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Art is Long and Time is Fleeting: The Current Problems and Future Prospects for Time-Resolved Enzyme Crystallography

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Art is long and time is fleeting: the current problems and future prospects for time-resolved enzyme crystallography

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I offer comments on the challenges and problems of the future based on the papers in this volume. First, the requirement of the Laue technique for a very well-ordered crystal is a major obstacle to many studies. Efforts to ease this problem are needed. Secondly, the fundamental issues in time-resolved crystallography are now chemical rather than crystallographic. Methods for the rapid initiation of many reactions must be developed. Thirdly, it is imperative that the kinetics of the process in question be studied in the crystal before any diffraction experiments are done. We need better ways to make those solid state kinetic measurements. Fourthly, we should make use of combined methods, such as cryoenzymology plus Laue diffraction or site-directed mutagenesis plus Laue diffraction, to bring many processes into the time régime in which we currently can work. Fifthly, we have to be able to deconvolute diffraction data that come from a mixture of two or three discrete species. Finally, no matter how powerful our synchrotrons get, it seems to me that some of the most important events in any enzymatic reaction are not going to be accessible: consider the formation and decomposition of a transition state as an example. I close by discussing the role of computational biochemistry in filling in those frames of our enzymatic movie that we cannot observe directly by time-resolved X-ray crystallography.

The title of this essay comes from Longfellow's *A Psalm of Life* (1839). I chose it because it seemed to capture the essential problem one faces in time-resolved protein crystallography. Of course the thought, like most insightful thoughts, is much older than that. Goethe remarks (*Wilhelm Meisters Lehrjahre*, 1786) that 'Art is long, life short'. But he's just borrowing from Seneca (A.D. 50): 'Vita brevis est, ars longa'. (Similar utterances are also made by Browning (*The Ring and the Book*) and Chaucer (*The Parliament of Fowls*), among others, but this can get out of hand.) And Hippocrates (420 B.C.) in his *Aphorisms* says, 'Life is short, the art long, opportunity fleeting, experience treacherous and judgement difficult'. If that doesn't sound like Laue crystallography, I don't know what does.

I called this paper an essay in the previous paragraph. Montaigne coined that word in 1580 from the French for 'attempt'. I will attempt in this paper to summarize the excellent presentations of this Discussion Meeting and from them to draw some conclusions about the problems of time-resolved crystallography, especially by the Laue method, and its prospects.

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1. Introduction

During the course of this meeting, one of its organizers, Sir David Phillips, speculated that in the future this first gathering devoted to time-resolved macromolecular crystallography would be regarded as a water-shed in structural biology, in much the same way as those physics conferences – whose black and white photographs of stiffly posed participants adorn modern textbooks – that, in the early part of this century, ushered in the age of quantum mechanics. Leaving aside the self-serving reasons for agreeing with this speculation – everyone wishes to be thought of as helping to foster a revolution – there is, in the work presented in this meeting, objective evidence that something special is happening. For the first time, scientists are using the techniques of time-resolved crystallography to try to answer specific biochemical questions. There are undercurrents of this theme in almost every paper in this meeting, but it is particularly strong in the presentations of Johnson, Liljas, Pai, Ringe and Sweet. Other applications are clearly in progress or being planned, all of which suggests that a fourth dimension is being added to structure determination.

Another theme that emerges from the meeting is that the technical problems in time-resolved macromolecular crystallography are becoming well-defined and can now be tackled by specialists in organic chemistry and diffraction physics. There is, in fact, remarkable unanimity as to what those problems are. In the remainder of this essay I will try to summarize the consensus on the current problems, suggest some possible approaches to solutions, and speculate on the prospects for this emerging field.

