A UNIFYING CONCEPT FOR DEGENERATIVE DISEASES

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■ In 1917, Jacques Loeb and Nobel laureate John H. Northrop determined the temperature coefficient of longevity. In a joint paper they showed that the longevity of the banana fly *Drosophila melanogaster* had the temperature dependence of chemical reactions generally.[1] This was strong evidence that longevity depends of molecular reactions.

The next logical question is, "What chemical reaction could cause the greatest damage in a living cell with the smallest possible input?" Without doubt, the polymer chemist answers, "crosslinkage." A single chemical crosstie connecting two giant molecules can negate their function, even if the molecular weight of the crosslinking agent is <50 and the molecular weights of the crosslinked molecules are in the millions.

Let us look at some specific examples, and begin with the basics. A crosslinking agent is shown attached to one strand of a DNA molecule (Figure 1). The DNA molecule is shown as a double helix, and a small crosslinkage molecule is represented as a little rod with a hook on each end. The crosslinking agent can be any small molecule that has at separate positions two groups, of which each can react with at least one site of a giant molecule. The defense mechanism can excise it, and one strand can then be repaired flawlessly, using the remaining intact strand as a template. The two strands are mirror images of each other, and each one has the ability to duplicate its companion. Such crosslinking agents are plentiful; they include aldehydes, peroxides, polyvalent metal ions, sulfur, free radicals, ozone, quinones, oxidation products of unsaturated fatty acids, and many others.

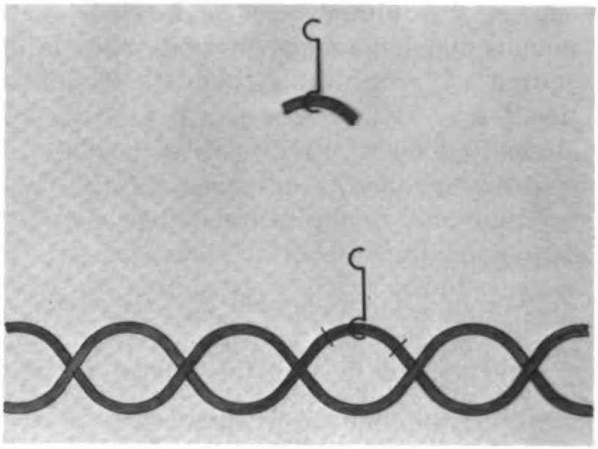

Figure 1. A crosslinking agent attached to only one strand of a DNA molecule. (From Bjorksten.[8] Used with permission.)

A certain time will inevitably lapse before the repair is completed, and in the meantime, the DNA molecule is vulnerable. If, in this critical

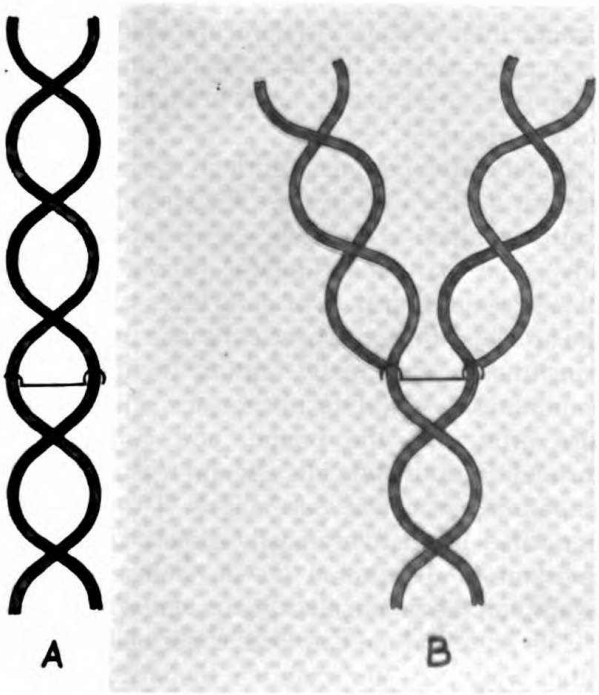

Figure 2. The crosslinkage has been completed before the defense mechanism could act. (From Bjorksten.[9] Used with permission.)

