# LONGEVITY
# • A QUEST •

**An Odyssey**
**by**
**Johan Bjorksten, Ph.D.**

Containing over 700 references related to gerontology.

**1981**

c/o Bjorksten Research Foundation P.O. Box 9444 Madison, Wisconsin 53715

# PREFACE

The slowness of editorial processing has been a major retardant of scientific progress. For example, the discovery of oxygen has been credited to Priestley, because the tardiness of Scheele's publisher delayed the Swedish researcher's publication three critical years.

From personal experience in recent years, two articles I wrote for books published by two different leading publishers were delayed one 3 years, the other 4 years in editorial processing.

This being a common, if not general practice, the delay in making scientific information available is staggering.

The present book is an attempt to secure more rapid publishing by using photo reproduction of the original publication wherever applicable, and modern reproduction methods. This has been made possible by the permission and cooperation received particularly from the JOURNAL OF THE AMERICAN GERIATRICS SOCIETY, REJUVENATION, LIPIDS, JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, CHEMICAL INDUSTRIES, and FINSKA KEMISTS. MEDD., which are gratefully acknowledged.

As a result of their cooperation, papers published as recently as 1981 are included in the bibliographies. This book contains over 700 references related to gerontology, all of them subsequent to Loeb

and Northrop's pioneering paper in 1917*, which strongly indicated that aging in Drosophila has the temperature coefficient of a chemical reaction.

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* Loeb, J. and Northrop, J.H. On the influence of food and temperature upon the duration of life. J. Biol. Chem. 32: 103, 1917.

ACKNOWLEDGEMENTS

Mrs. F. B. Korsgard has worked on our age associated problems since January 1945, and Dr. R. U. Schenk since 1970. Both have rendered essential assistance not only in assembling this book, but also on many of the component publications. To them both I express my sincere appreciation.

I greatly appreciate the permission to reprint copyrighted material granted us by the many journals in which we have published.

For the financial support in our studies on aging, which are the subject of this book project, I am indebted to Mr. Paul F. Glenn, Mr. Robert S. Morrison, the Paul F. Glenn Foundation, Mr. and Mrs. Martin Hilby, Mrs. Stanley J. Buckman, Mr. and Mrs. Marc A. Chavannes, Mr. Peter A. Benoliel, Mr. Lucius E. Burch, Dr. Floyd A. Eberly, and Dr. Myron A. Coler.

I am further indebted to all those other 135 persons who have contributed towards the research on which this book is based.

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# LONGEVITY - A QUEST

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## How it all Began

Having gained experience in the laboratories of Hans von Euler in Stockholm and A. I. Virtanen in Helsinki, both Nobel Prize winners, and done post-doctorate research with Dr. R. A. Gortner at the University of Minnesota under the auspices of the International Education Board of the Rockefeller Foundation, I found myself after a few years of industrial experience as Chief Chemist of Ditto, Inc. then the world's largest manufacturer of hectograph films and equipment.

A principal problem area was to increase the hydrothermal stability of hectograph films, so that these could withstand summer temperatures, exposure to water, mechanical stretching and friction.

To resolve that problem I worked 5 years with five assistants on the controlled stabilization of protein gels by cross-linking.

The closing paragraphs of a summary paper marked the beginning of a long quest.

"To those working on this type of precisely regulated tanning, a strange resemblance is apparent between the 'aging' or progressive

tanning of photographic or of hectograph duplication films and the aging of the human body. Both processes involve protein reactions, leading to reduced hydration, loss of elasticity; all the earmarks of slowly, but inexorably, progressing tanning reactions.

"And the chemist observing this strange analogy, must wonder if the aging of the human body might not largely, and even principally be an instance of a progressive tanning reaction akin to, or identical with, the "after-hardening" of proteins tanned with a slight trace of aldehyde, in contact with oxygen. The photographic and duplication industries know means of delaying these reactions which are fairly effective, and it does not appear altogether impossible that, at some future time, important contributions towards prolonging the span of human life may emanate from this industrial work."

This concept was expanded, clarified and supplemented. In 1942 I wrote again in the context of a summarizing paper on duplication industry: (Chemical Industry 50, 69, 1941):

"This problem of controlling tanning reactions may have considerable potentialities in medicine and gerontology.

"The amounts of acetaldehyde normally contained in human blood, would amply suffice to tan ..... Such tanning is a cause of the rigidity, which occurs within a few hours after death.

"In the living organism, this tanning is counteracted by (A)-the directional tanning under influence of repeated stretch and (B)-the continued state of flux in the protein molecules, which are continually split and re-synthetized. In this interplay of synthetic and splitting reactions, the protein molecules are broken down before tanning has gone very far, and re-synthetized in a non-tanned state.

"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 molecule. In either event, the result is that cumulative tanning of body proteins, which we know as old age."

From Theory to Experiment

This line of thinking gave a deeper significance to observations made in the course of commercially oriented activities. A question, commonly asked, was given a new twist: "Why don't we age much faster than we do, in view of the large number of potent cross-linking agents normally present in the human body? Our first paper on this subject was submitted to the Journal of the American Chemical Society.

It was, as usual, sent to referees. One of these considered the work trivial, unorthodox, not worth publishing. The other recommended publication, but with modifications and corrections. The editor was then Dr. Arthur B. Lamb. He wrote us: "Your work could be important. I will publish this paper as submitted."

[Reprinted from the Journal of the American Chemical Society, 64, 868 (1942).]

[Contribution from the Laboratories of Ditto, Inc.]

# Mechanical Influence upon Tanning

By Johan Bjorksten and William J. Champion

The aldehyde content in human blood is so high,[1] that in the light of experience with industrial protein gels, it should amply suffice to tan the body proteins to complete rigidity and loss of elasticity within a matter of months. While such progressive tanning is evidenced by the loss of elasticity of tissues such as for example the arteries[2,3] with aging, the rate of this tanning is much less than one would expect in view of the tanning substances present in body fluids.

Therefore, it appeared that this tanning might be retarded by factors not previously considered, and that a study of progressive tanning of protein gels during rhythmical motion simulating that of living arteries, might yield information bearing on this subject. The present investigation was made with this aim in view.

Since high Bloom gelatins are protein substances obtained by the mildest type of extraction, and since their behavior when tanned with slight amounts of tanning agents has been most thoroughly studied and understood,[4] this protein was selected for our initial study.

300 grams of 225 Bloom pig skin gelatin was allowed to swell in a mixture of 300 g. of glycerol and 450 g. of water, at room temperature. After one hour, the mixture was melted in a water-bath of 55° and mixed with 1500 g. of additional glycerol, pre-warmed in the same water-bath.

The resultant mix was divided in two 50% aliquots. To one of these we added 60 cc. of 33% acetaldehyde solution, as tanning agent; the other aliquot served as our non-tanned control.

The mixes were allowed to stand one-half hour at 55°, to eliminate air bubbles, and were then poured to form uniform sheets having the dimensions 330 mm. × 216 mm. × 2.5 mm. Two such sheets were obtained from each aliquot.

(1) K. Ri, *J. of Bioch., Tokyo*, **32**, 38–50 (1940).

(2) J. C. Bramwell, A. V. Hill and B. A. McSwiney, *Heart*, **10**, 233 (1923).

(3) J. M. Steele, "Abstracts of the Atlantic City Meeting of the American Chemical Society," September, 1941, p. B2.

(4) J. Bjorksten, *Chem. Ind.*, **48**, 749 (1941).

The sheets were allowed to stand for twenty hours at room temperature, in order to give them the firmness required.

After this period, one tanned and one non-tanned sheet were attached with one short end to a rigid metal holder, and with the other short end to a holder having a reciprocating motion, so that the sheets were continually stretched and relaxed at a rate of eight times per minute, to an extent of 10% elongation.

The other two sheets, one tanned and one non-tanned, were placed on a porous backing next to the stretching machine, and were thus exposed to room temperatures, humidities and ventilation identical with those of the stretched samples.

After twelve days, the stretching was interrupted, the sheets were kept overnight (sixteen hours) at 55% relative humidity and 21°. Test strips were cut from center and ends of each sheet having their length parallel to the direction of stretch, and others perpendicular thereto, and the tensile strength was determined in a conventional paper tensile strength testing machine.

The tabulated figures are averages of triplicate determinations, all of which were within 5% of the averages shown.

Table I

Tensile Strength in Grams per Sq. Mm.

| | | In direction of stretch | Across direction of stretch |
|---|---|---|---|
| Stretched tanned sheet | End | 171 | 144 |
| | Center | 192 | 146 |
| Stretched non-tanned sheet | End | 153 | 148 |
| | Center | 150 | 152 |
| Tanned non-stretched sheet | | 60 | |
| Non-tanned non-stretched sheet | | 155 | |

With the stretched sheets, determinations were made at the end and the center of each sheet, as the direction of stretch might have shown slight deviations near the edges.

The most striking facts shown by these data are: (I) that the stretched tanned sheet not only is vastly stronger (320%) than the non-stretched tanned sheet in the direction of stretch, but also in the direction perpendicular to the tension

(240%); (II) that the stretched tanned sheet is materially stronger in the direction of stretch than is the non-tanned sheet; (III) that in the non-tanned sheets there is no correlation between strength and direction of stretch, while in the tanned sheets this correlation is pronounced; (IV) that in the non-tanned sheets, stretching did not cause any observable increase in strength.

Since the strength of the stretched tanned sheet was several times greater than that of the non-stretched tanned sheet, it appeared advisable to investigate whether the tanning itself had been impeded by the stretching process. For this purpose, the softening points of the sheets were determined on circular samples of 25 mm. diameter, by the Ring and Ball method, in oil-bath.

The softening points of both of the non-tanned sheets were 60 ± 1°, while those of the stretched as well as the non-stretched tanned sheets exceeded 110°.

The elongation of the stretched sheets at the termination of the test was approximately 5%, and was thus insignificant in relation to the effects observed.

The condition of the gel sheet tanned without stretch was representative of similar gels tanned to a softening point exceeding 110°, regardless of the tanning agents employed, but the strength and elasticity of the stretched and tanned sheets far exceed anything the authors have ever seen in such gels having a softening point above 100° in their many years of experience with industrial protein gels of this general formulation. The high softening point conclusively proves that the tanning agent remained and was active in the tanned stretched sheets. Manifestly the continued stretch exerted a directional influence on the positioning of tanning bridges in relation to the protein molecules, so that the tanning bridges connecting the protein molecules were formed in positions not interfering with stretch and relaxation of the gel.

The condition of repeated stretching and relaxation is even present in the walls of the arteries of higher animals. The effect described above may be of considerable importance in delaying the aging of arteries, and may at least in part explain the slow progress of this aging in spite of the well-known presence of active tanning agents in the blood stream. The effect may also be a factor contributing to the beneficial effects of exercise, and to the atrophy of organs under conditions of continued absence of stretch.

Further work is in progress.

## Summary

Rhythmical stretching and relaxation of a protein gel containing a tanning agent, caused a very great increase of tensile strength over a similarly tanned non-stretched gel, *in all directions in the gel.* The effect did not take place in the absence of the tanning agent. The softening point of the stretched gel rose as in normal tanning.

Analogies with conditions in human arteries are discussed.

CHICAGO, ILL. RECEIVED NOVEMBER 10, 1941

Guiding Principles

On the threshold to what turned out to be a long and arduous path, I took stock of the time requirements, and the field ahead. The following points seemed germane:

1. The developed information and application data on cross-linkage was concentrated in the industrial field, therefore, I could best acquire the additional requisite know-how while remaining in industry.
2. The time factor in this study could be devastating. To reach the goal of this quest in my lifetime, I had better devote primary attention to time saving strategies, plot a path to the goal, and follow this doggedly without yielding to the temptations of digressions and without losing time on repetition or on greater accuracy than was needed for planning purposes and guidance at any one time.

These principles have guided us from the beginning. The following formalization, made only recently, has guided our steps for a long time.

# THE TIME FACTOR IN GERONTOLOGICAL RESEARCH

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## SUMMARY

A common object in gerontological research is to gain time for :

1. Enjoying a pleasant life already attained.
2. Adding new disciplines of knowledge and creativity to those already mastered.
3. Gaining the time necessary to complete a specific task to which one is wholly committed.
4. Such other purposes as may appear.

To achieve these objectives in time for those now active to enjoy, the following research strategy is suggested :

I. De-emphasize gerontological work with any animal which has substantially shorter life span than humans. Those chemical reactions which underly human physiological deterioration are largely different from those acting on shorter lived animals. Slow rate processes not affecting longevity seriously in less than 40 years are those against which evolution has **not** provided humans with adequate defenses.

II. Center work on humans in age far enough advanced to give meaningful results, but not so advanced that secondary damage overshadows basic aging effects.

III. Do key research in countries where this can be done with minimum harassment and legalistic formalities.

IV. Do not sacrifice speed of research for the sake of accuracy greater than the situation permits, or requires.
**To illustrate :** If a physician is even 90 % certain of his diagnosis he treats his patient accordingly, with life saving as the prime objective. He does not wait for the autopsy to make certain of the absolute correctness of his diagnosis.

In contradistinction, the researcher under academic standards is too often held to statistical accuracies desirable when time permits, but which applied without regard for the terrible urgency of the objective, in the aggregate lead to unjustifiable delays.

The simple fact is : If we want to have any chance at all of achieving a true breakthrough in geriatrics, giving us on the average at least a 50 year life extension in good health in time for those now 60 to enjoy, we must use realistic criteria in deciding when and how to proceed to the next step on the basis of a 70 to 90 % probability of basic assumptions being correct, thus taking a calculated risk to gain necessary time.

## DETAILS AND DISCUSSION

According to the 1978 Life Tables of the U.S. National Center for Health Statistics, the average life expectancy at age 65 was 16.3 years in 1978, against 14.4 in 1959-61. For white males it was 14.0 years in 1978 vs. 13.0 years in 1959-61. This is too slow progress.

An all-out effort on an adequate scale might be funded by suspending such work now being done with short lived animals, as is unlikely to result in major life extensions in **humans.**

Organisms are by evolution endowed with protective mechanisms, which give them enough time to have their offspring and to give it proper education and a start in life. For a mouse this is perhaps a year, for a human 60-70 years. The molecular events which result in progressive deterioration are, therefore, in humans largely those which require 60 years to become critical. These reactions are missed in work with animals which have lifespans shorter than about 40 years. Precious time is being wasted on a huge scale on gaining information which has no practical use. The answers, which we seek, are to be gained by study of humans old enough to show the critical events, but before these become obscured by secondary complications, preferably about 45-55 years.

While an 80 % probability would be totally unacceptable in most of the short range studies to which we are accustomed, the picture changes when the time required expands our present life expectancies.

A projection shows a probable duration of 400 years if the study is carried out with present test procedures and insistence on multiple animal tests prior to any work with humans. On that route, the probability of a breakthrough in our life time is nil.

It should be possible to complete this work in a very much shorter time, quite possibly within ten years, if we content ourselves with an 80 % probability in those steps where a higher precision would very greatly increase

time requirements, and start sequential tests as soon as the previous tests have given a fair qualitative indication of which way to go. The price we would pay for speed is perhaps a 20 % risk that a serious flaw may have been overlooked, and that we must backtrack some, at worst all the way to the beginning. The time to be gained may justify this risk.

This estimate presupposes capable overall direction, and responsibility centralized in one competent and inspiring leader.

If the necessary clinical work cannot be done where we are, let it be done elsewhere ! The world is wide and the instinct of self-preservation is universal to all life.

The Chinese gave an impressive demonstration of what they can accomplish, in abolishing the Keshan disease in 3 years of excellent clinical research, with 8,495 children aged 1-9 in 1974 ; 12,212 children in 1975 and 12,579 children in 1976. The Keshan disease, a myocardiac disease of children, with 50 % lethality, was conclusively proven to be a selenium deficiency disease, and was eliminated. 2), 3), 4). When we compare this with the great multitude of not yet generally accepted results of scattered, uncoordinated tests such as, for example, thouse with ascorbic acid in the Western literature, we cannot avoid thinking that a large enough well supervised test over 3 years with about 12,000 persons each year, would have settled all uncertainty, once and for all.

A most important part of international cooperation might well be to centralize major clinical research projects in countries which can apply the necessary organization and controls, and where researchers are not required to explain and obtain informed consent from the parents of each participating child ; to face interference from poorly informed outsiders ; the personal hazards of malpractice suits ; and the well intentioned yet very time consuming multiple distractions imposed by bureaucracy. Freedom in organizing and conducting clinical research, used skillfully and conscientiously, would be a great help.

However with or without this advantage, to gain the desired breakthrough in our lifetime, we cannot afford to work with higher precision than needed, when this leads to major loss of time, and have to adopt a strategy of concentrating support on work which, if successful, will be clearly relevant to the artificial extension of the human specific lifespan. We must move on with a belief that the target can be achieved and with a firm resolve to succeed in time to reap the rewards of a greatly extended life span in good health.

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Causal Relationship

Abiding by this principle, several studies were made to determine to our own satisfaction whether cross-linking could really simulate aging to a sufficient degree to make the postulated causal relationship plausible, and whether the progressive insolubilization of proteins would actually occur to an extent consistent with the role ascribed to it.

These questions were answered in the affirmative by the following papers:

Reprinted with Permission from the Journal of the American Geriatrics Society Volume VIII(8) 1960, pp 632-637

# FUNDAMENTALS OF AGING: A COMPARISON OF THE MORTALITY CURVE FOR HUMANS WITH A VISCOSITY CURVE OF GELATIN DURING THE CROSS-LINKING REACTION

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The mortality curve is an expression of the rate of aging, for it may be assumed that the rate of exposure to infections, accidents and other traumata is in the same order of magnitude in the various periods of life, and does not increase logarithmically or even linearly with age. Thus the rate of traumata being roughly constant, the mortality curve is an expression of the decreasing ability of the aging organism to overcome all kinds of stress. This decreasing ability to overcome stress is the principal expression of aging and is the basis for the shape of the mortality curve.

In view of the mounting evidence in favor of the theory that biologic aging is largely caused by the immobilization of active large molecules, it appeared of interest to compare the shape of a time/viscosity curve of protein immobilization by cross-linkage *in vitro* with the shape of the mortality curve for humans.

## MATERIALS AND METHODS

A 25-Gm. aliquot of Graylake gelatin having an isoelectric point of 8.0 and a Bloom value of 250 was dissolved in 250 ml. of distilled water and the pH was adjusted to 3.3 with sulfuric acid. The viscosity of the gelatin solution (at 45°C.) was measured with a Brookfield viscometer and found to be 65 centipoises, before addition of the cross-linking agent. Then 7.5 Gm. of a commercial basic chrome sulfate (Tanolin W2XD)[1] was added to the composition after the gelatin was placed in a delta blade Brabender Plastograph.[2] The mixing speed of the instrument was 50 rpm, the reaction temperature was 45°C., and the lever ratio was 1:1. The revolving rate of the chart was 1 cm. per minute.

## RESULTS

A typical Brabender viscosity tracing obtained under the conditions described is shown in Figure 1, and a plot of the data redrawn to linear coordinates is shown in Figure 3. If aging and the concomitant decreased resistance are indeed results of immobilization of proteins, it would be expected that a curve expressing a simple chemical reaction leading to such immobilization would have the same shape as the mortality curve. That this can be the case is shown by Figures 2 and 3.

## DISCUSSION

The theory that aging is due to immobilization of large active intracellular molecules (previously expressed by one of the present authors (2, 3)) has not

[1] Tanolin W2XD was obtained from Diamond Alkali Company, Kearny, N. J.

[2] The Brabender Plastograph is manufactured by the C. W. Brabender Instruments, Inc. of South Hackensack, N. J. It subjects the test material to the kneading action of two revolving blades, and records the power required, which increases with viscosity.

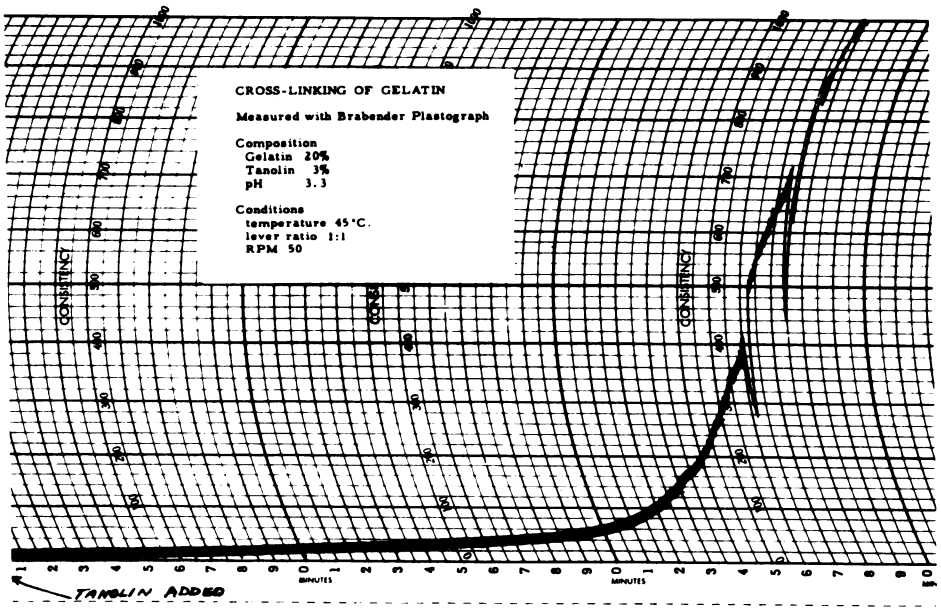


FIG. 1. Brabender Plastogram of the gelatin-Tanolin W2XD reaction

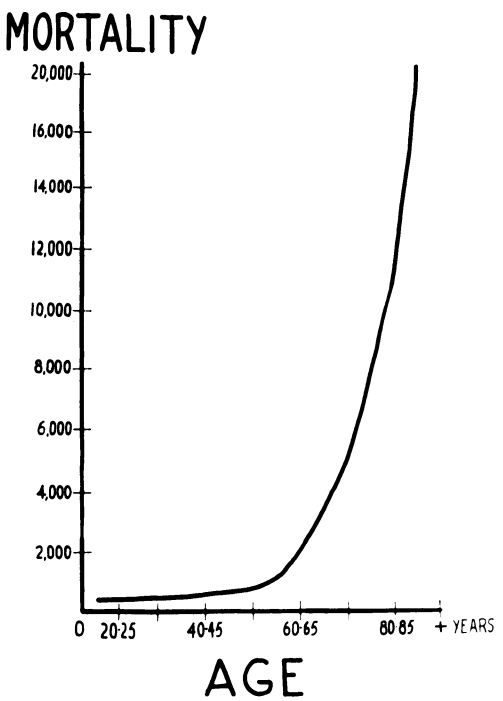


FIG. 2. Mortality rate plotted against time (age) for white American males (1)

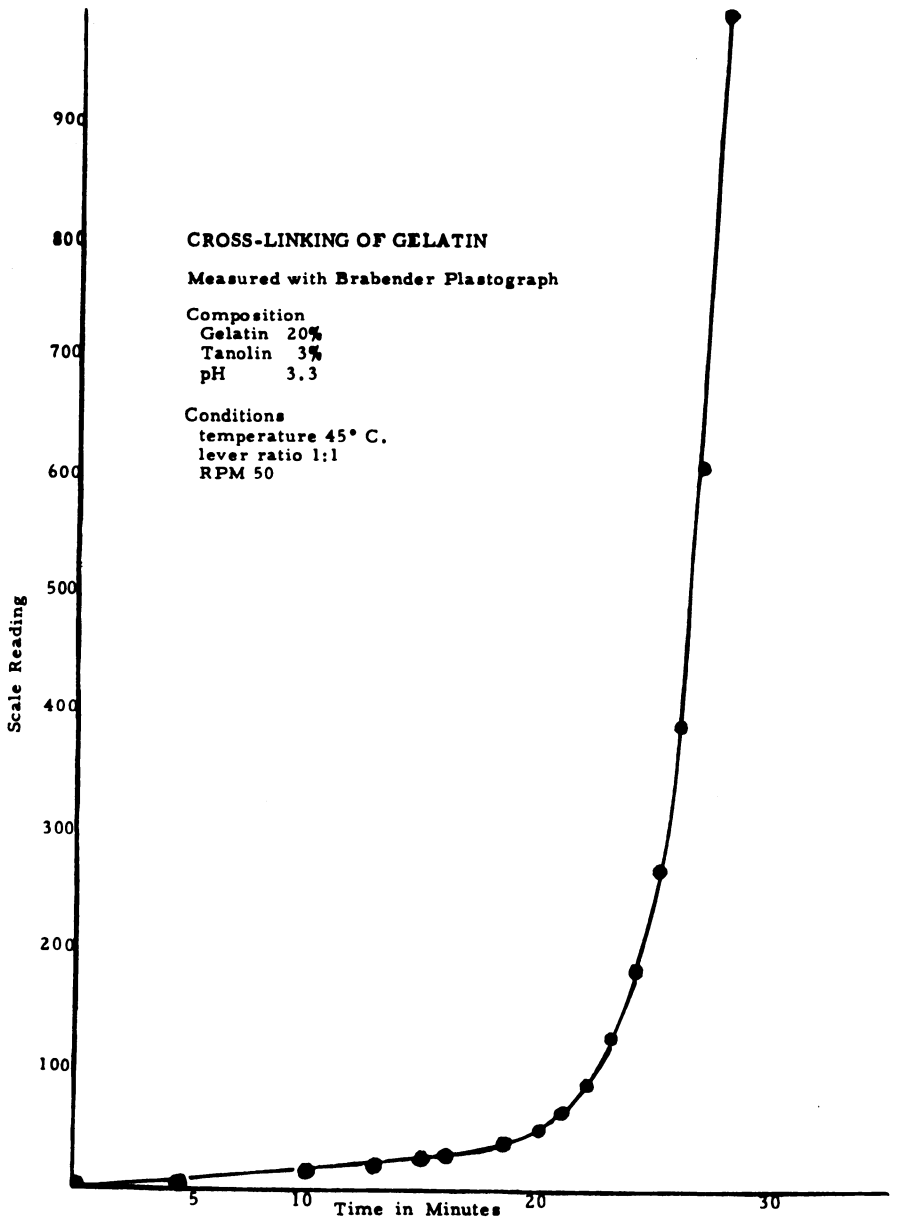


FIG. 3. The rate of progressive immobilization of gelatin under the influence of a cross-linking agent, as recorded on the Brabender Plastograph—transcribed from Figure 1 to straight-line coordinates.

