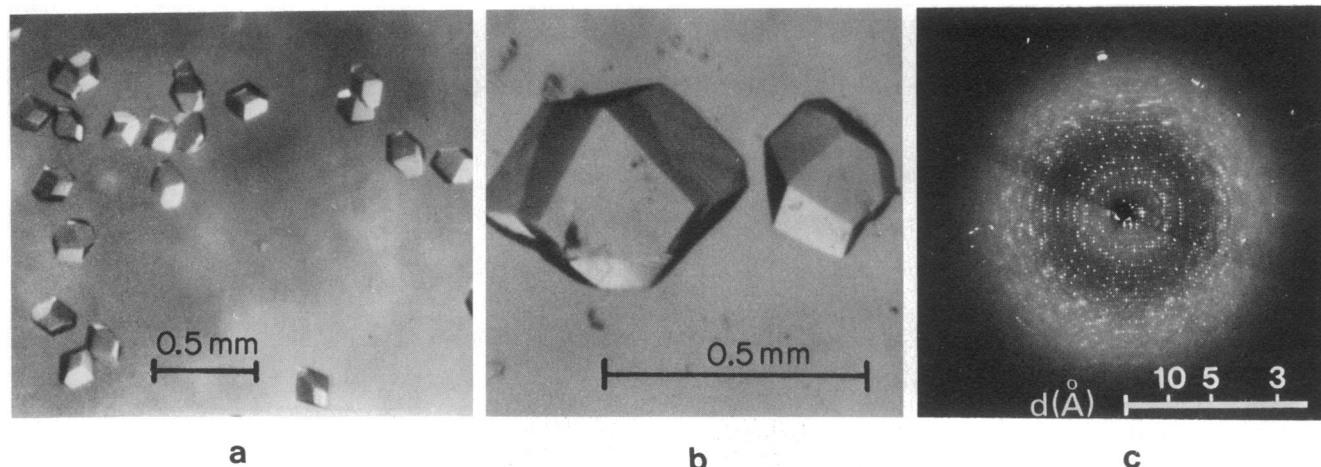


FIG. 1. (a) Small, well-formed, Form III crystals showing the pseudorhombic dodecahedral morphology. (b) Two crystals with fully developed 4-fold axes (at top of crystal on left and at left of crystal on right). The crystal on the left is similar in size to those used for x-ray diffraction experiments. (c) Two hour, still x-ray photo ($\bar{\mu} = 0^\circ$) with the x-ray beam nearly parallel to the $[110]$ direction. The pattern is strong to at least 3.0 \AA . The crystal to film distance is 35 mm .

olution of our x-ray precession photographs (about 4.4 \AA), space group $P4_22_12$ is favored, because reflections of the type $h00$ occur only for $h = 2n$.

The crystals diffract strongly to 3.6 \AA and the pattern extends at least to 2.7 \AA on 2-hr-still photographs (Fig. 1c). The average crystal lifetime in the x-ray beam is 20 hr.

Our arguments above that the unit cell must contain two molecules can be checked by measurements of the wet density of the crystals ($\rho = 1.184 \pm 0.007 \text{ g/cm}^3$). The number of molecules (n) can be calculated from the relationship:

$$n = \frac{N\rho VX_p}{M_r}$$

in which

$$X_p = \frac{[(1/\rho) - \bar{v}_s]}{(\bar{v}_p - \bar{v}_s)}$$

is the weight fraction of protein in the crystal, and M_r is the molecular weight of the enzyme ($M_r = 560,000 \pm 25,000$), $\bar{v}_s = 0.962 \pm 0.001 \text{ cm}^3/\text{g}$ is the partial specific volume of the crystallization solvent, and $\bar{v}_p = 0.734 \pm 0.01 \text{ cm}^3/\text{g}$ is the partial specific volume of the protein (estimated from the amino acid composition, see ref. 16). The value obtained is $n = 1.97 \pm 0.15$, confirming that n is two.

DISCUSSION

Several investigators (8–10, 12) have suggested that the crystalline inclusions observed in chloroplasts are microcrystals of ribulosebiphosphate carboxylase, because this enzyme accounts

for such a large fraction of the soluble protein of the organelle. This might imply that carboxylase can be crystallized readily *in vitro* at neutral pH. Kawashima and Wildman (17) were, in fact, able to grow single crystals as large as 1 mm on an edge from carboxylase isolated from tobacco leaves of *Nicotiana tabacum*, and this achievement has since been repeated for seven different species of *Nicotiana* and 14 reciprocal interspecific F_1 hybrids (14). We investigated these crystals (Form I) by x-ray diffraction and electron microscopy (18), but found that their lattice spacings (383 \AA) are much too great to be the same crystals as that found in chloroplasts. The unit cell dimensions of a second crystal Form (II, 13) which grows at slightly acid pH are also too great to be the chloroplast form.