time interval, the crosslinking agent touches the other strand of DNA, then the two strands will be bound together (Figure 2). Then the cell is doomed. If the defense mechanism cuts off the crosslinking agent, it will unavoidably cause complete breakage of the DNA molecule, and no template is left that could be used for repair or replication. On the other hand, if no repair is made and the crosslink is left in place, then in the next cell division, when the two strands part and each begins to replicate its former companion, the process can only proceed as far as the crosslinkage. It cannot go beyond that point because the crosslink prevents the necessary parting of the strands. The resultant molecule is a monstrosity, like a Siamese twin, and the cell will die. The importance of this mechanism is emphasized by Hart and Setlow, who showed that the lifespans of six out of seven species of mammals are directly correlated to their speeds of repair of the DNA (Figure 3).[2]

Important as the crosslinkages of DNA unquestionably are, though, it is possible that another mechanism may be equally or even more important. For every DNA molecule there are

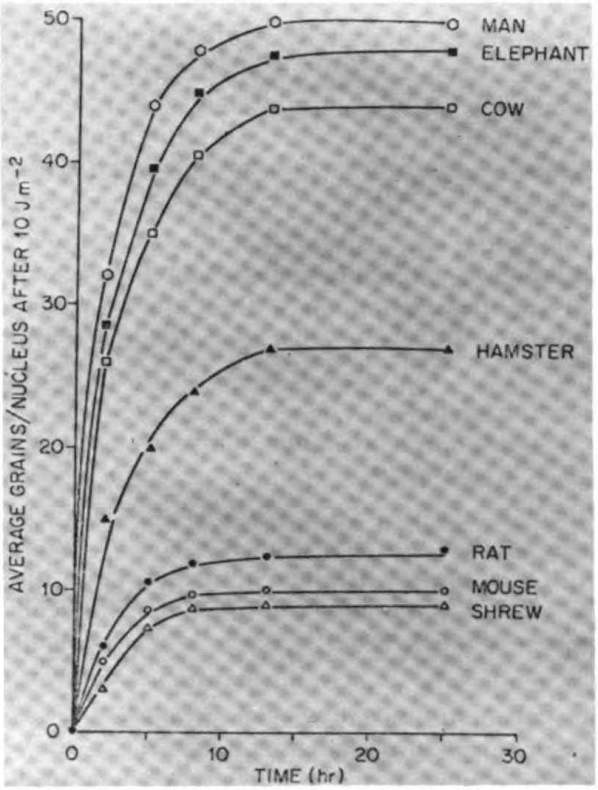

Figure 3. Lifespan compared to rate of repair of the DNA molecule. (From Hart and Setlow.[2] Used with permission.)

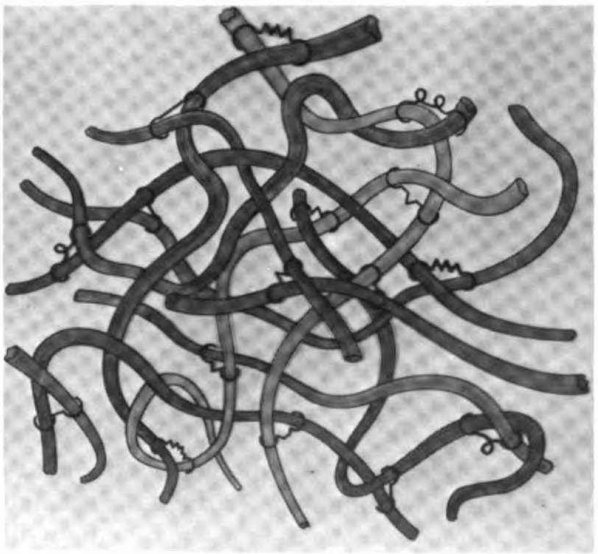

Figure 4. Random, accidental, mostly nonenzymatic crosslinkages accumulate over a lifetime to form dense aggregates of any available large molecules with any crosslinking agent. (From Bjorksten.[8] Used with permission.)

thousands of other vitally significant macromolecules: enzymes, immunoglobulins, antibodies, membrane, and structure proteins. These make up in numbers what they lack in uniqueness, as compared to DNA. Figure 4 illustrates aggregates formed over a lifetime by the crosslinking of nongenetic molecules. Here, too, the newly added linkages could be reversed, but these will become irreversible in a short time as each additional linkage or blockage makes it more difficult for the repair enzymes to penetrate to the critical sites.