been disputed. Various possibilities have been presented as to the way in which this immobilization could take place. Cross-linkage appears the most likely major mechanism, however, because through cross-linkage a very small amount of reactant can have a very large effect on the large molecules, and it is as uni-

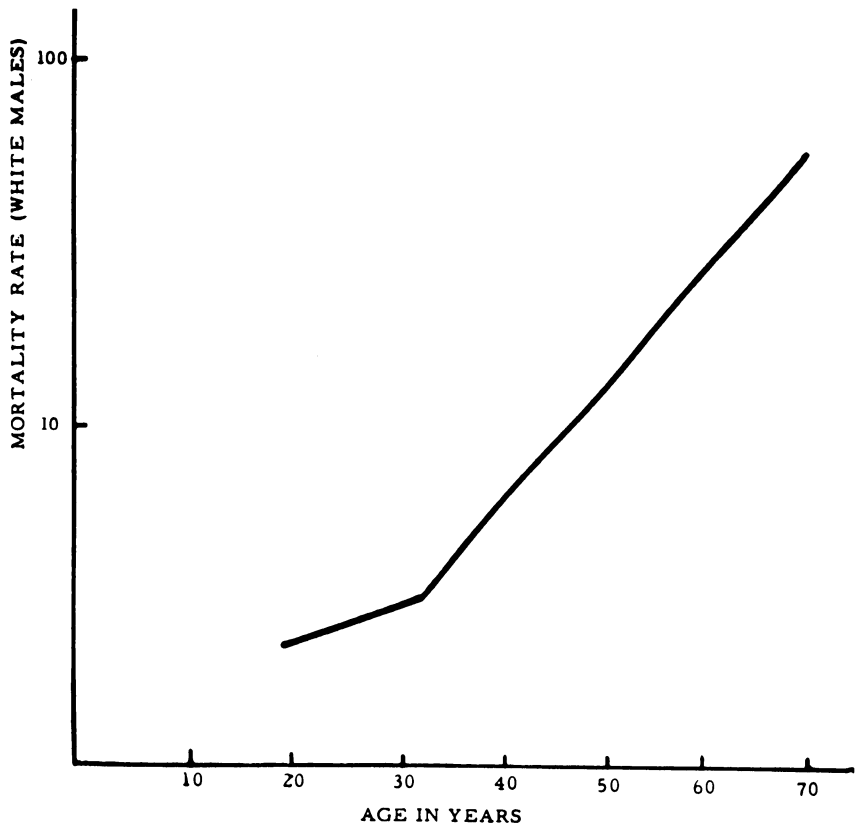


FIG. 4. Mortality rate plotted against time (age) for white American males, using semi-logarithmic coordinates.

versal as aging itself (4, 5). Cross-linkages can immobilize proteins in a way which renders them irreversibly inert toward body enzymes (6). Since cross-linking agents have been shown to be normally present in the organism (5), these highly reactive compounds are bound to interact with the body proteins. The recent work of Alexander and Stacey (7) has shown that cross-linkages of deoxyribonucleic acid may similarly occur.

The finding of Bjorksten and Gottlieb (6) that enzymatic digestion of a protein (gelatin) is blocked or severely distorted by cross-linkage, has been recently confirmed by Kohn and Rollerson (8) using collagen cross-linked with formaldehyde. These authors state: "The data suggest that the decrease (in digestibility of collagen) noted with advancing age may be due to a chemical reaction between collagen and some metabolite functioning as a tanning agent."

Radiation effects have been proposed or implied by Harman (9, 10) and by Szilard (11). Although radiation will break off free radicals which can cause cross-linkages, this can hardly be a major mechanism in aging, because even ten times the amount of radiation a person receives from cosmic sources during a lifetime is insufficient to cause the amount of immobilization of proteins observed in old animals (12).

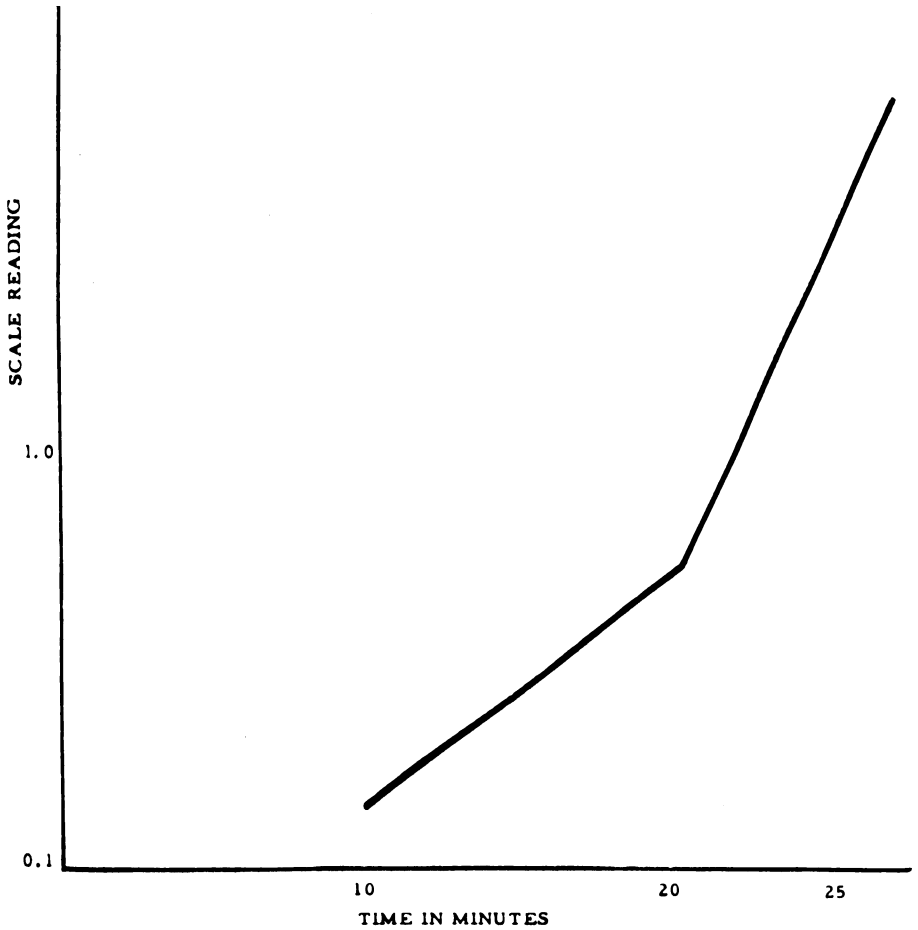


FIG. 5. The rate of progressive immobilization of gelatin under the influence of a cross-linking agent (from Figures 1 and 3) plotted on semi-logarithmic graph paper.

Thermal denaturation has been proposed as a form of immobilization of protein (13, 14) but this is also unlikely to be a major mechanism because denatured proteins are usually easily attacked by body enzymes and thus returned to the metabolic cycle.

It is not our wish to attempt any analysis or conclusion on the basis of the present limited data, but it is interesting that curves (Figs. 2 and 3) plotted on a logarithmic scale show similar breaks in the slope. This is apparent from Figures 4 and 5. The latter parts of these curves proceed in accordance with a steeper exponential slope. The reason in Figure 5 probably is, and in Figure 4 perhaps is, that the initial cross-linking fixes the protein chains in spatial proximity to each other, thereby increasing the probability of additional cross-linking and consequent final immobilization.

SUMMARY

The data of the present study demonstrate that a similarity exists between the curve expressing changes in mortality with time in humans and the curve expressing congelation of protein being cross-linked under the specified conditions. Plotted on semi-log paper, both curves show similar breaks and rate changes. This adds further support to the theory that immobilization of large molecules is a controlling factor of the aging mechanism.

*Acknowledgment*

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# FUNDAMENTALS OF AGING: IMMOBILIZATION OF PROTEINS IN WHOLE-BODY IRRADIATED WHITE RATS

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This study covers one phase of a larger program dealing in both natural and accelerated aging. In this instance, aging of rats has been accelerated using fractionated doses of whole-body X-irradiation at weekly intervals over a twenty-week period. Subsequent reports will cover biochemical findings on naturally aged and chemically aged animals.

The similarity between natural aging and the effects of ionizing irradiation has been emphasized by several authors (1–4). It therefore appeared of interest to determine whether or not immobilization of protein material was taking place in conjunction with continued whole-body irradiation, inasmuch as such immobilization by cross-linkage or otherwise, could be a common molecular basis for natural aging and irradiation toxicity (5–8).

Although excellent reports are available in the literature which indicate that irradiation induces precocious aging, there are few direct comparisons of irradiated tissues with non-irradiated old tissues at the biochemical level. It is also noteworthy that there has been little or no standardization of dose or type of ionizing irradiation employed in the experimental development of senescence in mammals. The problem becomes even more complex when it is realized that the study of aging has been largely empirical in nature, and that few analytical data have thus far been amassed. In short, there is little scientific background to guide the researcher in this area of study.

## MATERIAL AND METHODS

### *Animals and diet*

Sixty-five 70-day-old female white rats, obtained from the Badger Research Corporation, Madison, Wisconsin, were used for the irradiation phase of the program. The animals were originally derived from the Sprague-Dawley strain, but had the advantage of having been raised for several generations on Purina Laboratory Chow instead of a vitamin A and D-deficient diet used by Sprague-Dawley. The young animals under test were selected on the basis of weight as well as age, with a spread of 7 grams from the heaviest to the lightest animal. They were then separated into three test groups—25 young controls and two groups of 20 irradiated animals each.

At the onset of the program 55 females ranging in age from 12 to 18 months were selected to serve eventually as non-irradiated old control animals.

All of the animals in this study were fed Purina Laboratory Chow *ad libitum* and in addition were given an antibiotic (Polyotic, a product of American Cyanamid Company) in their drinking water three times weekly for a total of thirty-six weeks.

### *Irradiation*

The irradiation was administered as follows: Ten rats were irradiated as a group in a circular container divided into equal pie-shaped segments, with the head of each rat facing

toward the center. The cage was constructed entirely of wood and lucite, and the rats were pushed sufficiently snug into each compartment and held with outer wooden blocks, so that the whole unit was almost a solid cylinder of tissue or tissue-equivalent material. Irradiation of 1.3 mm. Cu H. V. L. was used, generated by a Picker 280 KVP self-rectified unit (Vanguard) and delivered at a distance of 126 cm. from the target to the top surface of the cage. The central axis of the cage was aligned with the central ray of the beam by means of a back pointer, and the cage rotated during treatment at 1 r.p.m. to reduce variation in dose to different rats, which might otherwise result from the target "heel" effect. The air dose at the center of the cage was 17.2 r per minute, and 17.0 r at the periphery. This yielded a central surface dose rate of 23.6 r per minute, decreasing to 16.6 r per minute at the bottom inside cage surface. The edge fall-off of the beam occurred primarily within the wooden holding bars peripheral to the rats. Irradiation time was eight minutes for group A and four minutes for group B, the air dose exposure being 133 r and 66 r respectively. Thus, group A received a maximum tissue dose of 189 r to the back and a minimum tissue dose of 133 r to the ventral surface; group B received half of this dose per treatment.

For the sake of clarity in subsequent sections of this report, group A will be referred to as the 189 r/week group, and group B as the 95 r/week group. All irradiation figures are based on the maximum tissue dose delivered to the back of the animal.

## RESULTS

During the course of the irradiation regimen the average weight gain was recorded weekly on 10 animals in each of the two irradiated groups, and in the young controls. These data indicate that after 10 whole-body irradiation exposures the cumulative effects of the irradiation became apparent, the results appeared to be independent of the dose level.

### *Physical condition*

Aside from a 12 per cent reduction in weight gain, the group of animals (group B) which received 95 r per week for twenty weeks showed little adverse effect as the result of exposure to this dosage of X-irradiation. After ten weeks of irradiation (total exposure 945 r) a few of the 95 r/week animals showed evidence of minor bleeding around the nose. This bleeding was slight and could not be detected twenty-four hours after irradiation. As the weekly exposure was continued, the number of bleeding animals increased, until all 20 rats in this group were affected to a slight extent.

Tumors developed in 6 animals (30 per cent) of the 95 r/week group. In 1 case the animal was sacrificed when the tumor had reached a size of 81 grams. All other rats in this group survived and appeared outwardly to be in good health.

The second group (group A) of animals, which received 189 r exposure per week began showing signs of irradiation sickness after receiving 1700 r. At this point bleeding from the nose and anorexia were evident for about a day after irradiation. The first of these animals died three days after receiving 2079 r, and the mortality rate increased gradually during the next eight weeks until the eighteenth and nineteenth weeks of irradiation were completed. Fifteen per cent of the rats in this group were still alive thirty days after reaching the 3591 r level. When the animals had reached the stage of listlessness, labored breathing, nasal bleeding, and emaciation, they were sacrificed by bleeding them during light

ether anesthesia. The 3 rats which survived the complete irradiation regimen were sacrificed forty-five days after the final dose. The non-irradiated young controls and 10 of the animals in the 95 r/week series were likewise sacrificed at that time, and the tissues were used in the analytical phases of the program.

*Histologic findings*

At the onset of this program, we were not familiar with any standard irradiation procedures which could be used to accelerate aging without the attendant complications of acute irradiation sickness. It was therefore deemed necessary to use two test exposures and include a brief comparative study of both non-irradiated old organs and irradiation-aged organs at the microscopic level. Since this program is not concerned with irradiation pathology, the details will be only briefly summarized here.

In general the irradiated animals exhibited a marked increase in pigment deposition within the spleen, an increase of connective tissue, and a distortion of corpuscular pattern. These same observations were made in non-irradiated old animals. The primary difference in tissues attributable to irradiation appeared to be degeneration of the megakaryocytes in splenic tissue and moderate fat infiltration in liver tissue. Observations on the heart, kidney, thoracic aorta, uterus and ovaries of irradiated animals did not demonstrate any great differences compared with naturally senile animals.

The fact that histopathologic comparison of tissues from old and from irradiated animals did not reveal any great differences was taken as an indication that the irradiation regimen adopted was essentially sound.

*Immobilization of protein, determined via trypsin hydrolysis (liver homogenates)*

A method of trypsin hydrolysis was developed, so that levels of protein immobilization could be compared in the various test groups. First, a series of kinetic studies on young rat liver was conducted to determine optimum concentration and hydrolysis time for Difco trypsin (1:250). Then rat liver homogenates from both irradiated and non-irradiated animals of the same age were incubated with the enzyme under the controlled conditions enumerated in Table 1.

After incubation with the enzyme, trichloroacetic-acid filtrates were prepared and analyzed for liberated amino nitrogen, using the Van Slyke alpha-amino nitrogen apparatus. If cross-linking or immobilization of protein occurs in natural or accelerated aging, it would be expected that less amino nitrogen would be liberated during tryptic hydrolysis. This fact is borne out in the experimental data amassed to date.

The values for liberated nitrogen in 10 irradiated animals (total dose 1890 r) and 14 non-irradiated animals were compared statistically, and the means were found to be significantly different at the 75 per cent probability level based on the distribution test with 16 degrees of freedom.

The nitrogen values obtained in the 189 r/week group of animals showed a significant difference as compared to the controls at the 99 per cent probability level. The enzyme data summarized in Table 1 clearly indicate that irradiation modified the liver protein in a manner strongly suggestive of cross-linking.

TABLE 1

*Liberation of Alpha-amino Nitrogen in Irradiated and Nonirradiated Rats after Incubation of Liver Homogenates with Trypsin**

| | Age at Death (wks.) | No. of Animals Sacrificed | Mg. Nitrogen Liberated (avge.) | Reduction in Solubilised Protein (%) |
|---|---|---|---|---|
| Young control rats | 36 | 14 | 1.99 | 0.0 |
| Irradiated rats (1890 r) | 36 | 11 | 1.86 | 6.5 |
| Irradiated rats (3591 r) | 24–36 | 16 | 1.58 | 20.6 |

These figures are based on 0.1 Gm. of liver wet weight.

* Ten ml. of liver homogenate (1.0 Gm. of liver) was mixed with 50 ml. of Sorenson's buffer (pH 7.7) and 20 mg. of Difco trypsin (1:250) in 125-ml. Erlenmeyer flasks. Each sample was set up in duplicate with one reaction mixture precipitated immediately upon mixing with 20 ml. of 20% trichloroacetic acid. The second flask was shaken for twenty hours at 38°C. and then precipitated with 20 ml. of 20% trichloroacetic acid. Both samples were filtered and washed with 5% trichloroacetic acid into 100-ml. volumetric flasks. The filtrates were used for alpha-amino nitrogen determinations with the Van Slyke apparatus. Each value for liberated nitrogen listed above has been corrected by subtracting the ml. of $N_2$ for the control sample from the ml. of $N_2$ liberated after hydrolysis, before the milligram values were calculated.

*Cross-linking of proteins studied by shrinkage temperature of collagen in rat-tail tendons*

Of the connective-tissue proteins, collagen and elastin are the most frequently studied because their physical and chemical properties have been more thoroughly characterized than those of other proteins also classed as albuminoids. In this program our interest in connective-tissue proteins was limited exclusively to a survey study of the shrinkage behavior of collagen in rat-tail tendons.

Shrinkage temperature has long been considered the most simple and direct method for determining the degree of cross-linking in collagen (9–11). This method is based on a peculiar property of collagen, namely, its ability to shrink sharply and reproducibly when heated to a critical temperature in an aqueous buffer solution.

In this laboratory a tensionless apparatus is used for the determination, and the test is carried out in an M/45 phosphate buffer adjusted to pH 6.9. Because the tail tendons are not held under tension, errors due to differences in cross-sectional diameter are minimal, and the values for comparable tendons usually agree within ±1°C. This equipment also allows for measurement of the extent of shrinkage in the individual tendons, and this may also be considered an index for cross-linking of collagen.

Shrinkage temperature measurements were made on 8 tendons from each of 18 control rats (7 months old), 10 rats irradiated with 95 r/week, 17 rats irradiated with 189 r/week, and 9 old control rats (3 years old). Each of these groups yielded shrinkage temperature values within ± 0.5° of the mean value of 60.3°C. for normal young controls. It was noted, however, that in spite of the closeness of the mean values, some physical changes had occurred in both the irradiated

and aged groups, *i.e.*, the irradiated tendons pulled apart or had less strength than normal tendons, and both irradiated and aged tendons shrank less sharply and to a lesser extent than normal. This suggests cross-linking similar in nature to that induced by certain tannins.

On the basis of prior experience in the field of protein chemistry, the authors believe that the collagen in the tendons of the irradiated animals had been cross-linked at sites in the polymer chains, and that simultaneously certain other naturally occurring bonds had been severed by X-irradiation.

## DISCUSSION

Study of the aging syndrome is complicated by the marked variations in response (either chemical or biologic) within outwardly homogeneous groups. Although inbred strains of animals of one sex are used under carefully controlled environmental conditions, the data, to be meaningful, must be handled statistically.

One of the simplest statistical experiments in this field is the determination of mortality rates of populations, and the first aging studies on experimental animals performed in this laboratory involved this approach. In a cooperative experiment with the Sprague-Dawley staff, 55 male and 55 female weanling rats selected from standard laboratory stock were fed and maintained for a period of three years. At the end of three full years, 7 per cent of the females and 12 per cent of the males were still alive. The survival half-times were not determined, since such data are of little value in the study of aging at the biochemical level. Both sexes were in the terminal stage of life, displaying all of the usual signs of senility. Tissues from these particular animals were used as "old controls" in the collagen shrinkage-temperature and histologic phases of the current research. Unfortunately it was not possible to hold the liver tissue long enough for use in the trypsin hydrolysis work.

On the basis of this study, it was decided to use females throughout the program, since their average life span is somewhat less than that of male animals. In retrospect, however, males might better have been used in order to remove one important variable, namely, the spontaneous rise in the incidence of mammary tumors in many females of the breed between the ages of 12 and 24 months.

The study of protein chemistry is fundamental for an understanding of the aging process. If ionizing irradiation is to be used safely as a tool for such study, it is necessary to exercise careful control over the number of roentgens used in order to minimize the specific trauma resulting from excessive toxicity during treatment. If acceleration of the aging process is accompanied by specific pathologic findings, aging cannot be adequately studied. For this reason two separate experimental groups were set up, and when the animals were sacrificed the vital organs were compared histologically with the organs of the senile group just described. The two groups were found to be reasonably comparable.

From the chemical standpoint, however, the amount of liver protein which resisted tryptic hydrolysis was significantly larger (20.6 per cent) in the 189 r/week animals than would be expected on the basis of visual observation. Al-

though it will be several months before a sufficient number of non-irradiated senile animals will be available for hydrolysis study, it is doubtful that so great a reduction in protein digestibility will be noted in the naturally aged liver. It is anticipated that the results will more closely approximate those obtained in the lower-dose irradiation group.

Quantitative measurements of the changes in collagenous tissue during irradiation, maturation and senescence, although not dramatic, are nonetheless significant. Distinct changes occur in the structure of collagen during maturation and again during senescence. In very young rats (70 days old) the collagen of the tail tendons dissolves almost completely when the critical shrinkage temperature is reached (approximately 60°C). Tail tendons from mature animals do not dissolve in buffer, but shrink sharply at a temperature close to 60°C. Tail tendons from 2-year or 3-year-old rats display a greater variability in thermal response. The tendons of a few animals in this group, upon shrinking, closely resemble those of ordinary mature rats. The majority, however, shrink less sharply, and the length of these tendons after shrinking is about 25 per cent greater than that of young adult tendons. In some 2- or 3-year-old rats, the shrinkage temperature value is about 64°C. These changes in shrinkage behavior with aging are clear indications of progressive protein immobilization and cross-linking, and are considered to be the most sensitive test for determination of physiologic age.

Although the shrinkage behavior of tendons from irradiated young animals resembles most nearly that of the transition group between maturity and senility, the tendons are not exactly the same. A significant weakening of the tendons was noted and it was thought that although cross-linking had occurred, the irradiation had simultaneously produced breaks in the collagen helix which made interpretation of the data more difficult. Further work on *in vivo* irradiated tail tendons using end-groups analysis could prove to be quite important for the following reasons: This method, which embodies the classic approach of Sanger (12), would reveal whether or not breaks had occurred in the polypeptide chains, and also which peptide bonds were ruptured. In this manner irradiation could be used as a tool in the evaluation of protein structure. Perhaps the most interesting prospect which comes to mind, however, is that this technique could be used quantitatively as a rapid clinical colorimetric method for determining the amount of whole body X-irradiation absorbed accidentally (13). This procedure could be employed easily on pure collagen, because in this protein in its native state there are no free alpha-amino groups available for reaction with end-group marking reagents (14).

Szilard (15) has shown mathematically that aging must be dependent upon a random process, and advances the thought previously expressed by one of us that ionizing irradiations may be among the causative factors of aging (8). The concept of a genetic change as a basis for aging is entirely compatible with the concept of immobilization of protein, inasmuch as this would be the logical starting point or cause for any genetic change (16).

The experimental data presented here indicate that under natural conditions

irradiation immobilization of the proteins is not the major factor in aging. Irradiation up to 1890 r produced only a relatively moderate degree of immobilization in liver protein and in collagen, and none of the animals died. This dosage did not have sufficiently strong effects to be readily confirmed by analysis of tissues in which only small amounts were present. Inasmuch as the amount of irradiation received by a person during his life span is only in the order of magnitude of 30 r, it would appear that natural cosmic or background irradiation could not account for more than 10 to 20 per cent of aging at the most.

Assuming that aging is basically due to an irreversible immobilization of proteins, which withdraws protein molecules one by one from the general interplay of catabolism and anabolism and causes them to become inert and gradually clutter up the biologic system of all cells in the body (5–8, 17, 18), such a result might be achieved by several mechanisms, all of which play some part.

The validity of this theory is borne out in the experimental work of several workers, using various tissues. Bernstein (19) and Dische (20, 21), among others, reported insolubilization of the protein of the lens with aging. Dische presented evidence indicating that gluthathione is an important component in a regulatory system which controls the speed of oxidation (insolubilization) of lens protein. Medvedeva (22) reported accumulation of insoluble protein and insoluble nonprotein nitrogen in the heart, kidney, muscle, intestine and lung. Harman (23, 24) postulated that the formation of free radicals accounts for natural aging. The free radicals, are potent cross-linking agents, and oxidation reactions involving thiols may be considered one type of cross-linking.

