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Ribonuclease II. Accuracy of Measurement and Shrinkage*

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A new type of shrinkage phenomenon is reported for crystals of the protein ribonuclease. It is shown that in some cases this produces appreciable alterations in the intensities of the X-rays diffracted by the crystal. The accuracy of the measurement of the X-ray intensities is also reported.

Introduction and outline of paper

This paper has two objects: first, to present some preliminary experimental studies on the accuracy of the measurement of the X-ray intensities of protein crystals; second, to report a new type of 'shrinkage' phenomenon for a protein crystal. The work has been carried out entirely on crystals of ribonuclease, and mainly on the monoclinic form ($P2_1$) known as ribonuclease II. However, it is probable that the results are broadly applicable to other forms of ribonuclease and to other protein crystals.

These studies have been made because of the great importance of the isomorphous-replacement method for protein crystals (Green, Ingram & Perutz, 1954). Since there are many atoms in a protein molecule, a rather heavy atom is required for a useful isomorphous replacement. It is natural to ask how heavy this atom must be for a given protein. In answering this and similar questions it is important to know the accuracy of the measurements. Hence the present work. Further papers, dealing with the theory of the method are in preparation.

The sources of inaccuracy in isomorphous replacement may be classed as follows:

(1) Errors due to technical limitations, e.g. variation of X-ray intensity of the source, absorption errors, setting errors, errors in measuring intensities, and so forth.
(2) Changes due to lack of strict isomorphism, apparently due to variation in the composition of the solvent and to the added molecules.
(3) Errors due to slight changes in the vapor pressure surrounding the crystal. This produces the new shrinkage phenomenon already mentioned.

We can summarize our results by saying that the errors due to (3) can under certain circumstances be considerable, but can be eliminated; that errors due to (1) are usually very small; but those due to (2) appear difficult to avoid in the case of ribonuclease II, and will probably be the limiting factor in a structure determination by isomorphous replacement.

The shrinkage phenomenon can be described briefly as follows: It has been shown for monoclinic ($C2$) horse haemoglobin (Perutz, 1946, Huxley & Kendrew, 1953) that the shrinkage of the crystals occurs in a number of discrete steps as the humidity of the surrounding air is lowered. This also seems to be the case for ribonuclease II.

Typically, in shrinkage of this type, the cell dimensions which change do so by several Ångströms. We have discovered that, in addition to discontinuous shrinkage, we can produce very small and apparently continuous changes of the cell dimensions up to, say, $\frac{1}{2}$ Å, and that *the intensities of the reflections also change*. Moreover, if the crystals have been mounted rather 'dry'—that is, with only a small amount of excess mother liquor in the mounting capillary—these changes occur spontaneously. We believe that they are due to small temperature gradients over the capillary, which induce local variations of vapor pressure inside it. The changes are more or less reversible and can be repeated a number of times.

We report the magnitude of the changes in cell dimensions, in the width of the reflections and in the $h0l$ intensities. We also show that a crystal mounted very wet displays little fluctuation (though the position of the crystal may shift in a troublesome manner) and that, for all crystals, fluctuations can be reduced by playing large jets of air, at room temperature, on to the capillary in order to reduce the temperature gradients.

Technique

(*a*) *Mounting*

The crystals of bovine ribonuclease were grown from mother liquor containing water and alcohol (or a similar substance), usually in about equal quantities. The methods used to grow the crystals will not be described here, as they form the subject of a separate paper (King, Magdoff, Adelman & Harker, 1955). Thus, in the case of ribonuclease, we have a solvent

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with two components, both of which are, in most cases, rather volatile.

The mounting of the crystals has also been described elsewhere (King, 1954), but a brief account is necessary here in order to bring out certain points required for this paper. The capillaries, of very thin-walled glass, are coated inside with a hydrophobic layer. The crystal clings to the wall, being in contact with a very small amount of mother liquor. A small piece of filter paper is included at the base of the capillary, and this is moistened with a drop of mother liquor. The reasons for this technique are as follows: The hydrophobic layer reduces any effects due to the alkalinity of the glass, and also makes for a cleaner mount, since the mother liquor tends to run off it. The small total amount of liquid in the capillary means that very few drops of liquid form on the walls (where they might produce X-ray absorption effects) and that the crystal, having little liquid in contact with it, shows hardly any tendency to change position as it does for a wetter mount. The disadvantage of the method, as we shall see, is that unless special precautions are taken, the amount of liquid in the capillary is not enough to buffer transient and local temperature gradients near the crystal.

