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Intensity Measurements from Insulin Crystals on the Goniostat; Sources of Error and Accuracy of Data

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An analysis of the intensity measurements from four crystals of insulin citrate, performed with the aid of a General Electric Goniostat, has revealed several sources of error, both random and systematic. Of the latter the most serious are those connected with absorption, which varies with migration of water surrounding the crystal, and those due to radiation damage to the crystal. Methods, which may also apply to proteins other than insulin, have been found for controlling or correcting for these and other errors. The method of intensity measurement compares favorably in speed and accuracy with photographic techniques.

Introduction

This study is part of a long-term project concerned with the elucidation of the three-dimensional molecular structure of insulin. The successful application of the method of isomorphous replacement to protein crystallography (Green, Ingram & Perutz, 1954) has emphasized the need for the rapid collection of many thousands of intensity measurements, with sufficient accuracy to determine relatively small differences between corresponding spectra of different chemical derivatives. To this end it was decided to purchase a Goniostat and to make a detailed analysis of the first few sets of intensity measurements, in order to determine the various sources of error, correct for these where possible, and assess the overall reliability of the data.

Several previous reports from this laboratory have been based on studies of insulin sulfate crystals (Low, 1952; Low & Richards, 1954; Low & Shoemaker, 1959). In this work, however, we have used insulin citrate, which has been shown to be isomorphous with type *A* insulin sulfate (Low & Berger, 1960), but appears to be more suitable for the preparation of heavy-atom derivatives of the protein.

We have used a commercial version of the Goniostat designed at the Protein Structure Project, Polytechnic Institute of Brooklyn, and, with relatively minor modifications, have followed the instrumental techniques first developed by this group for work on ribonuclease (Furnas & Harker, 1955; Magdoff & Crick, 1955; Furnas, 1957).

Experimental

All the data were collected from crystals of bovine insulin citrate grown from citric acid/sodium citrate buffer by a procedure described by Low & Berger (1960). The crystals all showed the same habit: diamond-shaped tables lying on (010) and bounded by {101}. Their maximum dimensions varied from 0·3 mm. to 0·5 mm. The crystals were mounted in thin-walled glass capillaries, being held to the walls of the capillaries by contact with small amounts of mother liquor. In order to maintain the crystals at 100% humidity, reservoirs of mother liquor were placed in the capillaries a few mm. above and below the crystals, where they were out of the path of the X-ray beam.

Measurements were made with nickel-filtered copper radiation and a General Electric SPG Spectrogoniometer fitted with an Eulerian cradle (Goniostat). The Goniostat enables all spectra within a complete hemisphere of reciprocal space to be recorded with a single mounting of a crystal. It was therefore possible to obtain a complete set of three-dimensional intensity measurements—out to the arbitrary limit of 2·5 Å—from a single specimen. Lattice parameters were measured with a narrow slit in front of the counter and a narrow source of X-rays. Integrated intensity measurements were collected with a wide uniform X-ray source and a wide counter aperture. Counts were taken for periods of ten seconds with the crystal and counter stationary. For each intensity measurement a correction was made for the background radiation, determined by a separate ten-second count with the crystal turned about 1·5° out of the reflecting position.

In order to minimize radiation damage to the crystals an automatic shutter was added to the instrument (Einstein, 1958). The shutter ensured that the crystals were exposed to X-rays only during the actual taking of counts, and for such operations as crystal alignment and the preliminary scanning of reflections, but not during the rather longer periods of time required to change the instrumental settings from one reflecting position to another. A clock timer in the automatic-shutter circuit enabled a record to be kept of the amount of exposure time each crystal had

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received. The room temperature was kept between 70 °F. and 75 °F. by means of air conditioning, and the crystals protected from local air currents by a shield built around the instrument. However, in the earliest work, before adequate precautions were taken, larger temperature variations did occur; the effects of these are discussed below.

Fourteen reflections, chosen along the three principal crystal axes, were monitored at frequent intervals throughout the course of intensity measurement. The intensities, together with associated background counts, of some or all of these reflections were recorded after approximately every hour of X-ray exposure. At each such check the cell dimensions were redetermined and the temperature and elapsed exposure time noted. In this way it was possible to observe and analyze any long-term variations in intensity due to changes in the specimen or experimental conditions.