A large number of publications have appeared in the literature concerning the mineral content of aged tissues. In general this work has been concerned with increases in calcium levels with advanced age, and there is only a meager amount of interpretation regarding the significance of the increase. More recently Zinsser *et al.* (25) showed that, with aging, aluminum and other potential cross-linking ions accumulate concurrently with a protein material in arterial tissues; X-ray diffraction evidence was presented, indicating that cross-linkage actually had occurred. It would be expected that polyvalent ions, if chemically combined with tissue proteins, would lead to cross-linkages which could not be neutralized by normal catabolic enzymes.

In gerontologic research the connective tissues have received more attention than any other type of tissue, and most of the emphasis has been in the field of collagen chemistry. There is no doubt that collagen levels are altered with advancing age (26–28), nor is there any doubt that physical changes occur in this protein with aging. Senescent changes in the physical properties of collagen are most easily determined by means of shrinkage temperature, the rate of swelling at various temperatures and pH's, and the use of special extraction techniques.

Shrinkage temperature, a distinctive property of collagen, has been used in the tanning industry for over fifty years to determine the extent of cross-linking (tanning) in animals skins during processing. Certain types of cross-linking agents, *i.e.*, chromium salts, aldehydes and diepoxides, raise the shrinkage temperature of normal collagen, whereas protein denaturants, heat and hydro-

lyzing chemicals lower it. Although cross-linking of collagen can occur without eliciting a rise in shrinkage temperature, the degree of shrinkage is usually reduced as the result of the tanning process. A rise in shrinkage temperature with aging, if only a few degrees above the normal (59–60°C), may be regarded as a clear indication that cross-linking has occurred. The British workers, Brown and Consden (29), recently published a highly significant paper in which they report a gradual rise in the shrinkage temperature of human collagen with progressive aging, thus indicating that cross-linkage (stabilization) has occurred.

This rise in shrinkage temperature with aging has not been reported by other workers, nor has it been observed consistently in this laboratory when using rat-tail tendons. We have noted, however, that tendons from both senile and whole-body X-irradiated rats exhibit a 25 per cent reduction in the degree of longitudinal shrinkage. This observation suggests that cross-linkage of some other type takes place in rat-tail collagen. Similarly, observations on the reduction of swelling in human connective tissues with progressing age have been reported (30–32).

The stabilization of collagen with aging has also been studied, using special extraction techniques. Of special interest is the work of Kao *et al.* (33), who reported a decrease of soluble protein in older connective tissues, as determined by the sponge-biopsy technique. These workers (34) also reported on the effects of age, sex and race upon the acetic-acid fractions of collagen. In the latter study it was found that the molecular weight of Eskimo connective tissue rose progressively from 96,000 for young subjects to a value of 427,000 for the aged. The rise occurs in a manner which suggests linear addition of a repeating unit. It was further reported that swelling of scleroprotein decreased with age, but the concentration of hydroxyproline remained constant. The authors suggested that increased stability and resistance to swelling might be the result of a cross-linking mechanism other than hydrogen bonding involving hydroxyproline.

The quantities of potential cross-linking agents in human blood represent a far greater potential immobilizing influence than the maximum attributable to free radicals due to ionizing irradiation. It is therefore plausible to assume that chemical cross-linkages play a far greater role than irradiation in the aging of humans. Sinex (35) postulates thermal denaturation as the insolubilizing influence. This would appear less likely, because thermally denatured proteins generally are digested by enzymes and should thus be remobilized by the organism, whereas this is not true of cross-linked proteins in which the cross-linkages have been placed so as to block the enzymal points of attack.

Although many reactions can effect the immobilization of proteins, it would appear that cross-linkage is likely to be the most important of these from the practical standpoint, both because of the known presence of substantial quantities of cross-linking agents as metabolites in normal human blood, and because no other known reaction can lead to the insolubilization of such large quantities of protein with a minimum amount of reactant.

The role of ionizing irradiation in the aging syndrome at the molecular level

may be summarized by quoting one brief statement from a recent publication of Henshaw (16): "At the present stage of understanding, a final choice cannot be made between mutation and accumulation of biochemical impediments as a basis for aging—radiologic or natural—nor indeed can it be said that these are the only possibilities. It would appear that these possibilities are not mutually exclusive, and that the evidence in favor of accumulation of immobilized protein as a cause of aging, is mounting rapidly."

SUMMARY

Whole body X-irradiation has been used to accelerate the onset of senescence in female white rats. Comparisons of non-irradiated and irradiated tissues have been made using histologic, physical, and enzyme techniques.

Conditions of irradiation were established under which rats receiving a total of 1890 r showed a minimum of specific adverse effects.

The data amassed to date indicate that X-irradiation causes a reduction of 20.6 per cent in the mount of alpha-amino nitrogen liberated by tryptic hydrolysis in liver homogenates.

Determinations of collagen shrinkage temperature in irradiated rat-tail tendons indicated that irradiation results in cross-linking of proteins in a manner resembling that observed in non-irradiated mature tendons. Apparently irradiation also severed peptide bonds in the collagen, which complicated interpretation of the data.

It is postulated that these observations may be useful both for a study of collagen structure and also as a clinical method for determining the amount of irradiation absorbed accidentally.

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# CHEMICAL MECHANISMS UNDERLYING THE BIOLOGICAL MECHANISMS OF THE AGING PROCESS

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In a recent paper, Curtis (1) discusses the biological mechanism of somatic mutations as an underlying factor of aging. It appears particularly timely, therefore, to discuss and present some new data having a bearing on the chemical mechanism which in turn underlies this biological mechanism.

## CROSS-LINKAGES IN AGING

In 1941 the senior author (2) pointed to the probable significance of cross-linkages in aging. In 1942 (3, 4) an hypothesis was presented that aging is due to irreversible cross-linking between macromolecules. The majority of such cross-linkages are correctable, particularly in rapidly metabolizing cells where cathepsins will usually break down the cross-linked material and resynthesize the molecules in their native active state. Cross-linkage has an irreversible effect, however, when it happens to form at a point which blocks enzyme action, or at a time when a key event in cell-life takes place (such as an anaphase just after the parallel chromosome DNA molecules have been cross-linked to each other).

In his initial papers Bjorksten discussed cross-linkage in the context of protein chains, though the same cross-linking reactions obviously apply to the nucleic acids which also contain amino groups and nucleophilic centers. Haddow and associates (5) in 1948 pointed out that the tumor growth-inhibiting activity of the nitrogen mustards was confined to polyfunctional compounds only, and that bifunctional cross-linking compounds were fifty times more active for the production of chromosome breaks than were monofunctional compounds (6). This led Goldacre and co-workers (7) to postulate that alkylating agents join chromosome threads together by a co-valent cross-linkage prior to mitosis. Alexander and Lett (8) showed that cross-linking of DNA occurs in contact with bifunctional nitrogen mustards.

## EFFECTS OF RADIATION

That the so-called radiomimetic substances are bifunctional substances capable of cross-linking is thoroughly discussed by Alexander (9) in his review of the reactions of carcinogens with macromolecules.

Charlesby (10) reported that ionizing radiation readily causes cross-linkage in large molecules, evidently by the preliminary formation of free radicals. Thus if cross-linkage is a mechanism in aging, it is to be expected that ionizing radiation would cause a similar phenomenon. As Curtis and others have pointed out, this is indeed the case. The lack of complete identity between irradiation and natural aging may be explained in part by the fact that the ionizing radiation also causes chain fission which leads to another series of reactions, quite different from cross-linkage.

Cross-linkage due to the free radicals generated by ionizing radiation will occur with any large molecules which have reactive side groups, and the amino groups and nucleophilic centers of nucleic acids are fully available for this purpose, as are the corresponding groups in proteins. Alexander's work has shown that nucleic acids are indeed cross-linked by substances analogous to known metabolites, and Curtis' report of bridged chromosomes in aged liver cells would appear to confirm that DNA frequently is cross-linked in these cells.

That the proteins in the liver cells of rats also are cross-linked by radiation is made apparent from the following series of tests, which are a continuation of those previously published (11) in that the 150-week old animals were recently sacrificed and their analytical data added, thus giving the correlation between irradiation and advanced aging under the conditions of this work.

A method of trypsin hydrolysis was developed so that levels of protein immobilization could be compared in the various test groups. First, several kinetic studies on young rat liver were conducted to determine the optimal concentration and hydrolysis time for Difco trypsin (1:250). Then rat liver homogenates

TABLE 1

*Liberation of Alpha-Amino Nitrogen in Irradiated and Nonirradiated Rats after Incubation of Liver Homogenates with Trypsin**

(Values are based on 0.1 gm of liver wet weight.)

| | Number of Animals Sacrificed | Mg. Nitrogen Liberated (avge) | Reduction in Solubilized Protein (%) |
|---|---|---|---|
| 36-week old control rats | 14 | 1.99 | 0.0 |
| 150-week old control rats | 10 | 1.57 | 20.6 |
| 36-week old irradiated rats (1890 r) | 11 | 1.86 | 6.5 |
| 24 to 36-week old irradiated rats (3591 r) | 16 | 1.58 | 20.6 |

* A 10-ml sample of liver homogenate (1.0 gm of liver) was mixed with 50 ml of Sorenson's buffer (pH 7.7) and 20 mg of Difco trypsin (1:250) in 125-ml Erlenmeyer flasks. Each sample was set up in duplicate with one reaction mixture precipitated immediately upon mixing with 20 ml of 20% trichloroacetic acid. The contents of the second flask were shaken for twenty hours at 38°C and then precipitated with 20 ml of 20% trichloroacetic acid. Both samples were filtered and washed with 5% trichloroacetic acid into 100-ml volumetric flasks. The filtrates were used for alpha-amino nitrogen determinations with the Van Slyke apparatus. Each value for liberated nitrogen listed above has been corrected by subtracting the ml of $N_2$ for the control sample from the ml of $N_2$ liberated after hydrolysis before the milligram values were calculated.

from both irradiated and nonirradiated animals of the same age were incubated with the enzyme under the controlled conditions enumerated in Table 1.

After incubation with the enzyme, trichloroacetic acid filtrates were prepared and analyzed for liberated amino nitrogen by use of the Van Slyke alpha-amino nitrogen apparatus. If cross-linking or immobilization of protein occurs in natural or accelerated aging, it would be expected that less amino nitrogen would be liberated during tryptic hydrolysis. This fact is borne out in the experimental data amassed to date.

The values for liberated nitrogen in 10 animals given 95 r of whole-body radiation per week (total dose 1890 r) and in 14 nonirradiated animals were compared statistically and the means were found to be significantly different at the 75 per cent probability level based on the distribution test with 16 degrees of freedom.

The nitrogen values obtained in the 189 r per week group of animals (total dose, 3591 r) were significantly different from those in the controls at the 99 per cent probability level. The enzyme data, as summarized in Table 1, clearly indicate that irradiation modified the liver protein in a manner strongly suggestive of cross-linking.

It is noteworthy that the degree of insolubilization of rat liver proteins caused by irradiation to the threshold of death almost exactly coincided with the insolubilization caused by aging to the same point of nonsurvival.

## CHEMICAL EVIDENCE

The numerous reports on indirect evidence of cross-linkage by progressively increasing molecular weight and decreasing swellability and elasticity have been previously summarized (12) and therefore need not be repeated.

Direct indications of cross-linkage were obtained by utilizing the observation of Katz (13) that anhydrous hydrogen fluoride dissolves a range of proteins without even destroying the activity of enzymes recovered from such solution but that the cross-linking agent, formaldehyde, causes insolubilization. The use of anhydrous hydrogen fluoride as a solvent to separate cross-linked from noncross-linked biologically active giant molecules seemed particularly attractive because of the possibility it offered of working at −78°C, where secondary reactions were unlikely to occur. Since the reagent and the conditions of its use are anhydrous, hydrolytic damage can be ruled out.

Table 2 shows that a series of normally noncross-linked proteins and two nucleic acids were all soluble in anhydrous hydrofluoric acid, but after cross-linkage with either of the two rapid cross-linking agents (formaldehyde or parabenzoquinone) were insoluble in the reagent. Protein isolated from the aged (average, 71 years) human heart contained an insoluble fraction which was likewise insoluble in anhydrous hydrofluoric acid (14).

As shown by Verzár (15), collagen is cross-linked as age progresses, co-valent bonds being particularly increased with aging (16).

Thus there is evidence for the accumulation of irreversibly altered, nonexcretable proteins in nondividing aged cells, as there is for nucleic acids in such

TABLE 2

*Solubility of Proteins and Nucleic Acids and Their Derivatives in Anhydrous Hydrogen Fluoride*

| Test Compound* | Solubility in Anhydrous Hydrogen Fluoride** | | | | | | |
|---|---|---|---|---|---|---|---|
| | Native controls | Denatured Proteins | | Acylated Proteins | | Cross-Linked Protein | |
| | | Urea 8M | TCA 5% | Hydroxy-ethyl der. | Thiohy-dantoin der. | Formal-dehyde | p-Benzo-quinone |
| Lysozyme | + | + | + | +(gel) | + | − | − |
| Edestin | + | − | + | +(gel) | + | − | − |
| Trypsin | + | + | + | ± | ± | − | − |
| Pepsin | + | + | + | + | + | − | − |
| Bovine serum albumin | + | − | + | +(gel) | + | − | − |
| Protamine sulfate | + | + | + | + | − | 0 | − |
| β-Lactoglobulin (bovine | + | + | + | + | + | − | − |
| Ovalbumin | + | + | + | +(gel) | + | − | − |
| Na-Desoxyribonucleate | + | + | N.T. | N.T. | N.T. | 0 | − |
| Na-Ribonucleate | + | + | N.T. | N.T. | N.T. | 0 | − |

* All pure compounds were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

** (+) indicates that sample is soluble; (−), that it is insoluble; (±), that it is partially soluble; (0), that the sample could not be isolated by acetone precipitation; (N.T.), that the sample was not tested.

cells. On the basis of facts now at hand, it is hardly possible to state which of these is the more important in causing senility and death. In any event both are secondary results of the cross-linking process which involves vital mechanisms, including but not limited to enzymes and chromosomal material. Cross-linking is the only known process by which extremely small quantities of any one of many known metabolites at the wrong place and time can, and indeed do, impair or destroy the function of two giant molecules.

Curtis uses the word "mutation" with reference to nondividing cells. The use of this word with reference to changes which thus cannot be propagated seems unusual. It would appear that Curtis is straining this term in an effort to express in the idiom of genetics something that has already been expressed by Alexander and co-workers and by Bjorksten: that nucleic acids in irradiated or old cells undergo changes which render them nonfunctional. We do not question Curtis' conclusion that chromosome damage occurs in nondividing cells on aging, since cross-linking has been shown to occur both with nucleic acids and proteins. However, Curtis' experimental data would have been more definitive had he employed a monohalo-substituted substance as the necrotizing agent to provoke cell division instead of a polyfunctional cross-linker such as carbon tetrachloride.

Cross-linking agents, present in all cells, act indiscriminately with reactive groups on both proteins and nucleic acids. Where turnover is rapid, most of the cross-linked molecules will be catabolized and resynthesized in their original

state. However, with 2.4 to 19.2 $\times$ $10^{19}$ molecules of potential cross-linkers at large in the body (17), every known type of cross-linkage will be among those formed, and some of these cannot be broken down by body enzymes. (18-21).

## COMMENT

Whether the irreversibly cross-linked proteins or the similarly changed nucleic acids exert the major influence toward the ultimate destruction of the cell is a moot question. In either event, however, cross-linkage is the primary step.

This gives us a somewhat more hopeful outlook on the possibilities of eventually counteracting the aging process than does the concept of a purposely built-in destructive mechanism.

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## Protein Insolubilization

The results of these studies, coupled with Medvedeva's data on progressive insolubilization of proteins, and with our tests with solubility in anhydrous hydrogen fluoride as an indication of covalent cross-linkage, which follows, was interpreted as provisionally justifying proceeding to the next task without delay.

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# Anhydrous Hydrogen Fluoride as a Tool in Studying Cross-Linkages in Proteinaceous Substances Accumulating with Age

*A Preliminary Report* *

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## Introduction

This study is based on the concept that cross-linkage of tissue proteins and nucleic acids initiates the chain of events known as "*aging*". This cross-linkage takes place continuously in all cells and insolubilizes viable molecules to the point of withdrawing them from their metabolic function. The resulting insolubilized molecules are thus made permanently immobile within the cells and can be neither excreted nor replaced with functioning molecules. This leads to the progressively reduced ability to function of all cells, which we know as "*aging*". (1, 2).

While overwhelming indirect evidence has been presented favoring this theory (3), there has not been any direct evidence

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that cross-linkage actually occurs in the proteinaceous substances accumulating in aging tissues. The object of this study is to furnish such proof.

J. J. Katz has demonstrated the solubility of proteins in anhydrous hydrogen fluoride and has shown that this is a true solvent action, not decomposition of any kind. Hydrolysis of course could not occur in the complete absence of water, and the true solvent nature was confirmed by the fact that enzymes dissolved in hydrogen fluoride upon precipitation retained their activity. (4). Katz also pointed out that, in some instances, cross-linked proteins were not soluble in anhydrous hydrogen fluoride in contradistinction to noncross-linked proteins.

In the present study, we have undertaken to test this concept on a number of native proteins (in uncross-linked state) with their cross-linked counterparts and have included insoluble heart proteins from five subjects, as well as three prolifuscin (*"age pigment"*) preparations.

## Experimental

Eight purified proteins and two nucleic acids * were used in this study. Each compound was tested for HF solubility before and after cross-linking with an aldehyde, a metal, an epoxide and a quinone. Each protein was also tested after acylation with ethylene oxide and phenyl isothiocyanate.

### *Methods and Materials*

#### *1. The test substances*

These included sodium desoxyribonucleate, lysozyme, edestin, trypsin, pepsin, bovine albumin, protamine sulfate, b-lactoglobulin, sodium ribonucleate and egg albumin.

#### *2. Aldehyde cross-linkage*

25 mg. of each test protein listed above were weighed into test tubes and 5 ml. of 10 % aqueous formaldehyde solution were added with shaking. The samples were allowed to cross-link for 22 days at room temperature, after which they were precipitated in acetone. The precipitates were filtered, washed with ether and then held in a $CaCl_2$ desiccator at room temperature *in vacuo* until tested for solubility. The nucleic acids and protamine sulfate failed to precipitate in acetone.

#### *3. Metal cross-linkage*

25 mg. of each test compound were weighed into test tubes

* Obtained from Nutritional Biochemicals, Inc., Cleveland, Ohio.

and 5 ml. of basic chromium sulfate ("*Tanolin W2XD*,"* 2mg./ml.) at pH3 were added with shaking. After 22 days at room temperature, each sample was prepared for solubility testing as already described. Only the protamine sulfate failed to precipitate.

*4. Epoxide cross-linkage*

25 mg. of the test compounds were weighed into test tubes and 5 ml. of 5 % epichlorohydrin (Shell) in 95 % ethanol were added with shaking. After 22 days reaction, the samples were dried under reduced pressure and held *in vacuo* over $CaCl_2$.

*5. Quinone cross-linkage*

25 mg. of the ten test compounds were weighed into test tubes and 5 ml. of ½% p-benzoquinone in Sørensen's buffer (pH7) were added with shaking. This group of samples was reacted 40 days before acetone precipitation and storage.

*6. Phenyl isothiocyanate acylation*

25 mg. of each test protein were reacted with phenyl isothiocyanate (Eastman) to form the thiohydantoin derivatives according to the method of Edman. (5).

*7. Ethylene oxide acylation*

25 mg. of each test protein were weighed into test tubes and 5 ml. of 1 molar ethylene oxide (Eastman) in 95 % ethanol were added with shaking. After 14 days reaction, the hydroxyethyl derivatives were prepared for solubility testing as described above.

*8. Insoluble heart proteins*

Five hearts from aged humans were fractionated with water, dilute potassium chloride and sodium hydroxide according to the method of Robinson. (6). The insoluble residue was then acetone extracted and dried *in vacuo*.

The proteinaceous nature of these insoluble heart fractions was verified by Kjeldahl nitrogen determinations in two instances (see Table 3). In addition, one sample (Sample 5, Table 3) was hydrolyzed with 3N hydrochloric acid and the hydrolyzate submitted to descending paper chromatography using butanol: acetic acid: water (4:1:4). Upon spraying with "*Ninhydrin*" eight spots with Rf values ranging from 0.31 to 0.97 developed.

The prolifuscin samples were prepared from the heart of a 69-year-old female who had died from leukemia and from a 50-

* Diamond Alkali Company, Kearny, New Jersey.

year-old male who had died of carcinoma. The pigments were isolated as described previously. (7, 8).

All of the insoluble heart protein fractions fluoresced under the ultraviolet microscope, though not as intensely as the prolifuscin preparation. (8).

The history and further data on the hearts from which the insoluble protein fraction were prepared are given from Table 3.

*9. Solubility test procedure*

Approximately 10 mg. of each native protein, as well as of the substances which had been cross-linked or acylated as described above, and of the insoluble protein fractions from hearts, were removed from the desiccator and placed in threaded tubes of transparent poly(trifluorochloroethylene) "*Kel-F*" having a bore of 8 mm. diameter and a depth of 60 mm. The Kel-F tubes containing the samples were dried in 0.5 mm. vacuum for 16 hours at a temperature of 25°C over $CaCl_2$ as desiccant. The tubes containing the samples were then cooled to —78°C and were attached each in turn to a cylinder of anhydrous hydrogen fluoride* by means of threaded refrigerator connectors. The HF line was swept free of moisture with dry nitrogen prior to attachment of the sample. Approximately 1 ml. of anhydrous hydrogen fluoride was condensed into the tubes containing the substances under test. The tubes were then removed from the lines, quickly capped and allowed to warm gradually to about 25°C in two hours. All of the control, cross-linked, and acylated compounds were observed for solubility after two hours. The heart fractions were observed after being held 16 hours in the anhydrous hydrogen fluoride.

## Results

The solubility data for the eight proteins and two nucleic acids, both native and cross-linked, are summarized in Table 1 below. All of the ten compounds in the native (noncross-linked) state dissolved quite rapidly in anhydrous hydrogen fluoride at —78°C, as was expected in view of Katz's findings referred to above.

None of the formaldehyde or p-benzoquinone cross-linked samples was soluble in HF although the tubes were slowly warmed for two hours to 25°C.

The much milder cross-linking agent, epichlorohydrin, and the metal cross-linker, chromium sulfate, which has a very high electronic affinity to HF, gave, in some instances, reaction pro-

* Obtained from The Matheson Company, Joliet, Illinois.

*Table 1.* Solubility of Native and Cross-Linked Proteins and Nucleic Acids in Anhydrous Hydrogen Fluoride at 25° C

| Test Substance | Solubility in Anhydrous Hydrogen Fluoride | | | | |
|---|---|---|---|---|---|
| | Control[a] | Formaldehyde[b] | Basic Chromium Sulfate[b] | Epichlorohydrin[b] | p-Benzoquinone[b] |
| Na-desoxyribonucleate | + | 0 | — | + | — |
| Lysozyme | + | — | — | — | — |
| Edestin | + | — | ± (gel) | — | — |
| Trypsin | + | — | ± | — | — |
| Pepsin | + | — | ± | ± | — |
| Bovine albumin | + | — | — | — | — |
| Protamine sulfate | + | 0 | 0 | + | — |
| b-Lactoglobulin (bovine) | + | — | + | — | — |
| Na-ribonucleate | + | 0 | — | + | — |
| Egg albumin | + | — | + | — | — |

a — All samples dissolve quickly at —78° C.
b — Solubility of the cross-linked preparations was read after two hours at approximately 25° C.
(+) indicates that sample is soluble; (—) that it is insoluble;
(±) that it is partly dissolved; (0) that the sample could not be isolated by acetone precipitation and was therefore not tested for solubility in HF.

ducts in HF, as shown in the table. This is further discussed below.

The solubility data for the hydroxyethyl and thiohydantoin derivatives of the test compounds are summarized in Table 2. All of the thiohydantoins with the exception of protamine sulfate and part of the trypsin dissolved completely in HF. The hydroxyethyl derivatives dissolved more slowly and several of the derivatives swelled greatly before dissolving in the HF.

*Table 2.* Solubility of Acylated Proteins in Anhydrous Hydrogen Fluoride at 25° C

| Test Substance | Solubility in Anhydrous Hydrogen Fluoride | |
|---|---|---|
| | Hydroxyethyl Derivative | Thiohydantoin Derivative |
| Pepsin | + | + |
| Lysozyme | + (gel) | + |
| Trypsin | ± | ± |
| Edestin | + (gel) | + |
| Protamine sulfate | + | — |
| Bovine albumin | + (gel) | + |
| b-Lactoglobulin | + | + |
| Egg albumin | + (gel) | + |

As shown in Table 3, all of the insoluble protein fractions isolated from human heart, as well as the prolifuscin samples, were completely insoluble in anhydrous HF, even on standing 16 hours at 25° C.

