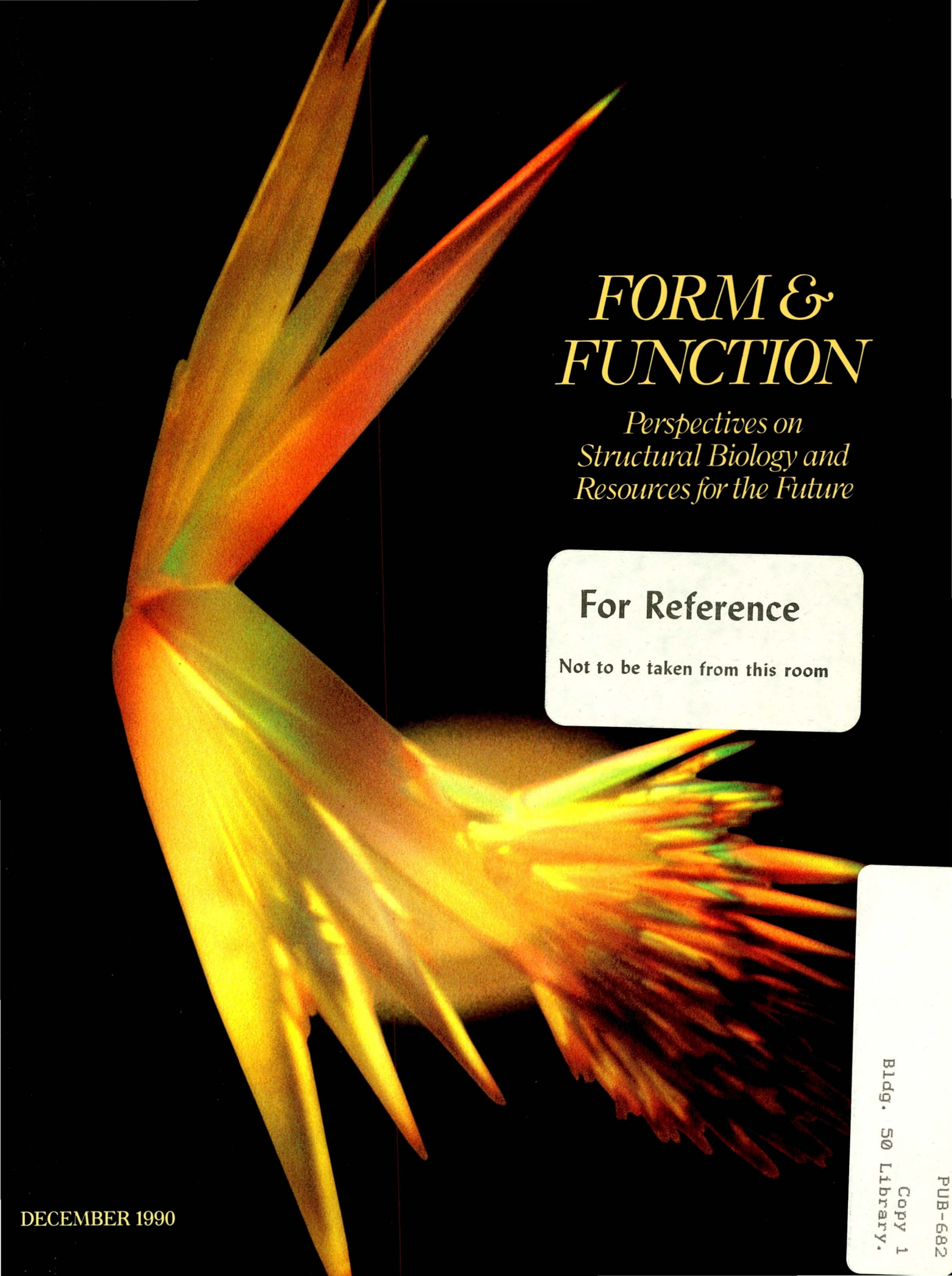

FORM & FUNCTION

Perspectives on Structural Biology and Resources for the Future

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On the cover: Crystals of an enzyme, $\triangle^5$-3-ketosteroid isomerase

FORM & FUNCTION

Perspectives on Structural Biology and Resources for the Future

DECEMBER 1990

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Foreword

A NATIONAL MISSION

A QUIET REVOLUTION IS IN progress in the life sciences. Not one that will overthrow established understanding, but rather one that is expanding the horizons of biological research into the realm of complex molecular structure. As part of this revolution, "big machines" that were once the province of physical scientists—synchrotron radiation facilities, neutron-producing research reactors, particle accelerators, and supercomputers—are now being turned to puzzle out the riddles of life. With this change come new opportunities, indeed new responsibilities, for the U.S. Department of Energy.

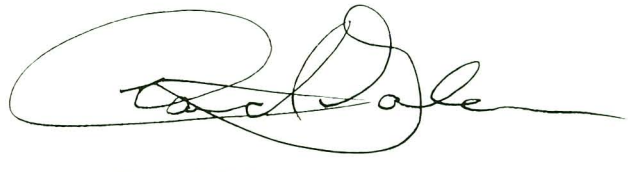
The impetus for this new approach to biological research is the universal recognition, voiced by scientists around the world and echoed by advisory panels both here and abroad, that the key to a full understanding of biology lies hidden in the molecular and supramolecular structure of living systems and that an essential path to understanding this structure now leads out of the lab and to major research facilities. Recent revelations about one of the molecular mechanisms of cancer induction and the first long strides toward fighting such viral afflictions as the common cold and acquired immune deficiency syndrome (AIDS) have been direct results of studies at national user facilities. These structural insights, described on pages 12–18, point toward dramatic advances in health care and are guiding early efforts in the rational design of new pharmaceuticals. The health research area is, of course, not alone in the benefit received—nor are such successes limited to the U.S. In Japan and Europe, intensive use of existing facilities and the active development of new ones already contest U.S. leadership in this field we call *structural biology.* Furthermore, in contrast to the best foreign facilities, DOE-operated facilities in the U.S. are often understaffed, underfunded, and inadequately equipped to keep pace in pursuing this kind of very basic biological research. The ramifications are practical as well as scientific: The nascent biotechnology industry is a direct beneficiary of advances in structural biology, which can be viewed as a field of research that will provide enabling technologies essential to the future of the industry.

At the same time, two new synchrotron radiation sources are under construction in this country, and the world's most advanced facility for neutron research could be built before the end of the decade. The door is thus open to a new commitment to structural biology research—a com-

mitment that will cement this nation's competitive position in biotechnology and, at the same time, guarantee the more basic benefits of understanding the nature of human disease and the processes of life itself. Without such a farsighted commitment, the vital centers of activity will inevitably shift to foreign shores, and the U.S. will play a diminishing role in structural biology.

The Department of Energy has a mandate to construct and operate major national user facilities for research and education. Beyond this basic responsibility, though, the themes of structural biology itself bear directly on the mission of the Department of Energy. The prospects for "customized" enzymes serve as a dramatic case in point: As our structural knowledge of proteins grows, so also does the hope that we may soon be able to custom-tailor enzymes to tackle such jobs as waste remediation, biomass-to-fuel conversion, and the production of novel new materials. As an endeavor basic to future biotechnology, it is clear both that the contributions of structural biology to the DOE's interests will be numerous and important and that they are largely unpredictable.

In conclusion, I want to emphasize that structural biology in the U.S. is, and must remain, a cooperative national enterprise. The National Institutes of Health, the National Science Foundation, and private philanthropies provide most of the support for structural biology research in this country. However, the Department of Energy, too, has an essential and growing programmatic interest in the continuing success of structural biology. In addition, the Department of Energy has one critical role that is largely unshared with the other agencies: to operate and upgrade the major, centralized user facilities where some of the most exciting structural biology research has been done and to construct new facilities for the next generation of breakthroughs.



David J. Galas
Associate Director for Health and
Environmental Research
Office of Energy Research
U.S. Department of Energy

Introducing Structural Biology

LINKING FORM AND FUNCTION

JUST AS HUMAN ARTIFACTS are constructed to suit their functions, so biological structures have been shaped by evolution to meet the needs of life. Not only hands and eyes and the other anatomical structures of the plant and animal kingdoms, but also the microscopic structure of each subcellular organelle and biological macromolecule must match its purpose. Molecules with just the right shapes are responsible for turning genes on and off, catalyzing the complex chemistry of life, defending against cellular invaders, and flipping the switches that initiate cell division and control development. To understand this intricate relationship between biological structure and function is the goal of the broad field of *structural biology.*

For centuries "natural philosophers" sought clues to behavior and function in the form they could discern with their unaided eyes. (Indeed, scientists even today seek insights in gross anatomy—as they unearth the fossils of early hominids, for example!) More recently, with the coming of the light microscope and then, in this century, the electron microscope, researchers extended the inquiry to the domain of cellular and subcellular organization. Now, with these tools and with the increasingly sophisticated tools of physical science, our vision extends to the level of molecules and atoms. Discerning form, however, continues to be the key to understanding function. Over the past several decades, practically every insight into subcellular and molecular biological structure has likewise enhanced our understanding of biological function. From the structure of an antibody's binding site, biochemists can often glean insights into the reasons behind its unerring specificity. The structure of a mutant protein implicated in cancer has suggested where biochemistry goes wrong in unregulated cell growth—and thus suggests a means of intervention. And understanding the structure of a virus can offer clues to its virulence, and thus be the first step toward combating it.

What we know and what we have yet to learn about our own genetic material dramatically illustrate the achievements and aspirations of structural biology. The encrypted recipe for each individual of our species consists of about two meters of DNA, apportioned among 46 chromosomes

in practically every cell of our bodies. The historic discovery of this natural polymer's double helical structure is surely the most famous of structural biology's achievements, made well before the field even had a name. As a result of Crick, Watson, and Wilkins' Nobel Prize–winning work, we can now describe in exquisite detail the three-dimensional structure of DNA's entwined spiral (Figure 1). And, as a direct consequence of this *structural* insight, we understand how highly specific interstrand linkages—adenine to thymine, guanine to cytosine—preserve the cell's genetic message during cell division. A picture can also be drawn, at least with broad strokes, of how the sequence of A's, T's, G's, and C's encodes the formulas of 100,000 enzymes, hormones, and structural proteins and how mRNA, the faithful messenger of this code, directs protein synthesis in the cell cytoplasm.

Much, though, remains to be learned. How, for example, are two meters of DNA packaged into 46 chromosomes, each a few micrometers long? And how is this intricate packing related to the organization of genes and to the regulation of gene expression? Every cell, after all, contains the same genetic prescription. What directs the differentiation of the embryo into muscle, bone, and brain? Are the organization and structure of the chromosomes somehow important in establishing and maintaining this cell diversity?

At a much deeper level of detail is the actual base-by-base text of the entire human genetic code—a text that is the ultimate goal of the human genome project. But even this highly visible, international effort will rely on structural biology for its ultimate, *practical* success. Three billion letters of genetic code, after all, will mean something only if they can be understood in terms of genes, which direct the synthesis of proteins, which function—and sometimes malfunction—as parts of a living system. How are we to identify the actual genes in this mass of data? Part of the answer, it turns out, is hidden in the structure of the RNA molecules that are the cell's genetic messengers. Further, how are we to relate the instructions of a gene, which merely dictate a linear sequence of amino acids, to the structure of the resulting functional protein. And finally, what does the protein's structure tell us about how it does its job? These are the questions that are the province of structural biology.

The purpose of the following pages is largely to explore and expand on the thesis embodied in these opening paragraphs: that form indeed follows function and that if we are to understand the workings of a living system, with all that such an understanding promises, we must first seek to describe the structure of its parts. Descriptions of a few achievements of structural biology lay the groundwork, but the substance of this booklet is a discussion of important questions yet unanswered and opportunities just beyond our grasp. The concluding pages then outline a course of action in which the Department of Energy would exercise its responsibility to develop the major resources needed to extend our reach and to answer some of those unanswered questions.

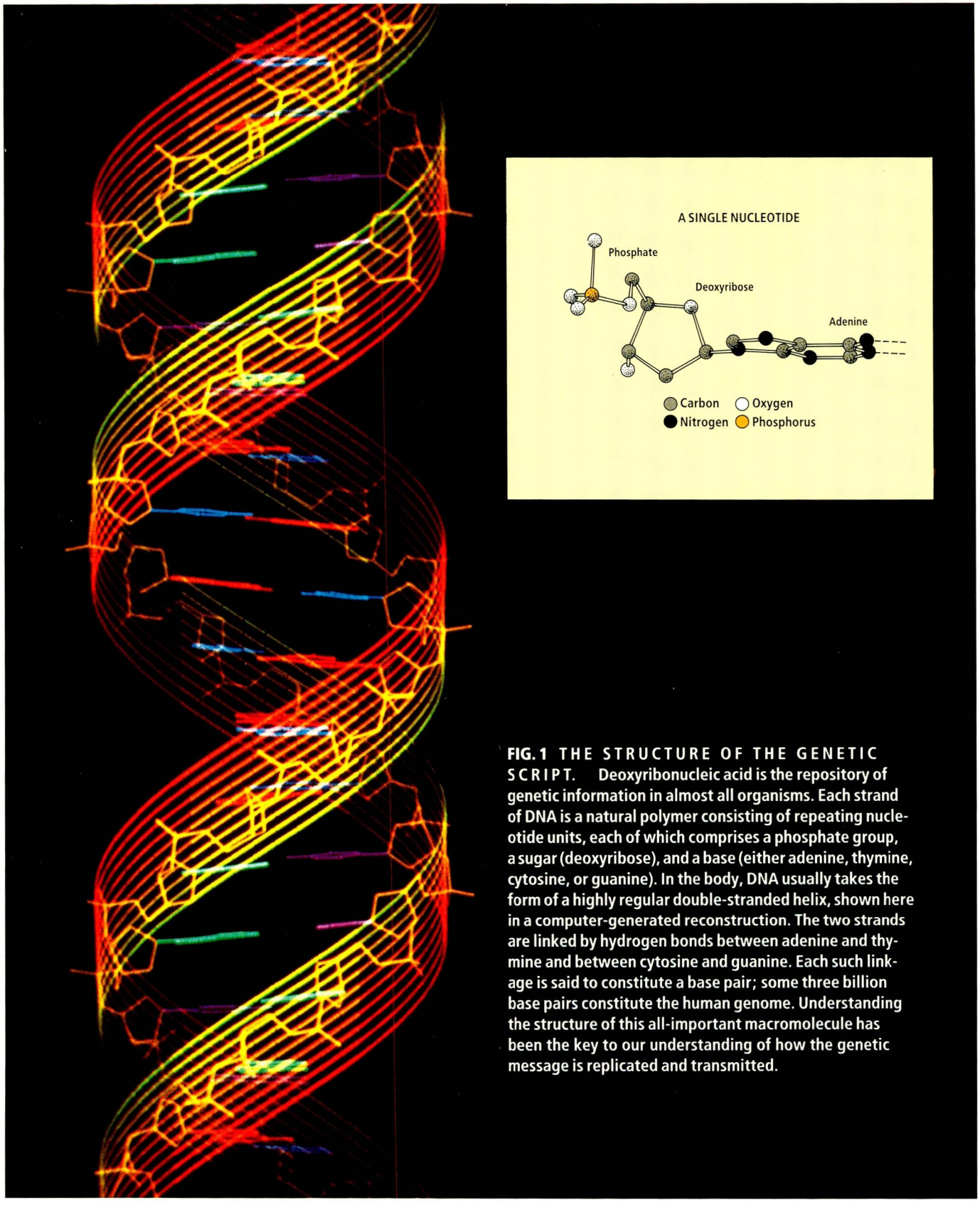


FIG. 1 THE STRUCTURE OF THE GENETIC SCRIPT. Deoxyribonucleic acid is the repository of genetic information in almost all organisms. Each strand of DNA is a natural polymer consisting of repeating nucleotide units, each of which comprises a phosphate group, a sugar (deoxyribose), and a base (either adenine, thymine, cytosine, or guanine). In the body, DNA usually takes the form of a highly regular double-stranded helix, shown here in a computer-generated reconstruction. The two strands are linked by hydrogen bonds between adenine and thymine and between cytosine and guanine. Each such linkage is said to constitute a base pair; some three billion base pairs constitute the human genome. Understanding the structure of this all-important macromolecule has been the key to our understanding of how the genetic message is replicated and transmitted.

THE SHAPE OF THINGS

ACHIEVEMENTS OF STRUCTURAL BIOLOGY

INTEREST IN STRUCTURAL BIOLOGY rests not only with its potential impact, but also with its real achievements over the past several years. A few examples serve as a foundation for the sections that follow. First, recent insights into a cancer-causing protein and the common cold virus are cited to underscore the importance of understanding structure in the quest to understand function. Then, the concerted effort to unravel the secrets of photosynthesis is used as an example to illustrate the synergism of structural biology research.

Structure At Atomic Resolution

PROTEINS AND VIRUSES: TWO CASE STUDIES

THERE IS OVERWHELMING evidence that a few key genes play a crucial role in orchestrating and regulating cell growth. Accordingly, loss of one of these genes, or even slight damage—caused, say, by radiation or environmental carcinogens—can be the key event leading to unregulated cell growth and cancer. Within this large class of genes are the cancer-causing *oncogenes,* and among these, the *ras* oncogene is one of the most common in human tumors. Indeed, it is found in about half of all colorectal cancers and in most pancreatic cancers, two of the five deadliest malignancies among U.S. cancer victims—and it has been implicated in many others.

The protein encoded by the normal *ras* gene is believed to play the all-important role of a molecular on-off switch, telling cells when to divide and when to cease. This normal *ras* protein apparently assumes its "on" state when some external growth signal induces it to exchange a molecule of guanosine diphosphate (GDP) for a molecule of guanosine triphosphate (GTP). In its GTP-complexed state, it signals cell growth. However, with the help of another protein, it quickly hydrolyzes the GTP to GDP, and thus reverts to an "off" state, awaiting another extracellular signal. The altered *ras* oncogene, by contrast, produces an oncoprotein that, once switched on, does not readily reassume its "off" conformation, apparently because it has lost its ability to hydrolyze GTP. With the signal thus stuck in the "on" state, unregulated, cancerous cell growth ensues.

Much remains to be learned about molecular signaling (see pages 30–31), but our current insight into the workings of the *ras* protein has greatly enhanced what we know of at least one important step in the process of oncogenesis. More importantly, this enhanced understanding suggests ways to shut off the defective molecular switch, thus perhaps controlling the progression of the cancer.

These functional insights owe much to molecular biological studies, but they are also indebted to three-dimensional structural studies of both the normal *ras* protein, in its "on" and "off" states, and the *ras* oncoprotein, using x-ray crystallography (Figure 2). Among techniques available to investigate biological structure and function at the highest resolution, x-ray crystallography has by far the most celebrated heritage: Almost 40 years ago, x-ray diffraction patterns from oriented fibers of DNA played a key role in settling the debate about the three-dimensional structure of the genetic material. Despite its distinguished history, however, x-ray crystallography has only recently taken on a full measure of importance as a tool for biological structure studies—stimulated by the growing availability of suitable sources of x-rays and by increasing success in crystallizing biological molecules. Today, the overwhelming preponderance of information we have regarding the three-dimensional arrangement of atoms in proteins, nucleic acids (DNA and RNA), and viruses has been obtained from the diffraction of x-rays from single crystals of these materials.

A second example with enormous potential impact can be found among the viruses, perhaps nature's most insidious creations. The first such targets for x-ray crystallography were plant viruses such as the tobacco mosaic virus, but the most exciting successes of the past few years have been structural determinations of several impor-

FIG. 2 NORMAL PROTEIN OR ONCOPROTEIN? This computer-generated image shows the structural backbone of the normal *ras* protein, as determined by x-ray diffraction studies. A molecule of a guanosine triphosphate (GTP) analogue appears in green. In this form, the protein plays the essential role of a molecular switch in the transmission of signals for normal cell growth. A single amino acid substitution at either of two critical locations produces a cancer-causing mutant that has been strongly linked to two of the most common fatal human cancers. These mutations, which only subtly change the conformation of the molecule, apparently reduce the protein's ability to hydrolyze GTP. In effect, the signal for cell growth thus gets stuck in the "on" position; the result is uncontrolled cell replication.

ANATOMY OF A PROTEIN

DNA and RNA continue to attract their share of attention in structural biology. The intricate packing of DNA in the chromosomes is the subject of pages 24–26, and discovery of RNA's catalytic activity has stimulated a burst of interest in this essential component of our biochemical machinery. Nonetheless, the greatest interest continues to focus on proteins, far more than on the genetic material itself. The source of this interest is the ubiquity and the variety of proteins. As enzymes they catalyze the chemistry of the living cell. As hormones they regulate development and orchestrate the activities of our organs. They transport oxygen in the blood, defend us against infection, transmute sunlight into life-giving carbohydrates in plants, and serve as the structural building blocks for contractile muscle fibrils and organelles of cell motility. At the same time, it is the protein coats of viruses that latch onto our cells and thus give diseases such as the common cold, influenza, and AIDS a foothold in our bodies. The underlying basis for the diversity and specificity of these functions, and the reason proteins have yielded their secrets so grudgingly, is the enormous variety of shapes these molecules can take.

