Glucose and Aging

Once considered biologically inert, the body's most abundant sugar can permanently alter some proteins. In doing so it may contribute to age-associated declines in the functioning of cells and tissues

by Anthony Cerami, Helen Vlassara and Michael Brownlee

As people age, their cells and tissues change in ways that lead to the body's decline and death. The cells become less efficient and less able to replace damaged materials. At the same time the tissues stiffen. For example, the lungs and the heart muscle expand less successfully, the blood vessels become increasingly rigid and the ligaments and tendons tighten. Older people are also more likely to develop cataracts, atherosclerosis and cancer, among other disorders.

Few investigators would attribute such diverse effects to a single cause. Nevertheless, we have discovered that a process long known to discolor and toughen foods may also contribute to age-related impairment of both cells and tissues. That process is the chemical attachment of the sugar glucose to proteins (and, we have found, to nucleic acids) without the aid of enzymes. When enzymes attach glucose to proteins, they do so at a specific site on a specific molecule for a specific purpose. In contrast, the nonenzymatic process adds glucose haphazardly to any of several sites along any available peptide chain.

On the basis of recent in vitro and in vivo studies in our laboratory at Rockefeller University, we propose that this nonenzymatic "glycosylation" of certain proteins in the body triggers a series of chemical reactions that culminate in the formation, and eventual accumulation, of irreversible cross-links between adjacent protein molecules. If this hypothesis is correct, it would help to explain why various proteins, particularly ones that give structure to tissues and organs, become increasingly cross-linked as people age. Although no one has yet satisfactorily described the origin of all such bridges, many investigators agree that extensive cross-linking of proteins probably contributes to the stiffening and loss of elasticity characteristic of aging tissues. We also propose that the nonenzymatic addition of glucose to nucleic acids may gradually damage DNA.

The steps by which glucose alters proteins have been understood by food chemists for decades, although few biologists recognized until recently that the same steps could take place in the body. The nonenzymatic reactions between glucose and proteins, collectively known as the Maillard or browning reaction, may seem complicated, but they are fairly straightforward compared with many biochemical reactions.

They begin when an aldehyde group (CHO) of glucose and an amino group (NH_2) of a protein are attracted to each other. The molecules combine, forming what is called a Schiff base [*see illustration on page 93*]. This combination is unstable and quickly rearranges itself into a stabler, but still reversible, substance known as an Amadori product.

If a protein persists in the body for months or years, some of its Amadori products slowly dehydrate and rearrange themselves yet again—into new glucose-derived structures. These can combine with various kinds of molecules to form irreversible structures we have named advanced glycosylation end products (AGE's). Most AGE's are yellowish brown and fluorescent and have specific spectrographic properties. More important for the body, many are also able to cross-link adjacent proteins.

The precise chemical structure of advanced glycosylation end products and of most AGE-derived cross-links is still not known. Nevertheless, some evidence suggests that AGE's are often created by the binding of an Amadori product to glucose or another sugar. Such end products would form bridges to other proteins by binding to available amino groups. In some instances two Amadori products may instead merge, creating an AGE that is also a cross-link. The one glucose-derived cross-link whose chemical structure is known appears to be just such a combination. It is 2-furanyl-4(5)-(2-furanyl)-1*H*-imidazole, or FFI. First isolated in the laboratory (from a mixture of the amino acid lysine, the protein albumin and glucose), FFI has since been found in the body.

The realization that the browning reaction could occur in—and potentially damage—the body emerged from studies of diabetes, a disease characterized by elevated blood-glucose levels. In the mid-1970's one of us (Cerami) and Ronald J. Koenig examined a report that the blood of diabetic individuals contained higher than normal levels of hemoglobin A_{1c}: a variant of the protein hemoglobin, which is the oxygen-carrying component of red blood cells. Curious about why the levels were elevated, the two investigators attempted to determine the molecule's structure.

