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THE CROSSLINKING THEORY OF AGING — ADDED EVIDENCE

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Abstract — The crosslinking theory of aging has been gaining acceptance at a steady pace, as evidenced by many independent rediscoveries. While several earlier studies were indicative, none seemed conclusive until it was shown, using Differential Scanning Calorimetry (DSC), that protein from young human brains could be made to closely resemble protein from old brains by exposing it to either of two entirely different crosslinking agents (glutaraldehyde and dipotassium diperoxy sulfate). This work has now been repeated with additional brain material, and a statistically more significant number of determinations. It is now shown that a treatment of brain protein with either one or two chemically totally different compounds which have no property in common except that both are crosslinkers, changes young brain protein so that it greatly resembles old, crosslinked protein. This shows that crosslinking reactions are involved in the age related changes in the studied proteins.

Key Words: crosslinking, aging, differential scanning calorimetry

INTRODUCTION

ACCORDING TO the crosslinking theory, aging is caused by the progressive linking together of large vital molecules. When many such molecules in a cell get linked together into huge tangles, intracellular transport is impeded. This causes life processes to go slower, and reduces the safety margins of vital functions.

The crosslinking theory was first expressed by Bjorksten (1942) as follows:

> The aging of living organisms I believe is due to the occasional formation, by tanning, of bridges between protein molecules, which cannot be broken by the cell enzymes. Such irreparable tanning may be caused by tanning agents foreign to the organism, or formed by unusual biological side reactions, or it may be due to the formation of a tanning bridge in some particular position in the protein molecules. In either event, the result is that cumulative tanning of body proteins, which we know as old age.

Tanning is an industrial term for *crosslinking*.

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This theory has been rediscovered many times (King, 1946; Verzár, 1955; Tanzer, 1973). Several other theories of aging have used the concept of crosslinking as an integral part of their proposed aging mechanism (Harman, 1956, 1984; Still, 1956; Carpenter, 1965).

Several studies (King, 1946; Verzár, 1955; Bjorksten and Andrews, 1960; Kohn and Rollerson, 1960; Zinsser, *et al.*, 1962; Bjorksten and Andrews, 1964; Bellows and Bellows, 1976; Zs.-Nagy and Nagy, 1980) have provided strong indications that the crosslinking theory is correct. Yet solid proof wasn't brought forth until 1984 (Bjorksten *et al.*, 1984). Tenhu and Sundholm (1986) added confirmatory findings and broadened the statistical base. The results obtained in 1984 are expanded upon in the work that follows.

MATERIALS AND METHODS

A good method of measuring crosslinkage is through the recording microcalorimeter, which measures the amounts of nonfreezing water in a macromolecule. This depends on the density of the network formed by macromolecules in an aggregate, which in turn depends on the crosslinkage density. In an aqueous protein sample there is a considerable amount of water which does not freeze even at very low temperatures. Proteins derived from linear chains with maximal exposure to ambient water permit an entry of water to structural layers, where they are constricted. This allows ice crystals to form to a much lesser extent than they could in crosslinked proteins, which are much more dense and rigid, and don't allow entry of water into structural layers to as great an extent (Privalov, 1980). Microcalorimetry measures crosslinkages indirectly. Other new tools are, however, much less direct. For example, measuring nuclear magnetic resonance is fine for work with pendant groups, but less applicable in measuring crosslinkages. This is why we chose the microcalorimeter as the best tool for our purposes, in spite of its high demands on time and meticulous care.

Our purpose was to verify that the reason why protein samples from old human brains held less nonfreezing water than samples from young brains (Bjorksten *et al.*, 1984) was because the protein in the old brains was more crosslinked. In this work raised temperatures and destructive chemicals were avoided, while the bulk of fats and phospholipids were removed.

Two autopsy samples (frontal lobe) in the ages of 17 and 27 years were extracted with DMSO. Frozen tissue was finely cut and extracted several times (until no foaming took place on shaking, and yet twice for additional safety's sake) with dimethylsulfoxide at room temperature. The samples were washed with water and dried in vacuum at 50° centigrade.

It is very important that the washing with DMSO be done thoroughly. Any trace of phospholipids still remaining after the DMSO wash would cause emulsification and serious disturbances in the water wash.

Thorough DMSO extraction effectively removed most carbohydrates and other water solubles. The remaining material contained nitrogen and amino acid quantities well within the range that would be expected for protein, as determined by the Kjeldahl and van Slyke tests. A typical elemental analysis for our isolated protein gave 58% carbon, 8.5% hydrogen, 9.6% nitrogen, 18% oxygen, and 5.8% sulfur.