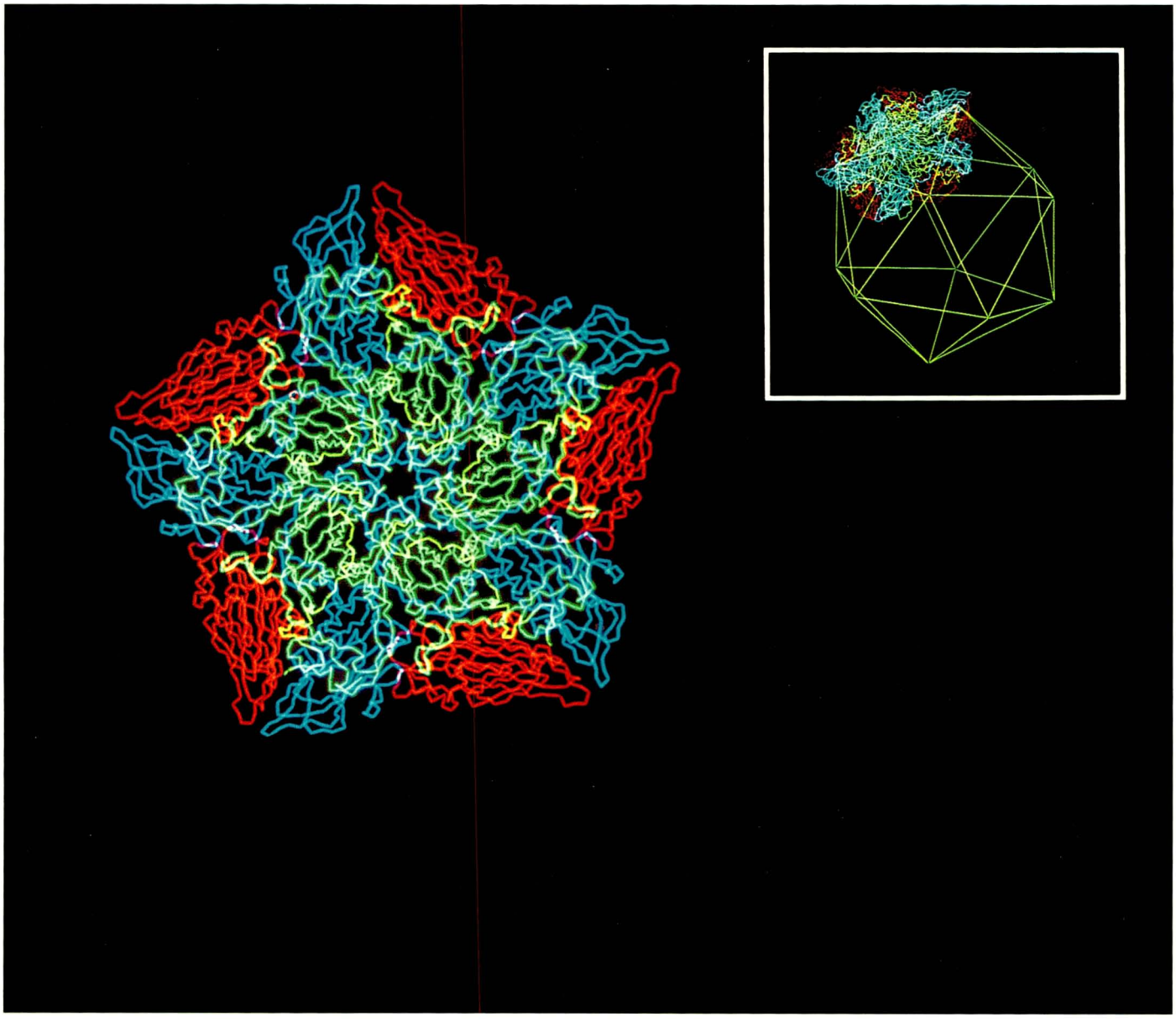
At a chemical level, each protein is merely a string of amino acids, a natural polymer (a *polypeptide*), whose sequence is ultimately dictated by the gene that directs its synthesis. (Many functional proteins comprise several subunits, assembled separately, but this does not alter the basic picture.) The mystery is how this string of amino acids—of which there are only 20 types to choose from—folds reproducibly to yield the protein's functional three-dimensional shape. Unless this "folding problem" can be solved (see pages 39–40), the structure of almost every interesting protein will demand a separate anaysis. One generalization that can safely be made is that the atomic details of a protein's spatial structure—dimensions, electrical charge, and surface chemistry—are essential to its role. The importance of this intricate architecture is depicted in many of the illustrations in this booklet. The point is made even more emphatically by a recent finding of the *ras* protein studies: We now know that a single amino acid substitution—producing a seemingly subtle transformation in three-dimensional structure—can turn a protein essential to life into a deadly messenger of cancer.

tant *picornaviruses* (literally, "small RNA viruses"), among them the viruses that cause hoof-and-mouth disease, polio, and the common cold.

One of the revelations of these studies has been the remarkable similarities seen among these viruses and others. In all of the picornaviruses, and in most of the plant and insect viruses, the protein coats share an icosahedral symmetry, and in each case the structural unit of the icosahedron has been a trio of identical, or nearly identical, proteins with slightly different three-dimensional folding patterns (Figure 3). This family resemblance and high degree of symmetry now allow virus crystallographers to take time-saving shortcuts in determining new viral structures—a convenience that has greatly simplified virus crystallography.

A second insight afforded by the viral structure studies has more profound economic and public health implications. It has been found in general that the viruses are subject to radical and rapid changes among the amino acids that constitute the outer surface of their coats. Indeed, such mutations explain the many varieties of cold viruses, as well as the periodic influenza outbreaks that plague us. These superficial changes render the body's previously synthesized antibodies useless, but have no impact on the viruses' infectious capabilities. These capabilities are instead embodied in certain structurally invariant regions, buried in clefts or pockets within the coats, inaccessible to the agents of the body's immune system. For instance, in the human cold virus HRV14 (for human rhinovirus 14), deep "canyons" are cut into the icosahedral faces in 60 places, giving access to the unvarying binding sites that allow the virus to attach itself to receptors on the surface of human cells. Armed with this knowledge, and with the detailed structure of the entire virus, researchers have now been able to identify pharmaceuticals that enter the canyon and attach to the HRV14 binding site (Figure 4). In so doing, these drugs reduce the flexibility of the virus's outer coat and thus inhibit "uncoating," a vital step if the virus is to disgorge its contents into a host cell.

A proven and highly effective cold remedy remains elusive, but these discoveries hold great



promise for the future. Indeed, they presage a revolution in "rational drug design," based on the premise that it should be possible to design and synthesize (or modify) a small molecule to inactivate practically any invading pathogen by binding to it in some way that inhibits its function. The steps taken with HRV14 establish a paradigm for future research: The protein target from the virus or bacterium must first be carefully characterized and the active site determined. To establish the detailed structure, the distribution of charge, and the arrangement of solvent molecules around the active site, several tools of structural biology

FIG. 3 THE MANY FACES OF COLD VIRUS HRV14. **The protein coat of the human rhinovirus HRV14 is composed of 12 "pentameric caps," each containing five copies of the three coat proteins, VP1, VP2, and VP3. The computer reconstructions shown here illustrate one of these fivefold-symmetric units, with red, green, and blue used to denote the three constituent proteins. One such unit occupies each of the 12 vertices of a regular icosahedron. A similar symmetry distinguishes many of the viruses whose structures have now been determined. [Michael G. Rossman, Purdue University]**

THE ART AND SCIENCE OF

Working out the three-dimensional structure of a protein by x-ray crystallography is a marriage of art and science. A good part of the art comes at the beginning, in the preparation of the crystals—a step that has resisted every effort at cookbook regimentation. Indeed, one of the triumphs of the work on photosynthetic reaction centers (see page 19) has been the successful crystallization of these recalcitrant membrane protein complexes. Further, it is usually necessary to prepare several types of crystals: the native biological molecule and then the same molecule containing different heavy-metal substitutions, whose known positions provide a starting point for the analysis. Once the crystals are in hand, data collection can begin. The data consist, basically, of the patterns of scattered x-rays obtained when the crystals are immersed in a highly collimated beam of x-rays. Patterns are recorded, on film or by electronic means, for both the native protein and the metal-doped ones, each in as many orientations as possible. From these patterns and the intensities of the individual spots, computer analysis yields an electron density map. And ultimately, again by application of some artful human insight, aided by computer graphics of increasing sophistication, a molecular structure emerges. Apart from the examples described in this booklet, notable successes of x-ray crystallography have included, among many others, structures crucial to understanding the mechanism of gene expression and repression (DNA-repressor complexes) and the involved process of protein synthesis (the recognition complex between transfer RNA and tRNA synthetase).

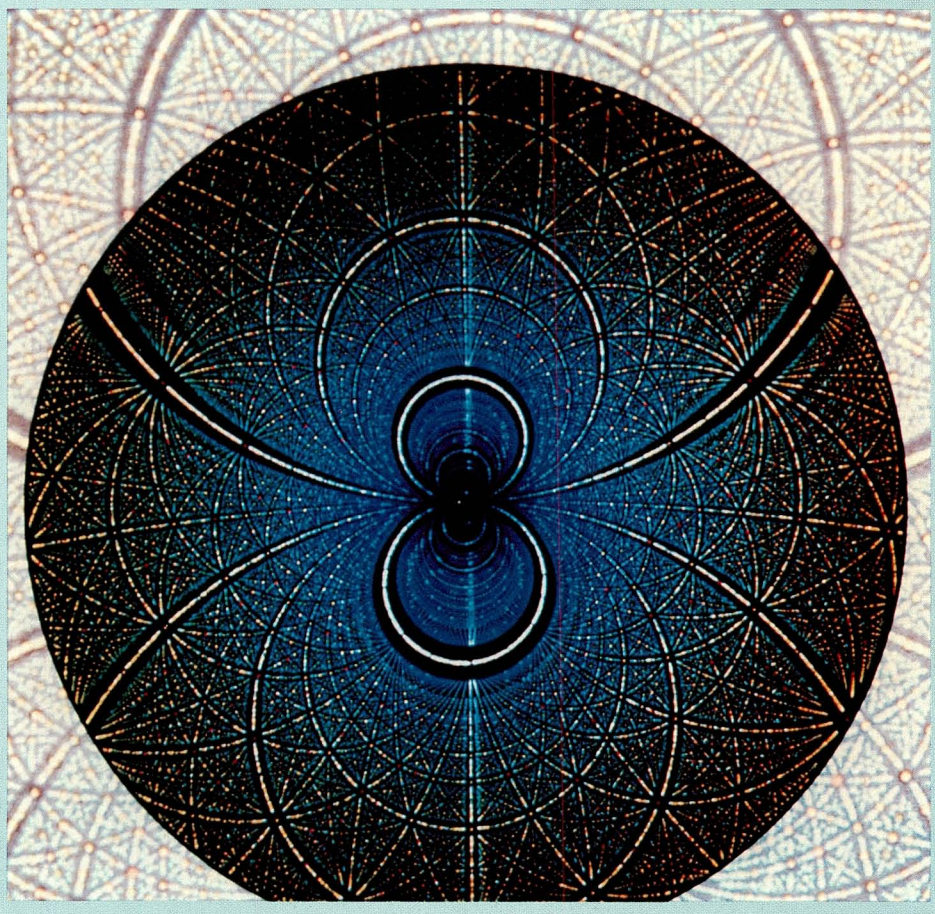
The 1930s saw the first glimmer of hope that biological molecules might be studied by x-ray crystallography. An enzyme was crystallized for the first time in 1926, and eight years later, the first good diffraction pattern was obtained from crystals of pepsin. By the late thirties, a small, but visionary, community had committed itself to making x-ray crystallography a useful biological tool. Nonetheless, it was not until 1958 that the first protein structure was solved—a three-dimensional model of myoglobin, described as a "visceral-looking object" with a "squashed orange" attached to it. Two years later, an improved structure at near-atomic resolution followed for the same molecule, together with a solution for the structure of hemoglobin. For these early successes, John Kendrew and Max Perutz shared the Nobel Prize for Chemistry in 1962. (Two years later, Dorothy Crowfoot Hodgkin, who also shares credit for the pepsin diffraction patterns, won the Prize for her earlier determination of the structure of vitamin B_{12}.) Along Kendrew and Perutz's tortuous path to success, perhaps the key discovery was the importance of *isomorphous replacement,* the technique of using heavy-atom derivatives to give an analysis a starting point.

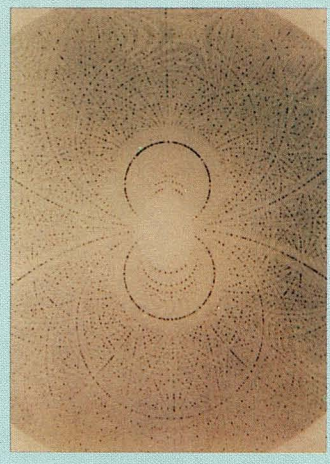
Since those early days, x-ray crystallography, though still largely an art, has taken many steps forward. One such stride has been made possible by the revolution in genetic engineering. Preparing samples in sufficient quantities and orchestrating amino acid substitutions are operations now routinely performed by bacteria engineered for the purpose. Perhaps the most dramatic advance, however, has come in the area of instrumentation, for today the most exciting prospects rest with synchrotron radiation, intense light from facilities that are the direct descendants of machines developed for high-energy physics research (see pages 42–46). The pinpoint intensity of this light, together with the ability to "tune" its wavelength, have slashed the time required to obtain crystal data, opened the door to the analysis of small or evanescent crystals, and catalyzed the development of at least two completely new approaches to x-ray crystallography.

One of these recently developed techniques is *multiple-wavelength anomalous dispersion,* a method that uses the synchrotron's virtue of wavelength tunability to get around the need to collect data from both native and metal-substituted crystals. The use of this technique has already made possible several structure determinations that would have been impossible with classical methods. A second technique that is possible only with synchrotron radiation is white-light Laue diffraction, which uses the raw, "un-monochromatized" beam from the synchrotron and allows a full set of data to be collected in a fraction of a second. Recently, snapshot pictures of enzymes actually in the process of catalyzing biochemical reactions have been obtained by this method.

It should also be mentioned that the use of synchrotron radiation poses challenges to match its promise. Espe-

X-RAY CRYSTALLOGRAPHY





cially at the facilities now under construction, continuing advances in the design of optical components will be needed if researchers are to realize the full potential of the new sources, with their intensity and high brightness. Likewise, among x-ray crystallographers, the further development of *area detectors* is one of the highest priorities. As the available flux of x-rays increases, it becomes correspondingly more important to gather data rapidly. To meet this need, area detectors act like the electronic analogues of photographic film, recording the entire pattern of diffracted x-rays simultaneously. These detectors have already had a dramatic impact on the field, but the new sources will put even higher demands on technological development.

MOLECULAR SNAPSHOTS. Whereas conventional diffraction patterns are obtained by exposing a crystal to monochromatic x-rays, Laue diffraction experiments use a wide and continuous spectrum of wavelengths from a synchrotron radiation source—the x-ray analogue of "white light." Shown here in color is a computer simulation of a Laue pattern for the enzyme glycogen phosphorylase. The actual data (smaller image) were obtained in a 1-second exposure, and much shorter exposures are now practical. [Louise Johnson, Oxford University]

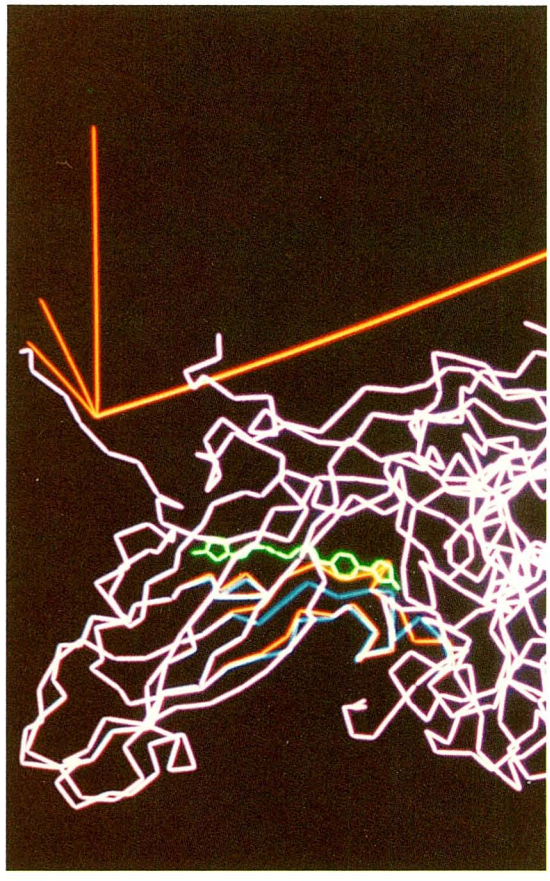


FIG. 4 ATTACKING THE COMMON COLD. The coat of HRV14 includes "canyons" encircling each of the fivefold vertices. The coats of different cold viruses show great variability, but many of them share the same structure at the canyon floors, which are the all-important binding sites for receptors on the surface of human cells. A compound with demonstrated antiviral properties is shown here bound within a cleft at the canyon floor, thus inducing a structural change in the virus itself. This structural shift, from blue to red in this computer-generated image, inhibits viral "uncoating," a necessary step if the virus is to inject its contents into the host cell. [Michael G. Rossman, Purdue University]

might be necessary—x-ray crystallography, neutron crystallography, NMR, x-ray spectroscopy, and others. (Each tool has its virtues, as the following pages aim to demonstrate.) With a complete physical chemical picture of the active site in hand, a suitable drug can then be designed—a molecule that fits snugly and binds tightly, even irreversibly. The drugs first tested with HRV14 were previously established antivirals, but in the future we can expect computers to suggest likely candidates never before synthesized.

Validating the drugs thus designed will ultimately be a task for biochemistry and pharmacology, but the process is again likely to involve a step taken with HRV14, namely, structural characterization of the pathogen-drug complex. A detailed picture of the interaction between drug and active site, together with biochemical evidence of the agent's effectiveness, provide a rational way to drug modification and refinement. This reasoned approach to drug design is in marked contrast to the guesswork that necessarily prevails when molecular structures remain hidden.

In conclusion, it should be emphasized that this scenario is more than the stuff of dreams. Active work is in progress to develop pharmaceuticals that will clog the influenza virus's binding sites, now that they have been well-characterized, thus preventing the virus from infecting human cells. Even more important efforts focus on the human immunodeficiency virus (HIV), which causes AIDS. HIV has not been crystallized, but some enzymes essential to its function have, as have parts of the cell receptor to which HIV binds in the human body. On both fronts, attempts to inhibit the activity of HIV—by getting in the way of its reproduction or by plugging up its binding sites—show encouraging progress.

To be sure, much remains to be done before this kind of drug design is a routine business. And in every instance, great care must be taken to guarantee that a new and effective drug has only its desired effect—just as in the drug approval process of today. Perhaps the way is not yet well-traveled, but the path to rational drug design is being prepared by the continuing successes of structural biology.

Turning Light Into Life

PROBING THE PHOTOSYNTHETIC PROCESS

THE PHOTOSYNTHETIC conversion of sunlight into chemical energy is the principal basis for life in the biosphere. Not surprisingly, then, the intricacies of photosynthesis have been probed for decades, both by biochemical means and, increasingly in recent years, by means of the physical tools of structural biology.

Broadly speaking, photosynthesis in higher plants is a means of "harvesting" sunlight, first trapping its energy to charge a kind of biochemical battery, then using the stored energy to power the conversion of water and atmospheric CO_2 into carbohydrates. In the process, the water yields O_2 in a little-understood step that provides all of the atmosphere's oxygen. This simple picture belies the complexity of the process, and fully understanding photosynthesis remains a profound challenge, further complicated by the fact that green plants, algae, and photosynthetic bacteria employ many different photosynthetic strategies. Nonetheless, owing to the enormous potential payoffs—improved crop yields, even new sources of energy—a worldwide effort continues in pursuit of a clearer structural and functional picture of this fundamental natural phenomenon.

Some of structural biology's most visible recent achievements have been successful structural studies of bacterial *reaction centers,* sites of the critical light-driven chemistry in photosynthetic bacteria. The breakthrough was the determination of the structure of the reaction center from the purple bacterium *Rhodopseudomonas viridis*—an effort by Hartmut Michel, Johann Deisenhofer, and Robert Huber, rewarded in 1988 with a Nobel Prize (Figure 5). The structure of this membrane protein complex is closely correlated with its function as a light-driven electron pump, propelling electrons along part of a cyclical path that crosses the photosynthetic membrane. By a series of steps, now far better understood as a result of this structural insight, a disequilibrium of protons is established, then used to synthesize the molecular energy packet adenosine triphosphate (ATP).

This current structural and functional picture is a culmination of efforts in many laboratories and is thus a striking example of the many-faceted nature of structural biology. Building on earlier lower-resolution studies using x-ray and neutron diffraction, x-ray crystallography provided a dramatic and integrative structural insight, but much that we know of the reaction center's function is a product of studies using very different techniques. Optical spectroscopy, for example, has been used to probe the sequence and timing of the first chemical steps of the photosynthetic process. Sub-picosecond laser flashes provide a unique probe of early events by allowing absorption spectra to be recorded for a sequence of transient excited states. Magnetic resonance has also played an important role. Apart from providing some of the earliest evidence for the presence of free radicals in the first steps of the process and for the presence of bacteriochlorophyll dimers in bacterial reaction centers, magnetic resonance has provided sound evidence that the structure seen by x-ray crystallography is the same as that in living cells.

