as a thin microscope cell. By means of a gear type pump the paraffin solution was circulated vigorously over the crystals, the direction of flow being reversed a few times. Pure liquid paraffin was then used to flush out the system, the crystals were washed with petroleum ether and dried with a current of dry air.

*Examples of growth systems on sucrose crystals*

The (100) face of a sucrose crystal grown from a pure solution is shown on photograph 3. This crystal was prepared by method 1 and shows a system of steps of varying heights, a feature often occurring on sucrose crystals.

Another example of a sucrose crystal on which is visible a growth hill having the asymmetric *D* shape characteristic on the (100) face is shown on photograph 4. The growth hill is remarkably even for such a large step height, this being 4000 Å. This crystal was grown in a solution containing 0·2% dextrose. On crystals grown in solutions of this composition, the step heights were about 10 times bigger than on crystals grown in pure solutions of sucrose.

The crystal shown on photograph 5 was isolated by method 2. This photograph also shows a (100) face, having a single spiral of step height 1700 Å on it. This crystal was removed from solution after being used for an experiment on the rate of growth.

Development of the methods of observation was assisted greatly by discussions with Mr K.W.Keohane on microscope techniques. We wish to thank the directors of Tate and Lyle Ltd. for their interest and support for the work described.

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# The Crystal Structure of Ribonuclease Comparison of Three Dimensional Patterson Vector Maps of Crystals Grown from Ethyl and Tertiary Butylalcohol Respectively

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Three-dimensional Patterson vector maps have been calculated for monoclinic crystalline ribonuclease grown from ethyl alcohol using approximately 13,000 slightly sharpened $F^2$ terms.

An analysis of the vectors within 5 Å of the origin shows that it differs structurally from haemoglobin and myoglobin.

## 1. Introduction

This paper describes briefly the main results of our analysis of the three-dimensional vector maps of crystalline monoclinic ribonuclease grown from ethyl alcohol. These findings are in broad agreement with a similar independent analysis of crystalline ribonuclease (ribonuclease II) grown from tertiary butyl alcohol already published by Magdoff, Crick & Luzzati (1956). It is now clear that the most promising way of determining the structure of crystalline proteins of low molecular weight is by the use of the isomorphous heavy-atom technique that was started by Green, Ingram & Perutz (1954) for haemoglobin and which has now been so ably exploited by Kendrew *et al.* (1957) for myoglobin, where some knowledge has been gained about the manner of folding of the polypeptide chains of this molecule. We are also undertaking work of a similar nature on the isomorphously related crystalline complex ribonuclease parachloromercuribenzoate; this was started four years ago and though actively in progress will be a long task.

In the meantime for two reasons we consider it worth while publishing our observations on the Patterson vector distributions of ribonuclease. We find that there is almost a one-to-one correspondence between

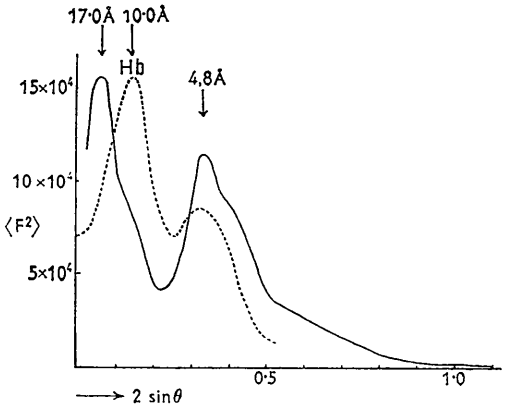


Fig. 1. The full line is a plot of the average $F^2$ for ribonuclease. The dashed curve shows the corresponding distribution of average intensities for methaemoglobin. A similar curve has been obtained for ribonuclease II by Magdoff *et al.* (1956).

our maps and those of ribonuclease II and that there is likely to be a close correspondence between the two sets of intensities for the general *hkl* reflections. The differences that are known to exist between the intensities on the one hand and the Patterson maps on the other of the respective crystals might possibly be due to the different solvents used in crystallization in the two cases—ethyl alcohol and tertiary butyl alcohol respectively. These might have given rise to crystals whose molecules have slightly different orientations with respect to each other.