There is ample evidence that such aggregates increase within the organism as time passes. Structures of this type can severely interfere with intracellular transport, as well as crowd out other important elements in the cell, thus progressively reducing the function of the cell. This can apply to all cells, whether they be excreting cells in a gland, hormone-synthesizing cells, immunoacting cells, or something less spectacular but nonetheless needed. Crosslinking contributes importantly, perhaps even principally, to what is often called *age-dependent deterioration.*

SPECIFIC SYNDROMES

Atherosclerosis

The most studied, most easily determined, and most generally recognized property of crosslinkage is loss of elasticity, Any carpenter knows that if he puts crossbracing between two boards, he stiffens the assembly. The same applies on a molecular scale, where the crosslinking agents build braces between molecules. Many crosslinking agents are normally present in blood (Table 1). A great many more have been found since, but even these should suffice to show the abundance of crosslinking agents.

As blood circulates, the crosslinking agents react very slowly with any molecule available, including the endothelia of the blood vessels. This causes a stiffening, particularly of the endothelium. Crosslinking in the media will likewise proceed steadily and inexorably through life. In the early part of life, the endothelia are still quite elastic and follow readily the pulsations of the arteries, but as time passes more and more crosslinking occurs, with consequent stiffening and loss of elasticity. Finally, the endothelia no longer follow the pulsations. Cracks then appear. A microrupture will form at a point where the hydrostatic pressure is high, such as in bends, or in branchings of an artery (Figure 5).

Blood serum can push through the crack and deposit a filter cake (Figure 6). This cake then contains blood corpuscles, chylomicrons, and any other particulate matter carried by the bloodstream. Ingrowth of cells takes place into the area of diffusion. These cells take up plasma constituents, principally liquids, to form cells sometimes resembling phagocytes. Fibroblasts also grow into the region, and collagen is formed. Endothelial cells attempt repair, which sometimes succeeds, particularly if the matter deposited is relatively easily dissolved. If it is resistant to dissolution (as is cholesterol, for example,) the damage will be progressive.

The endothelium can lose its permeability in other ways, too—for example, by parting of the seams between the cells or by the death of individual endothelial cells. Furthermore, as the medium hardens, the endothelial cells are squeezed more and more with every pulse wave. This can result in damage to the endothelial cells, which renders them permeable to flow.

These and other matters have been discussed in considerable detail by Harvey Wolinsky.[3] I am in accord with Wolinsky, taking exception

Table 1 • Potential Cross-Linking Agents in Blood

Agent	Concentration/100 ml
Acetaldehyde	<0.1 mg
Methylguanidine	0.2-0.3 mg
Alpha ketoglutaric acid	0.2-0.9 mg
Pyruvic acid	0.4-2.04 mg
Alpha keto acids, only generically identified	0-3.1 mg
Citric acid	1.3-6.0 mg
Malic acid	0.1-0.9 mg
Fumaric acid, in rat	<0.3 mg
Succinic acid	0.5 mg
Silicon	33-63 μg
Lead	18-49 μg
Aluminum	15-40 μg
Copper	73-115 μg
Iron	43-52 μg
Manganese	0-25 μg
Zinc	488-1272 μg

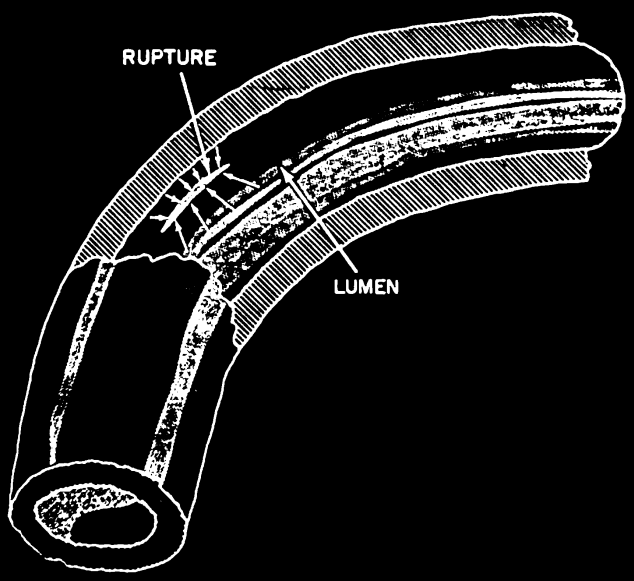

Figure 5. A rupture in the intima. Blood serum filters through and deposits particulate matter in the subintimal region.