*Table 3.* Insoluble Protein Fractions from Heart

| Sample No. | Age at Death | Sex | Kjeldahl Nitrogen | Cause of Death | HF Solubility * |
|---|---|---|---|---|---|
| 1 | 54 | F | 15.8 + | Cerebral hemorrhage | Insoluble |
| 2 | 64 | F | — | Carcinoma | Insoluble |
| 3 | 82 | F | 15.4 | Pneumonia | Insoluble |
| 4 | 72 | F | — | Stroke | Insoluble |
| 5 | 51 | M | — | Cerebral hemorrhage | Insoluble |
| 6 ** | 69 | F | — | Leukemia | Insoluble |
| 7 ** | 50 | M | — | Carcinoma | Insoluble |
| 8 *** | 51 | M | — | Cerebral hemorrhage | Insoluble |

* Solubility was read after 16 hours at approximately 25° C.
** Prolifuscin.
*** A dehydrated mitochondria preparation of Sample 5.

In a further experiment it was found that a freeze dried, twice-recrystallized hemoglobin was insolubilized in HF after being in contact with 100 mg. of autoxidizing methyl linoleate (dry $O_2$ atmosphere) for six weeks at room temperature. The control, treated identically except for the exclusion of methyl linoleate, was instantly soluble in HF.

## Discussion

It is noted that all of the proteins and nucleic acids studied were readily soluble in HF in the native state, and that none of them was found soluble after cross-linkage with formaldehyde or p-benzoquinone.

The fact that certain of the proteins cross-linked with chromium sulfate and epichlorohydrin are soluble does not detract from the general trend of the data. Chrome tannage depends upon formation of electrostatic bonds which may not necessarily be stable in the strongly electronegative HF in all instances. Epichlorohydrin as a protein cross-linking agent is relatively unknown outside the realm of collagen chemistry. It is possible that the cross-linking properties of this agent depend heavily upon a certain stereochemical configuration, which is not necessarily met in all proteins.

On the basis of the data summarized in Table 1, it is concluded that insolubility of a protein or nucleic acid in anhydrous hydrogen fluoride evidences that the material is cross-linked.

However, solubility in anhydrous hydrogen fluoride does not necessarily preclude some milder degree of cross-linking or some other chemical alteration of a protein.

On the basis of the data of Table 3 taken in view of those in Tables 1 and 2, it is shown that the insoluble proteins from aging heart tissues as well as the prolifuscins (*"age pigments"*) are firmly cross-linked, for none of these materials was at all soluble in HF, even though the solubilization time was extended to over 16 hours.

The utility of anhydrous hydrogen fluoride as a fractionating solvent for separating cross-linked from noncross-linked protein or nucleic acid is established. With this reagent it is now possible to obtain from tissues purified cross-linked proteins devoid of native or denatured materials which have previously hindered characterization of the cross-linking agent.

## Summary

The solvent effect of anhydrous hydrogen fluoride on native cross-linked and acylated proteins and nucleic acids, and on insoluble protein fractions from human hearts, has been studied.

Anhydrous hydrofluoric acid dissolves all of a wide range of native and acylated proteins and the two nucleic acids studied. Hydrofluoric acid does not dissolve firmly cross-linked proteins or nucleic acids, nor insoluble protein fractions isolated from human heart. On the basis of this study it is concluded that the insoluble prolifuscin or *"age pigment"* in aged heart tissue is composed of cross-linked protein. Further it is established that an additional quantity of colorless cross-linked protein is also present in the hearts of old persons.

Anhydrous hydrogen fluoride can be used as a fractionating solvent for separation of cross-linked from noncross-linked proteins and nucleic acids.

## Acknowledgment

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A Rapid Detection of Physiological Aging Effects

In any research, the speed of progress is limited by the speed of available assay methods. A year was spent on study of methods then available. Among the more promising were: skin fold resiliency tests, speed of recovering temperature balance after heating or cooling determined by no-lag high speed surface temperature radiation sensor systems, changes in the accommodation of the eye. None of these met the goal of immediate indication of any change in aging rates.

We attempted work with short lived insects but were soon discouraged by the lack of correlation between the insect data and the facts we already knew from observations with humans. Tests with small mammals were likewise not representative of human longevity, nor would they be sufficiently fast even if they were.

The solution of this need was found and reported in the following paper which summarizes work started 10 years earlier.

# A METHOD FOR DETERMINING THE GEROLYTIC EFFICACY OF ENZYMES

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**SUMMARY :**

A quick method is presented for determining the utility of treatments for removing normally insoluble gerogenic aggregates. Assay animals are used which received at the time of their birth large amounts of radiotracer. At the age of 600 days or later substantially all of the radioactivity is gone, except that which has become firmly fixed in normally immovable gerogenic molecules. The animals are now ready for assay. Any injected substance which can cause remobilization and excretion of the bound radioactivity will be apparent in a matter of minutes or hour from the increase in radioactivity in urine, exhaled air or faeces.

**DETAILS :**

The **time** required for assay has been a principal limiting factor for progress in Gerontology. Our knowledge of vitamins made a quantum jump when it became possible to substitute spectrochemical recognition and quick bacterial assays for the tedious bioassays previously necessary.

The spectacular progress of electronics science and technology has been possible only because once a proposed system has been assembled, closing a contact and reading a meter may suffice to tell whether it functions or not.

A principal target problem of Bjorksten Research Foundation has been to find similarly rapid means of recognition whether a system proposed to artificially extend the specific lifespan of mammals will, or will not function.

In **1957** Jos. Still[1] established that radioactivity introduced in certain organs is not completely excreted, but that some part of it remains locked into the frozen metabolic pool of insolubles as postulated by Zinsser et al.[2]. Bjorksten and Asham[3] confirmed this finding, and showed it to be true even of organs having a high degree of metabolic turnover, such as the liver. Whether these insolubles had been formed by crosslinkage, as postulated by Bjorksten[4] or by some other mechanism, it is clear that they are detrimental and that a lysis of them is desirable to remove impediment to intracellular transport and to cellular function generally. To probe candidate substances it would obviously be desirable to have available a rapid test capable of detecting quickly and with precision even those small effects which often provide the critical clues.

For this purpose, we prepared assay animals which contained radioactive components fixed in non-metabolizable gerogenic aggregates. An effective means to solubilize such aggregates will then be quickly recognizable by radioassay.

The time required for such an assay will be the time needed for the test animal to urinate, plus about 10 minutes for a scintillation count of the urine to detect any sudden strong release of the radioactivity. If the radioactive tracer is tritium, it may be detectable even sooner in the exhaled air. While the preparation of the assay animals is slow and expensive, each animal can be used many times for assay, and the assay time will be negligible compared to adequate assay for **maximum** life span of even the smaller mammals.

The preparation of the test animals has been described by Borksten and Ashman[3], p. 116, and ([5]), Bjorksten et al., p. 562. A pregnant rat was given 40 **milli**curies of tritium as acetate, and another 8 millicuries of tritium in tyrosin, beginning 7 days before giving birth, and ending 6 days after birth, thus with the lactation period.

The litters born thus received huge amounts of radioactivity from their mother. They never received any added radioactive material and were allowed to live out their life spans. Most of the animals lived at least 700 days after which most of them died from tumors. The tumors were free from detectable radioactivity, thus, the animals at the time of tumor formation had excreted rather completely the radioactivity they had received and were ready for test.

Substantial radioactivity was trapped in these animals in non-catabolizable molecules. During their lifespans we did not find any gerolytic substance capable of administration so as to cause a sudden release of the captured radioactivity.

Due to the cessation of funding, we could not prepare new animals for this assay. However, the carcasses of these test animals were dissected, the organs separated, and to the extent available funds permitted, were used to determine the distribution and disposition within the animal organisms of the compounds still retaining tagged molecules, known to have been introduced into the animals paranatally. The results have been partly published in the communications cited which also describe the methodologies employed.

Summarizing, the finding were : The amino

acids present in the insoluble nitrogenous fractions of the liver contained only the following amino acids, in the following percentages of the total radioactivity in the insoluble nitrogenous residue studied; Lysine 29.2%, Arginine 20.1 %, Aspartic acid 20.1 %, Glutanic acid 17.7 % and Serine 9.7 % and ether soluble substance not observed prior to destructive strong acid hydrolysis = 3.2 % (p. 121-122).

It is noted that these amino acids all have side chains, with a reactive end group.

The highest concentration of the tagged molecules at death was present in the brain.

The largest quantity of the tagged molecules was present in the muscles, because the total mass of the muscles greatly exceeded that of the brain.

Every organ tested contained some tagged molecules.

Neoplasms formed in the animals were totally free of radioactivity within the limits of detection by our instruments. The absence of proline and oxyproline indicates that collagen may not be a part of the most refractory component of the frozen pool of immovable molecules.

Components of both RNA and DNA were found in the insoluble fractions, co-valently associated with the radioactivity, RNA being by far the larger quantity (3) pp. 566-573. Such compounds comprising co-valent binding of amino acids with protein and nucleic acid had been reported by Yamamoto[6].

A substantial though not a major proportion of the radioactivity in the brain was extractable by chloroform-methanol. This points to the existence of immobile phospholipid derivatives. We should be particularly interested in exploring these in further details when funds for such research become available. We still have in deep freeze carcasses of several test animals.

Our main intent was to build up a source of assay animals to make possible instant detection and evaluation of even minute degrees of lysis of age dependent accretions formed by crosslinkage of large molecules and sterically inaccessible to normal lysis. The other observations were incidental.

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Frozen Metabolic Pool

The above rapid detection method served us well, and enabled us to quickly eliminate many seemingly plausible ideas so that we could keep on the main track with a minimum of diversions.

However, finally our assay animals were dead and we lacked the funds to start a new colony. However, the carcasses offered investigative opportunities. The remaining tritium obviously was contained in those components, which were stable, with near zero turnover. This is the "frozen metabolic pool" referred to by Zinsser, Bjorksten et al. 1962 (pages 45-68, particularly pp. 62, 65 and 67).

As can be seen in the subsequent two papers the main portion of this tritium was found in nitrogenous compounds which could not be solubilized without decomposition. The brain contained by far the highest concentration of these; however, the greatest total quantity was in the muscles. But even the liver, in spite of high turnover rate, contained a portion of these stable molecules in which some tritium, administered paranatally, could be found three years later. It contained no fatty acid in range $C_{10}$ - $C_{26}$. Infrared spectrum suggested the presence of carbonyl, aromatic structures and steroids.

Medical and Clinical Aspects of Aging
Edited by Herman T. Blumenthal, 1962
Columbia University Press
New York and London

# The Freezing Pool:
# A Unified Sequence of the Aging Process

HANS ZINSSER, JOHN BJORKSTEN, E. M. BRUCK, RICHARD F. BAKER, LESLIE KAEBURN, JEAN KINNEAR, ANNA COHEN, FREDERICK ANDREWS, IZAK SARFATI, AND IGO LIGHT

THE cardinal feature of the aging process represents an inability to adapt to variations in external conditions, physical, chemical, and psychological. From this point of view the early embryonic organism and the organism outclassed in physical dimensions by its surroundings are equally vulnerable. But this concept of loss of flexibility applies with all its implications to a steady-state process whose rate is a slow one rather than a rapid one. Substantial rearrangements in structure and in physiology occurring as repeated effects of Le Châtelier's principle result in an increasing structural and biochemical conservatism which progressively builds up structures and processes that we consider biological insult residua. Each adjustment taking place removes one degree of freedom for subsequent readjustment, so that further rigors or new environments find the aging subject not only unwilling but incapable of making adjustments.

On a macroscopic scale the phenomenon can be duplicated in the mechanical production of a felt hat. Here the fibrils with definite elasticity can be pounded by mechanical treatment, softened by heat treatment, compacted and fractured, tanned and intertwined so as to produce adaptation to the shape of the hatter's mold. The chemical structure of the individual fibrils can be affected by indigenous and exogenous radiation and the individual fibrils coated with materials protective against a variety of agents. The subsequent reblocking of this arrangement of wool fibers to duplicate the hat can be readily managed by the capable hatter. Reconstitution of this felt, however,

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to the free and adaptable structure which was present before the felting process took place is no longer feasible. Plastics, like wool, can be subjected to stresses which will produce long-range order, crystallinity, and crazing that may eminently fit them for their immediate purpose but remove from them the capacity to withstand mechanical or thermal shock which the native, unformed plastic originally had.

This is not true of the immature organism. In the young organism the expanding physical and chemical dimensions of the metabolic pool allow progressive dilution of exogenous agents which would tend to immobilize this metabolic pool. As the human reaches the age of thirty there is a progressive saturation of adaptive capacities, as shown in mortality plots, in the x-ray diffraction pattern of the connective tissue, in the drop-off of fertility, and in the capacities for renal and hepatic regeneration after injury reminiscent of the time sequence in the tanning of gelatin in the formation of lithographic printing plates.

The progressive hypertrophic responses of some body tissues to the influence of metabolizable toxic materials or to work loads, as seen in the myocardium and in the bladder, have a counterpart in the development in specialized tissue by successive mutations of new rapidly growing generations capable of resisting the toxic effects of butter yellow, for instance, aiming always to preserve a mutable chemical pool for further adaptive reserve at the expense of other functional capacities. This results in many instances in the dominance of the senescent conservative regulated tissue by rejuvenated unregulated and sometimes malignant hypertrophy.

Rather than the balance early predicated between rejuvenescence and senescence which were equilibrium concepts, we must think of a continuous attempt by the body to maintain the status quo in a real chemical processing sense as it continues to stay metabolically on stream. In this process, body substances susceptible to thermal and other types of radiation are denatured in their intimate chemical components, and it is the effects of this progressive exclusion from the general metabolic pool that will concern us primarily in some isolated instances.

## *MODES OF LOSS OF ADAPTIVE CAPACITY*

The simplest way in which this loss of adaptive capacity can be generated is by the slowing of the overall metabolic process so that greater and greater swings become necessary in the rates of associated phenomena to maintain the status quo. Little wonder then, that with

diminution in thyroid function the aging process becomes accelerated and with the diminution in overall body metabolism subsequent to premature ovarectomy, that similar phenomena occur.

As soon as the rate of destruction of tissue becomes more rapid than the rate of tissue regeneration, detritus and fragments of previously useful material litter the body like the pillage of armies left behind in the wake of conquest. The accumulation of fatigue pigments in the myocardium, of pigments in the liver, of connective tissue overgrowth, of insoluble lipid components, of incompletely calcified osteoid in the bone that waits like a bride at the church door for the calcium that never comes is evidence of the slowing of the chemical stream. The progressive insolubilization of otherwise easily metabolized materials due to a variety of processes and failure of specialized repair.

We may predicate many of the mechanisms active in this insolubilization. Successive antigen antibody formation in locations where resolubilization is difficult, the production of enzymes whose specificity for attack remains undaunted but whose ability to destroy the substrate is failing will tend to block the normal metabolic pathways. Successive accretion on the surfaces of discrete structures of the fibrils, cell walls, and mitochondria can block adequate transportation and translocation of smaller weight substances all the way down to the water molecule. The intimate relationship of enzymes tending to increase molecular weight in normal fashion as against those tending to depolymerize may result in abnormal gel to fiber ratios in interstitial spaces in unusual mucopolysaccharide molecular weight distributions, and can permanently clog detoxification and excretion avenues that can result in the fatal accumulation of a variety of agents.

Recently, we have put forward several statements of the degree of crosslinking demonstrable in tissues as manifested by the unavailability of some amino acids to enzymatic digestive methods as age progresses, by the duplication of mechanical properties with a variety of crosslinking agents such as acrylonitrile and radiation, and the progressive increase in metal content of many tissues which others of us have felt to be important in the progressive change in mechanical properties of aging tissue. Since 1955 we have worked in close harmony and with free interchange of information, and in many instances have studied identical tissues with multiple methods. The existence in several animals of dramatic vascular breakdown has afforded a ready tool to confirm basic assumptions regarding aging vascular tissue.

## *THE AORTIC MEDIA AS A KEY TISSUE IN THE AGING PROCESS*

### *X-Ray Diffraction*

The ready availability of fresh post-mortem human aortic media made it an attractive object for study from a variety of points of view. Preliminary experiments had shown in 1954 that as early as the age of thirty in some individuals a discrete x-ray diffraction pattern could be found in them (Tables I and II). As this pattern varies in characteristics somewhat with the direction of elastic fibers in the aorta, we were led to an investigation of the true arrangement of the elastic fibrils within it.

### *Felting of Fibrils*

Preliminary suggestions by Haas of almost ten years earlier were confirmed by successive thin sections using special elastin staining procedures, showing that there were indeed cylindrical condensations in the long axis of the aorta. These were confirmed in vitally stained aorta in dogs and rats, using azosulfadine and Prontosil. Two blocks of aortic media, one from a premature child and one from a sixty-year-old male, were subjected to serial sectioning and reconstruction by acetate tracing technique after iron staining. This led to the unequivocal impression that there was progressive condensation and felting of the elastin fibrils in the aging aorta, and it is apparent now that many of the elastic phenomena associated with aging aortic media are associated with little more than increasing mechanical complexity of the elastin network. Model experiments with uniformly elastic elastomers matted in tubular form would tend to bear out the contention verified independently by us and by Lansdowne by electrical analog applications, showing (*a*) that the hysteresis in the aorta resulted largely from extrafibrillar and extracellular mucopolysaccharide component, (*b*) that the collagen was so irregularly arranged as to have little directional elastic effect but rather a limiting one on the degree of extension, and (*c*) that the elastin in the dead aorta dominated the elastic behavior. Further, detailed study with a recording elastometer of our own design (Figs. 1 and 2) showed considerable variation in aortas from the same subject in location and in radial and circumferential elastic constants. In addition to progressive increases in elastic constant there is a loss in hysteresis with age, which

*Table I. Spacings found in some of the specimens*[a]

| 1 | | 2 | | 3a | | 3b | | 4a | | 4b | | 5a | | 5b | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *d/n* | *I*[b] | *d/n* | *I* | *d/n* | *I* | *d/n* | *I* | *d/n* | *I* | *d/n* | *I* | *d/n* | *I* | *d/n* | *I* |
| 3.40 | VW | 3.62 | M | 21.50 | S | 10.96 | M | 11.25 | M | 11.58 | S | 11.25 | S | 11.47 | M |
| 3.14 | VVW | 3.33 | W | 11.80 | S | 4.96 | W | 5.84 | W | 4.57 | M(D) | 5.11 | M | 5.08 | M |
| 2.85 | M | 3.00 | S | 4.98 | M | 3.31 | W | 5.26 | W | 2.76 | M | 4.58 | M | 4.48 | M |
| 2.58 | M | 2.83 | VW | 4.57 | W | 3.19 | NaCl | 4.34 | VW | 1.97 | W | 4.02 | W | 3.32 | VW |
| 2.14 | VW | 2.65 | W | 3.87 | W | 3.07 | W | 3.82 | S | | | 3.42 | M | 3.13 | M |
| 1.89 | VVW | 2.37 | VW | 3.33 | M | 2.78 | NaCl | 2.73 | VW | | | 3.15 | M | 2.98 | VW |
| 1.70 | M | 2.26 | VVW | 3.08 | M | 2.67 | VVW | 2.56 | VVW | | | 2.99 | W | 2.83 | M |
| | | 2.10 | VVW | 2.65 | VVW | 2.52 | W | | | | | 2.85 | S | 2.70 | VVW |
| | | 1.78 | S | 2.52 | M | 2.42 | VVW | | | | | 2.71 | VW | 2.53 | W |
| | | | | 2.43 | VVW | | | | | | | 2.69 | M | | |
| | | | | | | | | | | | | 2.48 | VW | | |
| | | | | | | | | | | | | 2.11 | VVW | | |
| | | | | | | | | | | | | 2.00 | VVW | | |
| | | | | | | | | | | | | 1.91 | VVW | | |

[a] Sample 1, age 71 years; fiber axis perpendicular to beam; dried in the air; natural length. Sample 2, age 80 years; fiber axis perpendicular to beam; stretched approximately 10%, dried thoroughly in the air. Sample 3a, age 84 years, rest length 6.5 mm extended 10%. Fiber axis parallel to beam. Sample 3b, same sample as 3a, extended 50%, parallel to beam. Sample 4a, premature baby (8 months) extension 10%. Fiber axis parallel to beam. Sample 4b, same sample as 4a, no extension. Fiber axis perpendicular to beam. Sample 5a, age 65 years, no extension, dried one week, fiber axis perpendicular to beam. Sample 5b, same as sample 5a, 10% extension, dried two weeks. Sample 6a, age 32 years, no extension, dried one week. Fiber axis perpendicular to beam. Sample 6b, same as sample 6a, no extension, dried one week. Fiber axis perpendicular to beam. Sample 7, age 91 years. Fiber axis parallel. Sample 8, fiber axis perpendicular to beam. Sample 9, fiber axis parallel to beam.

Of the lower value spacings some are close to atomic distances regularly found in organic compounds, thus 2.71 A, of Table II, is close to NH · · · O hydrogen bond, 1.90 and 3.82 A close to an Al-O bond, 1.86 A close to a C-O bond. Also, the distances of 5.14 A, 3.77 A, and 1.86 A seem to fall into one pattern.

[b] d/n, spacings in angstroms; I, intensity; VS, very strong; S, strong; M, medium; W, weak; VW, very weak; VVW, very, very weak; D, diffuse.

*Table I (Continued)*

| 6a | | 6b | | 7 | | 8 | | 9 | |
|---|---|---|---|---|---|---|---|---|---|
| *d/n* | *I* | *d/n* | *I* | *d/n* | *I* | *d/n* | *I* | *d/n* | *I* |
| 11.88 | M | 16.69 | S | 10.22 | M | 5.14 | W | 3.47 | VW |
| 7.97 | W | 11.36 | M | 8.56 | M | 4.12 | | 3.40 | VW |
| 6.24 | VW | 5.92 | M | 6.03 | M | 4.04 | W | 3.16 | W |
| 5.14 | M | 5.08 | W | 5.61 | M | 4.03 | W | 2.89 | M |
| 4.67 | M | 4.54 | M | 5.11 | S | 3.37 | W | 2.81 | S |
| 3.98 | W | 4.00 | VW | 4.79 | S | 3.15 | W | 2.79 | VVW |
| 3.77 | VVW | 3.82 | M | 4.45 | S | 2.99 | VVW | 2.58 | WM |
| 3.39 | M | 3.40 | M | 4.05 | W | 2.88 | S | 2.13 | VVW |
| 3.14 | M | 3.28 | VVW | 3.68 | VW | 2.81 | S | 1.99 | VVW |
| 2.95 | VW | 3.15 | M | 3.33 | VVW | 2.73 | VVW | 1.91 | VW |
| 2.81 | S | 2.95 | VVW | 3.11 | M | 2.54 | S | 1.85 | VW |
| 2.71 | VW | 2.83 | M | 2.79 | M | 2.47 | VVW | 1.73 | VVW |
| 2.56 | M | 2.71 | VVW | 2.53 | M | 2.27 | W | 1.71 | M |
| 2.37 | VVW | 2.56 | W | | | 2.25 | W | 1.65 | VVW |
| 2.23 | WW | 2.50 | VVW | | | 1.99 | VW | 1.51 | VVW |
| 2.14 | VW | 2.11 | VW | | | 1.91 | WW | 1.43 | VVW |
| 2.05 | VW | 1.90 | VVW | | | 1.85 | W | | |
| 1.98 | M | | | | | 1.77 | VVW | | |
| 1.90 | W | | | | | 1.70 | W | | |
| 1.86 | VW | | | | | 1.68 | W | | |
| | | | | | | 1.65 | VVW | | |
| | | | | | | 1.62 | WW | | |
| | | | | | | 1.51 | W | | |

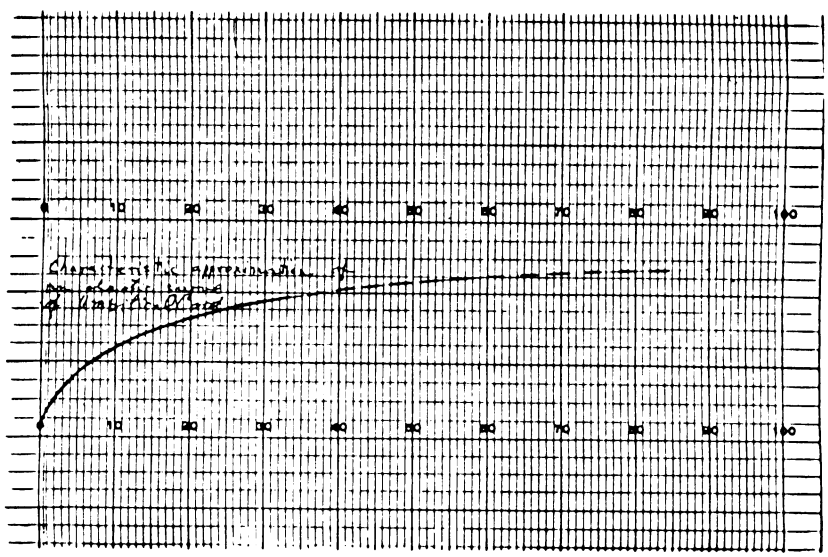


*Figure 1. An original recording of the automatic elastometer showing the type of curve obtained with it on the samples described*

*Table II*[a]. *Spacings of aortic specimens in diminishing order, compared with other materials; spacings are accounted for if they correspond to other spacings, or to multiples*

| *Aortas* | | *Glutamic acid salts* | *Scallop muscle* | *Collagen*[b] | *Ammonium oxalate* | *Al acetyl acetonate*[c] | *Spacings unaccounted for* |
|---|---|---|---|---|---|---|---|
| *Spacings* | *Intensity calcium* | | | | | | |
| 16.70 | S | | | | | | 16.70 S |
| 11.88 | M | | | × | | | |
| 11.80 | S | | | × | | | |
| 11.58 | S | | | × | | | |
| 11.47 | M+ | × | | × | | | |
| 11.36 | M× | | | | | | 11.36 M |
| 11.25 | S | | | | | | 11.25 S |
| 10.96 | M+ | | | × | | | |
| 10.22 | M | | | | | | 10.22 M |
| 7.97 | W | × | | | | | |
| 6.24 | W | | | | × | | |
| 6.03 | W | | | | | × | |
| 5.92 | M+ | | | | | | 5.92 M |
| 5.84 | W | | | | | | 5.84 W |
| 5.61 | M | | | | | × | |
| 5.26 | W | | | × | | | |
| 5.15 | | × | | | | | |
| 5.14 | W | | | × | | | |
| 5.11 | M | | | | | | |
| 5.10 | | | | | × | | |
| 5.08 | M− | | × | | | | |
| 4.98 | M | × | | | | | |
| 4.96 | W | | | | | × | |
| 4.79 | S− | | | | | | 4.79 S |
| 4.67 | W | | | | × | × | |
| 4.58 | W | | | | | × | |
| 4.57 | M | | | | | × | |
| 4.56 | W | | | | × | | |
| 4.54 | M | | | | | | 4.54 M |
| 4.45 | S | | | × | | | |
| 4.34 | VW | × | | | | × | |
| 4.26 | VVW | | × | × | | | |
| 4.05 | W | | | | | × | |

[a] The strong lines 10.2 A, 5.92 A, 5.84 A, 4.79 A, and 4.54 A remain unaccounted for by logical components in the aortas.