Our second consideration is that we find significant differences in the vector distributions within 5 Å of the origin between ribonuclease and haemoglobin and also between ribonuclease and myoglobin. This perhaps is best revealed by the very marked absence of strong 10 Å reflections in ribonuclease in contrast to their very definite presence in the diffraction patterns of haemoglobin and myoglobin. This dissimilarity suggests that the internal structure of globular proteins might be governed more by the sequence and stereochemical arrangements of reacting side groups and need not necessarily follow the geometrical laws of the simple packing of helices as obtained in the synthetic polypeptides.

## 2. Experimental procedure and general observations on the intensities of reflections from ribonuclease

The ribonuclease crystals used were those grown by Dr M. Kunitz. The cell dimensions are:

$$a = 30{\cdot}8,\ b = 38{\cdot}8,\ c = 54{\cdot}08\ \text{Å};\ \beta = 106°.$$

There are 2 molecules per unit cell with the space group $P2_1$. Two independent sets of X-ray data have been obtained with a Unicam Weissenberg goniometer modified to take a 6·00 cm. radius film holder. One striking feature of these crystals and of those subsequently grown in our laboratory both by Mr H. Dix and Mr M. Rosemeyer is the reproducibility of the X-ray patterns. This suggests that the differences which exist between the two chemically different types of ribonuclease *A* and *B* (Martin & Porter, 1951) must be very slight and is supported by the recent findings (Tanford & Hauenstein, 1956) that this difference is due solely to the presence of an additional carboxyl group in form *B*. As ribonuclease *B* contributes some 10 to 15% of the mixture we are clearly dealing with crystals which, for practical

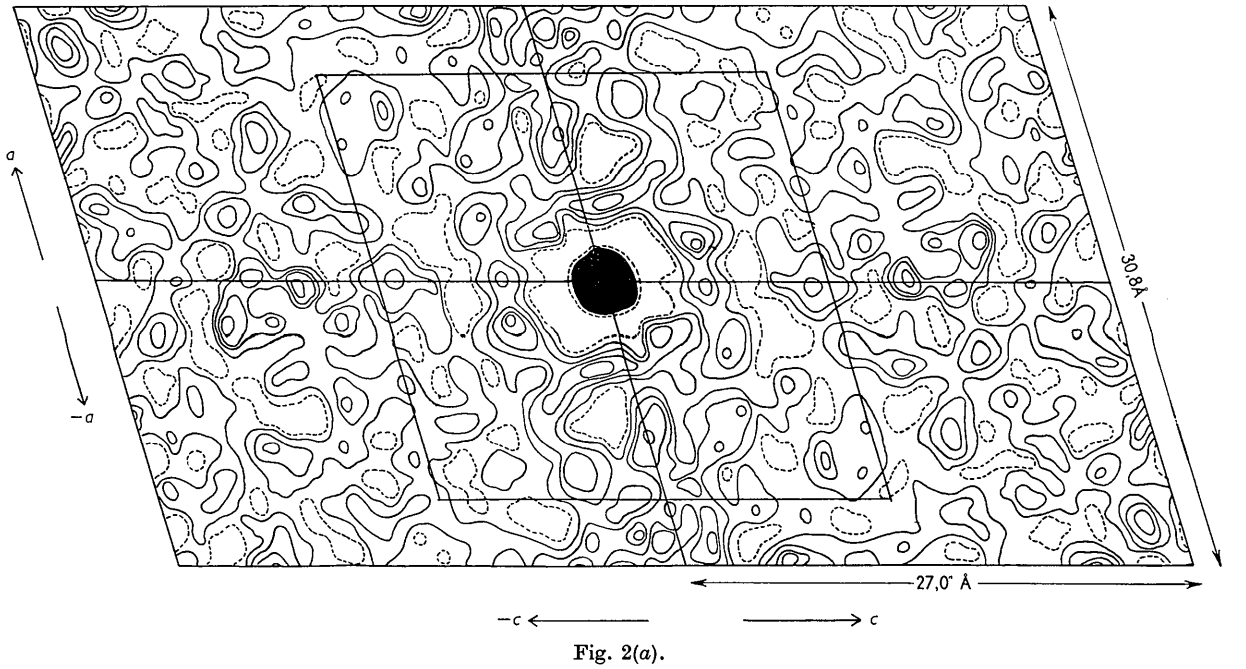


Fig. 2(*a*).

