A COMMON MOLECULAR BASIS FOR THE AGING SYNDROME

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The manifestations of aging are many, but there are certain underlying phenomena which they have in common.

The clinical symptoms of degenerative diseases are well known, and by the use of histologic techniques definite microscopic changes have been found. The chemical processes underlying these changes have not been properly elucidated, however. With advancing age, there is a slowing in metabolism, a loss of elasticity of the tissues, an increasing brittleness in bone and cartilage, a loss of water-binding capacity, and above all, a reduced resistance to disease and to trauma of all kinds.

This reduced resistance is represented graphically in Figure 1. The curve is based on the mortality rate for the total U. S. population (1).

In the exploration of possible causes for the progressive changes in body tissues which take place in aging, it appeared that certain of these might be interpreted in terms of polymer chemistry, especially with reference to known reactions of protein-type polymers. Reactions which result in exponentially progressive phenomena similar to those observed in the aging syndrome are found in the cross-linkage of proteins, and other reactions capable of changing their solubility characteristics and susceptibility to enzymatic hydrolysis. It appeared pertinent therefore to note reported evidence in the literature of the presence in the bloodstream of substances capable of causing changes in proteins, which *in vitro* in protein gels closely parallel the changes in elasticity, brittleness and water retention observed *in vivo* in senescent tissues. A search disclosed numerous well-documented reports of the presence in normal blood of substances which can cause aggregation or insolubilization of protein molecules by cross-linkage (Table 1).

CROSS-LINKAGE OF PROTEINS AND RESISTANCE TO ENZYMATIC HYDROLYSIS

A cross-linkage results when a molecule with 2 or more reactive groups reacts with one protein molecule at one of these groups and with another protein molecule at another reactive group. The result is the formation of a new molecule, the molecular weight of which is the sum of the molecular weights of the 2 component protein molecules, and the molecular weight of the cross-linking agent. From these data it appears that in the bloodstream there are enough cross-linking agents to cause progressive cross-linkage of some of the protein, and possibly also to enhance the oleophilic properties of protein materials in the tissues (19).

In view of the relative abundance of potential cross-linking constituents in human blood (Table 1), as well as the known cross-linking influence of ubiquitous ionizing radiation (20, 21, 22), it appeared of interest to explore the reason

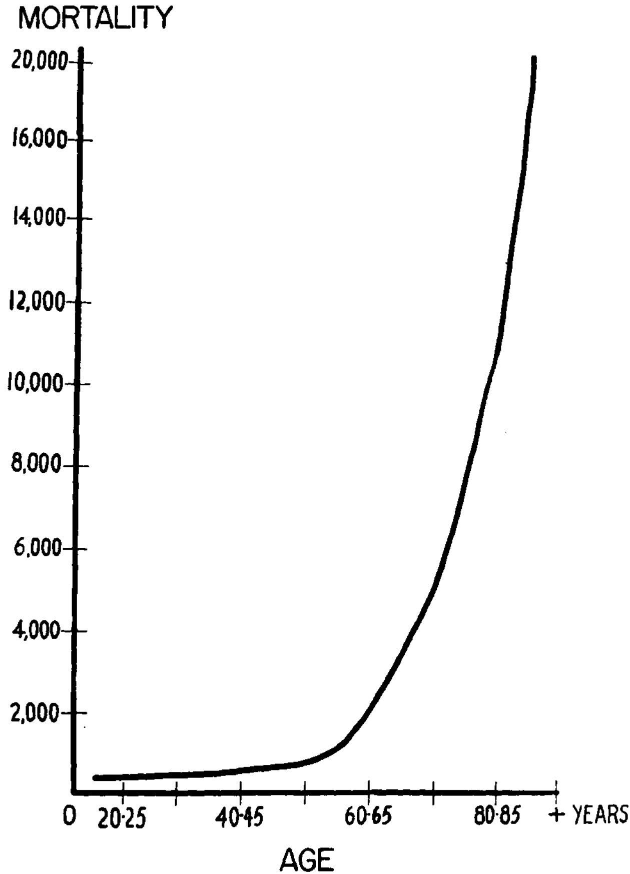

FIG. 1. Reduced resistance (mortality rate) with advancing age.