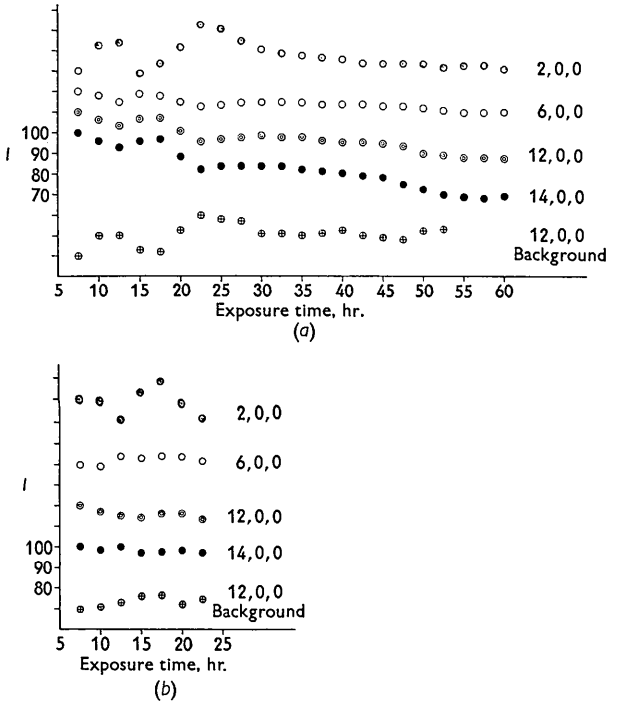

Fig. 1. Intensities (background subtracted) of 2,0,0; 6,0,0; 12,0,0 and 14,0,0 reflections, and background of 12,0,0 reflection, plotted against elapsed exposure time. All the curves are smoothed. The intensities and background are normalized so that the value at 7·5 hr. for each curve is 100. The values shown along the ordinate refer to the 14,0,0 reflection; the other curves are on the same scale, but with their origins displaced along the ordinate. (*a*) Data from Crystal 1. (*b*) Data from Crystal 2.

Intensity data were collected from four different crystals of insulin citrate, referred to below as Crystals 1, 2, 3 and 4. Measurements were made on Crystal 1 of all *hkl* spectra with spacings greater than 2·5 Å, some 4300 in all. The 0*kl* spectra were measured twice; after 10 hours and again after 60 hours X-ray exposure. During the course of these measurements the crystal suffered appreciable radiation damage, as judged from the intensities of the monitored axial reflections, Fig. 1(*a*). The thousand strongest reflections, which had been measured on Crystal 1 over the whole time range, were therefore remeasured on Crystal 2. During this second set of measurements the intensities of the monitored reflections, except for a few very low order spectra, remained approximately constant, Fig. 1(*b*). It was therefore possible to use the data from Crystal 2 as a standard of comparison in the later analysis of the various sources of error affecting the intensity measurements. More limited data were collected from Crystal 3, which was subjected to prolonged X-ray exposure so that the effects of radiation damage could be further investigated. Finally, the intensities of all the *h*0*l* reflections were remeasured on Crystal 4.

Classification of errors

In the analysis of the various possible errors in the recorded intensity data, it is convenient to distinguish between errors that are inherent in the method and essentially constant in time, and those that show long-term variations with time. To the former class belong such trivial and easily corrected systematic effects as the Lorentz and polarization factors and coincidence losses in the counter, as well as random errors due to counting and to short-term fluctuations in the incident X-ray intensity. More troublesome are certain systematic errors of which the effects are less readily estimated. Such, for example, is the possible loss of intensity due to inadequate breadth of X-ray source or of counter aperture, or to slight misalignments of the crystal. With a unit cell as large as that of insulin there is also an appreciable danger of overlapping reflections from adjacent spectra. Errors from this source can be quite large and often difficult to recognize. Finally there is the problem of absorption by the crystal, the mother liquor, and the capillary, for which the appropriate correction must be determined empirically.

To some extent this absorption effect overlaps the classification of errors as time-independent or time-dependent. Our data indicate that migration of water in the capillary is sometimes sufficient to cause appreciable changes in the absorption. This water migration also produces changes in background scattering and, moreover, seems to affect the intensities of some of the low-angle reflections by altering the salt concentration inside the crystal. The most pronounced time-dependent effect, however, is a monotonic decline in the measured intensities that is evidently associated with a loss of crystal quality on exposure to the X-ray beam. These two effects, of water migration and radiation damage, are the principal time-dependent factors that have been revealed by the comparison of data from different crystals as well as from the monitored reflections. A further time-dependent variation, cor-

related with temperature fluctuations, was found to be of minor importance under the experimental conditions employed.