crystallographic purposes, are exceedingly homogeneous.

Crystals selected for X-ray work were approximately 0·5 mm. × 1·0 mm. × 1·0 mm. and exposure times varied from about 80 to 100 hr. for each crystal. Precaution was taken to see that crystals were wet when mounted in capillaries, and this ensured a reproducibility of all X-ray photographs. Each layer line was obtained with a fresh crystal and all intensities were visually estimated. The two sets of X-ray data so obtained showed reflections down to spacings of 1·8 and 1·5 Å respectively. In the second case there are reflections with smaller spacings down to about 1·0 Å and less in the direction of the *c* axis but these were too weak to be estimated. A check between the intensities of the two sets of reflections showed that they were similar, and the second set was put on to an approximately absolute scale by Dr A. Klug using a modification of Wilson's statistical method (1942).

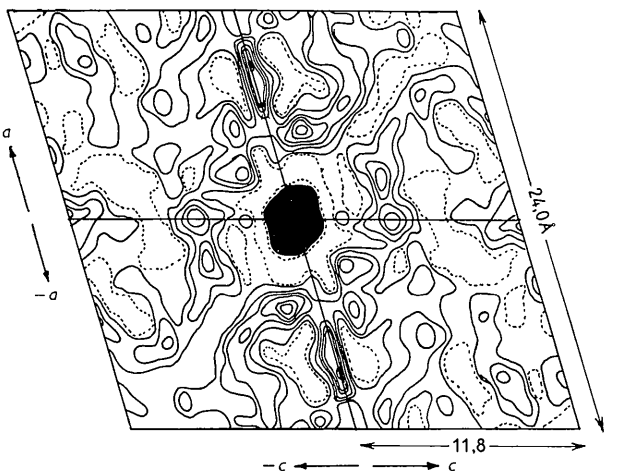


Fig. 2(*b*).

It was interesting to observe that the Geiger-counter measured intensities for the *h*0*l* reflections of ribonuclease II (Magdoff & Crick, 1955) when scaled and compared with the corresponding visually estimated intensities showed a disagreement of 10% estimated as a reliability index between the two sets of observations. When it is recalled that the respective observations come from crystals grown from different sources at different centres using different solvents, and under different degrees of wetness, it is remarkable that there is almost a one-to-one relationship between the two sets of intensities. However, although the unit cells of the two crystals are very nearly the same, there are differences of intensities in certain reflections which are outside experimental error, as for instance 200 and 1,0,11. It is quite reasonable to assume on the basis of this comparison that similar differences of intensity would have been noticed had the comparison been extended to the general *hkl* reflections. The physical difference between these crystals grown from different solvents appears to be small but real, possibly because the transforms of the molecules in the two