why these influences do not cause loss of elasticity and embrittlement in human tissues considerably more rapidly than is the case in normal aging. We have to assume that a defensive mechanism exists. The nature of this defense mechanism against progressive cross-linkage of proteins is suggested particularly by the work of Schoenheimer (23), Baldwin (24) and others, who have investigated the dynamic state of proteins in the body.

It was shown by these researchers, that protein molecules are continually broken down and re-synthesized.

The turnover is different for different tissues; the general range of half-lives is 5–200 days, with 80 days a general average for adult humans. Until recently it was thought that the proteins in the brain were metabolized slowly, but recent studies by Bakay (25, 26) have shown that this conclusion depended on failure to allow for the time necessary for passage of tagged amino acids through the blood-brain barrier, and that when the tracer substances are injected directly into the brain, there is a rapid rate of turnover. When a protein is broken down

TABLE 1. *Potential Cross-Linking Agents in Blood*

Agent	Concentration
Acetaldehyde (2)*	<0.1 mg./100 cc.
Methyl guanidine (3)	0.2–0.3 mg./100 cc.
Alpha ketoglutaric acid (4, 5)	0.2–0.9 mg./100 cc.
Pyruvic acid (6)	0.4–2.04 mg./100 cc.
Alpha keto acids, only generically identified (7, 8)	0–3.1 mg./100 cc.
Citric acid (9, 10)	1.3–6.0 mg./100 cc.
Malic acid (11)	0.1–0.9 mg./100 cc.
Fumaric acid, in rat (12)	<0.3 mg./100 cc.
Succinic acid (13)	0.5 mg./100 cc.
Silicon (14)	33–63 μg./100 cc.
Lead (15)	18–49 μg./100 cc.
Aluminum (15)	15–40 μg./100 cc.
Copper (16)	73–115 μg./100 cc.
Iron (17)	43–52 μg./100 cc.
Manganese (15)	0–25 μg./100 cc.
Zinc (18)	488–1272 μg./100 cc.

* Reference number.

to polypeptides and component amino acids, cross-linkages are dislodged; and when the protein is resynthesized, it is synthesized in its original state, since the synthesizing mechanism obviously would not replace the cross-linkages. If this defense mechanism functioned with complete efficiency, it appears that protein immobilization and its subsequent effects would be prevented. However, in this enzymatic mechanism there is an inherent imperfection, which, should it not suffice to explain the primary processes of aging, at least could account for phenomena which theoretically would result in changes similar to some changes observed in aging.

It is well known that proteolytic enzymes attack protein molecules at certain points. Assuming that molecules of cross-linking agents should block all the points of attack on 2 adjacent cross-linked protein molecules, then these protein molecules could not be broken down by the body enzymes, and they would remain immobilized. Since cross-linkage is a random process, dependent on chance contacts between the cross-linking agents shown to be present in the blood and the reactive groups in the protein molecules, it is apparent that in a certain statistically ascertainable percentage of such contacts the points of the molecules where body enzymes can attack will be blocked, and that the proteins thus blocked become fixed and immobile, and effectively withdrawn from participation in any metabolic reactions. As chronologic age progresses, an increasing amount of protein is thus made inert; it tends to clog the cells, interfering first slightly, then at an increasing rate, with the normal functions of the cell.

EXPERIMENTAL DATA

An important point in this theory, which is susceptible to experimental verification is:

Does cross-linkage of proteins in fact interfere with hydrolysis by enzymes, as postulated? This point was checked by cross-linkage of gelatin with known cross-linking agents, and subsequent treatment with a series of proteolytic enzymes. Yields and identity of liberated amino acids were studied by paper chromatography. It was shown that a portion of the cross-linked protein actually is made inaccessible to enzymatic action by pepsin, trypsin, papain and ficin (27, 28).

