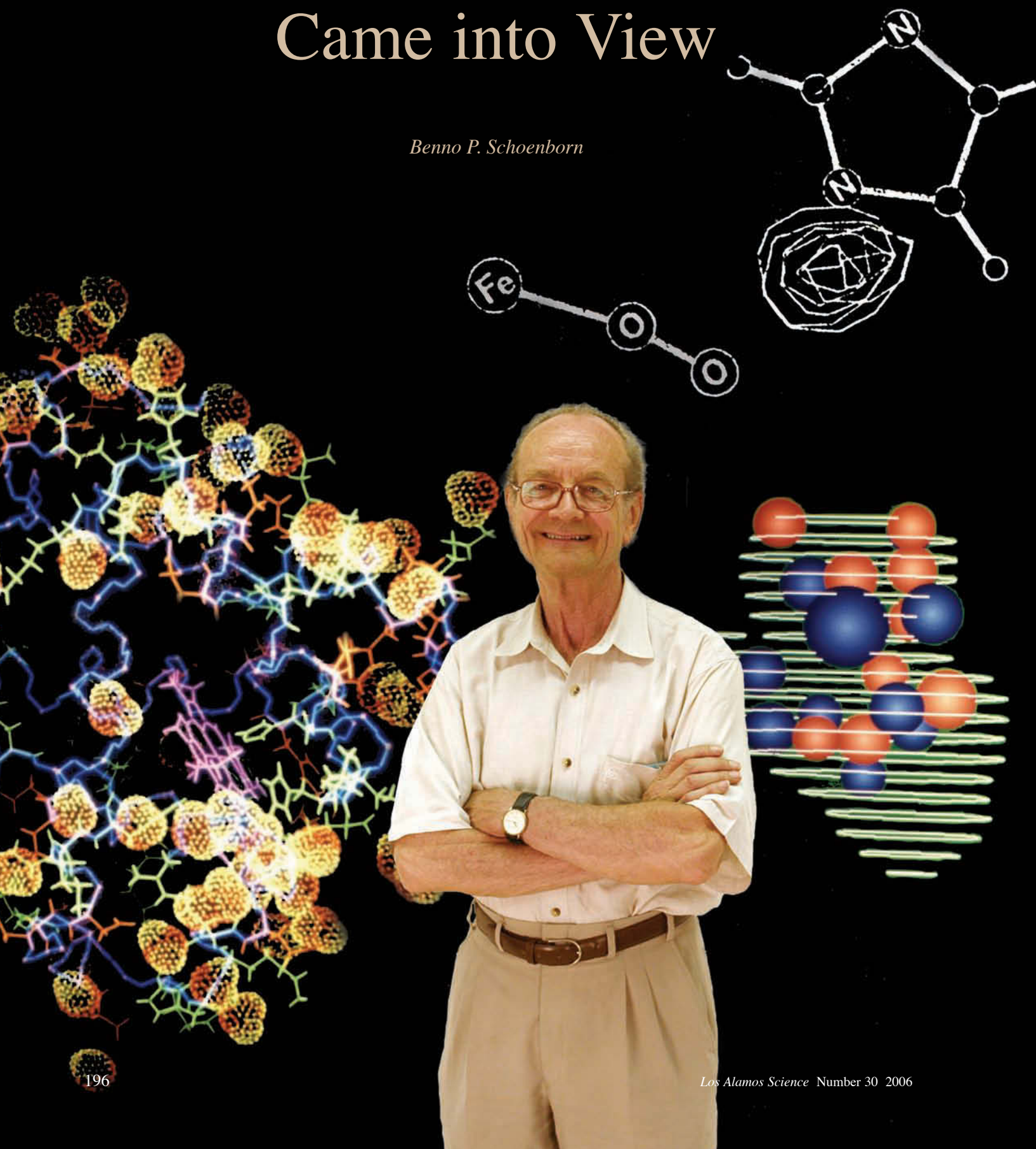
How Single Hydrogen Atoms Came into View

Benno P. Schoenborn



Forty Years Devoted to Neutron Techniques for Structural Biology

The father of neutron protein crystallography and contrast matching recounts his pioneering efforts to make neutrons a powerful tool in structural biology. The new Protein Crystallography Station at LANSCE—where scientists can finally pinpoint the specific hydrogen atoms that an enzyme moves to accomplish its catalytic work—marks the culmination of Benno's extraordinary achievements.

