JOURNAL OF THE AMERICAN GERIATRICS SOCIETY
Vol. 16, No. 4
Copyright © 1968 by the American Geriatrics Society
Printed in U.S.A.

THE CROSSLINKAGE THEORY OF AGING

JOHAN BJORKSTEN, PH.D.*

Bjorksten Research Foundation, Madison, Wisconsin

ABSTRACT: **For many decades the theory and practice of crosslinking (bonding that ties two or more large molecules together side to side) have been developed in industry, but only since the 1940's has the theory been considered in the field of medicine as a primary reaction underlying age-dependent changes.**

Crosslinking is damaging to the tissues and involves loss of elasticity, reduced swelling capacity, increased resistance to hydrolases and probably enzymes generally, and thus an increase in molecular weight and a tendency toward embrittlement. There is a growing amount of direct evidence and much indirect evidence for postulating the relationship between crosslinking and aging.

Crosslinking agents present in the living organism include aldehydes, lipid oxidation products, sulfur, alkylating agents, quinones, free radicals induced by ionizing radiation, antibodies, polybasic acids, polyhalo derivatives and polyvalent metals. The latter four types of compound are slow-acting but can also accumulate in the body to form a frozen metabolic pool. Sufficient amounts of all these potential crosslinking materials are present in the body to make the changes of aging unavoidable.

BACKGROUND

In 1887, Eisig observed in marine worms accumulations of colored particles, apparently "clinkers" of metabolism which had been accumulating within the organism. Thirteen years later, Mühlman (1900, 1901) advanced the hypothesis that "clinkers of metabolism"—metabolic ashes that cannot be combusted or excreted—may accumulate within the body and cause the visible and palpable changes so characteristic of aging.

Evidence of agglutination of colloids was presented by Ruzicka (1924) and ascribed by him to the effects of fluctuations of pH with age. Such a hysteresis theory was embraced by Bancroft and co-workers (1934), who championed the use of hydration-promoting ions such as sulfocyanates, as retardants for aging. Ruzicka's assumption that the hysteresis he observed was due to pH effects has not been confirmed, and Bancroft's work, although showing some measure of promise, was not carried forward after his death in 1934.

Meanwhile, intense industrial work had been proceeding for many decades in developing the theory and practice of crosslinking, that is, the causing, by any means, of bonds that tie together two or more large molecules side to side. Charles Goodyear's (1853) discovery that natural rubber could be crosslinked

* *Address:* Bjorksten Research Foundation, P.O. Box 265, Madison, Wisconsin 53701.

("vulcanized") by means of sulfur had provided the means of stabilizing the desired properties of rubber; this is the basis for the modern rubber industry. Like most important practical discoveries, this led to a vast body of both theoretical and practical studies on crosslinkages in the polymer industries; hundreds of papers related to this subject still appear every year.

In the tanning industry, it was observed that digestibility of proteins by enzymes decreases as crosslinkage proceeds (Thomas and Seymour-Jones, 1924; Gustavson, 1942; Lipsitz, Kremen and Lollar 1949). In polymer chemistry, Staudinger (1937) showed that a single crosslinkage between chains of 30,000 monomer units results in insolubility, thus establishing the great sensitivity of a key property of large molecules to crosslinkage; 0.003 per cent of the crosslinking agent divinylbenzene added to polymerizing styrene, would turn the benzene-soluble product into a gel of highly developed structural restraint.

In the photographic industry, the success of the film depends upon the use of crosslinkage to achieve and control a delicate balance between hydrophilic properties and temperature-humidity resistance. Likewise, in lithographic printing the crosslinking of gelatin has been developed to a high state of control. In hectograph duplication, not only mechanical resistance but also the speed of dye diffusion is controlled by crosslinkage, with a high degree of precision.

The field of sutures for surgical use likewise benefited from extensive research in crosslinking, for crosslinkage of macromolecules provided controllable delayed solubility of the sutures, and their resorption within a time interval consonant with the healing processes.

Looking at this background from the standpoint of 1940, the medical field then had only a hazy concept of loss of colloidal properties in proteins. On the other hand, the industrial fields had already at that time achieved highly developed practical and theoretical knowledge of the effects of crosslinking agents, both in high and very low concentrations, on a wide range of polymers.

It was inevitable that these two fields of knowledge should be brought together as soon as anyone chanced to become concerned in depth with both, simultaneously. It was my fate to be that "anyone." As Chief Chemist of the world's largest manufacturer of hectograph films, I was principally occupied with the problems of improving the hydrothermal properties of protein gels, by means of controlled low-level crosslinkage. It is a monument to myopia that it took four years to realize the connection; however once this was done, previously disconnected facts fitted together with precision and rapidity.

DEFINITION OF THE CROSSLINKAGE THEORY OF AGING

Inherent in the crosslinkage theory is the postulate that crosslinkage is a primary reaction underlying age-dependent changes. The numerous crosslinking agents known to be normally available in the organism will, by random uncontrolled action, slowly immobilize the large molecules in all cells and

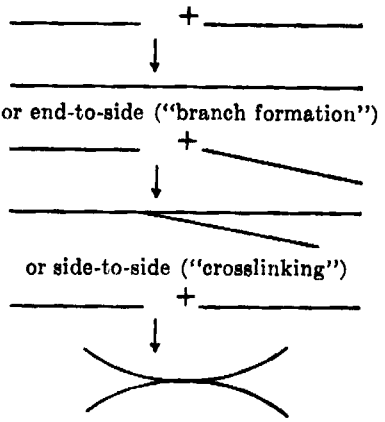
tissues by crosslinkages. Most of these will be removed in the metabolic processes, but such renewal is not 100 per cent efficient (Bjorksten, 1942). Reversal by metabolic processes of crosslinked molecules is unlikely in view of enzyme specificity, but by mathematically predictable chance (Carpenter, 1965; Carpenter, Carpenter and Loynd, 1967), crosslinks will once in a while take place in sufficient number or in critical positions where they block the catabolic reactions; thus the molecules so crosslinked will be withdrawn from metabolism, forming a frozen metabolic pool.

Crosslinking is most readily effected by means of a small molecule capable of reacting simultaneously with two macromolecules, but can also result by direct reaction between these macromolecules, whether this be by co-valent bond, hydrogen bond, hydrophobe bond, van der Waal forces, or in some other fashion.

Cells which are not renewed by division, such as the nerve and large muscle cells, are particularly susceptible to these types of action because the catabolic turnover inherent in cell division is greatly reduced in these cells.

DNA molecules are particularly sensitive to crosslinkage because any crosslinkage, even one which could be reversed given enough time for repair, is fatal if present in that form during mitosis. When two DNA molecules are lined up side by side, they form a perfect target for any molecule that happens to drift along and is capable of reacting at the same time with both molecules so as to link them together. If they are thus crosslinked when the anaphase begins, the cell will die, or possibly in some rarer cases a DNA molecule will rupture and a mutation will result. Thus, for molecules involved in cell division, a time factor is superimposed which renders them still more sensitive to crosslinkage than are molecules involved in functions where the time limit for restoration is less inexorable.

Interactions between macromolecules can occur end-to-end



In random interaction between biologically important macromolecules, crosslinkage is overwhelmingly predominant because of the relative improbability of "end-to-end" or "end-to-side" reactions as compared to "crosslinking" (or side-to-side) reactions in macromolecules with large numbers of reactive

groups distributed all along the chain. For practical purposes, very nearly all the random interactions between macromolecules which lead to an additive increase of molecular weight will therefore be crosslinkages.