Time-independent errors

Under conditions of uniform source intensity and uniform counter sensitivity, both of adequate width to ensure valid measurements of the integrated intensities, the recorded intensities must be corrected for Lorentz and polarization factors in the same manner as the equatorial reflections from a rotating crystal (Hirshfeld & Schmidt, 1953). The instrument had been adjusted so that both the incident intensity and the counter sensitivity were uniform to within 5% over an angular range of about 0·4°. Consequently these standard corrections were assumed to be adequate. The X-ray set was allowed to warm up for an hour before any measurements were made. It was found that after this period there were no appreciable fluctuations in counting rate apart from statistical counting errors. The counter response was checked and found to be linear to within about 2% at counting rates up to 10,000 per second. As no intensities greater than this were encountered, no correction for coincidence loss was applied.

The instrumental settings for the various reflections were computed from the measured cell dimensions on an IBM 650 computer. By checking periodically on the positions of selected standard reflections we were able to detect any movements of the crystal, as well as occasional small fluctuations in the position of the X-ray source, and to make any required adjustments of the crystal position or of the calculated instrumental settings. In fact, the relative dimensions of the counter aperture, X-ray source and crystals were chosen so as to allow a fair amount of tolerance in the instrumental settings, and serious misalignments, necessitating the remeasurement of a set of data, were very rarely encountered. The size of the counter aperture was sufficiently small to ensure resolution between neighbouring spectra. However, we were not able to obviate all possibility of interference from residual $K\beta$ and white radiation. In the small number of unfavorable cases, involving adjacent very strong and very weak reflections, where this could have caused appreciable errors, the necessary corrections, if any, were determined individually.

Correction of the measured intensities for absorption in the crystal, capillary, and mother liquor required an empirical determination of the variation of this absorption with the direction of the incident and reflected beams through the specimen. For certain reflections it was possible to measure the reflected intensity as the crystal was rotated about the normal to the reflecting planes (Magdoff & Crick, 1955). Additional information was obtained by comparison of intensities from crystallographically equivalent reflections. Since the recorded data were limited to Bragg angles, $\theta < 18°$, the variation of absorption with θ was considered unimportant. In fact a one-parameter function, varying only with the polar angle φ (Furnas, 1957), was considered adequate to describe the relative-absorption correction for all the measured reflections to within a maximum error of about 5%. From a separate measurement of the fraction of a narrow beam of monochromatic X-rays transmitted through the crystal and its surrounding liquid and capillary glass, in a known orientation to the beam, the *relative* correction was converted into an *absolute* absorption factor that was used in the determination of an approximate absolute scale for the measured intensities (see below).

From a comparison of the sets of data collected from the different crystals and a check of the weaker intensities against an earlier set of photographic measurements on insulin sulfate (Shoemaker, Einstein & Low, 1960), which has an intensity distribution similar to that of insulin citrate, a few 'human' errors in instrumental setting or data recording were detected and corrected.

Time-dependent errors

Fig. 1 shows values of reflected intensity (background subtracted) and background for several of the monitored reflections from Crystals 1 and 2 plotted against elapsed exposure time. These curves are all smoothed, the data from several successive counts being averaged and plotted in overlapping time intervals. The corresponding unsmoothed curves show only minor short-term intensity variations. These consist mainly of random fluctuations, such as would be expected from statistical counting errors, but they also include a few simultaneous changes in the various spectra which appear to be related to temperature fluctuations. With the aid of a heating/cooling air-conditioning system, the intensities of the axial reflections of Crystal 3 were measured at various temperatures between 65 °F. and 80 °F. The intensities were indeed found to decrease with increasing temperature, in accordance with the observed variations on the curves. This effect appears to be reversible and more pronounced for high- than for low-order spectra. However, as it was clear that this effect, over the temperature range employed, could not have caused errors of more than a few per cent in the intensity measurements, and as the data were inadequate for the determination of an appropriate correction factor, no correction was attempted.