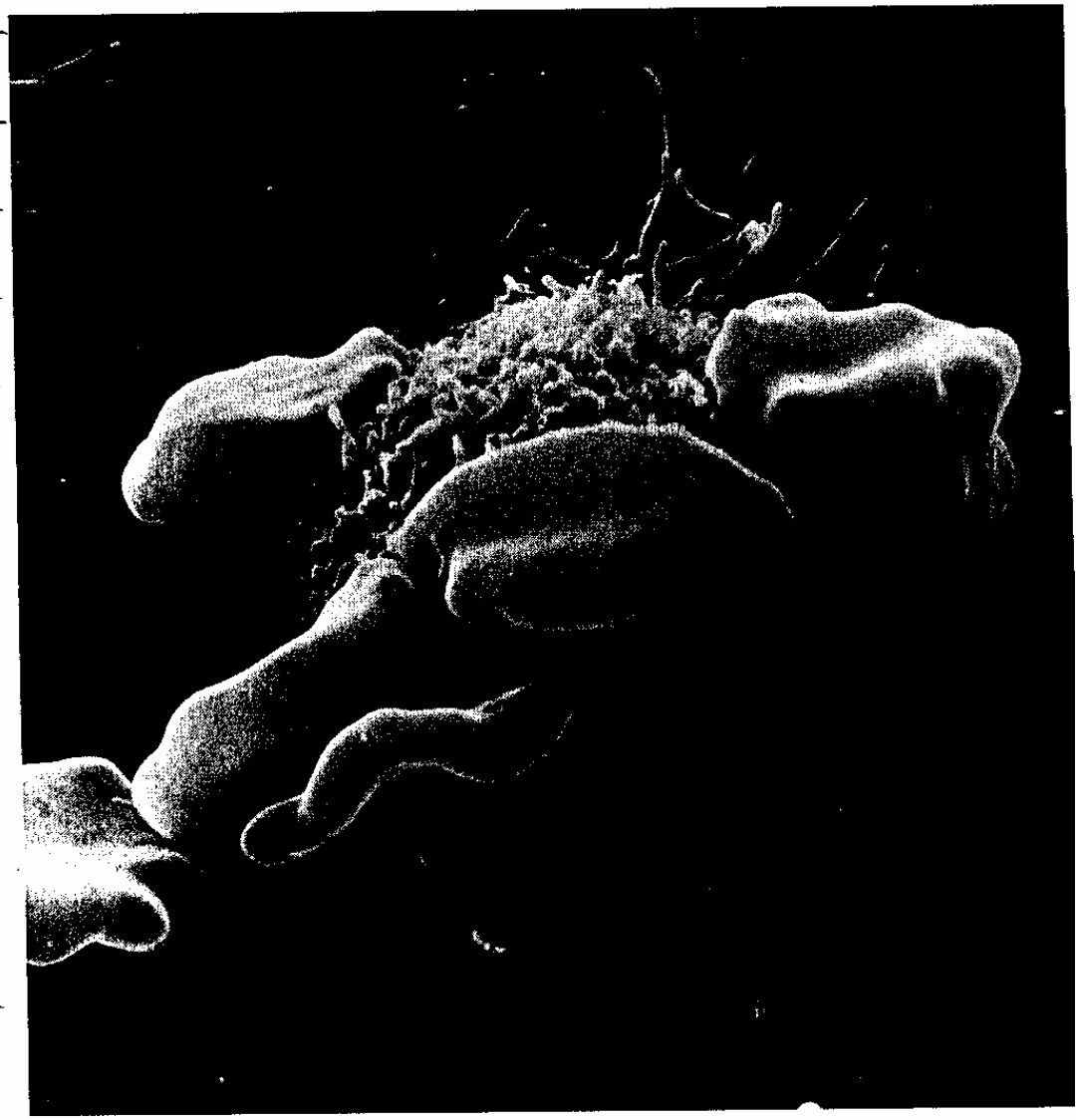
Hemoglobin A_{1c} is an Amadori product. Moreover, as is true for the amount of Amadori product formed in foods, the amount of hemoglobin A_{1c} formed is influenced by the level of glucose in the blood: when the glucose level is high, the amount of Amadori product is also high. (Workers in our laboratory and elsewhere have since identified more than 20 Amadori proteins in human beings and have consistently found two or three times as much product in people with diabetes as in nondiabetics.)

The hemoglobin findings reveal that glucose, which bathes tissues and cells throughout the body, is not the inert biological molecule most biologists thought it was. Although the sugar does not react while it is in its usual ringlike formation, the ring opens often enough to enable Amadori products and other substances to form. Glucose remains the least reactive sugar in the body, but it has the greatest

potential effect on proteins because it is by far the most abundant variety.

The fact that glucose is reactive suggested to Cerami that excess blood glucose in people with uncontrolled diabetes might be more than a marker of the disease. If the sugar could bind nonenzymatically to proteins in the body, he reasoned, excessive amounts could potentially contribute to diabetic complications: the host of disorders, ranging from impaired sensation to kidney failure, that often disable people with diabetes and shorten their life. In particular, it seemed possible that high levels of glucose could lead to an extensive buildup of advanced glycosylation end products on long-lived proteins. The accumulation of AGE's in turn might undesirably modify tissues throughout the body.