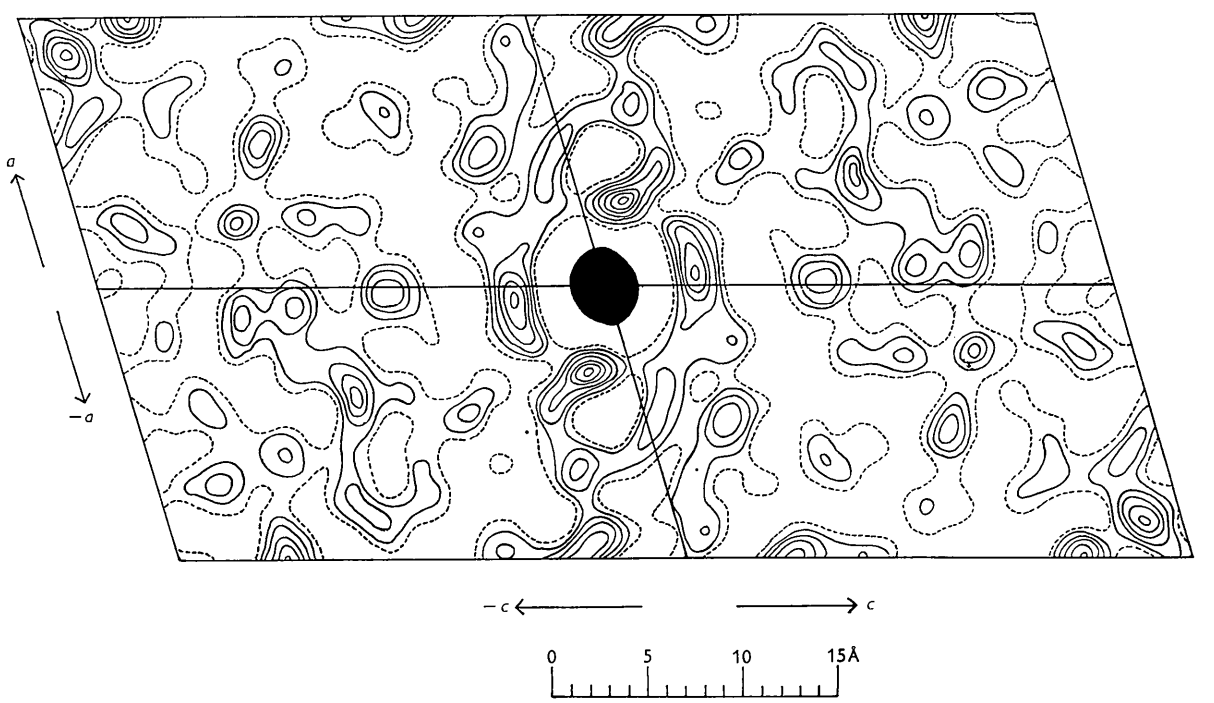


Fig. 2(*c*).

Fig. 2. (*a*) Bernal & Carlisle 1st set of X-ray data. (*b*) Bernal & Carlisle 2nd set of X-ray data. (*c*) Magdoff, Crick & Luzzati, 1956.

cases are being sampled at slightly different points in reciprocal space.

Fig. 1 is a plot of the average $F^2$ against 2 sin $\theta$ for Cu $K$ radiation for our second set of intensities; it shows two main peaks at about 17 Å and 4·8 Å respectively. A noticeable feature of this curve is the lack of a 10 Å peak, so clearly demonstrated by the corresponding dashed curve shown for haemoglobin. The rather weak bulge, indicated by the arrow marked Hb, shows the 10 Å region for ribonuclease indicating that the internal structures of these two proteins are not the same. The same argument can be applied to myoglobin (Kendrew *et al.*, 1950, 1956, 1957) which is also known to show strong reflections in the 10 Å region. This lack of predominating strong reflections in this region for ribonuclease has been confirmed by Arndt & Riley (1955) from their investigation of the X-ray scattering effects of a number of dried amorphous globular proteins, all of which except ribonuclease showed strong scattering in the 10 Å region. The complementary picture supporting this view is seen in the difference between the 5 Å vector distributions about the origins in the three-dimensional Patterson maps of haemoglobin, myoglobin, and ribonuclease; this is discussed in more detail later (Section 3).

As these crystals have been grown from aqueous alcohol we have not been able to ascertain the shape of the ribonuclease molecule by varying the solvent concentration as Bragg & Perutz (1952) have done in their determination of the shape of the haemoglobin molecule in crystals grown from ammonium sulphate solution. It would appear that for ribonuclease crystals grown from aqueous alcohol the shape of the molecule can only be determined when its detailed structure is known.