Of particular importance in photosynthesis research has been x-ray absorption spectroscopy (XAS) using synchrotron radiation. XAS is most often used to probe the environs of metal atoms, frequent constituents of important proteins. Sensitive measurement of the absorption spectrum

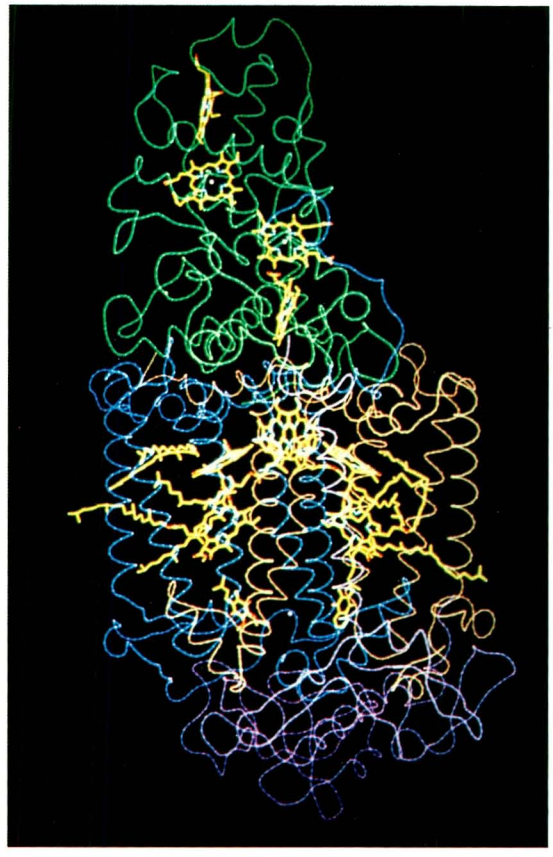
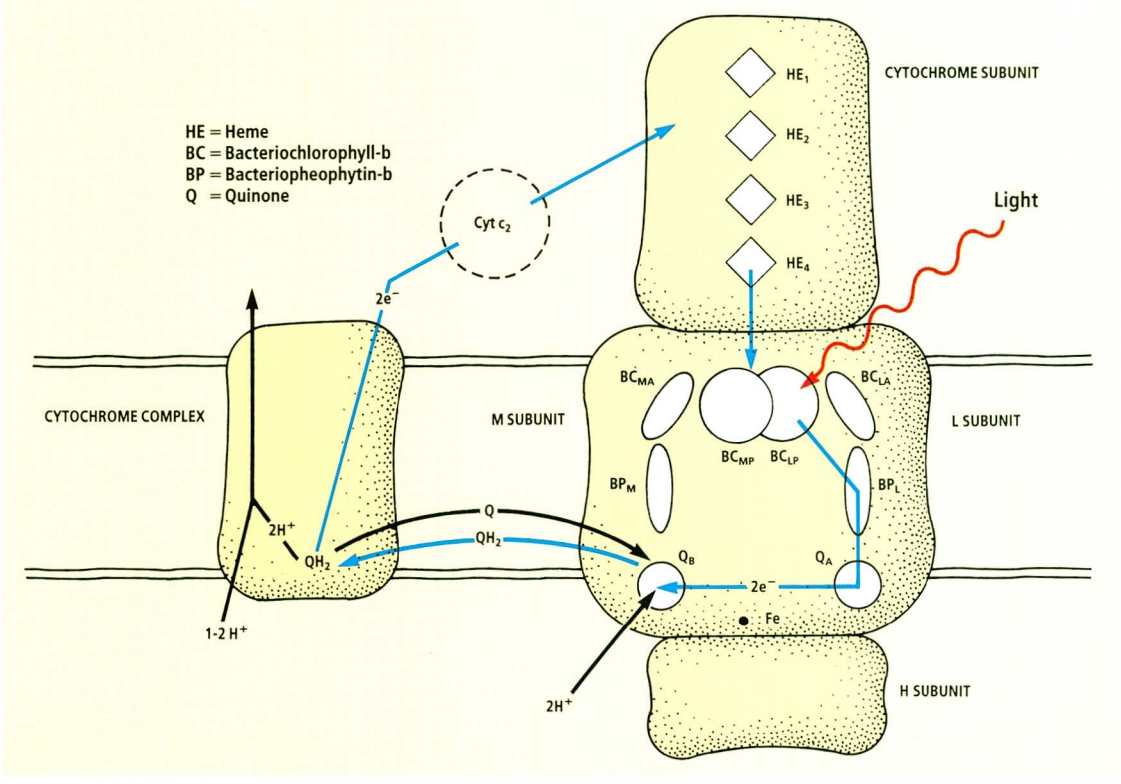


FIG. 5 THE PHOTOSYNTHETIC APPARATUS: STRUCTURE AND FUNCTION. The computer-generated image shown here depicts the structure of the photosynthetic reaction center from the purple bacterium *Rhodopseudomonas viridis.* The polypeptide backbones for the four subunits are traced in four colors: cytochrome, green; L, pale yellow; M, blue; and H, violet. The thicker, bright yellow lines represent various cofactors, such as iron-containing heme groups and light-absorbing bacteriochlorophylls. This structural determination, a dramatic success of x-ray crystallography, was an essential contribution to the current picture of the reaction center's functional role, depicted schematically below. The absorption of light by a pair of bacteriochlorophyll molecules (BC_{MP} and BC_{LP}) prompts a cyclic electron flow through the L subunit, to an independent cytochrome complex, and back through the cytochrome subunit. At the same time, there is a net efflux of protons across the membrane, producing a proton gradient that is used to synthesize molecules of ATP. [Johann Deisenhofer, University of Texas Southwestern Medical Center]



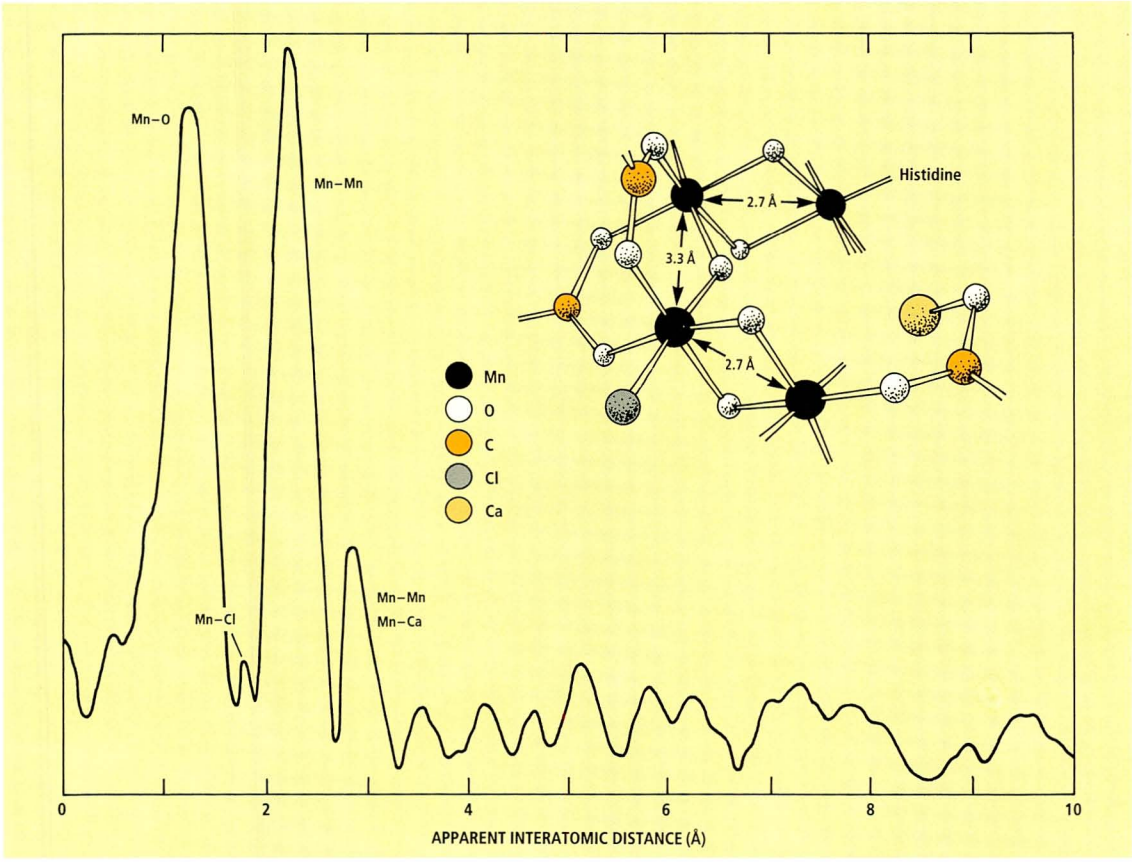


FIG. 6 MAPPING THE ATOMIC NEIGHBORHOOD. **Extended x-ray absorption fine structure (EXAFS) spectroscopy is a well-developed means of identifying the nearest neighbors of a strongly absorbing metal atom and charting their positions. Indeed, materials scientists have used EXAFS to establish interatomic distances to accuracies of 0.01 Å. In this example, the Fourier transform of an EXAFS spectrum reveals the "apparent" distances between manganese atoms and between manganese and its neighbors in the oxygen-evolving complex of photosynthetic membranes. These apparent distances are directly related to true interatomic distances. Structural changes associated with the cyclic events involved in oxygen evolution can also be observed by noting changes in EXAFS spectra such as this. The molecular structure shown here is a preliminary model consistent with the EXAFS data.**

of a particular metal can often reveal in extraordinary detail the arrangement of its neighbors. This information can, in turn, expose the particulars of functional activity in the region. In the context of XAS and photosynthesis, one critical element is manganese. In the photosynthetic membranes of green plants, the oxygen-evolving complex is an assembly of several proteins, with a cluster of four manganese atoms, together with calcium and chloride cofactors, at its center. In this complex, the energy of four photons is used to split two water molecules into hydrogen and oxygen, thus producing one molecule of O_2. This complex has not yet been crystallized, but the details of its structure are emerging nonetheless, thanks to the sensitivity of XAS (Figure 6). With a deeper understanding of the mechanism of oxygen evolution, it may even become possible to design synthetic catalysts that can produce hydrogen fuel and oxygen from nothing more than water and sunlight.

Another facet of photosynthesis research is the effort to uncover the functional details of a molecule known as RuBisCO (ribulose *bis*-phosphate carboxylase-oxygenase; Figure 7). Forms of this enzyme, the most abundant in all of nature, are responsible for "fixing" most of the CO_2 that is converted by photosynthetic organisms into sugars, starches, and other usable carbohydrates. Since this critical step is the source of all foodstuffs, understanding it has an obvious

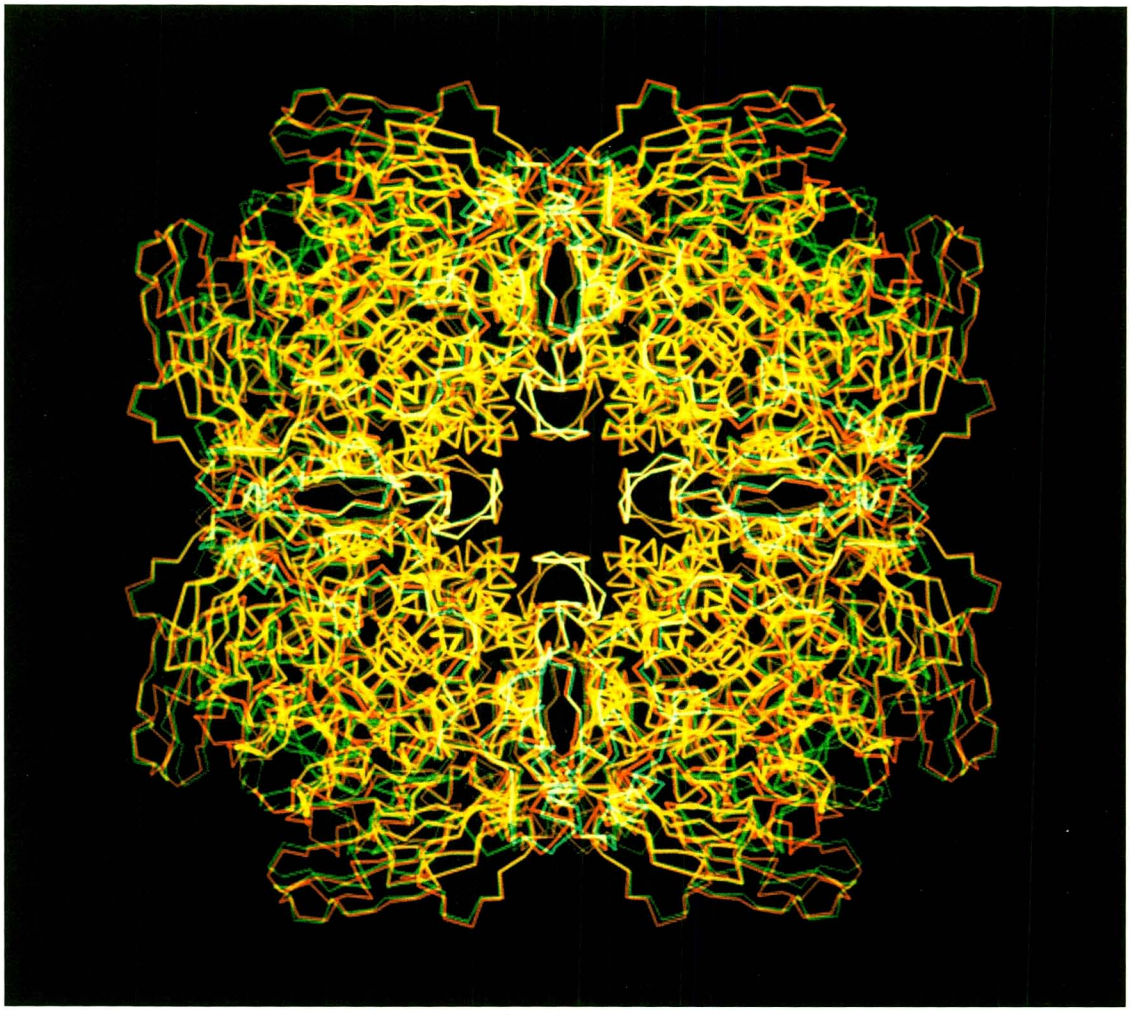


FIG. 7 NATURE'S MOST ABUNDANT ENZYME. Another essential element of the photosynthetic process is the conversion of stored energy and CO_2 into usable sugars and starches. This step is the responsibility of the enzyme RuBisCO, whose highly symmetrical three-dimensional structure in higher plants consists of eight copies of each of two subunits. In this image, the conformation of the active form of the enzyme (green) is superimposed on that of an inactive form (red). Regions that are identical in the two forms appear in yellow. The enzyme is activated by the presence of CO_2, which is then "fixed" in the form of carbohydrates. [David Eisenberg, UCLA]

attraction. Indeed, by somehow enhancing the activity of RuBisCO, one can envision increasing crop yields and food supplies. The other side of the coin, however, is RuBisCO's activity as an oxygenase. In darkness RuBisCO reverses its role and burns the sugars it has made, thus producing CO_2. To selectively inhibit this part of RuBisCO's catalytic activity is another plausible strategy for dramatically enhancing food production. As might be expected, this molecule, too, became an early target of crystallographers. Recently, the three-dimensional structure of this multifunctional enzyme was established, thus adding significantly to our understanding of how this economically important protein functions. X-ray crystallography showed how the 16 protein chains in one molecule are organized, with the catalytic sites in deep pits at the interfaces between chains. It also confirmed inferences from biochemical studies regarding the identity of some of the amino acids involved in activation of the molecule and in its catalytic activity. In addition, the detailed structure revealed the hitherto unsuspected roles of many other amino acids. On the basis of this work, biochemical experiments can now proceed, to see if the wasteful oxygenase reaction can somehow be suppressed.

UNANSWERED QUESTIONS

AT THE FRONTIERS OF BIOLOGY

THE NEXT FEW PAGES shift the focus slightly. The contributions of structural insights to our understanding of biological function will still show through, but the intent here is to suggest avenues of research that lie largely before us. In most cases, the lines of inquiry have been drawn, but much remains to be done. Formative development of new techniques and new technologies must be extended, and new facilities must be built. The concluding section of this booklet thus describes a course of action to provide for these needs of tomorrow's research.

Unraveling the Chromosome

A PUZZLE OF GENETIC ORGANIZATION

GIVEN ITS ROLE AS THE repository of the genetic script, the eukaryotic chromosome has attracted abundant scrutiny since its discovery in the mid-19th century. And understanding its structure, organization, and function remains central to biology today. In the continuing effort, many of the tools of structural biology have played a part: light and electron microscopy, neutron scattering, x-ray diffraction, and more. The resulting picture shows a fascinating hierarchy of poorly understood organizational levels, in which double-stranded DNA, in association with various proteins, is coiled and supercoiled into the fully condensed chromosome (Figure 8).

At the resolution available with the light microscope, several essential features of the chromosomes are visible, the most striking of which are their familiar and characteristic staining patterns, suggesting reproducible and highly ordered DNA folding arrangements within each chromosome. Hints of the complexity of this packaging emerge in electron microscopy images of human chromosomes (Figure 9). The second of the images shown here, though, also suggests a measure of order, and perhaps a functional basis for the structural organization of the chromosome. The estimated number of the observed DNA loops—still attached to a stable chromosomal scaffolding, but otherwise depleted of their organizing proteins—corresponds closely to the estimated total number of human genes. This raises the intriguing possibility that each loop defines a genetic unit of one gene, or perhaps a small number of linked genes, thereby providing a direct link between structure and function.

Upon even closer examination, now with such techniques as neutron scattering and x-ray and neutron crystallography, as well as electron microscopy, even deeper levels of order come into focus. It is now clear that a basic structure of the chromosome is a repeating subunit called the *nucleosome,* in which about 200 base pairs of double-stranded DNA are wound around a protein core. This string of nucleosome beads is then "supercoiled" into a solenoid-like configuration. The neutron scattering work has been especially illuminating. By altering the composition of an H_2O/D_2O mixture, it is possible to "contrast match" either the protein core or the DNA coiled around it. That is, conditions can be manipulated so that either one component or the other is largely invisible to the probing neutrons—blending in, as it were, with the surrounding water. Variations on this theme have made neutron scattering a tool of choice in studying the actomyosin complex that is the basis for muscle contraction, as well as multi-subunit enzymes and other macromolecular complexes. In the case of the nucleosome, this technique has produced a clear picture of the basic organization of chromatin, one structural step up from the double helix itself.

In complementary studies, the detailed structure of the nucleosome is being probed by x-ray and neutron crystallography. At a resolution of 7–8 Å, for example, much is revealed about the nature of the highly bent DNA wrapped around the protein core. In addition, the structure of the *histones* that constitute the core suggests possible mechanisms of unfolding in the nucleosome that may be related to the control of gene expression. Organization at the nucleosome level may therefore be crucial to switching genes on and off and to regulating cell division—processes central to our understanding of many disease states, includ-

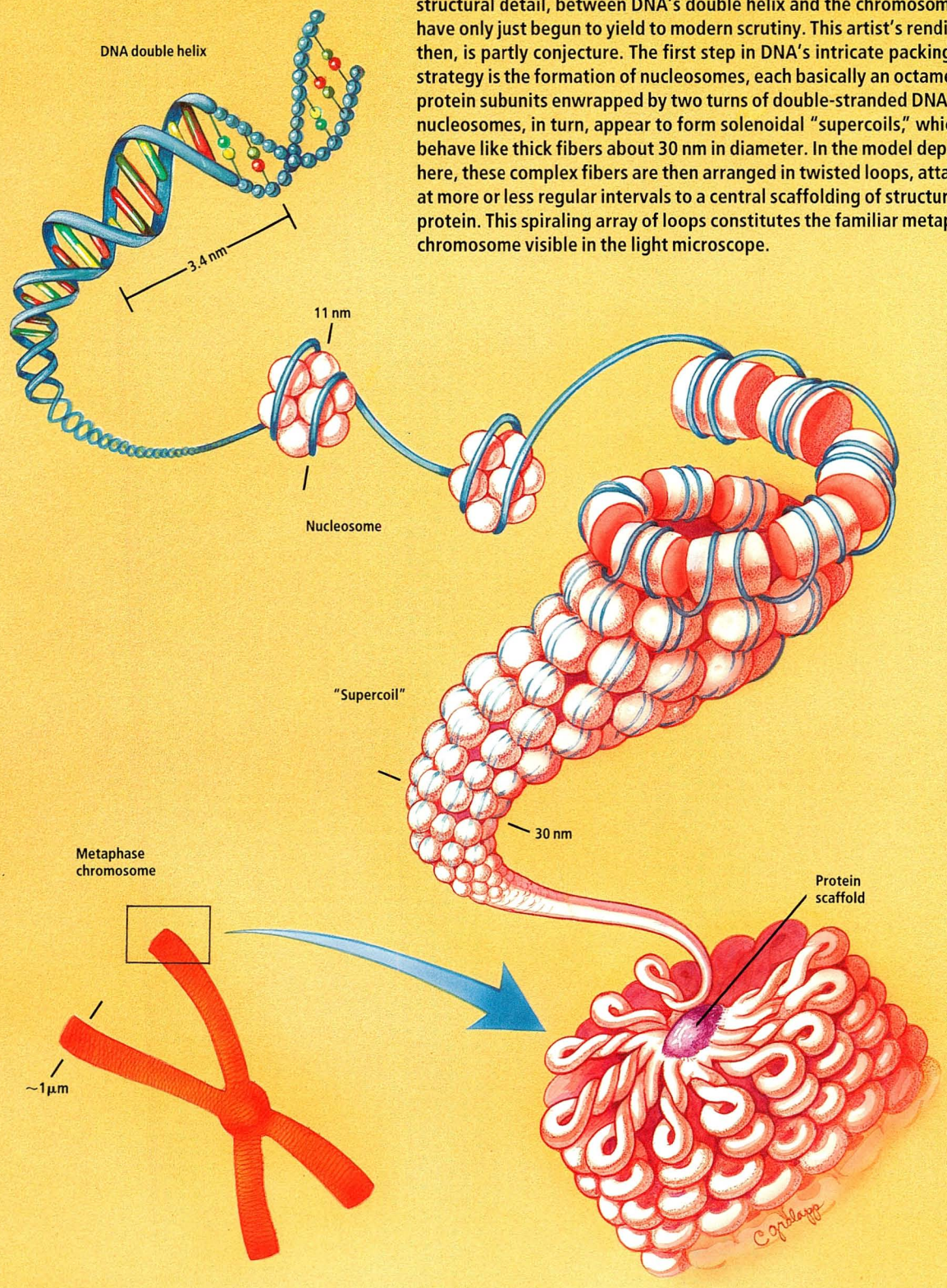


FIG. 8 LAYERS OF COMPLEXITY. The intervening levels of structural detail, between DNA's double helix and the chromosome, have only just begun to yield to modern scrutiny. This artist's rendition, then, is partly conjecture. The first step in DNA's intricate packing strategy is the formation of nucleosomes, each basically an octamer of protein subunits enwrapped by two turns of double-stranded DNA. The nucleosomes, in turn, appear to form solenoidal "supercoils," which behave like thick fibers about 30 nm in diameter. In the model depicted here, these complex fibers are then arranged in twisted loops, attached at more or less regular intervals to a central scaffolding of structural protein. This spiraling array of loops constitutes the familiar metaphase chromosome visible in the light microscope.