The first mention of using neutron scattering to investigate hydrogen bonding in proteins occurred in the spring of 1965, during a tea time discussion I had with Herman Watson, Uli Arndt, John Kendrew, and Chris Nobbs at the MRC Laboratory in Cambridge, England. I had just finished studying the binding of the inert gas xenon, an anesthetic, to the protein myoglobin and was trying to calculate the binding energies (Schoenborn et al. 1965). Thirty-two atoms were surrounding the xenon atom at van der Waals bonding distances, and structural assumptions suggested that most were likely to be hydrogen atoms. But that was only a guess. Clearly, a map depicting actual hydrogen atom locations was needed. We discussed a number of options for obtaining those locations, but only neutron diffraction was a possibility and a distant one at that because neutron sources have very low fluxes. Subsequent discussions with neutron-scattering experts at the Atomic Energy Research Establishment (Harwell, U.K.) were rather disappointing, and I shelved the idea at that time.

In 1967, during a seminar I gave on the binding of xenon to proteins at the Biochemistry Department at the University of California, Berkeley, I mentioned the neutron approach to mapping hydrogen atoms, and Prof. Daniel Koshland suggested I try to obtain some time on the neutron beam at the High-Flux Beam Reactor at the Brookhaven National Laboratory (BNL). On Koshland's recommendation and with help from Werner Hirs, I obtained a position at BNL and took another leave of absence from University of California, San Francisco, on what many considered a wild goose chase.

I was immediately faced with the problem that neutron sources have low fluxes and that, therefore, big crystals would be needed to obtain a measurable signal in a reasonable amount of time. Fortunately, millimeter-sized crystals are quite easy to grow for sperm whale myoglobin (this protein is the primary oxygen-carrying molecule of muscle tissue). In mid 1968, I obtained a few days of beam time at Walter Hamilton's single-crystal diffractometer, a monochromatic instrument with a single-neutron detector and one of the first computer-controlled data acquisition systems. Before making the diffraction measurement, I soaked the crystal in deuterated buffer (D_2O) to reduce incoherent background scattering from hydrogen atoms. It was already known that soaking would cause D_2O to replace water (H_2O) molecules in the crystal and that some

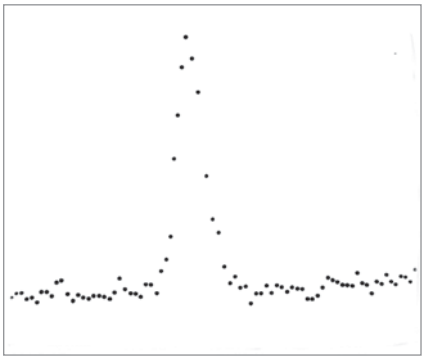


Figure 1. First Peak from Neutron Diffraction on a Protein Crystal
To collect this first reflection (6-0-3) from a single crystal of myoglobin, we used a single-crystal diffractometer on the High-Flux Beam Reactor at BNL. The vertical axis is the scattered intensity, and the horizontal axis is the scanning angle.

hydrogen atoms within the protein structure might be replaced by deuterium atoms as well. After a few hours of playing with the automation, we found the first Bragg reflection (refer to Figure 1), which turned out to be the 6-0-3 plane. Even with the large 25-cubic-millimeter crystal, it took 1 minute to collect each data point in Figure 1, and 15 points were needed to scan the width of a single reflection peak. It became obvious that completing the entire map would take an enormous amount of time. Over the next year, I was able to collect 4800 reflections and demonstrate that protein crystallography with neutrons was indeed possible and that hydrogen atom locations with a 2-angstrom resolution could be determined easily (Schoenborn 1969).