Such musings soon led to a suspicion that glucose could also play a role in the tissue changes associated with normal aging. The effect of diabetes on many organs and tissues is often described as accelerated aging because several of the complications that strike people with diabetes—including senile cataracts, joint stiffness and atherosclerosis—are identical with disorders that develop in the elderly; they merely develop earlier. If excess glucose



MACROPHAGE (*rough-surfaced body at center*), a cell that removes debris from tissues, is about to ingest red blood cells (*smooth disks*) to which advanced glycosylation end products, or AGE's, have been attached. AGE's are molecules derived from the combination of glucose and protein without the aid of enzymes. The authors postulate that AGE's gradually accumulate on long-lived proteins and cells, forming cross-links that impair tissue. Macrophages try to remove AGE-altered proteins but lose efficiency as people age. The cells are enlarged 10,000 diameters in the micrograph, made by David M. Phillips of the Population Council.

does in fact hasten the onset of these ills in people with diabetes, normal amounts could conceivably play a role in the slower onset seen in nondiabetics as they age.

Our laboratory's studies of senescence (which complement our ongoing studies of diabetes) began with an attempt to determine whether advanced glycosylation end products do in fact accumulate on, and form cross-links between, long-lived proteins in the body. Major constituents of the lens of the eye—the crystallin proteins—became the first objects of study because once these proteins are produced they are believed to persist for life; they therefore fit the profile of proteins that could amass advanced glycosylation end products. Also it seemed likely that a buildup of such AGE's and of AGE-derived cross-links could help to explain why lenses turn brown and cloudy (that is, develop senile cataracts) as people age. In support of this idea, workers elsewhere had previously found two types of cross-link in aggregates of crystallin proteins from human senile cataracts. One bridge was pigmented, suggesting that it could be an AGE. The other type was a disulfide bond formed between sulfhydryl (SH) groups of the amino acid cysteine.

In test-tube experiments Cerami, Victor J. Stevens and Vincent M. Monnier showed that glucose could produce a cataractlike state in a solution of the proteins. Whereas glucose-free solutions containing crystallins from bovine lenses remained clear, solutions with glucose caused the proteins to form clusters, suggesting that the molecules had become cross-linked. The clusters diffracted light, making the solution opaque. Analysis of the links between the molecules confirmed that both the disulfide and the pigmented types were present. The group has also discovered that the pigmented cross-links in human senile cataracts have the brownish color and fluorescence characteristic of advanced glycosylation end products. In fact, some cross-links can be chemically identified as the advanced glycosylation end product FFI.

Combined with other evidence, the above data suggest that nonenzymatic glycosylation of lens crystallins may contribute to cataract formation by a two-step mechanism. Glucose probably alters the conformation of proteins in ways that render previously unexposed sulfhydryl groups susceptible to combination with nearby sulfhydryl groups. Hence disulfide bonds develop, initiating protein aggregation. Later Amadori products on the proteins become rearranged, enabling FFI and other pigmented cross-links to form, discolor the lens and make it cloudy.

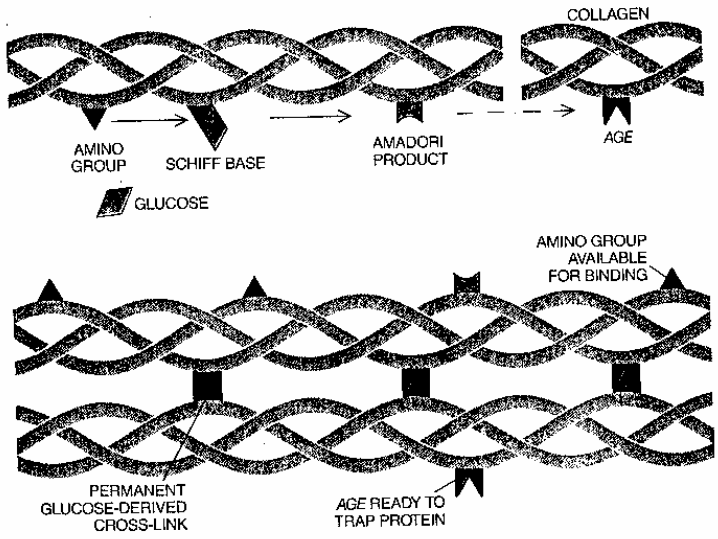
Convinced that at least one class of proteins undergoes the browning reaction and forms undesirable cross-links, we and our colleagues turned to the body's most abundant protein: collagen. This long-lived extracellular protein glues together the cells of many organs and helps to provide a scaffolding that shapes and supports blood-vessel walls. It also is a major constituent of tendon, skin, cartilage and other connective tissues. In the past 25 years various investigators have shown that collagen builds up in many tissues, becoming increasingly cross-linked and stiff as people age.