Figures 2 and 3 illustrate the data on which this conclusion is based. These data are taken from a report to the Office of Scientific Research of the U. S. Air Force, presented by Bjorksten and Gottlieb (29).

To a 5 per cent aqueous solution of 375 Bloom pig-skin gelatin was added formaldehyde in the molar ratios shown below Figure 1.

After forty-eight hours, the amino acids were determined by a paper chromatographic method.

Six-ml. aliquots of the reaction solutions were spotted on Whatman #1 chromatography paper. Reference spots of known amino acids were added to each strip before development. The developments were continued for approximately twenty hours in 4:1 (W/V) phenol-

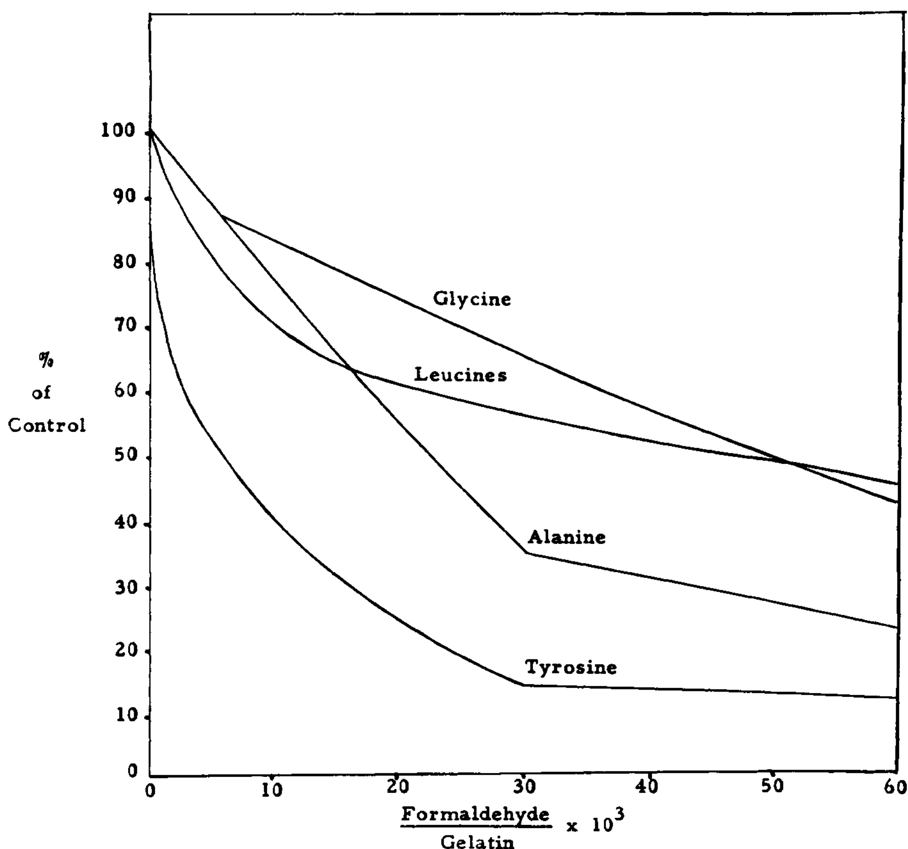

FIG. 2. The amino acids of formaldehyde-treated gelatin after 48-hour hydrolysis with trypsin.

water, 4:1:1 (V/V/V) butanol-water-acetic acid and 75:35:25 (V/V/V) methyl ethyl ketone-water-propionic acid at room temperature.

After development, the strips were air dried and sprayed with 0.25 per cent Ninhydrin in water-saturated butanol. The color reaction was allowed to continue several days at room temperature before carrying out qualitative and quantitative evaluations of the amino acids. The measurements were made by careful visual inspection at the height of the color intensities. The discernible spots were graded on a six-point intensity scale. The color scores for the amino acids from the untreated gelatin were used as the standards. The coloration produced by the amino acids from treated gelatin was scored as the percentage of the corresponding coloration produced by the amino acids from untreated gelatin after correcting for the coloration from the reagent controls for each of the hydrolytic reagents.