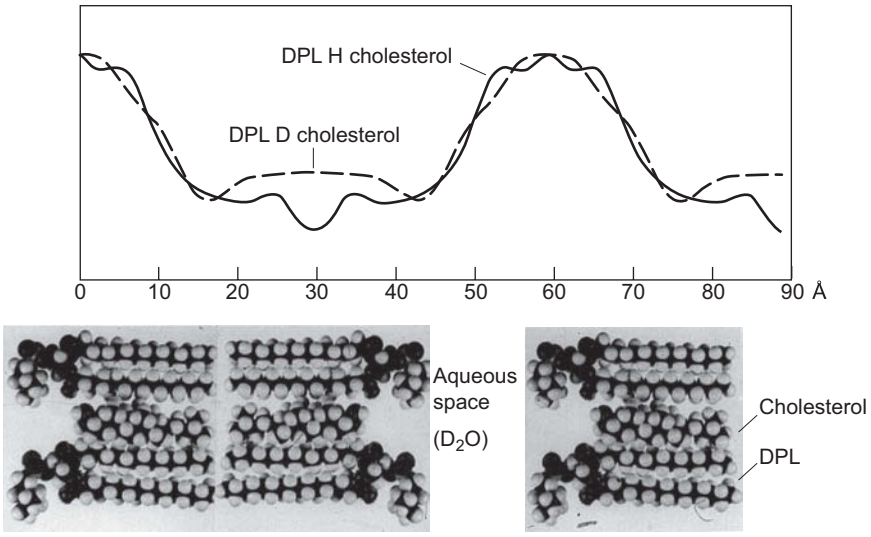


Figure 2. Neutron Scattering Data for a Reconstituted Membrane
Results are shown of the measured neutron-scattering density as a function of distance through a reconstituted membrane of dipalmitoyl lecithin with and without cholesterol. (Reprinted from *Chem. Eng. News*, January 24, 1977, 55, pp 31–41. Published 1977 American Chemical Society).

Expanding the Use of Neutrons in Structural Biology

I soon realized that the different neutron-scattering lengths of deuterium ($b = +6.65$) and hydrogen ($b = -3.71$) could be used to enhance contrast in neutron diffraction and neutron-scattering studies of biological structures, especially if only gross features were to be determined. This idea is analogous to the use of heavy (high Z) atoms to enhance contrast in x-ray scattering. By using different percentages of D_2O and H_2O in the solvent, one can match the average scattering-length density of the solvent to one or another component of a structure. The component then looks like the solvent and fades into the background whereas the components of different scattering-length density stand out.

I did the first diffraction experiment using contrast (or density) matching in collaboration with Donald Casper. It was on frog sciatic nerves. After soaking the excised nerve in D_2O and mounting it, we observed an extraordinarily large signal, of about 80 percent of the collimated incident beam. At first, we looked for a leak in the shielding and collimation since we were working very close to the direction of the incident beam. We found nothing wrong and eventually realized that we were observing a very intense 2-0-0 Bragg reflection. The reflection was caused by constructive interference from the membrane bilayer structure, which approximated a positive neutron-scattering layer (D_2O) next to a negative neutron-scattering layer composed of one carbon and two hydrogen atoms ($n CH_2$). The two layers were of about equal thickness. This work led to the development of and a patent for multilayer monochromators (Schoenborn et al. 1974), which select single-energy neutrons and x-rays and are therefore widely used now in x-ray and neutron-diffraction equipment. Similar principles are used today in studying surface layers with neutron reflectometry. (For example, see studies of the hydrophobic effect on page 164 of this volume.)

The technique of combining diffraction experiments with H_2O/D_2O exchange through soaking was soon used by some scientists to elucidate membrane structures such as the one shown in Figure 2 (Zaccai et al. 1975) and large biological complexes, including the structure of filamentous bacteria. A novel use of hydrogen/deuterium exchange, which involved extracting and perdeuterating whole proteins and then reinserting them into ribosomes, was proposed by Donald Engelman and Peter Moore. Together, we were hoping to elucidate the structure of ribosomes, the complex biological machines that manufacture proteins from genetic instructions (Moore et al. 1974). Perdeuterating means producing the protein in vivo by using genes cloned in *E. coli* and deuterated water and nutrients at every step. Figure 3a illustrates our strategy for perdeuterated proteins in neutron scattering studies of the ribosome structure. Our strategy reached

(a)