(b) X-rays

The X-ray intensities were measured using Cu $K\alpha$ radiation and a General Electric Geiger-counter spectrometer specially modified for single-crystal work by the construction of an Eulerian cradle for mounting the specimen. This instrument has been described elsewhere (Furnas & Harker, 1955) and, again, only the pertinent features will be mentioned here. The instrument can be used under two extreme conditions of collimation: fine and coarse. The former is used for measuring cell dimensions, the latter for measuring intensities. Under coarse conditions the widths of the slits are arranged so that the integrated intensity can be measured with the crystal stationary, provided that its size is not greater than $\frac{1}{2}$ mm. Counts are taken for a constant period of 10 sec. Reflections giving counts over 10,000 are reduced by the addition of nickel filters in steps of factors of about 3. The smoothed value of the counting rate is simultaneously recorded by a pen recorder, so that changes of intensity, on a logarithmic scale, can be followed visually.

The settings of the instrument necessary to bring a given reflection into the Geiger counter are calculated from the measured cell dimensions on IBM machines (Magdoff, unpublished). Under ideal conditions, it is only necessary, having once set the crystal, to adjust the angle control dials to the calculated readings and to count for 10 sec. However, in these experiments we have found it necessary to check the main settings every five readings or so, and to apply a running correction to the setting figures whenever necessary. We believe that in this way we have eliminated most of the errors due to mis-setting.

From the figure for the number of counts for any given reflection is subtracted the count due to the background. This is taken at some point in reciprocal space a short distance from the position of the reflection. We have not completely systematized this procedure for these preliminary studies. For very accurate work the effects of the β component and of the white radiation streaks (from the stronger reflections further out in reciprocal space) need to be watched.

Almost all our studies have been made on the $h0l$ reflections of ribonuclease II ($P2_1$). The crystal is customarily mounted with the b axis roughly parallel to the axis of the capillary. The b axis is then adjusted by X-rays to be accurately vertical, and the intensities are measured by rotating the crystal about the b axis, with the X-ray source, the crystal and the Geiger counter in the same horizontal plane. Thus, in measuring the complete set of $h0l$'s, the X-rays pass through the walls of the capillary in very different ways and the correction due to absorption by the wall varies considerably with the angular setting. The (approximate) value of this correction can be obtained by the following method (T. C. Furnas, unpublished). The b axis (and therefore the capillary axis) is adjusted by means of the Eulerian cradle to be *horizontal*, and the intensity of an $0k0$ reflection (usually 020) is measured as the capillary is rotated about this horizontal axis. The paths of the X-rays can thus be made very similar to, though not quite identical with, the paths at the various angular settings used while measuring the $h0l$'s. From the variations in the intensity of the chosen $0k0$ reflection, the *relative* absorption factor is thus found. This factor, in the best cases, varies only from 1·0 to 1·4, but a variation of 1·0 to 2·0 is not uncommon. It is therefore important not to omit it.

Reproducibility of intensities

We report first the comparison between sets of $h0l$ intensities for two crystals which we should expect to be identical. They were both grown using a solvent containing 50% by volume of tertiary butyl alcohol. Their volumes were in the ratio of about 1 to 2, and their capillary absorption corrections were 1·0–1·5 and 1·0–2·1 respectively. Their cell dimensions were the same within the experimental error.

The measured relative intensities were put on the same basis by adjusting the scale factor so that the sum of all the intensities measured ($h0l$'s out to $2\theta = 30°$) was the same in each case. In addition, the intensities were summed in groups, each having a small range of $1/d$, and the ratio of these sums for the two sets was plotted against $1/d$ (Fig. 1(*a*)). The fact that these ratios show no systematic deviation from unity means that the 'temperature factors' for the two crystals were essentially the same.

