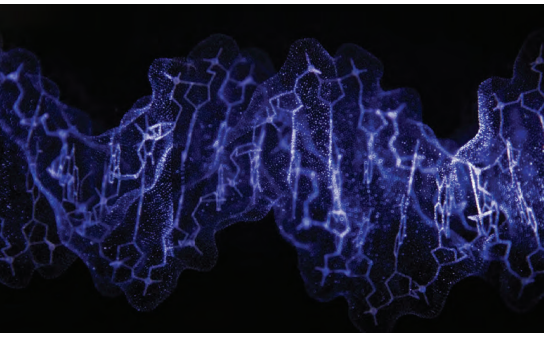
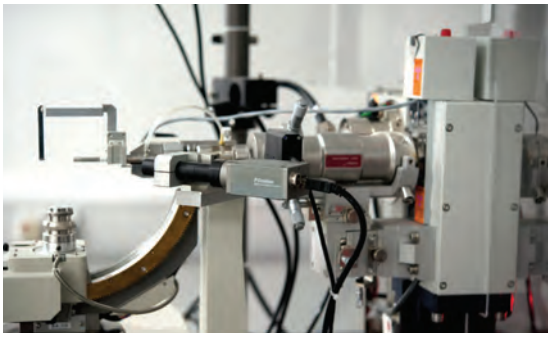
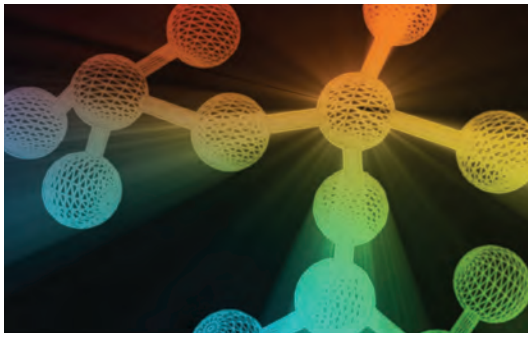
Crystallography: Understanding the Nature of Chemical Bonds and Molecular Structure

A white paper examining how crystallography yields key insights into the molecular structure and properties of organic and inorganic material, the latest methods for imaging new structures, and how it drives the development of new materials by chemical scientists across a variety of disciplines.

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Crystallography: Understanding the Nature of Chemical Bonds and Molecular Structure

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ABOUT THE AUTHOR

Kellyn Betts has written about environmental science and chemistry for two decades for publications including *Chemical & Engineering News, Environmental Health Perspectives, Environmental Science & Technology, Natural History,* and *Science News for Students*. She graduated from the Science Health and Environmental Reporting Program at New York University's Graduate School of Journalism and studied environmental science at Westminster College.

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X-ray crystallography machine. Jon Wilson/Science Photo Library

I. INTRODUCTION: A CENTURY OF PROGRESS

Over the last century, crystallography has dramatically expanded human understanding of the underlying structure and function of both natural substances and man-made materials. At the same time, the science has improved our ability to capitalize on crystals. The crystals in toothpaste, the liquid crystal displays of alarm clocks, mobile phones, and computer screens, and the crystalline beads in catalytic converters in cars all help improve everyday life.[1] Virtually any image of a molecule you've ever seen has been generated by crystallography.[2]

Crystallography originated with studies made possible by X-rays a century ago, but modern crystallographers use many other methods, including neutron diffraction, electron crystallography, and molecular modeling.[3] The future for scientists with a firm grounding in crystallography is bright in terms of both expectations for advances in the science and employment outlook.

II. OCCUPATIONAL & EMPLOYMENT OUTLOOK

Crystallographers investigate how the atoms in a material are arranged in order to understand the relationship between atomic structure and properties of these materials. They work in many disciplines, including biology, chemistry, geology, materials science, metallurgy, and physics.[4] Crystallographers study diverse substances, from living cells to superconductors, from protein molecules to ceramics. It is a field many believe to be particularly welcoming to women because much of the work is collaborative.[5]

Studying crystallography enables chemists and other scientists to use tools that can bring to light the invisible molecules that make up almost every aspect of our world, including everyday substances, high-tech materials, minerals, pharmaceuticals, and biological material such as proteins.[6] The properties and inner structures of crystals help scientists to examine the arrangement of atoms in the solid state, and this knowledge is used in fields including chemistry, physics, and biology.[7]

The techniques that crystallographers use include X-ray, neutron, and electron diffraction for identifying and characterizing solid materials, including biological samples. To construct detailed models of the atomic arrangements in solids, crystallographers often employ information from other analytical techniques, including X-ray fluorescence, spectroscopic techniques, microscopic imaging, and computer modeling and visualization. Taken together, data resulting from these analyses can provide valuable information on

a mineral, material, or biological sample's chemical makeup, polymorphic form, defects or disorder, and electronic properties. It can also help show how solids perform under temperature, pressure, and stress conditions.[8]

How crystallographers are employed has shifted significantly over the past few decades. Twenty years ago, there were many academic labs dedicated to X-ray crystallography because only specialists with years of training and expensive equipment could perform the complex techniques required in crystallography. In the intervening years, many labs have shifted their focus to specific scientific inquiries. X-ray crystallography has become one of a suite of tools used to find answers to these questions.[9]

GEOSCIENCES

The U.S. Bureau of Labor Statistics (BLS) continues to track the employment of crystallographers with geoscientists; employment for that group as a whole is expected to grow 16% between 2012 and 2022, significantly better than the national average of 11% in this time frame.[10] The need for energy, environmental protection, and responsible land and resource management is projected to spur future demand for geoscientists, including crystallographers. Greater demand for clean energy is also expected to increase the need for biochemists who research and develop alternative energy sources, such as biofuels, and crystallography can also play a role there as well.[11]

How crystallographers are tracked by the BLS may need to be updated, according to the American Chemical Society (ACS). Research by ACS shows that, although crystallographers historically have been associated with the geosciences, metallurgy, and ceramics engineering, the largest areas of demand for crystallographers today are in the medical and life sciences.[12] That has some positive ramifications. The BLS's projections for biophysicists and biochemists are even rosier than for geoscientists: 19% employment growth between 2012 and 2022.[13] The aging baby-boomer population and the demand for lifesaving new drugs and procedures to cure and to prevent disease likely will drive demand for biochemists and biophysicists involved in biomedical research. The demand for both geoscientists and biochemists and biophysicists is expected to grow at a faster pace than demand for chemists, which the BLS predicts to grow by 6% in the same time frame.[14]