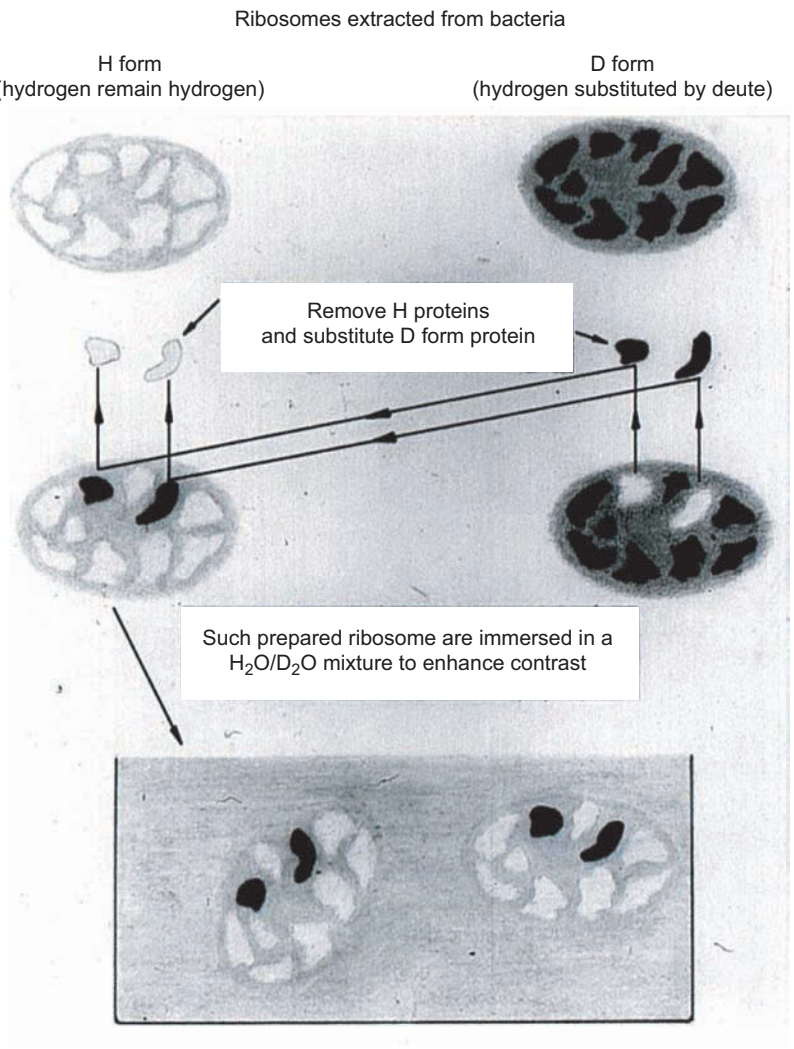
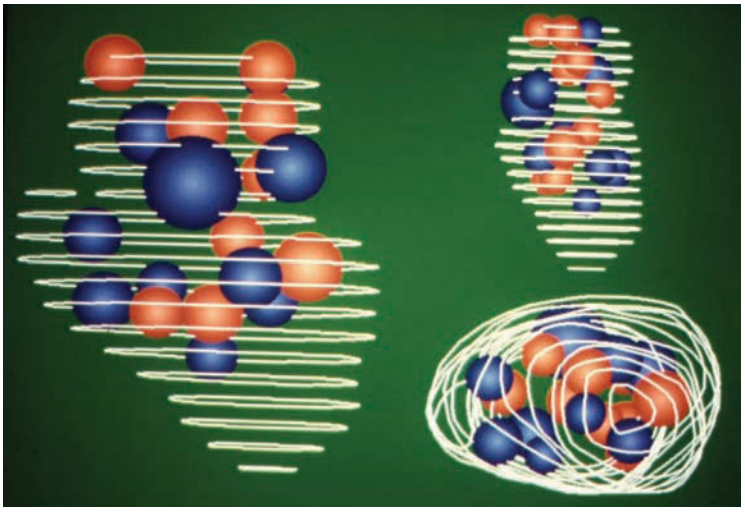


Figure 3. Strategy for Determining Protein Arrangement in the 30S Ribosomal Subunit
(a) Shown here schematically is the removal of pairs of proteins, their reinsertion in a perdeuterated form, and then the use of contrast matching and small-angle neutron scattering to make their sizes and relative positions visible in the 30S ribosomal subunit of *E. coli.* (b) Three orthogonal views are shown of the low-resolution structure of the 30S ribosomal subunit. (Courtesy of *Neue Zuercher Zeitung*, April 18, 1979, #89.)