These postulates were first arrived at by elimination. Of all known reactions involving proteins or nucleic acids, none even comes near the crosslinking reactions in sensitivity to small quantities of causative reactants. Although a functional macromolecule could be damaged by a single monovalent molecule, this would require a precise hit and would therefore be improbable in random reactions. Crosslinkage is damaging regardless of which of the large number of receptive groups of the protein or nucleic acid molecules happens to be involved, and regardless of how the crosslinkage has been effected.

To illustrate—an elephant might be killed by a rifle shot fired at random in the jungle, but the chances are against it. Conversely, if two elephants were chained together, they probably would not survive in the jungle and it would not matter at which points they were bonded together and of what materials the links were made, so long as they were strong enough.

Sometimes the crosslinkage theory has been connected with the clinker theory or the hysteresis theory. They have little in common. The crosslinked molecules are not clinkers, but important functional molecules which are immobilized by crosslinking accidents. Such accidents are not an inherent part of a normal metabolic process, comparable to clinkers resulting from combustion.

Hysteresis is a very general term, devoid of the specificity required of a theory, or an explanation.

EVIDENCE OF CROSSLINKAGE IN AGING

Certain changes which occur in aging have been thoroughly established in polymer chemistry as common to all crosslinkage processes. These are: loss of elasticity, reduced swelling capacity with consequent reduced ability to retain bound water, increased resistance to hydrolases and probably to enzyme action, which can lead generally to an increase in molecular weight and a tendency toward embrittlement.

This generic similarity, coupled with the fact that crosslinkage is the only process known to cause these changes with an extreme variety of different large molecules is already a good reason for the consideration of crosslinkage as the principal reaction initiating the chain of events which leads to human aging. It is also known that the content of many polyvalent metals—known crosslinkers—increases progressively with age (Schroeder, 1960) particularly in the circulatory system (Zinsser, Butt and Leonard, 1957; Zinsser, Bjorksten, Bruck et al., 1962; Schroeder, 1960), and that the insoluble fractions of proteins increase with age (Medvedeva, 1939).

In the years between the first presentation of the crosslinkage concept (Bjorksten 1941, 1942) and the present time, much additional evidence has been presented which supports the theory. King (1946) showed that the chain lengths of protein molecules (elastin) in human aorta are reduced on the average by a factor of nearly two-thirds during the interval from 20 to 80

years, and suggested that this is due to bonds becoming established between chains (crosslinkages). X-ray diffraction findings on human aorta show indications of crosslinking spacings characteristic of metal oxide bridges (Zinsser, Butt and Leonard, 1957). Accumulation of mucoprotein takes place in tissues with aging (Sulkin, 1955). Ionizing radiation causes symptoms resembling those of natural aging (Upton, 1957). Ionizing radiation is a powerful crosslinking influence, industrially widely used to increase temperature resistance or reduce the solubility of polymers (Charlesby, 1953). The same is true broadly of radio-mimetic agents, which are crosslinking agents (Alexander et al., 1952). Ionizing radiation crosslinks DNA (Lett and Alexander, 1961). LaBella and Lindsay (1963) have shown that aortic elastin undergoes a continuous in vivo crosslinkage with age.

The protozoan, Tokophrya infusionum, has provided unusual opportunities for studying phenomena associated with aging in a single-cell organism, because the cell retains its identity throughout reproduction and survives for from several days to two or three weeks (Rudzinska, 1951).

In Tokophrya, precipitation of organized protein is visible in the electron microscope as these organisms age, and overfeeding which reduces the life span will increase the visible accumulation commensurately (Rudzinska, 1955, 1956, 1961). By treatment with crosslinking agents, arterial walls can be made absorbent for cholesterol or any other lipids in vitro (Bjorksten, 1952; Bjorksten and Gottlieb, 1954). In vivo experiments have been reported showing local degenerative effects caused by injection of crosslinkers occurring in normal metabolism (Milch, Jude and Knaack, 1963). Kincaid, Baker and Milch (1965) have shown that radioactive glyceraldehyde, a potent crosslinking agent of both metabolic and dietary origin, can be selectively bound by the aortic wall.

Crosslinkages generally increase the oleophilicity of macromolecules, because the crosslinking agents generally act on hydrophilic radicals, thereby causing the macromolecules to be attached to each other in a position which ties together the hydrophilic areas and maximizes the exposure of the oleophilic areas. Thus the affinity to cholesterol and all oleaginous matter increases with progressive crosslinkage.

The molecular weight of collagen increases with aging (Boucek et al., 1958) and reduction in swellability with aging has been observed in several collagenous tissues (Kohn, 1959; Kohn and Rollerson, 1958; Banfield, 1956; Brown and Consden, 1958). Reduction in extractability of collagen is also apparent in aging (Bakerman, 1962).

An increase in the shrinkage temperature of collagen with age has been observed in collagen from humans (Brown and Consden, 1958), rabbits (Verzár and Huber, 1958), and rats (Bjorksten et al., 1960). That shrinkage temperature is related to crosslinkage is well established (Gustavson, 1956).

The time-viscosity curve in a crosslinkage reaction has been shown to match closely the time-mortality curve for humans (Bjorksten and Andrews, 1960).

Solubility in anhydrous hydrogen fluoride is characteristic for non-crosslinked proteins and nucleic acids. With progressing age, the amount of hydrogen fluoride-insoluble material increases, both in heart and tendons (Bjorksten, Andrews and Prahl, 1962). All the proteins and nucleic acids tested readily dissolved in this reagent in their noncrosslinked state; none did so after treatment with a potent crosslinker such as formaldehyde or benzoquinone.

The process of increasing crosslinkage has been directly observed in extracellular material such as collagen (Verzár 1963) and in gross DNA extracts from old as against young bovine thymus (von Hahn, 1963, a and b). Von Hahn (1963 a) found evidence that age-dependent crosslinkages in bovine thymus involve the nucleoprotein as an entity including histones rather than DNA specifically. Alexander, Swarcbort and Stacey (1959) showed the feasibility of co-valent bridges between DNA strands. Given enough time for random action, every possible reaction will take place.

This applies whether crosslinkages between nucleic macromolecules are direct or are between histones which in turn are bonded to the nucleic acids, as made plausible by von Hahn (1963) and von Hahn and Fritz (1966). The latter type of crosslinkages, however, would be more easily repaired by the mechanism indicated by Bjorksten (1942) when time between mitoses suffices. Thus the histones could mitigate, though never fully cancel, the effect of crosslinking on genetic material. Even though the histones act as shields for DNA-absorbing molecules of migrant crosslinkers, a 100% effective shielding would not permit the DNA molecules to perform their functions.

The indirect evidence implicating crosslinking in aging is also significant. The extreme ease with which radical changes in macromolecules are caused by addition of even very small additions of nonspecific crosslinking agents compels the conclusion that it would be impossible for such crosslinkage not to take place in vivo. Such crosslinkage, random and uncontrollable, is bound to interfere progressively with most, if not all, biochemical systems of the complex living organism.