Figure 2 shows that the proteins are affected by the cross-linking process in such a way that trypsin no longer can release more than a portion of the amino acids normally hydrolyzable, and that the amount which can be released is greater for glycine and leucine than for alanine and tyrosine.

Figure 3 shows the degree the refractoriness of protein to trypsin hydrolysis when para-benzoquinone is used as the cross-linking agent instead of formaldehyde. The procedure was the same as in the preceding experiment.

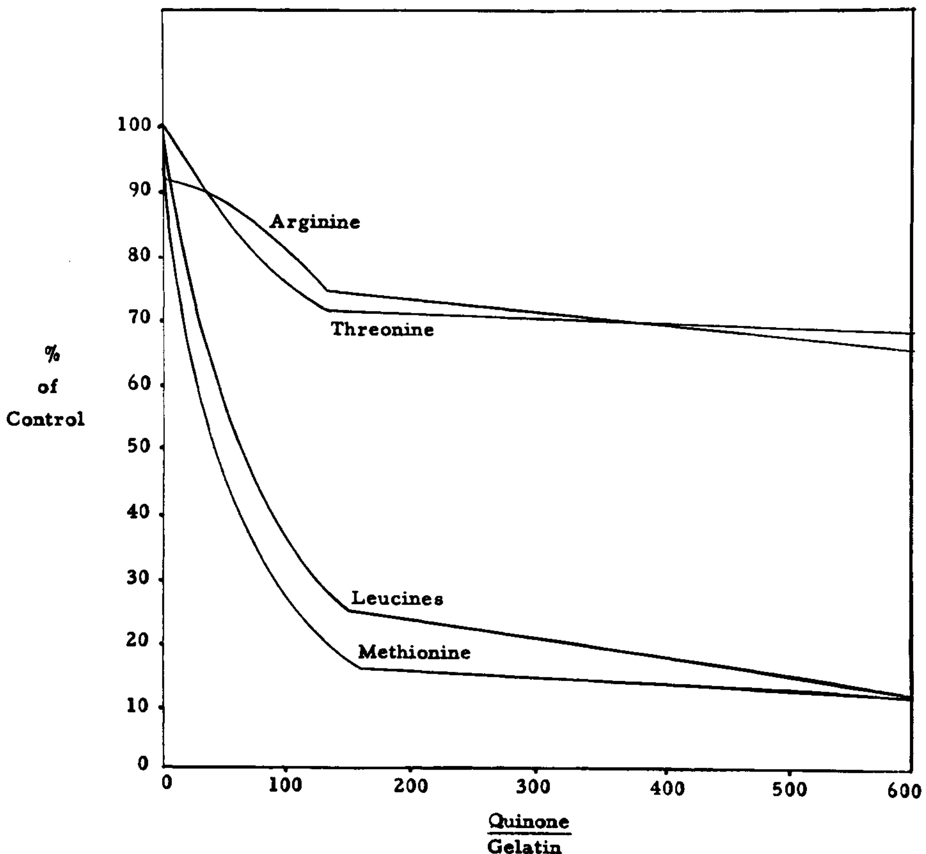

FIG. 3. The amino acids of quinone-treated gelatin after 48-hour hydrolysis with trypsin. The values on the abscissa are the molar ratios of quinone/gelatin.

Hundreds of determinations have been made and are being published separately (29). The results are substantially similar when different protein substrates, proteolytic enzymes and cross-linking agents are used. The foregoing data, however, should suffice to show that progressive cross-linking does indeed render a protein progressively resistant to enzymatic hydrolysis.