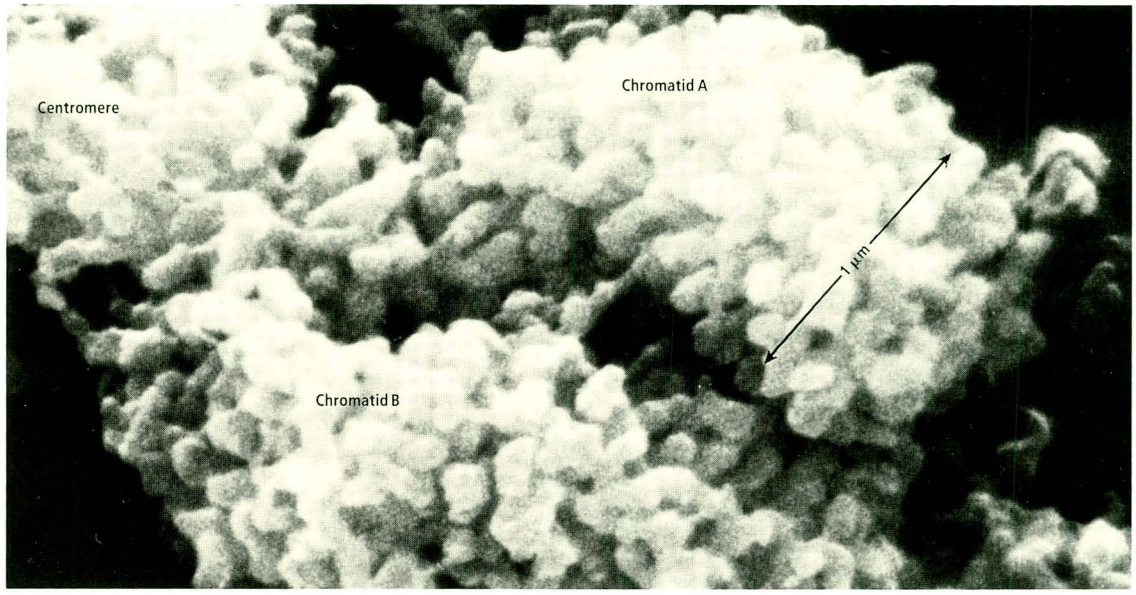
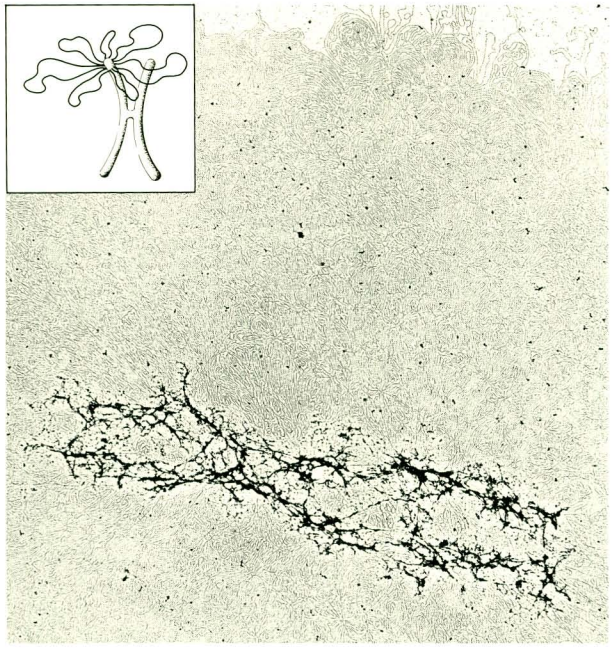


FIG. 9 CLUES TO CHROMOSOME STRUCTURE. Electron micrographs provide some of the evidence that underlies the current picture of chromosome structure. In the picture above, a scanning electron micrograph of a human metaphase chromosome shows compact projections thought to be condensed loops of thick chromatin fibers. In the image below, a human chromosome has been depleted of its organizing histones. The remaining nonhistone proteins form a scaffolding that retains the shape of an intact chromosome. Loops of naked DNA fibers form a halo around the scaffolding and remain attached to it at intervals (see inset). Judging from the size of the loops, researchers have suggested that each may represent a single gene or a set of linked genes. [U.K. Laemmli, Université de Genève]



ing cancer. To elucidate the functional role of the nucleosomes further, work continues in an effort to improve the resolution of these structures to near 3 Å.

Unfortunately, a large hiatus in our structural knowledge lies between the 10-nm scale of the nucleosome and the micrometer scale of the chromosome; even the supercoiled string of nucleosomes is merely the simplest model consistent with very sparse structural data. At the same time, this is a structural scale of great functional importance. How, for example, might different packaging arrangements at this level of organization affect gene expression? Might some genes in a given cell type be permanently suppressed by packaging them so that they are inaccessible to the proteins that control transcription? And what is the relationship between higher orders of chromatin structure and the process of DNA repair? DNA is forever susceptible to both environmental and spontaneous damage; DNA repair is therefore a vital and universal process, common to all species from bacteria to man. But we know little about how the chromosome's damage surveillance system works, or how the repair proteins gain access to the damaged DNA and go about their work. We need to know much more about the structure of both normal chromatin and chromatin that contains damaged DNA, as well as the three-dimensional structure of the repair proteins themselves.

Doing Things Nature Never Thought Of

CUSTOM-MADE ENZYMES AND ANTIBODIES

ENZYMES ARE THE ubiquitous workhorses of the living world, equally essential for replication, synthesis, digestion, and decay. As the biosphere's catalysts, these proteins can increase the rates of essential biochemical reactions by factors of a billion or more. The key to their speed and specificity lies, of course, in their three-dimensional structure. The structural configuration and charge distribution in the neighborhood of an enzyme's active site are precisely suited to its task. In the simplest cases, the substrate molecule (and no other) docks at this highly specific site, is chemically changed, and is then released in its transformed state. To understand this process in the most important enzymes is a fascinating prospect; to shape it to new purposes, though, would be the greatest triumph.

As with so many proteins, x-ray crystallography has been used to give us detailed pictures of enzyme structure. However, since the greatest interest focuses on only a small part of each enzyme—namely, the active site—other techniques have also proved invaluable as structural probes. One such technique is nuclear magnetic resonance spectroscopy (see page 32); another is x-ray absorption spectroscopy (XAS). As in photosynthetic membranes (see page 19), XAS has been used to investigate the environs of metal atoms in enzymes. Since these atoms are commonly found near the active sites, the resulting information can often be related to catalytic function. Iron, for example, is involved in several classes of enzymes that catalyze the oxidation of organic molecules. These include heme enzymes such as lignin peroxidase (where the iron is complexed in a multi-ring, nonprotein structure), and nonheme enzymes such as methane monooxygenase (MMO). Both of these enzymes are being studied by XAS, and in both cases, a more complete understanding of catalytic function might mean more than just adding to our basic knowledge: Both may prove useful in the fight against environmental contamination.

MMO shows particular promise. As a constituent of many bacteria, this enzyme oxidizes methane to methanol, which then serves as a metabolic source for the host. In addition, MMO is capable of degrading more complex hydrocarbons, including those found in crude oil, as well as such environmental pollutants as trichloroethylene and vinyl chloride. In recent pilot studies, MMO-containing bacteria have been used to purify aquifers polluted by industrial waste and to help in the cleanup of major oil spills, including the 1989 Valdez spill. These initial efforts at *bioremediation* are just a start, but they presage great advances in environmental restoration as we learn more about the structure and function of nature's catalysts.

The greatest promise, though, is going nature one better—modifying her enzymes and substrates to do society's bidding. Among the envisioned products of enzymatic catalysis are new materials for everything from clothing and skin grafts to adhesives and spacecraft. One approach to customizing enzymatic reactions is to feed a native enzyme a new substrate. By knowing the detailed structure of the active site of glycogen phosphorylase, for instance, researchers were able to infer that the enzyme might polymerize substrates it wasn't designed to accept. The products in this case—fluorinated glucose polymers, for example, which might have novel elec-

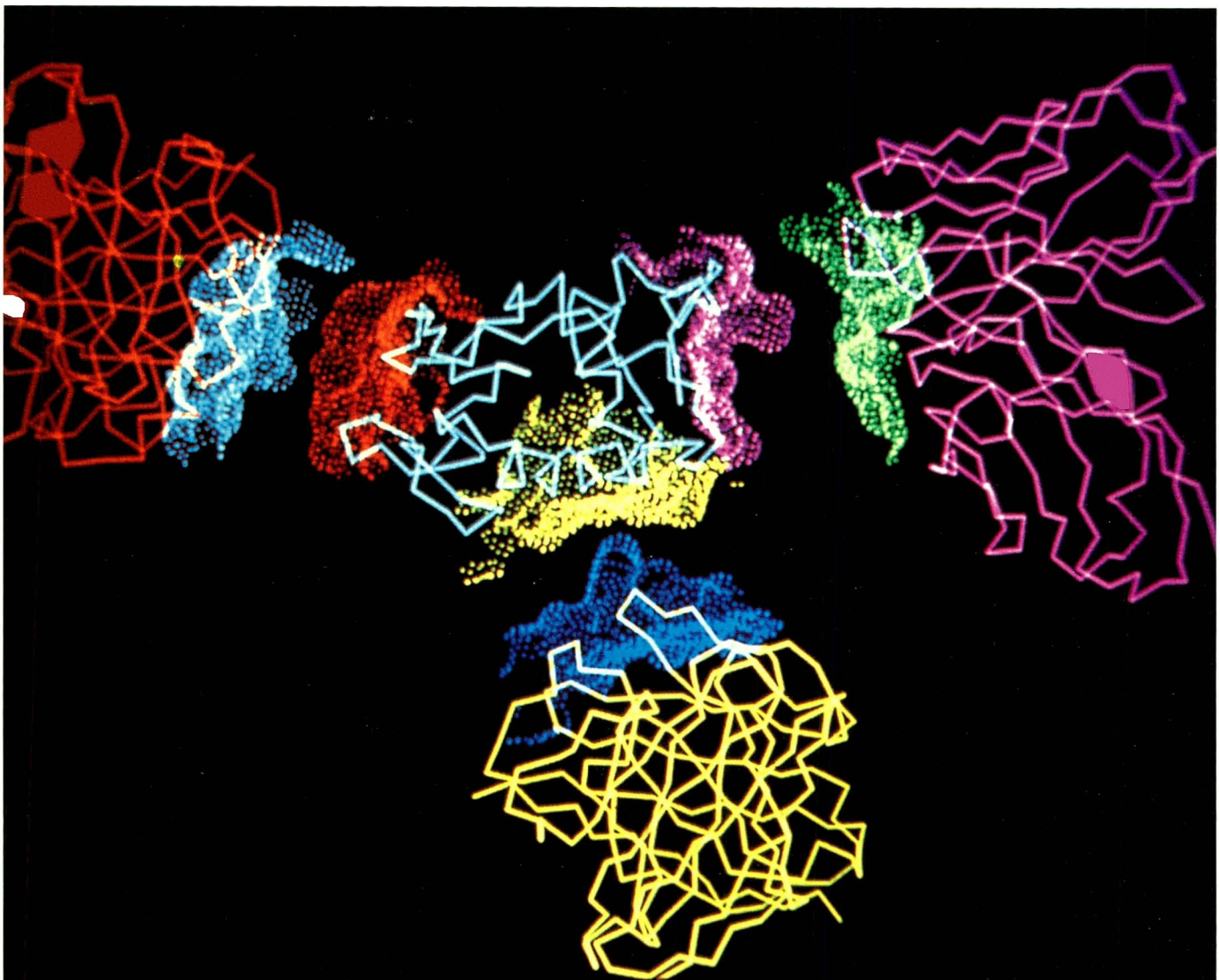


FIG. 10 DETAILS OF THE BODY'S DEFENSE. Antibodies serve at the front line of the body's defense system. Their production is stimulated by the presence of foreign substances, or antigens, to which the antibodies then fasten as part of the body's counterattack. The most common antibodies are Y-shaped proteins, two arms of which are tipped with highly specific binding sites. The image shown here summarizes the results of three independent x-ray crystallographic studies of antigen-antibody complexes. In each, the structure of a different complex was determined. The antigen here is lysozyme, an enzyme found in egg whites. Shown are a single lysozyme molecule (blue) and portions of three different antibodies from mouse (red, yellow, and violet). The complementary binding domains of both the antigen and the antibodies are depicted as stippled surfaces. This kind of detailed information can be used to engineer antibodies that act like enzymes or that bind only to certain cancer cells. [David Davies and Eduardo Padlan, NIH, and Steven Sheriff, The Squibb Institute for Medical Research]

tronic or surface properties—have potential industrial applications. Another approach, among several others, is *site-directed mutagenesis*. With this technique, amino acid substitutions within a native enzyme are orchestrated by changing the corresponding codons in the gene that codes for the enzyme. By knowing the structure of the enzyme, one can, in principle, prescribe amino acid substitutions to selectively alter the enzyme's function, thus customizing it to produce new products or to destroy toxic chemicals. Indeed, the first steps have already been taken toward realizing this futuristic scenario: Several enzymes have been successfully tailored to modify their specificity, enhance their efficiency, and increase their stability under adverse conditions.

Still another approach to "natural" catalysis is the development of *catalytic antibodies*. Antibodies are the immune system's foot soldiers, defending the body against alien substances such as viruses and toxic chemicals. By binding to these invading antigens, antibodies counteract their effects, either neutralizing them directly, immobilizing them, or setting them up for further biochemical attack. Much that we know about antibodies, including the structure of the all-important antigen-binding site and how antibodies recognize antigens in the first place, has emerged from studies using x-ray crystallography. In particular, the structures of the complementary surfaces of antigen-antibody complexes illustrate the reason for the exquisite specificity of antibodies (Figure 10). Though we do not yet know all the rules that govern this specificity, structural biology has revealed much about the crucial process of antibody-antigen recognition.

To turn these antibodies into catalytic agents, the key step is to determine the *transition state* for a chemical reaction of special interest, that is, to identify a transient intermediate near the energetic hump between reactants and products. Next, an organism is exposed to a "transition-state analogue," whereupon the animal's defense system is induced to make antibodies against it. By enhancing the formation of the transition state, these antibodies, in many cases, can then serve as effective catalysts for the reaction whose transition state was mimicked.

It might be said that we now know enough about enzymes and antibodies to understand their importance and to foresee their great promise—a promise to be realized only by learning a great deal more. The prospects for customizing enzymatic processes to aid in environmental cleanup and in the synthesis of novel materials are taken seriously by a great many chemists, biochemists, molecular biologists, and materials scientists. The key to each step forward, however, must be based on an accelerated effort in structural biology to understand protein structure and function.

Tackling Membrane Proteins

NEW DIMENSIONS IN CRYSTALLOGRAPHY

MOST PROTEINS GO about their business in the aqueous milieu of the cell cytoplasm, but some of the most important are more at home in the fatty environment of the cell membrane. Lipid bilayer membranes isolate the living cell from the extracellular environment and serve also to enclose specialized intracellular compartments, the cell nucleus being the most conspicuous example. Impermeable to most water-soluble molecules, these membranes allow the cell to concentrate and sequester critical metabolites and enzymes. As signal transducers and channels for ions and metabolites, the *membrane proteins* embedded in these membranes, in effect, modify and regulate this impermeability. They also conduct electrical impulses, play central roles in synthesizing and maintaining the membrane itself, and, in photosynthetic membranes, convert light to chemical energy.

Within the large class of membrane proteins that regulate membrane activity, *transmembrane receptors* constitute an especially important group. In the living cell, a "signal" is typically a cascade of biochemical events, leading from some extracellular messenger molecule to a secondary messenger molecule within the cell. A transmembrane receptor receives the primary signal and somehow transmits it across the membrane, thus precipitating a series of intracellular events whose product is the secondary messenger and, ultimately, a suitable biochemical response to the initial message (Figure 11).

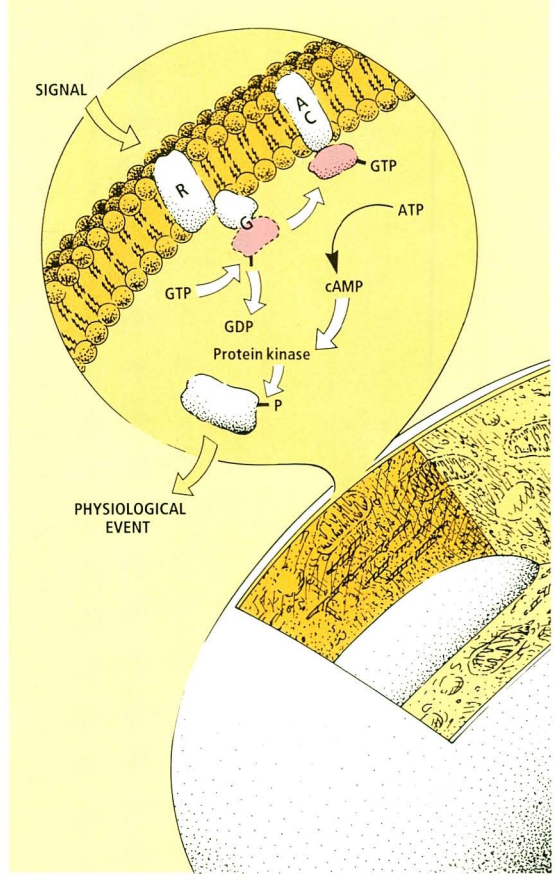


FIG. 11 GETTING THE MESSAGE THROUGH. Broadly, signal transduction in the cell requires three elements: a primary messenger whose role is to set the chain of events in motion, a receptor (R) that receives the signal and transmits it through the cell membrane, and an intracellular "second messenger" that relays the message to its destination in the cell. In this example, a transmembrane receptor prompts the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) by a subunit of a closely associated G-protein. The subunit, with its GTP, then migrates to adenylyl cyclase (AC) and produces the second messenger, cyclic AMP, which activates cAMP-dependent protein kinase, the necessary trigger to produce the physiological response. Whereas the water-soluble proteins of the primary and second messenger systems are amenable to analysis by classical crystallographic techniques, the vital transmembrane proteins can rarely be crystallized.

At either end of this signaling pathway, classical techniques of structural biology have made major contributions. For example, the structure of insulin—an extracellular messenger whose message is "metabolize sugar"—was one of the first to be determined by x-ray crystallography. The *ras* protein described on page 12, on the other hand, is one of a class of molecules known as *G*-proteins, intracellular components responsible for transmitting signals to their ultimate destinations within the cell. Again, our knowledge of the structure is a fruit of x-ray crystallography.