As crosslinkage of proteins proceeds, there is a change toward oleophilic and hydrophobic behaviour. This is because the groups participating in the formation of co-valent crosslinkages are preponderantly polar hydrophilic groups (amino, hydroxyl, phosphate, carboxyl); as these become locked together with similar groups in another macromolecule the tendency is for the hydrophilic portions of these molecules to become oriented toward each other, so that relatively hydrophobic areas are turned "outward" and exposed to the ambient medium.

OBJECTIONS TO THE CROSSLINKAGE THEORY

Against this extensive and steadily mounting evidence in favor of crosslinkage being a principal reaction causative of aging, only two objections have been raised. These are:

1. "Bjorksten has used his theory of crosslinking to explain the aging of all

proteins which then become insoluble. This—he says—may disturb metabolism and cause aging. In contrast to this view, it is shown that only nonrenewed substances age. Most cells being continually renewed are not aging" (Verzár, 1964 a, p. 918).

2. Both Verzár and Sinex state that it has not been proved that a sufficient number of crosslinking agents are present in a normal organism to account for the phenomena which occur in aging (Verzár, 1964, p. 918; Sinex, 1964, p. 173).

The first of these objections was anticipated and answered as follows: "In the living organism this tanning (= crosslinkage) is counteracted by the continued state of flux in the protein molecules which are continually split and re-synthesized. In this interplay of synthetic and splitting reactions, the protein molecules are broken down before tanning has gone very far and re-synthesized in their non-tanned state" (Bjorksten, 1942). This mechanism of regeneration by continued breakdown and re-synthesis is inherently carried on most rapidly in cells of high metabolic rate. This explains why such cells are much less susceptible to aging than the the non-dividing cells.

In addition, crosslinkage of DNA leads to death or mutation of the cell in the next mitosis, thus eliminating its candidacy for aging.

The second question is crucial. It is obvious to all that crosslinked large molecules cannot function normally and that crosslinkage on a large scale unavoidably must cause a broad deterioration in all organs, given enough time. It is equally obvious that if crosslinking influences or agents are not present in a quantity sufficient to cause appreciable crosslinkage in vivo, then the crosslinkage theory must be abandoned.

A really comprehensive survey of all reports of crosslinking mechanisms effective in vivo would expand this presentation far beyond allowable limits. However, even an incomplete and summary presentation of pertinent findings should suffice to establish that crosslinking agents and influences are abundantly present in living organisms.

PRESENCE OF CROSSLINKING AGENTS IN THE LIVING ORGANISM

We are dealing with two groups of crosslinking agents: those which crosslink slowly over periods of years, and those which react instantaneously with proteins or nucleic acids. These latter agents can never accumulate in the tissues because they react with the macromolecules present almost as rapidly as they are formed. However, the formation of many of these crosslinking agents as metabolites is fully established, as will be detailed below.

The slow-acting crosslinking agents can and do accumulate. These include most compounds of polyvalent metals such as aluminum, lead and cadmium, polycarboxylic acids such as citric acid and its esters (used in industry to crosslink soluble nylon-type compounds) or indeed any polymers which have amino groups outside of the carbon "backbone" chain. This includes all proteins and nucleic acids. It is probable that silicon compounds also act as slow crosslinkers.

Data from the literature on the occurrence of some of these compounds in the body have been previously tabulated (Bjorksten 1958). No attempt has been made to update this list, since the data available in 1958 sufficed to show the constant presence in the body of substantial quantities of crosslinking agents of this type. However, it may be added that the presence of lead in blood at a concentration of 0.05 mg per 100 ml is accepted as normal (Gradwohl, 1963), that the amount of aluminum normally present in a 70-kg man is 100 mg, and that the normal dietary intake of man approximates 36.4 mg (Hawk, 1965). Lead, titanium and aluminum are reported in the above references as increasing steadily with age. Detailed data on aluminum are reviewed by Sviridov (1966).

Like aluminum, silicon is present in all higher plants, so continuous intake is unavoidable. It is found in all tissues in relatively high concentration from 18 to 130 mcg/gr wet weight. Mammalian blood contains about 0.5 mg/100 ml (Hawk, 1965).

The significance of the metal ions as crosslinkers is enhanced by the work of Tyler (1953, 1965), who has shown that the life span of spermatozoa of widely divergent species can be drastically increased by removal of polyvalent metal ions by means of chelation.

Aldehydes

The most rapid crosslinker (though not the one giving the most stable bonds) is formaldehyde (Theis and Ottens, 1940). Even the primary amide groups participate in this reaction (Fraenkel-Conrat et al., 1945). A small amount of formaldehyde added to a large amount of protein or nucleic acid reacts almost completely within seconds. Therefore, it would hardly be expected that free formaldehyde would be found in the organism. Yet, it *is* formed in vivo, at least in the following reactions:

1. Dimethyl glycine + H_2O → Sarcosine + formaldehyde.
2. Sarcosine + FAD → glycine + formaldehyde + $FADH_2$ (MacKenzie and Hoskins, 1962).
3. N-methyl-1-amino acids + O_2 → 1-amino acids + formaldehyde.
4. Erythrulose-1-phosphate → dehydroxy acetone phosphate + formaldehyde (Charalampous, 1962; Peanasky and Lardy, 1962).
5. D-ribose-5-phosphate → erythrulose-1-PO_4 + formaldehyde.
6. Alpha keto gamma hydroxybutyrate → pyruvate + formaldehyde.
7. Serine aldolase → glycine + formaldehyde.
8. Deoxycytidylate + formaldehyde → 5-hydroxy methyldeoxycytidylate (Pizer and Cohen, 1962).

Since no biological reaction is absolutely quantitative, it is a sound assumption that at least some of the formaldehyde thus formed will crosslink whichever amine or hydroxy-containing macromolecules happen to be in the path of a diffusing formaldehyde molecule. Over a lifetime, resultant crosslinkage resulting from even a very small diversion of formaldehyde would be appreciable. Certainly enough formaldehyde is formed from the foregoing

reactions to account for more crosslinkages than all those induced by free radicals from "fallout" or other incident ionizing radiation.

Whereas formaldehyde is so extremely reactive that it is not easy to determine when formed, and thus could not accumulate in the body, acetaldehyde is somewhat less reactive and has been found in the blood stream (Ri, 1940; Stotz, 1943). In their bibliography of the formation of acetaldehyde in metabolism, Pansini, Fersini and Ripa (1960) quote 149 references, of which many show occurrence of acetaldehyde in vivo.

Substantially equal in rapidity in crosslinking proteins and nucleic acids are the other aldehydes which are water-soluble monomeric C_1 to C_3 aliphatic mono-aldehydes and C_2 to C_5 di-aldehydes; these have an electrophilic substitution on the alpha C atom, and an unsubstituted terminal hydrogen atom (Gustavson, 1949; Milch, 1964). These include several metabolically active aldehydes such as glyoxal, glyceraldehyde, and pyruvaldehyde, in addition to those already mentioned. Lipid oxidation is another source of crosslinking agents, the most active of which are aldehydes, but which also include peroxides and epoxides.

Lipid oxidation products

This important group of crosslinking agents comprises the tanning (= crosslinking) substances formed by oxidation of unsaturated fats. They have been most thoroughly investigated in connection with chamois tanning. Gustavson (1956 b, p. 305) ascribes the crosslinking effects both to aldehydes, of which acrolein is the most conspicuous, and to peroxides; the aldehydes react more rapidly with the basic groups of collagen to impart hydrothermal stability, by crosslinking, and the peroxides interact with the non-ionic protein groups, probably the numerous peptide bonds.