We next obtained a measure of the percentage difference in the intensities as follows: Let I be the value of the intensity of a given $h0l$ reflection for the

first crystal, and I' that for the second. We calculated for each group of intensities, over a small range of $1/d$, the value of $\Delta = \Sigma|I-I'| \div \Sigma I$. This is seen plotted against $1/d$ in Fig. 1(*b*) curve *A*. This curve, and

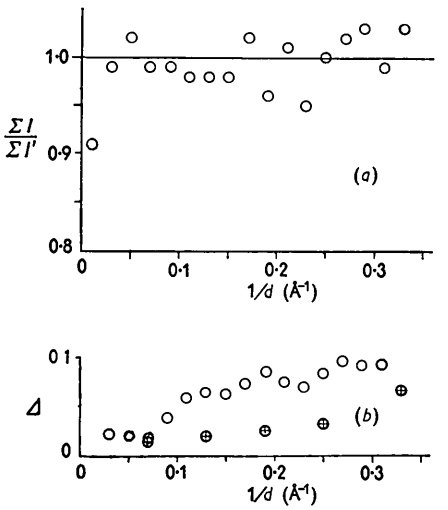

Fig. 1. (*a*) Plot of $\Sigma I/\Sigma I'$ against $1/d$ for a pair of ribonuclease crystals grown from aqueous tertiary butyl alcohol.

(*b*) ○: Curve *A*, plot of $\Delta = \Sigma|I-I'| \div \Sigma I$ against $1/d$. ⊕: Curve *B*, plot of calculated Δ for statistical counting errors only.

Both are for a pair of ribonuclease crystals grown from aqueous tertiary butyl alcohol.

similar curves of Δ against $1/d$ shown in Figs. 2 and 3, have been calculated by grouping the intensities into batches, each covering an interval of 0·01 Å^{-1} in $1/d$, and summing in turn over each batch plus the two adjacent batches, the latter being given half weight.

It can be seen that at low $1/d$ the agreement is excellent—within a few percent—and that it gradually gets worse, rising to an average deviation of about 10% around $1/d = 0{\cdot}3$ Å^{-1}. To estimate how much of this was due to statistical counting errors we took the figures for the counts of the smaller of the two crystals, and calculating an error* of $\sqrt{(4C/\pi)}$ (where C is the actual count recorded) for both the intensity count and the background count, we computed for each reflection of the group the average error due to these two errors combined at random. The total error, expressed again as $\Sigma|\Delta I| \div \Sigma I$, is plotted for a few selected groups in Fig. 1(*b*), curve *B*. (The true statistical error may be a little less than this curve, but it is unlikely to be greater.)

The general shape of this curve can be understood as follows: at low $1/d$ the errors would tend towards zero, except for the fact that nickel filters were used to keep the counts below 10,000. This makes the curve tend to a fixed value of a little over 1%. At intermediate $1/d$ the curve increases slowly as both the

* Statistical theory shows that the r.m.s. error expected in this case is $\sqrt{(2C)}$, and the mean of the modulus of the error is $\sqrt{(2/\pi)}$ times this.

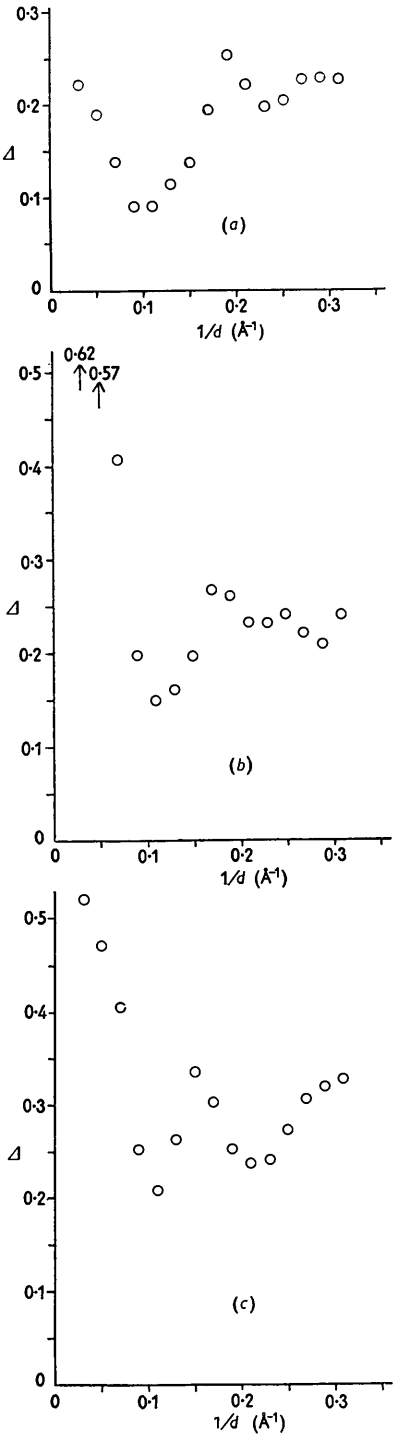

Fig. 2. (*a*) Plot of $\Delta = \Sigma|I-I'| \div \Sigma I$ against $1/d$ for two ribonuclease crystals, one grown from aqueous tertiary butyl alcohol and the other from aqueous ethyl alcohol.

