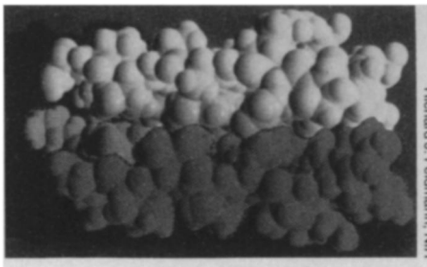
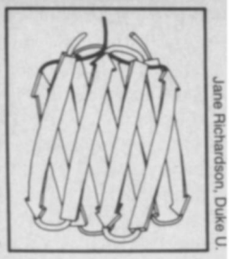
Redesigning Molecules Nature's Way

By JULIE ANN MILLER



Richard J. Feldmann, NIH



Jane Richardson, Duke U.

Researchers use computers to design new molecules. This computer model (above) of a newly created, proteinlike chemical called betabellin shows the major domains. It shows the close packing of atoms more effectively than does the line drawing.

Enviable toolboxes of biochemical techniques—but, alas, few detailed plans—are now available to those who would custom-design proteins for industry and medicine. For more than two decades imaginative biochemists have envisioned improving upon the natural proteins, especially enzymes, to increase their efficiency, make them more stable, change the range of the molecules on which they act or even introduce new catalytic functions.

"Protein engineering is one of the most exciting, but one of the most oversold, aspects of biotechnology," Michael Smith of the University of British Columbia in Vancouver said at the recent Dahlem Workshop on biotechnology in West Berlin. "We are not yet at a stage where we can predictably make a new enzyme, but I think it will come."

Scientists look forward to the day when they can routinely select a natural enzyme and tailor it for a variety of uses. Natural enzymes are generally fragile and function optimally only within a limited range of temperatures and chemical conditions. For example, in medicine, protein engineering might alter beneficial enzymes so that they are not destroyed under the normal conditions of the bloodstream or digestive tract. Therapeutic proteins might also be made more efficient so that smaller doses could be used. In industry, enzymes might be redesigned to withstand high processing temperatures and harsh conditions. In addition, enzymes could be tailor-made for chemical reactions for which there is currently no suitable catalyst.

Current research falls into three categories: the *de novo* design of a molecule; the assembling of functional units from different natural proteins; and the introduction of small changes, such as the replacement of individual amino acids, into a natural protein. Engineering attempts have been reported on more than a dozen proteins so far.

Although only six years ago protein engineering was still considered by many to be "fanciful and quixotic," a variety of chemical and biochemical techniques have been developed to alter proteins or construct them anew. Of these, genetic engineering opens the greatest vistas. Given the sequence of amino acids in the protein desired, scientists can now assemble and make copies of the corresponding gene, then put the gene into cells and create potentially unlimited amounts of any imaginable protein.

The imagination can freely compile characteristics of desirable proteins, but these visions do not easily translate into amino acid sequences. "The rules by which sequence determines structure, or structure determines function, are not known," explains Greg Winter of the Medical Research Council Laboratory of Molecular Biology in Cambridge, England. "So while sequence can be changed to order, changing the function of a protein to order is much more difficult."

While many protein engineers work diligently to apply what little is known about enzyme function, others propose a novel way to circumvent the limited understanding. This approach attempts to simulate evolution to create new proteins. "You take ... the molecular biology approach," said John Richards of the California Institute of Technology in Pasadena at the recent meeting in Los Angeles of the American Association for the Advancement of Science (AAAS). "You produce a large number of variants, then you test the structures until you find one that does what you want."

While mimicking natural selection in strategy, protein engineers have recently adopted a technique that expands the range of variants beyond that generally available to evolution. Each of the 20 amino acids found in protein is encoded in genes as a triplet — a sequence of three nucleotides, the "building blocks" of DNA. A natural mutation, or one induced by traditional laboratory techniques, typically will alter only one member of a triplet. But the technique called "site-directed mutagenesis" (SN: 2/26/83, p. 139) uses short, laboratory-synthesized chains of nucleotides to create any new triplet at a given location.

With this method scientists can explore in a given protein the effects of all 19 potential replacement amino acids (rather than just four to seven) at a site, or even combinations of amino acids at different sites. Richards calls this technique "cassette mutagenesis" because it can snap different "cassettes" of nucleotides into a site.