2. Problems and possible solutions

(*a*) *The requirement for a well-ordered crystal*

A major obstacle to the application of the Laue method to a wide variety of problems is its extreme sensitivity to even the slightest degree of disorder in the crystal (Hajdu *et al.* 1991). Specimens that would produce excellent data-sets by monochromatic techniques often give hopelessly streaked Laue photographs. This problem could be circumvented by screening a large number of crystals were it not for the fact that a well-ordered crystal can become disordered by the very chemical and/or structural event that is of interest. Although disordering is sometimes transient, it tends to happen at the most interesting time: when species that are inaccessible to conventional time-average techniques are forming and converting. Of course, if the changes that occur in the protein are large enough to crack the crystal or abolish order, they will be a problem for any data collection method, but the Laue method is especially sensitive.

Participants at the meeting suggested a number of approaches to coping with crystal disorder. Prominent among them were better software to allow streaked and partly overlapped reflections to be integrated, and very fast data acquisition techniques to record the diffraction before disordering occurs. To these I would add cross-linking of the crystal with agents such as glutaraldehyde (Quiocho & Richards 1964). Cross-linking itself can often disorder a crystal, but in favourable cases it can, instead, render the crystal immune to physical damage. Although there is fear that cross-linking may inhibit conformational changes, evidence on this point is lacking. I believe the technique has been unduly neglected.

Given the high likelihood that reactions in the crystal might induce disorder, and the high demand for synchrotron beam time, it is desirable to test in one's own

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

laboratory the diffraction behaviour of the crystals after reaction, so that suitable conditions for stabilization can be worked out. Unfortunately, monochromatic techniques are not adequate for such testing, since the Laue method is more demanding of crystal perfection. The recent demonstration by Moffat and co-workers that Laue patterns of protein crystals can be obtained (albeit very slowly) with laboratory X-ray sources suggests that in-house debugging of Laue experiments is now possible, and should be encouraged (Brooks & Moffat 1991).

(*b*) *Incomplete data-sets*

Except for crystals of very high space-group symmetry, it is not possible to record a complete data-set with a single Laue exposure (Clifton *et al.* 1991). Yet very fast time resolution is impossible if many orientations of the crystal are needed, because changing the crystal position is very slow compared with most processes of interest. Even if this problem is solved, say by merging data from several independent experiments, Laue data-sets will always be incomplete. Low-resolution reflections suffer from harmonic overlap, and many other reflections are discarded because of spatial overlap or weak intensity (usually $I < 2$ or $3\,\sigma_I$).

There was much discussion about the effects of systematically incomplete data on the quality of difference electron density maps. The contributions of Johnson and Helliwell offer a number of suggestions for improved software and experiment design aimed at recovering a larger percentage of the data. Yet, the quality of electron density maps presented by Pai, Ringe and Sweet was high, despite no more than 50 % completeness in the best case. The participants concluded that these studies were favourable because they used strongly diffracting crystals from small proteins where the bound substrate represented a reasonable fraction of the total scattering matter. Also important for good results was the high occupancy of the bound material in a single conformation.

I would add two suggestions for increasing the apparent completeness of the data in a time-resolved experiment. First, filling in systematically missing regions of data with calculated Fs from, for example, solvent flattening, should reduce spurious effects. (Use of the point-spread function as described by Hajdu *et al.* (1991) and by Johnson in this volume is also worth pursuing.) My second suggestion is that the weaker data should not be rejected cavalierly. They are clearly imprecise (poor agreement R-values) but may, given adequate redundancy and no systematic errors, be more accurate than is supposed (see, for example, Liddington *et al.* 1991). Inclusion of more of the weak data will have a big effect on the quality of difference electron density maps. The completeness of the data-set will be increased, but there is another reason. Coefficients of the form $(F_{\mathrm{obs}}-F_{\mathrm{calc}})$ contribute little to the difference density when F_{obs} is small and F_{calc} is small. But when F_{obs} is small and F_{calc} is large, the difference between them will be large. Weak observed reflections that calculate large are as important for difference maps as strong reflections. I suggest that those who use the Laue method should consider retaining more – perhaps nearly all – of the weak observations.

Of course, if the lifetime of the species of interest is long, the experimenter will have time to reposition the crystal and collect data from different orientations. I conclude that stretching out the lifetime of the species of interest is a strategy worth pursuing. This theme will reappear later.