Studies of the dura mater, the collagen sac separating the brain from the skull, provided early evidence that advanced glycosylation end products could collect on collagen. Monnier, Cerami and the late Robert R. Kohn of Case Western Reserve University found that the dura mater from elderly individuals and from diabetics displays yellowish brown pigments whose fluorescent and spectrographic properties are similar to those of advanced glycosylation end products formed in the test tube. As would be expected, protein from people with diabetes had accumulated more pigments than the protein of nondiabetics. In nondiabetics the amount of pigment attached to the protein increased linearly with age.

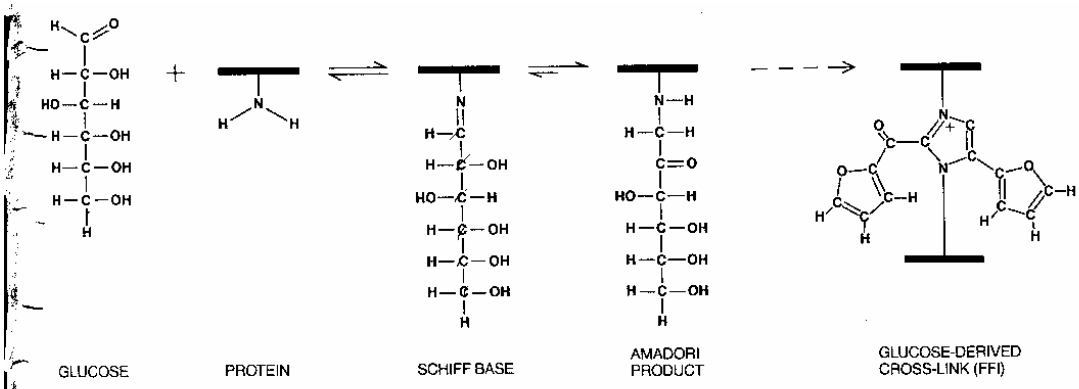
Evidence suggesting that glucose induces collagen not only to form AGE's but also to become cross-linked comes from several studies. On the basis of work by other investigators, it has long been known that fibers from the tail tendons of older rats take longer to break when they are stretched than fibers from younger animals, indicating that the older fibers are more cross-linked and less flexible. Monnier, Cerami and Kohn therefore attempted to mimic the effects of aging by incubating tendon fibers of young rats with various sugars. The fibers gradually accumulated advanced glycosylation end products and showed a concomitant increase in breaking time.

More recently we have evaluated the cross-linking of both purified and aortic collagen. In the first instance the protein was incubated with glucose in a test tube; in the second instance it was essentially incubated in the body of diabetic animals that had high blood-glucose levels. In both conditions our chemical tests unequivocally showed that the glucose led to extensive cross-linking.

Although we suspect that the formation of glucose-derived cross-links between long-lived proteins helps to account for many symptoms of aging and for many complications of diabe-



FORMATION of glucose-derived cross-links, shown highly schematically, begins when glucose attaches to an amino group (NH_2) of a protein (*top*), such as collagen. The initial product, known as a Schiff base, soon transforms itself into an Amadori product, which can eventually pass through several incompletely understood steps (*broken arrow*) to become an AGE. In many instances AGE's are like unsprung traps (*red symbol*), poised to snap shut (*bottom*) on free amino groups of any nearby protein and to form cross-links.



CHEMICAL STRUCTURE is known for glucose-protein Schiff bases and Amadori products. Workers have yet to learn the structure of most AGE's and AGE-derived cross-links, but one link has been identified: 2-furanyl-4(5)-(2-furanyl)-1*H*-imidazole, or FFI.

tes, such bridges are not the only ones that can potentially damage the body. We have shown that AGE's on collagen in artery walls and in the basement membrane of capillaries can actually trap a variety of normally short-lived plasma proteins. Even when collagen is incubated with glucose and then washed so that no free glucose is present, the long-lived protein can still covalently bind such molecules as albumin, immunoglobulins and low-density lipoproteins.

This binding may help to explain why both people with diabetes and the aged are prone to atherosclerosis: a buildup of plaque in arterial walls. The plaque includes smooth-muscle cells, collagen (which is produced by the smooth-muscle cells) and lipoproteins (the cholesterol-rich proteins that are the primary source of fat and cholesterol in atherosclerotic lesions).

No one yet understands the exact processes leading to atherosclerosis. It is conceivable that glucose contributes to plaque formation by causing advanced glycosylation end products to develop progressively on collagen in the vessel walls. Once those substances form, collagen may trap low-density lipoproteins from the blood—which in turn can become attachment sites for other lipoproteins.