Form I and II are also of limited usefulness for structural studies. They allowed us to establish that the enzyme molecule is roughly $115 \times 115 \times 100 \text{ \AA}$ in size, with a stoichiometry of L_8S_8 , and with the eight large subunits arranged in two layers about a 4-fold axis (Figs. 3 and 4). The symmetry of the molecule is $D_4 [422]$. But beyond providing these gross features, the earlier crystal forms are not sufficiently ordered for protein crystallography.

Form III has the dual interest of being the first crystal form of the enzyme suitable for structural studies at medium to high resolution, and the first crystal form that is compatible with the spacings observed in the crystalline inclusions. It also has the attractive feature that the molecular packing can be inferred from the crystallographic dimensions and symmetry.

The suitability of Form III for protein crystallography is evident from the entry of Table 1 that gives the finest resolution

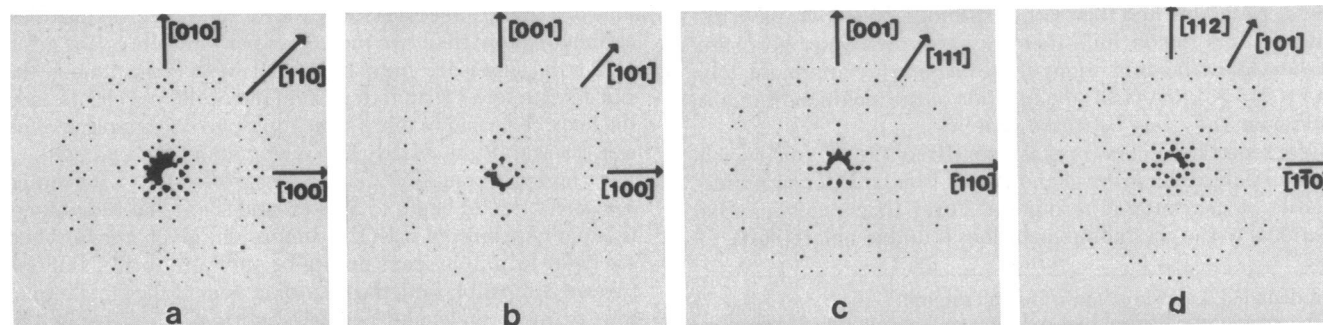


FIG. 2. Precession photographs ($\bar{\mu} = 5^\circ$) of the (a) $hk0$, (b) $h0l$, (c) hhl , and (d) $h, k, h+k$ reciprocal lattice zones. Exposure times were approximately 12 hr for (a), (c), (d), and 4 hr for (b). The limiting resolution for these photos is 8.8 \AA .