(c) *Rapid initiation*

Perhaps the greatest obstacle facing someone who wishes to carry out a time-resolved crystallographic experiment is the problem of initiating the reaction rapidly and simultaneously throughout the crystal. Although pressure – and temperature – jump experiments may work in some cases, as might pH-jumps (see the contribution from Sweet), for most reactions only photochemical initiation (Kaplan *et al.* 1978) will be fast enough. Trentham has addressed the design of caged substrates with suitable quantum yields and uncaging kinetics; the related, ingenious strategy of caging the enzyme has been developed by Ringe (this volume).

Despite the spectacular success of photoactivation in the work reported by Pai on H-ras p21 complexed with GTP (Schlichting *et al.* 1989, 1990) and by Ringe on the reaction of chymotrypsin with a suicide substrate (Stoddard *et al.* 1991), caged compounds do not exist for most reactions of interest. Here is an important challenge for the synthetic organic chemistry community.

Yet once again, I note that all of the problems associated with synchronous reaction initiation become easier to solve if the lifetime of the species of interest is long. Initiation does not have to be fast in absolute terms; it only need be fast relative to the lifetime of the desired state.

(d) *Rapid data collection*

The more rapid the data collection, the shorter-lived the species of interest can be. The work presented at the meeting indicates that reasonable single-exposure data sets can now be obtained in 10^{-4} s for strongly diffracting crystals. Moffat reported encouraging progress toward the use of synchrotron X-rays from single bunches of electrons to obtain useful Laue photographs. These bunches give pulses of about 100 ps duration. Spectacular as these timescales are, they only serve to point out the inadequacies of other aspects of our time-resolved technology for ultra-fast studies. Hardly any photochemical events are fast enough to serve as triggers on a picosecond timescale except those associated with chromophore excitation. And repeated (as opposed to single) picosecond measurements would seem pointless if it takes seconds to reorient or translate the crystal between exposures.

Increasing the speed of data collection is a worthwhile goal, but the limitation of our initiation methods suggest that an equally important line of research is the design of methods to stretch out the lifetime of the species of interest into the range of seconds, where our current data collection times are fast enough to be used.

(e) *Kinetics of reactions in the crystal*

No time-resolved crystallographic study should be attempted until the kinetics of the process of interest in the crystal have been established. The reason for this requirement is obvious: Laue data-sets do not usually provide true atomic resolution and it will often be difficult, if not impossible, to deduce the identity of a bound species from the electron density map alone. Knowledge of the rates of transformation of substrate into intermediates and of intermediates into product, after initiation of the reaction, will allow one to design the time-resolved experiment so that a given Laue data-set represents a single species of known chemical structure.

The consequence of failing to do this homework can be imagined. For a simple irreversible reaction of the form $A \rightarrow B \rightarrow C$ whose rate constants are $k_a = 10\ k_b$ in the crystal, the concentration of each species with time following initiation is given in

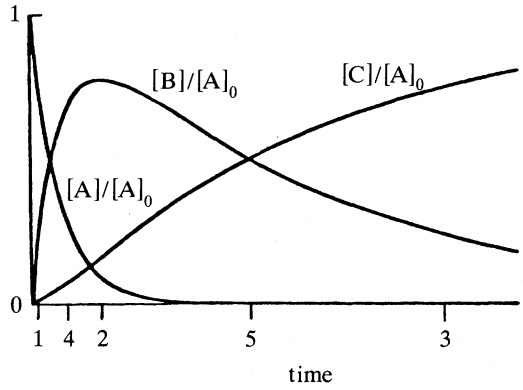

Figure 1. Relative concentrations of A, B and C in the consecutive reaction scheme $A \rightarrow B \rightarrow C$, where the rate constant for $A \rightarrow B$ is 10 times that of $B \rightarrow C$, i.e. conditions under which the intermediate B accumulates. Time-resolved data sets taken at time points 1, 2 and 3 provide reasonably homogeneous pictures of A, B and C respectively. Data-sets taken at time points 4 and 5 will represent complex mixtures of species.