(b)



fruition 13 years later, when we completed the structure of the ribosomal subunit shown in Figure 3b (Capel et al. 1987).

With the rapid growth of neutron science in structural biology during the 1970s and 1980s, the nuclear reactor sources at BNL and the Institut Laue-Langevin in Grenoble, France, emerged as two major centers of activity.

Seeing Hydrogen Atoms with High-Resolution Neutron Diffraction. Once two-dimensional (2-D), position-sensitive detectors had been developed, we were able to use high-resolution neutron diffraction at BNL to reveal hydrogen atom positions on both the protein and the solvent. We were also able to distinguish nitrogen from carbon or oxygen because of their different scattering lengths. Our neutron-diffracton data on the positions of nitrogen atoms in proteins were used to resolve ambiguities in x-ray diffraction studies, particularly the orientation of

the amino acid residues known as histidines (see Figure 4). To reduce background scattering during data collection and enhance the localization of exchangeable hydrogen atoms, we exchanged the H_2O solvent in most single crystals with D_2O, using a soaking procedure similar to that for the membrane studies. This soaking exchange procedure allowed us to observe the exchange rate of labile hydrogen atoms. Labile hydrogen atoms that do not exchange indicate some structural stability in a given region. Structural studies as a function of soaking times give insight into diffusion effects within proteins. Thus we realized almost immediately that H_2O/D_2O exchange could provide some information on protein dynamics and enhance protein-solvent contrast in diffraction and small-angle neutron-scattering experiments.

The solvent analysis in myoglobin resolved a major controversy between nuclear magnetic resonance (NMR) studies that showed only three water molecules bound to myoglobin and x-ray studies that showed over 80 bound water molecules. The neutron studies proved that, indeed, only three water molecules are bound to the protein with three hydrogen bonds, which makes them irrotationally bound, that is, they are unable to rotate. All other water molecules exhibit only two or fewer hydrogen bonds and thus can rotate, tumble, and jump about (see Figure 5). Studies with x-rays reveal only the central oxygen atom of water molecules and therefore cannot provide any indication of permanent binding. By contrast, NMR studies locate only irrotationally bound water molecules.

Need for Improved Instrumentation

Collecting neutron-diffraction data had a very slow start because the low flux at neutron reactors made it time-consuming. Because increasing the flux was not a real possibility, it seemed that developing improved instrumentation would be the only way to increase data collection. The 2-D position-sensitive detectors previously discussed are an example of improved instrumentation. They allow studying macromolecules such as myoglobin routinely. It seemed that increased data-collection rates would be possible only by improved instrumentation, particularly by increasing detector coverage. But then another possibility opened up. In the 1990s Tom Kitchens and John Browne, then Physics Division Leader, invited me to Los Alamos, and I became aware of the power of spallation neutron sources to provide dramatic increases in usable neutron flux. Following that visit, I slowly developed plans for a protein station at a spallation source. With the help of Morton Bradbury of Los Alamos, these ideas were presented to the Department of Energy's Office of Health and Environmental Research and eventually led to the development of the Protein Crystallography Station (PCS) at LANSCE (Schoenborn 1996).

Designing a PCS for a Spallation Neutron Source

One way to increase neutron flux and thus increase data-collection rates is to apply the Laue diffraction technique, which uses white radiation instead of monochromatic radiation. Unfortunately, this technique increases the background significantly and degrades data quality. Therefore, it has limited use at reactor-based installations. The Laue technique can, however, be advantageously tailored to spallation neutron sources at accelerators, which produce extremely short bursts, or pulses, of spallation neutrons having a broad range of energies. As the neutrons in a pulse travel down the beam line to the spectrometers, they develop a time-dependent wavelength spread with the shortest-wavelength (highest-velocity) neutrons arriving first and the longest-wavelength (lowest-velocity) neutrons arriving last. Data can be collected as a function of time, yielding sequential diffraction data sets at increasing wavelengths and each set mimicking a monochromatic diffraction experiment (Schoenborn 1992).