STRUCTURAL BIOLOGY

Because crystallography has become an important tool for studying structural biology, many crystallographers now refer to themselves as structural biologists.[15,16] Proteins and other biological materials (including viruses) may be crystallized to aid in studying their structures and composition. Many important pharmaceuticals are administered in crystalline form, and detailed descriptions of their crystal structures provide evidence to verify claims in patents.[17] Crystallography can also help scientists understand how to devise new drugs and improve existing ones.[18]

INSTRUMENTATION

Crystallography specialists also find opportunities working in instrument and software development, customer support for instrument manufacturing companies, user support at national laboratories, or working in crystal-growing laboratories.[19] When employed by instrument manufacturers, crystallographers are often involved in customer sales and support functions, including instrument repair and assisting customers with special projects. Staff crystallographers at the national laboratories develop and maintain leading-edge research instruments and software capabilities. They may assist users who visit the lab in setting up and running experiments using specialized techniques, including synchrotron X-ray diffraction and neutron diffraction. Universities employ staff members to maintain and operate their research laboratories, as well as to train students to use the instruments.[20]

Crystallographers may develop instrumentation and software for collecting, analyzing, and visualizing data, and for translating these data into crystal structure models. Some crystallographers maintain and develop archival databases at industrial and academic institutions, as well as some nonprofits and government laboratories.[21]

SERVICE LABORATORIES

Although the number of service laboratories that provided crystallography services was on the wane for a time, there is a growing list of contract companies that specialize in crystallography.[22] Service laboratories hire diffraction technicians to prepare and catalog samples, collect data, and prepare routine reports on the results. Technicians may also be called on to perform routine instrument maintenance and simple repairs.[23]

FORENSICS

Forensics laboratories use crystallography to investigate cases involving product adulteration or counterfeiting. Those with crystallography training working in such labs may identify minerals, metals, or other materials found at crime scenes. They may also aid in the investigation of industrial accidents by identifying corrosion products or other residues found at such sites to help verify the events leading up to the accidents.[24]

CRYSTAL GROWING

Because crystallography requires the substances being analyzed to be crystallized, crystal-growing specialists use a variety of techniques to produce crystalline forms of compounds for use in research or manufacturing. They may be experts in working with hard-to-crystallize materials, or they may grow crystals to exacting specifications for use in computer chips, solar cells, optical components, or pharmaceutical products.[25]

MATERIALS SCIENCE

Crystallographers have been associated with materials design, metallurgy, and ceramics engineering.[26] One of the principal factors determining the properties of any material is its crystal structure. New techniques for investigating materials' structures and defects are constantly being developed using X-ray, neutron and electron diffraction methods of

crystallography.[27] The work of crystallographers is crucial for designing new materials, such as quasicrystals and grapheme, which are expected to play important roles in the future.

AND MORE

Crystallographers also can play a role in space exploration, farming, art conservation, formulating beauty products, and improving how foods taste.[28] The science laboratory on NASA's Curiosity rover, which began collecting and analyzing samples on Mars in 2012, includes an X-ray diffractomer, which provided the first quantitative data on the minerals that make up the Martian surface.[29]

Fewer and fewer scientists may be calling themselves crystallographers now and in the future, but the use of X-ray diffraction as a routine tool is expected to accelerate in nanotechnology, synthetic chemistry, biochemistry, and cell biology.[30] Scientists will also help determine how crystallography is used in the future, and the possibilities are wide open.

III. A BRIEF HISTORY

The modern discipline of crystallography is an outgrowth of the X-ray diffraction experiments on crystals conducted by Max von Laue in 1912 and William Henry Bragg and William Lawrence Bragg in 1913.[31,32,33,34,35] Von Laue's achievements were recognized with the Nobel Prize in physics in 1914, and the Braggs earned the same honor in 1915. Their discoveries enabled the scientific study of the arrangement of atoms in solids.

Crystallography was what initially allowed scientists to illuminate the basis of ionic, covalent, metallic, and hydrogen bonds. The first crystal structure to be definitely identified through crystallography was that of table salt. X-ray diffraction has also been a major driving force in modern materials science and other fields.[36] Scientific achievements related to, or involving the use of, crystallography have led to the award of 29 Nobel Prizes.[37]

Among the first of the landmark achievements enabled by crystallography was the 1913 confirmation of the tetrahedral structure of carbon atoms in a diamond. In 1923, the first organic molecule, hexamethylenetetramine, was imaged.[38] This showed that molecules, not just atoms, can make up the repeating elements of a crystal. Two years later, the determination of the structure of silicate minerals, beginning with quartz, was fundamental to the field of mineralogy.[39] A few years later, researchers showed that polycrystalline powder samples also diffracted X-rays, which opened up X-ray analysis to many more types of crystals.[40,41] This was also the time that X-ray diffraction revealed the structure of hexamethylbenzene, which provided insight into the nature of aromatic compounds.[42,43] Scientists also used the then-new technique to resolve debate about the structure of water in its crystalline form.[44,45]

Over the years, the science of crystallography has provided important information about biological structures such as enzymes, proteins, and active sites. Modern drug discovery owes a huge debt to the determination of the structure of penicillin, which was completed by a group led by Dorothy Crowfoot Hodgkin, of the University of Oxford, during World War II but suppressed from publication. The structure's 1946 publication marked the first time the structure of a whole molecule had been calculated from X-ray data.[46,47]

Some of crystallography's initial findings sparked objections from chemists at the time.[48,49] By 1951, however, the acceptance of crystallography as a discipline by chemists was such that a group attending the Second International Congress of Crystallography agreed that the science was opening "a new window… into the realm of organic structure through studies at low temperatures." At the time, scientists at the conference were discussing how to analyze protein structures using crystallography.[50]

Soon after, a research group published a widely read paper that described how, in principle, X-ray diffraction could be used to directly determine a protein's structure.[51] The recognition of the double-helix structure of DNA was aided by Rosalind Franklin's 1952 X-ray diffraction images of the DNA molecule, especially the image known as photo 51.[52,53] This was also around the time when the work of a Dutch crystallographer made the first definitive determination of a chiral structure with mirror-image forms, sodium rubidium (+)-tartrate tetrahydrate.[54,55,56]