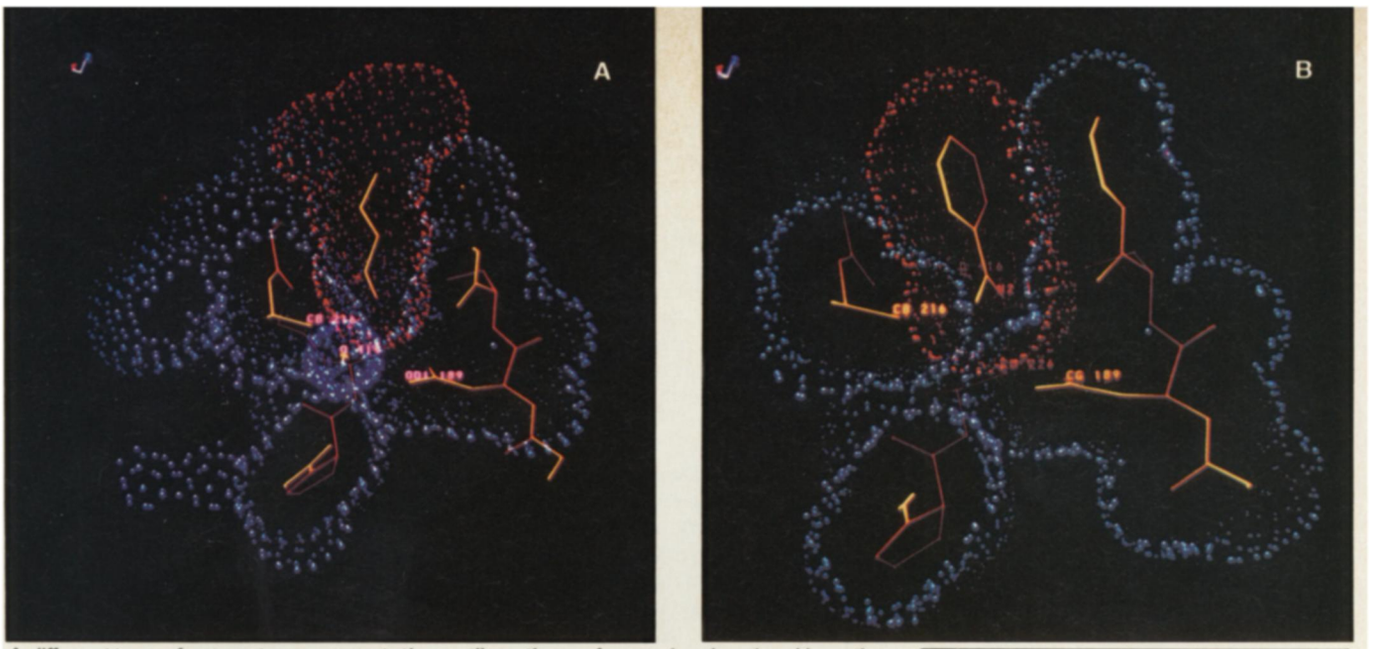
Richards reports "surprising results" when he applied this technique to the study of beta-lactamase, an enzyme that endows microorganisms with resistance to penicillin and some other antibiotics. He has focused on sites that, because they have the same amino acids in many species, appear to be critical to the function of the enzyme. But Richards finds that a large variety of different amino acids can be inserted in some of these positions and the enzyme still works, although there is some variation in what antibiotics the enzyme breaks down.

In one case, Richards did further remodeling of the enzyme. After the first amino acid substitution, the enzyme did not allow the breakdown of an antibiotic called cephalothin. But in the presence of cephalothin, some bacteria containing this enzyme underwent a second mutation. This alteration, at a second site, made the bacteria even more resistant to the antibiotic than were bacteria containing the native enzyme.

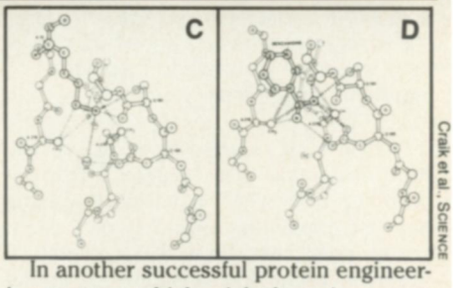
"This indicates that we can train enzymes to do things that they don't normally do," Richards says. "It may hold out hope that you may make an enzyme better than normal. All enzymes are not evolved to be perfectly efficient."