Both samples were treated in an identical manner, except that different crosslinking agents were used. The isolated material was crosslinked with glutaric aldehyde and in another parallel experiment with potassium sulfate.

1. One hundred milligrams of the protein was swelled in 0.1 M phosphate buffer solution (pH 7). An excess amount of 25% aqueous glutaric aldehyde solution (40 ml) was added, and

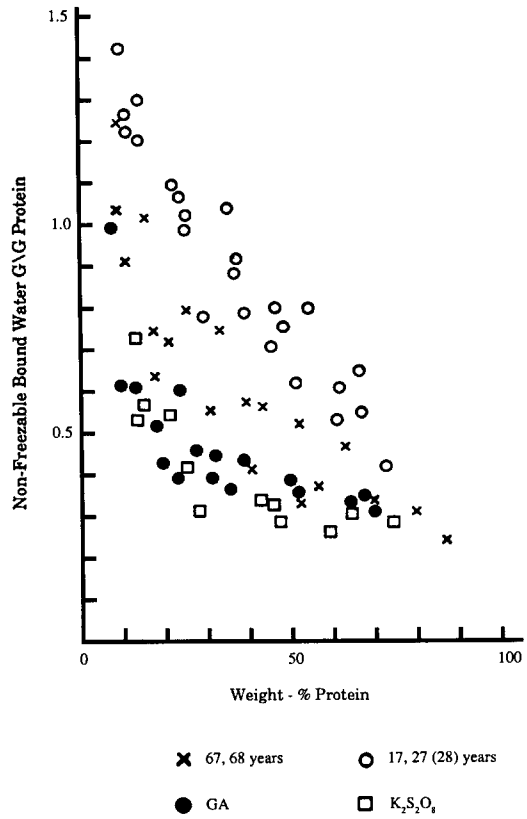

FIG. 1. This figure incorporates data comparing the crosslinkage status, as reflected by nonfreezing water content, of comparable protein fractions from old brains and young brains. Protein samples from young brains clearly bind more nonfreezing water than similar samples from old brains. Treating samples from young brains with either of two entirely different crosslinkers (glutar aldehyde and dipotassium peroxysulfate) causes a reduction in their capacity to bind nonfreezing water, in a manner analogous to the samples from old brains. This shows that crosslinking occurs in brains as they age.

allowed to react 3 h at room temperature. The material was washed repeatedly with water and dried in vacuum.

2. One hundred milligrams of the protein was swelled in 5 ml of distilled water. After deaeration with nitrogen 50 mg of dipotassium peroxysulfate ($K_2S_2O_8$) was added and the sample kept at 60° centrigrade for 20 min, and then washed and dried as above.

The quantities of glutaric aldehyde and dipotassium peroxysulfate were very large in order to ensure rapid and fairly complete reaction. In biological systems, the amounts of crosslinking agents present are much smaller; however, reaction times in ageing measure in decades. Our excessive amounts of crosslinkers compensate for the greatly reduced reaction times.

Calorimetric measurements

The measurements were performed with a differential scanning calorimeter (Perkin Elmer DSC 1B). Samples of weighed amounts of proteinaceous material and water were kept in sealed

sample pans at 5 °C for 24 h before running the DSC. The samples were cooled to −50 °C in the calorimeter and kept at that temperature 5 to 10 min, and then heated to room temperature at a rate of 4 to 8 ° per minute. The enthalpy of melting of water in the samples was compared to that of pure water, the amount of freezable water in the protein was estimated from the area of the melting peaks. The amount of nonfreezable water was calculated as the difference between the total amount of water and the freezable water. Because the amount of nonfreezable water increases with increasing water content in the sample, the measurement was repeated with several protein/water ratios. The resulting nonfreezing water content is expressed in g/g protein as a function of protein content of the samples.

The crosslinkage characteristics of two young brain samples are radically altered to a curve similar to that of old brain samples by exposure to either one of two chemically different substances, which have nothing in common except that both are crosslinking agents.

The points on Fig. 1 show the resolution of the points now determined from the brains used for the graphs in the above reference and of two additional brains. The added data broaden the statistical base (without changing the conclusion).

CONCLUSION

Since two totally different crosslinking agents have caused the identical change of the crosslinkage status of young brain protein, making this indistinguishable from the corresponding protein fraction from old brains, it appears that crosslinkage is indeed an important causative factor in aging.

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