The smoothed data show marked systematic variations in the intensities from Crystal 1, especially during the first 25 hours of X-ray exposure, with much smaller variations for Crystal 2. The smaller variability in the data from Crystal 2, which is presumably due to better temperature control and to the fact that these data were collected during a shorter total span of exposure time, suggests that the intensities recorded from this

crystal are relatively free of errors of the time-dependent sort. Thus the comparison of intensities of corresponding reflections from the two crystals permits an evaluation of the time-dependence of the intensities from Crystal 1 based on far more extensive data than are provided by the monitored reflections alone.

As a first step, reflections were grouped into several ranges of θ and, for each group, average values of the ratios $|F|^2$(Crystal 1)/$|F|^2$ (Crystal 2), both values corrected for absorption, were plotted against exposure time for Crystal 1. The fluctuations on these curves approximately matched those shown in Fig. 1(*a*) for the monitored reflections. Thus there could be no doubt that the intensities from Crystal 1 were all subject to a general time variation of rather serious magnitude. The key to a portion, at least, of this effect was provided by the concurrent variation in the background intensities, of which that associated with reflection (12,0,0) shown in Fig. 1(*a*) is typical. This background variation is clearly opposed to the variation in reflected intensities in a manner that agrees perfectly with the supposition that the effect is produced by migration of water in the vicinity of the crystal. Evidently, an increase in the amount of water surrounding the crystal increases the background scattering while decreasing the reflected intensities by increasing the absorption. Since the relative intensity fluctuations appeared to be about equal for the monitored reflections along all three principal axes, it was concluded that the absorption variations were approximately isotropic, requiring no change in the *relative* absorption correction.

Exceptional behavior, however, was noted for several of the very low-angle reflections, such as (2,0,0) shown in Fig. 1(*a*). This may be ascribed to variations in the salt concentration of the mother liquor, which cause changes in electron density in the regions of the unit cell between protein molecules. Salt-concentration changes of comparable magnitude in hemoglobin crystals have been shown to produce large changes in the amplitudes of low-order structure factors (Perutz, 1954). Such an explanation implies that the migration of liquid responsible for this salt effect proceeds by evaporation and condensation of water in different parts of the capillary rather than by the rolling around of mother liquor. This is entirely consistent with the observation that the effects we have attributed to this cause were greatest during the early period of the work on Crystal 1, when temperature fluctuations were most severe.

Once the diagnosis had been made, the next step, apart from a determination to seek ways of minimizing the water migration in future experiments, was to find a method of allowing for this effect in further analysis of the data already obtained. For this purpose it was necessary to distinguish as far as possible between the effects of water migration and of radiation damage. Apart from the sensitivity to changes in salt concentration of the very low-order reflections, the effects of water migration might be expected to be independent of Bragg angle. This in fact appeared to be the case, as can be seen from Fig. 1(*a*), where the relative intensity fluctuations of (6,0,0), (12,0,0) and (14,0,0) are approximately equal during the first 20 hours of exposure. Radiation damage, however, was found to affect primarily the high-angle spectra (see below). Consequently a number of moderately low-angle reflections, e.g. (6,0,0), which appeared to be little affected either by radiation damage or by changes in salt concentration and the monitored intensities of which showed essentially similar variations with time, were chosen to provide an estimate of the changes in absorption factor due to water migration. The resulting time-dependent absorption correction was then applied to all the intensities recorded from Crystal 1.

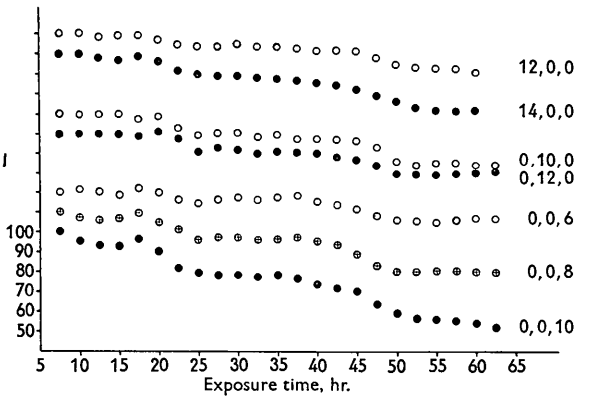

Fig. 2. Intensities (background subtracted) of some monitored reflections from Crystal 1, corrected for absorption variations due to water migration and plotted against elapsed exposure time. All the curves are smoothed. The intensities are normalized so that the value at 7·5 hr. for each curve is 100. The values shown along the ordinate refer to the 0,0,10 reflection; the other curves are on the same scale, but with their origins displaced along the ordinate.