(*b*) Plot of $\Delta = \Sigma|I-I'| \div \Sigma I$ against $1/d$ for two ribonuclease crystals, one grown from aqueous tertiary butyl alcohol and the other from aqueous monoacetin.

(*c*) Plot of $\Delta = \Sigma|I-I'| \div \Sigma I$ against $1/d$ for two ribonuclease crystals, both grown from aqueous tertiary butyl alcohol, but one containing the dye brom-phenol blue.

Lorentz factor and the 'temperature factor' reduce the number of counts. At higher values of $1/d$ the error increases rapidly owing to the fact that the average intensity is not far above the background. Notice that the statistical error depends on the volume (V) of the crystal, being roughly proportional (except at very low $1/d$) to $1/\sqrt{V}$.

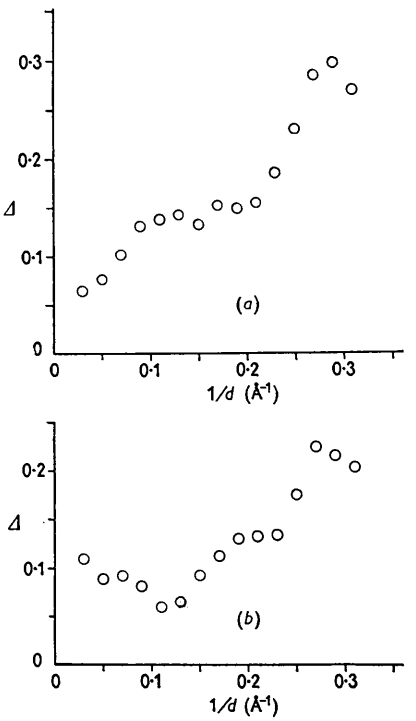

Fig. 3. (*a*) Plot of $\Delta = \Sigma|I-I'| \div \Sigma I$ against $1/d$ for a single ribonuclease crystal grown from aqueous ethyl alcohol, with the crystal in the 'wet' and in the 'damp' conditions. (*b*) Plot of $\Delta = \Sigma|I-I'| \div \Sigma I$ against $1/d$ for two ribonuclease crystals grown from aqueous tertiary butyl alcohol, one set of intensities measured under controlled conditions, and the other when the crystal was allowed to fluctuate.

It can be seen that the whole of the error is not due to the statistical effect. The additional inaccuracy may be due to slight differences in the two crystals, or possibly in part to inaccuracies in the absorption correction. Since it is small, we have postponed further investigation of it for the moment.

These results show (1) that up to $1/d = 0{\cdot}3$ Å^{-1} one can achieve a mean error of 10%, and rather less for low values of $1/d$; (2) that this error is not wholly due to the statistical counting error.

Changes due to the solvent

The fact that the nature of the solvent produces very small changes in cell dimensions for ribonuclease II is being reported elsewhere (King *et al.*, 1955). Cell dimensions for a number of cells are given in Table 1.

As a rough estimate the r.m.s. error for the wet crystals expressed in angular measure is about 0·02° at a 2θ of 18°, or about 0·1% in cell dimensions, so that these differences are certainly real.

Table 1. *Cell dimensions of ribonuclease*

Solvent % by volume	a_0 (Å)	b_0 (Å)	c_0 (Å)	β (°)
50 % tertiary butyl alcohol	30·28	38·39	53·16	105·83
55 % ethyl alcohol	30·49	38·32	53·30	105·94
65 % monoacetin	30·24	38·37	52·99	105·87
50 % tertiary butyl alcohol plus brom-phenol blue	30·49	38·37	53·22	106·08
Shrinkage stage	30·4	33·4	53·0	113

Associated with these changes in cell dimensions there are changes in the intensities of the reflections. For convenience we have again computed, for the *h*0*l* reflections, $\Delta = \Sigma|\Delta I| \div \Sigma I$ as a measure of the percentage error, grouping together intensities with similar values of $1/d$. In all cases we have found that the temperature factors do not differ significantly.