16.7 A is probably a diagonal reflection of the 11.8 A long chain of 1.98 metal oxygen linkages arranged linearly in pairs of which the first, second, third, and fourth multiples are present. These are apparently arranged at right angles such as to present the diagonals 2.82, 5.60, 11.28, as other multiples as well.

Similar considerations probably explain the 11.3–5 A spacing as a multiple of the 1.90–1.95 Ca-O distances of which the first multiple, and the first diagonal 2.69 A appear.

Axial orientations in aortic media have already been described.

[b] Spacings from rat tail tendon (unpublished).

[c] Sample from MacKensie Chemical Works. Possibly contaminated with iron.

*Table II (Continued)*

| *Aortas* | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Spacings* | *Intensity calcium* | *Glutamic acid salts* | *Scallop muscle* | *Collagen* | *Ammonium oxalate* | *Al acetyl acetonate* | *Spacings unaccounted for* |
| 4.04 | W | | | × | | | |
| 4.03 | W | | | × | | | |
| 4.02 | W | | | × | | | |
| 4.00 | VW | | | × | × | | |
| 3.98 | W | | | × | | | |
| 3.82 | S | | | × | × | | |
| 3.80 | | × | | | | | |
| 3.77 | VVW | | | | | × | |
| 3.62 | | × | | | | | |
| 3.47 | VW | × | | | | | |
| 3.46 | M | | × | | | | |
| 3.42 | M | | | | × | | |
| 3.40 | M | × | | | | | |
| 3.39 | M | | | | | × | |
| 3.37 | | | | | | × | |
| 3.33 | VVW | × | | | | | |
| 3.32 | VW | × | | | | | |
| 3.31 | W | × | | | | × | |
| 3.28 | VVW | | | | | × | |
| 3.15 | S | | | | | × | |
| 3.14 | VVW | | | | | × | |
| 3.13 | M− | | | | | | |
| 3.11 | VVW | | | | | × | |
| 3.00 | | | | | | | |
| 2.99 | VW | | | | | × | |
| 2.98 | VW | × | | | | | |
| 2.96 | | | | | | | |
| 2.95 | M | × | | | | | |
| 2.88 | S | × | | | | × | |
| 2.85 | S− | × | | | × | | |
| 2.83 | M | × | | × | | × | |
| 2.81 | S | | × | | | | |
| 2.78 | VVW | × | | | | | |
| 2.76 | M | | | | × | | |
| 2.73 | VW | × | | | | | |
| 2.71 | VW | × | | | | | |
| 2.70 | VW | × | | | | | |
| 2.69 | M− | × | | | | × | |
| 2.65 | W | × | | | | | |
| 2.64 | VW | | | | | | |
| 2.58 | M+ | × | | | | | |
| 2.56 | W | | | | | × | |
| 2.54 | | | | | | | |
| 2.53 | M− | × | | | | | |
| 2.52 | M | × | | | | | |
| 2.50 | W | | × | | | × | |
| 2.48 | VW | × | × | | | | |
| 2.47 | VVW | × | × | | | | |

*Table II (Continued)*

| *Aortas* | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Spacings* | *Intensity calcium* | *Glutamic acid salts* | *Scallop muscle* | *Collagen* | *Ammonium oxalate* | *Al acetyl acetonate* | *Spacings unaccounted for* |
| 2.42 | VVW | | | | | | |
| 2.37 | M | × | | | | × | |
| 2.27 | W | | | | | × | |
| 2.26 | VVW | | | | | × | |
| 2.25 | W | × | | | | × | |
| 2.23 | | × | | | | | |
| 2.22 | VVW | × | | | | × | |
| 2.19 | VVW | | | | | | |
| 2.18 | VW | × | × | | | | |
| 2.14 | VW | × | | | × | | |
| 2.13 | VW | × | | | | × | |
| 2.12 | V | × | | | | | |
| 2.11 | VW | × | | | | | |
| 2.10 | VVW | | | | | × | |
| 2.07 | V | × | | | | × | |
| 2.06 | VVW | × | | | | | |
| 2.05 | VVW | × | | | | × | |
| 2.02 | VVW | × | | | × | × | |
| 2.00 | VVW | | | | | × | |
| 1.99 | VVW | × | | | | | |
| 1.98 | M | × | | | | × | |
| 1.97 | W | × | | | | × | |
| 1.95 | M | × | | | | × | |
| 1.92 | W | × | | | | | |
| 1.91 | VVW | × | | | | | |
| 1.90 | VW | × | | | | | |
| 1.89 | VVW | × | | | × | | |
| 1.85 | VW | × | | | | × | |
| 1.78 | S− | × | | | | | |
| 1.77 | VVW | × | | | | | |
| 1.73 | W | × | | | | | |
| 1.72 | M | × | | | | | |
| 1.70 | W | × | | | | × | |
| 1.68 | W | × | | | | | |
| 1.65 | VVW | × | | | | × | |
| 1.63 | VW | × | | | | × | |
| 1.62 | VVW | | | | | × | |
| 1.59 | M+ | × | | | | | |
| 1.55 | VVW | × | | | | × | |
| 1.51 | W | × | | | | × | |
| 1.50 | VVW | × | | | | × | |
| 1.46 | VVW | × | | | | | |
| 1.44 | VVW | × | | × | | | |
| 1.43 | VVW | × | | | | | |
| 1.42 | VVW | × | | | | | |
| 1.39 | VVW | | | | | × | |
| 1.32 | VVW | | | | | × | |
| 1.24 | VW | | | | | × | |

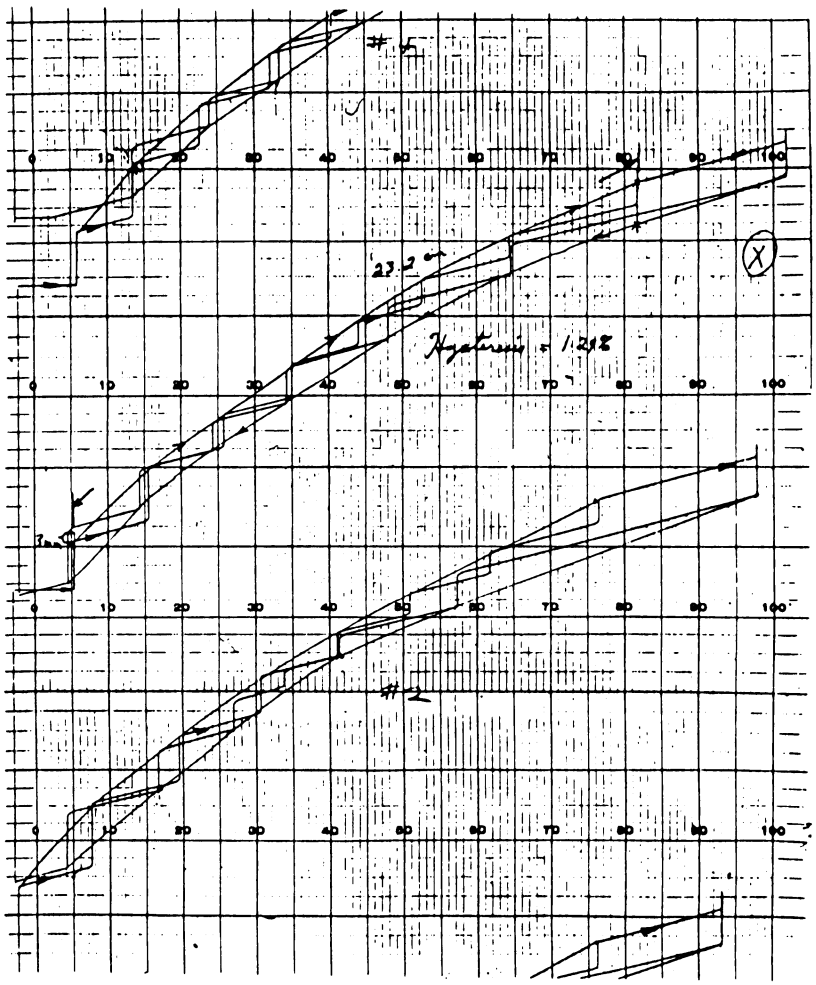


*Figure 2. Recording of an aortic sample being run in the elastometer showing the way in which hysteresis data were derived*

is most marked in the abdominal aorta, but can be shown throughout (Table III). This hysteresis is frequency dependent, but the implications of this remain to be thoroughly explored. Salt concentration and degree of polymerization of interstitial material have additional effects (Figs. 3–5).

*Fracture of Fibrils*

Analogies of the fracturing of elastin fibrils to the crazing in some elastomers secondary to ultraviolet and radiation were brought out, and electron microscopic evidence of some condensation on the surface of elastin fibrils (Fig. 6) as well as extracellular crystalline inclusions in the aging aorta have already been described. Simultaneous study of a number of specimens by x-ray diffraction, emission spectrophotometric and electron microscopic and mechanical elastometer methods showed that there was correlation of the elevated elastic constant (Ke), the clarity of the specific x-ray diffraction pattern, accompanying aging, the increased density of the elastin fibrils by electron microscopy, and concomitant erosion (Fig. 7), and the increase in dicarboxylic amino acid content previously reported by others.

*Table III*

HYSTERESIS, LONGITUDINAL SECTIONS

| *Age* | *Ascending* | *Arch* | *Thoracic* | *Abdominal* |
|---|---|---|---|---|
| 40–49 | + | | + + + | + + |
| 50–59 | + + + + | + + + | + + | |
| 60–69 | + + + | + + + | + | |
| 70–79 | + | + + + | + + | + + + |
| 80 and over | + + | + | + + | |

HYSTERESIS, TRANSVERSE SECTIONS

| *Age* | *Ascending* | *Arch* | *Thoracic* | *Abdominal* |
|---|---|---|---|---|
| 40–49 | + | + + | + | + + + |
| 50–59 | + | + | + + + | + + + |
| 60–69 | + + + | + + | + | + + |
| 70–79 | + | | + + + | + |
| 80 and over | + + + + | + + + | | |

COMPARISON OF GROUPS UNDER 65 AND 65 AND OVER

| | 40–64 | | 65 AND OVER | |
|---|---|---|---|---|
| | *Longitudinal* | *Transverse* | *Longitudinal* | *Transverse* |
| Ascending | 5.38 | 5.01 | 5.12 | 5.27 |
| Arch | 5.17 | 5.02 | 5.11 | 4.84 |
| Thoracic | 5.34 | 4.89 | 4.69 | 4.58 |
| Abdominal | 4.50 | 5.42 | 4.69 | 5.05 |

TOTAL, ALL AGES

| | *Longitudinal* | *Transverse* |
|---|---|---|
| Ascending | 5.24 | 5.15 |
| Arch | 5.14 | 4.94 |
| Thoracic | 4.99 | 4.72 |
| Abdominal | 4.60 | 5.29 |

*Table III (Continued)*

| ASCENDING | | ARCH | | THORACIC | | ABDOMINAL | |
|---|---|---|---|---|---|---|---|
| *L* | *C* | *L* | *C* | *L* | *C* | *L* | *C* |
| | | | *Age 40 to 49* | | | | |
| 9.37 | 14.81 | 12.25 | 12.47 | 15.30 | 13.92 | 7.77 | 14.86 |
| 17.06 | 13.10 | 10.82 | 16.60 | 23.52 | 15.45 | 23.81 | 25.02 |
| 26.43 | 27.91 | 23.07 | 29.07 | 38.82 | 29.37 | 31.58 | 39.88 |
| + 6 | 6 | 6 | 6 | 7 | 7 | 6 | 7 |
| 4.40 | 4.65 | 3.84 | 4.84 | 5.55 | 4.20 | 5.26 | 5.70 |
| | | | *Age 50 to 59* | | | | |
| 5.80 | 3.21 | | 3.21 | 6.89 | 5.80 | | |
| 19.91 | 17.76 | 16.41 | 23.28 | 19.76 | 22.79 | 3.86 | 12.01 |
| 25.71 | 20.97 | 16.41 | 26.49 | 26.65 | 28.59 | 3.86 | 12.01 |
| + 4 | 5 | 3 | 6 | 5 | 5 | 1 | 2 |
| 6.43 | 4.19 | 5.47 | 4.41 | 5.33 | 5.72 | 3.86 | 6.00 |
| | | | *Age 60 to 65* | | | | |
| 22.17 | 6.44 | 23.30 | 12.90 | 18.34 | 16.50 | 3.21 | 4.50 |
| 11.73 | 14.79 | 14.89 | 9.93 | 6.89 | 13.19 | 1.84 | 3.21 |
| | | | *Age 65 to 69* | | | | |
| 14.21 | 18.62 | 6.72 | 12.39 | 9.91 | 16.72 | 12.47 | 16.83 |
| 16.45 | 11.39 | 8.50 | 11.45 | 7.07 | 10.93 | 2.50 | |
| 64.56 | 51.24 | 53.41 | 46.67 | 42.21 | 57.34 | 20.02 | 24.54 |
| + 11 | 9 | 9 | 9 | 9 | 13 | 6 | 5 |
| 5.87 | 5.69 | 5.93 | 5.19 | 4.69 | 4.41 | 3.34 | 4.91 |
| | | | *Age 70 to 79* | | | | |
| 19.08 | 6.69 | 13.46 | 3.86 | 13.59 | 6.11 | 11.91 | |
| 21.01 | 24.84 | 21.51 | 12.09 | 29.19 | 37.98 | 16.78 | 13.49 |
| 40.09 | 31.53 | 34.97 | 15.95 | 42.78 | 44.09 | 28.69 | 13.49 |
| + 9 | 7 | 6 | 5 | 9 | 8 | 5 | 3 |
| 4.45 | 4.50 | 5.83 | 3.19 | 4.75 | 5.51 | 5.74 | 4.50 |
| | | | *Age 80+* | | | | |
| 16.36 | 18.04 | 8.88 | 8.87 | 12.02 | 9.87 | | |
| 15.36 | 9.93 | 12.42 | 19.15 | 21.97 | 1.17 | 3.21 | |
| 31.72 | 27.97 | 21.30 | 28.02 | 33.02 | 11.04 | 3.21 | |
| + 6 | 4 | 5 | 5 | 7 | 3 | 1 | |
| 5.29 | 6.99 | 4.26 | 5.60 | 4.86 | 3.68 | 3.21 | |

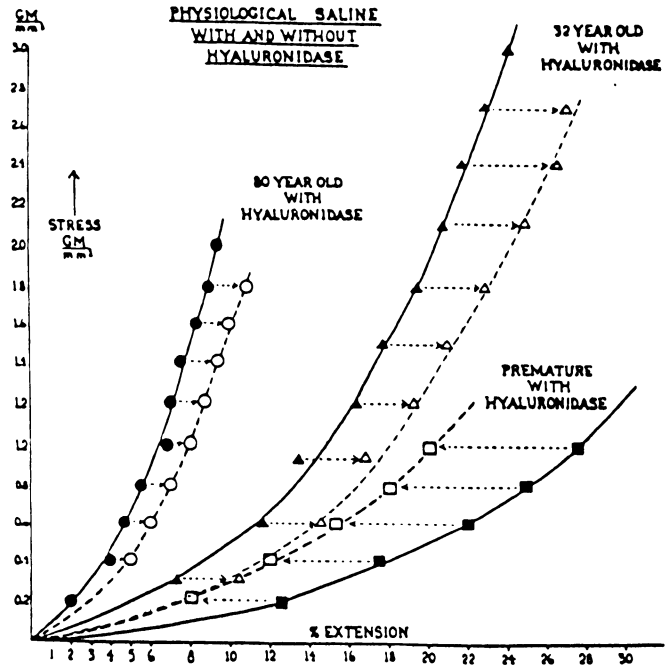



*Figure 3. Variations with age and the application of hyaluronidase to typical aortic samples are shown*

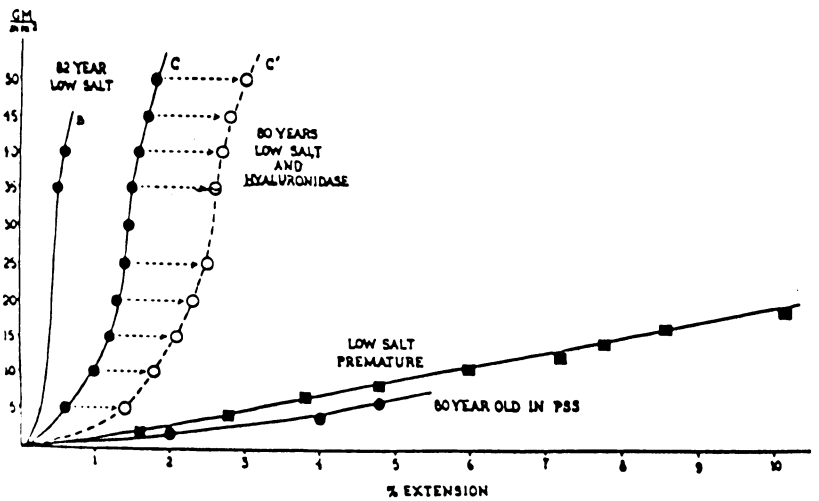



*Figure 4. Effect of age and varying concentration of sodium chloride on the elastic constant and hysteresis of individual aortic samples is shown*

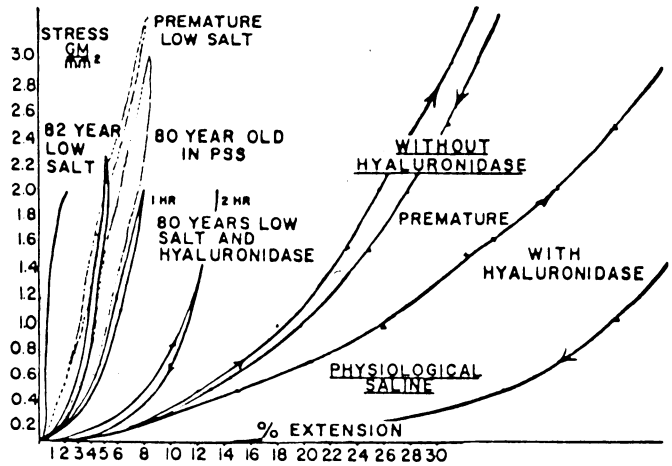



*Figure 5. Effect of both varying salt concentration and hyaluronidase treatment on aortic samples can be shown to give somewhat similar results which both reinforce and attenuate each other.*

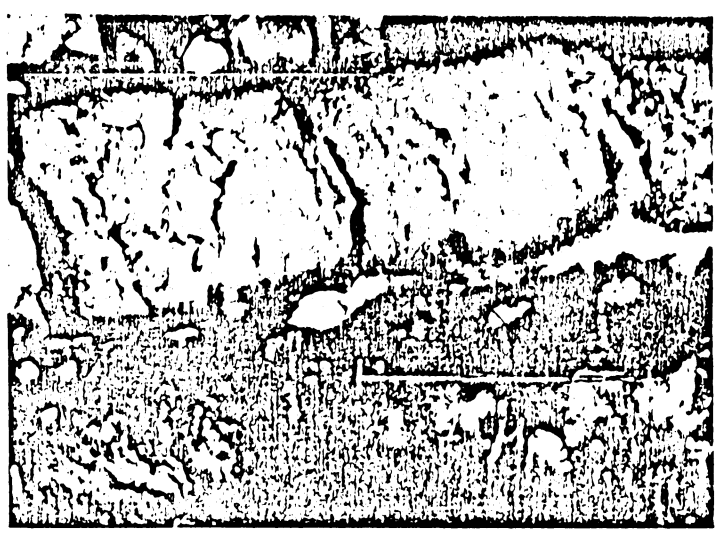


*Figure 6. Electron micrograph of an individual elastic fiber in aortic media showing condensation of electron dense material at the edge of the fiber in an old specimen.*

It then seemed logical that the association of metals and dicarboxylic acids was a meaningful one and perhaps accounted for many of the observed phenomena. The similarity of this x-ray diffraction pattern to the salts of glutamic acid (Tables VI and VII), and the susceptibility of the elastic constant to monovalent cations, led to the thesis

*Figure 7. Electron micrograph of a similarly aged elastic fibril in human aortic media showing the lacunae secondary to the effect of elastase treatment*

that metal accumulation might be one of the stabilizing or stiffening reactions taking place in the body over many years, resulting in crosslinking of dicarboxylic acid residues in a variety of tissue components. Direct measurement of the stability constants of metal glutamates made it seem unlikely that aluminum could be an important factor in spite of previous evidence to the contrary (Fig. 8). Both copper and iron ($Fe^{+++}$) were found to form stable glutamates and their concentration in the tissues studied made such crosslinkage entirely possible. The inability of calcium to duplicate either the x-ray

diffraction pattern or the elastic phenomena made it seem unlikely that this was the crucial cation. It seems entirely likely now that the successful competition for calcium with phosphate to form hydroxyl-apatite perhaps reduces calcium in its key position of importance, in spite of the considerable quantities present in aging tissue of several species. The stability of aluminium chelates, the small ionic radius

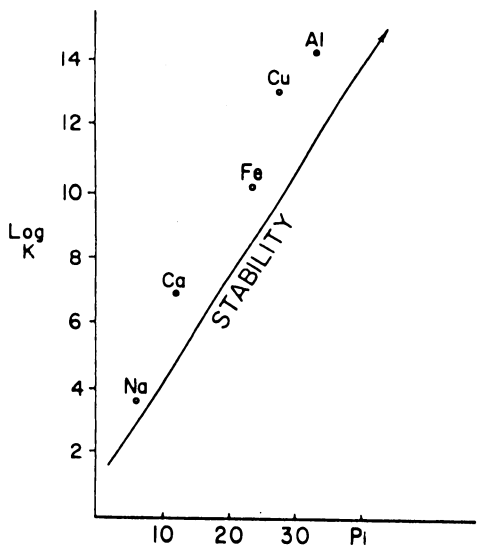



*Figure 8. Range of stabilities of various metal complexes is shown relating them to their electronegativity*

(Fig. 9), and the peak in aortic concentration at 40 to 50 (Fig. 10) make it still a likely culprit.

Detailed study of the mucopolysaccharides and the enzyme systems of the aorta and work indicating that the smooth muscle component of the aorta remains relatively intact until late stages of aging have taken place have inclined us to minimize the importance of changes occurring in these components of the aortic media.

The intimate sequence of events which should be rather directly susceptible to study by potential measurement methods of the endothelium would seem to be that preliminary phases involved intimal crosslinking, resulting in an increase in elastic constant of the intima itself. It is likely that when this occurs sheer stresses previously described in more detail, coupled with a relatively hydrophobic resultant endothelial surface, in part perhaps produced as the result of

phosphoprotein adsorption would produce fracture in the intima and subsequent admission of lipid substances subintimally.