The crosslinking efficacy of the oxidation products of unsaturated oils has been demonstrated in a model system designed as an analogue to skin. Bjorksten and Collbring (1964) exposed a suspension of 0.17% unsaturated oils in a gelatin film to ultraviolet radiation from a mercury lamp, or to direct sunlight, and found a resultant crosslinking that steadily progressed for weeks subsequent to short exposures to ultraviolet rays. Thus crosslinkages were effected by relatively stable products formed at the time of irradiation and not by short-lived free radicals nor by radiation directly.

The rate of increase in melting point of the carrier protein gel from 53°C to more than 100°C within a month matches that obtained with aldehydes. Tappel (1955, 1962, 1964) emphasizes peroxides; Witting (1965) postulates that in vitro lipid autoxidation and in vivo lipid peroxidation are kinetically comparable regardless of the anti-oxidants present. Gustavson (1956, p. 305) believes aldehydes to be the primary, and peroxides the secondary contributor to crosslinkage by fat oxidation products. Be this as it may, it is now established that as little as 0.17 per cent of unsaturated lipid on brief irradiation will generate crosslinking agents amply sufficient to crosslink a protein dramatically in three weeks in the dark. Both the irradiation and the presence

of unsaturated fat and oxygen are necessary for the process. This source of crosslinking agents also would alone suffice to cause the changes observed with age in body proteins and nucleic acids.

In the living cells, although the quantity of crosslinking agents formed from unsaturated fatty acids is substantial, the site of their release in the cell membranes is also important. The formation of crosslinking agents from unsaturated fatty acids is significant far beyond the quantities formed, even though these are substantial, because these crosslinking agents are largely released in cell membranes. A large proportion of the unsaturated fatty acids in the cell are part of the phospholipid molecules in the lipoproteins contained in cell membranes, where phospholipid molecules are aligned adjacent to protein and nucleic acid molecules. The membranes contain all the intracellular enzymes as well as the nucleic acids. The enzymes, their substrates and interacting large molecules generally are positioned in closest proximity to each other as well as to the oxidizable unsaturated components of the phospholipids. The crosslinking agents released from these on oxidation will react immediately with the nearest receptive groups. The probability is very great, then, that the resultant crosslinkage will involve and inactivate critically important proteins including enzymes, and nucleic acids. Crosslinkage between two strands in a nucleic acid is a particularly serious injury because it simultaneously involves the corresponding sites of adjacent strands. Such an injury could not be repaired by the mechanism postulated by Howard-Flanders and Boyce (1966).

Sulfur

Oeriu has stressed the significance of disulfide crosslinkages in senescence. Many of these may be readily manipulated by body enzymes, but the accumulation of such bonds with aging indicates that under certain conditions this form of crosslinkage can also be irreversible under in vivo conditions (Oeriu, 1964).

These linkages may result by direct action of the proteins in question involving cystein or methionin, or they may result from the crosslinking action of sulfides. Crosslinkages from addition to double bonds of elemental sulfur, or reaction with carbon disulfide, are the subject of a vast literature, particularly in elastomer technology.

Alkylating agents

The chief reason why radiomimetic agents so often are crosslinkers of the alkylating type is that the more rapidly-acting crosslinkers (active aldehydes, quinones, heavy metals) react and are trapped before they have the time to penetrate into the cells. The alkylating agents are generally sufficiently slow-acting to be able to penetrate the cell membranes, yet sufficiently fast-acting to cause quickly apparent results. Bifunctional alkylating agents which can be expected to occur in normal metabolic reactions include di-esters of co-enzyme A (e.g., succinyl, citryl, malonyl), diacetyl glutamate, and pos-

sibly some polymers of acetyl glutamate, acyl adenylates, and peptides with more than one mono-methyl substituted amino group. Alexander and Connell (1962) report that bifunctional mutagens (crosslinkers) shorten the life span of mice much more than do monofunctional mutagens, and that this is unrelated to the mutagenic effect.

Quinoid crosslinkers

The excellent hydrothermal stability of quinone-tanned pelt was stressed by Wilson (1929) and by Theis and Blum (1942). On this basis and on the basis of experience with gelatin gels, Bjorksten (1951, p. 344) stated, with a view to crosslinking in aging: "Quinoid structures are particularly intriguing since their crosslinkages are more stable than those caused by aldehydes, and since structures of this type may also be formed in biochemical reactions." Gustavson (1956 b, pp. 283–294) has thoroughly discussed the crosslinkage reactions and efficacy of quinones.

Puett, Ciferri and Rajagh (1965) and also Ciferri and Rajagh (1964) have shown that quinone crosslinkages even exceed chrome tannage linkages in stability.

Ubiquinone, or di-methoxy methyl polyisoprenoyl benzoquinone is present in every living cell as an indispensable link in the oxidation chain. We do not know how this substance is synthesized in vivo, but it seems probable that crosslinking quinones are intermediates, or possible by-products. Since the quinone crosslinkages are irreversible, even the slightest trace of a quinoid crosslinker could have permanent effects.

By oxidation of the isoprene chain of ubiquinone, quinonealdehydes could be formed.

The quinone methides are even more powerful crosslinking agents than the extremely effective benzoquinones. They are intermediates in reactions previously thought to be free radical reactions.

That catechol derivatives are oxidized to orthoquinones by peroxidase was suggested by Szent-Györgyi (1925). Ball and Chen (1933) presented strong evidence that adrenaline is biologically oxidized to an orthoquinone. This was confirmed by Green and Richter (1937), who prepared this adenochrome in crystalline form. Orthoquinones are exceedingly powerful crosslinkers, leading to the formation of permanent bonds.

As an instance of reactions of a quinone to crosslink DNA molecules, mention is made of the excellent study of mitomycins as crosslinkers for DNA by Szybalski and Iyer (1964).

Uyama, Ogino and co-workers (1953–56) suggest that senile cataract can be caused by quinone. Quinone imine carboxylic acid, the oxidation product of 5-oxyanthranilic acid, which can be isolated from the urine of senile cataract patients, causes cataract in Vitamin C-deficient guinea pigs. The 5-oxyanthranilic acid is a metabolic product of tryptophan.

Integrally formed crosslinkages

In addition, we have to consider the crosslinkages which are formed as integral parts of the reacting molecules without any external reactants. Piez

(1966) has shown that specific lysine residues can be converted to aldehydes, which react to crosslink collagen.

Evolution selected the path of quinone crosslinking for the purpose of creating the extremely tough and resistant exoskeletons of insects. Phenols, e.g., protocatechuic acid or 3-hydroxykynurenine, are oxidized to quinone imines, and these form crosslinkages by condensing with amino or aromatic hydroxyl groups in the 4, 5 positions. Tanning by o-quinones is widespread in hardening certain structures and organs among insects, worms, crustaceans and some structures of the lower vertebrates. The fundamental paper was published by Pryor in 1940; subsequent developments have been reviewed by Gustavson (1956 b, pp. 346–356).

LaBella and Paul (1965, p. 58) showed convincingly that progressive crosslinkage with aging takes place in collagen, in part by oxidation of constituent tyrosine residues to form reactive quinoid structures capable of binding adjacent functional groups co-valently. Quantitative study of fluorescence peaks demonstrated that only a portion of the quinones may be derived from the oxidation of tyrosine residues in the peptide chain. The balance, state LaBella and Paul, must be assumed to be deposited by the circulating body fluids (presumably having been formed by one or more of the mechanisms discussed above).