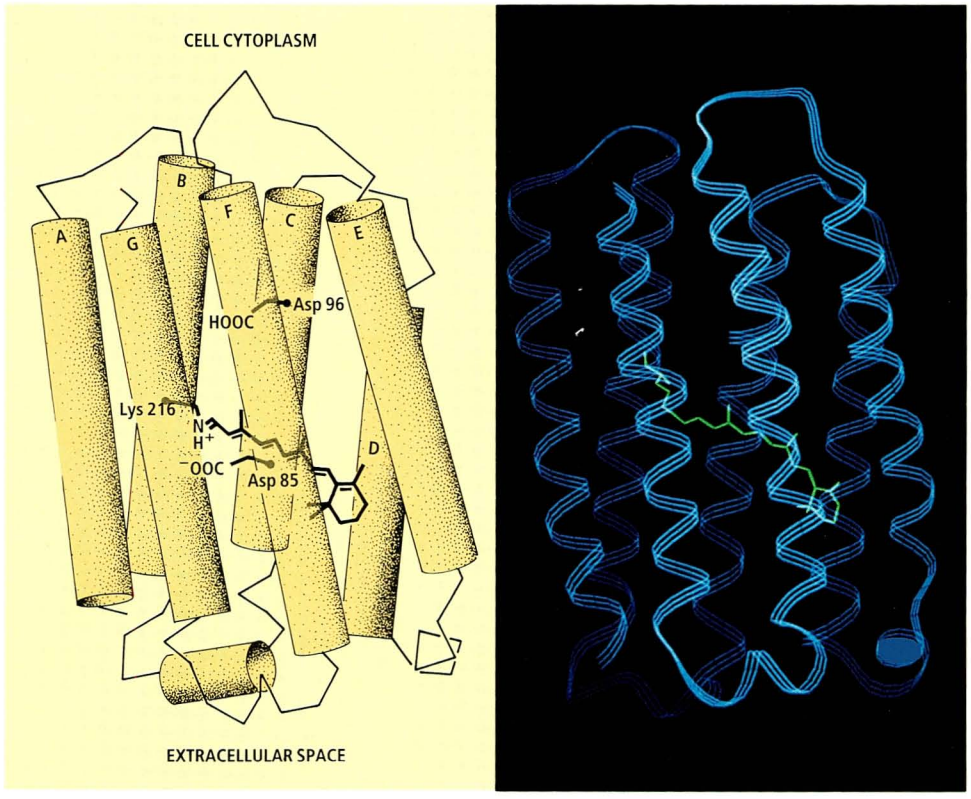


FIG. 12 IMAGES OF MEMBRANE STRUCTURE. **Membrane proteins rarely crystallize in a form suitable for x-ray crystallography, but they can sometimes be made to form two-dimensional crystals that yield to analysis by electron diffraction. A recent triumph of this technique was the elucidation of the structure of bacteriorhodopsin, a light-driven proton pump from *Halobacterium halobium*. This ribbon diagram of the protein backbone, showing the light-absorbing retinal molecule in yellow, suggests the presence of a proton channel down the center of the structure. A schematic view into this channel can be used to describe the conjectured mechanism of proton transfer. The absorption of a photon causes a *trans*-to-*cis* isomerization of the retinal and prompts the transfer of a proton from lysine-216 (on helix G) to aspartic acid-85 (helix C). The proton is subsequently transferred to the extracellular space, and the lysine-216 is reprotonated from the cytoplasm, via aspartic acid-96 (helix C). [Richard Henderson, MRC, Cambridge, England]**

The transmembrane receptors themselves, however, represent a missing link in this family of structural studies. The obstacle is one that impedes progress on practically all membrane proteins: Most of these proteins, essential elements of cellular architecture and activity, have so far resisted efforts to crystallize them in a form suitable for classic x-ray diffraction studies. (A rare success story has been the crystallization of photosynthetic reaction centers—see page 19.) Luckily, some of them *can* be crystallized in two dimensions, thus forming a membranelike monolayer. And in this form, they are again amenable to analysis by certain diffraction techniques. In particular, single-crystal diffraction patterns can be obtained by using electrons. Structure determination by this means is in its infancy, by comparison with x-ray techniques, but it has already scored several notable successes. A recent example was the structural determination, at near-atomic resolution, of bacteriorhodopsin from *Halobacterium halobium* (Figure 12). Neutron scattering work with deuterated specimens, together with results from a number of other techniques, had established some of the general features of the molecule, but electron diffraction provided the details. As in the case of the photosynthetic reaction center, this success led directly to a functional model that elegantly ties together a great deal of earlier data.

There are, then, two major avenues to be pursued aggressively if we are to add significantly to our successes with membrane proteins. The first is to expand efforts to crystallize membrane proteins in a form that will allow classic x-ray crystallography studies. The second is to continue efforts based on diffraction by two-dimensional crystals. Without the knowledge to be gained by one means or the other, much that we would like to know about cellular structure and function will remain unexplained—including an essential step in the biological signal pathway.

Structure and Dynamics of the Living Cell

IN PURSUIT OF THE WHOLE PICTURE

IN SOME SENSE, the history of structural biology is a story of increasing "visual" acuity, an ever-increasing facility to discern structural detail and thus to infer functional mechanisms. The ultimate goal, though, might be to retrace history's steps—to reassemble, at least conceptually, the pieces of the cellular apparatus and thus gain a clearer picture of the intact living cell. This is a formidable challenge; indeed, x-ray crystallography, the most commonly exploited technique for high-resolution structural study, requires orderly crystalline samples, deprived of their natural environment, and thus cannot reveal all of the dynamic properties of molecules. Accordingly, some of structural biology's newest ways of seeing—as well as its most venerable—take as their subjects more complex, functional assemblies, or at least molecules in solution, where molecular dynamics is less constrained.

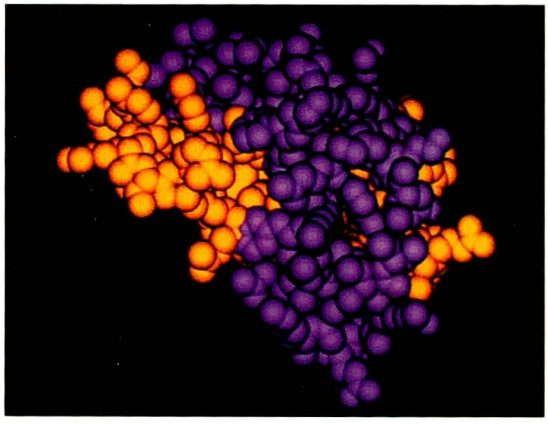
Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) spectroscopy is a logical complement to crystallographic methods: The goals are similar, but the approaches very different. NMR is a well-established method of chemical analysis, but only within the past few years has it emerged as a way to resolve the three-dimensional structure of molecules as large as small proteins. In NMR's early days, it allowed one to infer only the structures—often only the empirical formulas—of small, relatively simple molecules. Over the past five years, however, thanks in large part to advances in the technology of superconducting magnets and in computer modeling techniques, NMR has been used not only to elucidate three-dimensional molecular structure, but also to uncover details of molecular motion—the bending and flexing of molecules in solution that are vital to physiological processes.

By subjecting a sample solution to a strong magnetic field, then probing the local electromagnetic fields around the atoms of the sample molecule, the decades-old technique of "one-dimensional" proton NMR produces a spectrum whose peaks can be assigned to the different species of protons in the molecule. Further, the relative sizes and positions of the peaks yield clues to the number and environment of each type of proton. By virtue of advances in instrumentation and computer analysis techniques, two-dimensional NMR now goes one large step further. Instead of a simple spectrum, each two-dimensional experiment yields a kind of contour map on which "connectivities" can be established between NMR peaks (Figure 13). These correlations, in turn, indicate the spatial proximity of the protons that gave rise to the peaks, and provide an estimate of the number of chemical bonds that separate them. With enough such clues, the investigator can construct a consistent three-dimensional structure.

Much information has come in this way from two-dimensional NMR spectra, but within the past few years, further advances have taken place: the practical implementation of three-dimensional NMR and, in 1990, the first successful four-dimensional NMR experiment. These increasingly sophisticated techniques now yield information about spatial relationships among three and four nuclei, respectively. The advantage of three-dimensional NMR is a dramatic increase in spectral resolution, compared to one- and two-dimensional methods, thus making larger, more complex biochemicals amenable to structural study. Four-dimensional NMR, a method whose benefits lie almost entirely in the future, represents yet another step forward.

In practical use, both three- and four-dimensional NMR techniques require that the sample molecule be uniformly labeled with stable isotopes such as ^{15}N and ^{13}C. (NMR is sensitive only to *paramagnetic* nuclei, which include the ubiquitous proton, but not the most abundant isotopes of carbon and nitrogen.) With the combination of three- or four-dimensional NMR and isotopic labeling, the prospects are bright for analyzing much larger biomolecules



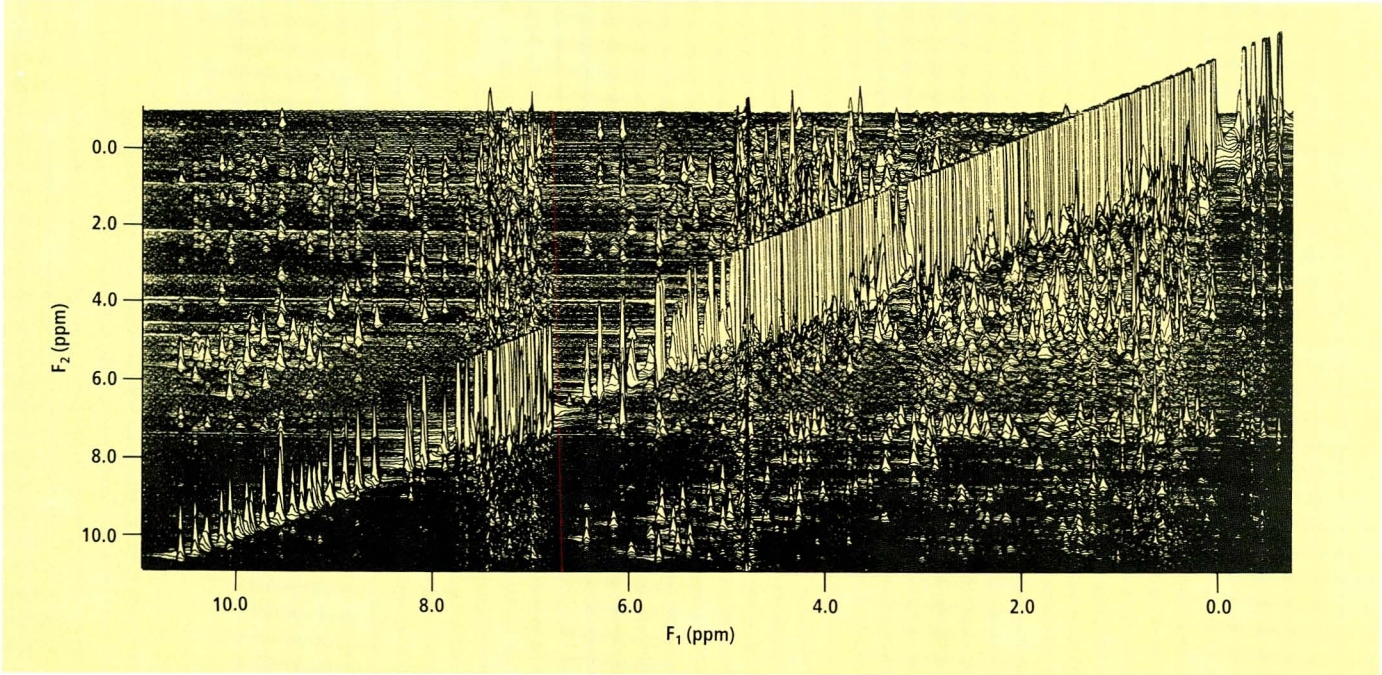
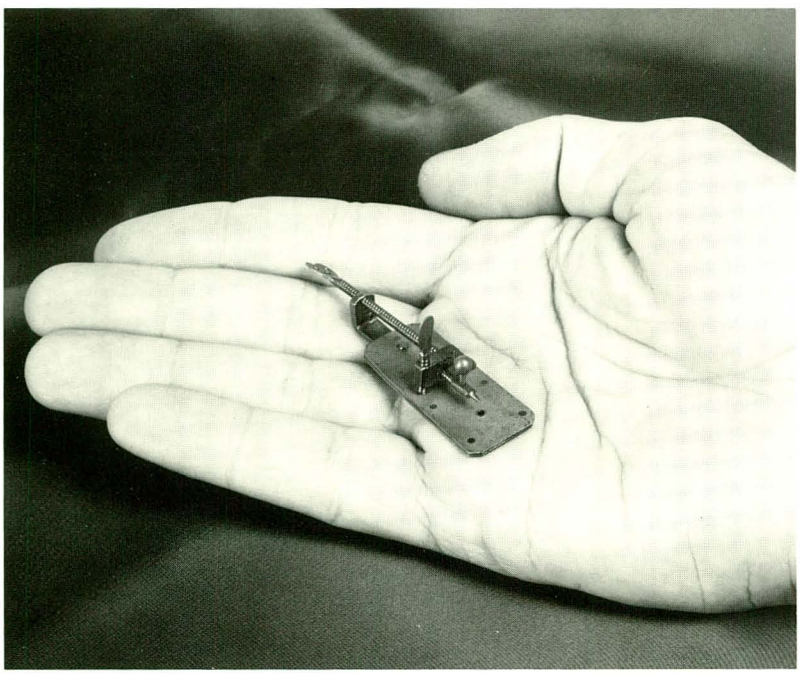


FIG. 13 AN NMR LANDSCAPE OF MONELLIN. Traditional one-dimensional NMR spectra are presented as conventional graphs; two-dimensional spectra are shown either as depicted here or as "contour maps." Three-dimensional NMR spectra, requiring an additional dimension for the display of their three-dimensional analogues of "peaks," are presented within a cube, or as a series of two-dimensional slices through a cube. The peaks observed in multidimensional spectra arise from interactions among the magnetic fields of atomic nuclei, either via chemical bonds or because of the nuclei's proximity in physical space. The peaks in this two-dimensional spectrum of a protein called monellin arise from interactions between protons that are spatially separated by less than about 4 Å. The intensities of the peaks provide estimates of spatial separation. These and other NMR results have provided information about the effects of engineered modifications to the protein and thus offer hints about the way the protein folds into its stable three-dimensional shape. The structure of the unaltered monellin (above) was determined by crystallographic means.

FIG. 14 SIMPLE ORIGINS. With a modest instrument such as this, Antony van Leeuwenhoek founded the science of microbiology. One of the nine extant examples of Leeuwenhoek's original microscopes has a magnifying power of 266 × and is easily capable of resolving smaller-than-average bacteria. The sample is affixed to the pointed spike and is viewed through a lens mounted in the small hole below it. Three thumbscrews and the knob on the sample holder provide focusing and sample-position adjustments. [Replica courtesy of Caroline Schooley, Robert D. Ogg Electron Microscope Laboratory, UC Berkeley]



in solution and for a new understanding of physiological dynamics at the molecular level. As an example, one of the central puzzles of biology, the complex problem of protein folding, may yield some of its secrets to NMR, which can be used to identify intermediates in the folding pathway (see pages 39–40).

Imaging: The Classical Microscopies

Over the years, the study of cellular structure and dynamics has been dominated by two techniques, light and electron microscopy. Indeed, the cell itself was a discovery made possible by light microscopy (Figure 14). Today, despite their well-known limitations, both microscopies continue to evolve, and both remain essential parts of the arsenal of methods available to structural biology.

Light microscopy enjoys the great virtue of requiring little by way of sample preparation. It is thus perhaps the most faithful probe of functionally intact living systems. The disadvantage is that light microscopy cannot resolve details separated by much less than 1 μm, a limit imposed by the wavelength of visible light. The banding patterns of the chromosomes thus press the limits of the technique. At the same time, new developments have opened new doors, and there are even tricks that appear to subvert the physical limitations of resolution. Single DNA molecules, for example, have been detected and their dynamics studied by tagging them with fluorescent dyes. Thus, a strand of material only 2 nm wide can be made "visible," although its shape cannot be resolved.

Among the significant new developments has been the introduction of confocal scanning optics, which yields a highly resolved image from a single in-focus plane. Applications include noninvasive optical "sectioning" of biological specimens and the investigation of structure deep within turbid samples—both likely to allow more detailed observations of natural structure and dynamic processes.

Although light microscopy was the first of the structural biologist's probes, electron microscopy surely shares with it the distinction of being the most productive. In particular, most of what we know today about the ultrastructure of the cell, albeit usually in a dehydrated state, has come by way of this versatile technique. With the development of low-voltage scanning electron microscopes, cell surface structures have now been imaged at resolutions of 2 nm, and scanning transmission electron micrographs have revealed functional detail at the macromolecular level (Figure 15). Techniques are also now available for viewing hydrated samples, both in a frozen state and under near-ambient conditions. In addition, by use of related electron diffraction techniques, suitable samples can be structurally characterized at near-atomic resolution (see pages

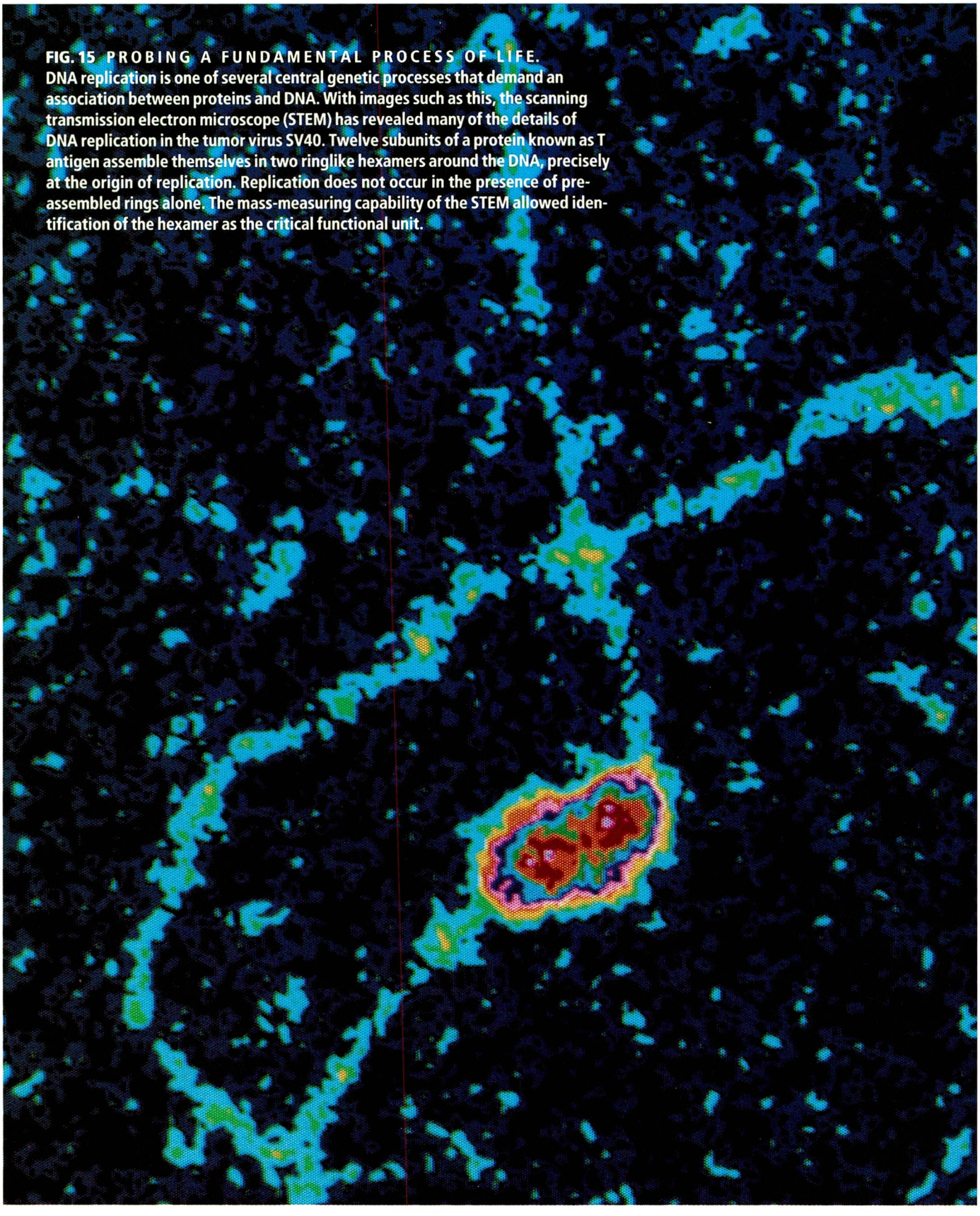


FIG. 15 PROBING A FUNDAMENTAL PROCESS OF LIFE. DNA replication is one of several central genetic processes that demand an association between proteins and DNA. With images such as this, the scanning transmission electron microscope (STEM) has revealed many of the details of DNA replication in the tumor virus SV40. Twelve subunits of a protein known as T antigen assemble themselves in two ringlike hexamers around the DNA, precisely at the origin of replication. Replication does not occur in the presence of pre-assembled rings alone. The mass-measuring capability of the STEM allowed identification of the hexamer as the critical functional unit.

30–31). Further advances will take this latter tool even further, to yield high-resolution images of such difficult specimens as helical fibers, icosahedral viruses, and other symmetrical assemblies. The limitations of electron microscopy, however, invite the development of complementary methods—methods that will go beyond light microscopy in their discernment of detail, yet preserve the structural integrity of living systems.

Imaging: New Ways of Seeing

It has long been realized that the limitations of light microscopy might be overcome by using shorter-wavelength light, thus lowering the lower limit on resolvable dimensions. X-rays would be ideal. The impediment, until very recently, was the resistance of x-rays to being focused or reflected. Today, microlithography, a technique of the semiconductor industry, has ushered in a revolution in x-ray optics, and intense new sources of x-rays have catalyzed a new interest in x-ray imaging (Figure 16). Further, with even brighter sources of synchrotron radiation on the horizon and with further developments in microfabrication techniques likely, x-ray images resolved to 10 or 20 nm appear possible within a few years. Along a parallel path, the technology of x-ray holography has already yielded images of subcellular organelles at a resolution of about 50 nm, and prospects for the future include x-ray laser–produced images that provide three-dimensional information about biological structure.

The attraction of x-ray microimaging is severalfold. First, the penetrating properties of the x-rays permit the viewing of unsectioned objects, suspended in an aqueous solution similar to their native environment. In addition, high-resolution x-ray "snapshots" of biological systems, obtained with exposure times of less than a millisecond (less even than a nanosecond with an x-ray laser), may soon become feasible, offering the opportunity to probe dynamic subcellular events, such as those associated with secretion, muscle contraction, cell motility, and chromosomal folding and unfolding. And finally, x-rays offer the potential for elemental mapping—that is, establishing the distribution of specific chemical elements within a biological specimen by observing differences in x-ray absorption.

