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MACROMOLECULAR X-RAY DATA COLLECTION ON A ROTATING ANODE DIFFRACTOMETER

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1. Introduction

For many biological macromolecules such as proteins and nucleic acids, because of their large unit cells and weak diffracting power, a conventional sealed tube diffractometer is often not the preferred instrument for X-ray data collection. Instead, film methods with a rotating anode or even a synchrotron source are often used. Film methods in general do not come close to the accuracy obtainable with conventional scintillation detectors. They also involve a great deal more difficulty in handling and processing. Although synchrotron radiation [1] makes possible experiments on extremely small crystals and/or ones that are short lived, synchrotrons are in short supply and often there is a mong waiting period to get time on such a source, even for preliminary tests for determining whether an experiment is feasible. It would be very desirable if there were an affordable in-house instrument that would allow accurate and rapid data collection on difficult crystals.

Recent developments in instrumentation, such as an integrated system consisting of a powerful and reliable rotating anode coupled to a fast and convenient to use diffractometer carry a promise to meet many of the needs of macromolecule crystallographers.

Although we considered such a system to be very attractive, there has been very little published experience on its use with biological macromolecules. In order to ascertain its utility, we thought it necessary to answer a few often asked questions:

1) Due to the anticipated variability in beam intensity, would the data collected be of sufficient quality?
2) Would such an instrument be much more difficult to align and use than a conventional sealed tube diffractometer?
3) Rotating anodes have had a reputation for being expensive to maintain, essentially requiring a full time technician to support them. Would the potential advantages outweigh the drain on personnel resources?
4) Due to the complexity of such a system, and the potential for breakdowns, what fraction of the time would it be available for production runs?
5) Due to the relatively remote location of our laboratory, how difficult would it be to arrange service and obtain spare parts?

In this article we will describe a set of experiments designed to answer these questions.

2. Description of the Equipment

2.1 AFC-5R

A Rigaku AFC-5R diffractometer with RU-300 X-ray generator was used for these experiments. It has a χ circle with diameter of 190 mm, and a 400 mm evacuated detector arm. An RU-300 rotating anode, with a Cu target, 0.5 x 1 0 mm forcal spot and a graphite monochromator provided the source of X-rays. In general the rotating anode was run at 15 kW (50 kV and 300 mA). A 100 mm thick transparent (PVC with lead) radiation shield was constructed locally to provide protection against any scattered radiation. The diffractometer is controlled by a microVAX-II [Digital Equipment Corp. (DEC), Maynard, MA], with 9 M-bytes of memory and a 71 M-byte Winchester Eisk, currently running VMS version 4.3. The computer was interfaced to the AFC-5R by a standard DRV11-J parallel interface board (DEC) installed in the microVAX-11. The software control programs to run the diffractometer were provided by Rigaku Corp., Tokyo and Molecular Structure Corp. (MSC), College Station, TX. The system is shown in Fig. 1 and 2.

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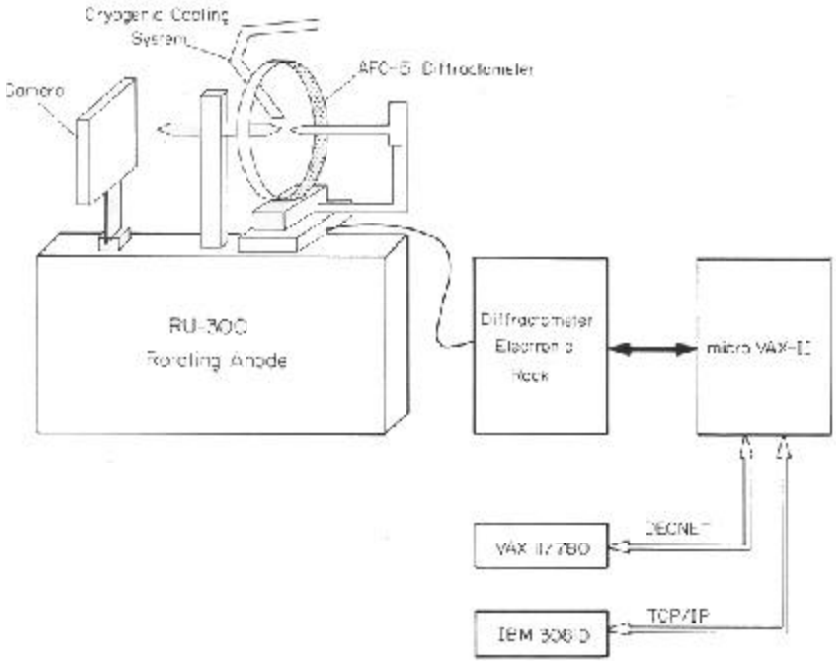

Fig. 1 . System block diagram of the Rigaku AFC-5R rotating anode diffractometer and microVAX II control system.