Scientists anticipate using this approach not only in modifying natural proteins but also in creating *de novo* forms and composite proteins. For example, Bruce Erickson of Rockefeller University in New York City recently created a synthetic structure, called betabellin, intended to be the framework for a new class of molecules with proteinlike functions (SN: 6/8/85, p. 357). To add sites to the betabellin molecular backbone where the synthetic protein can bind molecules and perform catalytic functions, Erickson plans to vary the amino acids at several positions simultaneously, creating a mixture of up to 1,000 slightly different proteins. For example, if three amino acids are under consideration at each of six positions, there will be 729 different proteins in all. "Then we go fishing," Erickson says. He plans to use a binding assay to select from the mixture the best candidate for the binding site or catalytic site desired.

The strategy of mimicking evolution also applies to the research that is fusing together parts of genes to create composite proteins. In a discussion at the Dahlem conference, Albrecht Sippel of the University of Heidelberg in West



A different type of computer representation outlines the surfaces of molecules. Here, the enzyme surface is blue, and the chemical that binds is red. Backbones of the molecules are visible in yellow and orange. The substrate-binding pocket of an altered trypsin molecule is depicted. Bound are an inhibitor (A, C) and benzamidine (B, D).



Craik et al., SCIENCE

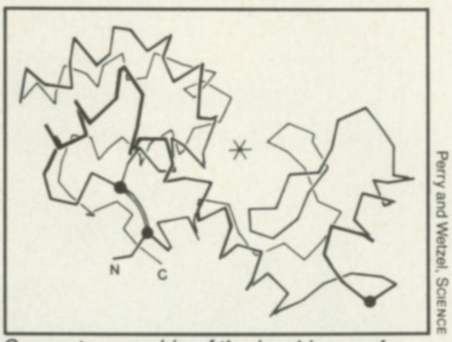
Germany called this strategy "domain engineering." This, he says, is what nature does — there is evidence that during evolution functional regions of proteins appear in new combinations.

"In principle, it's a nice idea," Winter says, "but it's difficult to recombine segments and get the packing right." Heinz Schaller of the University of Heidelberg suggests that scientists fuse together protein domains, then make variants of the hybrid protein and select any with the desired interactions. Some of the initial work in recombining subunits involves adding segments of antibody molecules to catalytic regions of other enzymes, so that the new molecule will be easier to purify.

Most progress in protein engineering to date, however, has come not from the evolutionary approach but from the more traditional "chemical" tactics. Instead of creating hundreds of variants and sifting through them, the chemical approach is to try to deduce—from what is known about a protein — structural changes likely to give a single variant or a small group of variants with the desired characteristics. "You use *insight*," Richards says, "to alter the function of a protein in an interesting way."

Ideally, that insight would come from a thorough understanding of the structure of a protein and the function of its binding and active sites. Improved computer graphics have had an enormous impact on analyzing protein structures and investigating potential alterations. But the X-ray crystallographic data needed for such analysis exist for only about 100 proteins.

One example of such protein engineering — and one that could have implications for industrial biotechnology — is the stabilization of an enzyme by the introduction of a new intramolecular bond. L. Jeanne Perry and Ronald Wetzel of Genentech, Inc., in South San Francisco have engineered the protein called T4 lysozyme, which breaks down the cell walls of bacteria infected with the virus named T4. To make the enzyme stable at higher temperatures, Perry and Wetzel decided to create a cross-link within the molecule. Theoretical calculations suggested that the bond should link amino acids far apart on the protein chain, but with side chains close together in the globular, three-dimensional structure. The structure was known in detail from crystallography, and the scientists used computer graphics to choose an appropriate bridge to create.



Perry and Wetzel, SCIENCE

Computer graphic of the backbone of a T4 lysozyme molecule shows the location of the active site (star) and of a new bridge (double lines connecting dark spots). The cysteine amino acid (dark spot) at the lower end of the bridge was introduced by protein engineering.

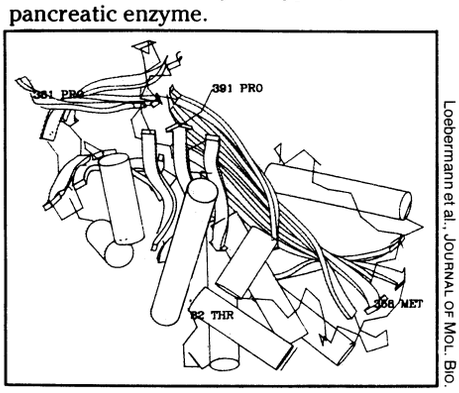
The resultant cross-linked enzyme has the same catalytic activity as the original T4 lysozyme, and it is more stable than the original at elevated temperatures, a potentially important attribute in industry. "Our results show that introduction of disulfides into proteins that have evolved without them is possible and that such derivatives can retain activity and also have enhanced stability," Perry and Wetzel reported in the Nov. 2, 1984 SCIENCE (Vol. 226, p. 555).