figure 1. If Laue data-sets are taken rapidly at time points 1, 2 and 3 one will obtain clean structures for the enzyme complex with A and with C at points 1 and 3 respectively. The data-set taken at time point 2 will have a substantial contribution from the intermediate EB complex, but this will be superimposed onto electron density from smaller, but non-negligible, amounts of EA and EC. If the kinetics are known accurately, one may be able to subtract the appropriate fractional amounts of the homogeneous EA and EC structures from this intermediate electron density map and obtain a relatively clean image of the EB structure. Without the kinetic data, one must guess the correct fractional occupancies of EA and EC, or worse, one might have taken the data sets at only time points 4 and 5 instead. As figure 1 illustrates, data-sets taken at these time points are complex mixtures of species. If they are the only X-ray data available, it will not be possible to deconvolute them and obtain the structures of the individual species on the reaction path.

The Rossi paper describes microspectrophotometry techniques suitable for studying reactions in crystalline enzymes. The few examples of such studies in the literature need to be supplemented with many others. It would also be helpful to have commercial apparatus available for this purpose at a reasonable cost. Failing that, it is sometimes possible with clever experimental design to obtain kinetic data on reactions in the crystal by indirect means (Schlichting *et al.* 1989).

In many cases, however, the need for monitoring the reaction will not cease when the experiment starts. Heating effects generated by the synchrotron beam as well as by any powerful light source that is used in triggering the reaction imply that the conditions during the time-resolved experiment may be different – and unpredictably so – from those in the in-house mock runs. There is also the problem of crystal size effects, which make every specimen behave somewhat differently with regard to diffusion times and absorption. I see no alternative in many instances to monitoring the reaction spectroscopically during the experiment. The cautionary tales of Moffat and Ringe at this meeting reinforce that conclusion, and indicate the need for the design of suitable apparatus.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

(*f*) *Radiation damage*

Synchrotron radiation can destroy a specimen in less than a second. Up to now experimenters have comforted themselves with the notion that radiation damage was slower than Laue data collection times. They were probably right. Radiation damage is largely a secondary process requiring formation of hydrated electrons, hydroxyl radicals and superoxide ions (Ringe *et al.* 1983). Yet, as synchrotrons get more powerful, it seems likely that this problem will worsen. And for time-resolved studies in which one needs to take several data-sets from one crystal, either to cover reciprocal space adequately or to follow a reaction, radiation damage severely limits the time resolution. If only one white-beam photograph can be taken at each position of the crystal because of damage, the only hope is to focus the beam to a size smaller than the length of the crystal, and then to translate the specimen between exposures. This is a common strategy, yet the time required to move the crystal is much longer than the time of an exposure.

Solutions to the radiation damage problem are lacking, but some data are emerging. Sweet discussed the influence of beam intensity on the problem and Blow pointed out that proper choice of wavelength range has reduced the effects. Low temperatures clearly help (Petsko 1975), but the necessity for a fluid medium in most time-resolved studies will impose a rather high lower limit. Chemical protection has not been investigated as thoroughly as it should be. We have noted in the laboratory that protein crystals mounted in flow cells (Petsko 1985) usually suffer 3–4 times less damage than those mounted conventionally. Perhaps the damaging chemical species are being 'flushed out' of the crystal. We have also noted that crystals whose mother liquor contains high concentrations (100 mM) of reducing agents such as dithiothrietol suffer less damage than crystals of the same protein when the reducing agents are omitted. These observations suggest that chemical protection deserves more study.

(*g*) *Stretching out the timescale*

Since so many of the problems associated with a time-resolved diffraction experiment become less severe when the reaction is slow, it makes sense to try to stretch the timescale of faster reactions out to the range of tens of seconds or longer. It is probably not accidental that the few successful time-resolved studies that have been accomplished thus far all involve very slow reactions (these are summarized in the papers by Pai, Ringe, Johnson and Sweet in this volume).