The potential of x-ray microimaging for structural biology can be suggested with a few questions: What is the appearance of mitochondria, chloroplasts, secretion granules, and the nucleus when probed in their native states at a resolution beyond the reach of light microscopy? What can be learned of the organization of the macromolecular aggregates contained within and making up these structures, as well as those found elsewhere in the cell—microtubules, ribosomes, muscle fibrils—when they are viewed without preparative alteration? What can we discover about the organizational structure of chromatin, at those scales where the current picture is largely speculative? And what can be learned about the distribution of specific chemical elements in the cellular inclusions found in various disease states, such as the calcium-containing spicules of certain degenerative disorders?

Interestingly, synchrotron radiation has yet another, very different use in the area of biomedical imaging. The goals of structural biology are best served by images at the subcellular level, but images at much lower resolution may also prove to be an important benefit of research at synchrotron radiation facilities. In particular, *coronary angiography* at such facilities shows great promise. About a million coronary angiograms—in effect, x-rays of the heart—are performed each year in the U.S. to diagnose the extent of symptomatic coronary artery disease. Owing to the invasive nature of this technique as currently practiced, mortality is high: Thousands of patients die annually as a direct result of the procedure. The promise of a method based on synchrotron radiation lies in the fact that it is far more sensitive to the iodine dye that reveals the state of the coronary arteries. As a consequence, the dye can be administered by routine intravenous injection, rather than by a risky aortic catheter. The first experimental studies with dogs, and more re-

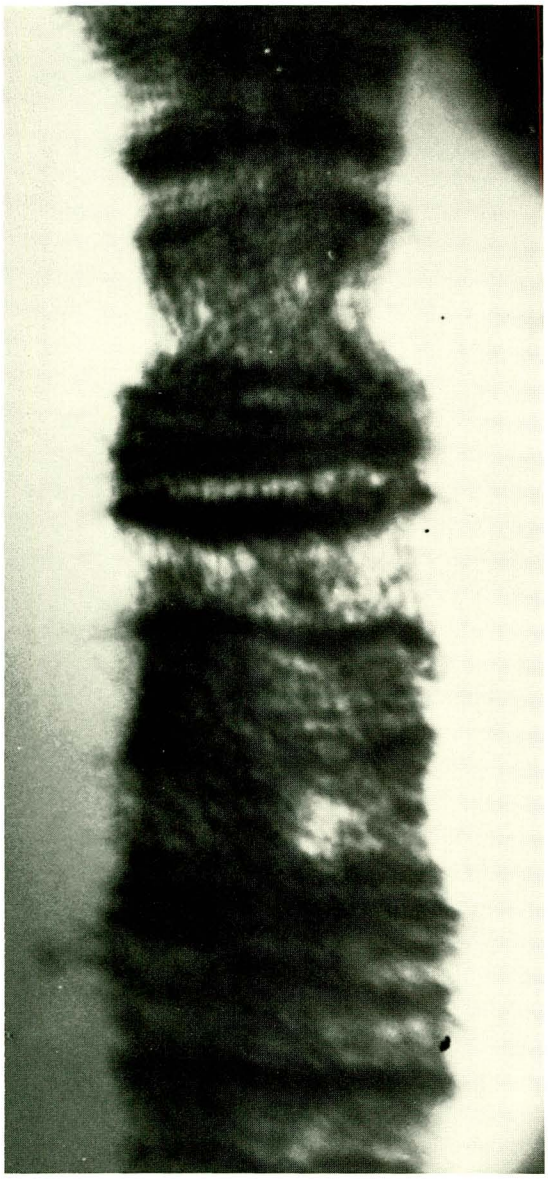
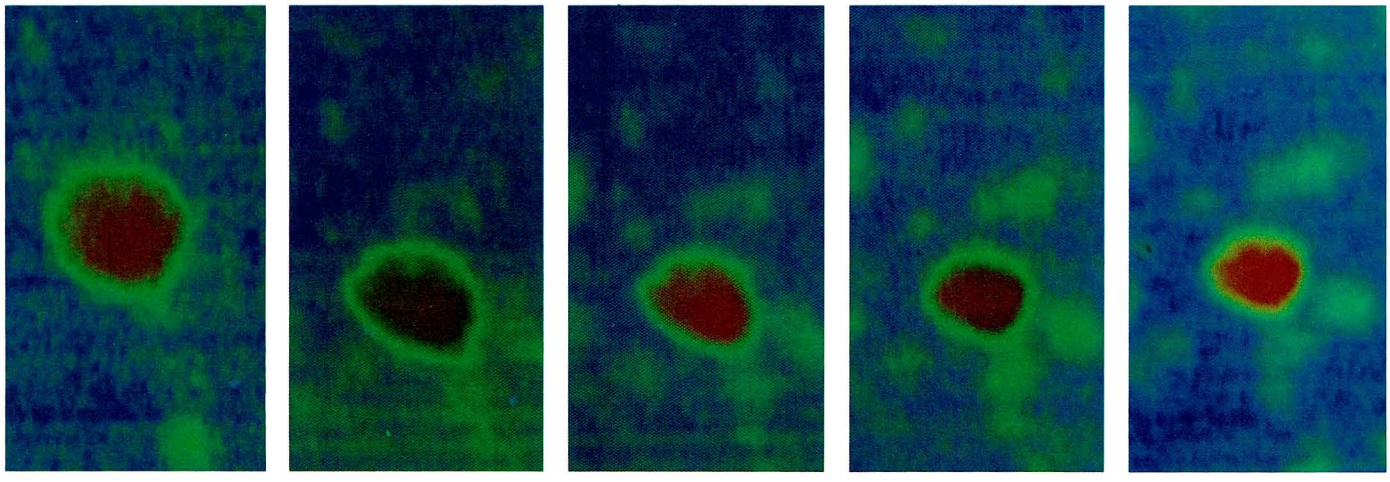


FIG. 16 IMAGING WITH X-RAYS: TOOLS AND RESULTS. The idea of an x-ray microscope long resisted practical application, for lack of suitable optical elements. The recent development of Fresnel zone plates, however, has now opened the door to imaging with soft x-rays. Each zone plate is a tiny diffractive disk, fabricated by microlithography to focus x-rays to a spot whose radius is approximately equal to the width of the zone plate's outermost rings. This dimension can now be as small as 30 nm. To the left is an x-ray micrograph of a fully hydrated chromosome from a larva of the midge *Chironomus thummi.* This image suggests the promise of x-ray microimaging in elucidating subcellular structure. Also shown is a sequence of x-ray micrographs of a subcellular secretory structure known as a zymogen granule. This sequence, a unique achievement of x-ray microscopy, shows dynamic changes in the granule that cannot be attributed to radiation damage—most notably shrinkage, accompanied by protein loss across the granule membrane. The transmitted x-ray intensity can be used directly to estimate protein content. The resolution of the granule images is about 50 nm. [Chromosome image: G. Schmahl and M. Robert-Nicoud, Universität Göttingen]



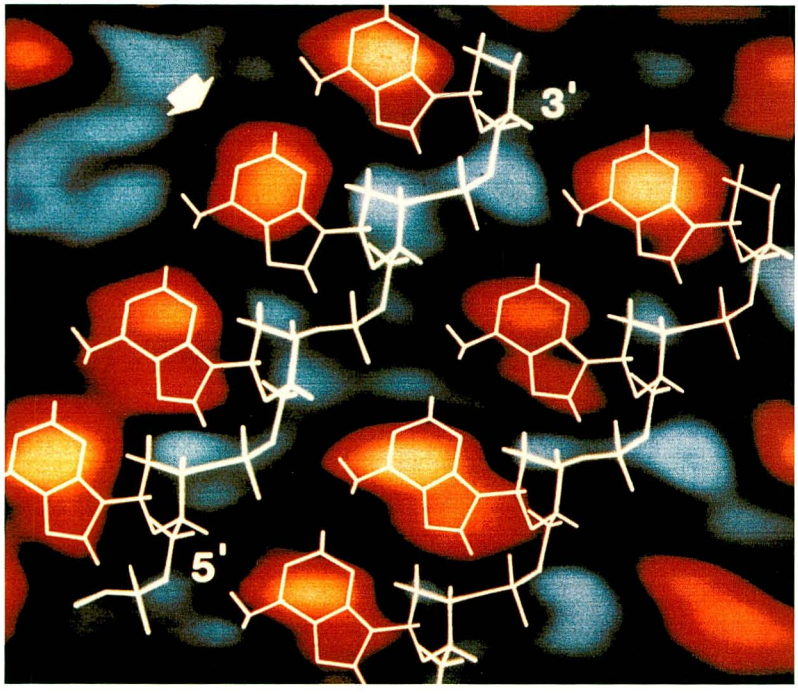


FIG. 17 MOLECULAR IMAGES. Scanning tunneling microscopy, capable of atomic resolution with conductive samples, has now been successfully applied in the more challenging arena of biological imaging. The resolution does not yet approach that obtained with conductors and semiconductors, but the individual bases of a single-stranded DNA-like polynucleotide have been discerned. In the image shown here, the areas coded yellow and red correspond to molecules of adenine, the A of ATGC and thus single characters in the genetic code. The structure of poly-A is shown in the overlay. [Carlos Bustamante, Institute of Molecular Biology, University of Oregon]

cently with human volunteers, offer realistic hope that this technique might someday replace the dangerous catheterization method, or perhaps even serve as a routine screening procedure for asymptomatic, but high-risk, individuals.

On a very different front, dramatic advances have been made in exploiting the *scanned-tip microscopies*. The shared characteristic within this family is the use of a fine stylus or probe, which is passed over the sample, allowing the measurement of some spatially varying physical property. The result is a surface profile of the sample, which might be, for example, a single molecule on a flat substrate. The original instrument in this class is the scanning tunneling microscope (STM). Capable of atomic resolution on surfaces of conductors and semiconductors, it has now yielded recognizable images of fully extended, single-stranded polynucleotides (Figure 17). Logical spin-offs of the STM include the atomic-force microscope, the near-field optical microscope, and a number of other innovative proposals for imaging at molecular, even atomic, resolution. A measure of their promise is the success of atomic-force microscopy in imaging both single protein molecules and the polar head groups of lipid molecules.

From Sequence to Structure

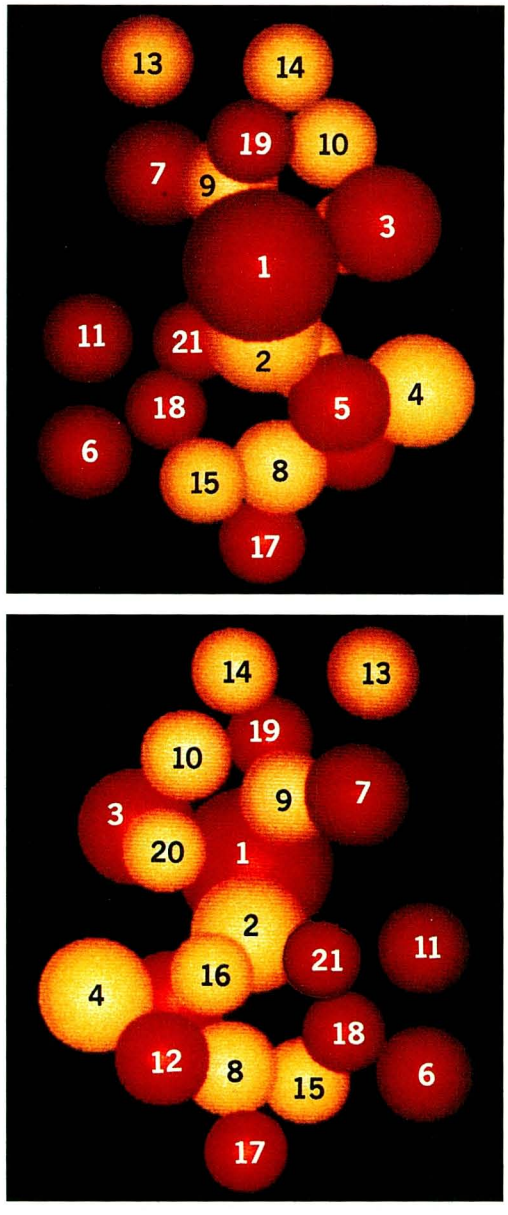
THE RIDDLE OF PROTEIN FOLDING

DETAILED THREE-dimensional structural reconstructions are now in hand for a few hundred proteins, each the product of considerable, even decades-long, effort. By contrast, the list of proteins whose "mere" amino acid sequences are known is a hundred times as long. However, these sequences *dictate* protein structure. Each protein sequence thus embodies all the information we need—in principle at least, if not yet in practice—to predict the final functional structure of the protein. To derive from the tenets of thermodynamics and kinetics the general precepts by which such predictions might be made is perhaps the central problem of structural biology. A complete solution would provide instant insight into the structure—and in time, the function—of tens of thousands of proteins. Even a minor breakthrough could considerably advance our understanding of protein structure. Most important of all, this insight will be the key to translating the product of the human genome project into the currency of practical knowledge. From the letters of the genetic code will emerge structural and functional information about thousands of pieces in the body's intricate biochemical puzzle.

In approaching the so-called folding problem, two strategies are possible. The more fundamental of these is to consider protein folding a kinetic problem and to ask *how* the protein assumes its final configuration. What conformation does the polypeptide chain assume when first synthesized, and by what sequence of events does it collapse and rearrange to give the final, biologically active structure? A general answer to this question is, naturally, beset with many challenges. First among them is the difficulty of characterizing the short-lived intermediates in the folding process, transient species usually present in only tiny quantities. All the same, exciting progress has been made. By changing the properties of the solution, an unfolded protein can be made to fold on cue into its stable, active form. If a "pulse" of deuterium is added during the folding, labile hydrogens that have not yet formed hydrogen bonds are replaced by deuterium. The labeled protein continues to fold, but by identifying the sites of substitution in the stable molecule, NMR can then provide a picture of the conformation of the intermediate. Applying deuterium pulses at different times reveals different intermediates. By such means, in fact, we are accumulating clues to the steps in the folding process.

The second strategy forsakes any hope of understanding the mechanisms of protein folding and asks instead, Given a string of amino acids, what final, stable structure does thermodynamics demand? Perhaps the most basic way to approach this question—which seeks only a correlation between sequence and final structure, not a path from one to the other—is to search for the most energetically stable conformation of the molecule, taking account of all the many interatomic forces that might come into play. Such conformational searches are far too cumbersome to apply to the problem of predicting protein structure from its sequence alone, but they have yielded insights into some of the interactions that stabilize protein structure. Furthermore, the extent of the search can often

FIG. 18 THE MACHINERY OF PROTEIN SYNTHESIS. In all cells, the ribosome, a complex assemblage of proteins and RNA, is responsible for the translation of the genetic code into functional proteins. The organization of the smaller of two ribosomal subunits from the bacterium *Escherichia coli* has now been revealed in a 15-year-long series of experiments using small-angle neutron scattering. The relative positions of the 21 constituent proteins, shown in these "front" and "back" computer-generated images, were determined by measurements of 115 different interprotein distances. Each distance determination relied on selective deuteration of a pair of proteins, followed by neutron scattering measurements in a solution that "contrast matched" the rest of the subunit. A sufficient number of such measurements ultimately yielded an unambiguous three-dimensional picture. [Peter B. Moore, Yale University]



be limited by referring to the growing data base of known protein structures. Whenever a newly determined sequence bears a significant resemblance to that of a solved protein structure, a fairly realistic model of the new protein can usually be derived. Indeed, even if the similarity exists only for limited domains, useful predictions can be made. In effect, a number of folding "motifs" have been identified and correlated with amino acid sequences. In addition, *site-directed mutagenesis,* which allows prescriptive replacement of key amino acids, has permitted systematic studies of sequence-structure relationships.

Clearly, though, we do not yet understand the full set of rules that govern protein folding. But as synchrotron radiation facilities make data collection easier, and as advances in data analysis accelerate the solution of structural puzzles, further patterns are sure to emerge.

The folding problem, however, does not end with the protein. One can also ask how larger macromolecular aggregates assemble themselves. Such assemblies are common functional components of the cell, and some have roles of central importance. Hemoglobin, for instance, is a tetramer of polypeptide subunits; anything less will not carry out its essential physiological function. A second, especially impressive example is the ribosome, which provides the molecular machinery for translating genetic instructions into amino acid sequences. Each ribosome consists of more than fifty proteins and three RNA molecules, organized in two subunits (Figure 18). In a number of cases, these larger functional structures, even including icosahedral viral coats, assemble themselves spontaneously in solution, when the appropriate components are present.

Fortunately, and perhaps not surprisingly, this self-assembly appears to be impelled by the same forces that drive protein folding. Protein-protein interfaces are stabilized by the same forces that stabilize the interiors of folded proteins. The riddle of protein folding thus has ramifications that extend well beyond the structure of isolated proteins. Progress toward its solution promises enormous insight into the living machinery of the cell.

SEIZING THE OPPORTUNITY

A COURSE OF ACTION

THE MESSAGE OF THE PRECEDING pages is clear: The prospects for structural biology are exciting. The vitality of the field has attracted the brightest minds; advances in molecular biology, instrumentation, and theory have fueled important discoveries; and the benefits to be gleaned from further insights into biological structure are now widely acknowledged. Indeed, the promise of practical payoff has encouraged investment by pharmaceutical firms and other industries. Nonetheless, progress in structural biology still depends largely on coordinated support from the National Institutes of Health, the National Science Foundation, the Department of Energy, and private philanthropic organizations.

The DOE has historically supported a broad range of biomedical research, both to fulfill its statutory responsibilities in the areas of health and safety, and to ensure that the resources of the national laboratories are effectively used to advance biomedical science and biotechnology. In line with the latter goal, the mandate for the national laboratories is, in large part, to pursue research that demands a confluence of interdisciplinary talent and to operate large facilities for a national community of users.

Research programs at the national laboratories exploit all of the techniques described on the previous pages, at the same time focusing on the refinement of those techniques and on the development of new technologies. National laboratories contributed the first images of DNA ever obtained with an STM, they have been responsible for seminal advances in NMR and electron microscopy, and they are major players in the development of techniques to produce customized enzymes. Accordingly, especially in programs coordinated with major research universities, the national laboratories have been fertile training grounds for future researchers in structural biology.

The next pages describe four areas critical to the advance of structural biology—areas that underscore the uniqueness of resources at the national labs. Then, in the concluding pages, the focus is on the future—in particular, on a course of action that will ensure the continued productivity of structural biology research at the DOE's national user facilities and thus help realize the profound promise of an exciting quest.

The National Laboratories and Structural Biology

BUILDING ON A UNIQUE ASSET

X-Rays for Structural Biology

THE MAJOR ROLE OF x-ray crystallography in elucidating the chemistry of life is well-illustrated by several of the examples in this booklet. X-rays are also the key to complementary probes of biological structure, especially x-ray absorption spectroscopy and x-ray microimaging. Taken together, x-ray techniques cover a range of structural scales from atoms to cells. And for all of these techniques, the essential resource today is *synchrotron radiation.*

Charged particles (usually electrons) forced to follow a curved trajectory emit light—synchrotron radiation—whose characteristics depend on the energy of the electrons and the radius of their curved path. In an electron storage ring, therefore, where electrons circulate for many hours at a constant energy and where magnets constrain the particles to nearly circular orbits, the electrons emit sweeping beams of light—like the headlight of a train on a circular track. It is this mechanism that produces most of the synchrotron radiation at present-day facilities.

However, a more purposeful manipulation of the electrons' trajectory is also possible. Indeed, the quality of synchrotron radiation can be significantly enhanced by inserting *undulators* or *wigglers* in the straight sections of an electron storage ring. These special magnetic structures, collectively known as insertion devices, bend the electrons along an undulating path, whereby the emitted light emerges as a more tightly constrained beam, centered on the axis of the electrons' motion (Figure 19). In particular, the radiation from undulators is compressed into a very narrow cone, sharply peaked at discrete wavelengths and with substantial laserlike properties of coherence. Furthermore, the wavelength of the light can be readily tuned by mechanically opening and closing the gap between the undulator faces. In contrast to undulators, wigglers produce a broad spectrum of synchrotron radiation, typically at higher photon energies.

Two synchrotron radiation facilities now under construction—the Advanced Light Source (ALS) at the Lawrence Berkeley Laboratory (LBL) and the Advanced Photon Source (APS) at the Argonne National Laboratory (ANL)—will be optimized for insertion devices such as these and will deliver beams of x-rays of unprecedented quality for basic research (Figure 20).

With regard to structural biology, the promise of these new sources of x-rays has two aspects. First, they will provide beams of greater *flux* and higher *brightness* (or "brilliance"). Greater flux means more photons per second; higher brightness, a direct consequence of the light's coherence, means more of the photons can be tightly focused to a small spot. Whether flux or brightness is the greater virtue depends on how the photons are to be used. For x-ray microimaging, focusability is the key virtue, and the ALS, with its high-brightness beam of soft x-ray photons, will be the ideal machine. In crystallography both brightness and flux can be important factors; which is more critical depends on the size of the molecule or supramolecular complex under investigation and on the size of the sample crystals that can be coaxed out of solution. The twin payoffs, however, are shorter exposure times and successful studies of smaller crystals. The implications of the re-

duced exposure times—as short as a nanosecond or less—are especially dramatic. It may become routinely possible to study dynamic processes in molecular crystals or supramolecular assemblies by establishing the three-dimensional structure of transient intermediate species. (It is surprising, perhaps, but many of the most important enzymatic reactions proceed efficiently in crystallized samples.) Short exposures also open the door to the analysis of highly labile specimens.