## 3. Computation of the three-dimensional Patterson vector maps and comparison with corresponding maps of ribonuclease II

Three-dimensional Patterson sections parallel to (010), at intervals of $\frac{1}{60}\times(a, b$ and $c)$, were calculated with the first set of X-ray data using some 5000 reflections. The peak positions seen on these sections are almost identical with peak positions on the three-dimensional maps calculated from our second set of more complete data. Fig. 2(*a*) shows the section at $V = 0$ employing the first set of intensities.

The Patterson sections calculated from the second set of X-ray data used about 13,000 reflections. Here again sections were calculated parallel to (010) but at intervals of $\frac{1}{64}\times(a, b$ and $c)$. The terms in this Fourier series were slightly sharpened by dividing them through by the $f$ values for carbon taken from the second volume of the *International Tables for Crystallography* (Gebrüder Borntraeger, Berlin, 1935). We limited the calculations in this case to a region of 12 Å from the origin. This restricted region is outlined by the small rectangle on Fig. 2(*a*), and Fig. 2(*b*) shows the Patterson section at $V = 0$ obtained from this second set of data. The aim here was to carry out a more detailed analysis of this region particularly with respect to the distribution of vectors within 4–5 Å of the origin, in order that some light may be thrown on the nature of the molecule (see Section 4).

In the comparison of our Patterson maps with those of ribonuclease II, two main points are to be considered: in the first place reflections used in the calculation of the Patterson Fourier series for ribonuclease II extend only to spacings of about 2·5 Å whereas our Patterson maps contain data extending at least to 1·5 Å. In the second place a smoothing function was used on the X-ray data of ribonuclease II in order to avoid diffraction effects due to cut off in the X-ray data whereas our Patterson Fourier series has its terms slightly sharpened in order that more detail be revealed in the 5 Å region. Apart from these differences we find that the distribution of the larger peak positions is almost identical in the two sets of maps but as would be expected they do not agree in peak height. Fig. 1(*c*) shows the section of ribonuclease II at $V = 0$. Much of the bareness so characteristic of the ribonuclease II map is relieved by the presence of more peaks in ours due to the use of more terms. It is difficult in such a case to do more than point to the general similarity of Figs. 2(*a*) and 2(*c*).

One striking difference between them, however, is seen in the section $V = 0$ where at about 9 Å along the $a$ axis in ours there is a heavy peak which is not so well indicated on the corresponding section for ribonuclease II. The sharpness of this peak, Fig. 2(*b*), which we thought at one time was due in part to errors in our work, is also seen on the corresponding Patterson section obtained from our first set of X-ray data, Fig. 2(*a*). We have subsequently confirmed the genuineness of this peak by calculation on a different mesh and its physical reality is further attested by the strength of the planes $h = 3, 6, 9$ and 10 easily seen on an $a$ axis rotation photograph. These planes indicate a prevalence of vectors of about 9 Å in this direction.

## 4. The 5 Å shell

As the Patterson sections parallel to (010) are similar to those already published for ribonuclease II (Magdoff, Crick & Luzzati, 1956) we have refrained from showing the three-dimensional data in this form, and concentrated our arguments instead on the prominent feature of the 5 Å shell shown as a stereographic projection on (010), Fig. 3(*a*). For comparison we have also shown the stereograms of ribonuclease II, haemoglobin and myoglobin, Figs. 3(*b*), (*c*), (*d*) respectively. The first two are identical, taking into account the greater resolution of 3(*a*) due to the inclusion of planes of large unitary structure factors with spacings less than 3·0 Å. Figs. 3(*c*), (*d*) show

substantial differences no matter in what way the projections are oriented.