In another successful protein engineering venture, which might have important medical applications, scientists decided what specific amino acid changes might be profitable by considering the characteristics of several related molecules that had been chemically synthesized. A natural protein called *alpha* 1-antitrypsin defends the lungs against damage by the enzyme elastase. (The protein's name is a misnomer because another protein better inhibits the related enzyme called trypsin.) Physicians use large doses of *alpha* 1-antitrypsin to treat acute inflammatory respiratory conditions and emphysema, an irreversible lung disease characterized by loss of lung elasticity. At Chiron Research Laboratories in Emeryville, Calif., scientists used site-directed mutagenesis to construct derivatives of the molecule resistant to oxidation, a chemical reaction that inactivates the protein's protective function and limits its clinical effectiveness.

The site on the protein where the damaging oxidation occurs is also the site that determines what enzyme the 394-amino-acid molecule will inhibit. Chiron scientist Philip J. Barr and his collaborators replaced the natural amino acid, methionine, in that position with a valine. Experiments by other investigators using chemically synthesized shorter chains of amino acids had indicated that a valine would resist oxidation but still allow the protein to inhibit elastase. Chiron's valine-containing molecule lives up to these expectations.

Other amino acids at the key position permit the protein to inhibit still other enzymes. An arginine makes it inhibit thrombin, an enzyme crucial to normal

blood clotting; a lysine makes it inhibit trypsin, a pancreatic enzyme; a leucine makes it inhibit chymotrypsin, another pancreatic enzyme.



Loebermann et al., JOURNAL OF MOL. BIO.

The structure of alpha*1-trypsin inhibitor. Protein engineers have altered the amino acid at the position labeled 358 MET. In this diagram, helices are shown as cylinders, sheets as arrows and irregular segments as thin lines.*

"In the short term, I see two uses of the modified inhibitors," says Chiron researcher Robert A. Hallewell. First, the arginine-containing form may be a much better treatment than those currently available for natural deficiencies in the protein that normally inhibits thrombin. Second, he sees a "strong possibility" that the valine form will find use in adult respiratory distress syndrome, a disease that resembles a very rapidly developing emphysema.

In perhaps the most advanced cases of "insightful" protein engineering so far, scientists have redesigned enzymes to alter catalytic function. For example, at the AAAS meeting scientists described work with an enzyme, dihydrofolate reductase (DHFR), that is essential to nucleic acid synthesis and cell growth. This enzyme normally catalyzes the transfer of a hydrogen ion from a chemical known as NADPH, rather than from the closely related chemical called NADH. Joseph Kraut and his colleagues at the University of California at San Diego examined mutants created with site-directed mutagenesis. One variant can be made to form a disulfide bridge, which increases resistance to denaturation. Another variant, containing two amino acid changes, reverses the enzyme's preference for NADPH over NADH.

"We've also gotten the beginnings of some important information about structure-function relationships," Kraut says. The work has already corrected an "error" in scientists' idea of how the substrate, the molecule on which an enzyme acts, is bound to the enzyme, he reports.

In other research, scientists at the University of California at San Francisco modified trypsin, which usually digests proteins by cutting them at an arginine amino acid. This enzyme less often cuts at a lysine. The three-dimensional structure of trypsin, which has been known for about 10 years, has a pocket into which arginine and lysine fit. With computer graphics, Charles S. Craik, Robert Fletterick and William Rutter determined alterations in the protein that they suspected would influence the enzyme's preference. The researchers then produced a modified version that cuts preferentially at lysine.

"At the moment our ability to achieve a predicted, beneficial change in protein function is not great, but this has to increase and with it there should be a significantly useful increase in our ability to produce useful new protein functions," Smith says, in summarizing the Dahlem Workshop discussion. The current status of protein engineering, and of much of biotechnology, is reflected in a story Smith told to the Dahlem conference. A savvy Swiss company, he said, decided to convert cow manure into chocolate, so they commissioned a biotechnology firm. After a year, the biotechnology company enthusiastically reported the success of the first stage of research. Now, for the second stage, they declared, all that remained was to improve the color, texture and flavor. □

This is the third of a series of articles on current developments in biotechnology.