Maximizing Neutron Flux. Maximizing flux and reducing background were the main criteria guiding the design of a protein crystallography station for LANSCE. Neutrons having relatively low energies (long wavelengths) suitable for diffraction experiments of all kinds are typically produced by sending the high-energy spallation neutrons through a moderator containing light elements such as water. The interactions with those light elements cause the neutrons to slow down. The typical moderators at LANSCE contain poison layers (elements that absorb neutrons) and decoupling layers to maintain a pulse containing a narrow band of neutron wavelengths for high-resolution diffraction experiments. Unfortunately, this narrow wavelength bandwidth is achieved at the expense of the neutron flux.

Our calculations showed, however, that high-wavelength resolution is not necessary for protein crystallography. Rather, a wavelength bandwidth of +/–0.12 angstrom is ideal for protein crystallography. This wavelength bandwidth is determined by the typical disorder (mosaic) in protein crystals of +/–0.1°. To achieve this broader bandwidth, we studied the placement of poison layers surrounding the moderator by performing Monte Carlo calculations with the Lahet code (Schoenborn 1992, 1996). The backscattering moderators at

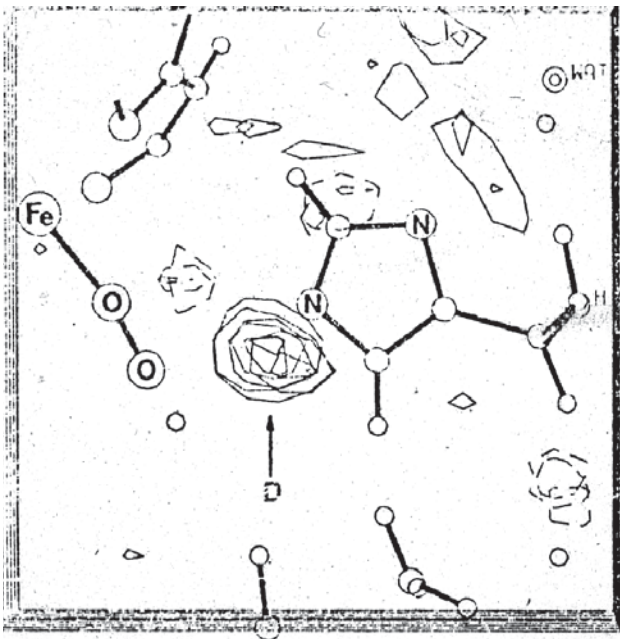


Figure 4. Single Hydrogen Atom Binding Oxygen to Myoglobin
A neutron-density map (fine lines) is superimposed on a ball-and-stick model of a histidine residue in oxygen-carrying myoglobin. The map shows a hydrogen atom bound to the nitrogen atom of the histidine residue and indicates a hydrogen bond between an oxygen atom and the histidine. These structural data were the first to reveal the biological role of a specific hydrogen bond.