The 1950s also saw the birth of structural biology, which is widely credited to the X-ray crystallography studies of hemoglobin conducted by Max Perutz, an Austrian-born molecular biologist at the University of Cambridge in the U.K.[57] In 1956, Dorothy Crowfoot Hodgkin led a team that capitalized on the presence of cobalt in vitamin B-12 to image its structure for the first time, the most complex molecule yet tackled by crystallographers.[58,59] By 1958, the first protein, myoglobin, a blood protein from a sperm whale, was imaged.[60] In 1960, Perutz was finally able to publish the structure of hemoglobin, a feat requiring the analysis of thousands of reflections with the aid of the computers available at the time.[61,62] A few years later, in 1965, hen egg whites were used as the source of the first image of an enzyme.[63,64]

Improvements in technology over the years have accelerated the pace of discovery. The successful reconstruction of the three-dimensional structure of the tail of the T4 bacteriophage from a limited set of electron microscopy images inspired the development of electron crystallography.[65] In the 1970's, scientists began to use X-rays generated by a synchrotron in crystallography studies. The first image published using this source was a study of insect muscle at the German Electron Synchrotron (DESY) in Hamburg.[66] During this decade, crystallography helped explain why polymeric organic compounds could conduct electricity to produce what we now know as semiconductors.[68] In 1978, the first atomic-scale image of a complete protein was made of a plant virus, the tomato bushy stunt virus.

In 1984, researchers identified the first crystals with atomic arrangements that do not repeat exactly.[69] The existence of such quasicrystals defied the current understanding of the nature of crystals. In 1985, the first structure of a membrane protein, the photosynthetic reaction center of a photosynthetic bacterium, was imaged.[70] The resolution of crystallographic images of proteins passed a critical threshold for discriminating single atoms in the 1990s.[71] This was also the decade when X-ray crystallographers had their first successes imaging short-lived, excited-state molecules, such as the nitroprusside ion.[72,73]

In 2000, the improved resolution possible with crystallography led to more images that rocked the science world by showing the structure of the ribosome, the molecular machine that assembles proteins from instructions encoded in DNA.[74,75] Also beginning in 2000, the U.S. National Institutes of Health's Protein Structure Initiative (PSI) inspired more than 5,300 distinct protein structures to be solved. The initiative also spurred innovations in crystallographic methods.[76] Crystallography played a key role in the 2004 discovery that the main mineral of the lower portion of the earth's mantle (which lies between the top crust and molten core), iron-bearing magnesium silicate ($(Mg,Fe)SiO_3$) perovskite, transforms to a compact configuration known as post-perovskite at conditions similar to those at the core–mantle boundary.[77,78]

Today, tens of thousands of new structures are imaged every year, and newer X-ray sources promise images of challenging proteins that are hard or impossible to grow into large crystals.[79]

IV. THE SCIENCE OF CRYSTALLOGRAPHY

The scientific study of crystals dates back centuries.[80] Scientists have long understood that the constituent molecules that make up a crystal are arranged in an ordered pattern. Over the years, mathematically inclined researchers worked out all of the possible distributions of atoms to account for all the theoretically possible classes of crystals.[81]

William Roentgen's discovery of X-rays in the late 19th century also played a key role in creating the science of crystallography. The discovery inspired many scientists to study the behavior of X-rays.[82] Max von Laue and William Henry and William Lawrence Bragg (a father-son team) were among these scientists.

Von Laue's original insight was that when X-rays passed through a crystal, they would scatter off the atoms in the sample and then interfere with each other like waves passing through a breach in a shore wall. Von Laue and his colleagues proved his theory in 1912 with a copper sulphate crystal.[83]

The mathematical equation used to determine how the locations and intensities of the spots seen in a diffraction pattern can be transferred back to identify the smallest repeating unit in

a solid material is known as Bragg's Law.[84] It is based on William Lawrence Bragg's insight that the spots shown in von Laue's experiment were due to the reflection of X-ray pulses by sheets of atoms in the crystal.[85] As von Laue and the Braggs determined, the waves add to each other in some places; in others, they cancel each other out. The location of the atoms that scattered the original X-rays can be back-calculated from the resulting diffraction pattern. These ideas inspired William Henry Bragg, William Laurence's father, to build the first X-ray spectrometer.[86] Using that new equipment to investigate the structure of crystals such as table salt revealed that crystals could be made up of repeating atomic lattices rather than molecular ones.[87] With the help of spectrometry, researchers can now solve the structures of materials with more than 1,100 atoms per unit cell, such as $Gd_{117}Co_{56}Sn_{112}$.[88]

Crystallographic studies allow the determination of the positions of atoms in solids and lead to understanding of structure-property relationships of materials, which have health, environmental, energy, and technological applications.[89] Over the last century, the discipline has informed almost every branch of the natural sciences.[90]

POWDER CRYSTALLOGRAPHY: INSIGHTS INTO MINERALS

Many of the insights that crystallography has produced about the nature of minerals result from powder crystallography.[91] This branch of crystallography is based on the recognition that the diffraction of X-rays is due to their interaction with the electrons of the atoms in the lattice. Because electrons are in a defined spatial location relative to the nucleus, this suggests that diffraction occurs in randomly oriented crystals.[92,93] Powder diffraction spectra provide a means to directly compare scattered intensities coming from all crystal planes, which was not possible with early studies using the Bragg spectrometer.[94]

In the 1930s, researchers began to examine more complex structures, thanks to the insight that the intensities of the diffractions caused when X-ray radiation is scattered by the atoms present in a crystal can be used to determine the crystal's interatomic distances.[95,96] During the same decade, researchers recognized that neutron rays could be diffracted and scattered similarly to X-rays.[97] By the end of the 1940s, the instruments built for nuclear fission research during World War II showed that neutrons interact equally well with light and heavy elements (unlike X-rays, which scatter against electrons and barely register a light element such as oxygen).[98]

Crystallography has often surprised scientists by what it reveals about structures. For example, before X-ray crystallography definitely showed the structure of ferrocene to be akin to the layers of a sandwich, many scientists didn't believe that such a shape was likely.[99,100]

IDENTIFYING AND STORING STRUCTURAL DATA WITH X-RAY CRYSTALLOGRAPHY

Crystallography is now routinely used to determine the three-dimensional structures of biological molecules to provide information about biomolecular processes. In fact, most of the structures included in the Protein Data Bank (PDB) archive maintained by scientists at universities in New Jersey and California were determined using X-ray crystallography.[101]