Free radicals induced by ionizing radiation

Free radicals have long been known to be powerful crosslinking agents (Charlesby, 1953), and free radical-generating catalysts such as peroxides are used routinely in the plastics industry to cause crosslinkage. A part of the crosslinkages in the body may be due to cosmic radiation, radio-potassium, and incidental contact with fallout, x-ray equipment or phosphorescent dials in watches or other instruments. These are special cases of crosslinkage.

However, all these taken together will not account for more than 5–10 per cent of the observed aging in test animals; also the attempts of Harman (1956, 1957) to use free radical-neutralizing chemicals to prolong the life of test animals have not led to major results, particularly considering that the AKR mice normally die earlier than other mice. Anti-oxidants as such are more effective, but this may be ascribed more plausibly to suppression of lipid oxidation.

Slow-acting crosslinking agents

Since we are dealing with very long time spans, certain slower-acting but abundantly available potentially active crosslinking agents cannot be disregarded. However, because of the slower activity, these remain largely unexplored as crosslinkers.

They include polybasic acids and their esters (Allen, 1949; Coffman, 1954). Citric acid, an abundantly available metabolite, has been used industrially to crosslink polyamide resins.

Silica could have similar effects, and any polyhalo derivative that is to any extent hydrolyzable is a potential slow crosslinker. Some condensed hydro-

carbon in which the electron distribution could give rise to strong hydrogen bonding could effect crosslinkages not breakable in vivo. Some of the aldehydes generally regarded as nonreactive with proteins might also contribute to crosslinking, when time is no limitation.

Antibodies

Walford, who pioneered the auto-immunity concept of aging, points out that antibodies may be ideal crosslinking agents; that antigen-antibody precipitates are quite insoluble and could well form a frozen metabolic pool; and that at least in the case of antibody to bovine serum albumin, the protein is significantly shielded from digestion by papain (Walford, 1964).

Metallic crosslinking agents

All polyvalent metals are potential crosslinkers. Living organisms are equipped to handle certain of these efficiently: iron, zinc, magnesium, manganese, cobalt, copper, and possibly chromium and calcium with some reservations. But aside from these, polyvalent metals are capable of accumulating with age, particularly in the circulatory system. Even the essential metals show some such tendency. This becomes particularly apparent when the percentage of each metal is calculated on the basis of organic matter, so as not to be overshadowed by the large increases in ash due to calcification.

Most detrimental are probably those metals which are present in traces small enough so that the individual can proliferate before being severely damaged, yet large enough to attain serious proportions over a lifetime. Among these particularly suspect metals are cadmium—which accumulates in arterial tissues (Zinsser, Bjorksten, Bruck et al., 1962)—aluminum, silicon, lead, tin and titanium. Schroeder (1960) states that all hypertensive agents not acting on nerves and thus presumably restoring elasticity, appear to be metal-binding agents. This may be viewed in context with Zinsser and coworkers' (1962, p. 482) demonstration of metal oxide crosslinkages in arterial tissue. Tyler's finding (1953, 1965) that the life span of spermatozoa from widely different species is extended by treatment with metal chelating agents has been previously discussed.

ARE QUANTITIES OF CROSSLINKING AGENTS AVAILABLE IN THE BODY?

This question is more easily answered for those crosslinking agents which do not react instantly and thus can be determined in body fluids. A summary of data available at the time (1963) indicated these to be present to the extent of 1.4 grams or 2.5 to 19.2 $\times$ 10^{19} molecules of crosslinking molecules in a human weighing 70 kilograms (Bjorksten, 1963 b, p. 182).

Considering that a single crosslinkage between two macromolecules composed each of 30,000 monomer units (amino acids in the case of proteins) is sufficient for radically changing their solubility and diffusion characteristics, and probably also their immunological behaviour, this alone should suffice to account for senescence. However, we have then not taken account at all of the

very large crosslinking potential of the fast-acting crosslinking metabolites—aldehydes, quinones and lipid oxidation products—which react with macromolecules as soon as they are formed. When these are also considered, we must ask not: "Is this sufficient to cause aging?" but rather: "How is it possible that aging proceeds as slowly as it does?"

APPLICATIONS OF THE CROSSLINKAGE CONCEPT

The crosslinkage theory meets the criteria recommended by Shock for screening aging theories (Shock, 1960; Bjorksten, 1962 a). Moreover, it provides simple explanations for several observations:

1. The life-shortening effect of overeating. With a low caloric diet, oxidation proceeds rapidly to the innocuous end-products, carbon dioxide and water. On overeating, intermediate products accumulate at the metabolic "bottlenecks," and many of these are crosslinking agents.
2. The life-shortening effect of ionizing radiation and the aging effect of ultraviolet irradiation of the skin. The free radicals are known crosslinking agents. That ultraviolet induces formation of tanning agents from unsaturated fatty acids present in all cell membranes is fully proved.
3. Crosslinkage changes the immunological behavior of proteins. This is a prelude to auto-immunity.
4. Crosslinkage causes changes of proteins from hydrophilic to oleophilic. This is a prelude to atherosclerosis. Another prelude is that crosslinkage destroys elasticity, thus causing microfractures in organs, including arterial endothelia.

COMMENT

It has been postulated (Sinex, 1957) that damage to macromolecules might be caused by thermal denaturation due to thermal irregularities on a micro scale. From a practical standpoint, this is answered by the fact that DNA and protein molecules tolerate without damage even a very long time in solution at normal temperatures, whereas they are inactivated or profoundly changed in hours or seconds by solutions of crosslinking agents demonstrably present in the body or formed in its normal processes.

It seems improbable that an irreversible phenomenon like aging could be initiated by the formation of denatured macromolecules which are readily hydrolyzed to form excretable fragments. This was brought out already by Anson and Mirsky (1934) and the literature of the years following is replete with papers reporting instances where denaturation of proteins resulted in enhanced protein digestibility, but none showing the reverse. This has been reviewed, for example, by Putnam (1953) and by Green and Neurath (1954). In contradistinction, crosslinkage will result in the formation of steric hindrances, which prevent breakdown or removal of the aggregate molecules, as previously discussed.

It is immaterial at what sites the crosslinkage takes place, or whether the linkage is a hydrogen bond, a hydrophobe bond, a van der Waal bond, a co-

valent bond or a combination of these, so long as the macromolecules are tied together irreversibly.

The expectation that chance thermal distribution might inactivate DNA is akin to the thinking of the legendary California farmer who sat on a fence waiting for an earthquake to come along and shake his potatoes out of the soil. Neither event is thermodynamically impossible, but both demonstrably unlikely. It seems far fetched indeed to seek the primary causative factor of senescense in so circuitous a reasoning when an effective demonstrable cause is before us in the crosslinking agents normally available in the human body in quantities sufficient to make the changes observed in aging not only understandable, but unavoidable.

(For Summary, see Abstract at beginning of article.)

Acknowledgment

The author expresses his appreciation of the courtesies extended by the libraries of The Upjohn Co. and the University of Wisconsin; of several suggestions made by Dr. David E. Green and Dr. K. H. Gustavson, and of a contribution of $500 from the Glenn Foundation for Medical Research.