After this correction for water migration, the monitored reflections showed residual time variations of a more gradual nature (Fig. 2). These variations were greater for high-angle than for low-angle reflections and showed long periods of fairly constant intensity punctuated by two rather sudden intensity drops after about 17 hours and about 45 hours of irradiation. In accordance with these indications, reflections which had been measured on both Crystal 1 and Crystal 2 were sorted according to the time of the Crystal 1 measurements, and for each of the three periods of approximately constant intensity, as shown by Fig. 2, values of

$$\log_{10}(\Sigma|F|^2(\text{Crystal 1})/\Sigma|F|^2(\text{Crystal 2}))$$

were plotted against $\sin^2\theta$ (Fig. 3); the Crystal 1 data being corrected for water migration, and the summations extended over small overlapping intervals of $\sin^2\theta$. It is evident from the fact that the points lie on straight lines of different slopes that the three

curves of Fig. 3 correspond to three different values of an apparent temperature parameter B, and it is natural to attribute the changes to increases in disorder induced in the crystal by radiation. Similar radiation-damage effects have been observed photographically for crystals of many other proteins. Though there have been up to now no quantitative investigations of this phenomenon, appreciable deterioration in crystallinity has generally been observed only after prolonged irradiation, and it has been reported (Magdoff, 1953) that in the case of ribonuclease the deterioration occurs quite suddenly. The behavior reported here suggests that the radiation damage to this particular insulin crystal occurred in two well-defined stages, perhaps through the rupture of different types of linkage in the crystal structure, either intermolecular or intramolecular, differing appreciably in their number or in their vulnerability to X-ray damage. A possible loosening up of the crystal structure is also indicated by sudden increases in the length of the c axis by about 0·1 Å that coincided with the two sudden decreases in intensity shown in Fig. 2. The a and b axial lengths changed less, if at all.

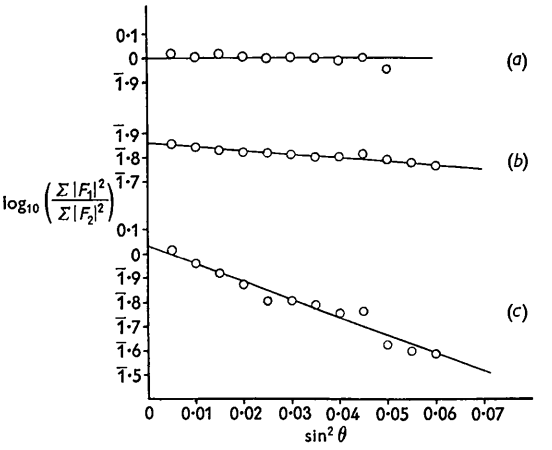

Fig. 3. $\mathrm{Log}_{10}(\Sigma|F|^2(\text{Crystal 1})/\Sigma|F|^2(\text{Crystal 2}))$ plotted against $\sin^2\theta$. The summations included reflections measured on both Crystals 1 and 2 and extended over small overlapping intervals of $\sin^2\theta$; the values of $|F|^2$(Crystal 1) used in the summations were corrected for water-migration effects, but not for radiation damage, nor for absolute scale. The three curves derive from reflections measured at different times on Crystal 1; (*a*) up to 17 hr. exposure; (*b*) between 24 and 42 hr. exposure; (*c*) more than 60 hr. exposure.

Table 1. *Values of ΔB_r for Crystal* 1

hkl	2θ	B_r(35 hr.)$-B_r$(12 hr.)	B_r(60 hr.)$-B_r$(12 hr.)
12,0,0	18·4	2·1	8·5
14,0,0	21·5	3·7	10·3
0,10,0	17·2	7·8	16·2
0,12,0	20·7	4·7	10·1
0,0,6	13·9	2·7	13·6
0,0,8	18·6	4·5	14·6
0,0,10	23·3	5·1	14·9
From Fig. 3(*b*)		4	
From Fig. 3(*c*)			16
Values used in correction factor		4	14

The variation of B_r, the radiation-induced part of the apparent temperature parameter B, was estimated both from the curves of Fig. 3 and from the observed variations in the monitored intensities (Table 1). These data suggest that B_r may not have been completely isotropic but, limited as they are to low Bragg angles, they do not permit a reliable estimate of the anisotropy. A reasonable approximation was obtained by the assumption of two sharp increments ΔB_r equal respectively to 4 Å^2 and 10 Å^2. The intensities from Crystal 1 were accordingly corrected to the appropriate pre-radiation values by the factor $\exp(2B_r \sin^2\theta/\lambda^2)$, intermediate values of B_r being used for reflections recorded during the two periods of rapid intensity decline.