Under the localized stresses in these situations it is likely that the elastic fibers become overly compacted, under increased diastolic

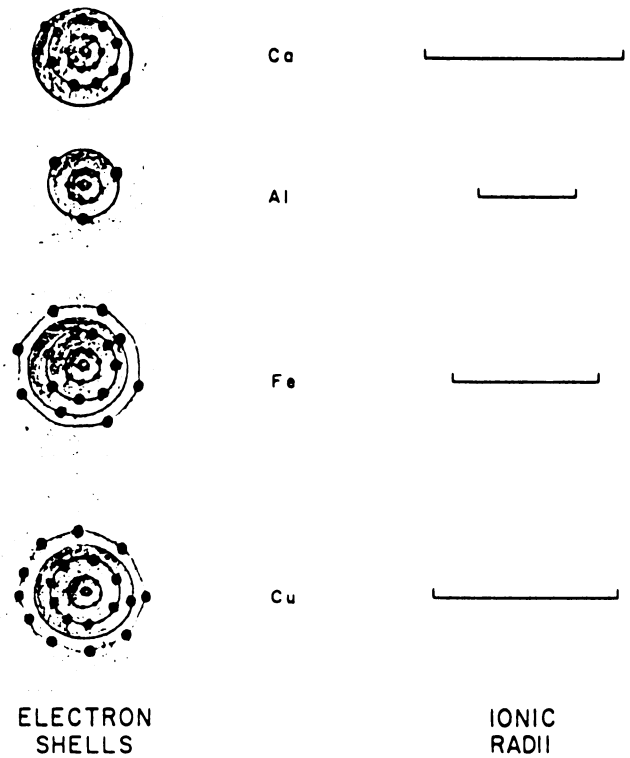



*Figure 9. Relative electron radii of metals thought to be important in crosslinking of aortic tissue showing the most likely candidates for ions to get entrapped in the maze of chemical binding sites existing in aging tissue*

pressures and analagous to the formation of bone, will undergo preferential orientation and preferential adsorption of cations. A few synthetic polymers have been shown to be cation sensitive in their elastic properties, but their behavior is close enough to elastin to make them useful to our argument.

*Loss of Local Circulation—Autograft Model*

If we presume that the circulation of the aortic media depends in large part on regular compression and expansion of the wall itself,

one can imagine that increasing elastic constants will lead to microscopic ischemic areas. Such areas can be produced by complete transection and resuturing of an aortic autograft. This healed segment duplicates many of the elastic phenomena. There is as a result of tissue anoxia and failure of perfusion, metabolic standstill, with loss of adjacent metabolic turnover sites and a microfatality of the tissues with saturation with end products of metabolism such as oxalic acid, glutamic acid, cystine, and other overly oxidized organic acids and potential crosslinking agents which are adsorbed and accept cationic crosslinkage.

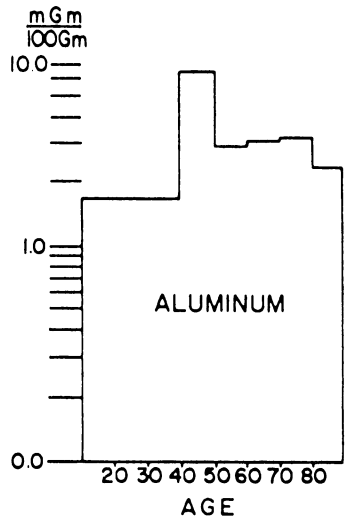



*Figure 10. Plot of aluminum concentration versus increasing age in aortic tissue*

It can be seen that this reaches a peak in the 40 to 50 age group and as this is derived from patients who have died, it is perhaps not surprising that the aluminum content falls off after the peak of early cardiovascular death.

The coincidence that transamination is necessary for the mobilization of lysine glutamic acid and arginine, and that the pyroxidal transaminase needs iron, copper, or aluminum for activity is hard to ignore. The possibility that metal excesses may contribute to inactivation of the enzyme system is an attractive one.

*Loss of Perfusion—Millipore Model*

An interesting analog capable of much further study than it has thus far been our fortune to pursue involves the fate of millipore

implanted in tissue. Thanks to the efforts of Dr. C. Andrew L. Bassett, who has been using millipore as a protective covering for nerve suture in his work with Dr. J. B. Campbell, a progressive aging process can be seen microscopically to take place in the interstices of the millipore filter over six months to a year. We had known that millipore adsorbed some material, presumably mucopolysaccharide, from urine in the process of filtration, making millipore almost impenetrable with high hydrostatic pressures within a matter of a few minutes of use. If we presume that after adsorption on a relatively acidic surface amino groups from the mucopolysaccharide are still available for dicarboxylic acid adsorption and that cations such as calcium in turn accrete thereon, one has a simple model system for the type of freezing over of the metabolic pool and the transport passages in the body with increasing time.

Some of these phenomena have been studied in other systems, and it is obvious that this is not the only mechanism which can explain the sequence of events.

The effects of radiation on protein, mucopolysaccharides, and on polymeric plastics would seem in many ways to reproduce the phenomena we have discussed, but mere loss of circulation in the aortic media would seem to duplicate most of the observed phenomena quite closely.

## *OTHER ORGAN AND CHEMICAL SYSTEMS UNDERGOING AGING*

Some of the most profound minds to approach the problem of aging, the late Dr. Szilard, Dr. Failla, and others have emphasized successive radiation insult. Dr. Szilard, at least, perhaps overemphasized the increase of entropy in the gene as a result of radiation and random crosslinking. Many of these radiation effects can be imitated by metals even within the chromosome systems, and we would prefer to think rather than a "randomization" of a "fixation" in a useless configuration of chromosomal material. Many of the phenomena associated with radiation damage and shortening of life span have basic dissimilarities to conventional aging on life span. Perhaps the crosslinking induced by radiation is still susceptible in most instances to normal enzymatic metabolic turnover (Table IV). The diminution in serum levels of many substances with aging more likely is a reflection of the diminution in turnover of all types of metabolic components in the plasma of aging individuals. In skin, in the eye, and in

the kidney, there are characteristic and successive changes. The disproportion in viscosities between extracellular and intracellular components in several cellular species can be shown to vary with age, as well as the degree of polymerization of them.

*Table IV. Liberation of alpha-amino nitrogen in irradiated and nonirradiated rats after incubation of liver homogenates with trypsin*[a]

| | *Age at death (weeks)* | *No. of animals sacrificed* | *Mg nitrogen liberated (average)* | *Reduction in solubilized protein (%)* |
|---|---|---|---|---|
| Young control rats | 36 | 14 | 1.99 | 0.0 |
| Irradiated rats (1890 r) | 36 | 11 | 1.86 | 6.5 |
| Irradiated rats (3591 r) | 24–36 | 16 | 1.58 | 20.6 |
| Old controls | 27 mos | 15 | 1.57 | 20.6 |

[a] These figures are based on 0.1 g of liver wet weight. Ten ml of liver homogenate (1.0 g of liver) was mixed with 50 ml of Sorenson's buffer (pH 7.7) and 20 mg of Difco trypsin (1 : 250) in 125-ml Erlenmeyer flasks. Each sample was set up in duplicate with one reaction mixture precipitated immediately upon mixing with 20 ml of 20% trichloroacetic acid. The second flask was shaken for twenty hours at 38° C and then precipitated with 20 ml of 20% trichloroacetic acid. Both samples were filtered and washed with 5% trichloroacetic acid into 100-ml volumetric flasks. The filtrates were used for alpha-amino nitrogen determinations with the Van Slyke apparatus. Each value for liberated nitrogen listed above has been corrected by subtracting the milliliter of $N_2$ for the control sample from the milliliter of $N_2$ liberated after hydrolysis, before the milligram values were calculated.

## *POTENTIAL IMMOBILIZING AGENTS AND THEIR ANTAGONISTS: THOSE ALREADY IN USE AND SOME AS YET UNTRIED*

The ideal agent to resist or even reverse the immobilization process, as sodium, magnesium, lithium, potassium, and other metals incapable of multiple linkages, should displace more firmly bound metals. (Figs. 9 and 10; Tables V and VI). Once displaced, however, the metals present (Table VII) must be kept in solution in readily excreted form and bound firmly enough to the carrier to avoid being trapped again by the tissue binding sites from which they have been released. Such an agent should be degraded by the body, little if at all, should be absorbed after oral administration, rapidly excreted with the metal attached more firmly to it than to any competing fixed body site. Low dosage and low cost would aid in spreading its usage. None of the present agents comes close to matching these requirements.

*Table V. Trace Metals—Liver and aorta. Milligrams per 100 g dry tissue*[a]

| *Organ* | *Ca* | *Al* | *Co* | *Ag* | *Cr* | *Cd* |
|---|---|---|---|---|---|---|
| | | | *Age to 40* | | | |
| Liver | 34.0 | 15.0 | 0.24 *5 | 0.055 | 0.62 *1 | 4.20 |
| | (14) | (14) | (9) | (14) | (10) | (11) |
| Aorta | 188.0 | 2.4 | 0.28 *8 | 0.017 *2 | 0.41 *1 | 1.92 *2 |
| | (12) | (14) | (6) | (12) | (10) | (9) |
| | | | *Age 41 to 50* | | | |
| Liver | 23.8 | 10.0 *1 | 0.45 *11 | 0.138 | 0.99 *4 | 3.80 *1 |
| | (14) | (13) | (3) | (13) | (5) | (8) |
| Aorta | 9.5 | 6.5 *1 | 0.29 *10 | 0.030 | 0.29 *3 | 3.90 |
| | (14) | (13) | (4) | (13) | (6) | (9) |
| | | | *Age 51 to 60* | | | |
| Liver | 37.1 | 7.9 | 0.54 *16 | 0.031 *1 | 0.40 *1 | 3.30 |
| | (25) | (25) | (8) | (23) | (13) | (14) |
| Aorta | 1564.0 | 2.87 *3 | 0.31 *15 | 0.030 | 0.32 *1 | 4.58 |
| | (24) | (22) | (7) | (23) | (11) | (14) |
| | | | *Age 61 to 70* | | | |
| Liver | 30.9 | 9.9 | 1.28 *12 | 0.042 | 0.67 | 3.40 |
| | (17) | (17) | (5) | (14) | (6) | (6) |
| Aorta | 1747.0 | 3.2 *2 | 0.91 *14 | 0.046 *1 | 0.22 | 6.10 |
| | (17) | (15) | (3) | (13) | (6) | (6) |
| | | | *Age 71 to 80* | | | |
| Liver | 17.2 *1 | 16.2 | 4.2 *11 | 0.030 *1 | 0.53 *1 | 1.40 *2 |
| | (12) | (13) | (2) | (12) | (5) | (4) |
| Aorta | 1644.0 | 3.35 | 0.10 *10 | 0.033 | 0.30 *1 | 4.56 |
| | (12) | (13) | (2) | (13) | (4) | (6) |
| | | | *Age 81 to 90* | | | |
| Liver | 19.6 | 18.9 | *6 | 0.026 | 0.39 *1 | 3.7 |
| | (7) | (7) | | (7) | (2) | (3) |
| Aorta | 2990.0 | 2.46 | *6 | 0.039 | | 9.7 |
| | (7) | (7) | | (7) | | (3) |

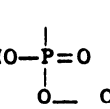
[a] Parentheses indicate the number of cases; asterisks indicate the number of cases in which the metal was not detected.

*Table VI*

| *Insitu metal binding groups* | *Order of binding* |
|---|---|
| Disulfide | $Hg^{++}$ $Cu^{++}$ $Zn^{++}$ $Fe^{+++}$ |
| Imidazole | $Hg^{++}$ $Cu^{++}$ $Zn^{++}$ $Fe^{+++}$ |
| Dicarboxylic | $Hg^{++}$ $Cu^{++}$ $Zn^{++}$ $Fe^{+++}$ $Ca^{++}$ |
| Hydroxyl | $Fe^{+++}$ $Hg^{++}$ $Cu^{++}$ $Zn^{++}$ $Ca^{++}$ |
| Amino | $Hg^{++}$ $Cu^{++}$ $Zn^{++}$ $Fe^{+++}$ |
| Sulfonic | $Fe^{+++}$ $Ca^{++}$ $Cu^{++}$ $Hg^{++}$ $Zn^{++}$ |
| Phosphate | $Hg^{++}$ $Cu^{++}$ $Fe^{+++}$ $Zn^{++}$ $Ca^{++}$ |

*Unified Sequence of the Aging Process*

*Table VII. The relative binding capacities of certain ionic groups for certain metals indicating the diverse binding sites for metals present in most proteins and polysaccharides*

| |  |  |  |  |  | |
|---|---|---|---|---|---|---|
| $Na^{+}$ | 0 | 0 | 0 | 0 | 0 | 0 |
| $Ca^{++}$ | + | + | 0 | 0 | + | 0 |
| $Fe^{+++}$ | + | + | + | + | ++ | + |
| $Zn^{++}$ | + | + | + | + | + | + |
| $Cu^{++}$ | + | + | + | + | + | ++ |
| $Hg^{++}$ | + | + | + | + | ++ | ++ |

If one accepts the thesis that progressive crosslinking or reaction to resist solubilization is taking place in the process of aging, one can construct a table of agents perhaps important in various locations (see Table VIII). Their chemical antagonists, some of them of very low toxicity, can be postulated as well (see Table IX), and one is struck by the fact that one agent at least has been in common usage for a long period of time, notably thiocyanate. The results of prolonged salicylic acid and penicillamine feeding in attempts to combat heavy metal accumulation, the possible effects of ethanol and antabuse in the aging population are fertile fields for investigation. We must be aware

*Table VIII. Metal complexing*

| *Agents now in use* | *Chief ligands* |
|---|---|
| Urea and thiourea | H-O-C H-N-C |
| Ammonia | $Ag^{+}$ $Cu^{++}$ |
| Thiocyanate | $Fe^{+++}$, $Cu^{++}$ |
| Ethanol | $Zn^{++}$ |
| Citrate | $Ca^{++}$ $Mg^{++}$ $Zn^{++}$ |
| Glutamate | $Cu^{++}$ $Ca^{++}$ $Mg^{++}$ |
| Sulfamate | $Ca^{++}$ $Mg^{++}$ $Fe^{+++}$ $Al^{+++}$ |
| Salicylate | $Cu^{++}$ $Fe^{+++}$ $Al^{+++}$ |
| Cysteine, BAL, penicillamine | $Fe^{++}$ $Fe^{+++}$ $Cu^{++}$ $Hg^{++}$ |
| Vitamins C, $B_6$, nicotinic | $Zn^{++}$ |
| Hormones, thyroxine, adrenaline | $Cu^{++}$ |
| Antibiotics | $Cu^{++}$ |
| EDTA, HEDTA, ACTA, DTO | $Ca^{++}$ $Pb^{++}$ $Be^{++}$ |

of the underlying disease leading to metal accumulation and the metal oxygen skeleton in aged tissue (Fig. 11) and that simple removal of metal or exposure to the metal may not be curative. The potential groupings responsible for metal binding, must be blanketed, rearranged, or prevented by changes in diet or environment so that

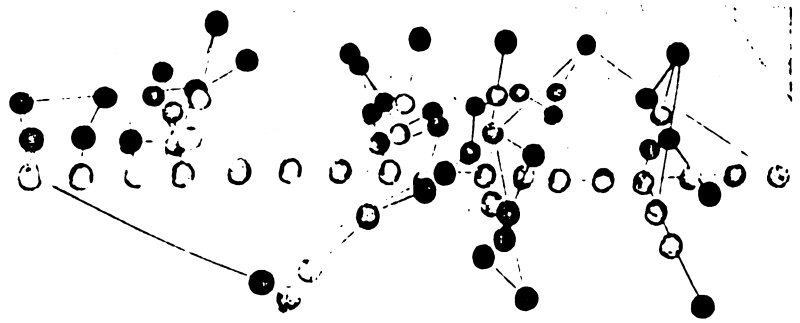


*Figure 11. Skeleton of metal oxygen linkages to be found by x-ray diffraction in aging tissue*

The oxygen molecules are black, the metal atoms are silver and can be seen to run in a continuous line down the longitudinal axis of the elastic fibers and for short periods, in the two other axes of the fiber as well, although there is evidence of an increasing degree of disorder away from the central axis. A nitrogen, hydrogen, oxygen diagonal linkage can be seen crisscrossing this central spine of metal-oxygen-metal-oxygen bonds.

*Table IX. Aluminum chelating agents*

| | *Competitors* |
|---|---|
| 1. Acetylacetone | $Fe^{+++}$ |
| 2. Salicylic acid | $Fe^{+++}$ |
| 3. 1,2-Diaminocyclohexane NNN'N tetracetic acid | $Hg^{++}$, $Cd^{++}$ $Lu^{+++}$ $Pb^{++}$ |
| 4. Morin 3,5,7,2',4'-pentahydroxy-flavone | $Be^{++}$, $Zr^{++++}$ |
| 5. 8-Dihydroxy-2-phenylazonaphthalene-3-disulfonic acid | $Fe^{+++}$ |
| 6. Aurintricarboxylic acid (ATCA) | $Be^{++}$ |
| 7. Pontachrome blue black R (4-sulfo-2, 2'-dihydroxyazonaphthalene) | None |
| 8. Hydroxytriphenyl methane dyes | $Fe^{+++}$ $Cr^{+++}$ $Zr^{+++}$ |

reattachment cannot occur. Other modes of crosslinking must be prevented as well, and our attack on them demand further work. Several agents for removing aluminum are attractive. These naturally occurring crosslinking agents are: acetoacetic acid, oxalacetic, acetaldehyde, oxalic acid, aspartic acid, cystine, hydroxyglutamic acid, ascorbic acid, quinones, VMA, and kynurenin.

## *SUMMARY*

A considerable body of work, primarily on the immediate fresh post-mortem aortic media has been described, all of it compatible with the concept that the aortic media undergoes progressive changes as a result of the accretion of relatively nonmetabolizable metal salts, affecting the mechanical properties of the aorta with consequent destructive effects secondary to the change in elastic constant within the aortic media. The mechanical effects of such increase in elastic constant have been adequately reviewed elsewhere.

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# NITROGENOUS COMPOUNDS IMMOBILIZED IN AN AGED RAT

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ABSTRACT: **A pregnant rat received 8 mc of tritiated tyrosine at the time of giving birth (from seven days before, to six days after). No radioisotopes were ever given directly to the litter born. A male from this litter died from pneumonia at age 809 days. After removal of water and acetone solubles and of phospholipids, hydrolysis of the residue released the following radioactive amino acids, parts of molecules fixed until death and containing tritium present at birth: lysine, arginine, aspartic acid, glutamic acid, serine, listed in order of decreasing radioactivity, with lysine carrying 29 per cent of the total tritium present.**

Still (1) has reported that radioisotopes administered to mice at birth remain in their brain, heart and skeletal muscles to a very measurable degree after 164 days, but that kidney and liver within this time are free from radioisotopes.

The work by Thompson and Ballou (2) with Sprague-Dawley rats given tritium for six months (dosage, 5 microcuries per milliliter of their drinking water supply) and analyzed after nine months, is not directly comparable with ours because of the long feeding period.

Still's report that the liver was cleared of isotopes in 164 days appears irreconcilable with the finding of Bjorksten, Andrews, Bailey and Trenk (3) who reported that an enzymatically nonhydrolyzable nitrogenous substance accumulated in the liver as a result of exposure of the animal to ionizing radiation; and the further finding (4) that a comparable quantity of similar material accumulated in the control animals from this experiment, when they reached senility about three years later.

It seemed important to clarify these observations, because the turnover in the liver is so much more rapid than in any other major organ that the liver presents a highly accelerated condition compared with the organism as a whole.

Therefore we repeated Still's experiment using a higher dosage of radioactivity and instrumentation not available in 1957.

While we essentially confirmed Still's important findings regarding retention of radioactivity in brain, heart and muscle, we also found appreciable quantities of radioisotope retained in the liver. These were further resolved.

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## EXPERIMENTAL DETAILS AND RESULTS

A pregnant Sprague-Dawley female rat received 2 mc of tyrosine-$H^3$ orally seven days before giving birth to a litter of six. The first and second days after giving birth she received 2 mc of tyrosine-$H^3$ daily in her drinking water, and the fifth and sixth days she received 1 mc daily. Thus the total dose (before and after the birth) was 8 mc.

The animals lived a normal laboratory life, being fed with Purina Lab. Chow and tap water ad lib. Eight hundred and nine days after birth one of the male offspring died of pneumonia. Its excised liver weighed 14.56 gm. This liver was homogenized for twenty-five minutes in a Virtis homogenizer, at half speed, to facilitate temperature control. A few large pieces had been wrapped around a blade. They were detached and chopped with a scalpel; the homogenization was resumed for an additional twenty-five minutes. A few larger particles still required manual chopping, whereupon the material was again blended in the Virtis homogenizer for thirty minutes. Then 1-ml samples were taken out, dried in air in dialyzing bags, and combusted in oxygen to water, according to the Thomas-Ogg procedure (5). The resultant water which contained all the tritium of the sample free from all organic substance was mixed with 5 ml of scintillation fluid and counted (0.5 ml of sample to 15 ml of scintillator fluid).

The remaining homogenate was washed ten times with water and centrifuged at 17,000 rpm between washings. Acetone (40 ml) was added to the residue and the mixture was blended fifteen minutes with the Virtis homogenizer. The slurry was centrifuged, washed with additional acetone, and vacuum dried; it yielded a residue that weighed 691 mg. This material was suspended in 40 ml of 2:1 chloroform:methanol. The mixture was blended for fifteen minutes at high speed in the Virtis homogenizer, and centrifuged at 17,000 rpm. This procedure was repeated three times, after which no radioactivity was apparent in the wash liquid.

The residual solid was vacuum dried to yield a residue which weighed 675 mg.

This material was hydrolyzed at 37°C and pH 9.3, in 25 ml of phosphate buffer. Two 0.5-ml aliquots were added to the scintillation liquid and counted:

| | | |
|---|---|---|
| Sample 1 | 86 CPM (above background) | 101 DPM |
| Sample 2 | 87 CPM (above background) | 102 DPM |

Taking the average of these samples, the total radioactivity of all the material processed was 4,975 disintegrations per minute (DPM). An insoluble residue weighing 84 mg remained; this contained a total of 360 DPM.

The liquid sample was dialyzed for removal of buffer, and was subjected to further study as follows:

A part of the original hydrolysate was used for probing, before the detailed procedure was evolved. This investigation is confined to the nitro-

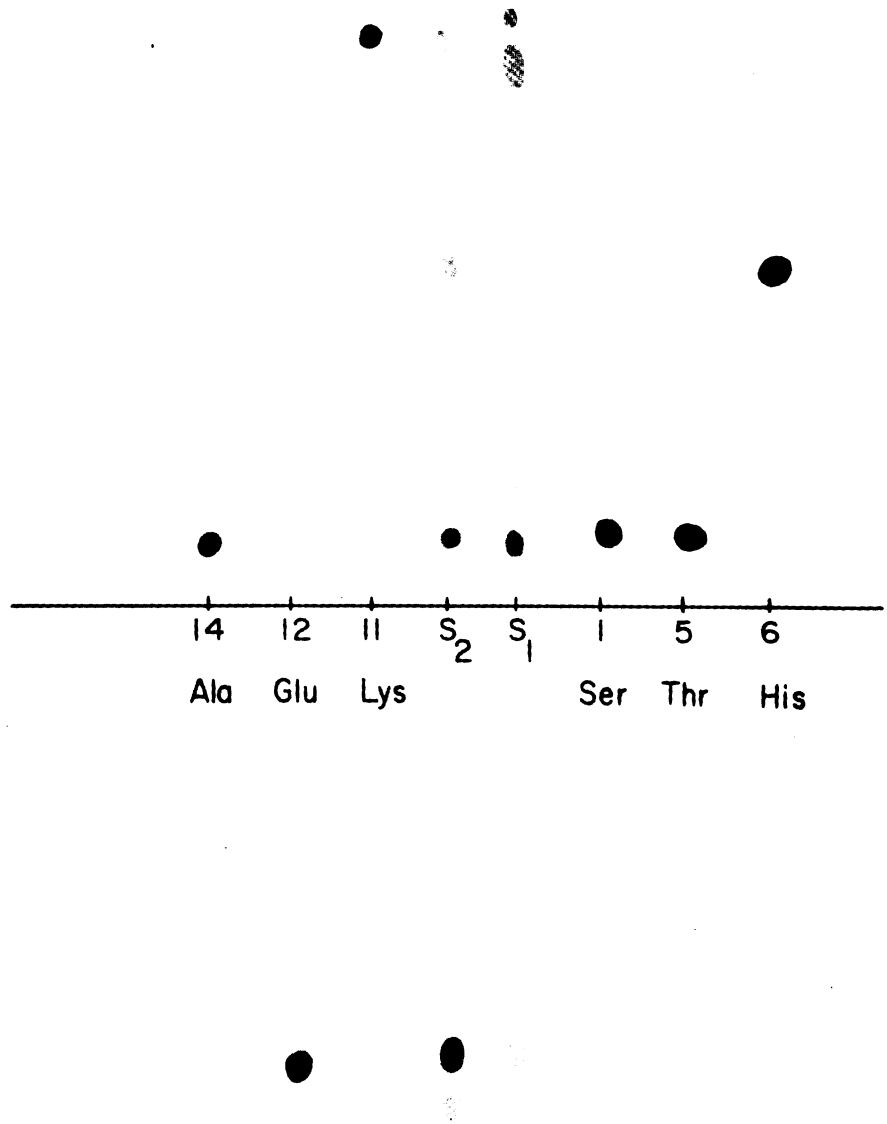


FIG. 1. Electrophoresis of peptides.

$S_1$ = primary hydrolysate fractions separated into 5 parts not corresponding to any amino acids, at pH 3.7.

$S_2$ = amino acids obtained after further hydrolysis of the primary hydrolysate.