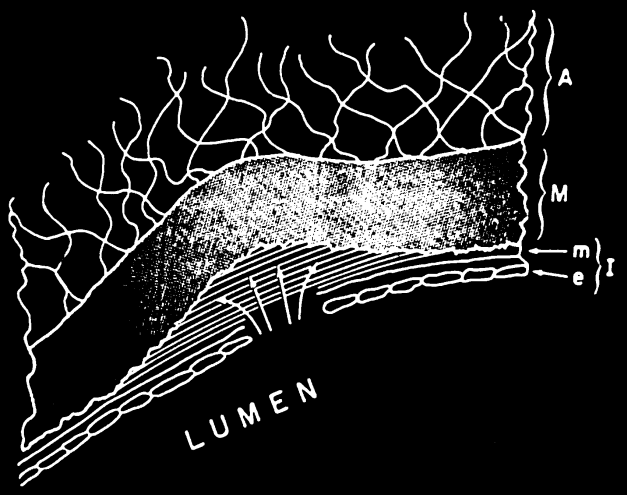

Figure 6. The rupture in section. A = adventitia; M = media; m = membrane; e = endothelium; I = intima.

only to his statement that no mechanisms yet described in cells grown in tissue culture can explain the lipid accumulation. This occurs at an accelerated rate in hypercholesterolemics who have a total lack of specific receptor uptake.

I believe the crosslinkage theory provides an explanation of the earliest precellular stage of atherosclerosis, which, as Wolinsky shows, is characterized by an enormous accumulation of cholesterol ester (in the smooth muscle cells). I propose that this molecular mechanism is also a direct result of crosslinking.

One side of an unspecified protein molecule contains more hydrophilic sites than the other side (Figure 7). (It rarely happens that both sides are in complete balance.) Consequently, these hydrophilic sides of the protein molecules will be preferentially crosslinked, facing each other, thus turning the more lipophilic sides outward. This is a cause of progressively increasing lipophilia as a consequence of crosslinking. It leads to progressively more ready absorption of cholesterol and, indeed, any other lipid. Lipids differ in reversibility; cholesterol is among the more difficult to remove.

Two aspects are paramount in the inception of atherogenesis: (1) the endothelium becomes permeable to liquid, and (2) the protein molecules in the affected tissues and deposits become progressively lipophilic. Both can result from progressive crosslinking. Crosslinkages must be expected to occur in every living cell, because all life requires the presence of reactive giant molecules and of several known crosslinking agents.

Senile Cataracts

Another specific area where crosslinking appears to cause problems is senile cataracts (Figure 8). The results of extensive crosslinkages leading to progressive insolubilization and optical density have been documented.[4]

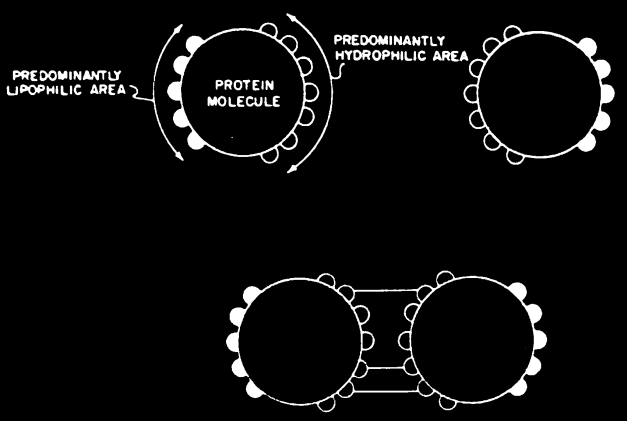

Figure 7. Two protein molecules. A. With lipophilic (dark) and hydrophilic (white) sites. B. Crosslinked preferentially at the hydrophilic sites, so as to turn the lipophilic sites outward, thus becoming lipophilic.