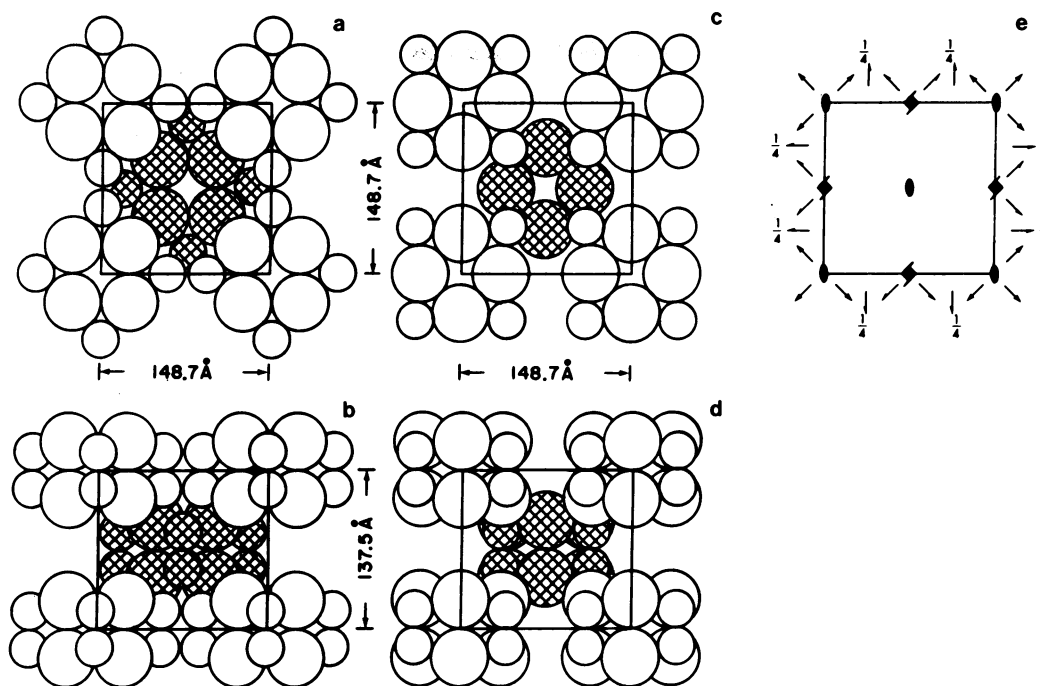


FIG. 3. Molecular packing diagrams for space group $P4_22_2$. Each molecule is depicted as two eclipsed layers of four large and four small spherical masses whose volumes are proportional to the large and small subunit masses. This model, whose gross features of mass distribution agree with electron microscopic images (13), does not necessarily represent the actual disposition of large and small subunit masses. Both (a) and (b) show one possible packing and (c) and (d) show the other. In (a) and (c) the unit cell contents are projected along the c axis and in (b) and (d) along the b axis. Cross hatched molecules are situated at the unit cell center, and the others are displaced by $1/2, 1/2, 1/2$. The crystallographic symmetry elements for $P4_22_2$ are shown in (e) for the cell viewed along c .

x-ray reflection. It is 2.7 \AA for Form III, compared to 14 \AA for Form II and 50 \AA for Form I. In Form III, the molecular symmetry required by the space group is $D_4 [422]$ at low resolution and $D_2 [222]$ at higher resolution. This indicates that there are eight quite similar subunit pairs (LS)₈, but that these are actually grouped as four identical copies of two similar pairs (LL'SS')₄. While it is conceivable that preformed molecules of D_2 symmetry in solution are incorporated into the Form III crystals, it is perhaps more likely that molecules of D_4 symmetry are slightly distorted to D_2 as they grow into a crystal structure having sites of D_2 symmetry.

Form III and Chloroplast Inclusions. Lattice spacings of the microcrystals from chloroplast inclusions are by and large compatible with lattice spacings in our Form III crystals. In each of the eight studies of chloroplast inclusions, some reported spacings are consistent with spacings of lattice planes of our crystal, but also in each study there is at least one spacing reported that is not one of the expected lattice planes. Discrepancies between spacings in Form III and in electron micrographs may result for one or more of the following reasons: (i) the preparation of specimens for electron microscopy (fixation, dehydration, infiltration of polymer, sectioning, staining, beam damage, etc.) may distort the lattice; (ii) the chloroplasts are usually sectioned at random. Therefore, the micrographs are random views of the crystal which may not be identical to views along rational directions (often there is only one evident spacing in the micrographs so it is not possible to determine the direction of view). Even in freeze-etched experiments, the replica of the cleaved plane may not lie exactly normal to the direction of view; and (iii) the reported crystalline inclusions may be of more than one type, depending on the conditions prevailing in any given experiment.

The report of inclusions in spinach chloroplasts by Larsson *et al.* (9) is of particular interest because at least two micro-

graphs appear to correspond to principal projections of Form III crystals. These authors sectioned an inclusion crystal with a hexagonal outline. This may suggest that the crystal was sectioned normal to a rational direction. They found a quasi-hexagonal arrangement of molecules. Their measurements gave lattice plane spacings of $85\text{--}95 \text{ \AA}$ with interaxial angles of $54\text{--}66^\circ$. Just such a hexagonal arrangement of molecules would be expected for a view along the 111 direction of our pseudocubic Form III crystals. The principal vectors for this net are d_{101} (101 \AA) and d_{110} (105 \AA), with angular separation of 59° . Figs. 7–9 of the same report show a micrograph which is similar to a view along the 001 direction of Form III. The reported dimensions for the inclusions in these micrographs are 98 and 110 \AA with an angle of 85° between them. For Form III, we would expect $d_{110} = 105 \text{ \AA}$ and $d_{1\bar{1}0} = 105 \text{ \AA}$, with an angle of 90° between them. Other micrographs show spacings of $75\text{--}85 \text{ \AA}$, approximately those of the d_{111} (84 \AA) spacing of Form III.