The numbers represent amino-acid standards.

genous compounds. A smaller radioactive part, tentatively considered phospholipids because of solubility in chloroform:methanol, may become the subject of a separate study.

The material remaining after the foregoing dialysis was lyophilized and subjected to electrophoresis on 3-mm Whatman paper at 2,500 v and 150 amp. for forty-five minutes at pH 6.5 (Fig. 1) and at pH 3.5 (Fig. 2). At the higher pH we obtained 4 components; at the lower pH, 5 compo-

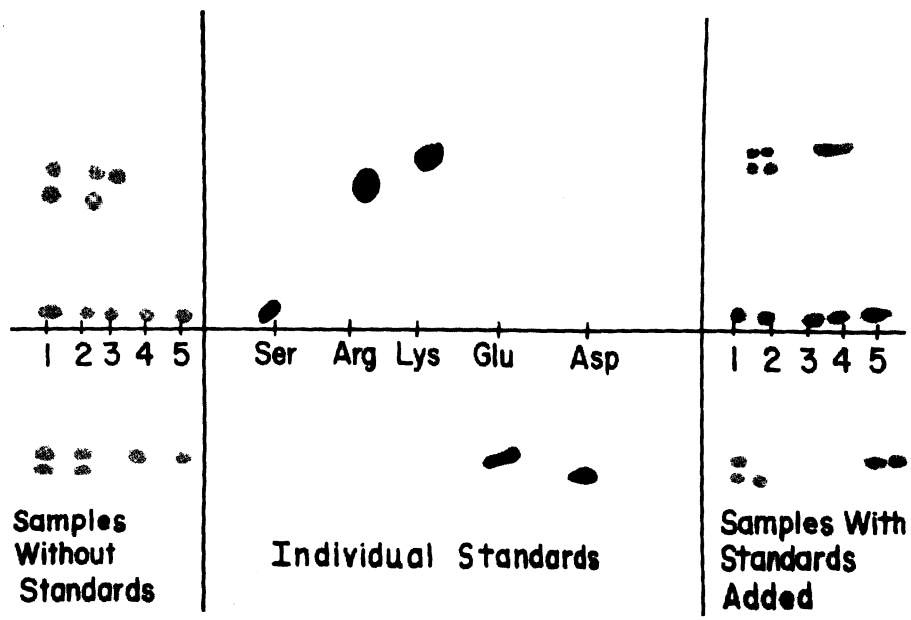


FIG. 2. Electrophoresis of amino acids of the five fractions of Sample S. On the left, amino acids in the hydrolysate; on the right, amino acids in the hydrolysates plus standard amino acids.

The following standard amino acids were added to the fractions on the right of the electropherogram: Fractions 1 and 2, ser, lys, arg, glu and asp; Fraction 3, ser and lys; Fraction 4, ser and glu; Fraction 5, arg and glu.

nents. These showed the smear characteristic of peptides. The buffers employed were made up in the following proportions by weight: 1) pyridine 2.4; acetic acid 60; water 540; and 2) pyridine 1; acetic acid 10; water 287.

Preparative electrophoresis was applied, followed by acid hydrolysis of the peptides at 110°C for twenty-four hours with 6 M HCl, the amount of acid being 0.1 ml per mg. Chromatography of the hydrolysates was then carried out on Whatman grade 1 paper previously washed with isopropanol and water (2:1) made slightly alkaline with ammonia. Two different solvent systems were used: 1) butanol:formic acid:water—225:45:30, and 2) butanol, pyridine, water—1:1:1; each system for eighteen hours.

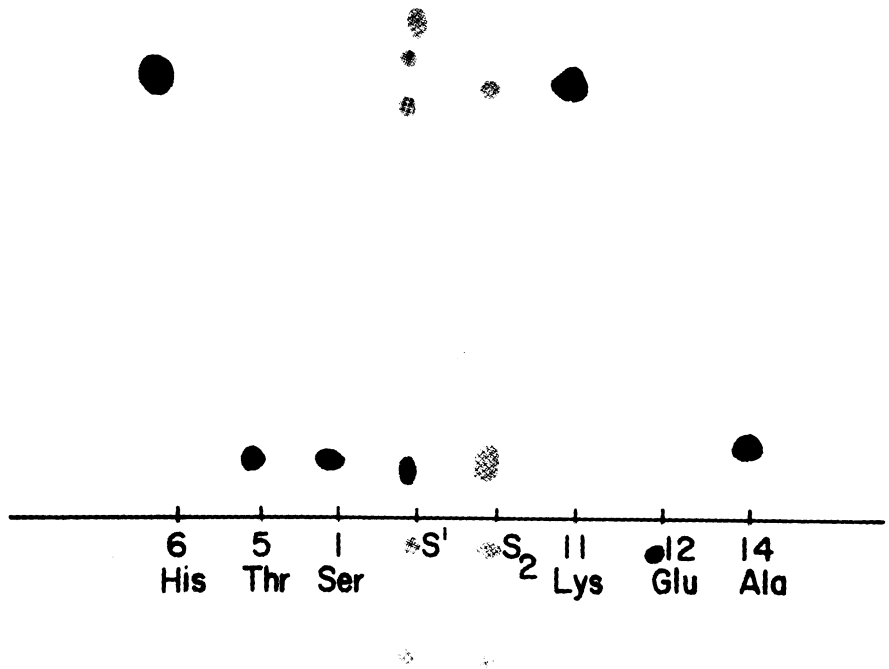


FIG. 3. Electrophoresis.
$S_1$ = the same as for Figure 1, at pH 6.7.

The peptides were separated and resolved as follows:

| Peptide No. | Weight Separated (mg) | Amino Acids |
|---|---|---|
| 1 | 1.3 | Ser, Glu, Asp, Lys, Arg |
| 2 | 1.2 | Ser, Glu, Asp, Lys, Arg |
| 3 | 13.0 | Ser, Lys |
| 4 | 12.0 | Ser, Glu |
| 5 | 0.6 (some lost, quantity estimated) | Ser, Glu |

To quantitate the amino acids, we combined the hydrolysates of the peptides, because the quantities at hand seemed too small for individual resolution.

| | CPM above Background | % of Total Radioactivity |
|---|---|---|
| Lysine | 425 | 29.2 |
| Arginine | 293 | 20.1 |
| Aspartic acid | 291 | 20.1 |
| Serine | 141 | 9.7 |
| Glutamic acid | 258 | 17.7 |
| Ether-soluble substance not observed before strong acid hydrolysis | 46 | 3.2 |
| | 1454 | 100.0 |

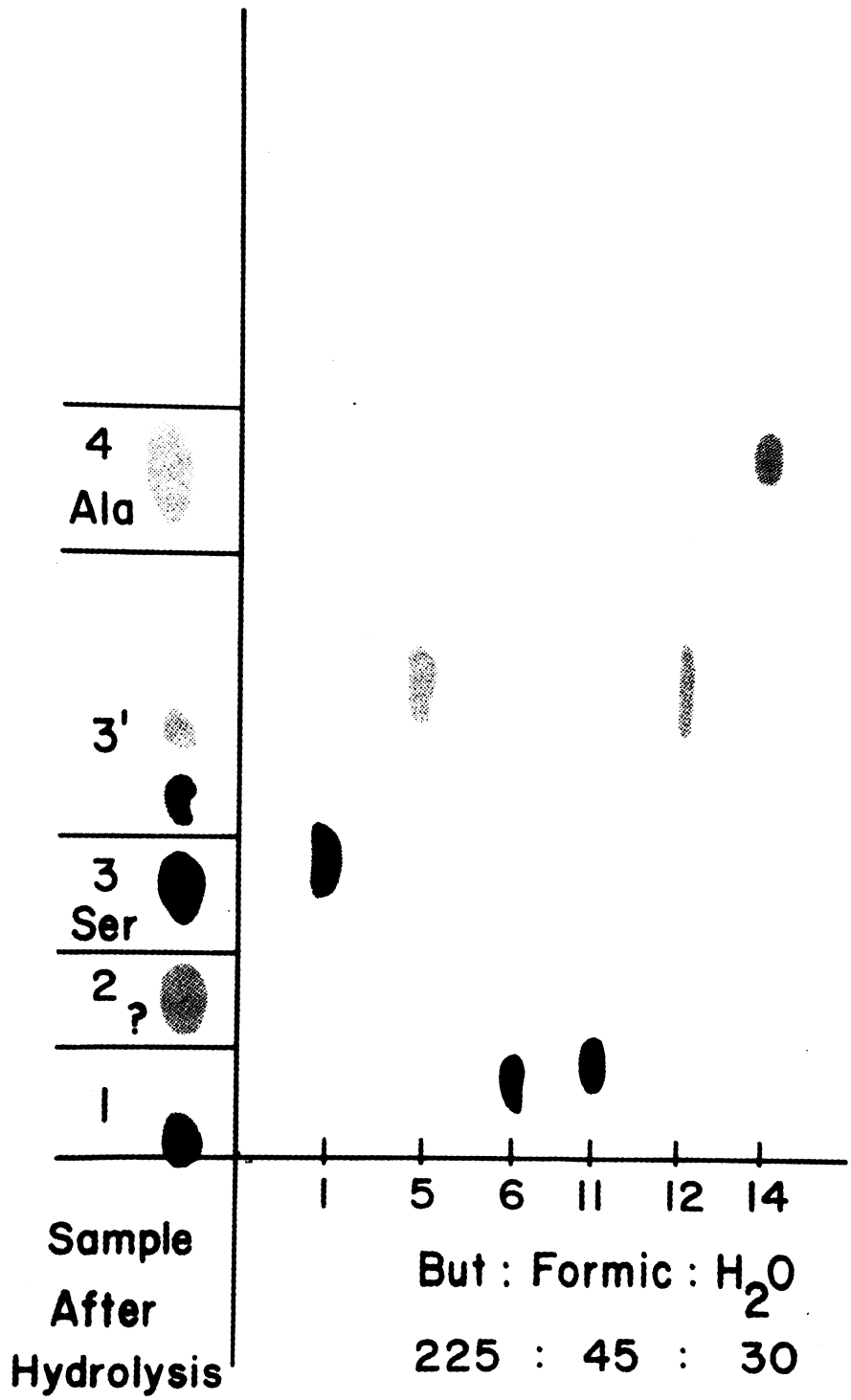


FIG. 4. Paper chromatography of amino acids of mixture S, with butanol:formic acid:water (225:45:30).

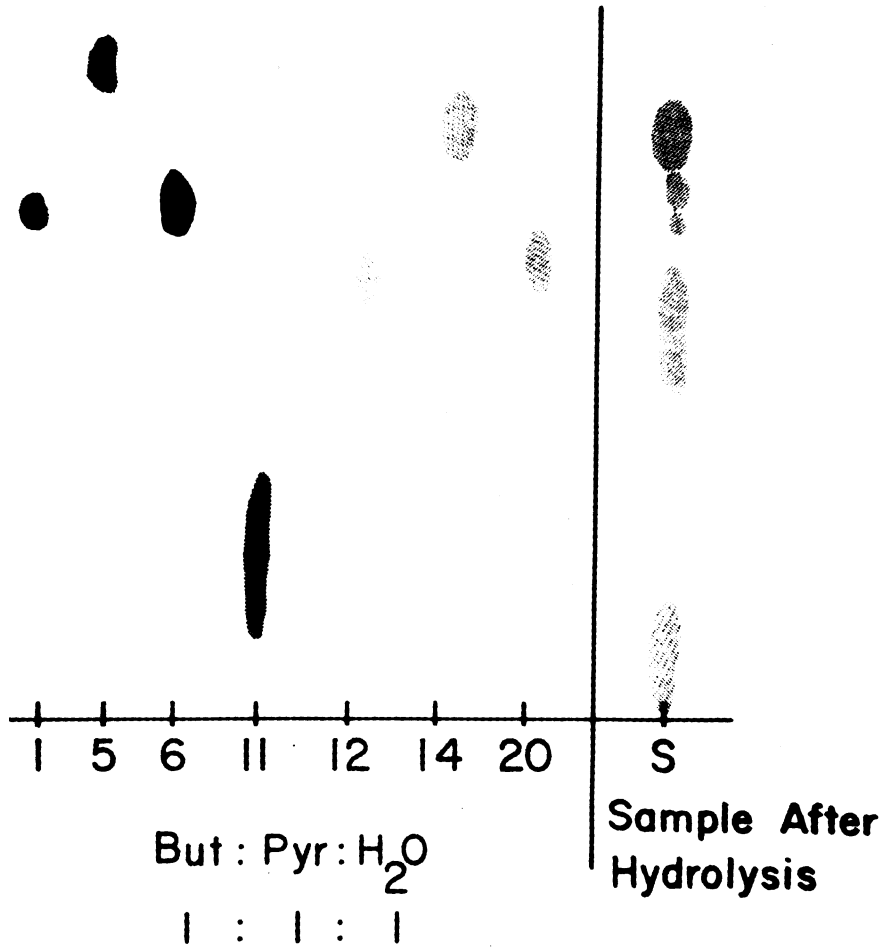


FIG. 5. Paper chromatography of amino acids of mixture S, with butanol:pyridine: water (1:1:1).

The identity of the amino acids was confirmed by co-electrophoresis with the known amino acids at 1,000 v and 150 amp. for two hours.

An attempt was made to identify the ether-soluble fraction, but the quantity at hand (less than 1 mg) proved insufficient. Gas-liquid chromatography was performed on a diethyleneglycol isophosphate column after clarification of lipids with $CH_2N_2$ and temperature programming from $110^0$ to $270^0$C. There was no known fatty acid in the range $C_{10}$–$C_{26}$.

The infra-red spectrum showed a strong band at 1,725 $cm^{-1}$, suggesting the presence of carbonyl groups. A band between 1,430 and 1,500 $cm^{-1}$ indicated aromatic structures. A strong band between 750 and 800 $cm^{-1}$ indicated steroids. The $CH_2$ stretching band at 3,000 $cm^{-1}$ was slight.

Nuclear magnetic resonance (NMR) (on a 60 MHz spectrometer) gives only rough approximations when application to sub-milligram quantities is attempted. Peaks at 48, 72, 84 and 108 cps could be attributed to steroid structures.

From these data it is apparent that the radioactive ether-soluble material was not a single simple fatty acid, but multiple, or complex, or both. Larger quantities and a substantial effort will be necessary for positive identification. Although the extraction and washing procedures used were thorough, it is not entirely certain whether the radioactive ether-soluble substance was chemically bound prior to hydrolysis, or only strongly adsorbed.

## DISCUSSION

The radioactive substances present in the liver of a rat 809 days after the administration of tritium to its mother can be regarded as stabilized in the organism in a manner not susceptible to normal in vivo removal. They are representative of the substances which have accumulated irreversibly in the liver, on aging of this organ.

From the analysis of these substances, the following negative and positive conclusions can be drawn:

The amino acids whose R groups could participate in hydrophobic bonding of a protein are alanine, valine, leucine, isoleucine, phenyl alanine, tryptophan, methionine, tyrosine and proline. None of these was present. Therefore hydrophobic bonding was not involved.

Of the 20 amino acids present in collagen, 15 (including oxyproline) were missing in the radioactive peptides obtained, and those present were in proportions different from their proportions in collagen. Thus there was no indication that collagen or a collagen derivative might be among the irreversibly accumulating substances in the liver of the rat.

The sulfur-containing amino acids, cysteine and methionine, were absent. It is inferred that in the case of the rat liver, the enzymes present are capable of resolving these amino acids, including disulfide cross-linkages.

The following 4 amino acids are regarded as playing the most significant role in the hydrogen bonding of tertiary or quaternary structures: lysine, histidine, aspartic acid, and serine (6). Three of these were among the 5 amino acids found in our peptides. Therefore, hydrogen bonding could be significantly involved.

The following 5 amino acids can bear a charge at physiological pH: lysine, arginine, aspartic acid, glutamic acid, histidine (6). Four of these amino acids were among the 5 found. Accordingly, it is indicated that cross-linkages based on co-valent bond reactions of the charge-carrying side groups of these amino acids are involved in the lifetime immobilization of radioactivity administered at birth.

The 5 amino acids found are those which have a reactive group positioned at the end of an extended side chain. The most abundantly present,

lysine, is the one which has the most reactive group on the most extended side chain.

These findings are consistent with the cross-linkage theory of aging (7) and have no apparent relation to any of the other theories advanced (8).

## *Acknowledgments*

We are indebted to The Upjohn Company for the donation of a liquid hydrolysate of a radioactive liver of known history, a by-product of Upjohn's project with Bjorksten Research Laboratories, Inc.; and to the Marcus and Bertha Coler Foundation for a donation to defray part of the expenses of this work. We acknowledge with thanks the valuable help of Dr. Roswell Boutwell in preparing animals, of Dr. P. V. N. Acharya in analysis, and of Mr. Raymond Kandler in preparing some of the samples.

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# GEROGENIC FRACTIONS IN THE TRITIATED RAT

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ABSTRACT: **A rat that had received tritiated acetate perinatally was killed at the age of 609 days, and was found to have retained substantial quantities of tritium in all organs examined. This study was focussed on the liver, which—after a succession of extractions with a series of various solvents followed by catalytic hydrolysis at body temperature—yielded a residue that was insoluble in a wide range of common solubilizing media. Treatment with hot mineral acid partially dissolved this residue and electrophoretic fractionation further led to 4 fractions of which a single fraction contained most of the tritium in the insoluble residue.**

**Our analyses showed that the insoluble residue contained a variety of common amino acids and a considerable amount of phosphorus. The solubilized fractions derived from the insoluble residue all contained substantial concentrations of pentose, deoxypentose, and phosphorus. They showed ultraviolet absorption spectra qualitatively similar to those of nucleic acids. From their chromatographic behavior on crosslinked dextran columns, all 4 solubilized fractions showed molecular weights greater than 5000. In addition, these fractions showed substantially greater resistance to hydrolytic degradation than do authentic RNA and DNA. Taken together, this is interpreted as evidence that the gerogenic insoluble residue is composed of a highly crosslinked network of at least RNA, DNA and protein, which is stabilized by covalent crosslinkages of unusual stability. Formation of these crosslinked structures could easily interfere with the function of certain critical molecules of RNA, DNA or other polymers, leading to impaired cell function and death.**

In a preceding paper (1) from this laboratory, it was reported that

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nitrogenous molecules were found in the liver of a rat whose mother received tritium-labeled tyrosine at the time of giving birth.

In the present study a pregnant Sprague-Dawley rat received 40 mc of 3H-acetate, administered orally with drinking water as follows: 10 mc seven days prepartum, 10 mc postpartum, 10 mc two days postpartum, 5 mc five days postpartum, and 5 mc six days postpartum.

The litter consisted of 8 rats, all apparently healthy. A male of this litter was sacrificed 609 days after birth. The only radioactivity this animal received was that from its mother. Its liver was examined first because this is a large organ with an active metabolism.

## MATERIALS AND METHODS

1. Substances that could be extracted from the wet liver with water, acetone, chloroform:methanol, and which could be catalytically hydrolyzed (about body temperature), were further resolved into various subfractions by preparative electrophoresis on Whatman 3 MM paper at 2500 V, 150 m A, for forty-five minutes in a buffer system of pH 6.5 containing pyridine, acetic acid, and water in the proportions (v/v/v) 300:12:2700 [Michl (2)].

2. Individual amino acids from the foregoing substances were obtained by 6 *M* HCl hydrolysis at 110°C for twenty-four hours, and were detected and isolated both by preparative paper electrophoresis under the above conditions and by preparative paper chromatography with use of a solvent system n-butanol:formic acid:$H_2O$; 245:45:30.

For measuring the color that each of these separated amino acids formed with ninhydrin, a corresponding authentic amino acid was taken as standard. The quantitative ninhydrin reaction (3) was determined on 10λ of the 1-ml solution of the sample.

3. Tritium activity of each amino acid was determined in a Packard Tricarb (Model 3365) scintillation counter on the 0.99-ml of the sample remaining from operation 2, after mixing with 15 ml of BBOT-dioxane.

Radioactivity in various extractable and catalytically hydrolyzable fractions of the liver was, however, determined from aliquots of these fractions in scintillation medium "Diotol" (Berdick & Jackman Laboratories)—except the insoluble fraction, which was combusted in oxygen to water which was then counted in the same manner (4).

4. Ribose was determined according to the method of Dische (5) after a mild hydrolysis of the samples with 0.1 *N* HCl in the amount of 0.1 ml/1 mg at 110°C for one hour and passing over a Dowex-50 column.

5. *Isolation of soluble fractions from the insoluble residue of liver* was carried out as follows: 50 mg of the insoluble residue was hydrolyzed with 10 ml of 1 *M* $H_2SO_4$ at 100°C for one hour. Insoluble material was removed by filtration. The filtrate and several washings were made up to 17 ml. The pH of the solution was brought to 1.0 by addition of 11 *N* KOH. Then 3-ml aliquots were heated to 90°C, and 0.3 ml of 20% $AgNO_3$ was added to each. Silver salts of the bases were precipitated. After standing overnight, the samples were centrifuged, and the precipitate washed with 1-ml portions of 0.1 *N* $H_2SO_4$; the original supernatants and the washings were filtered and combined. These solutions and the precipitates were

separately subjected to HCl treatment to free the bases. The insoluble silver salts of the bases were heated four times with 3 ml of 0.1 *N* HCl in boiling water each time for five minutes; the solids sedimented by centrifugation and the supernatants were combined. The combined supernatants after filtration were made up to 17 ml each. After suitable dilution, their ultraviolet absorption spectra were measured with a Gilford spectrophotometer (6).

The soluble silver salts of the bases were warmed to 70°C, 1 ml of 1 *N* HCl added in each, and the precipitate washed three times by suspension with 1-ml portions of 0.1 *N* HCl. The combined filtrates were diluted to 15 ml. The pH of these solutions was brought to 4.7 with NaOH and the volumes brought to 17 ml each. They were then incubated with 3 ml of acid phosphatase (wheat germ, Sigma) solution (containing 1 mg/1 ml) at 38°C for three hours to bring about dephosphorylation.

The solutions from the three assay samples were mixed, the pH brought to 8.3 with NaOH, and the solution filtered through Dowex-1 (bicarbonate) column 1 cm × 40 cm. The column was finally washed with 2% $NaHCO_3$.

The effluent was suitably concentrated, brought to pH 1 and put on Whatman 3 MM paper for electrophoresis in a pyridine acetate buffer of 3.5 pH ($H_2O$:pyr:HOAC; 287:1:10) [Michl (2)] at 2500 V, 80 m.a., for forty-five minutes. Zones were detected under ultraviolet radiation in the preparative procedure. They were eluted with 0.1 *N* HCl and each fraction so eluted was subjected to a second dephosphorylation treatment and passage over Dowex-1 (bicarbonate) columns as described above.

Throughout the procedure a control was run with authentic RNA (core-pancreatic, Sigma) (7).

6. *Characterization of the soluble fractions* was performed in three stages: (a) Analysis and determination of pentose, deoxypentose, and phosphorus. Pentose was determined according to Dische (5) technique, deoxypentose according to the method of Burton (8), and phosphorus according to the method of Bartlett (9).

(b) Estimation of the molecular size of these complexes and their sub-units obtained after perchloric-acid treatment in relation to those of authentic RNA-core and DNA (Calbiochem) respectively which have undergone similar experimental procedures. The perchloric-acid digestion was performed according to the method of Wyatt (10). The size estimation was made by elution with water. The Sephadex we used has an exclusion limit of 5000 m.w. The bed volumes of columns ranged from 30 ml to 42 ml and their void volumes from 13 to 18 ml.

Molar quantities of ultraviolet-absorbing bases in these substances were estimated by dividing their absorbancies at 260 nm by a mean molar extinction coefficient of 10,000.

(c) For purposes of comparison of these 4 nucleic acid complexes with RNA (core), DNA, and a physical mixture of RNA and DNA, ultraviolet spectra of these substances were also obtained at pH 1 and pH 11, on Beckman scanning spectrophotometer Model 10 within the region 320 nm to 210 nm.

## THE INSOLUBLE RESIDUE

The insoluble residue, which weighed 195 mg, proved to be insoluble in all solvents that are usually employed to dissolve proteins, e.g., dilute acids and alkalies, methyl formamide. acetamide, peracetic acid, dimethyl sulfox-

ide, 8 *N* urea, 6 *N* guanidine with and without the addition of detergents, anhydrous hydrofluoric acid, and the like.

*Preliminary fractionation*

The liver weighed 21.75 grams wet. It was comminuted with 135 ml of water in a Virtis disintegrator and exhaustively extracted with a series of solvent systems. The results are shown in Table 1.

From the hydrolysate (6 *N* HCl, 110°C, twenty-four hours) of a portion of the insoluble residue, we identified and estimated a number of amino acids. The results are shown in Table 2.