Osteoporosis

The relationship of crosslinkage to osteoporosis may be twofold. Since all collagen in the body is exposed to the crosslinking agents in the circulating blood, the collagenous binding matrix in the bone must gradually become embrittled, to a degree that will reduce the shock resistance and elastic recovery.

Furthermore, the bone structure is sensitive to anoxia, a sequela to any appreciable degree of arterio-atherosclerotic processes within the circulatory system. Anoxia severely curtails the catabolic reactions that act to repair incipient damage to the large organic molecules within the bone structure, even a mild degree of anoxia gives rise to partial oxidation products that are powerful reactants. The anoxia may be due to restriction of blood supply, e.g., by sclerosis. Or it may be caused by a lack of tocopherols, selenium, or other antioxidants. In their absence, non-enzymatic reactions wasteful of oxygen are favored. Anoxia may also be caused by ingesting disproportionate amounts of fat in the diet.

Diabetes

Diabetic metabolism enhances the probability for crosslinkage by eliminating, in a large part of the organism, the most direct and least inter-

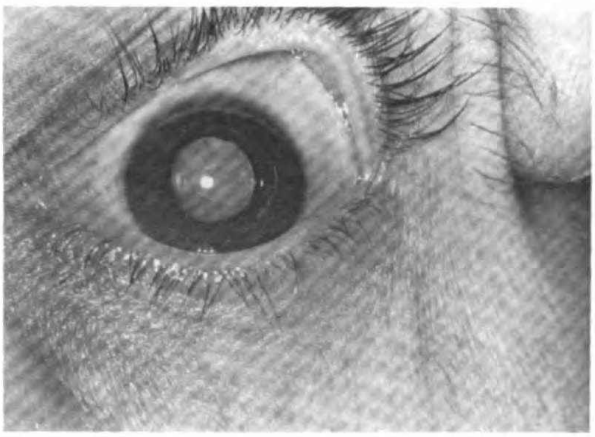

Figure 8. Senile cataract. (From Bellows.[4] Used with permission.)

mediate-byproduct-producing pathway for energy release (Figure 9).[5] The basic principle here is that the brain requires glucose for its nutrition. It can, to some degree, when necessary, utilize some of the ketone bodies: pyruvic acid, acetonylacetate, and gamma oxybutyric acid. But the brain cannot utilize fat at all, in any form. Therefore, evolution has developed a safeguard for the brain's glucose supply. If this supply is threatened, the metabolism will immediately change to preempt the essential glucose for the brain. The very first step is the loss of the ability of the muscle to use carbohydrates, thus forcing the muscles to a fat metabolism. The muscles, in contradistinction to the brain, are very capable of utilizing fats. The organism controls the ability of the muscles to use fats by inactivating the muscle response to insulin and, alternatively or additionally, by curtailing the production of insulins. At the same time, protein amino acid catabolism yields more pyruvic acid, which in turn goes to fatty acids to increase the supply of these for the muscles, and in this process also steps up cholesterol production.

Fatty-acid and cholesterol formation are thus both favored by the diabetic metabolism. Fatty acids and cholesterol both have a well-known positive correlation with atherosclerosis; fur-