Perhaps the simplest method for stretching out the timescale of a reaction is to select a slow substrate. This technique has a long and honourable history, starting with the studies of Johnson and Phillips of tri-NAG bound to hen-egg-white lysozyme (Blake *et al.* 1967). Care must be taken that the substrate is slow because of chemical, rather than binding, factors; a slow substrate that chiefly binds non-productively or that only binds weakly will not give a high-occupancy productive complex in a time-resolved experiment. Papers by Johnson and Ringe in this meeting illustrated the value of this approach.

Slow enzymes are equally useful. Pai illustrated the power of current time-resolved techniques on a reaction that takes minutes at room temperature (see also Schlichting *et al.* 1990). He also pointed out that mutant enzymes can have reaction times orders of magnitude slower than those of the wild-type, sometimes so slow that monochromatic data collection methods (*vide infra*) may suffice for observing the

enzyme–substrate complex. Given the ease of site-directed mutagenesis, and the spectacular successes of protein engineering in producing enzymes with lower catalytic activity than their wild-type counterparts, this approach deserves wide use. It is imperative, however, to establish that the mutant enzyme operates by the same mechanism as the wild-type, unless the aim of the study is only to study the mutant for its own sake. An enzyme with a turnover number of 1000 s^{-1} can be studied by current time-resolved techniques if its activity is reduced by a few thousand-fold. Such reductions are easily accomplished with single-site mutations that do not change the mechanism (see, for example, Strauss *et al.* 1985), although the work involved in proving mechanistic integrity can be formidable. Unfortunately, mutants that cause even smaller losses of activity have been known to alter the mechanism profoundly (see Nickbarg *et al.* (1988) for a particularly subtle example).

Extreme pH values may also lead to a slower reaction rate, but this technique is not ideal. Time-resolved crystallography still involves spatial averaging over all of the molecules in the crystal; only that species present at highest occupancy during the data collection time will be observed. Enzymes are less active at pH values far from their optimum because the concentration of correctly protonated species is low. Observation of enzyme–substrate complexes under these conditions may give misleading results; it is well known that protonation or deprotonation of a single amino acid side-chain can lead to significant changes in conformation, so the species observed at non-optimal pH may have a non-productive arrangement of catalytic groups.

Blow pointed out in this meeting that in many cases monochromatic techniques may be used, especially if an equilibrium can be established that favours a single bound species on the reaction pathway. Knowles and Phillips first realized that single-substrate/single-product enzymes could always be studied in this way (Banner *et al.* 1971). Crystallographic studies of isomerases (Alber *et al.* 1981; Collyer *et al.* 1990; Farber *et al.* 1989) in particular have used this strategy to obtain structures of productive enzyme–substrate complexes by monochromatic techniques. All isomerases, racemases and mutases are accessible to this approach, which only requires knowledge of the internal equilibrium constant for identification of the bound species that is observed.

But the easiest method conceptually for stretching out the timescale of the reaction so that the species of interest has a long lifetime is to reduce the temperature. Low-temperature techniques in protein crystallography have been around for decades (see Petsko (1975) for early references) but they have not been widely used for the stabilization of reaction intermediates. Recent developments in rapid-freeze techniques (Hartmann *et al.* 1982; Dewan & Tilton 1987; Hoppe 1988) have led to the adoption of sub-zero temperatures as a means for reducing radiation damage. Unfortunately, rapid-freeze methods are not suitable for most time-resolved studies because they do not yield a fluid mother liquor at low temperature.

I think the reason low-temperature techniques have not found more favour in crystallographic studies of enzyme reactions is the inherent difficulty in finding suitable cryoprotective mother liquors and holding a crystal at a stable temperature far below zero during data collection. But these problems only arise because it takes days to collect X-ray data by conventional techniques, and so one must work at temperatures far below the freezing point of the normal mother liquor in order to stabilize transient species for such long times.