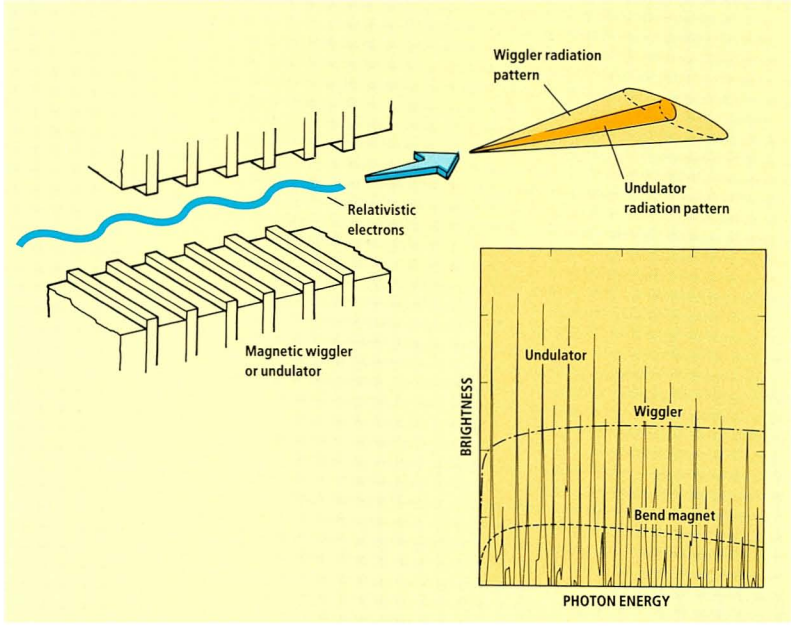
The second virtue of these new facilities is that they offer a first opportunity to integrate life sciences research into the programmatic plans of major synchrotron light sources from the start and to provide a fully adequate infrastructure for structural biology research. Resources for the life sciences are underdeveloped at today's facilities, at least in the U.S., and the facilities that are available are sorely oversubscribed. Addressing this problem means, for both the ALS and the APS, insertion devices and beamlines matched to the needs of the life sciences; experimental facilities designed expressly for x-ray diffraction, x-ray spectroscopy, and x-ray imaging; and ancillary laboratory and office space.

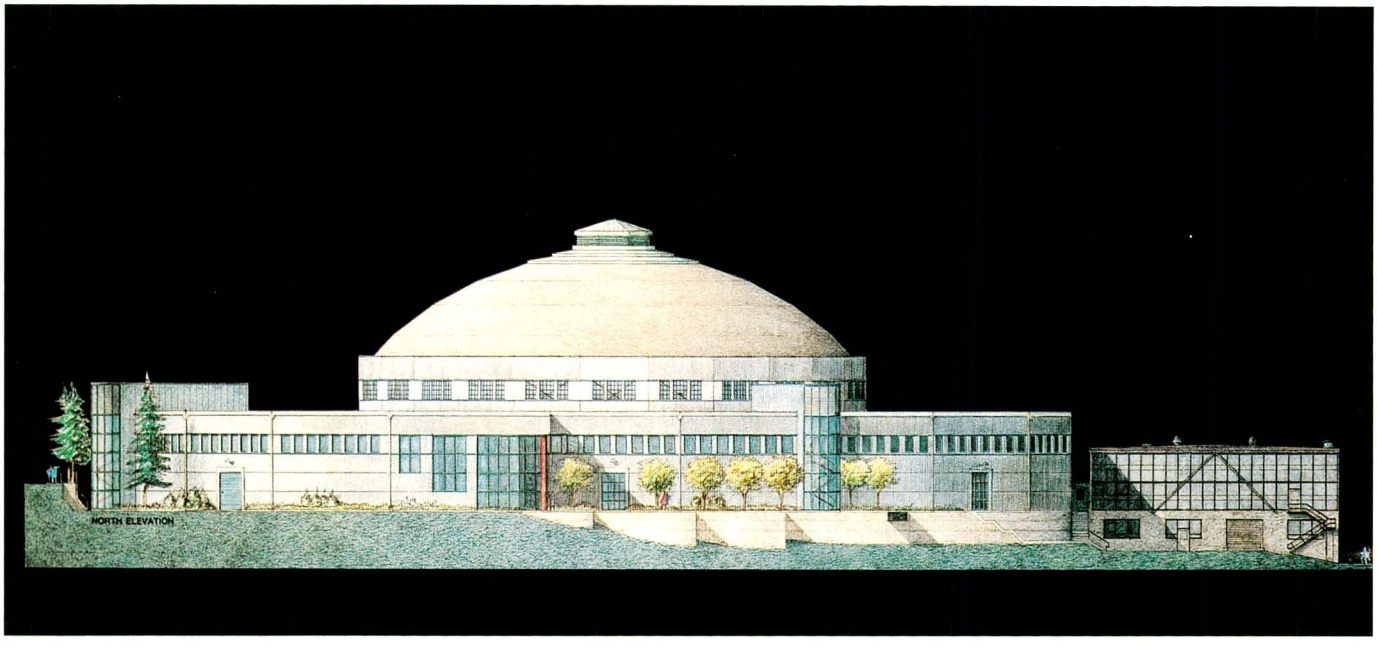
Plans to fully exploit the ALS and APS are, however, not enough. The productive use of x-rays for structural biology—for diffraction and scattering, spectroscopy, and microimaging—demands more than those two new facilities. It also requires making the best use of facilities already on-line: continuing to support productive facilities and taking full advantage of capabilities that are not being effectively exploited.

Many pioneering achievements in x-ray crystallography and x-ray microimaging are now emerging from facilities at the National Synchrotron Light Source (NSLS) at the Brookhaven National Laboratory (BNL), and continued development of NSLS is an important element of any future plan for structural biology. As its name implies, NSLS is a research center fully dedicated to studies based on the use of synchrotron radiation. Two electron storage rings currently serve as one of the world's brightest continuous sources of x-rays and ultraviolet radiation for basic and applied research.

One station at the x-ray ring is devoted to protein crystallography, and others are expected to come on-line during 1991. At the station already operational, the brightness of the x-ray beam, together with a highly computerized x-ray diffractometer, allow diffraction data to be collected as rapidly as at any facility in the world. This speed means more than mere convenience for the investigator: It makes a much wider range of experiments practical. As an example, it allows the comparison of many similar structures—site-directed mutants, for example, or enzymes with different drugs or effectors bound to them—whereas before, only a few such structures could be compared on a reasonable time scale. It also

FIG. 19 PATTERNS OF SYNCHROTRON LIGHT. Synchrotron radiation is always produced by the deflection of charged particles (usually electrons), but several variations can be played on this basic theme. Bending magnets, used to constrain the particles to a roughly circular orbit, produce a sweeping beacon of light and a broad distribution of radiation wavelengths. Insertion devices, on the other hand, cause the charged particles to "wiggle" or "undulate" as shown here, and thus they produce a more directed beam of light. A wiggler of N magnetic periods is, in effect, a series of $2N$ bends; consequently, it produces a spectrum that looks like that of a bending magnet, but its brightness is $2N$ times as great. (As a practical matter, wiggler magnets are invariably stronger than bending magnets, so the radiation spectrum is also shifted to higher photon energies.) Light from the successive bends of an undulator, by contrast, interferes constructively at certain wavelengths. The result is a radiation spectrum that is sharply peaked at these wavelengths, and the brightness increases in proportion to N^2.





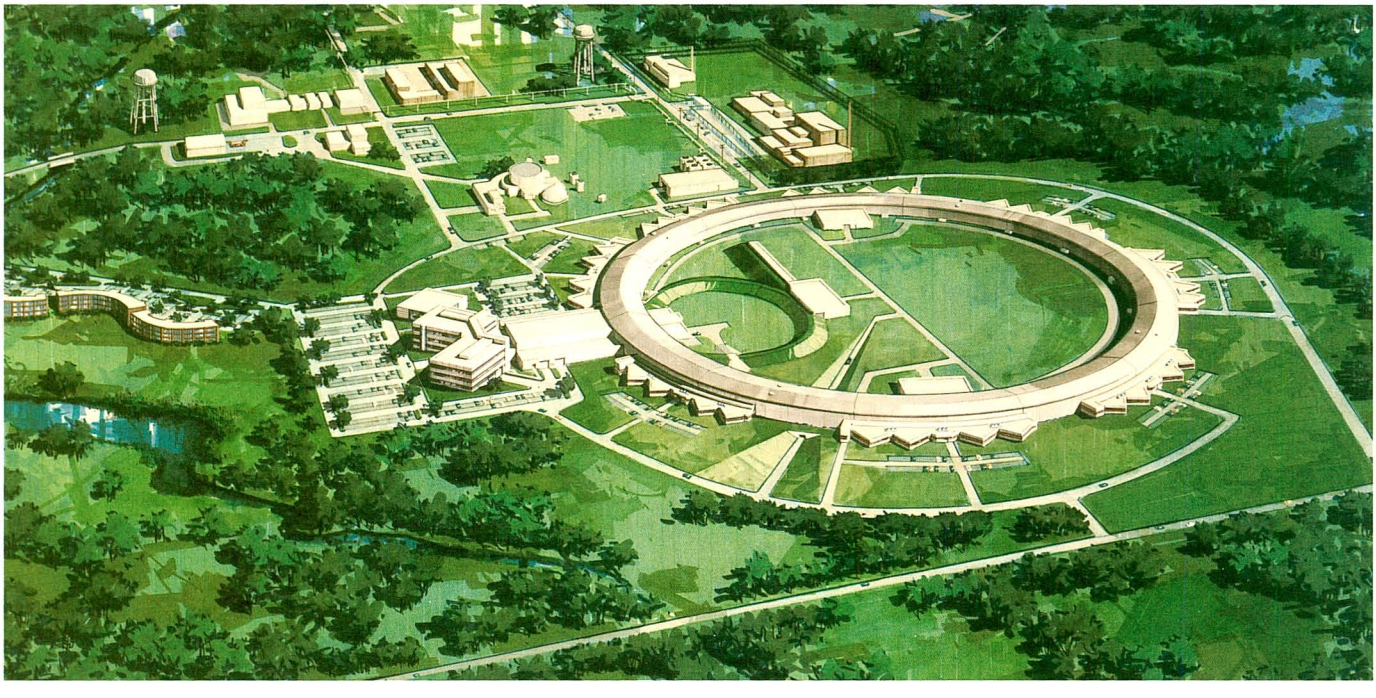


FIG. 20 X-RAYS FOR ALL OCCASIONS. The 1.5-GeV Advanced Light Source at LBL (upper illustration) and the 7-GeV Advanced Photon Source at ANL, shown here in architects' renderings, were designed as complementary facilities to span the full range of useful x-ray energies. The ALS will provide the world's brightest beams of soft x-rays, whereas the APS will be the nation's brightest dedicated source of hard x-rays, in the range between 1 and 100 keV. (Unique among facilities in the U.S., the APS will store positrons—antielectrons—but this difference will have no practical consequences for the user.) The ALS will be operational in 1993; experiments at the APS will begin in 1996.

simplifies modern techniques such as multiple-wavelength anomalous dispersion. Among recent studies have been efforts focused on photosynthetic reaction centers, virus-pharmaceutical complexes, and the RuBisCO enzyme.

Other stations at NSLS are used for Laue diffraction studies and for pioneering experiments in microimaging with soft x-rays. In a few cases, by use of the Laue technique, sampling in the millisecond range has already made possible the study of dynamic enzymatic processes, triggered to occur in the crystal simultaneously with the collection of the diffraction data. At the "softer" end of the x-ray spectrum, NSLS has provided light for x-ray microscopy experiments, including the studies of subcellular dynamics illustrated on page 37. The undulator used for these experiments is currently the world's brightest source of soft x-rays, and it is a key proving ground for techniques to be explored further at future light sources.

In addition, in the fall of 1990, the Stanford Synchrotron Radiation Laboratory (SSRL) began to operate its SPEAR storage ring as a dedicated synchrotron light source. Historically, structural biology has constituted about a third of the experimental effort at SSRL, and dedicated operation of SPEAR will significantly increase national access to intense beams of x-rays. Earlier, SSRL served its synchrotron radiation users by tapping two storage rings designed and used primarily for high-energy physics research. The second ring, PEP, remains a high-energy physics facility, but even in its "parasitic" mode, it provides the world's brightest x-ray beams to two synchrotron radiation experimental stations. SSRL has been a leader in the development of insertion devices for synchrotron radiation research, and it continues to be the site of some of the most advanced crystallography work using multiple-wavelength anomalous dispersion. Pioneering work is also being done there in applying x-ray absorption spectroscopy to biological problems, and the first experiments using synchrotron radiation–based coronary angiography were also performed at SSRL.

SSRL provides scientists access to 25 experimental stations, many of which are available for structural biology studies. A wide range of instrumentation is available for conventional and Laue x-ray crystallography, for x-ray scattering from solutions and membranes, and for x-ray absorption spectroscopy. Nonetheless, as with NSLS, demand for facilities and beam time far outstrips available resources.

KEEPING PACE?

International cooperation in science is the talk of the times. In fields from molecular genetics to high-energy physics, collaborative multinational enterprises are encouraged as a way to share both the costs and the profits of expensive ventures in basic research. In structural biology, too—though the field does not depend on the organizational cohesiveness of a human genome project—progress has been an international achievement. The danger is that the U.S. may be yielding not only its leadership of the worldwide effort, but even its position as an equal partner.

Of eight synchrotron radiation facilities around the world being used for significant structural biology research, half are in the U.S. However, for lack of operating support and beam time, many U.S. researchers are going abroad to do their work. In addition, the first "hard x-ray" ring optimized for insertion devices will be the European Synchrotron Radiation Facility (ESRF) in Grenoble—not the APS. Furthermore, construction of the ESRF will include beamlines, instrumentation, and research facilities for structural biology research, whereas full utilization of the APS is not guaranteed by construction alone. Other sources of funds must be sought if structural biology is to move ahead vigorously there. Likewise, Japan, where the Photon Factory at Tsukuba already operates productively, has recently authorized construction of a $750 million facility near Osaka, again fully outfitted for research. At longer wavelengths, the ALS will be the first third-generation machine on-line, but here again no support has been guaranteed for life sciences research facilities. Machines abroad, under construction and with experimental facilities fully funded, could well prove more productive.

Continued on next page

KEEPING PACE?...

With respect to neutron-based research, the picture may be even bleaker. Virtually all of the neutron scattering research in the U.S. relies on major user facilities. Currently, however, only Brookhaven's HFBR is a well-established resource for structural biology efforts in the U.S., and smaller or newer programs are under way at two other facilities. Abroad, neutron facilities are more numerous, more accessible, and more advanced; in Western Europe alone, operating expenditures for neutron scattering at research reactors are roughly three times those in the U.S. At the Institut Laue Langevin (ILL), instruments for ultrahigh-resolution spectroscopy offer energy resolutions orders of magnitude greater than those possible in the U.S. Further, major efforts are under way at ILL to develop and construct monochromators, polarizers, reflecting "supermirrors," environmental control systems, and more. Other reactor centers are in Saclay, Berlin, and Japan. A pulsed source more intense than LANSCE is currently in operation in Great Britain, and another is planned for Japan.

Most importantly, in parallel with the situation at synchrotron radiation facilities, investment in instrumentation is the weakest link in the U.S. effort. Owing to an investment gap that has widened over the past two decades, many U.S. researchers now look to Europe or Japan for neutron beam time.

On a different front, the development of the x-ray laser is a unique contribution of the Lawrence Livermore National Laboratory (LLNL). The intensity of this device, if it could be harnessed for x-ray holography, would allow exposure times of a few tens of picoseconds, thus arresting molecular processes in living cells or organelles and allowing images to be obtained before molecular disaggregation or thermal expansion distorts the structure.

Neutron Diffraction and Scattering

In broad outline, the practice of neutron crystallography is similar to x-ray crystallography. However, there are differences that set the techniques apart and thus establish them as complementary tools. Low-angle neutron scattering further enhances the important role that neutrons play.

The x-ray diffraction patterns that are the basic data of x-ray crystallography yield electron density maps of the crystalline samples that produced the patterns. Rarely, though, are these maps detailed enough to define the positions of the all-important hydrogen atoms, each with its lone, weakly scattering electron. Fortunately, neutrons scatter from atomic nuclei, not from electrons as x-rays do. In fact, neutrons scatter from protons almost as strongly as from carbon, oxygen, or nitrogen nuclei. As a result, high-resolution neutron crystallography can provide vital insights into the locations of hydrogen bonds and the nature of macromolecule-solvent interactions (Figure 21). At lower resolution, the significance of neutrons again hinges on the scattering properties of hydrogen—in particular, on the differences between ^{1}H and ^{2}H. An example was cited on page 40.

On the basis of these contributions and others, neutrons are now universally regarded as one of the fundamental probes of biological structure. Activity in the field, however, is currently centered in Europe, and extensive facilities are now under construction in Japan. To reestablish U.S. preeminence in the use of neutrons in biology, physics, and materials science, the Advanced Neutron Source (ANS) has been proposed for construction at the Oak Ridge National Laboratory (ORNL; Figure 22). When built, the ANS will be the world's most powerful research reactor; its facilities for structural biology will include stations for both high-resolution neutron crystallography and neutron scattering. As with synchrotron light sources, ancillary on-site biology laboratories are an essential ingredient of plans to make productive use of the ANS.

For the present, important work is being done at the High Flux Beam Reactor (HFBR) at BNL, at the Manuel Lujan Jr. Neutron Scattering Center (LANSCE) at the Los Alamos National Laboratory (LANL), and at the High Flux Isotope Reactor (HFIR) at ORNL. In light of the important role of neutrons, full utilization of these

national facilities must remain an important element of a balanced structural biology effort.

Brookhaven's HFBR supports the nation's most active neutron-based structural biology program. Unique in the country is the small-angle spectrometer using subthermal (liquid hydrogen temperature) neutrons. Beams of these long-wavelength neutrons are especially well-suited for structural studies of multicomponent macromolecular assemblies such as ribosomal subunits, chromatin filaments, contractile proteins, viruses, and microemulsions. This instrument is complemented by a membrane diffraction spectrometer that accepts thermal neutrons. A third instrument is optimized for protein crystallographic investigations, providing a maximum thermal neutron flux at the specimen in the wavelength range between 1 and 2 Å. This diffractometer is equipped with a high-resolution area detector for neutrons so that dozens of reflections can be measured simultaneously.

A second structural biology effort centered around neutrons is under way at LANSCE. LANSCE is a pulsed source providing a very high peak neutron flux, meaning that experiments can be done more quickly, using smaller samples, than at other, similar facilities. Available neutrons have wavelengths between 0.2 and 15 Å, a range especially suitable for small-angle scattering and low-resolution diffraction experiments. One experimental station, the Low-Q Neutron Diffractometer, is devoted to such studies. Available instrumentation allows data to be collected from specimens either in solution or in ordered arrays. A recent success with this instrument was a series of seminal small-angle scattering studies of the calcium-binding protein calmodulin, using $^{240}Pu^{3+}$ as an analog to bound Ca^{2+}.

The Oak Ridge Center for Small-Angle Scattering Research comprises facilities for neutron scattering studies at ORNL's HFIR and an independent small-angle x-ray spectrometer. At HFIR the experimental stations include a 30-meter small-angle neutron spectrometer and a single-crystal neutron diffractometer. The spectrometer, one of only four or five such instruments in the U.S., has been extensively used in the study of chromatin, nucleosomes, and histones. The diffractometer is a higher-resolution instrument, used primarily to complement x-ray crystallography studies by supplying the "missing" hydrogens. One of the principal efforts under way at this facility is the study of hydrogen bonding in cyclodextrins, model compounds for studying active-site catalysis in enzymes.