Smoothed values of Δ plotted against $1/d$ are shown in Fig. 2. Examine first Fig. 2(*a*), showing the difference between the tertiary butyl alcohol case and the ethyl alcohol case. Notice that (except near the origin) the value of Δ increases roughly linearly with $1/d$, as might be expected if there were minor shifts of the molecules between one case and the other.

Next compare tertiary butyl alcohol with monoacetin, shown in Fig. 2(*b*). Here the biggest values of Δ occur at very low values of $1/d$. This is due to the difference in the electron densities of the two solvents, that of 50% tertiary butyl alcohol being 0·303 and that of 65% monoacetin as high as 0·373 e.Å^{-3}. Examination of the low orders shows that in all cases the monoacetin reduces the intensity. (There is even a small solvent effect between the tertiary butyl and the ethyl alcohol crystal. Density measurements (King *et al.*, 1955) show that the former is slightly heavier, and the low orders of the ethyl alcohol are on the average slightly larger than those of the tertiary butyl. This explains why Fig. 2(*a*) does not tend to zero for small $1/d$.)

In Fig. 2(*c*) is shown the effect of adding a dye molecule. As can be seen from Table 1, this has altered the cell dimensions slightly, and it is not clear how much of Δ is due to this and how much to the heavy atoms in the dye. This problem is examined further in the theoretical paper.

We see, therefore, that *precise* isomorphous replacement may not be easy to achieve in ribonuclease II, because the changes of intensity produced by very slight shifts in the molecule may confuse the changes produced by the heavy atom. Instead of errors giving a value of Δ of 10% or less, we are more likely to have some 20 or 30% as background error.

Changes due to 'shrinkage'

(*a*) *Exploratory work*

We report first some qualitative experiments which show the general nature of the effect. For this purpose

we studied the counts from two *adjacent* reflections, $80\bar{9}$ and $8,0,\bar{1}0$. It happens that one of these ($8,0,\bar{1}0$) varies considerably, while the other varies very little, or, if it does, in the opposite direction, going up while the first one goes down. Thus, by comparing two such reflections, many possible explanations, such as a change of intensity of the X-ray source, of mosaic structure, or of local absorption effects, are eliminated.

A small jet of cool air, some 5° C. below room temperature, was played on the top of the capillary, far from the crystal. After a short time, if the crystal had been mounted fairly dry, the intensities of some of the reflections would start to change. Typical figures for counts in ten seconds were

	$80\bar{9}$	$8,0,\bar{1}0$
Initially	4300	3700
With cold jet	5300	1600

If the jet were now turned off the intensities would return to their original values, or ones close to them, with a time-constant of, say, 2 min. to go two-thirds of the way. If the jet were now turned on again the behavior could be repeated. Measurement of the cell dimensions showed that the cold jet caused them to *decrease* slightly. Because of this, care was always taken to set accurately on the reflection being measured on each occasion.

A small jet of air played through the collimator on to the capillary in the neighborhood of the crystal could produce similar effects, although its temperature was estimated to differ from room temperature by not more than 1° C. The direction of the effect depended on the position of the crystal in the capillary with respect to the jet. This could be studied by measuring $\bar{8}09$ and $\bar{8},0,10$ instead of $80\bar{9}$ and $8,0,\bar{1}0$.

For one crystal, it was found that under favorable circumstances the intensity of $8,0,\bar{1}0$ could be reduced by a factor of 2 by the heat of a hand held for a minute or so 4 or 5 in. from the capillary on the side nearest the crystal. The effect was repeated a number of times.

The effects can also be produced by the general conditions of the X-ray room. (This is fed by an air-conditioning plant which keeps the temperature not far from 25° C. Owing to this, and to the fans of the cooling systems of the X-ray units, there is a considerable movement of air in the room.) These effects were in fact first discovered by noting the spontaneous variations of $8,0,\bar{1}0$. Typical figures taken over the course of a day were 2300, 3600, 3500, 2900, 3600, 2700 counts. This shows that for a crystal mounted rather dry, as this one was, the spontaneous variations of intensity are large enough to be important.

The effect has been demonstrated qualitatively for *all* crystals of ribonuclease II which have been examined for it, including those having been crystallized from different solvents. All crystals for which results are quoted in this paper show the effect.