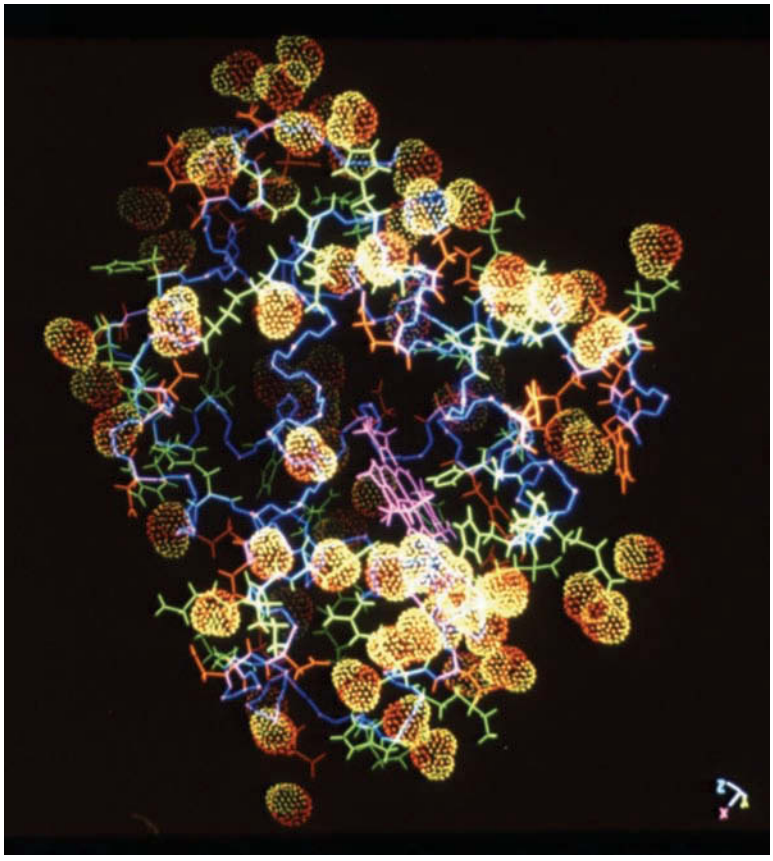


Figure 5. Water Molecules on Myoglobin
This neutron-density map showing 87 water and ion molecules (space-filling dotted clusters) are superimposed on the surface structure of myoglobin. The iron (heme) group is purple; the backbone is blue; the acidic residues of the amino acid are orange; and the basic residues of the amino acid are green.