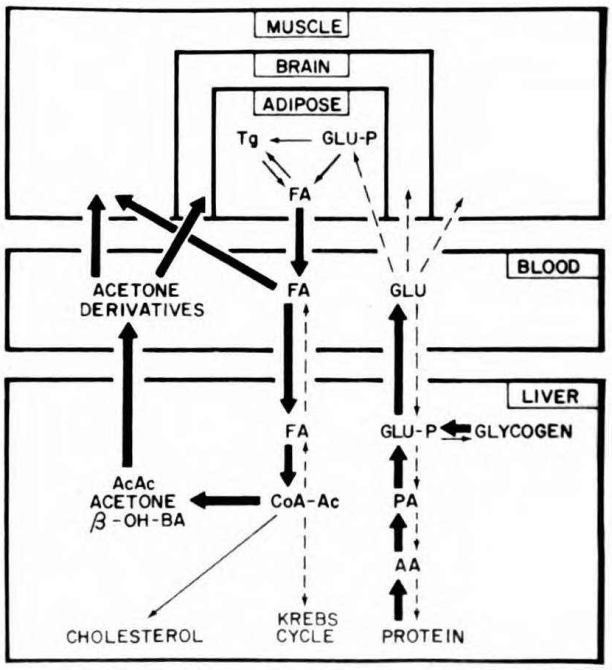

Figure 9. Diabetic metabolism.——— = metabolic pathways restricted in diabetes; → = pathways increased; AA = amino acids; AcAc = autoacetic acids; β-OH-BA = β oxybutyric acid; CoA-AC = coenzyme A acetate; FA = fatty acids; Glu = glucose; **Glu P** = glucose phosphate; PA = pyruvic acid; Tg = **triglycerides**. (After Miettinen.)[5]

thermore, the oxidation products of the fats comprise both aldehydes and peroxides, of which many are highly active crosslinking agents.

Immunologic Impairment

The mechanism shown in Figure 8 (in connection with lipid absorption by proteins) illustrates how very profoundly surfaces and immunologic behavior can be changed by crosslinkages. Not only may the immune system recognize crosslinkages as such, but it may also react to crosslinking agents incipiently attached at only one point and may react particularly to the gross changes that occur when giant molecules become further aggregated by multiple crosslinking.[6] The prevalence of crosslinked aggregates will interfere greatly with intracellular transport, slowing it down or even making normal transport impossible.

Cancer

The data now at hand are too complex to permit conclusions as yet. However, it may be pointed out that crosslinkages will cause DNA anomalies that have been associated with cancer. Almost all known carcinogens either are themselves potential crosslinking agents or have metabolites that are. A high correlation exists between cytotoxicity and crosslinking capacity.[7]

COUNTERMEASURES

I have discussed elsewhere the literature on reducing exposure to unavoidable random crosslinking.[8-10] Methods for minimizing crosslinking include such well-known steps as nutrition planning to avoid sudden overloads or any bottleneck in any metabolic path, favoring paths that involve the minimum amount of chemical processing in the body, and avoiding exposure to noxious environmental situations.

A simple experiment published in 1952 indicated that in one single instance a cholesterol-absorbent spot made with a specific metallic crosslinking agent could be made non-cholesterol-absorbent by subsequent treatment with (1) a chelating agent, presumably pulling out the metal component; or (2) a protease, which presumably undercut the treated surface.[11-13] I chose to pursue the enzyme lead for the following 25 years, because it seemed applicable to organic as well as metallic crosslinking agents.

ENZYME PROCEDURE

In order to screen microorganisms for activity on crosslinked gerogenic materials, we used human heart muscle from six hearts ranging in age from 64 to 74 years. A preliminary acid and fat extraction was followed by exhaustive hydrolysis with trypsin, papain, collagenase, elastase, acid reflux, and a final extraction, as shown in Figure 10. The resultant product was insoluble in anything we could think of except anhydrous hydrazine—a substance never encountered in nature. No corresponding fraction was found in young hearts. We now started the quest for an enzyme capable of dissolving and removing this highly resistant powder. In the course of this work we shifted to the similarly prepared substances from old brains, which gave us a higher yield of crosslinked residues than did the hearts.

Basically, our premise was that some soil organism must be able to dissolve and consume the gerogenic insolubles. for otherwise these would have formed geologically significant fossil layers. Following the method of René Dubos, we therefore suspended the gray insoluble powder in a transparent agar gel, and inoculated it with mixed soil bacteria in the form of an infusion of rich garden soil (Figure 11). Here and there a colony developed surrounded by a clear halo, a circular zone around the colony in which the suspended so-called insolubles had been dissolved. We made subcultures, ran fermentation tests, and found some organisms that grew well, could be handled, and produced an enzyme that could, indeed, dissolve these gerogenic particles.

Toxicity tests showed at least one such enzyme was surprisingly well tolerated by rats and mice. Massive dosages led to hemorrhage, but there seemed to be quite a practical margin in which application seemed possible. However, we were not in a position to go into larger-scale production, so we turned over the procedure and cultures to Worthington Biochemical Corporation, in Freehold, New Jersey, which can furnish it to researchers.