Fig. 2. The AFC-5R rotating anode diffractometer as set up in the X-ray laboratory at the Weizmann Institute of Science.

2.2 Low temperature attachment

At the time the diffractomter was ordered all macromolecule data collection was expected to be carried out at or just below room temperature.

However, by the time the equipment was delivered it had been shown that many crystals of biological macromolecules can be cooled to cryogenic temperatures, while preserving their crystallographic integrity [2], [3]. It therefore became necessary to improvise a low temperature attachment in order to utilize this development. A basic Enraf-Nonius low temperature apparatus was available for this purpose. The apparatus is capable of supplying a regulated stream of freshly boiled, cold N_2 gas. A combination of evacuated glass tubing and styrofoam was used to construct an outlet tube to provide a fixed position

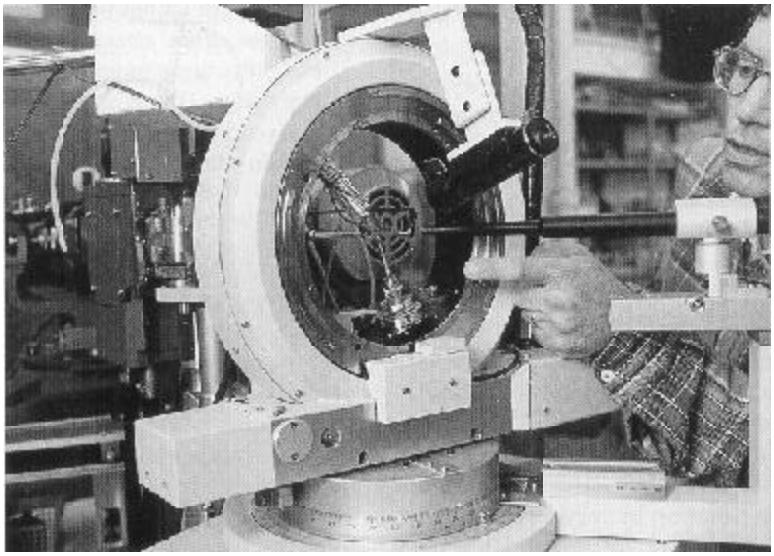

Fig. 3. The provisional fixed position liquid N_2 low temperature cooling system nozzle, at approximately 11:00, in place on the AFC-5R diffractometer.

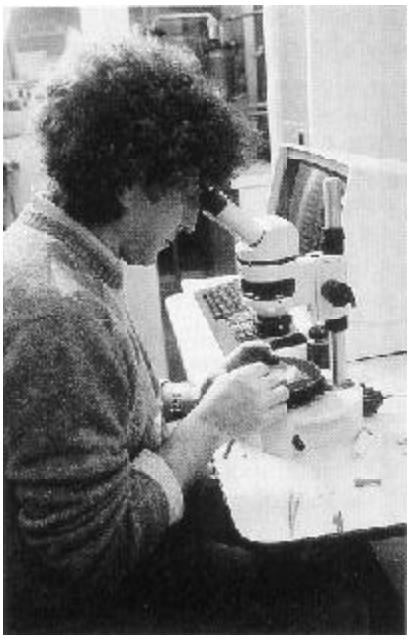

Fig. 4. The low temperature crystal mounting technique is shown using a low power stereo n\microscope coating the crystals with a viscous oil in the crystallization droplet. After all mother liquor solution covering a crystal is removed, it is picked up with a thin glass fiber, and then placed directly in the stream of cold N_2 to shock freeze it [2], [3]. It should be emphasized that the crystal is mounted *without putting it in a capillary.*

cold gas stream that would allow relatively free motion of the χ circle (see Fig. 3). The apparatus used initially gave a temperature near 115K. In its present form it gives a steady temperature of about 90 K at the crystal site, with a liquid nitrogen consumption of 0.7 *l*/hr. This apparatus is now being used for nearly all macromolecular data collection in our laboratory.