The existence of open repositories for sharing crystallographic data is an important support for the science of crystallography. When the PDB opened in 1971, receiving and distributing structural data required shipping paper punch cards or magnetic tape, and the computer hardware and software needed to visualize or analyze these data were rare.[102] Edgar Meyer of Brookhaven National Lab, where the PDB was housed, created the first general software tools for handling and visualizing protein structural data.[103] A few years later, he published software for storing and searching protein structures in the PDB.[104] At the end of the 1970s, scientists in the U.K., founded the Collaborative Computational Project Number 4 (CCP4) to provide protein crystallographers with software tools for processing and analyzing crystallographic diffraction data. CCP4 evolved into a suite of programs that are still in use today.[105,106]

X-ray crystallography can provide very detailed atomic information about biological molecules. It can show every atom in a protein or nucleic acid, along with atomic details of ligands, inhibitors, ions, and other molecules that are incorporated into the crystal.[107] Max Perutz of the University of Cambridge played a key role in discovering how X-ray diffraction could be used for the direct determination of a protein structure. Perutz and his colleagues used the isomorphous replacement method to first solve hemoglobin's structure. This method involves introducing "heavy" atoms (such as mercury) into the crystal, and taking advantage of the complexes formed between the heavy-atom-containing compounds and the free molecule groups present in the protein. By comparing the differences in intensities between the diffraction spots from the heavy-atom-containing crystal and the normal crystal, researchers determine the location of the heavy atoms and from that information the phases of the X-rays. As William Lawrence Bragg pointed out, this heavy-atom technique works because "the molecule takes no more notice of such an insignificant attachment than a maharaja's elephant would of the gold star painted on its forehead."[108]

X-ray crystallography works particularly well for determining the structures of rigid proteins that form ordered crystals. But some other proteins are more difficult to study by this method, and some proteins cannot be crystallized at all. For example, because crystallography relies on having many molecules aligned in exactly the same orientation, like a repeated pattern in wallpaper, flexible proteins can be difficult to study. The flexible portions of protein will often be invisible in crystallographic electron density maps because their electron density will be smeared over a large space.[109]

X-ray crystallography's broad utility for scientists can even extend to the business and legal realms. For example, studies using X-ray crystallography recently showed that a drug patented by the Oncoceutics company was not based on the correct chemical structure. The Scripps Research Institute California has applied for a patent on the corrected structure and licensed it to another company, Sorrento Therapeutics. This may lead to an unprecedented legal case.[110] Experts believe that crystallography is also key to driving innovation and helping grow economies throughout the world.[111]

CUTTING EDGE X-RAY SOURCES AND THEIR IMAGING PROMISE

In recent years, instruments that enable new sources of X-rays have revolutionized the field. Neutron sources, synchrotrons, and X-ray free-electron lasers are enabling diffraction measurements to be made that were not possible just a few years ago. These technologies can also help characterize materials that do not even form single crystals.[112]

NEUTRONS

Scientists have known that neutron rays could be diffracted and scattered similarly to X-rays since the 1930s.[113] Applying that theoretical knowledge was possible by the end of the next decade, thanks to the advances in the understanding of nuclear fission achieved during World War II, and the instruments built to support that research. The then-new field of magnetic crystallography capitalized on the recognition that unlike X-rays, neutrons interact equally well with light and heavy elements.[114,115]

Neutron scattering research was initially confined to a limited number of nuclear reactors. The practice of allocating time at these facilities for research, which began in the U.K. in the mid-1950s and was quickly adopted by other countries, led to their widespread availability by the early 1970s, when the first facility dedicated to basic research opened in France.[116] Many neutron facilities now exist throughout the world, and neutron scattering is considered an essential tool in materials research. It is valuable for helping scientists optimize energy-storage materials, as well as for unraveling the structure of viruses and proteins.[117]

SYNCHROTRONS

Over the past three decades, synchrotron radiation sources have helped catalyze many important discoveries in crystallography. Synchrotrons produce light by using radio frequency waves and powerful electromagnets to accelerate electrons to nearly the speed of light. Energy is added to the electrons as they accelerate so that, when the magnets alter their course, they naturally emit a very brilliant, highly focused light.[118]

In the 1980s, high-intensity synchrotron X-ray sources allowed crystallographers to begin to conduct experiments that take advantage of the characteristics of synchrotron radiation, including the broad distribution of wavelengths, high intensity, low divergence, strong polarization, and a pulsed time structure. The equipment allowed the development of new types of crystallographic studies, for example, energy-dispersive and surface diffraction studies. The equipment's ability to allow diffraction experiments to be performed on very small crystals or on large biological molecules was revolutionary, as was its facility for permitting weak magnetic scattering to be detected.[119]

The importance of synchrotron radiation research for challenges such as resolving the structure of proteins led to the construction of a large number of dedicated synchrotron radiation facilities.[120] Today, the most advanced, so-called third-generation facilities are based on undulators, and are used by thousands of scientists around the world.[121]

X-RAY FREE-ELECTRON LASERS

X-ray free-electron lasers (XFELs) take advantage of the fact that additional information can be harvested beyond the peaks conventionally measured in X-ray diffraction patterns, a concept that dates back to the 1950s.[122] Free-electron lasers work by sending highly compressed bunches of electrons through a periodic magnet array to generate photons. These photons interact back with the electrons, which causes the bunch to be microstructured in such a way that they radiate coherently. This produces extremely bright and short pulses of X-ray radiation, so bright that they obliterate most samples placed in their path.[123]

A key insight about how to overcome the highly destructive nature of XFELs was published in 2000.[124] Researchers realized that a molecule exposed to an ultrashort X-ray pulse from an XFEL begins to explode at about 10 fs, and that it is possible to pass light pulses through a molecule to capture images before it is destroyed to collect diffraction patterns strong enough to be measured. The concept was proved in principle in 2006.[125] Over the next five years, the technique's utility was buttressed by new technologies developed for delivering samples. New processing algorithms have been devised for piecing together diffraction patterns from the molecules imaged with the technique to produce a complete image. Experts believe that the technique has promise for imaging a wide array of structures, including cells and viruses. The pulsed nature of XFELs also offers the potential for creating movies of molecular processes.[126]