To show that the variations are not merely due to temperature, we took measurements on a small group of reflections at a temperature of about 14° C. instead of the usual 25° C. This was done by playing two large fast jets of 14° C. air onto the capillary. The results, together with those for the same crystal in a slightly shrunk state ('damp', Table 3) are shown in Table 2.

Table 2

	$\bar{8}06$	$\bar{8}07$	$\bar{8}08$	$\bar{8}09$	$\bar{8},0,10$
Normal state, 25° C.	720	2850	1000	3680	3000
Normal state, 14° C.	1250	2790	840	3980	2250
Slightly shrunk state	1870	2620	500	3860	800

The slightly shrunk state was in this case produced by a jet, through the collimator, whose temperature differed from room temperature by about 1° C. Some of the variations at 14° C. were probably due to slight temperature gradients caused by the large jets whose temperature differed considerably from room temperature, since the results varied a little with the aspect of the crystal. In any case the differences in intensity produced by an 11° C. change in temperature were smaller than those produced by small temperature *gradients*.

We have found that crystals mounted very wet show little if any spontaneous variation, and that it is much harder to produce changes in them by the manipulation of local jets of air. It is often possible to observe visually the movement of drops of solvent from one part of the capillary wall to another due to the local jets.

Taking all these facts into account we believe that the phenomenon is due to changes in vapor pressure over the crystal due to temperature gradients over the capillary. A local cold patch at one end of the capillary will condense solvent and lower the vapor pressure over the crystal, while the latter remains at the original temperature. Thus solvent will distil out of the crystal, the crystal will contract slightly, the protein molecules shift, and the intensities change. If the cold jet is now turned off, uniformity of temperature is restored, the crystal takes up solvent and returns to its original condition.

(*b*) *Quantitative work*

We now report a few more quantitative studies. We attempted to keep a crystal in a fixed condition by playing a very fine cold jet on to the top of the capillary and adjusting its rate of flow so that the $8,0,\bar{1}0$ intensity was kept at roughly half its normal intensity. We shall call the crystal in this condition 'damp' to distinguish it from the normal 'wet' condition. The $h0l$ intensities (out to $2\theta = 30°$) were collected for both the wet and the damp stages of the same crystal, which was of the ethyl alcohol type. The values are reported in Table 3. To enable the general character of these results to be grasped more easily we have evaluated, as before, $\Delta = \Sigma|\Delta I| \div \Sigma I$, and the smoothed values of this are plotted against $1/d$ in Fig. 3(*a*). As might be expected it shows a roughly linear in-

Table 3

	W*	D*	W	D	W	D	W	D	W	D	W	D	W	D	W	D	W	D
l / $h\rightarrow$	$\bar{1}\bar{0}$	$\bar{1}\bar{0}$	$\bar{9}$	$\bar{9}$	$\bar{8}$	$\bar{8}$	$\bar{7}$	$\bar{7}$	$\bar{6}$	$\bar{6}$	$\bar{5}$	$\bar{5}$	$\bar{4}$	$\bar{4}$	$\bar{3}$	$\bar{3}$	$\bar{2}$	$\bar{2}$	$\bar{1}$	$\bar{1}$
0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1	—	—	4	3	52	93	1	2	3	3	23	19	91	111	210	180	178	143	170	180
2	31	30	57	41	181	203	72	82	144	172	22	12	36	47	30	37	6	10	268	265
3	7	14	12	41	309	279	28	46	19	7	25	15	67	54	168	212	124	129	—	—
4	50	30	44	38	100	59	6	4	1	—	81	78	15	17	767	691	13	16	91	94
5	53	74	8	13	18	5	30	12	120	87	1	—	101	120	46	41	221	242	—	—
6	13	3	82	50	7	47	357	408	11	13	52	58	17	12	21	16	15	12	73	79
7	46	41	2	6	118	139	87	43	84	83	10	14	26	33	9	11	62	73	5	4
8	—	—	2	—	34	21	146	164	51	64	76	84	67	61	7	8	178	167	12	9
9	—	—	51	67	158	211	13	1	229	210	38	26	141	165	6	9	63	64	14	15
10	—	—	5	1	208	120	14	3	36	36	80	102	9	4	38	47	4	4	11	7
11	—	—	—	4	5	11	—	1	21	26	28	23	29	—	204	202	120	128	85	85
12	—	—	11	3	26	40	36	28	24	25	43	47	89	100	94	89	151	148	401	384
13	—	—	—	—	3	2	59	83	34	34	93	107	22	21	231	264	—	—	26	25
14	—	—	—	—	1	—	15	4	206	139	28	35	154	170	6	4	1	—	86	84
15	—	—	—	—	—	—	—	—	71	60	—	—	69	107	30	30	21	26	3	2
16	—	—	—	—	—	—	—	—	6	9	114	134	14	4	221	229	20	18	—	—
17	—	—	—	—	—	—	—	—	—	—	17	12	33	28	21	26	25	28	22	27