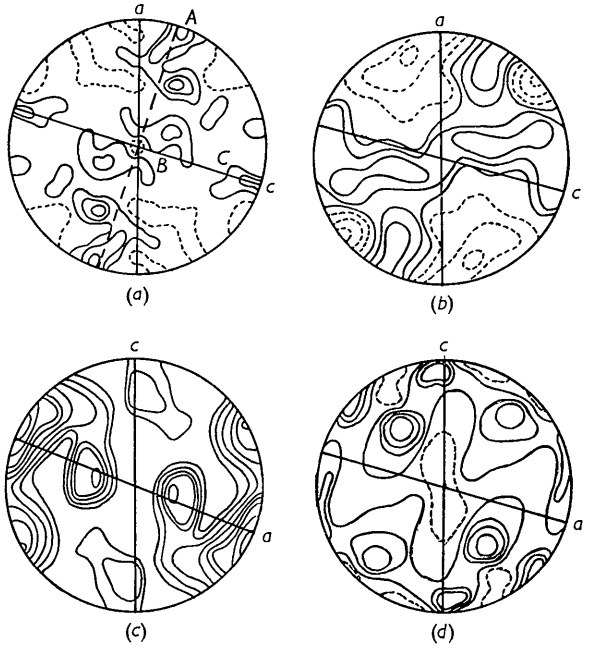


Fig. 3. Stereograms of the Patterson vectors in the 5 Å shells of: (*a*) Ribonuclease ex aqueous ethyl alcohol (Bernal & Carlisle). (*b*) Ribonuclease ex tertiary butyl alcohol (Magdoff, Crick & Luzzati, 1956). (*c*) Methaemoglobin (Perutz, 1949). (*d*) Myoglobin (Kendrew, unpublished).

Fig. 3(*a*) is a projection of a spherical distribution of vectors only to a first approximation; the distances of the maxima in Patterson space to the origin do differ, varying from 2·7 Å to 5·4 Å. The most obvious feature of the projection is the concentration of the vectors into three main groups referred to as *A*, *B* and *C* which correspond to the shaded areas displayed on 3(*b*) for ribonuclease II. The remaining areas for 3(*a*) have a lower density. The group *A* lies at a distance of about 4·5 Å from the origin and appears to be more widespread than the others with a tendency to rise nearly parallel to the *b* axis from the plane $V = 0$ for about 4 Å. This is shown in Fig. 4(*a*) by the section taken approximately through the plane containing the *A* and *B* group of vectors indicated by the dashed line on the stereograms. The second group *B*, at a mean distance of about 4·8 Å from the origin and lying in a plane almost parallel to $\bar{1}01$ have their vectors extending in an arc of about 40° on either side of the *b* axis but are clearly not as heavily concentrated as those in *A*. The third group *C* lying in the *bc* plane and starting from the *c* axis has an angular spread of about 25° on either side of the *c* axis at a mean distance of 5·2 Å from the origin. The relation of this peak to the rest of the shell is shown in Fig. 5 which is the section at $U = 0$.

The nature of the difference between haemoglobin and ribonuclease becomes clearer if we take the orientation of Fig. 3(*c*) with its largest vector in the *a* direction which lies parallel to the presumed direction of the polypeptide chains of haemoglobin. This has been concluded by Perutz (1949) essentially from considerations of reflections in the 10 Å region and it would indicate major concentrations of scattering matter at 5 Å intervals along the chain. This corresponds roughly to the picture presented by the keratin group of fibrous proteins where the 10 Å and 5 Å reflections are found respectively parallel to and perpendicular to the fibre axis.

In ribonuclease the situation is different. There is no such single predominating 5 Å vector but rather a belt of such vectors all normal to the crystallographic *c* axis, together with one not so strong 5 Å vector lying along it. It would appear that this direction is related to some unique feature of the molecular structure. Further evidence which makes this likely is provided by the sections $U = 0$ (Fig. 4(*b*)) and $V = 0$ (Fig. 2(*b*)) which show a definite peak at about 2·7 Å limited to a volume close to the *c* axis. It continues as an inward protuberance of the 5 Å shell. These are the only vectors found inside the 5 Å cage corresponding to a set of planes of large unitary structure factors nearly normal to the *c* axis.