NMR and Isotopic Labeling

Using new superconducting materials, it is now possible to develop NMR spectrometers that operate at magnetic fields 50% to 60% higher

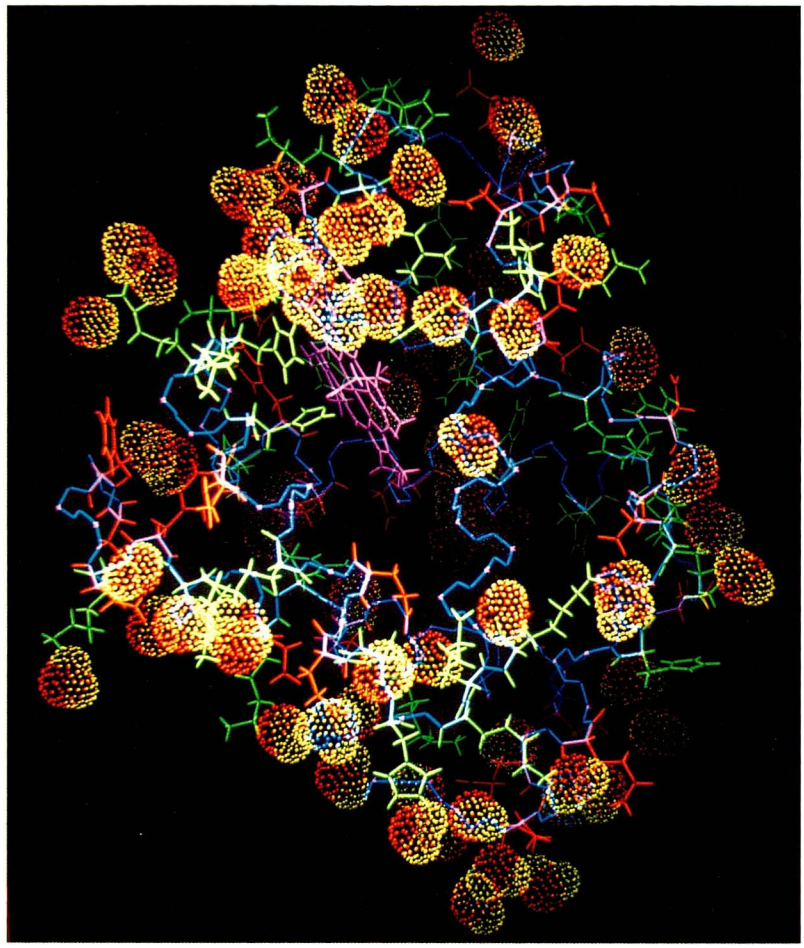


FIG. 21 PUTTING WATER IN ITS PLACE. The power of neutron diffraction in detecting hydrogen atoms, and thus determining the positions of hydrating water molecules, is shown in this image of hydrated carbon monoxide myoglobin. The protein is depicted as a stick model, whereas the water molecules are shown as space-filling stippled structures. Note that the access route (center of picture) to the CO binding site on the pinkish-colored heme group is unobstructed by water molecules.

than those used in available commercial instruments. For example, the world's first 1000-MHz NMR spectrometer for the structural analysis of large macromolecular species in solution is under development at the Pacific Northwest Laboratories (PNL). This instrument, when used with three- and four-dimensional NMR techniques, will more than double the size of macromolecules accessible to NMR analysis. For such studies as these, as well as for neutron scattering and diffraction studies, isotopically labeled samples are an essential resource. Selective deuterium labeling, for example, is often a critical step in low-resolution neutron scattering experiments, and labeling with paramagnetic isotopes of carbon, nitrogen, and oxygen is a necessary prelude to multidimensional NMR studies. The Stable Isotope Resource at LANL is the sole source for many of the labeled molecules used in these studies; facilities include isotope-separation equipment, reaction and fermentation facilities, and facilities for the biosynthesis of labeled macromolecules. Full support and utilization of this facility must remain a high priority.

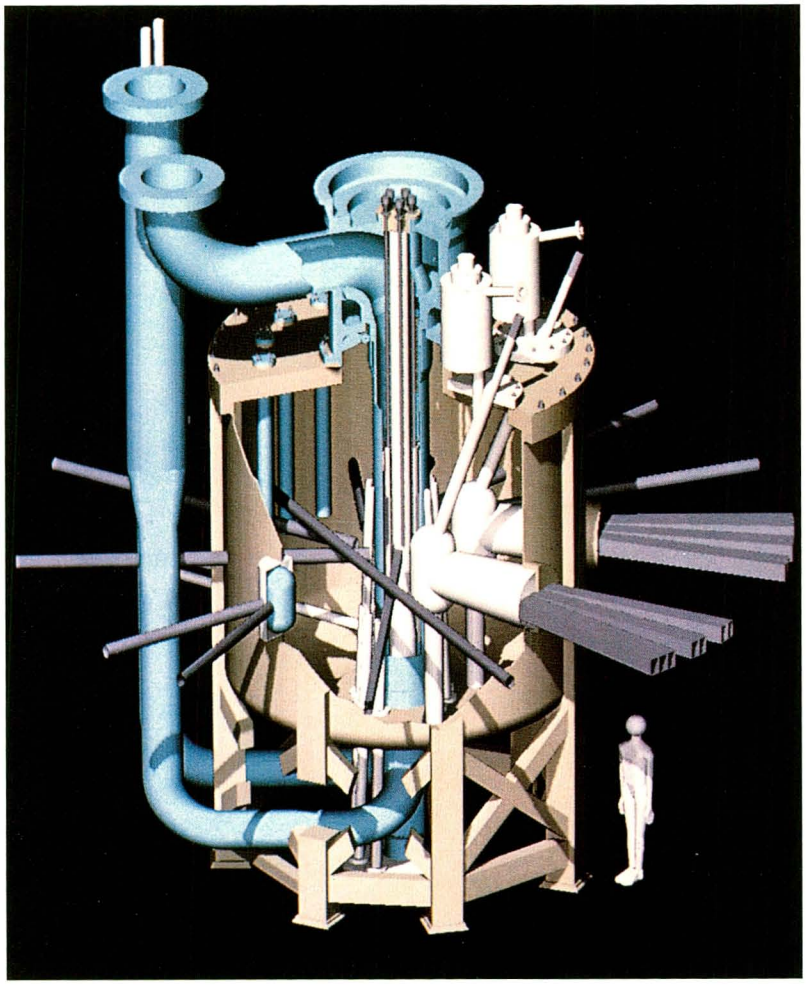


FIG. 22 A DESIGN FOR THE FUTURE. The Advanced Neutron Source at ORNL will produce the world's most intense continuous beams of neutrons. In this computer drawing of the "reflector tank," several neutron beam tubes can be seen emerging from the reactor. Scheduled for completion in the late 1990s, the ANS is expected to attract more than 1000 industrial, academic, and government researchers each year.

Theoretical and Computational Support

Crystallography and NMR studies place high demands on computational resources, in terms of both access to powerful computers and the availability of sophisticated processing algorithms. Furthermore, as larger and larger molecules are studied, and as data acquisition becomes more rapid, these demands will escalate. In addition, the protein folding problem is a puzzle that will yield only to sufficient computational power. Whether protein structure is to be predicted by "searching" through enormous numbers of plausible structural conformations or by homology with the structural motifs of known structures, computational muscle is essential. More generally, structural biology has come to rely ever more heavily on computer graphics. Improved algorithms for image processing and three-dimensional reconstruction are thus critical resources for the field.

Reliable access to the nation's supercomputers is therefore becoming increasingly important, and efforts to develop efficient networks and parallel processing architectures are likely to pay handsome dividends. Computational facilities built around state-of-the-art supercomputers are especially well-developed at LLNL and LANL, and theoretical efforts are well-developed there and elsewhere. The further development and use of advanced computing resources for structural biology must be an essential part of the national effort.

Resources for Tomorrow

SERVING NATIONAL NEEDS

IN RESPONSE TO THE promise and the needs of structural biology, the responsibilities of the Department of Energy are clear: to enhance the opportunities for biology research at operating national user facilities, to ensure that future facilities are adequately equipped and supported for such research, and to promote the development of promising technologies using the unique facilities of the national laboratories.

Accordingly, the DOE's capital-intensive user facilities will require special resources if they are to contribute their full measure to the advancement of structural biology, biomedical science, and biotechnology. It will be necessary to upgrade resources at operating facilities, to equip the ALS and APS for biomedical research, and to plan for facilities at the ANS. Expansion of operations at existing facilities is needed to relieve the immediate, and growing, problem of oversubscription, whereas the outfitting of new facilities, *before they become operational,* is required in anticipation of even greater user demand in the future—and in recognition of the unique capabilities to become available with the next generation of machines.

More than 80% of the required support is needed for biomedical research stations and for DOE research at synchrotron radiation sources. Most of the remainder is needed to ensure full exploitation of operating neutron sources and to prepare for the opportunities to be offered by the ANS at the end of the decade. Also necessary is enhanced support for LANL's Stable Isotope Resource, for Brookhaven's STEM facility, and for development of the 1000-MHz NMR spectrometer at PNL.

As the homes of major facilities for structural biology research, either in operation or under construction, or as the sites of unique resources for technology development, eight national laboratories will play critical roles in the continuing advance of structural biology. Exploiting the unique facilities at these laboratories entails, in many cases, supporting construction projects to provide adequate research facilities, followed by continuing operating support. Where facilities already exist, a level of operating funding must be assured that will optimize the utility of the resources for structural biology.

In addition to providing physical facilities, it is also essential to improve the infrastructure for user support of biomedical researchers at existing facilities and to establish improved methods for involving the broad scientific community in the design, development, and conduct of biomedical research at facilities coming on-line. The DOE's advisory committee on health and environmental research has recommended that consortia comprising scientists from the industrial and academic communities be formed to provide the interdisciplinary stimulus required to fully exploit the potential of these resources. In addition, the Department plans to work aggressively to assure that plans for the development and use of national laboratory resources are fully integrated with the needs of the other government agencies and private organizations who share responsibility for the future of structural biology research.

The next two pages describe more specifically the needs and opportunities that lie ahead at eight national laboratories.

ARGONNE NATIONAL LABORATORY When experiments begin at the Advanced Photon Source in 1996, they will take advantage of the brightest fully dedicated source of x-rays in the U.S. X-ray crystallographers, in particular, will benefit from the greatly enhanced access to synchrotron radiation; oversubscription to beam time at current facilities greatly inhibits progress in this critical field. To ensure that this prospect is fully realized, a Structural Biology Center has been proposed at the APS. This national center for structural biology research will equip and operate a sector of the APS, furnishing for the use of a national community of researchers all necessary x-ray optical equipment, experimental facilities, computer equipment and software, and support staff. Facilities proposed as part of the Center include two fully equipped beamlines. The first, outfitted for both Laue diffraction studies and conventional crystallography, originates at a wiggler designed for both experimental modes and includes an experimental station equipped with large-area detectors, computer hardware and software, and lasers for photolysis and sample heating. The second beamline originates at a bending magnet and is equipped with electronic detectors and high-speed computers.

Also proposed at the APS is a Medical Imaging Center, a national center to be available to all medical scientists interested in the application of x-ray imaging to basic questions in medicine and physiology. This second center will comprise the facilities of a completely equipped wiggler beamline, together with a bending-magnet beamline to be used largely for animal studies.

BROOKHAVEN NATIONAL LABORATORY Enhancements proposed at BNL aim primarily at expanding the productivity of facilities that are already proven resources for structural biology, especially NSLS, HFBR, and the STEM. Several such enhancements would be embodied in the proposed Molecular Biotechnology Unit. This collaborative undertaking among BNL staff, universities, and industry is aimed at facilitating the entire process of analyzing macromolecular structure and function. Among its resources will be a protein engineering facility for synthesizing analyzable quantities of scarce or specifically altered proteins. A facility for the deuteration of proteins and protein–nucleic acid complexes will also be established, as a source of materials for neutron diffraction and neutron scattering experiments. Also as part of the Biotechnology Unit, computational resources will be enhanced, a dedicated Laue crystallography station will be developed at NSLS, and badly needed laboratory space will be developed at NSLS for use by structural biologists. The Unit will also support the design and fabrication of advanced detectors for x-ray diffraction studies, and it will develop and support educational programs for postdoctoral students and active researchers.

LAWRENCE BERKELEY LABORATORY The Advanced Light Source will be the world's first synchrotron light source explicitly optimized for undulators. It thus offers structural biology a unique opportunity to exploit high-brightness radiation, at both soft and hard x-ray wavelengths. To make full use of the ALS, however, life scientists require beamlines and experimental stations dedicated to their use, as well as ancillary laboratory space for sample preparation and characterization. Accordingly, a Life Sciences Center has been proposed at the ALS; its elements will include two insertion devices (an undulator for microimaging and holography and a wiggler for diffraction and spectroscopy), specially designed and dedicated beamlines and experimental stations, and, in contiguous space, sufficient office and laboratory space to accommodate users from institutions around the country.

Operational and programmatic support is also necessary to ensure optimal use of the ALS by researchers from universities, private industry, and the national laboratories. Support will encourage both collaborative research efforts on the life sciences beamlines (involving, for example, regional biotechnology companies), and further development of detectors, x-ray optical components, and computational tools for structural biology research at synchrotron radiation facilities nationwide.

LAWRENCE LIVERMORE NATIONAL LABORATORY At LLNL effort will focus on the development of x-ray laser imaging, especially three-dimensional holography, of macromolecular structures ranging in size from 10 to 200 nm. This capability is being developed at the Nova Laser Facility. The x-ray laser currently operates at wavelengths between 3.5 and about 30 nm, with high coherent energy in a 100- to 200-ps pulse. Goals include shortening the wavelength to about 1.2 nm, providing limited wavelength tunability, and developing a stand-alone user facility capable of high shot rates. The central elements of this user facility will be two high-repetition-rate, 1-kJ neodymium-glass lasers to pump the x-ray laser. Support is also needed to assure the productive use of these and other unique LLNL structural biology facilities by LLNL biologists and by researchers from academia, private industry, and other national laboratories.

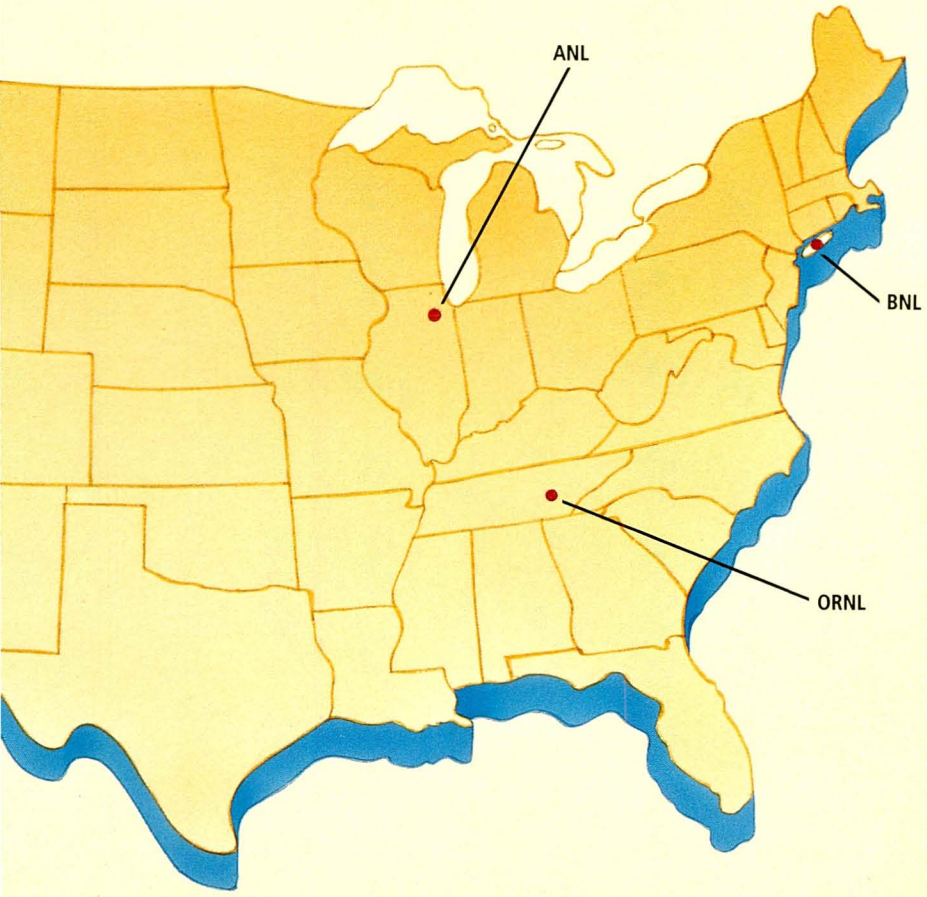
LOS ALAMOS NATIONAL LABORATORY Continued productive use of facilities at LANL requires support on three fronts: (i) facility enhancements and operating support for LANSCE and for x-ray scattering research, (ii) increased support for isotopic labeling and multidimensional NMR research, and (iii) support of efforts in the area of theoretical and computational biology. The most significant needs in the areas of neutron and x-ray scattering include a second low-Q neutron diffractometer at LANSCE, a complementary high-

resolution small-angle x-ray scattering station, a station for neutron diffraction studies of protein crystals, and a fully equipped laboratory for sample preparation at LANSCE. To allow visiting scientists to fully exploit the Stable Isotope Resource, additional staff are needed, especially to pursue further developments and applications of multidimensional NMR. Additional support would also enhance the transfer of labeling and separation technology to private industry.

OAK RIDGE NATIONAL LABORATORY Neutron-based research is increasingly being dominated by facilities abroad. The Advanced Neutron Source could restore the balance by the end of this decade by providing the U.S. with the world's most powerful and versatile research reactor. With suitable planning, the life sciences can expect to be a full participant in the research program at the ANS, with a research emphasis on structural studies of macromolecular complexes, oriented fibers, membranes, and single crystals. Envisioned experimental stations for structural biology research at the ANS will include instruments for small-angle scattering, a 10- to 20-meter high-resolution small-sample instrument, diffractometers for macromolecular neutron crystallography, and a neutron microscope. Necessary ancillary facilities will include a complete biochemistry wet lab, a deuterium-labeling facility, and office space for visiting researchers.

As a bridge to ultimate utilization of the ANS, support must continue for ongoing use of the HFIR for structural biology research. Such programmatic support will provide necessary guidance to the designers of the ANS and serve as a foundation for crystallographic and small-angle scattering research there.

PACIFIC NORTHWEST LABORATORIES In 1989 PNL established the Molecular Science Research Center (MSRC) for fundamental chemical research on molecular structure and interfacial phenomena relevant to materials science and structural biology. When completed in 1994, the Environmental and Molecular Science Laboratory, part of the MSRC, will serve as a national user facility for advanced theoretical and experimental research in molecular science. The five research areas to be emphasized are theory, modeling, and simulation; computer and information science; chemical structure and dynamics; macromolecular structure and dynamics; and materials and interfaces. Of particular relevance to structural biology, continuing support is needed for the development of a high-resolution 1000-MHz NMR spectrometer for multidimensional NMR spectroscopy.



STANFORD SYNCHROTRON RADIATION LABORATORY

Further development of resources at SSRL has three aspects: (i) increasing productive user access to existing structural biology facilities at SPEAR, (ii) developing instrumentation to enable studies in new scientific areas, and (iii) exploring the capabilities of the PEP storage ring. Dedicated operation of SSRL for synchrotron radiation research has dramatically increased its availability to the structural biology community, but increased support is necessary to translate this availability into productive use. In addition, beyond the use of existing facilities, SPEAR has available a number of straight sections that can accommodate new insertion devices. Consequently, a state-of-the-art wiggler and wiggler beamline have been proposed for structural biology research. The wiggler will be designed to provide high-brightness radiation for both Laue x-ray crystallography and time-resolved diffraction experiments. Coupled with beamline development, a vigorous program to develop new area detectors will allow x-ray absorption spectroscopic measurements on highly dilute samples and will extend crystallographic studies to smaller samples (or allow data to be collected more quickly). Finally, even further ahead, lie the frontiers to be explored with PEP. Recent experiments have demonstrated that PEP's high brightness offers the potential to do experiments that are not possible elsewhere, particularly those involving small samples, tightly focused beams, or short exposures. Continued development of PEP, together with the detector development program, also offer experience and instrumentation that will be directly applicable to the new generation of synchrotron radiation facilities represented by the ALS and the APS.

USERS OF DOE NATIONAL USER FACILITIES FOR STRUCTURAL BIOLOGY RESEARCH

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Boston College	Massachusetts
Boston Dental College	Massachusetts
Boston University	Massachusetts
Brandeis University	Massachusetts
Bridgewater College	Virginia
California Institute of Technology	California
California State University	California
Carnegie-Mellon University	Pennsylvania
Case Western Reserve University	Ohio
Columbia University	New York
Cornell University	New York
Elizabethtown College	Pennsylvania
Georgetown University	Washington, D.C.
Georgia Institute of Technology	Georgia
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Harvard University	Massachusetts
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Indiana University	Indiana
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Kentucky Western University	Kentucky
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University of Chicago	Illinois
University of Cincinnati	Ohio
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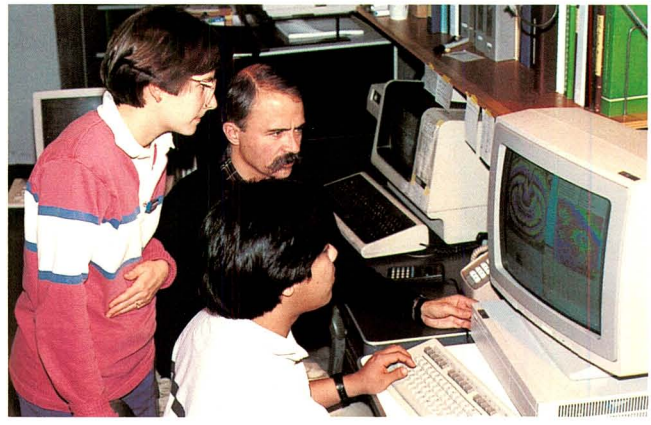

TOOLS FOR EDUCATION. **Facilities at the national laboratories are essential resources for undergraduate and graduate education. Here, Robert Sweet (seated, in the dark sweater), of Brookhaven's Biology Department, describes the analysis of data from NSLS's video-based x-ray detector to Katy Forest and Ron Shigata, chemistry graduate students at Princeton University.**

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This booklet was prepared at the request of the U.S. Department of Energy, Office of Health and Environmental Research, as an overview of achievements and prospects in the field of structural biology, and as a summary of a course of action that would address the resource needs of this vital field. Scientists from eight national laboratories* collaborated actively in the compilation of the material presented here, which was then edited and produced by the Lawrence Berkeley Laboratory. A final draft was reviewed by prominent researchers from national laboratories, universities, independent research institutions, and private industry, and by program managers from the DOE, the National Institutes of Health, and the National Science Foundation.

DOUGLAS VAUGHAN
Editor

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