The Linac Coherent Light Source at the SLAC National Accelerator Laboratory in Menlo Park, California, is one of the world's most powerful XFELs.[127] It produces pulses of X-rays that are more than a billion times brighter than the most powerful synchrotron sources, which are also based on large electron accelerators.[128]

SERIAL FEMTOSECOND CRYSTALLOGRAPHY

One new technique which uses higher energy XFELs is known as serial femtosecond crystallography (SFX).[129] The concept was initially proposed in 2000 as a way to obtain structures from microcrystals that are too small for analysis by conventional crystallographic instruments.[130] It was recently used to take structural snapshots of proteins with near-atomic resolution, demonstrating its promise for revealing the high-resolution structures of many biomolecules that have resisted traditional crystallographic analysis.[131] The technique capitalizes on the fact that X-rays travel through the sample at the speed of light, whereas damage propagates at slower speeds. Eventually the sample is completely vaporized by the high-energy X-rays, but not before data about its structure can be obtained.[132]

Earlier this year, scientists reported that they had used SFX at the Linac Coherent Light Source (LCLS) to analyze protein microcrystals deposited on an ultra-thin silicon nitride membrane and embedded in a preservation medium at room temperature. They found that data could be acquired at a high acquisition rate to overcome radiation damage, and the consumption of the sample was dramatically reduced. They wrote, "This demonstration opens the door to ultra-low

sample consumption SFX using the technique of diffraction-before-destruction on proteins that exist in only small quantities and/or do not produce the copious quantities of microcrystals required for [other] methods."[134]

In the future, researchers believe that using SFX with still-higher-energy XFELs could also make it possible to structurally analyze biomolecules without having to crystallize them at all and to make molecular movies of reactions.[133]

CRYSTAL-FREE CRYSTALLOGRAPHY

When scientists want to determine a molecule's structure but the substance can't be crystallized, or not enough of the substance exists to get good crystals, they used to face a roadblock. In 2013, researchers found a way to solve this problem using nanoscale scaffolds to capture the molecules they want to visualize.[135] A Japanese research team described how to use porous metal frameworks with large cavities as "crystalline sponges" that soak up guest molecules within their voids, putting the molecules in an ordered array that can be studied via X-ray crystallography. The technique works on as little as 80 ng of material.[136]

One example of the new technique's use was to soak up just 5 µg of miyakosyne A, a scarce marine natural product with a central methyl group that had defied stereochemical assignment. Using their crystal-free crystallographic technique, researchers were able to identify the compound's stereochemistry.[137] However, the work didn't live up to all its promise, because the team realized that the method could not unambiguously determine the stereochemistry of a marine sponge metabolite, as they had previously claimed.[138]

In 2013, a second research group at MIT independently verified that the method works for determining atom connectivity.[139] The hardest part of the crystal-free method was getting the reagent inside the metal-organic framework (MOF) scaffold, according to one of the MIT researchers. Experts believe the new technique may prove to be useful in natural products chemistry, food and perfume science, drug discovery, forensic science, and the daily research of synthetic chemists.[140] The approach has the potential to have a major impact on the speed with which natural compounds are identified.[141] It may one day enable researchers to mine marine life, soil bacteria, and other organisms for compounds that might have uses in, for example, cancer drugs, because it is often difficult to determine the shape of these molecules from the small quantities found in nature.[142,143]

The small molecules that can be studied with this technique are currently limited to those that fit within the sponges' pores. For example, in its current form, the technique isn't applicable to proteins, because the pockets in the crystalline sponge are not big enough. However, the potential exists to design and make other crystalline sponges with different sizes and pore environments, so that the technique can be extended to the analysis of larger molecules, such as proteins.[144]

THE VALUE OF REJECTED DATA

A growing body of evidence suggests that X-ray crystallographers have been tossing out data during their quests for biomolecule structures that are useful after all, according to recent research.[145] This research reports strategies for using traditionally ignored information to improve the quality of structures.

When solving an unfamiliar protein's structure, crystallographers obtain phase information to interpret X-ray diffraction patterns. For that task, they normally employ heavy atoms, such as selenium, but this isn't always straightforward. For four different proteins, a team of scientists led by a Columbia University researcher combined data from multiple crystals, demonstrating that typically neglected information in a protein's own sulfur atoms provides phase assistance with no heavy atoms needed.[146] A second team of U.S. and German scientists developed a statistical routine that they claim does a better job of identifying at what point the outer edges of diffraction patterns should be cut from calculations compared with established criteria.[147,148]

Another recently identified method that takes advantage of crystallographic data normally discarded as noise aids in the identification and analysis of enzymes involved in catalysis.[149] A team of researchers from Brandeis University and the University of California, Berkeley used high-resolution, room-temperature X-ray crystallography data on a human enzyme known as cyclophilin A (CYPA).[150] Their results directly demonstrate that both chemical interactions and conformational states are necessary for catalysis, the researchers say. They believe that the approach may prove to be a general strategy to identify and evaluate the roles of hidden, higher energy conformations in other enzymes.[151]

USE IN DRUG DESIGN AND DISCOVERY

Crystallography has been used for more than a decade in what is known as structure-based drug design (SBDD).[152] This approach to drug design involves using protein crystallography to image the three-dimensional structures of bioactive agents and their targets as the basis of drug discovery. Protein crystals can be challenging to grow, and very fragile when grown. In the past, most proteins were crystallized manually, but in recent years, the automation of crystallography screens has improved the technique's success rates, as well as how quickly it can be accomplished.[155]

X-ray crystallography plays an important role here because accurate analysis of crystal structures of target macromolecules and macromolecule-ligand complexes is critical at all stages.[153] Among the dozens of drugs discovered this way are the AIDS medications Crixivan and Viracept, the flu drug Tamiflu, the leukemia therapy Gleevec, and the cancer agent Tarceva.[154]

X-ray crystallography is often used in combination with other techniques to enhance its usefulness in drug design.[156] For example, scientists at Schering-Plough used it in combination with structure-activity relationship (SAR) analysis to develop novel hydroxamates as potent

inhibitors of tumor necrosis factor-α converting enzyme (TACE), an enzyme implicated in autoimmune disorders such as rheumatoid arthritis. At Merck, researchers used molecular modeling and X-ray crystallography to identify potent and selective inhibitors of β-site amyloid precursor protein cleaving enzyme (BACE-1) as potential Alzheimer's treatments. Another team using molecular modeling and X-ray crystallography developed a potent, selective oral chymase inhibitor that entered clinical trials for asthma and dermatitis.