In theory, glucose-altered collagen could also trap von Willebrand factor, a protein that is believed to promote the aggregation of platelets (sticky bodies involved in blood clotting). The platelets may release a factor that stimulates the proliferation of smooth-muscle cells, which produce extra collagen. Other glucose-related events may further promote plaque formation [*see illustration on page 95*]. More studies are needed to determine the extent to which any of the postulated events take place and how they might interact with various other processes that contribute to atherosclerosis.

Protein trapping and cross-linking may also help to explain the thickening seen in the basement membrane of capillaries as people grow older (and the more rapid thickening in people with diabetes). In people with diabetes, thickening of a specialized basement membrane in the kidney, the mesangial matrix, promotes renal failure. In nondiabetics the consequences of renal basement-membrane thickening are less clear, although we suspect the process may help to decrease the aged kidney's ability to clear wastes from the blood. Elsewhere in the body thickened capillaries become particularly narrow or occluded in the course of time in the lower extremities, where gravity increases the rate of protein trapping by vessel walls. Such narrowing can contribute to the impaired circulation and loss of sensation often found in the feet and legs of both diabetics and older nondiabetics. In order to function properly, the sensory nerves need an adequate supply of blood.

Because aging takes place at the level of the cell as well as of tissue, our laboratory has recently begun to examine the effects of glucose on the material that controls cell activity: the genes. At least in resting cells, the nucleic acid DNA, which contains amino groups, is long-lived. It therefore can potentially accumulate advanced glycosylation end products. These AGE's might then contribute to known age-related increases in chromosomal alterations or to declines in the repair, replication and transcription of DNA. Such genetic changes are believed to impair the body's ability to replace proteins critical to normal cell function and survival. Nonenzymatic glycosylation might also cause mutations that affect the activity of the immune system or lead to some types of cancer.

Richard Bucala, Peter Model and Cerami have found that incubating DNA with glucose does indeed cause fluorescent pigments to form. The pigments do not build up as quickly as they do on proteins, because the amino groups of nucleic acids are significantly less reactive than the amino groups of proteins.

No one has yet investigated the effects of AGE's on the nucleic acids of mammalian cells, but the group's studies of bacteria suggest that nonenzymatic glycosylation may well interfere with the normal functioning of human genes. When a bacteriophage (a bacterial virus) with a DNA genome was incubated with glucose and then inserted into the bacterium *Escherichia coli*, the phage's ability to infect *E. coli* cells was shown to be reduced. The degree of reduction depended on both the incubation time and the concentration of the sugar.

Bucala and his fellow workers also found that adding the amino acid lysine to a mixture of DNA and glucose hastened the loss of viral activity. Presumably the sugar reacted with the amino acid, forming an "AGE-lysine" that quickly bound to the DNA. Because both protein and glucose are present in mammalian cells, it seems likely that a similar reaction might account for the finding that protein covalently attaches to the DNA of aged cells. The effects of such protein binding to genetic material are not known.

Just how the attachment of glucose or a glycosylated protein to DNA interfered with the bacteriophage's normal activity is also not clear. In anoth-

er study, though, sugar was shown to cause a mutation in DNA. The workers isolated plasmids (extrachromosomal pieces of bacterial DNA) carrying genes that make *E. coli* resistant to the antibiotics ampicillin and tetracycline. Then they incubated the plasmid with glucose-6-phosphate, a sugar that reacts more quickly than glucose, returned the DNA to bacterial cells and exposed the cells to an antibiotic. Most of the cells exposed to tetracycline died, whereas most exposed to ampicillin lived. Clearly some of the incubated plasmids kept the ampicillin-resistance gene but had lost the activity of the tetracycline-resistance gene.

Further study showed that most of the tetracycline-resistance genes had been altered by deletions or insertions of DNA. We suspect that those genes had collected advanced glycosylation end products and that the resulting mutations arose when the bacteria attempted to repair the DNA altered by AGE's. This conclusion is supported by the finding that bacterial cells lacking a DNA-repair enzyme did not have mutations in the DNA.

In order to better determine the effects of advanced glycosylation end products on the DNA of human cells, we are developing new methods for measuring both AGE's and glycosylated proteins on DNA. In addition we need to learn more about the cell's mechanisms for repairing glycosylated nucleic acids.

The ultimate goal of our research into both aging and diabetes is to find ways of preventing or delaying their debilitating effects. If our glycosylation hypothesis is correct, such effects might be mitigated either by preventing the formation of glucose-derived cross-links or by increasing the activity of biological processes that remove AGE's.

On the first front we, along with Peter C. Ulrich in our laboratory, have developed a promising drug called aminoguanidine. This small molecule, in the class of compounds called hydrazines, reacts with Amadori products. It apparently binds to carbonyl groups and in so doing prevents the Amadori products from becoming advanced glycosylation end products.