The advent of the Laue method, however, reduces the data collection times to

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

seconds or less. One therefore needs only to slow the reaction down to the point where the species of interest has a lifetime of a few seconds in order to observe it by Laue diffraction, and such a modest reduction in rate can often be achieved at easily accessible temperatures. For example, a reaction whose rate-limiting transition state has an activation energy of 12 kcal mol^{-1}† (a typical value for many enzyme-catalysed reactions) will be slowed by a factor of 2 for every 10 °C reduction in temperature. A species with a half-life of 1 s at 37 °C will be stable for over a minute at −13 °C if this relation holds, and −13 °C is a very easy temperature to achieve. Most protein crystal mother liquors will supercool to between −10 °C and −20 °C. So a fluid medium can be maintained at a temperature that does not require expensive cryogenic equipment to reach, without the use of cryoprotectants. Bartunik presented very successful techniques and applications of moderately low temperature work.

I think that the best approach to increasing the lifetime of the species of interest in a time-resolved experiment will involve a combination of methods: moderately low temperature plus a slow substrate or moderately low temperature plus a mutant enzyme, for example. Given the difficulty of developing very fast methods for triggering reactions in the crystal, these strategies are likely to be needed for a long time to come for many enzymes, regardless of how rapid our data collection rates become.

(*h*) *The importance of monochromatic methods*

The possibility of stretching out the timescale of interesting reactions into the minute range raises the question of whether white-beam methods are always needed. The answer may well be no. Blow's paper illustrates the wealth of information that can be obtained by clever application of more 'conventional' techniques. And one should not forget that the first successful time-resolved crystallographic study, that of Hajdu and Johnson on the phosphorylation of the slow substrate heptenitol by phosphroylase, was carried out by rapid monochromatic data collection methods (Hajdu *et al.* 1987).

Technical problems presently limit the application of fast monochromatic techniques. Film changers are not as fast as they should be, and one would like to be able to replace film with image plates, so cheaper off-line image plate scanners would also be helpful. But the biggest problem is that the most suitable geometry for rapid monochromatic data collection, the Weissenberg method, requires cameras that are not available at most synchrotrons. I hope that instrument manufacturers and beam-line managers can be persuaded to remedy this situation.

(*i*) *Synchronicity*

Several papers, especially those of Moffat and Ringe, stress the problem of loss of synchronization as the reaction proceeds. Maxwell–Boltzmann statistics require that a distribution will be created rapidly following initiation of a reaction. The width of this distribution is hard to estimate, given that in many crystal systems there will be otherwise identical molecules with different packing environments. Whatever the details, it seems reasonable to expect there to be many different microscopic rates of approach to any stable intermediate. If the lifetime of that intermediate is long compared with the data collection time, asynchrony will not matter too much, since

† 1 cal = 4.184 J.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

the intermediate occupancy can be allowed to build up. But the longer one tries to follow the reaction, the worse the problem will become.

Ringe has concluded from these considerations that the proper strategy for time-resolved studies is not to try to follow a reaction in real time in a single experiment, except in rare cases (e.g. ras p21, see the paper by Pai in this volume). She argues that the best procedure is to divide and conquer: to devise conditions under which each low-energy species along the reaction path will accumulate in the crystal and be stable for at least a few seconds. She uses the analogy of film making: we want to obtain real-time, atomic resolution movies of enzymes as they act. But real movies, she points out, are never made in a single continuous filming. Scenes are shot separately and out of order, to fit the convenience of the project, and then spliced together at the end to make the chronologically correct movie. It seems like a good strategy for making our movies, too.

2. Prospects

With so many remaining problems, it is no wonder that success stories are few. Thus, the safest prediction is probably that time-resolved macromolecular crystallography will only be applicable to a small number of systems for the immediate future. Considering what has been learned already, however, from the few systems studied so far, it is probably also safe to predict that the results will have a significant – perhaps revolutionary – impact on our understanding of the structure/function relation in enzymes and photoactive proteins.

Rather than speculate further, I will indicate here what I think *should* happen. Our efforts ought to be directed along three lines: better methods to get more data at high resolution, better methods for following reactions in the crystalline state, and better methods for initiating more reactions in the crystalline state.

There is no substitute for being sure of the chemistry. Those systems that are well-characterized kinetically and that yield high-resolution Laue data will probably give the most useful results in the future. It also seems safe to conclude that our data collection times will decrease rapidly, with picoseconds being the ultimate goal.