More recently, researchers have used neutron crystallography, a technique that has been around for decades, to determine the structure of a human enzyme.[157] The first team to use the technique determined the structure of carbonic anhydrase bound to a clinical drug, acetazolamide.[158] The researchers said that neutron crystallography makes it possible to visualize hydrogens (or deuteriums), hydrogen bonding, and charge states—molecular features that are not visible with conventional X-ray crystallography and are potentially valuable for drug design.

While there has been significant progress in improving methods of structural biology, particularly in X-ray crystallography, corresponding progress in the development of computational methods (such as in silico high-throughput screening) is still on the horizon. Crystal structures can be overinterpreted and thus bias hypotheses and follow-up experiments. As in any experimental science, the models of macromolecular structures derived from X-ray diffraction data have their limitations, which need to be critically evaluated and well understood for structure-based drug discovery.

RACEMIC CRYSTALLOGRAPHY

Racemic crystallography has gotten attention recently for its promise to help structural biologists obtain high-quality protein crystals from tough-to-crystallize proteins.[159] The technique works with chiral molecules that lack an internal plane of symmetry and therefore have both right and left "mirror image" versions, or enantiomers. A racemic mixture contains equal numbers of both enantiomers. The idea that a racemic protein mixture would crystallize more readily than either enantiomer alone first dates back to 1995.[160]

A few years ago, chemists used a protein known as BmBKTx1, which is found in the venom of a scorpion native to Asia, to show that they could readily obtain high-quality crystals from a chemically prepared racemic solution.[161] Previously, this protein resisted extensive crystallization attempts by multiple labs. The racemic crystallography method requires that proteins made purely of D-amino acids be created to mirror conventional L-amino acid-containing proteins. D-Amino acid proteins must be chemically synthesized, a task that has become less challenging as the process has evolved.[162]

More recently, researchers used native chemical ligation, mirror-image phage display, and racemic protein crystallography to crystalize a much larger protein.[163] Their successful identification of the first D-protein antagonist of vascular endothelial growth factor type A

(VEGF-A) could lead to improved cancer and macular degeneration drugs.[164] Two injectable drugs, the anticancer drug Avastin and the macular degeneration agent Lucentis, are conventional L-protein VEGF-A antagonists. Because enzymes don't recognize D-protein drugs, such drugs resist proteolytic breakdown and are potential oral medications. Another advantage is that the immune system doesn't attack D-proteins because it doesn't recognize them either.[165] The researchers used racemic protein crystallography to determine the structure of the ligand/VEGF-A complex. The protein complex they analyzed structurally is more than 10 times the size of any determined previously by racemic crystallography.[166] Experts believe that racemic crystallography is likely to become a general approach to getting structures of difficult-to-crystallize proteins.[167]

ELECTRON CRYSTALLOGRAPHY

Electrons have been used in diffraction experiments for more than 80 years, but electron crystallography really came into its own after the invention of the electron microscope.[169] This development capitalized on the recognition that the phase information present in diffracted electron beams can be recovered by focusing them back into a two-dimensional (2-D), real-space projection by means of the magnetic lenses of an electron microscope. The first successful demonstration of this was the reconstruction of the three-dimensional (3-D) structure of the tail of the T4 bacteriophage from a limited set of electron microscopy images by a team including Aaron Klug of the University of Cambridge.[170] Electron crystallography has become a vast field, and it is widely employed in materials science.[171] It is an important technique for studying and monitoring nanoparticles, and it is useful for obtaining structural information for proteins that form 2-D crystals.[172] Unlike data collection in X-ray crystallography, which is now typically fast and straightforward, data collection in electron crystallography can take months or years and requires substantial expertise.[173]

A unique modified transmission electron microscope interfaced with an ultrafast laser located at the California Institute of Technology is capable of capturing four-dimensional pictures of molecules (3-D structural changes over time) as they form and break apart. Researchers at Caltech are using ultrafast electron crystallography to make movies of structural dynamics of, for example, interfacial macromolecular assemblies, nanostructures, and crystalline fatty acid bilayers. These movies have subnanometer spatial and subpicosecond temporal resolutions.

The researchers have also used ultrafast crystallography to study the structure and dynamics of interfacial water, a thin layer of water in close proximity to a surface.[175] It behaves differently than bulk water and is difficult to investigate experimentally. Hydrophobic and hydrophilic interactions of macromolecules with the thin water layer around them, however, play an important role in deciphering research questions such as how proteins fold. The group's ultrafast electron crystallographic study of a monolayer of ice on different substrates yielded long-sought information on bond distances.[176] The method was also used to investigate structural dynamics of a cuprate superconductor that has fleeting phase transitions.[177] Because the team was able to directly watch the evolution of a lattice structure, they discovered

a photoinduced structural phase transition that cannot be seen with conventional techniques. Another study demonstrated that crystals undergo sequential atomic motions as they pass through phase transitions, which is similar to what happens with individual molecules during chemical reactions.[178] A few researchers have demonstrated the feasibility of combining the 2-D high-resolution electron microscopy images to produce a 3-D reconstruction. Some researchers, including a group at Stockholm University's Department of Materials and Environmental Chemistry, are exploring ways to use 3-D electron crystallography to image crystallized proteins too small to be imaged with X-ray crystallography.[180]

COMBINING X-RAY CRYSTALLOGRAPHY WITH OTHER TECHNIQUES

X-ray crystallography can provide very detailed atomic information. It can show every atom in a biological molecule such as a protein or nucleic acid, along with atomic details of ligands, inhibitors, ions, and other molecules that are incorporated into the crystal. However, the process of crystallization is difficult and can impose limitations on the types of substances that may be studied by this method.[181] Information from X-ray crystallography is often combined with other techniques such as electron micrographic studies and nuclear magnetic resonance (NMR) spectroscopy to sort out the atomic details. This has proven very useful for elucidating multimolecular biochemical structures such as complexes of ribosomes, transfer RNA (tRNA), and protein factors.[182]

In the 1980s, researchers found a way to combine X-ray diffraction with absorption spectroscopy. Resonant magnetic X-ray diffraction techniques grew out of an experiment involving tuning the energy of X-rays impinging on a thin holmium crystal so that they match, or resonate with, the "absorption edge" characteristic of a specific electronic binding energy, greatly enhancing the magnetic signal.[183,184,185] Like those of many other rare-earth elements, the magnetic structure of holmium is characterized by a helical ordering of the magnetic moments, an arrangement known as a spin spiral. Using the resonant X-ray technique, it was possible to map out this structure.