REFERENCES

Alexander, P., and Connell, D. I.: The failure of the potent mutagenic chemical, ethyl methane sulphonate, to shorten the life-span of mice. UNESCO Symposium on Cellular Basis and Aetiology of Late Somatic Effect of Ionizing Radiations. New York, Academic Press, Inc., 1962.

Alexander, P.; Fox, M.; Stacey, K. A., and Smith, L. F.: The reactivity of radiomimetic compounds. I. Crosslinking of proteins. *Biochem. J. 52:* 177–184, 1952.

Alexander, P.; Swarcbort, A., and Stacey, K. A.: The reactivity of radiomimetic compounds. III. Crosslinking of nucleoprotein, *Biochem. & Pharmacol. 2:* 133–145, 1959.

Allen, S. J., and Napier, J. G.: Production of Basic Linear Polymers. Washington, D. C., U.S. Patent No. 2,483,513. Col. 9, Oct. 4, 1949.

Allen, S. J.; Napier, J. G., and Bryans, F.: Production of Basic Linear Polyamides Formed from Polyamines and Oxalic Esters. Washington, D. C., U.S. Patent No. 2,483,514. Col. 5, Oct. 4, 1949.

Andrews, F.; Bjorksten, J.; Trenk, F. B.; Henick, A. S., and Koch, R. B.: The reaction of an autoxidized lipid with proteins, *J. Am. Oil Chemists Soc. 42:* 779–781, 1965.

Anson, M. L., and Mirsky, A. E.: The equilibrium between native and denatured hemoglobin in salicylate solutions and the theoretical consequences of the equilibrium between native and denatured protein, *J. Gen. Physiol. 17:* 407, 1934.

Assael, M., et al.: Blood acetaldehyde levels in affective disorders, *Israel Ann. Psychiat. 2:* 28–34, 1964.

Atomic Bomb Casualty Commission: First Report, pp. 56–57.

Bakerman, S.: Quantitative extraction of acid-soluble human skin collagen with age, *Nature 196:* 375–376, 1962.

Ball, E. G., and Chen, T. T.: Studies on oxidation-reduction. XIX. Epinephrine and related compounds, *J. Biol. Chem. 102:* 691–718, 1933.

Bancroft, W. D.; Farnham, E. C., and Rutzler, J. E., Jr.: Preliminary tests with sodium rhodanate on rabbits and chickens, *Science 80:* 549, 1934.

Bancroft, W. D., and Rutzler, J. E., Jr.: Reversible coagulation on living tissue. XII, *Proc. Nat. Acad. Sc. 20:* 501–509, 1934.

Banfield, W. G.: age changes in the swelling capacity of the human Achilles tendon, *J. Gerontol. 11:* 372, 1956.

Bjorksten, J.: Recent developments in protein chemistry, *Chem. Industries 48:* 746–751, 1941.

Bjorksten, J.: Chemistry of duplication, *Chem. Industries 50:* 69, 1942.

Bjorksten, J., and Champion, W. J.: Mechanical influences upon tanning, *J. Am. Chem. Soc. 64:* 868–869, 1942.

Bjorksten, J.: Cross-linkages in protein chemistry, *in* Advances in Protein Chemistry, ed. by Anson, Edsall and Bailey. New York, Academic Press Inc., Vol. 6, p. 343, 1951.

Bjorksten, J.: A mechanism of cholesterol deposition on arterial walls, *Proc. Soc. Exper. Biol. & Med. 81:* 350–353, 1952.

Bjorksten, J., and Gottlieb, H.: Protein Structure and Aging—Crosslinking in Gelatin. I. Formaldehyde-Induced. Washington, D. C., Air Force Office of Scientific Research Tech. Report No. OSR-TN-54-304, November, 1954.

Bjorksten, J.: Cross-linking, key to aging? *Chem. Eng. News 33:* 1957, 1955.

Bjorksten, J., and Gottlieb, H.: A Study of Cross-Linkages in Gelatin. Washington, D. C., Air Force Office of Scientific Research Tech. Report No. 57-26, March, 1957.

Bjorksten, J.: A common molecular basis for the aging syndrome, *J. Am. Geriatrics Soc. 6:* 740–748, 1958.

Bjorksten, J., and Andrews, F.: Fundamentals of aging: a comparison of the mortality curve for humans with a viscosity curve of gelatin during the cross-linking reaction, *J. Am. Geriatrics Soc. 4:* 632–637, 1960.

Bjorksten, J.; Andrews, F.; Bailey, J., and Trenk, B.: Fundamentals of aging: immobilization of proteins in whole-body irradiated white rats. *J. Am. Geriatrics Soc. 8:* 37–47, 1960.

Bjorksten, J.: Aging: present status of our chemical knowledge, *J. Am. Geriatrics Soc. 10:* 125–139, 1962.

Bjorksten, J.; Andrews, F. A., and Prahl, H. F.: Anhydrous hydrogen fluoride as a tool in studying cross-linkages in proteinaceous substances accumulating with age, *Finska Kemists. Medd. 71:* 69–76, 1962.

Bjorksten, J., and Andrews, F.: Cross-linkages in the aging cycle. Sixth Internat. Congress Gerontology, Copenhagen, 1963.

Bjorksten, J.: Aging; primary mechanism, *Gerontologia 8:* 179–192, 1963.

Bjorksten, J., and Collbring, T.: A chemical model of ultraviolet radiation effects on skin collagen, *Proc. Sci. Section Toilet Goods A. 42:* 1–5, Dec. 1964.

Boucek, R. J.; Noble, N. L.; Kao, K., and Elden, H. R.: The effects of age, sex and race upon the acetic acid fractions of collagen, *J. Gerontol. 13:* 2–9, 1958.

Brown, P. C., and Consden, R.: Variation with age of shrinkage temperature of human collagen, *Nature* (London) *181:* 349–350, 1958.

Carpenter, D. G.: Diffuse theory of aging, *J. Gerontol. 20:* 191–195, 1965.

Carpenter, D. G.; Carpenter, J. A., and Loynd, J. A.: Computer solutions for diffusion theory equations on the accumulation of cross linked molecules, *Gerontologia 13:* 111–123, 1967.

Charalampous, F. C.: Phosphoketotetrose aldolase from rat liver, *in* Methods in Enzymology, ed. by Colowick and Kaplan. New York, Academic Press Inc., vol. 5, pp. 283–286, 1962.

Charlesby, A.: Effect of high energy radiation on some long chain polymers, *Plastics* (London) *18:* 70, 142–145, 1953.

Ciferri, A., and Rajagh, L. V.: The aging of connective tissue, *J. Gerontol. 19:* 220–224, 1964.

Coffman, D. D.: Cross-linked Polyamides by Reaction of Polymeric Amines with Oxalic Acid. Washington, D. C., U.S. Patent No. 2,682,524, June 29, 1954.

Curtis, H. J.: Biological mechanisms underlying the aging process, *Science 141:* 686–694, 1963.

Eisig, H.: Monographie der Capitelliden. Fauna et Flora des Golfes von Neapel, Vol. 16, 1887.

Failla, G.: Considerations bearing on permissible accumulated radiation doses for occupational exposure. The aging process and cancerogenesis, *Radiology 69:* 23–29, 1957.

Fraenkel-Conrat, H.; Cooper, M., and Olcott, H. S.: The reaction of formaldehyde with proteins, *J. Am. Chem. Soc. 67:* 950–954, 1945.