Table 3 (*cont.*)

	W	D	W	D	W	D	W	D	W	D	W	D	W	D	W	D	W	D	W	D
l / $h\rightarrow$	0	0	1	1	2	2	3	3	4	4	5	5	6	6	7	7	8	8	9	9
0	—	—	241	219	208	216	2	4	42	46	1	1	213	249	39	41	2	5	7	3
1	43	43	184	163	63	66	2	2	155	152	2	—	133	138	14	12	9	4	1	1
2	57	54	44	38	11	14	86	98	4	1	71	86	330	263	1	1	152	113	1	4
3	218	229	102	82	—	1	65	53	44	44	43	61	294	392	1	4	37	77	36	32
4	47	39	2	2	18	24	1	1	2	2	—	—	57	27	157	71	72	75	—	—
5	12	13	3	—	150	133	9	7	12	19	252	234	53	33	137	183	20	6	—	—
6	6	7	43	29	44	52	20	16	16	24	—	—	195	280	4	3	25	24	—	—
7	39	32	98	82	3	1	4	7	—	2	37	21	38	10	142	124	—	—	—	—
8	11	11	6	5	11	12	5	2	—	—	60	63	—	2	—	3	—	—	—	—
9	22	26	36	27	13	18	—	—	1	1	19	30	16	14	8	2	—	—	—	—
10	11	10	57	43	146	144	48	60	48	63	11	4	—	—	—	—	—	—	—	—
11	179	180	87	210	7	8	42	30	17	31	1	2	—	—	—	—	—	—	—	—
12	2	3	8	7	63	65	109	109	13	—	1	7	—	—	—	—	—	—	—	—
13	3	2	5	13	39	52	177	153	52	28	—	—	—	—	—	—	—	—	—	—
14	15	12	37	29	12	2	37	39	—	—	—	—	—	—	—	—	—	—	—	—
15	2	2	1	—	23	16	—	—	—	—	—	—	—	—	—	—	—	—	—	—
16	6	4	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
17	26	27	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* D: damp; W: wet.

crease of Δ with $1/d$, rising to a figure of about 30% for $1/d = \frac{1}{3}$ Å^{-1}.

The changes in cell dimensions were measured under 'fine' conditions. The b_0 dimension was not attempted, as we could not be confident that we could keep the condition of the crystal the same during the radical change of position of the capillary required for this measurement. The values for the damp crystal are a little less accurate than usual, owing to the experimental difficulties, but there is no doubt that the change of cell dimensions is real. The results are shown in Table 4.

The approximate breadth of the reflections was also investigated, under fine conditions, to see if the reflections broadened as the crystal shrank. This was found to be the case. In Table 5 we have recorded the approximate width of the diffraction peak for three states of the crystal, as well as the spacing of the $30\bar{4}$ reflection and the relative intensity of $8,0,\bar{1}\bar{0}$, to give some idea how much the crystal had shrunk in each state.

Table 4. *Cells of wet and damp crystals*

	a_0 (Å)	c_0 (Å)	β (°)
Wet crystal	30·49	53·30	105·94
Damp crystal	30·17	52·96	106·03

Table 5. *Peak widths*

State of crystal	Peak width (°)	2θ for $30\bar{4}$ (°)	Counts for $8,0,\bar{1}\bar{0}$
Normal	0·05	9·68	12,500
Slightly shrunk	0·06	9·75	8,300
Shrunk further	0·16	9·82	3,500

The breadth under our 'damp' condition was still sufficiently small to enable us to measure the integrated intensity correctly under the normal coarse conditions.

If the cold jet was adjusted so that the crystal con-

tinued to shrink, the intensities would start to fall rapidly, as the breadth of the reflections became very great, and finally the crystal would shrink to the first shrinkage stage. On turning off the cold jet it eventually returned to normal. Nevertheless, we have been cautious about overshrinking a crystal under study. The 'damp' condition chosen for detailed examination was such that the crystal had still some way to shrink before changing over to the first shrinkage stage.