Resonant magnetic X-ray diffraction is element specific. Initially, the technique took advantage of the second- and third-

AN UNUSUAL ROUTE TO PRODUCING A CRYSTAL—AND A CHEMICAL

A recent achievement involving crystallography with practical implications relates to scientists' success in developing a way to make the antifreeze protein that enables billions of Canadian snow fleas to survive frigid winter temperatures.[168] The study was the first to use the two mirror-image forms of the so-called "snow flea antifreeze protein (sfAFP)" to determine the previously unknown crystal structure of this unique protein. Scientists had tried for years unsuccessfully to decipher the molecular structure so that they could reproduce it from chemicals in a laboratory. Those steps are critical for obtaining larger amounts of the protein, which exists naturally in only minute quantities in snow fleas.

The researchers made synthetic sfAFP and showed that it has the same activity as the natural protein. They also produced variants, including one form of sfAFP with a molecular architecture that is the reverse, or mirror image, of natural sfAFP and different from any other protein found in living things on earth. The mirror-image form of sfAFP appears to be less likely to trigger harmful antibodies and is more resistant to destruction by natural enzymes, the scientists note, making it potentially more effective than the native form for use in organ and tissue preservation. The researchers believe that the potential medical and commercial uses of their first-of-a-kind proteins include extending the storage life of donor organs and tissues for human transplantation.

generation synchrotrons available at the time, which made it possible to selectively tune intense X-ray beams at energies close to element absorption edges. As the interest in nanoscale magnetic structures grew, the technique's ability to investigate magnetic and electronic surface and interface effects proved valuable.[186] More recently, resonant magnetic X-ray diffraction techniques have evolved to include inelastic X-ray scattering, which has become an important tool for probing collective excitations in solids.[187,188]

In the 1990s, researchers used X-ray crystallography combined with a then-new technique called single-particle cryo-electron microscopy (cryo-EM) to work out the key details of the structure of the ribosome, the primary site in cells where proteins are synthesized.[189] Cryo-EM is a technique for imaging sets of individual molecules embedded in a thin layer of ice. Although it can be used to observe a greater variety of functionally interesting forms of biomolecules than is possible with crystallography, cryo-EM can't normally attain atomic resolution. Crystallographic data can help researchers refine their cryo-EM maps. By applying this combined approach to study ribosomes, researchers were able show how tRNA can act like a molecular spring in some situations: the tRNA can distort as it contacts messenger RNA (mRNA) upon entering the ribosome, then straighten.[190]

A decade ago, combining electron crystallography and X-ray crystallography helped researchers from a number of groups uncover a better understanding of bacteriorhodopsin, a small protein found in the cell membrane of *Halobacterium salinarum,* an organism that tolerates high concentrations of salt.[191,192,193] The protein contains a light-sensitive pigment, called retinal, that when illuminated triggers events that lead to the release of a proton from inside the cell to the outside against a gradient, thereby converting light to chemical energy. This research revealed the value of combining information from electron crystallography of 2-D crystals with that from X-ray crystallography, according to members of the research teams.[194] Combining X-ray crystallography with NMR can be particularly useful for elucidating the structures of complicated protein assemblies. The sensitivity of NMR spectroscopy in detecting the conformational properties of individual atoms in proteins and their complexes, without any prior knowledge of conformation, is highly valuable for obtaining the high-quality crystals necessary for structure determination by X-ray crystallography.[195]

Together, X-ray crystallography and NMR spectroscopy have provided important insights into portions of DNA-containing guanine molecules located close enough together that they can form planar hydrogen-bonded arrangements known as guanine quartets (G-quartets), which can aggregate together to form G-quadruplexes that are roughly cylindrical in appearance.[196] G-quadruplexes are believed to play an important role in human telomeres and promoter regions of genes. (Telomeres protect the ends of chromosomes, and promoters are chromosome sequences that control gene expression.[197]) The G-quadruplexes are also suspected to play an important causative role in Fragile X syndrome, the most common form of inherited mental retardation, and they have been implicated in the cause of two premature-aging conditions, Bloom and Werner syndromes. DNA quadruplexes in telomeres have been

shown to inhibit the activity of telomerase, an enzyme expressed selectively in cancer cells. Researchers believe that directing drugs to such sites might be a way of artificially regulating gene expression and thus providing medicinal benefits such as anticancer activity. Some scientists are using X-ray crystallography combined with NMR to guide the design of drugs that interact with G-quadruplexes.

INSIGHTS INTO BIOCHEMISTRY

Over the last decade, crystallography has enabled scientists to glean many insights into biochemistry. In 2003, researchers using X-ray crystallography reported that they trapped a transition state of an enzyme-catalyzed reaction involving phosphor groups, which are involved in many enzyme-catalyzed metabolic reactions.[198,199] Some aspects of the finding's interpretation were debated, but the work was lauded for showing crystallography's ability to capture transient species along an enzyme's reaction pathway in addition to the easier task of probing the structures of enzymes bound either to their substrate or product.[200,201]

In 2012, crystallography enabled researchers to visualize how B vitamins transfer a methyl group to produce an amino acid.[202] The study showed that a dramatic conformational change is required for the methyl transfer between folate, also known as vitamin B-9, to vitamin B-12. The transfer is a key step in the synthesis of the amino acid methionine in humans. It also underlies the process by which certain bacteria manage to subsist on carbon dioxide. In both cases, huge protein complexes facilitate the seemingly simple methyl transfer step.[203] At the time, it was the largest protein conformational change ever observed in the crystalline state, according to the study's authors. They said that their work "helps explain why such an elaborate protein framework is required for such a simple, yet biologically essential reaction." [204]