The low temperature crystal mounting technique consists of first coating the crystals with a viscous oil in the crystallization droplet, removing all the mother liquor solution covering it (see Fig. 4). A single crystal is then picked up with a thin glass fiber, and placed directly in the stream of cold N_2 [2], [3]. In our laboratory, this low temperature mounting procedure usually takes about 1/2 hour, which is comparable to the time required to mount a protein crystal, inside a capillary with its mother liquor, in a conventional manner.

3. Performance Tests

After installation of the equipment we aligned the diffractometer. This was much simpler than we had anticipated, and in fact took only a few hours. This is primarily because a convenient method for moving the AFC-5 diffractometer relative to the fixed Xray source was provided in the design of the system.

After alignment we performed a number of tests to answer some of the questions raised above. In this section we describe these tests in some detail.

3.1 Overall system stability

A single crystal of ruby, ground to spherical shape, was oriented on the diffractometer. A reflection with peak intensity near 100,000 counts/ sec was measured at random intervals (63 individual measurements) over a two-day period. The scan speed was selected to yield about 100,000 counts/scan. The

incident beam monitor was not used. From the measured intensities an estimated standard deviation $\sigma(I)$ for the scan intensity was obtained from the expression

$$\sigma(I) = \sum(I_i - \bar{I})^2/(n-1),$$

where I_i is the number of counts in scan number i, $\bar{I}$ the mean for the sample, and *n* the number of individual measurements. It is common in crystallographic practice to assess stability from the expression

$$\sigma^2(I) = \bar{I} + (p\bar{I})^2$$

where *p* is an adjustment factor ('fudge factor'), sometimes referred to as uncertainty factor. For ideal Poisson statistics p is zero. A value of 0.005 for p is normally considered excellent. For the measurements on ruby the value for p is about 0.001, indicating essentially perfect stability. In this context it is appropriate to mention that use of the incident beam monitor as a time standard would lead to significant deterioration in apparent stability unless timing were based on at least 100,000 counts. We believe that the extraordinary system stability virtually obviates the need for an incident beam monitor.

During the initial period of testing our laboratory was plagued by numerous power failures. Reproducibility of the beam intensity following restart of the rotating anode after shutdown was therefore determined. *No intensity change couldbe detected by statistical tests.*

Data from production intensity measurements also support the stability findings described above. A data set for the protein ferredoxin from *halobacteria* of the Dead Sea (see below), was measured with the crystal cooled to 115 K. During data collection two monitor reflections were repeatedly measured for approximately 100 times over three days of data collection (see Fig. 5). Their scan intensities were about 10,000 and 7,000 counts. These reflections gave p values of 0.004 and 0.006, respectively. This is extraordinary for protein data, and would be excellent for high-precision small-molecule data collection. Here it must be emphasized that this stability could not have been attained without the low temperature technique.

3.2 Comparison with sealed tube intensity

Intensity measurements of one crystal were performed both with a sealed tube source, and with the rotating anode source. Graphite monochromated CuKα radiation was used in both cases. The sealed tube was operated at 50 kV, 30 mA, and the rotating anode at 50 kV, 300 mA. The ratio between recorded intensities is approximately 1:10, in keeping with the current ratio. This tenfold increase in intensity is extremely important for macromolecule measurements, where large numbers of reflections must be reocrded, many of which are relatively weak. For the stronger intensities the meausring speed can be increased, while weaker intensities will be enhanced,