In test-tube studies of the drug we incubated albumin either with glucose alone or with glucose and aminoguanidine. Advanced glycosylation end products formed in the first mixture within half a week and increased progressively with time. In contrast, the aminoguanidine mixture produced an equal amount of Amadori product but resulted in marked inhibition of AGE formation. Similarly, when we incubated collagen with glucose, the protein became extremely cross-linked, whereas the addition of aminoguanidine blocked nearly all glucose-derived intermolecular bridges.

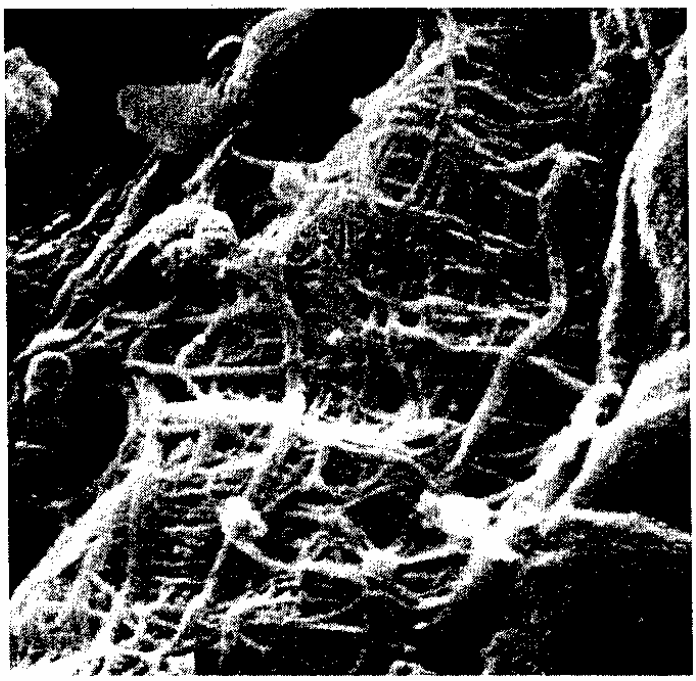
Parallel findings come from studies of diabetic rats. Animals treated with aminoguanidine amassed fewer advanced glycosylation end products in the aorta, and fewer cross-links, than untreated rats. In a separate group of diabetic rats we have shown that aminoguanidine prevents both the trapping of immunoglobulin in the basement membrane of renal capillaries and the trapping of plasma lipoproteins in the arterial wall.

We are now planning trials of aminoguanidine in human subjects. If the drug is shown to be safe, we hope to conduct long-term trials of its ability to prevent diabetic complications. Because diabetes is in some ways a model of aging, success in such trials might eventually help to justify studying the ability of aminoguanidine (or similar compounds) to prevent disorders related to age in nondiabetics.