Later in 2012 , researchers devised a way to use time-resolved Laue crystallography, a technique involving the use of a synchrotron light source, to watch a light-induced signaling protein as it functioned.[205] Experts believe that the technique could make it possible to better understand step-by-step mechanisms of other signaling proteins, which control processes such as the movement of leaves toward light, vision in higher animals, and possibly the mechanisms of enzymes as well.[206] In this study, researchers used time-resolved Laue crystallography to take structural snapshots of bacterial photoactive yellow protein (PYP) in action. When PYP is activated by potentially harmful light frequencies, it provides signals that induce the bacteria to swim away. The researchers used the Laue method, which harnesses polychromatic X-ray pulses to rapidly obtain structural information. The technique's 150-ps time window made it possible for the researchers to view previously unobservable structural changes in a *p*-coumaric acid unit that undergoes *trans*-to-*cis* iso-merization when the protein complex is activated. The study also visualized effects of hydrogen bonding, strain, and water in the activation process.[207]

CRYSTALLOGRAPHY DATA BANKS

The crystallography community has a long-standing tradition of openly sharing code and software designed to help the community as a whole.[208] In the mid-1960s, researchers in

the crystallography group led by Olga Kennard of the Department of Organic, Inorganic, and Theoretical Chemistry of the University of Cambridge began to collect published bibliographic, chemical, and crystal structure data for all small molecules studied by X-ray or neutron diffraction. With the rapid developments in computing taking place at this time, this collection was encoded in electronic form and became known as the Cambridge Structural Database (CSD), one of the first numerical scientific databases to begin operations anywhere in the world. [209]

In 1971, protein crystallographers attending a Cold Spring Harbor Symposium on "Structure and Function of Proteins at the Three Dimensional Level" began a discussion that culminated in an official announcement of the establishment of the Protein Data Bank (PDB). This data bank is a repository for protein crystallographic data, and it was initially run as a collaboration between the Brookhaven National Laboratory and the Cambridge Crystallographic Data Centre.[210]

In the 1960s and '70s, sharing crystallographic data was a fundamentally challenging endeavor. Receiving and distributing structural data required shipping paper punch cards or magnetic tape through the mail, and computer hardware and software needed to visualize or analyze these data were still rare.[211] By the end of the 1980s, the value of the PDB had become sufficiently evident that structural biologists began to argue that deposition of structural data to the PDB should be required of all scientists in the field.

The PDB now hosts more than 100,000 structures, of which more than 87,000 are derived from X-ray crystallography. All structures are provided freely and without restriction, and many journals routinely require deposition of protein structures and the associated experimental data to the PDB as a prerequisite for manuscript publication. According to the International Union of Crystallography, there are nine major public databases containing crystal structures, including the CSD and PDB (see sidebar).

• **Bilbao Crystallographic Server (BCS)** contains crystallographic symmetry information and is maintained by the Materials Library of the University of the Basque Country in Spain. It has provided access to crystallographic data and solid state programs and utilities since 1997.[214] *http://www.cryst.ehu.es*

• **Biological Macromolecule Crystallization Database (BMCD)** is maintained by the U.S. National Institute of Standards and Technology. It archives crystallization data from published reports for all forms of biological macromolecules that have produced crystals suitable for X-ray diffraction studies. The information includes the crystallization conditions, crystal data, comments about the crystallization procedure, and information on the biological macromolecule or biological macromolecule complex.[215,216] *http://xpdb.nist.gov:8060/BMCD4/index.faces*

• **CRYSTMET** contains information about metallic and inter-metallic structures, and it is maintained by a private Canadian company, Toth Information Systems, Inc. The database contains chemical, crystallographic, and bibliographic data, together with associated experimental details. The database can be used to derive powder pattern calculations.[217] *http://www.tothcanada.com/databases.htm*

• **Cambridge Structural Database (CSD)** of organic and metal-organic structures was established in 1965 by researchers at the University of Cambridge. It contains the results of more than a half-million X-ray and neutron diffraction analyses. CCDC Software Limited (founded in 1998) currently administers the database and retains close links with the University of Cambridge.[218] *http://www.ccdc.cam.ac.uk/Solutions/CSDSystem/Pages/CSD.aspx*

• **Inorganic Structural Database (ICSD)** bills itself as the world's largest database for completely identified inorganic crystal structures. It contains nearly 170,000 peer-reviewed data entries, including atomic coordinates, dating back to 1913. The database is maintained by FIZ Karlsruhe – Leibniz Institute for Information Infrastructure, a nonprofit organization with the mission to make scientific and technical information from all over the world publicly available.[219] *http://www.fiz-karlsruhe.de/icsd.html*

• **Nucleic Acid Database (NDB)** contains information about experimentally determined nucleic acids and complex assemblies. It was founded by researchers from Rutgers and Wesleyan Universities in 1992, and it is maintained at Rutgers, the State University of New Jersey, with funding from the National Institutes of Health.[220] *http://ndbserver.rutgers.edu*

• **The Pauling File** is a materials database containing physical properties, crystallographic, and structural data, as well as binary phase diagrams information. It is maintained by Materials Design, Inc. a private company with employees and partners working on three continents.[221] *http://www.paulingfile.com*

• **Protein Data Bank (PDB)** has been collecting resolved 3-D structures of large biological structures, including proteins and nucleic acids, since it was originally established at the U.S. Brookhaven National Laboratory in 1971. It is now maintained by the Research Collaboratory for Structural Bioinformatics (RCSB), which is staffed by researchers at Rutgers and the University of California, San Diego. It now contains nearly 100,000 entries.[222] *http://www.rcsb.org/pdb/home/home.do*

• **Powder Diffraction File (PDF)** contains nearly 800,000 unique material datasets associated with powder diffraction, including crystallographic, bibliographic, and experimental data. It is maintained by the International Centre for Diffraction Data, a non-profit scientific organization.[223] *http://www.icdd.com*

V. LOOKING AHEAD: THE FUTURE OF CRYSTALLOGRAPHY

In the coming decades, experts expect crystallography to be used routinely as a tool in nanotechnology, synthetic chemistry, biochemistry, and cell biology.[224] Among the current challenges that experts predict crystallography may help address in the future are better ways to grow crystals and screen membrane proteins, which represent approximately 30% of proteins coded by the human genome. Scientists' ability to use synchrotrons to determine structures at room temperature is also expected to improve, as is the eventual ability of X-ray free-electron lasers to image single molecules.[225]

As the science inspired by crystallography progresses, X-ray diffraction as we currently know it may become obsolete. Whether or not that actually happens, experts believe that ideas and methods from crystallography will continue to exist as components of many, if not most, of the newer techniques that replace it.[226]

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