I would urge those interested in using time-resolved crystallography to consider reactions that are inherently slow, or to use some of the methods discussed earlier to make reactions slow; there is no better way to stop focusing on extending the technique and to start thinking about the questions one wants to address. I would also urge adoption of the divide and conquer strategy; it seems the best way to guarantee that the species being observed is the desired one!

Finally, let me offer a few brief comments about the rest of the movie. Crystallography, even at very fast data collection rates, is still a spatial averaging technique. Only species that accumulate synchronously throughout the crystal are observed. Transition-states and intermediates whose formation are rate-limiting may never be seen no matter how fast we can collect the data. How do we fill-in these missing frames of our enzymatic motion picture?

Recent developments in computer simulation of enzyme reactions offer a possible route to a complete film. Methods for coupling a quantum-mechanical potential function to the empirical potential energy function used in molecular dynamics and molecular mechanics calculations, developed for the study of reactions in condensed phases (Field *et al.* 1990), have now been applied to enzyme reactions (Bash *et al.* 1991). Combined quantum mechanics/molecular mechanics potential functions can

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

be used to simulate an entire catalytic pathway, from formation of the enzyme–substrate complex through release of product. For the enzyme triose-phosphate isomerase, such a simulation has led to unexpected insights into the state of protonation of active site residues and the role of charged residues remote from the active site in transition-state stabilization (Bash *et al.* 1991).

Such simulations can fill-in the missing frames, but they must be guided. High-resolution structures of ES and EP complexes, and of stable intermediates along the reactions path, obtained by time-resolved crystallography, are exactly the sort of guides that are needed. In the future, I expect to see this computational approach allied with time-resolved diffraction to give complete picture of all of the steps in the reactions of several enzymes. Ras p21 would seem to be a particularly good candidate. Such movies could transform how we think about enzymes.

All experimental science is an attempt to overcome the limitations of the human senses. Predominant among the sensory organs is the eye. If the eye were perfect – if we could see things infinitely small or far away – there would be no need for microscopes or telescopes. We could just sit back and watch things happen in real time. To observe things in real time on a fast timescale, all we would need is a camera with this same perfect vision and very fast film.

In principle, crystallography is such a camera. Its characteristic time for scattering is so rapid that it freezes all motion, and it has perfect microscopic vision; it can see individual atoms. But it is a camera whose shutter is stuck open for a long time. The pictures we normally obtain are time averages, to which species only present transiently make no contribution. The advent of synchrotron radiation and the Laue method has changed all this. Our shutter speed is now 0.1 ms; it will get even faster. Now our problems have become chemical, not crystallographic: how to initiate reactions rapidly and synchronously throughout a crystal containing 10^{15} molecules, and how to study the kinetics of that reaction so that we know when to collect our data. As these problems are solved for more systems, the applicability of time-resolved crystallography will increase.

In 1971 David Phillips wrote a famous and prophetic article in which he argued that protein crystallography had come of age (Phillips 1971). The explosive growth of structural biology since then, and the adoption of its results as guides by molecular and cell biologists and biochemists, have proven him right. I think he has once again found the pulse of our field with his comments on the significance of this meeting. Considering the results presented by the participants and the agreement on the problems that remain and how to tackle them, it seems reasonable to conclude that this symposium does mark a watershed in structural biology: the transformation of a static technique into a dynamic one. But as we contemplate the future we ought to remember the past. History is filled with techniques, from ultracentrifugation through site-directed mutagenesis, that were heralded as providing the answers to all the important remaining questions. No technique can ever do that. As we begin to look at proteins at work in real time at atomic resolution, we will need all the information that kinetics and spectroscopy and simulations and molecular biology can provide if we are to make any sense out of the fuzzy images that we obtain. For our art is still long, and time is fleeting.

In developing the ideas presented here I have relied heavily on discussions with many colleagues. My special thanks to K. Moffat, E. F. Pai, J. Helliwell, R. S. Goody, L. N. Johnson, G. Farber, S. Almo, B. Stoddard and P. L. Howell. I also thank J. Hajdu for helping my laboratory become

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

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