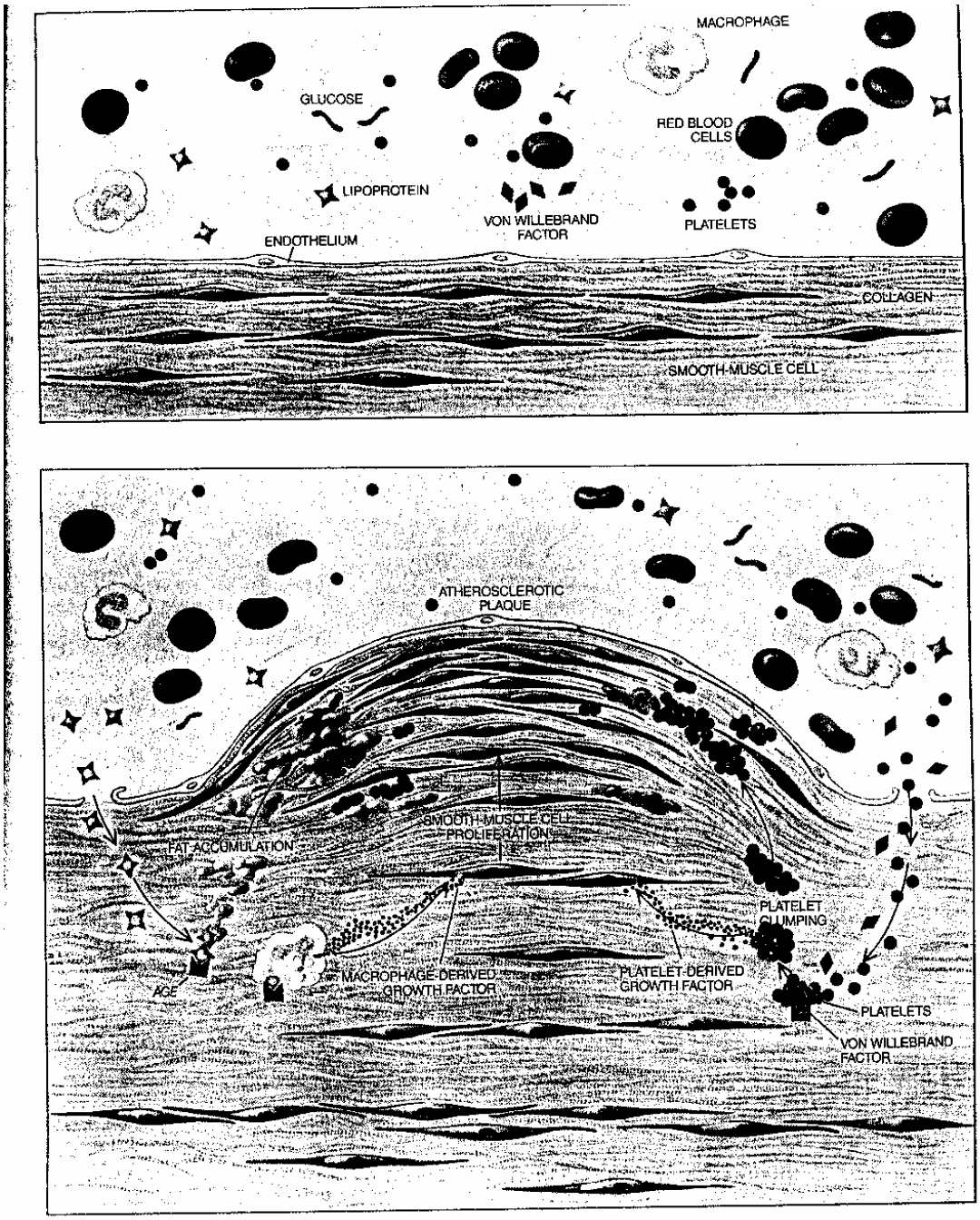
We are also studying the other approach to treatment: increasing the activity of the body's AGE-removal system. Even if the production of advanced glycosylation end products could not be prevented, an effective AGE-removal system might help to counteract any dangerous buildup on proteins. Macrophages, the scavenger cells that remove debris from tissues, apparently constitute one such removal system.

This property of the scavenger cells became clear about three years ago when we examined peripheral-nerve myelin: the complex mixture of long-lived proteins that forms an insulating sheath around nerve fibers. We incubated isolated myelin with glucose for eight weeks to mimic the effects of long-term exposure to glucose in the body. Then we introduced macrophages into the mixture. The cells ingested more myelin than they did when the substance had not been exposed to sugar. They also took up more myelin from diabetic animals than from nondiabetic ones, presumably because the diabetic animals had a greater amount of advanced glycosylation end products.