We have made a preliminary examination of the values of ΔF representing the change between wet and damp, and have also calculated a difference Patterson (using ΔI), ignoring the changes in the dimensions of the cell. As might have been expected, this Patterson is rather flat in the region of the origin.

Without describing our studies in detail we may summarize our tentative conclusions: (1) The changes in intensity appear somewhat greater than would be expected from a 'breathing movement' of the crystal, i.e. one in which the molecules remain parallel and in which the fractional coordinates of their 'centers' remain unchanged. (2) There is some evidence of a very small rotation of the molecules about an axis not far in direction from the c axis of the crystal.

To complete our quantitative studies we have compared a set of data for the $h0l$'s of a tertiary butyl crystal, taken without any special precautions before this effect was discovered, with an accurate set of data. The values of $\Sigma|\Delta I| \div \Sigma I$ are plotted against $1/d$ in Fig. 3(b). It can be seen that the error is usually about twice that shown in Fig. 1(b). Some of this additional error may be due to other causes, but it seems probable that the major part was due to spontaneous variations in the crystal.

As implied earlier, we have found two methods of reducing the spontaneous variation in the crystal. The first is to mount the crystal very wet; the second to play large jets of air, at room temperature, on to the capillary. A pair of broad jets was used, mounted on either side of the capillary and having a fast flow of air, so that the capillary was in the middle of a region of turbulent mixing. The first method is unsatisfactory because many drops of water condense on the capillary wall, and more particularly because the crystal, if mounted very wet, is apt to rotate as much as 1° or 2° from time to time, which both makes for inaccuracies and maddens the experimenter. The large jets, while they do not always abolish completely the intensity variations, reduce them to a very small value even in the worst cases, and in favorable cases the intensities are very steady. A reasonable compromise would be to avoid very dry mounts for the crystal, and to use large jets as a matter of routine in all accurate work.

Discussion

The implications of these results for isomorphous replacement are discussed in the theoretical paper. We shall restrict ourselves here to a few comments and to a discussion of the shrinkage phenomenon.

The possibility of *small* changes in cell dimensions due to solvent effects and other causes underlines the importance of *accurate* measurements of cell dimensions. Although there is no guarantee that when the cell dimensions are identical to within 0·1% the position of the protein part of the crystal is also identical, it is at least probable. Consequently, we feel that in all cases of isomorphous replacement the cell dimensions should be measured to the highest possible accuracy.

It would also seem good practice to study the reproducibility of intensity measurements by the most direct method. That is, by taking measurements on two (or more) crystals which are believed to be completely identical, and carefully comparing the results. In no other way is it possible to form an adequate estimate of the reproducibility, and this is really essential for any convincing use of the isomorphous-replacement method, except perhaps when the atom is very heavy.

We have made no quantitative studies using X-ray photographs, but we think it unlikely that the normal visual method of estimation could give reproducibilities of as little as 5 or 10%, as is possible with a Geiger counter. It remains to be seen what accuracy can be achieved with a photomultiplier to measure the intensity of good X-ray photographs. While the Geiger counter has the advantage of showing temporal changes of intensity rather easily, the photographic methods give an integrated value and this may reduce the effects of fluctuations caused by a rather dry mount.

We should like to draw attention to the considerable inaccuracies which may arise from the absorption of X-rays by the walls of the capillary. This effect will always be present if a Weissenberg is used. If a precession camera is used in such a way that the crystal faces either the film or the X-ray source, the effect is very small, but if the crystal is 90° to these positions the errors will be greater. The accidental use of two capillaries of rather different thickness could greatly upset an isomorphous-replacement experiment if the correction were omitted in the cases where the camera arrangement is unfavorable.

It would be of interest to know whether the new shrinkage phenomenon is peculiar to ribonuclease II, or whether it occurs with other protein crystals. Preliminary results on ribonuclease VI ($C2$) suggest that it also occurs there, but lack of a further supply of good crystals prevented us from establishing it beyond doubt. The phenomenon is unlikely to depend upon there being *two* volatile components to the solvent, since the water–monoacetin system and the water–2,5-hexanediol system showed it, although both monoacetin and 2,5-hexanediol are comparatively non-volatile. We think it very probable that it occurs for all protein crystals.

It is possible to calculate the approximate number

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.

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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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