The deduction of chain directions from Patterson vector maps of protein crystals now becomes a little tenuous in the light of Kendrew's recent findings on myoglobin where it would have been difficult to

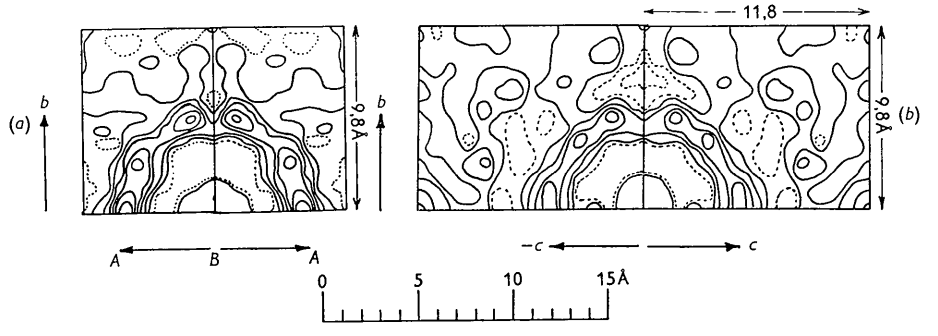


Fig. 4. (*a*) Patterson vector distribution on plane approximately perpendicular to *c* axis. (*b*) Patterson vector distribution at $U = 0$.

deduce from Fig. 3(*d*) that there were two or more chain directions in this molecule. Furthermore, from evidence of the Patterson sections alone, it is impossible to make any positive statements about the type of chain systems present in ribonuclease.

Whatever the structure of ribonuclease is, it would appear on other evidence to be complex. Hvidt (1955) finds that deuteration of this enzyme proceeds at an instantaneous and then at a slow rate and interprets these results in terms of a molecule whose loose outer part shields an inner hydrogen-bonded folded structure. From their studies on the effect of LiBr on the specific optical rotation of native and oxidised ribonuclease, Harrington & Schellman (1957) come to the conclusion that the molecule does not make maximum use of its peptide hydrogen bonds, and Yang & Doty (1957) suggest from measurements of the dispersion of the optical rotation of protein solutions that ribonuclease cannot claim more than 15% of the polypeptide in an $\alpha$-helix configuration. Taken in conjunction with the three-dimensional Patterson vector distribution of this crystal, all this evidence merely emphasises the need of obtaining a direct X-ray structure analysis of this protein—one involving the minimum of hypothesis.

## 5. Conclusions

The three-dimensional vector maps calculated for the monoclinic ribonuclease crystals grown in aqueous alcohol are in general agreement with the vector maps of crystals grown from tertiary butyl alcohol. There are significant differences in certain intensities of reflections between the crystals grown from the different solvents which suggest that the relative orientation of the molecules in the two cases is not quite the same. This is supported by differences in the vector distribution along the *a* axis of the two crystals, although there is a general agreement in their respective vector maps.

These investigations have clearly revealed the limitations of Patterson analysis in the study of protein crystals. This does not, however, negate the use of such analyses in the early stages of such work particularly when comparisons are made between the vector maps of different crystalline proteins. The full Patterson evidence obtained for ribonuclease will be more fittingly used in conjunction with the early three-dimensional electron-density maps obtained by use of the heavy-atom technique which is now in progress.

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This investigation has entailed an enormous amount of calculation, and many at one time or another have given us assistance. We should like first of all to express our appreciation to Mr T. B. Boss of the National Physical Laboratory for giving us a Master Pack of Hollerith Cards for Fourier summations, and to Dr A. D. Booth for allowing us to use a Senior Hollerith Tabulator. We would like to thank Mrs Marianne Ehrenberg, Mrs Margaret Levy, Dr Helen Scouloudi, and Messrs. G. S. D. King, R. Pascal, M. Rosemeyer, and P. D. Shukla for their invaluable help at various stages in the work.

Finally, we would like to thank Dr J. C. Kendrew for allowing us to show Fig. 3(*d*) from his unpublished material.

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