A recent study of chloroplast inclusions in tobacco by Willison and Davey (12) is largely, but not completely, compatible with the hypothesis that the inclusions are Form III crystals. They observed square patterns in micrographs of sectioned inclusions, consisting of two sets parallel lines of molecules at angles close to 90° , each set spaced at about 100 \AA . Transmission electron micrographs of sectioned material are projections of the sections. Thus, this observation is consistent with the view of Form III crystals along the 001 direction which would show in projection the square pattern of the 110 planes ($d_{110} = 105 \text{ \AA}$). Micrographs of freeze-etched inclusions also showed square arrays of molecules but with larger spacings, about 120 \AA . Such shadowed, freeze-etched samples show a surface rather than a projection. Thus, the view of Form III crystals along the 001 direction would display a square lattice of larger spacings ($d_{100} = d_{010} = 149 \text{ \AA}$). The view along the 100 direction would show

Table 1. Properties of ribulosebisphosphate carboxylase crystals

	Crystal form		
	I	II	III
Crystallization conditions			
pH	7.4–8.8	6.0–6.2	4.8–5.2
Buffer	0.025 M Tris·HCl	0.025–0.05 M Tris·HCl or K phosphate	None
Other	None	None	0.2 M K phosphate, 0.2–0.3 M (NH ₄) ₂ SO ₄
Protein (mg/ml)	10–20	5–15	3–5
Birefringence	None	Strong	Weak
Density (ρ , g/cm ³)	1.058 ± 0.005	1.096 ± 0.006	1.184 ± 0.007
Crystal system	Cubic	Tetragonal	Tetragonal
Space group	I4, 32	P4 ₂ , 2*	P4 ₂ , 2†
Dimensions a=b (Å)	383 ± 3	230 ± 2	148.7 ± 0.2
c (Å)	383 ± 3	315 ± 3	137.5 ± 0.2
$\alpha = \beta = \gamma$	90°	90°	90°
Cell volume (Å ³ × 10 ⁻⁶)	56.2 ± 0.8	16.7 ± 0.3	3.04 ± 0.01
Molecules per cell (<i>n</i>)	12	6	2
Solvent content (Å ³ /dalton)	8.36 ± 0.39	4.96 ± 0.24	2.71 ± 0.12
Weight fraction protein (<i>X_p</i>)	0.21 ± 0.02	0.32 ± 0.02	0.51 ± 0.03
Molecules per asymmetric unit	1/4	1/2, 1/4‡	1/4
Minimum molecular symmetry	D ₂	C ₂ , C ₄	D ₂
Finest x-ray resolution (<i>d</i> _{min} , Å)	50	14	2.7
Reference	18	13	This paper

* At higher resolution (<14 Å) the space group is either P4₂, 2 or P4₂, 2 (13).

† Space group is I4₂, 2 for *d* > 10 Å, and either P4₂, 2₁, 2 or P4₂, 2₂ for finer resolutions.

‡ Molecules occupy two types of crystallographic special positions in this form (13).

a nearly square rectangular array ($d_{001} = 137 \text{ \AA} \times d_{010} = 149 \text{ \AA}$). Thus, these inclusion crystallites correspond to Form III in that square spacings are greater for a surface view than for a projection, but the ratio of spacings is less than expected for Form III. Willison and Davey also present hexagonal views of freeze-etched samples, but these are of less convincing quality than the square views. These authors measured center-to-center separations of molecules in these views as 120 Å (corresponding to interplanar spacings of $120 \times \sqrt{3}/2 = 104 \text{ \AA}$). This is close to the separations of 126 Å (along the 111 direction) and of 138 Å (along the 001 direction) that we would expect in the surface normal to the 110 direction. In this surface, molecules are related quasi-hexagonally: the interparticle vectors form angles of 66.4° and 56.8° with each other.

On the basis of their observation of equal molecular spacings (120 Å) for square and hexagonal arrays in the inclusions, Willison and Davey concluded that a face centered cubic lattice is the only lattice possible for the inclusion. While it is true that the tetragonal lattice of Form III requires different spacings for these two views, Willison and Davey do not provide enough information about calibration of their magnifications and preservations of their hexagonal specimens for one to be confident that the Form III lattice is incompatible with the inclusions. This is especially true in that the tetragonal lattice of Form III accounts for the observed inequality of spacings in the square views of the sectioned and freeze-etched specimens, which the face centered cubic lattice cannot explain.

In short, the Form III structure accounts for many, but not all reported aspects of inclusion crystallites. The discrepancies may arise from artifacts of electron microscopy of the inclusions, or from the fact that the inclusion crystals resemble Form III but are actually distinct from it.