IMMOBILIZATION OF PROTEINS AS A BASIC CAUSE OF AGING

Other effects obtainable by cross-linkage may be related to phenomena of aging. As discussed in previous publications (19, 30), arterial tissues exposed to several known cross-linking agents become oleophilic; this may have some bearing on interaction between fatty material (including cholesterol) and arterial surfaces. Also, the progressive loss of elasticity of certain tissues exposed to continuous pulsation (31) may be caused by cross-linkage and may in turn result in minute ruptures which initiate further damage.

In the years preceding the first publication in which the working hypothesis of immobilization of proteins as the basic cause of aging was stated (32), observations had been brought forth which were compatible with this hypothesis. It may be pertinent to review supporting observations:

1) Loss of elasticity in many body tissues is a characteristic of old age. Loss of elasticity is a characteristic effect of cross-linking.

2) Increased brittleness of rigid tissues is a characteristic of old age. Increased brittleness is a characteristic effect of cross-linking.

3) Decline of the percentage of bound water is a characteristic of old age. Decline of the percentage of bound water is a result of cross-linking in water binding proteins.

4) Diabetics are particularly susceptible to degenerative circulatory disease. Diabetics have what is known as a ketogenic metabolism, most likely to give rise to potential cross-linking agents in the organism.

5) Animals receiving very low caloric diets have a substantially increased life span (33, 34). A very low caloric diet makes for a minimum of metabolic by-products which might act as cross-linking agents.

6) Aluminum, a potent cross-linking ion, accumulates progressively with age in tissues of dogs (35).

In the sixteen years since this working hypothesis was first reported, it has been elaborated and further developed (36, 37). Observations in several laboratories have produced the following experimental evidence which is compatible with the hypothesis:

1) Ionizing radiations, known to cause cross-linking of large molecules (20–22, 38–40), also cause symptoms resembling those of natural aging (41–45).

2) On aging, a large aggregate protein substance accumulates in the arteries progressively (46).

3) On aging, aluminum, a potential cross-linking ion, accumulates concurrently with a protein material in arterial tissues (47).

4) Accumulation of mucoprotein takes place in tissues with aging (48).

5) In a certain unicellular organism, the macronucleus increases greatly in size with age, indicating compensation for immobilization of proteins; and a

concurrent precipitation of organized protein is visible in the electron microscope (49).

6) These "chromatin bodies" appear earlier, in Tokophrya organisms in which the life span has been shortened by overfeeding (50–53).

7) On aging, colloidal material, presumably of protein nature, accumulates in kidneys (54).

Further supporting evidence:

1) Chemical cross-linking agents alter the hydrolysis of proteins by body enzymes (19, 27, 28, 30).

2) Proteins irradiated to the point of altered aggregation exhibit alterations of proteolysis by enzymes (28).

3) By treatment with certain cross-linking agents, arterial walls can be made absorbent for cholesterol or any other lipids (19, 30).

How the immobilization of proteins occurs, and its relationship to aging are subjects for further study. At present, cross-linkage seems to us the most likely mechanism. However, polymerization of proteins without cross-linking, denaturation or crystallization could also be the cause of immobilization and contribute to withdrawal of proteins from their normal functions.

The next phase of the study should be directed to obtaining more specific knowledge of the reaction which leads to the immobilization of proteins. When this information is available, specific controls and counter-measures may become possible. For example, if a certain type of cross-linkage should be found to account for a large part of the aging syndrome, a search for suitable enzymes or agents capable of breaking that cross-linkage would be indicated.

For a frontal attack on the problem, increased efforts and funds are needed. As pointed out by E. V. Cowdry (55), studies directed toward the fundamental chemistry of aging on a molecular level are likely to prove rewarding in the elimination of degenerative diseases, as they would strike directly at the cause of these diseases.

SUMMARY

Degenerative changes must have a basic cause on the molecular level. For example, the possible role of protein immobilization by means of progressive cross-linking reactions is critically examined in the light of known data on potential cross-linking agents present in the blood stream, and of related physiologic facts.

Acknowledgment

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