The possibility of a more complicated change was checked by a comparison of Patterson projections along the a axis computed from two sets of data collected from Crystal 1 after about 10 hours and after about 60 hours exposure. The Patterson functions are practically identical, except that the peaks on the latter are generally only about 75% as high as the corresponding peaks on the former. A difference Patterson projection computed from the same two sets of data shows positive peaks, surrounded by negative regions, at much the same positions as the peaks on the other two projections but with height only about 5%, on the average, of those on the 10-hour projection. This is what would be expected if the two sets of data corresponded to identical structures with different temperature parameters, and indicates that the increase in crystalline disorder was not accompanied by important specific structural rearrangements.

Crystal variability

A comparison of the data from the four crystals of insulin citrate used in the intensity measurements not only helped to indicate various sources of error, but also made possible a quantitative assessment of the reproducibility of the results.

Measurements of cell dimensions on the four crystals were identical to better than one part in 300. The mean values were:

$$a=57{\cdot}72,\ b=51{\cdot}44,\ c=38{\cdot}12\ \text{Å}.$$

Insulin citrate has the orthorhombic space group $P2_12_12_1$.

Independent determinations of the absolute scale of intensity were made with three of the crystals. This involved measuring their volumes and absolute absorption factors and then scaling their measured intensities in accordance with intensity measurements made on standard crystals of known structure and

volume (Robertson, 1933). Beryllium acetate (Tulinsky & Worthington, 1959) and anthracene (Mason & Milledge, 1960) were used as standard crystals for this purpose. Most of the crystals were rather irregularly shaped and it proved very difficult to measure their volumes accurately. Presumably because of this difficulty, the scales obtained from the various determinations differed considerably, and the scale finally adopted may be in error by as much as 20%.

The effects of radiation damage on Crystal 1 have been described above. Crystal 3 showed a similar small and sudden deterioration in crystallinity, coinciding with a small elongation of the c axis, after about 15 hours exposure. However, Crystal 2 showed no appreciable changes in the intensities of the monitored reflections up to 22 hours exposure (Fig. 1(b)). Crystal 4 was exposed for only a few hours and did not show changes. It should be noted in this connection that the amount of radiation received by a crystal depends on the collimation as well as the exposure time, and that the times quoted above for the onset of radiation-damage effects may consequently not be pertinent to experimental arrangements different from that which we have used.

After the derivation of observed $|F|$'s from the corrected intensity measurements, values of the ratios $\Sigma|F|^2/\Sigma|F|^2$(Crystal 2) were calculated for Crystals 1, 3 and 4; the summations extended over groups of reflections measured on both crystals and corresponding to small ranges of $\sin^2\theta$. Reflections affected by radiation damage were not included in these summations. Fig. 3(a), the curve for Crystal 1, is typical of the plots of the logarithms of these ratios against $\sin^2\theta$. The ratios do not vary significantly with Bragg angle, indicating that the four crystals all have the same 'temperature factor' before being affected by radiation damage. These ratios were also used to put the data from all four crystals on a common approximately absolute scale.

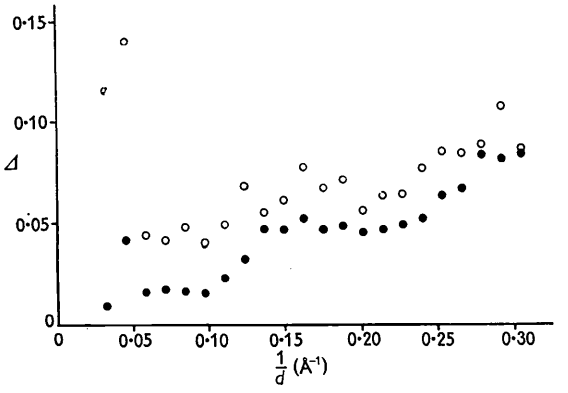

Fig. 4. Plot of $\Delta=\Sigma||F$ Crystal 1$|-|F$ Crystal 2$||/\Sigma|F$ Crystal 1$|$ against $1/d$. ○ denote experimental values; ● denote calculated values of Δ due to statistical counting errors.