Packing in Form III. There are two possible packings of

enzyme molecules in the unit cell, and one in favored marginally by our x-ray data. In space group P4₂, 2 with two carboxylase molecules per unit cell, the molecules must be centered at positions of 222 symmetry, at 0,0,0, and 1/2, 1/2, 1/2. This determines the positions of the molecules, but two orientations are possible, depending on which of the two molecular 2-fold axes is parallel to the unit cell edge. The two packings are shown in Fig. 3, in which molecules are depicted by a model proposed in another paper (13). The same two packings are possible in the low resolution space group I4₂, 2; the only difference is that in I4₂, 2, each molecule would contain a 4-fold axis of symmetry.

Evidence favoring the packing of Fig. 3a and b over that of Fig. 3c and d can be found in the intensities of low order x-ray reflections. The distribution of intensities on the (*h*, *k*, *h* + *k*) zone (Fig. 4a) is pseudo-hexagonal at low resolution. We would expect such a distribution only if the molecule itself has a pseudo-3- or 6-fold mass distribution at low resolution when projected along the 111 direction. A simple model (Fig. 4b) places the eight large subunits (about 80% of the total mass) in a cubic arrangement. This octomer appears pseudo-hexagonal when viewed in projection along the cube body diagonal. Note that this occurs only if the two layers of four large subunits are eclipsed or nearly eclipsed as in the "cubic" octomer. Notice also that the 400 reflection (Fig. 2a) is weak or absent, as are most *h*,0,0, reflections (other than 2,0,0; 12,0,0; and 14,0,0). This indicates that the mass distribution is nearly continuous for the projections of the unit cell contents on to the *a* or *b* crystal axes. Yet it can be seen by viewing Fig. 3c and d, at glancing angle, that this packing should result in a strong 4,0,0 reflection (provided that the gross features of the mass distribution of our model are correct). We conclude that the packing of Fig. 3a and b is somewhat more likely than that of Fig. 3c and d. The actual

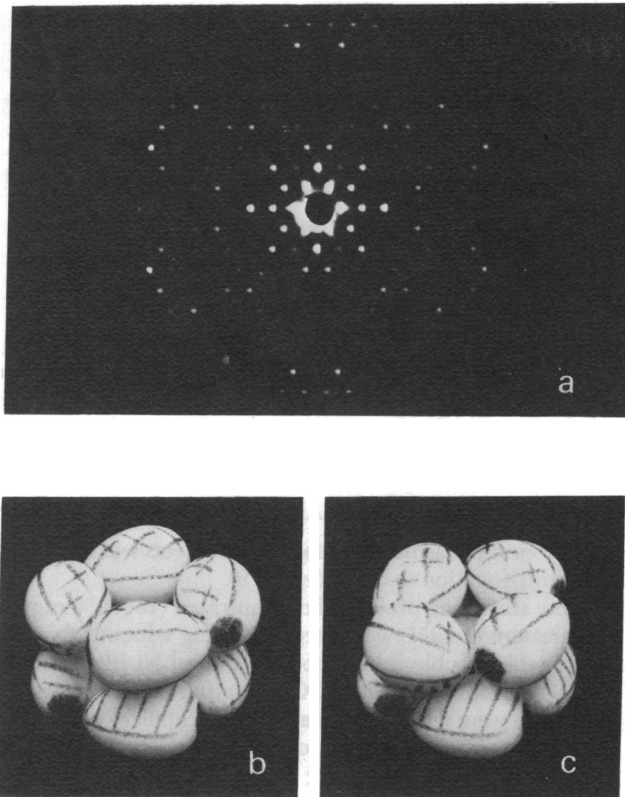


FIG. 4. (a) Low resolution (13 Å) precession photo ($\bar{\mu} = 3.5^\circ$) of the $h, k, h + k$ reciprocal lattice zone, showing a pseudo-6-fold intensity distribution. Because the motif in the crystal is one molecule (at low resolution in space group I422), the diffraction pattern is simply the molecular transform sampled by the reciprocal unit cell lattice. The pseudo-6-fold character of the $h, k, h + k$ zone implies, therefore, that molecule has a 6-fold character when its mass is projected along the 111 crystal direction. (b) Eclipsed octomer ("cubic") model of ribulosebiphosphate carboxylase large subunits viewed in the [111] direction of the packing arrangement of Fig. 3a and b. (c) Staggered octomer model viewed as in (b).

arrangement should emerge from a low-resolution electron density map.

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