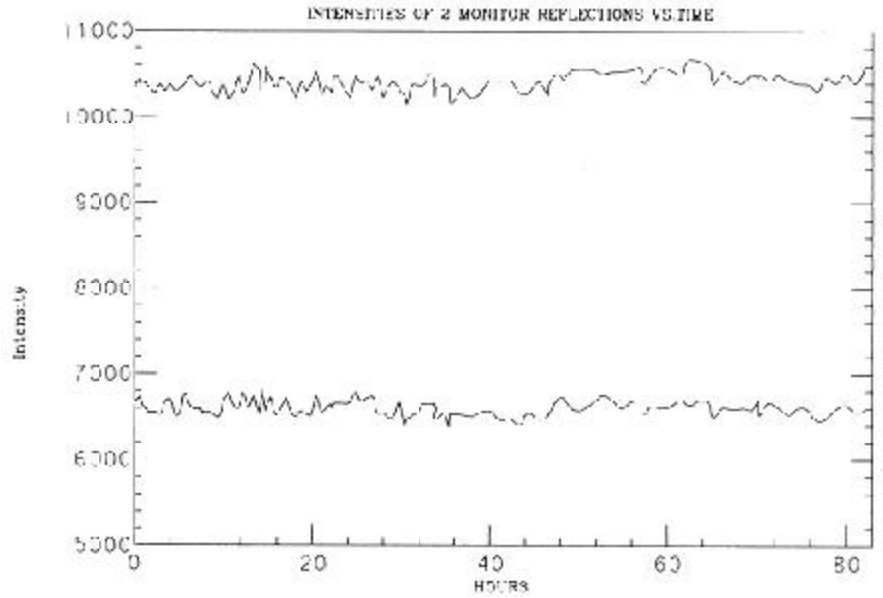

Fig. 5 Stability of the intensity of monitor reflections as a function of time for a protein molecule. Measurements of two monitor reflections (413 top, and 0.0.16 bottom) were repeated approximately 100 times over three days of d ata collection for the 2Fe-2S ferredoxin from *Halobacteria* of the Dead Sea.

thereby increasing the resolution limit that can be attained within a reasonable time span.

Following the initial alignment of the system, we collected data on it, 24 hours a day, running the Xray generator at 15 kW, for approximately 3 months with no down-time (except for power failures). Following this we had a period of several months of the system being up and down due to a failure of the high voltage system in the rotating anode. The problem was diagnosed by the factory engineers together with our local technicians (communicating via FAX) to be due to a faulty ceramic insulator in the rotating anode assembly. Once this was replaced by a modified insulator, the system has again been operating without any downtime for approximately 3 months till now.

3.3 Attenuation factors

In order to prevent serious coincidence losses at high count rates the diffractometer is equipped to insert up to three different attenuation filters in the beam. In addition there is an electronic coincidence correction circuit. This ciruit can be activated or deactivated manually. Prior to use the attenuation factors were measured. For this we used the automatic procedure provided in the MSC/Rigaku software. Apparent factors were measured for several reflections at decreasing values of the anode current. Two series of measurements were carried out, one without the electronic coincidence loss correction, and one with the correction activated. The apparent attenuation factors were plotted as a function of intensity. A straight least-squares line could be fitted to the points measured without electronic coincidence correction. The intercept for zero intensity can be taken to be the true value for the attenuation factor. The slope yields the dead time. In this manner we determined the natural dead time for the counting chain to be τ=1.4μs, in accord with specifications. (τ is defined by the equation $I_{true} = I_{obs}(1+ (1 + \tau I_{true}) .)$

With the coincidence correction activated, the value of t should ideally be zero at all count rates. However, the attenuation factors obtained did not fit a straight line. We found the apparent dead time to vary with the count rate, from τ=-0.4μs at 15,000 counts/sec (i.e. overcorrection) to some significant positive value (undercorrection) at count rates above 30,000 counts/sec. For the most accurate measurements it therefore seems best to work with the correction circuit inactive, and carry out a numerical correction based on scan profiles.

3.4 Data collection performance

The results of the initial testing were generally very satisfactory, and we proceeded with full scale data collection for a number of crystals of biological macromolecules.

It is important to emphasize that the MSC/ Rigaku software controlling various stages of searching for reflections, determining and refining the crystal parameters and orientation matrix, as well as the data collection procedure itself is very user friendly. Most commands are given in menu type format, with the default parameters (which can easily be overriden) shown on the terminal screen. It is possible to use the diffractometer control programs without having to learn the VAX/VMS operating system. For the biological macromolecules that we investigated thus far, the setup time before beginning data collection, once the crystal was mounted, usually ranged from 1 to 3 hours.

In the following section we discuss the most important aspects of our experinece obtained from these measurements.

4. Biological Macromolecules

Three-dimensional X-ray diffraction data have been collected on the AFC-5R system described above for a series of different proteins and nucleic acids, including ferredoxin from the extreme *Halobacteria* of the Dead Sea [4], [5], the DNA tridecamer d (CGCAGAATTCGCG) [6] and octamer d(GGGCGCCC) [7], human carbomonoxy hemoglobin [8], colicin immunity protein [9], and soy bean agglutinin (SBA) [10]. In general the space group and unit cell parameters of the proteins and nucleic acids as determined at this cryogenic temperature on the AFC-5R are all within 1% of the parameters as measured at room temperature or 4°C.