At low angles the intensities from different crystals exhibited some marked discrepancies (Fig. 4). Since it is in this low-angle region that the anomalous intensity fluctuations, due to the salt effect, were observed, it was assumed that the variations from crystal to crystal were similarly due to differences in salt concentration. Such differences must be expected, even for crystals drawn from the same preparation, because of uncontrolled evaporation of water during the selection and mounting of the crystals. Support for this hypothesis was provided by a remeasurement of intensities from Crystal 3, the capillary of which had been broken and then resealed after most of the water around the crystal had evaporated. The second set of data showed sharply reduced intensities for about 20 of the strong very low-angle reflections, whereas the intensities of higher-order reflections agreed with the earlier measurements apart from smaller, fairly uniform changes attributable to the change in absorption factor.

In order to estimate the reproducibility of the intensity measurements we have used the structure moduli calculated from the fully corrected intensity data to compute discrepancy factors of the form $R=\Sigma||F|-|F\text{(Crystal 2)}||/\Sigma|F|$ between Crystal 2 and Crystals 1, 3 and 4. Values of R between Crystals 1 and 2 were computed for groups of reflections covering small ranges of $1/d$. For comparison, estimates were made of the corresponding discrepancy factors due to the statistical counting errors alone.

The contributions of the statistical counting errors to the R values were calculated as follows: the standard deviation in the intensity count, I (i.e. with background subtracted) is $(I+2B)^{\frac{1}{2}}$, where B is the background count, and the mean absolute error, η is $(2/\pi)^{\frac{1}{2}}$ times this. As $|F|^2$ is proportional to I, the mean absolute error in $|F|$,

$$\eta(|F|)=|F|/2\,.\,(2/\pi)^{\frac{1}{2}}\,.\,(I+2B)^{\frac{1}{2}}/I$$

and hence

$$\eta(||F_1|-|F_2||) = 1/(2\pi)^{\frac{1}{2}} \times\left[\left(\frac{|F_1|}{I_1}\right)^2(I_1+2B_1)+\left(\frac{|F_2|}{I_2}\right)^2(I_2+2B_2)\right]^{\frac{1}{2}},$$

where subscripts 1 and 2 denote Crystals 1 and 2. Taking $|F_1|=|F_2|$ and rearranging,

$$\eta(||F_1|-|F_2||) = 1/(2\pi)^{\frac{1}{2}} \times\left[\left(\frac{I_2}{I_1}\right)^2\left(\frac{I_1+2B_1}{I_2+2B_2}\right)+1\right]^{\frac{1}{2}}\cdot\frac{(I_2+2B_2)^{\frac{1}{2}}|F_2|}{I_2}.$$

As I_1 was always considerably greater than I_2, (I_1/I_2 varied from 4 to 12), and the background counts were fairly constant for each range of $1/d$, the factor

$$\left[\left(\frac{I_2}{I_1}\right)^2\left(\frac{I_1+2B_1}{I_2+2B_2}\right)+1\right]^{\frac{1}{2}}$$

was treated as a constant for each group of reflections.

The contribution of the statistical counting errors to the R values was therefore calculated as

$$\frac{\Sigma[|F_2|(I_2+2B_2)^{\frac{1}{2}}/I_2]}{\Sigma|F_2|}$$

multiplied by a constant.

In Fig. 4 both the experimental and the statistical discrepancy factors between Crystals 1 and 2 are plotted against $1/d$. The curves reflect the fact that, like other proteins, insulin has relatively strong intensities at spacings of about 5 Å and 10 Å. Apart from very small values of $1/d$, which are affected by differences of salt concentration, the calculation of $(R^2(\text{experimental})-R^2(\text{statistical}))^{\frac{1}{2}}$ shows a fairly uniform discrepancy of about 4% not accounted for by statistical counting errors. The effects of short-term fluctuations in experimental conditions, inaccuracies in the various correction factors, and possible slight differences between the two crystals would all contribute to this residual discrepancy. Discrepancy factors between Crystals 1 and 2 were also calculated for the three groups of reflections corresponding to different stages of radiation damage. For $B_r=0$, $0<B_r\leq 4$ and $4<B_r\leq 14$, R was found to be 6·2%, 6·0% and 7·5% respectively. The discrepancy factor between Crystal 2 and the very small Crystal 3 was 10·3%, and between Crystals 2 and 4, 7·0%. The higher discrepancy factors for Crystal 3 and for the $B_r>4$ group of reflections from Crystal 1 is largely explained by the smaller intensities and consequently larger statistical counting errors in these two cases. Reflections for which the standard deviation in I exceeded the observed I were excluded in the calculation of R values.