While we wait for the academic community and pharmaceutical industry to carry out the

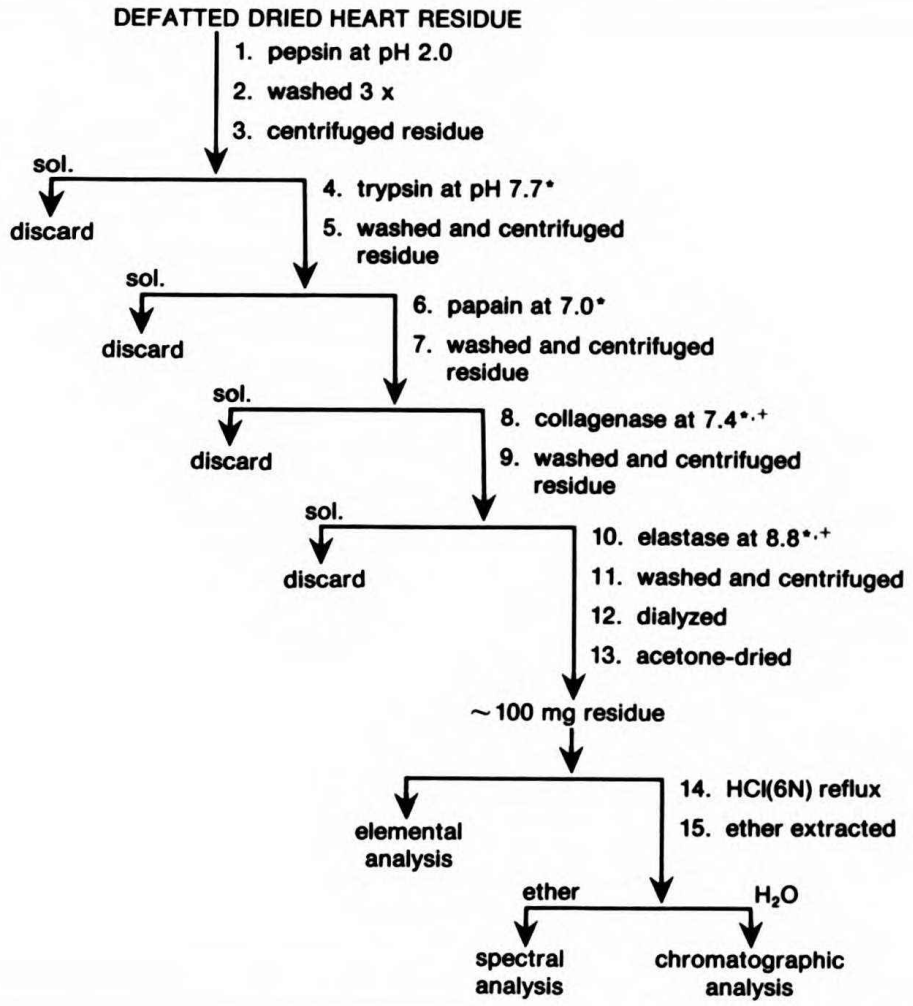

Figure 10. Enzyme hydrolysis procedure. * = Tris buffers, ionic strength 0.05 used throughout. † = Both collagenase and elastase hydrolyses were run twice.

animal tests, we shall be exploring the chemistry of some other related but nonidentical enzymes, while also studying chelation and considering the possibilities of other, nonenzymatic ways of breaking down macromolecules. The findings of Robinson and coworkers—that breakdown products of ascorbic acid can destroy large molecules containing peptide bonds—indicate attractive possibilities for practical application and may open new areas of exploration.[14]

CHELATION

Though restricted to metallic crosslinkages, chelation still has considerable significance. This was emphasized by Tyler, who showed that the survival of sperm cells in tap water could be increased several hundred percent by removing the polyvalent trace elements using chelation.[15]