Although most of the macromolecules that we have tested can be shock cooled and X-ray data can be collected on them, SBA is an example of a protein that just does not diffract at all once it is cooled. We are testing alternative methods of shock cooling protein crystals to try to overcome these problem cases.

Examples of two data sets that benefitted enormously from the rotating anode diffractomter together with the low temperature system are discussed in more detail below.

4.1 Ferredoxinf rom Halobacteria of the Dead Sea

In order to survive in the saline environment of the Dead Sea, the saltiest body of water found on earth, the extreme Halobacteria of the Dead Sea maintain internal concentrations of the monovalent cations Na^+ and K^+ as high as 0.7M and 4M, respectively. Proteins from extremely halophilic organisms are not only able to perform their functions in multimolar concentrations of salt, but some of them have been shown to require salt for their stability. What are the structural requirements for a protein to render it soluble and active at practically saturated salt solutions?

As a model for the adaptation process of a protein to an extrmely salty environment, we are investigating the crystal structure of a 2Fe-2S ferredoxin from the *Halobacteria* of the Dead Sea. It was chosen in part because the three-dimensional structures of related *non-halophilic* ferredoxins with the same 2Fe-2S prosthetic group from *Spirulina platensis* [11] and *Aphanotheca sacrum* [12] are know. A complete sphere of X-ray data was collected at 115 K from a single crystal of the ferredoxin out to 1.9 Å resolution. It should be stressed that the cryogenic technique appears to have preserved virtually indefinitely the diffracting power of the crystal. It was possible therefore to collect an entire set of approximately 20,000 reflections from one single crystal in about 3 days (approximately 6,000 reflections/day). Previously, on a conventional sealed tube diffractometer, at room temperature, this took about a month and the use of many crystals because of radiation damage. These data are of unusually high quality, with over 89% of the Fobs greater than 6 times their estimated standard deviation, and with an overall Rsyn=2.5%.

Based on the low-temperature data, an electron density map was calculated, initially out to 3.3 Å resolution using previously determined phases [13]. This new map (see Fig. 6) was much clearer than the one previously calculated using room temperature data. The protein backbone was traced through this improved map and after a preliminary least-squares refinement with CORELS [14] and PROFFT [1 5, 1 6] the current R factor, for data to 2.5 Å resolution data, is 27.3% (see Fig. 7) [17].

4.2 DNA molecules containing inserted bases

Recent advances in DNA synthetic methods [18] have made it possible to study by X-ray crystallography single crystals of DNA, 4-12 nucleotides long, with predefined sequences [19]. Until now, apart from one example [20], only self-complementary DNA sequences or those containing mismatched base-pairs [21] have been examined. From these studies, DNA appears to exhibit enormous conformational flexibility.

The conformation of extrahelical bases in nucleic acid duplexes has been a problem of long standing interest because of their relevance to frame shift mutagenesis [22, 23]. We are studying the tridecamer sequence, d(CGCAGAATTCGCG), by X-ray crystallography [24]. Except for the inserted adenosine at position 4, it has a sequence identical to the previously determined B-DNA dodecamer, d(CGCGAATTCGCG) [25]. This structural analysis will help determine whether the extra residue is stacked inside the helix as precicted from NMR data [26] or looped out.

Initially X-ray data were collected on crystals at room temperature and at 4° C [27]. However, the