Discussion

Any discussion of sources of error would be incomplete without a consideration of means for avoiding them or mitigating their effects.

As was shown in the later work, the absorption variations caused by water migration can be made negligible by keeping the specimen at a constant temperature. This also avoids the changes in intensity with temperature that were observed in the unsmoothed plots of the monitored intensities from Crystal 1.

Yet another temperature effect has been reported by Magdoff & Crick (1955), who found in their studies of ribonuclease that small temperature fluctuations in the neighborhood of the crystals were capable of producing large changes in the intensities as well as changes in cell dimensions of up to $\frac{1}{2}$ Å. These effects, however, were observed only with crystals that had been mounted dry, i.e. with very little mother liquor in the capillary. As indicated above, all our crystals were mounted with a considerable amount of mother liquor, and we did not observe these effects.

The effects of radiation damage, at least in the case of insulin citrate, could also be avoided by discarding a crystal as soon as appreciable changes in the monitored reflections are observed. There are, however, disadvantageous aspects to such a policy. Considerable time is spent in the mounting, alignment, and the preliminary examination of specimens, as well as the computation of instrumental settings for the various spectra. Also a considerable proportion of the specimens examined on the Goniostat are found to be unsuitable for the collection of data. While the form of correction we have applied is essentially an approximation, the calculation of discrepancy factors and an individual comparison of the data from Crystals 1 and 2 indicate that this approximation does not greatly affect the overall accuracy of the data for the early stages of radiation damage. So far as our data are concerned, the use of such a correction factor seems to compensate adequately in saving of time and effort for the possible slight improvement in accuracy that might result from the limitation of data collection to about 1000 reflections per specimen. This may be even more true for heavy-atom derivatives, where errors due to variations in the occupancy of heavy-atom sites in different crystals may well outweigh the inaccuracies in the correction factor. However this type of correction for radiation damage may of course not apply to proteins other than insulin citrate, has yet to be tested for spectra with spacings less than 2·5 Å, and is likely to be an increasingly poor approximation for the more advanced stages of radiation damage, for which the increase in the proportion of low intensity counts would in any case cause a serious deterioration in the quality of the data.

The statistical counting errors could of course be diminished by taking longer counts on the weak reflections, though this would hasten the advent of radiation damage. It would not, however, be particularly advantageous to measure the time required for a constant number of counts, rather than counting over a constant time interval, because of the large background of scattered radiation.

In assessing the advantages and disadvantages of the Goniostat for intensity measurements from proteins it is interesting to make a comparison with photographic techniques. There are some differences in the sources of error that have to be considered. Whereas photographic measurements may have to be corrected for rotation factor and variations in spot shape, they are not affected by variations in experimental conditions (e.g. X-ray output) which influence the intensities of all reflections equally, and they are also less liable to 'human' errors. However, though the effects of radiation damage and temperature variations might result in relatively small errors in the averaged intensities on any one photograph, they could lead to serious incompatability between data collected on different photographs. Photographic data are generally also subject to errors from absorption, though,

with special precautions, the relative-absorption factor can be made negligible for photographs taken on a precession camera (Blow, 1958). Undoubtedly the potential effects of some of the usual sources of error are aggravated by the use of an instrument on which different reflections are measured at different times, but we feel there is adequate compensation in the greater ease with which it is possible to observe and correct for these factors. The overall accuracy of our intensity measurements seems comparable with that attained with the most careful techniques involving photographs and is probably considerably better than the accuracy normally achieved with visual estimations.

With experience in the use of the Goniostat, it is possible to record 40 to 50 intensity measurements (with the corresponding background counts) in an hour. The measurement of 4,300 reflections on Crystal 1, including frequent checks and realignments, took us some five weeks. To this should be added another three weeks approximately for finding a suitable specimen, preliminary measurements, the computation of instrumental settings, and the supplementary measurements required to determine the absolute intensity scale. This compares very favorably with the time required for the collection of data of comparable accuracy by photographic methods.

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