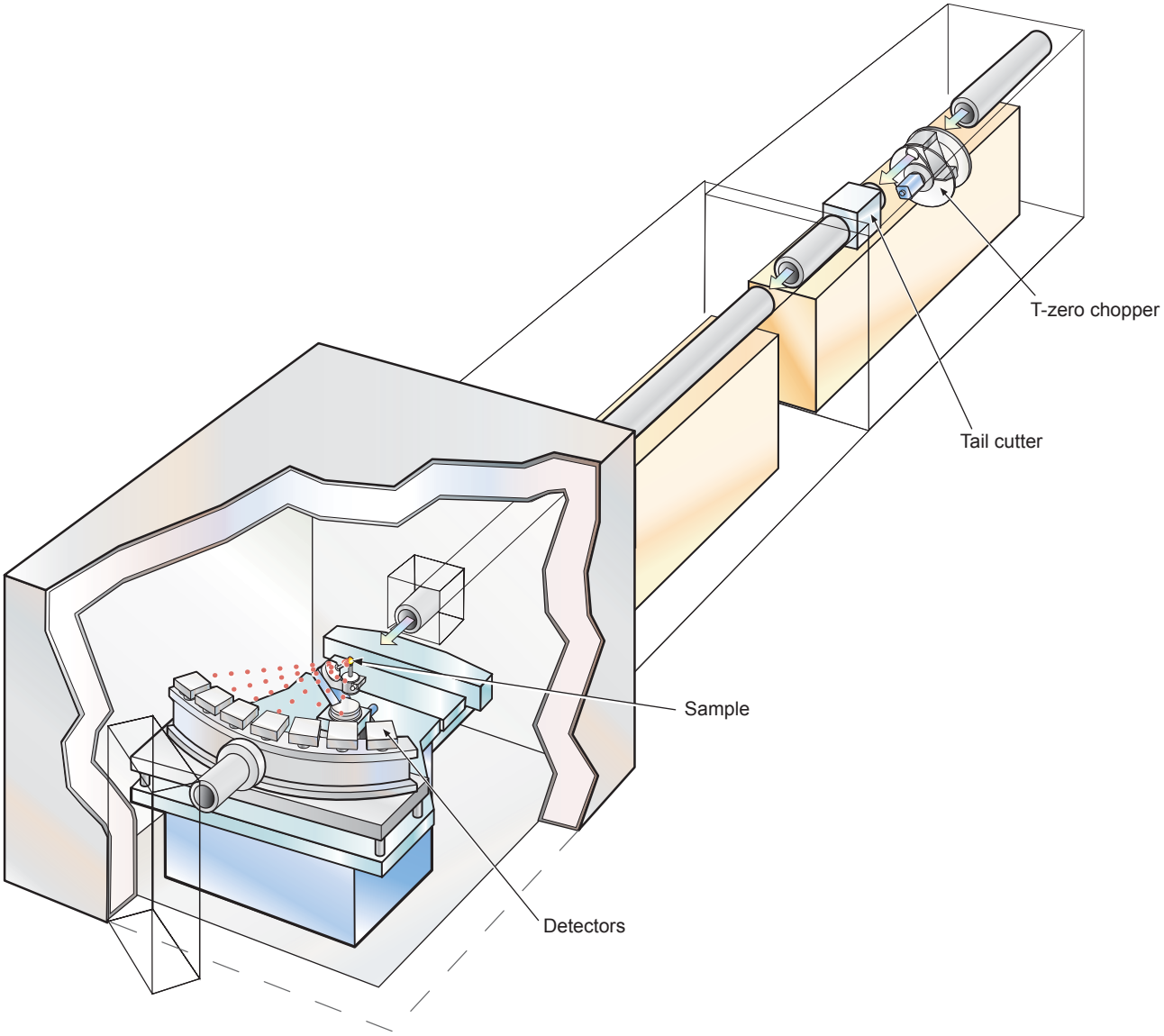


Figure 6. The Protein Crystallography Station at LANSCE
This drawing shows the layout of the PCS at LANSCE's Lujan Center. The large cylindrical position-sensitive detector was built by the Instrumentation Division at the BNL for the PCS at LANSCE. This collaboration marked the beginning of a productive relationship with the Instrumentation Division.

LANSCE were thus modified to produce pulses with a broader bandwidth and increased flux. With a pulse frequency of 20 hertz, a 28-meter flight path allows us to collect data between 0.6 and 7 angstroms with 96 time slices and a bandwidth of about +/– 0.1 angstrom. With a required beam divergence of +/–0.1°, this 28-meter flight path allows the complete view of the moderator; that is, it collects all neutrons within the given angular divergence. The beam line consists of a conical focusing tube with beam-defining apertures placed at calculated distances aimed at maximizing neutron flux at the given divergence. The beam tube itself is lined with boron poly inserts to minimize beam scattering and thus background. A large concrete shield surrounds the evacuated beam tube to reduce room background. To avoid any crystal and detector damage a beam chopper eliminates the front end of the pulse with its fast neutrons and gamma radiation. Radiation with wavelengths longer than 7 angstroms is eliminated by a second disk on the chopper.

Minimizing Background. Background radiation on the detector arises from a number of contributors: room background, air scattering from the direct beam, crystal scattering from incident radiation not in

the diffraction (Bragg) condition, and incoherent scattering from hydrogen atoms.

Room background radiation has been minimized by a well-shielded beam pipe and experimental hutch. Both are lined with boron poly and steel. Air scattering from the direct beam can be reduced by minimizing the air gap between the neutron collimator, the crystal, and the detector. A helium or argon gasbag between the crystal and the detector is unfortunately not easy to achieve without structural elements interfering with diffracted beams. Protein crystals often contain more than 50 percent water, which produces large incoherent scattering from the hydrogen atoms. As discussed earlier, this incoherent scattering can be eliminated by soaking the crystal in heavy water to exchange most labile hydrogen atoms in the proteins and replace all hydrogen atoms in water with deuterium. The ultimate background-elimination scheme involves the exchange of all hydrogen atoms by perdeuterating the protein (Shu et al. 2000). Modern biochemical techniques have been perfected to allow perdeuteration of most proteins if the clone of the corresponding gene is available. We have now established a deuteration laboratory at LANSCE to produce fully deuterated proteins.

Operation of the PCS. This first neutron PCS based on the previously-described design using time-of-flight techniques has now been operating as a user facility for nearly three years and has produced superb data for a number of proteins, notably D-xylose isomerase and dihydrofolate reductase (see Figure 6 as well as the preceding article “Finding out How Enzymes Work” on page 186). ■

Acknowledgments

I wish to acknowledge the numerous students and colleagues that made the described work possible. I thank Paul Langan and Gayle Greene for major contributions in the development and fabrication of the Protein Crystallography Station at Los Alamos National Laboratory.

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Further Reading

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