Freund, G., et al.: Acetaldehyde concentrations in alveolar air following a standard dose of ethanol in man, *J. Lipid Res. 6:* 471–477, 1965.

Goodyear, C.: Gum-Elastic and Its Varieties. New Haven, Conn., 2 Vols. (1853–55).

Gradwohl's Clinical Laboratory Methods and Diagnosis, ed. by Frankel and Reitman. St. Louis, Mo., Mosby Medical Books, 6th edition, p. 395, 1963.

Green, D. E., and Richter, D.: Adrenaline and adenochrome, *J. Biochem. 31:* 595–616, 1937.

Green, N. M., and Neurath, H.: Proteolytic enzymes, *in* The Proteins ed. by H. Neurath, and K. Bailey. New York, Academic Press, Inc., Vol. II B, Chapt. 25, pp. 1172–1173, 1954.

Gustavson, K. H.: Behaviour of collagen in combination with tanning agents towards trypsin, *Svensk. Kem. Tidskr. 54:* 251, 1942.

Gustavson, K. H.: The behaviour of some long-chained glycoldialdehydes towards collagen, *Svensk Kem. Tidskr. 61:* 114–123, 1949.

Gustavson, K. H.: The Chemistry and Reactivity of Collagen. New York, Academic Press Inc., p. 230, 1956.

Gustavson, K. H.: The Chemistry of Tanning Processes. New York, Academic Press Inc., 1956.

Hahn, H. P. von: Age-dependent thermal denaturation and viscosity of crude and purified desoxyribonucleic acid prepared from bovine thymus, *Gerontologia 8:* 123–131, 1963.

Hahn, H. P. von: The role of desoxyribonucleic acid (DNA) in the aging process, *Gerontologia 8:* 168–178, 1963.

Hahn, H. P. von, and Fritz, E.: Age-related alterations in the structure of DNA. III. Thermal stability of rat liver DNA, related to age, histone content and ionic strength, *Gerontologia 12:* 237–250, 1966.

Harman, D.: Aging: a theory based on free radical and radiation chemistry, *J. Gerontol. 11:* 298–300, 1956.

Harman, D.: Prolongation of the normal life span by radiation protection chemicals, *J. Gerontol. 12:* 257–263, 1957.

Hawk, P. B.: Physiological Chemistry. New York, Blakiston Div. McGraw Hill, 14th ed., pp. 541, 561, 1965.

Howard-Flanders, P., and Boyce, R. P.: DNA repair and genetic recombination: studies on mutants of E.coli defective in these processes, *Radiation Res.* Suppl. 6: 156–184, 1966.

Kincaid, D. T.; Baker, L. D., and Milch, R. A.: Tissue distribution and aortic binding of dl-glyceraldehyde-C^{14}, *J. Surg. Res. 5:* 120–122, 1965.

King, A. L.: Pressure-volume relation for cylindrical tubes with elastomeric walls: the human aorta, *J. Applied Physics 17:* 501–505, 1946.

Kohn, R. R.: Age and swelling in acid of perivascular connective tissue in human lung, *J. Gerontol. 14:* 16–19, 1959.

Kohn, R. R., and Rollerson, E.: Relationship of age to swelling properties of human diaphragm tendon in acid and alkaline solutions, *J. Gerontol. 13:* 241–248, 1958.

Kohn, R. R., and Rollerson, E.: Aging of human collagen in relation to susceptibility to the action of collagenase, *J. Gerontol. 15:* 10–15, 1960.

LaBella, F. S., and Lindsay, W. G.: The structure of human aortic elastin as influenced by age, *J. Gerontol. 18:* 111–118, 1963.

LaBella, F. S., and Paul, G.: Structure of collagen from human tendon as influenced by age and sex, *J. Gerontol. 20:* 54–59, 1965.

Lett, J. T., and Alexander, P.: Crosslinking and degradation of deoxyribonucleic acid gels with varying water contents when irradiated with electrons, *Radiation Res. 15:* 159–173, 1961.

Lipsitz, P.; Kremen, S. S., and Lollar, R. M.: Studies of vegetable tanning. VIII, *J. Am. Leather Chem. A. 44:* 371–387, 1949.

MacKenzie, C. G., and Hoskins, D. H.: Sarcosine dehydrogenase, *in* Methods in Enzymology, ed. by Colowick and Kaplan. New York, Academic Press Inc., vol. 5, pp. 738–742, 1962.

Medvedeva, N. B.: The variations in nitrogen compounds and proteins in the tissues of the aged, *J. Med. Ukraine 9:* 325–338, 1939.

Mertz, W.: Biological role of chromium, *Fed. Proc. 26:* 186–193, 1967.

Milch, R. A.; Jude, J. R., and Knaack, J.: Effects of collagen reactive aldehyde metabolites on the structure of the canine aortic wall and their possible role in atherogenesis, *Surgery 54:* 104–123, 1963.

Milch, R. A.: Reaction of certain aliphatic aldehydes with gelatin, *Gerontologia 10:* 117–136, 1964.

Mühlmann, M.: Über die Veränderungen der Nervenzellen in verschiedenem Alter, *Verhandl. d. deutsch. path. Gesellsch. 3:* 138–157, 1900.

Mühlmann, M.: Weitere Untersuchungen über die Veränderungen der Nervenzellen in verschiedenem Alter, *Arch. f. mikr. Anat. 28:* 231–246, 1901.

Northrop, J. H.: Biochemists, biologists, and William of Occam, *Ann. Rev. Biochem. 30:* (Preface) 1–10, 1961.

Oeriu, S.: Proteins in development and senescence, *in* Advances in Gerontological Research, ed. by B. Strehler. New York, Academic Press Inc., Vol. 1, pp. 23–85, 1964.

Pansini, R.; Fersini, C., and Ripa, R.: Nozioni e quesiti sul ruolo dell acetaldeide nel ricambio intermedio, *L'Arcispedale S. Anna di Ferrara 13:* 1147–1192, 1960.

Patterson, C. C.: Contaminated and natural lead environments of man, *Arch. Environ. Health 11:* 344–360, 1965.

Peanasky, R. J., and Lardy, H. A.: Bovine liver aldolase, *in* Methods in Enzymology, ed. Colowick and Kaplan. New York, Academic Press Inc., Vol. 5, pp. 310–317, 1962.

Piez, K. A.; Martin, G. A.; Kang, A. H., and Bornstein, P.: Heterogeneity of the α chains of rat skin collagen and its relation to the biosynthesis of cross-links, *Biochemistry 6:* 3813–3820, 1966.

Pizer, L. I., and Cohen, S. S.: Deoxycytidylate hydroxymethylase from virus-infected E. coli, *in* Methods in Enzymology, ed. by Colowick and Kaplan. New York, Academic Press Inc., Vol. 6, pp. 131–136, 1963.

Pryor, M. G. M.: Hardening of the oötheca of Blatta Orientalis, *Proc. Royal Soc. B. 128:* 378–393, 1940.

Pryor, M. G. M.: Hardening of the cuticle of insects, *Proc. Royal Soc. B. 128:* 393–407, 1940.

Puett, D.; Ciferri, A., and Rajagh, L. V.: Interactions between proteins and salt solutions. II. Elasticity of collagen tendons. *Biopolymers 3:* 439–459, 1965.