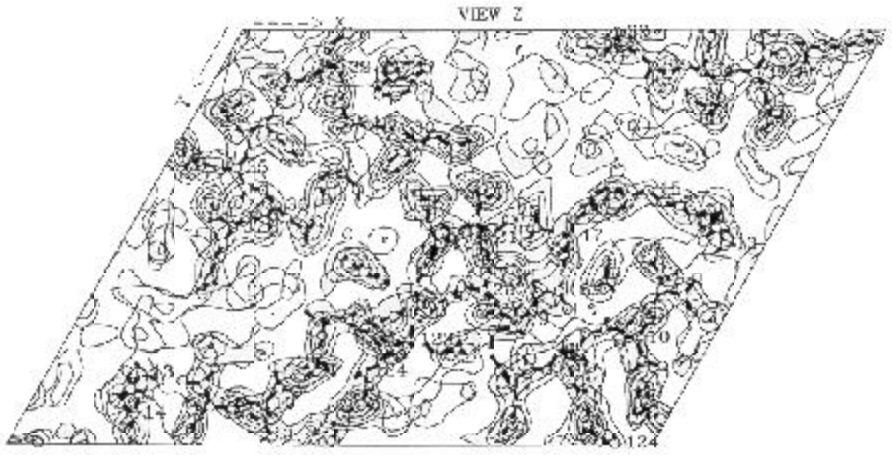

Fig. 6 Five Angstrom thick slice through the 2.5 Å resolution 2Fo-Fc map of ferredoxin from *Halobacteria* of the Dead Sea as determined from data obtained on the AFC-5R system at 115 K. C_α atoms for each amino acid appearing in this portion of the map are labeled.

Fig. 7 ORTEP [29] drawing of the three-dimensional backbone structure of ferredoxin from *Halobacteria* of the Dead Sea as determined from data obtained on the AFC-5R system at 115 K. The 2Fe-2S prosthetic group liganded to the protein appeas in the lower left portion of the illustration.

crystals decomposed in the X-ray beam within a few hours, and showed significant shrinkage in one of the unit cell dimensions. Using the techniques described above for extreme low temperature X-ray data collection, it was possible to prolong the lifetime of the crystals practically indefinitely in the X-ray beam.

A complete set of X-ray data was collected, from *a single crystal*, out to the limit of its diffraction, i.e. 2.5 Å resolution, on the Rigaku AFC-5R at 115K. As in the case of ferredoxin, described above, the monitor reflections showed no intensity loss through-out the data collection, implying that there was no crystal damage.

Molecular replacement methods as well as the use of covalently linked heavy atoms markers to various cytosine residues in the sequence are being used to solve the three-dimensional structure of the tridecamer [28].

5. Conclusions

Our main conclusion is that all questions posed in the introduction have been answered in a manner that dissipates all implied concerns.

Installation and maintenance are easily handled with our personnel and technical resources, and when required, factory support has been very satisfactory. After some initial problems with the rotating anode, that have now been solved, the entire system seems to be as reliable as a conventional sealed-tube diffractometer.

When used in conjunction with our cryogenic attachment, the system has yielded data of exceptional stability for a number of biological macromolecules. Data acquisition speed has been satisfactory, but not as high as one could expect, given the high maximum drive speeds for the axes. Presumably this is related to somewhat conservative ramp tables for the stepping motors in our system, resulting in relatively long travel times between reflections. Careful tuning of the drive systems could result in significantly increased speed. Additional, undesirable delays are seen when use of attenuation filters has been selected in data collection setup. In the latter case substantial time is spent for each reflection in determining what attenuation to use, if any. It would be more productive to respond to a need for attenuation only as a result of too high a count rate registered by the detector.

Although it is difficult to assess actual data quality, or accuracy, the indicationswe have (stability of monitor reflections, Rsym, quality of electron density maps) suggest that the data we have obtained with our system are of far higher quality than any data we have obtained previously for comparable samples (see Figs. 5 & 6). We believe that the diffractometer/ rotating anode source as well as the low-temperature technique are necessary components if high-quality data are to be obtained.

We are confident that for data sets of moderate size (up to 100,000 reflections) our approach provides dramatic advantages over traditional, film based methods, both in technical simplicity and data quality. We also have strong indications that the use of our system in many cases can obviate the need for a synchrotron source.

An intriguing problem is the question of the relative merits of a conventional four-circle diffractometer versus an area detector diffractomter. In terms of simplicity of use the four-circle diffractometer at present has obvious advantages; these, however, may diminish in importance as area detector systems continue to improve. For very large data sets the area detector appears to have an advantage. We do not yet have sufficient information to allow a comparison of data quality for area detector data sets that can also be reasonably handled with the four-circle diffractometer.

For small to moderate data sets a system constructed along the lines described in this paper is currently the best, in overall performance. We expect that for a number of years to come such systems will continue to provide the overall best approach to high-quality data acquisition for biological macromolecules, other than the very large ones.

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References

[1] R. Helliwell, The Rigaku Journal, **3**, (1986), 3-12.

[2] H. Hope, Amer. Cryst. Assoc., Abstract Ser. **2, 13**, (1985), abstract no. PA3.

[3] H. Hope, Acta Crysta., **B43**, (1987), in press.

[4] J. L. Sussman, M. Shoham, M. Harel, F. Frolow and H. Hope, International Meeting on Biochemistry, Beijing, abstract. (1 987), B-Al -0453, p. 386.