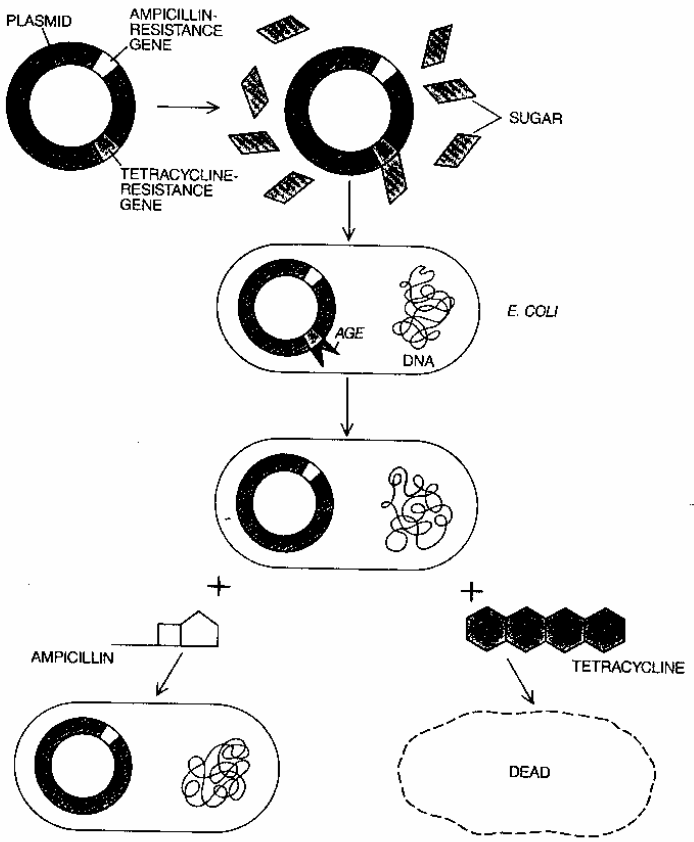
More recent evidence indicates that



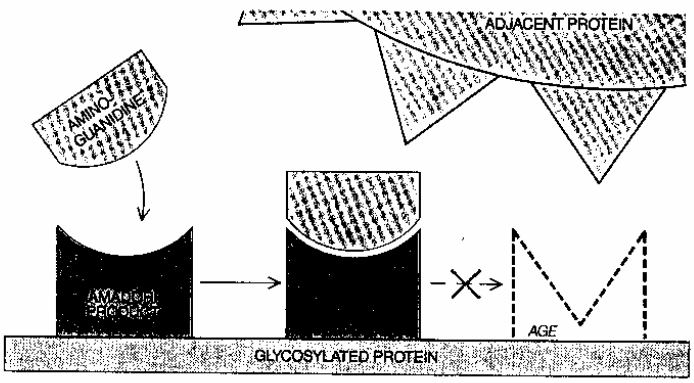
FIBRILS of collagen, the most abundant protein in the animal world, are enlarged 26,000 diameters in this scanning electron micrograph of chick-embryo collagen made by Christine McBride and David E. Birk of the University of Medicine and Dentistry of New Jersey in Piscataway. As animals and people age, cross-linking of the protein molecules in such fibrils causes tissues throughout the body to stiffen. The exact nature of all cross-links is not known, but evidence suggests that many of them may be AGE-derived.



GLYCOSYLATION END PRODUCTS are suspected of contributing to atherosclerosis, and thus to coronary disease, by several pathways. When the inner lining of a healthy blood vessel (*top*) is damaged, plasma proteins leak into the arterial wall (*bottom*). AGE's on collagen in the wall then trap low-density lipoproteins (LDL), which accumulate to form cholesterol deposits in atherosclerotic plaques. Macrophages attempt to remove the captured lipoproteins and in the process secrete a factor that stimulates smooth-muscle cells to proliferate and make new collagen (*blue*). Finally, AGE's on collagen may trap von Willebrand factor, a protein that causes platelets to adhere to the vessel wall. Like macrophages, activated platelets secrete a cell-proliferation factor.



PLASMID, an extrachromosomal circle of bacterial DNA, underwent mutation as a result of being incubated with sugar, suggesting that glucose may contribute to some of the genetic damage seen in humans as they age. After incubation, plasmids carrying genes that render the bacterium *Escherichia coli* resistant to the antibiotics ampicillin and tetracycline were inserted into *E. coli*. In the presence of ampicillin the bacterial cells reproduced normally, but in the presence of tetracycline most cells died. Apparently bacterial enzymes attempted to repair tetracycline-resistance genes that accumulated AGE's.



AMINOGUANIDINE, an experimental drug developed in the authors' laboratory, interferes with the ability of Amadori products to undergo changes that could normally result in the formation of cross-links. The drug's safety and efficacy in humans are under study.

the signal for protein uptake by macrophages is specifically the advanced glycosylation end product. We have found, for example, that a mouse macrophage has an estimated 150,000 receptors for the AGE's that form on albumin. Macrophages attempt to ingest any protein attached to the advanced glycosylation end product FFI, but the cells' receptors do not appear to react with any non-AGE substances that accumulate on proteins, including Amadori products.

The affinity of macrophages for FFI, and for advanced glycosylation end products in general, became dramatically apparent when we attached FFI and other AGE's to membrane proteins of normal red blood cells. Mouse macrophages took up the altered cells much more avidly than they take up normal cells. (In addition to supporting the contention that macrophages are an AGE-removal system, this discovery suggests that advanced glycosylation end products have at least one constructive role in the body: they may indicate that a cell is aged and should be removed.)

Why do AGE's build up on proteins if the body has a system for removing them? We do not have an answer, but a few explanations seem likely. For one thing, the end products may generally form in locations that are not readily accessible to macrophages. Moreover, the highly cross-linked proteins that eventually accumulate appear to be increasingly difficult to remove. Also, as people age, their macrophages may become less efficient as a disposal mechanism. In support of this last notion we have very recently discovered that the number of AGE receptors on mouse macrophages declines as mice grow older.

We are currently seeking drugs that increase the removal rate of unwanted advanced glycosylation end products, but a successful treatment will have to dissolve the end products without excessively damaging irreplaceable proteins. In the case of myelin, for instance, excess AGE-stimulated uptake of old or damaged protein could erode the myelin sheath, which is essential for nerve functioning.

Additional evidence must be gathered before we can say with certainty that nonenzymatic glycosylation of proteins contributes to the cell and tissue changes characteristic of aging. The data collected so far do indicate that our hypothesis is a promising one. More important, the findings raise the exciting possibility that treatments can one day be developed to prevent some of the changes that too often make "aging" synonymous with "illness."