Putnam, F. W.: Protein denaturation, *in* The Proteins, ed. by H. Neurath, and K. Bailey. New York, Academic Press Inc., Vol. IB, Chapt. 9, pp. 825–826, 1953.

Ri, K.: Influence of various kinds of nutrition on the formation of acetaldehyde in liver, *J. Biochem.* (Japan) *31:* 215–241, 1940.

Rudzinska, M. A.: The influence of the amount of food on the reproduction rate and longevity of a suctorian (Tokophrya infusionum), *Science 113:* 10, 1951.

Rudzinska, M. A.: Observations on the fine structure of the macronucleus of Tokophrya infusionum, *J. Biophys. & Biochem. Cytol. 1:* 421, 1955.

Rudzinska, M. A.: Similarities between old and overfed organisms in Tokophrya infusionum. Libro de Resumenes, Primer Congreso Panamericano de Gerontologia, Ciudad Universitaria, Mexico, pp. 198–200, 1956.

Rudzinska, M. A.: The use of a protozoan for studies on aging. II. The macronucleus in young and old organisms of Tokophrya infusionum: light and electron microscope observations, *J. Gerontol. 16:* 326–334, 1961.

Ruzicka, V.: Beiträge zum Studium der Protoplasmahysteresis und der hysteretischen Vorgänge (zur Kausalität des Alterns). I. Die Protoplasma-Hysteresis als Entropieerscheinung, *Arch. mikr. Anat. 101:* 459–482, 1924.

Schroeder, H. A.: Possible relationship between trace metals and chronic diseases, *in* Metal Binding in Medicine, ed. by M. J. Seven. Philadelphia, J. B. Lippincott Co., pp. 59–67, 1960.

Shock, N. W.: Some of the facts of aging, *in* Aging—Some Social and Biological Aspects, ed. by N. W. Shock. Washington, D. C., Am. Assoc. Adv. Science, pp. 250–251, 1960.

Sinex, F. M.: Aging and the lability of irreplaceable molecules, *J. Gerontol. 12:* 190–198, 1957.

Sinex, F. M.: Cross-linkage and aging, *Adv. Gerontol. Res. 1:* 173, 1964.

Staudinger, H.: Zur Entwicklung der Chemie der Hochpolymeren. Berlin, Verlag Chemie p. 152, 1937.

Still, J. W.: Are organismal aging and aging death necessarily the result of death of vital cells in the organism? A cybernetic theory of aging, *Med. Ann. Distr. Columbia 25:* 119–203, 1956.

Stotz, E.: Photometric method for determination of acetaldehyde in blood, *J. Biol. Chem. 148:* 585, 1943.

Sulkin, N. H.: Histochemical studies on mucoproteins in nerve cells of the dog, *J. Biophys. & Biochem. Cytol. 1:* 439, 1955.

Sviridov, N. K.: Aluminum in human pathology, *Lab. Delo.* (Russ.) *12:* 699–702, 1966.

Szent-Györgyi, A. V.: Zellatmung. IV. Ueber den oxydations Mechanismus der Kartoffeln, *Biochem. Ztschr. 162:* 402, 1925.

Szybalski, W., and Iyer, V. N.: Cross-linking of DNA by enzymatically or chemically activated mitomycins and porfiromycins, bifunctionally "alkylating" antibiotics, *Fed. Proc. 23:* 946–957, 1964.

Tappel, A. L.: Studies of the mechanism of vitamin E action. III. In vitro copolymerization of oxidized fats with proteins, *Arch. Biochem. & Biophys. 54:* 266, 1955.

Tappel, A. L.: *in* Lipids and Their Oxidation, ed. by H. W. Schultz. Westport, Conn., Avi, p. 367, 1962.

Tappel, A. L.: Lipids linked to aging process, *Med. World News* pp. 115–116, Aug. 14, 1964.

Theis, E. R., and Ottens, E. F.: Studies in aldehyde tannage. III. The free amino groups of collagen before and after formaldehyde treatment, *J. Am. Leather Chem. A. 35:* 330–351, 1940.

Theis, E. R., and Blum, W. A.: Significance of shrink temperatures in vegetable tanning, *J. Am. Leather Chem. A. 37:* 553–577, 1942.

Thomas, A. W., and Seymour-Jones, F. L.: Action of trypsin upon diverse leathers, *Indust. Eng. Chem. 16:* 157, 1924.

Trnavská, Z.; Sit'aj, S.; Grmela, M., and Malinsky, J.: Certain intermediary metabolites and the formation of fibrils from collagen solutions, *Biochim. et biophys. Acta 126:* 373–381, 1966.

Tyler, A.: Prolongation of life span of sea-urchin spermatozoa, and improvement of the fertilization-reaction, by treatment of spermatozoa and eggs with metal-chelating agents (amino acids, versene, DEDTC, oxine, cupron, *Biol. Bull. 104:* 224–239, 1953.

Tyler, A.: Longevity of gametes; histocompatibility—Gene loss and neoplasia, *in* Aging and Levels of Biological Organization, ed. by A. M. Brues and G. A. Sacher. Chicago, Univ. of Chicago Press, Sect. II (Genetics and Environment) Part II, pp. 50–86, 1965.

Upton, A. C.: Ionizing radiation and the aging process, *J. Gerontol. 12:* 306–313, 1957.

Uyama, Y.; Ogino, S., and Ichihara T.: The cataractogenic agent in the urine of senile cataract patients, *Acta Soc. Ophthalmol.* (Japan) *58:* 1100–1108, 1954; and *Am. J. Ophthalmol. 39:* 125, 1953.

Uyama, Y., and Ogino, S.: The metabolism of crystalline lens. IV. *Med. J. Osaka Univ. 6:* 813–852, 1956.

Verzár, F.: The aging of collagen, *Scientific American 208:* 110–114, 1963.

Verzár, F.: Aging of connective tissue, *Gior. di Gerontol. 12:* 915–921, 1964.

Verzár, F.: Aging of the Collagen-fiber, *Internat. Rev. Connect. Tissue Res. 2:* 243–299, 1964.

Verzár, F., and Huber, K.: Thermic-contraction of single tendon fibres from animals of different age after treatment with formaldehyde, urethane, glycerol, acetic acid and other substances, *Gerontologia 2:* 81–103, 1958.

Walford, R. L.: Further considerations towards an immunologic theory of aging, *Exper. Gerontology 1:* 73, 1964.

Wilson, J. A.: Chemistry of Leather Manufacture. New York, Chem. Catalogue Co. a: pp. 740–741, 1929.

Witting, L. A.: Lipid peroxidation in vivo, *J. Am. Oil Chemists Soc. 42:* 908, 1965.

Zinsser, H. H.; Butt, E. M., and Leonard, J.: Metal content correlation in aging aorta, *J. Am. Geriatrics Soc. 5:* 20–26, 1957.

Zinsser, H.; Bjorksten, J.; Bruck, E. M.; Baker, R. F.; Kaeburn, L.; Kinnear, J.; Cohen, A.; Andrews, F.; Sarfati, I., and Light, I.: The freezing pool: a unified sequence of the aging process. *in* Medical and Clinical Aspects of Aging, ed. by H. Blumenthal. From Proc. 5th Congress of the International Association of Gerontology, 1962. New York and London, Columbia Univ. Press, pp. 460–483, 1962.