[5] M. Shoham, J. L. Sussman and M. Harel, lxth International Congress of Biophysics, Jerusalem, abstract (1 987), p. 26.

[6] J. L. Sussman, L. Joshua-Tor, M. Hirshberg, M. A. Saper, F. Frolow, H. Hope and E. Appelia, Transactions of the Beijin Meeting on "Molecular Structure: Chemical Reactivity and Biological Activity", (1 987), in press.

[7] D. Rabinovich, M. Eisenstein, T. E. Haran and Z. Shakked, IX International Congress of Biophysics, Jerusalem, abstract (1 987), p. 113.

[8] B. Shaanan, (1987), unpublished results.

[9] M. Shoham, lXth International Congress of Biophysics, Jerusalem, abstract (987), p. 115.

[10] B, Shaanan, (1987), unpublished results.

[11] T. Tsukihara, K. Fukyama, M. Nakamura, Y. Katsube, N. Tanaka, M. Kakudo, K. Wada, T. Hase and H. Matsubara, J. Biochem. (Tokyo), **90**, (1981), 176-1773.

[12] T. Tsukihara, M. Mizushima, K. Fukuyama and Y. Katsube, International Summer School on Crystallographic Computing II, Kyoto, (1983), pp. 63-64.

[13] J. L. Sussman, J. H. Brown and M. Shoham, Iron-Sulfur Protein Research (eds. H. Matsubara, Y. Katsube and K. Wada), Japan Scientific Socieites Press, Tokyo/ SpringerVerlag, Berlin. (1986), 69-82.

[14] J. L. Sussman, S. R. Holbrook, G. M. Church and S.-H. Kim, Acta Crysta., **A33**, (1977), 800-804.

[15] W. A. Hendrickson and J. H. Konnert, Biomolecular Structure, Function, Conformation and Evolution (eds. R. Diamond, S. Ramaseshan and K. Venkatesan), 13.01-13.25, Indian Academy of Sciences, Bangalore. (1980).

[16] B. C. J. Finzel, Appl. Cryst., **20**, (1987), 53-55.

[17] M. Shoham, M. Harel and J. L. Sussman, submitted to Science. (1987).

[18] K. Itakura, N. Katagiri, C. P. Bahl, R. H. Wightman and S. A., Narang, J. Amer. Chem. Soc., **97**, (1975), 7327-7332.

[19] R. E. Dickerson, H. R. Drew, B. N. Conner, R. M. Wing, A. V. Fratini and M. L. Kopka, Science, **216**, (1982), 475-485.

[20] M. McCall, T. Brown, W. N. Hunter and O. Kennard, Nature (London), **322**, (1986), 661-664.

[21] T. Brown, O. Kennard, G. Kneale and D. Rabinovich, Nature (London), **315**, (1985), 604-606.

[22] G. Streisirger, Y. Okada, J. Emrich, J. Newton, A. Tsugita, E. Terzaghi and M. Inouye, Cold Spring Harbor Symp. Quant. Biol., **31**, (1966), 77-84.

[23] J. W. Drake, B. W. Glickman and L. S. Ripley, American Scientist, **71**, 1983), 621 -630.

[24] L. Joshua-Tor, J. Hope, E. Appella and J. L. Sussman, Fifth Conversation in Biomolecular Stereodynamics, Albany, NY, abstract (1987), pp. 42-43.

[25] R. Wing, H. Drew, T. Takano, C. Broka, S. Tanaka, K. Itakura and R. E. Dickerson, Nature (London), **287**, (1980), 755-758.

[26] D. J. Patel, S. A. Kozlowski, L. A. Marky, J. A. Rice, C. Broka, K. Itakura and K. Breslauer, J. Biochemistry, **21**, (1982), 445-451.

[27] M. A. Saper, H. Eidar, K. Mizuuchi, J. Nickol, E. Appella and J. L. Sussman, J. Mol. Biol., 1**88**, (1986) 111 -113.

[28] L. Joshua-Tor, D. Rabinovich, E. Appelia and J. L. Sussman, (1987), unpublished results.

[29] C. K. Johnson, A FORTRAN Thermal-Ellipsoid Plot Program for Crystal Structure Illustrations, ORNL-3794 (Second Revision), Oak Ridge National Laboratory, Oak Ridge, TN., (1965).