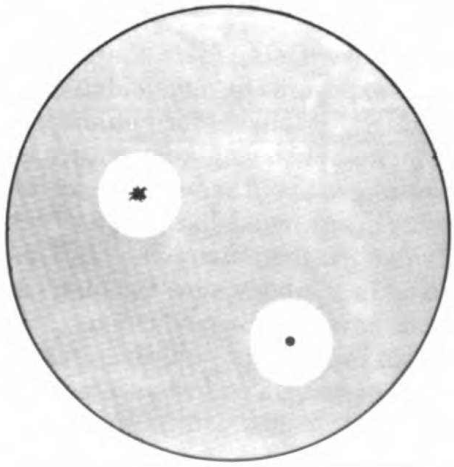

Figure 11. Dense aggregates (Figure 4) were isolated from old human brain, pulverized, and dispersed. A suspension of mixed soil organisms was used as inoculum.

Chelation is currently being practiced at several clinics as a necessary treatment for acute lead or plutonium poisoning, and is a still-debated treatment for removal of excess calcium.[16]

Chelation is not, of course, directly effective on fat deposits. But it is quite plausible that a certain part, possibly a major part, of organic nonchelatable crosslinking is favored by primary metal-mediated crosslinking. Otin and Alexa showed in 1938 that hide powder treated with basic aluminum sulfate fixed 2.14% of the aluminum measured as oxide, but that this absorption of aluminum, which formed aluminum crosslinkages of collagen, increased the absorption of additional phenolic crosslinkers from 38% to 82%.[17] This was evidently an effect of orienting the protein molecule (Figure 7), and also of fixing protein molecules in the proximity of each other, thereby making them easier targets for organic crosslinking agents of wider span. If a primary metal crosslinkage increases the absorption of fatty substances as indicated, which appears quite plausible, then it could play a major part in atherosclerosis as well as in arteriosclerosis.

The significance of chelation was dramatically re-emphasized by the recent finding that injecting minute quantities of aluminum ion intracranially in cats leads to a slowly developing permanent fibrillary degeneration similar to that previously recognized in Pick-Alzheimer disease.[18] Crapper and co-workers found that the aluminum was absorbed by the chromatin in all brain cells, and it affected, in particular, neural cells with large dendritic trees. The observations of the effect of aluminum ion on the electrical impulses from brain cells might be interpreted as supporting Still's cybernetic concept of aging.[19] Clinical observations in humans have recently been tied to the accumulations of aluminum.[20,21]

It would seem feasible to remove aluminum already established in the nervous system by the use of chelation agents, such as those suggested by Zinsser, Bjorksten, et al.[22] The problems of the blood-brain barrier seem surmountable, partly because (1) no biologic barrier is absolute, and nanogram quantities of the chelating agent in the correct site would suffice; and (2) lactic acid, a chelating agent active on aluminum, is a normal metabolite accumulating on muscular exhaustion. The favorable results reported from physical exercise to the point of exhaustion in treatments to combat degenerative disease may be due to the metal-chelating potency of the lactic acid thus generated, in addition to the resulting physical relaxation and vasodilation.[23]

CONCLUSION

The crosslinkage theory of aging formulated in 1942 has withstood the test of time. It is compatible with the known facts. Crosslinking could well be a basic underlying cause common to degenerative diseases heretofore viewed separately. It provides a unified guideline that could be useful in formulating preventive steps.

Crosslinking itself can hardly be prevented, because the agents are largely metabolites, the complete exclusion of which would be incompatible with life. It may, however, be reversed—in the case of metallic crosslinkers, by means of chelation; and in the case of organic crosslinkers (aldehydes, quinones, oxidation products of unsaturated fats of catecholamines, free radicals, and many other reactive di-substituted compounds), by means of enzymatic destruction of the backbones of the macromolecules involved, so that the normal anabolic processes can effect replacements. In the case of genetic molecules, a complete interstrand crosslinkage is probably irreversible, but increased enzyme activity could potentially assist the organism in effecting excision of the initial bonding site of the crosslinking agent from one strand before the other strand becomes engaged, thereby enhancing the probability of repair of the incipient lesion before the crosslinkage is complete.

Thus, the crosslinkage theory, viewed as a common denominator for divergent forms of degenerative disease, gives us some new guidelines. It points the way to advancement beyond anything commonly accepted as possible today. ▲

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