*Amino Acid analyses of hydrolysate of 1.3 mg of insoluble residue from liver*

The neutral amino acids were calculated on the basis of the equivalent weight and color yield of isoleucine. Separations were made by paper electrophoresis; quantitative estimation was performed photometrically on the color produced with ninhydrin. Standard solutions of the same amino

TABLE 1

| | Dry Weight (mg.) | Total Radioactivity in Fraction (DPM) |
|---|---|---|
| 1. Water extract | 1590 | 3120 |
| 2. Acetone | 200 | 3060 |
| 3. $CHCl_3$:MeOH (2:1) Soxhlet extraction, 64 hrs. | 131 | 4280 |
| 4. Hydrolysis, room temp., analogous to Andrews et al. (11) | 250+ | 2670 |
| 5. Insoluble residue | 195 | 4350 |

TABLE 2

*Amino Acid Analyses of Liver Insoluble Residue*

| Amino Acid | O.D. at 570 mμ | μg | mμM | Molar Ratio |
|---|---|---|---|---|
| Standards | | | | |
| Ile | 0.142 | 4.9 | | |
| His | 0.138 | 3.2 | | |
| Arg | 0.290 | 10.0 | | |
| Lys | 0.220 | 2.5 | | |
| Glu | 0.098 | 3.7 | | |
| Asp | 0.098 | 3.2 | | |
| Samples | | | | |
| Neutral a.a's | 1.354 | 5.2 | 370 | 2 |
| His | 0.150 | 3.5 | 23 | 0.12 |
| Arg | 0.420 | 14.0 | 80 | 0.42 |
| Lys | 0.238 | 2.7 | 185 | 1 |
| Glu | 0.458 | 1.7 | 115 | 0.64 |
| Asp | 0.186 | 6 | 46 | 0.24 |
| Total | | 33 | | |

acids were carried through the whole separation and estimation procedure as controls.

In a separate experiment the neutral amino acids were resolved chromatographically, showing predominantly phenyl alanine, isoleucine and valine. Quantitation of these was not attempted. We found no evidence of either proline or oxyproline in the neutral amino acid fraction. The total of the amino acids measured (Table 2) was about 2 per cent of the total weight of the insoluble residue. Material available was insufficient to make adequate tests of the specific activity of the amino acids.

## SEPARATION AND CHARACTERIZATION OF SOLUBLE FRACTIONS FROM THE INSOLUBLE RESIDUE

A 50-mg aliquot of the insoluble residue of the liver was subjected to hydrolysis with 10 ml of 1 *N* $H_2SO_4$ at 100°C for one hour. A large portion of (presumably) the peptides remained insoluble and was filtered off. Subsequent steps such as reaction with silver nitrate, dephosphorylation by acid phosphatase and chromatography on Dowex-1 (bicarbonate) column were carried out as described in the Methods section. The effluent of the Dowex-1 (bicarbonate) column was concentrated and the pH adjusted to 1.0 with $H_2SO_4$ and put on Whatman 3 MM paper for electrophoresis. Four dark zones were detected under ultraviolet radiation, three moving to cathode and one to anode (Fig. 1).

After paper electrophoresis in a preparative system, the separated zones were eluted with 0.1 *N* HCl and lyophilized. Each fraction was subjected to a second dephosphorylation treatment and again passed over Dowex-1 (bicarbonate) columns (1 cm × 40 cm) and precipitated with acetone. Aliquots of each fraction were taken for the determination of pentose, deoxypentose, and phosphorus and for the measurements of their absorption spectra.

Table 3 gives the results of these analyses. As may be seen, the molar quantities of pentose and deoxypentose together exceed the molar quantities

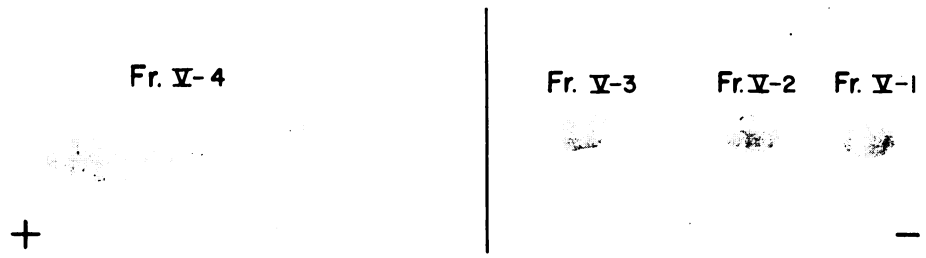


FIG. 1. Electrophoretic separation of soluble fractions of the insoluble residue of liver obtained by hydrolysis with 1 *N* $H_2SO_4$ at 100°C for one hour, on Whatman No. 3 paper. The electrophoresis was carried out for 45 minutes at 2500 V, 80 m.a. in a buffer containing $H_2O$:pyr:HoAC:: 287:1:10, and of pH 3.5. Fr.V-4 moved to the anode. Dark zones were detected under ultraviolet light.

TABLE 3
*Composition of Electrophoretically Separated Fractions Isolated from 50 mg of Insoluble Residue of Liver (mμ moles)*

| Sample | Pentose | Deoxypentose | Phosphorus | Bases |
|---|---|---|---|---|
| Fr. V-1 | 61 | 55 | 108 | 74 |
| Fr. V-2 | 109 | 126 | 142 | 41 |
| Fr. V-3 | 320 | 133 | 440 | 198 |
| Fr. V-4 | 1100 | 46 | 315 | 146 |

of bases in all the fractions. The values of phosphorus are also greater than those of the bases.

Figures 2 and 3 show the ultraviolet absorption spectra obtained for these 4 fractions. The strong similarity between these spectra and the spectra of authentic nucleic acids (12) is apparent. However, since these electrophoretic fractions had already been subjected to several steps (beginning with 1 *N* $H_2SO_4$ hydrolysis, 100°C for one hour) in which fractionation in all likelihood occurs, no particular significance can be attached to the stoichiometry. What is surprising, however, is the finding of nucleic-acid components in these materials after the initial strong hydrolysis, in spite of the known acid lability of deoxyribose and (to a lesser degree) its phosphate ester linkages. Indeed, both RNA and DNA are hydrolyzed to the level of monomers by 1.5 *N* HCl at 100°C for one hour (13). Table 3 also shows that substantial amounts of phosphorus remained after acid hydrolysis and two cycles of acid-phosphatase treatment.

## GEL FILTRATION CHROMATOGRAPHY OF THE 4 ELECTROPHORETIC COMPONENTS

Figures 4, 5 and 6 Show the elution patterns of these 4 components on Sephadex G-25 columns. In these figures we see that whereas the uridine and cytidine bases obtained as soluble silver salts from standard RNA (core) are eluted well beyond the void volumes of their respective columns, our samples (even after 1 *N* $H_2SO_4$ treatment at 100°C for one hour) invariably come within the void volumes. Since the exclusion limit of the Sephadex G-25 is generally accepted to be m.w. = 5000, these results show that the nucleic-acid complexes we have in hand are macromolecules having molecular weights greater than 5000. When samples of these 3 electrophoretic components (the fourth was not available in sufficient quantity) were hydrolyzed with 60% perchloric acid at 100°C for one and a half hours, gel filtration chromatography showed *only partial* degradation to units of lower molecular weight.

The results of these experiments are shown in Figures 4, 5 and 6. It is seen that this very strong acid hydrolysis results in breakdown to the point where the ultraviolet-absorbing products are retarded beyond the void volume, but not to the extent shown by the products from the control in which DNA was treated in an identical manner.

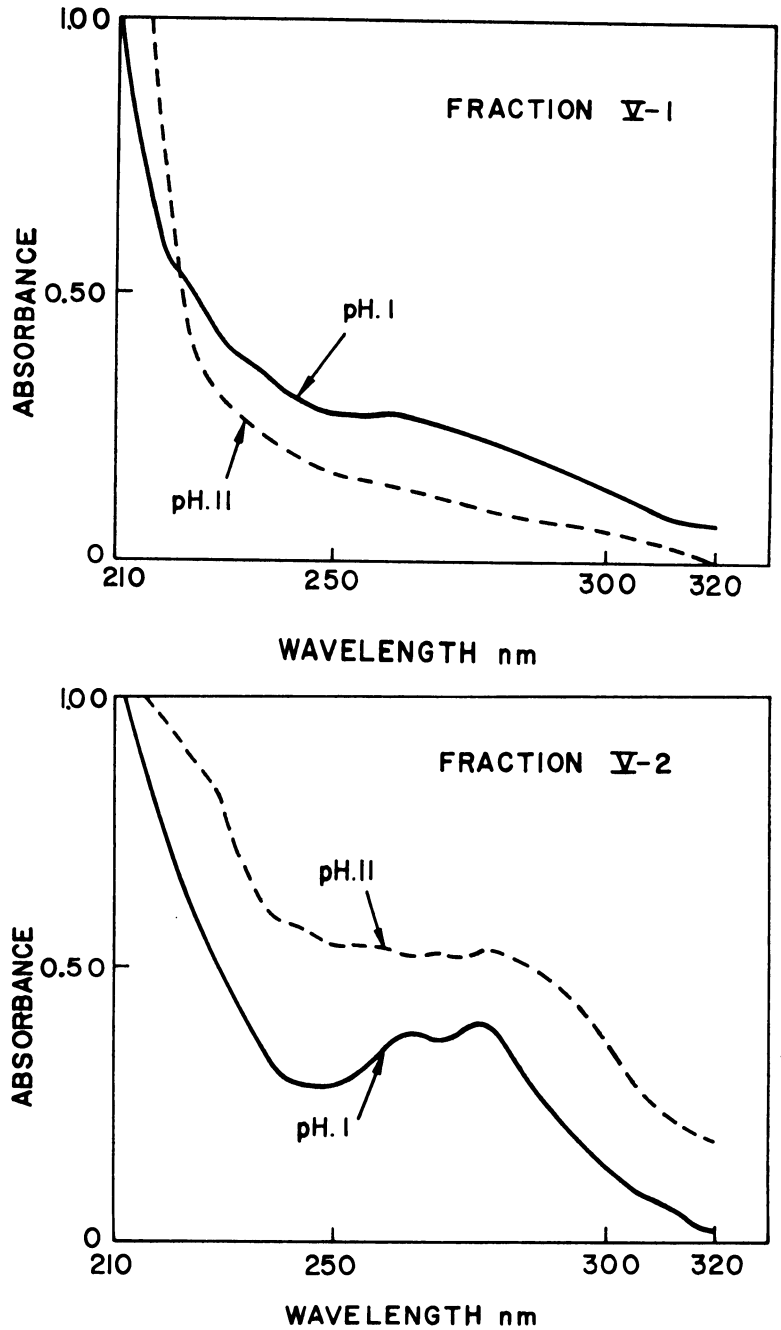


FIG. 2. Ultraviolet absorption spectrum of electrophoretically separated fractions of insoluble residue of rat liver, after hydrolysis with 1 *N* $H_2SO_4$, 100°C, one hour. Above, Fr.V-1; below, Fr.V-2. The spectra were taken on Beckman Model 10. The solid line shows the spectrum at pH 1, and the broken line shows the spectrum at pH 11. 0-1A is the measure of absorbance selected.

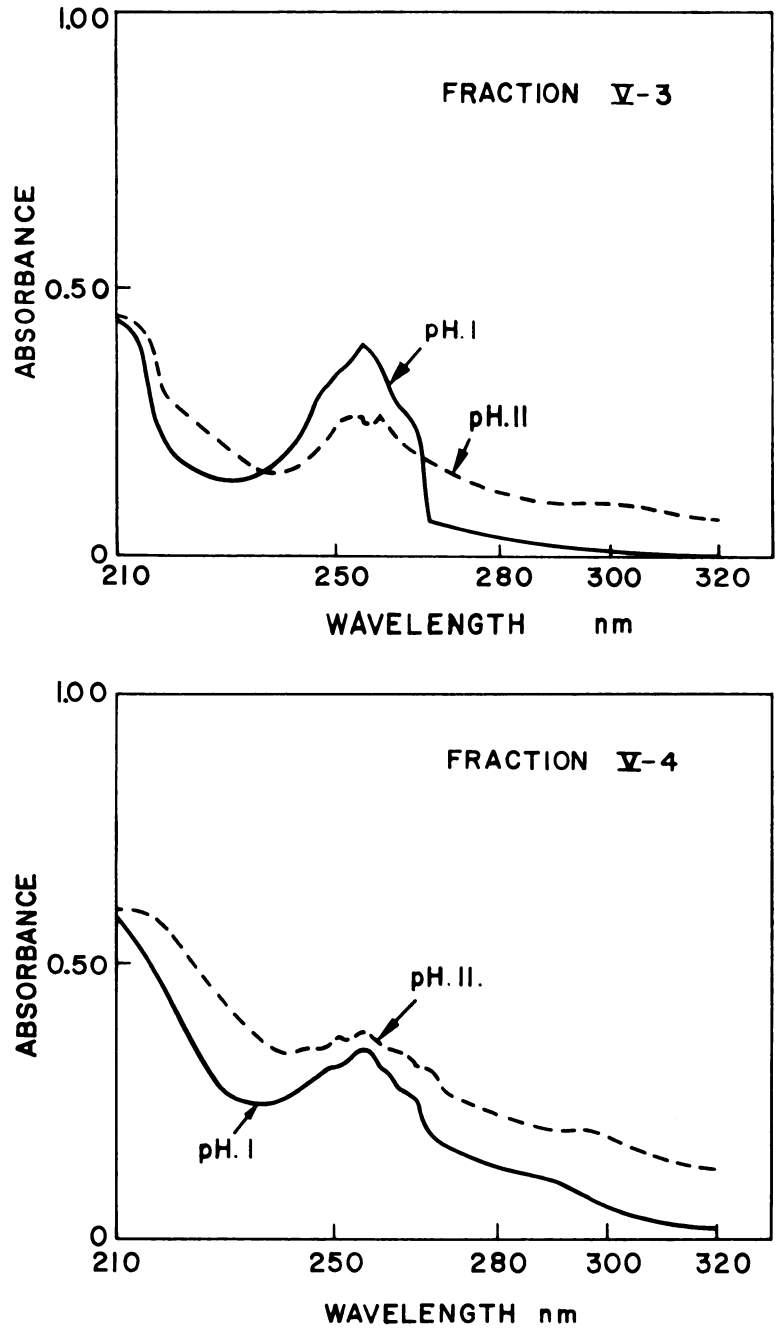


FIG. 3. Ultraviolet absorption spectrum of electrophoretically separated fractions of insoluble residue of rat liver after hydrolysis with 1 *N* $H_2SO_4$, 100°C, one hour. Above, Fr.V-3; and below, FR.V-4. The spectra were taken on Beckman Model 10. The solid line shows the spectrum at pH 1 and the broken line shows the spectrum at pH 11. 0-3A is the measure of absorbance selected. This means with 0-3A, three anodes of the ultraviolet-absorbing material give as much absorption as 1 mole of the same material with the selector at 0-1A.

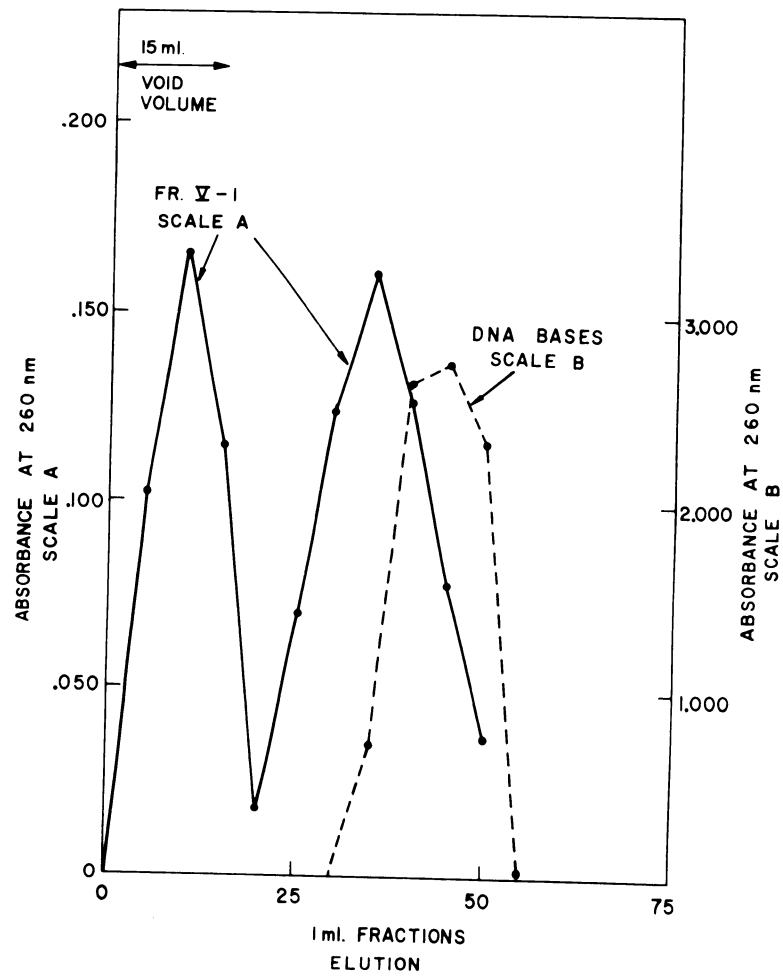


FIG. 4. Elution chromatogram of Fraction V-1 treated with 60% $HClO_4$, 100°C, ninety minutes, on a Sephadex G-25 column, along with that of DNA bases liberated by treatment of DNA with perchloric acid under similar conditions. Fraction V-1 gives two peaks, one substantially within the void volume, and the other outside the void volume but substantially before that of the DNA bases.

## TRITIUM ACTIVITY IN THE ELECTROPHORETICALLY SEPARATED FRACTIONS

Tritium activity was examined only in the 4 soluble electrophoretic complexes, the silver salts of which were soluble in acid (0.1 *N* HCl) medium, and which were eluted within the void volumes of their respective Sephadex G-25 columns. Their composition and ultraviolet spectra have already been discussed.

Table 4 gives the tritium activity of the 0.1-ml portions of 1-ml solutions of the 4 substances. The counting was done for ten minutes. DPM was calculated by taking the efficiency of the Packard scintillation counter at 60

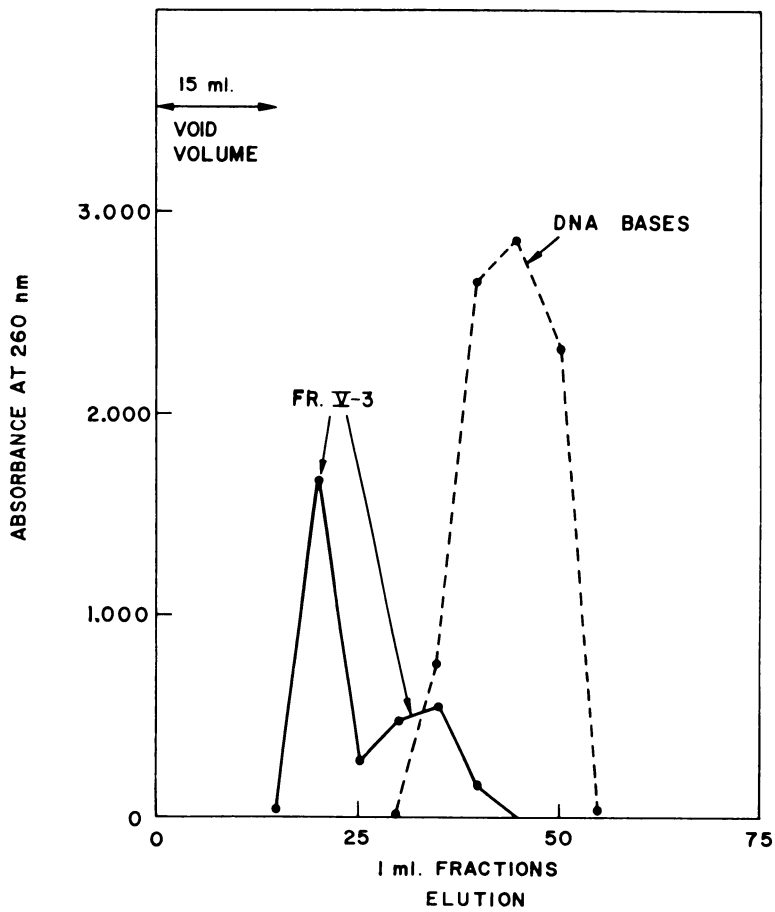


FIG. 5. Elution chromatogram of Fraction V-3 treated with 60% $HClO_4$, 100°C. ninety minutes, on a Sephadex G-25 column, along with that of DNA bases liberated by treatment of DNA with perchloric acid under similar conditions. Fraction V-3 gives two peaks, one at the end of the void volume, the other far beyond but substantially before the peak given by DNA bases.

per cent. It may be seen in Table 4 that even though small amounts of activity were present in Fractions V-1 and V-2, almost the entire activity originally found in the liver-insoluble material was in the negatively charged Fraction V-4.

The apparent recovery of 110 per cent of the starting radioactivity (cf. Table 1) is considered to be within the experimental error for 100 per cent. on a conservative estimate of sampling and counting errors.

## DISCUSSION

Radioactivity was found in soluble fractions prepared by solvent extraction under mild conditions, with a succession of solvents. Radioactivity

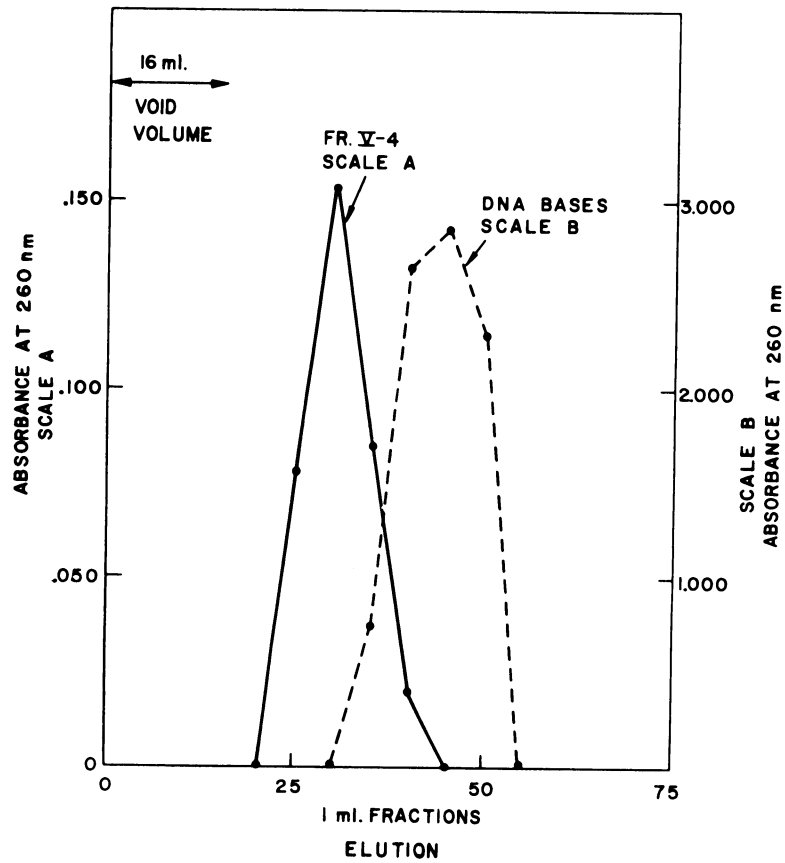


FIG. 6. Elution chromatogram of perchloric-acid treated (60% $HClO_4$, 100°C, ninety minutes) Fr.V-4 of Sephadex G-25 column along with that of DNA bases liberated by treatment of DNA with perchloric acid under similar conditions. Fr.V-4 gives one peak coming beyond the void volume but substantially before that of the DNA bases.

TABLE 4

*Tritium Activity in the Nucleic Acid Complexes*

| Substance | CPM in 0.1 ml/1 ml Solution (calculated from 10 min. counting) | Total DPM Accounting for 195 mg of Liver Hard Core |
|---|---|---|
| 145 mg of liver hard core | — | 4350 (Table 1) |
| Fraction V-1 | 19 | 210 |
| Fraction V-2 | 18 | 140 |
| Fraction V-3 | 15.7 | 0 |
| Fraction V-4 | 85.3 | 4500 |
| Background | 15.7 | — |

also was found in the insoluble residue remaining after the successive extractions. The specific activity was greatest in the chloroform-methanol extract, and next greatest in the insoluble residue. The other 3 fractions showed substantially lower specific activities. We first concentrated our attention on the insoluble residue, for the obvious connection it may have with the theory from this laboratory about the molecular mechanisms of aging (14–16).

Our analyses, although not yet extensive, show that the insoluble residue contains amino acids and the components of both DNA and RNA (Table 2 and Figs. 2 and 3). We found about 2 per cent of the total weight of the insoluble residue to be identifiable amino acids, although the nitrogen content of such insoluble residues in our experience corresponds to about 40–60 per cent protein. Appreciable quantities of pentose and deoxypentose were found in the soluble fractions V-1 to V-4, prepared by acid hydrolysis of the insoluble residue.

The absence of proline and oxyproline can be taken as evidence that there was no appreciable collagen component in the insoluble residue.

The second and third lines of evidence depend on the high molecular weight of the electrophoretic fractions, and on their resistance to strong hydrolytic procedures. The behavior of these soluble fractions on Sephadex columns before concentrated $HClO_4$ hydrolysis indicates a molecular weight greater than 5000 for all 4 electrophoretic fractions. The behavior after $HClO_4$ hydrolysis shows the persistence of some ultraviolet-absorbing fragments, of molecular weight greater than that of DNA monomer. Taken together, these two lines of evidence indicate that the solubilized fragments of the insoluble residue are of high molecular weight and that they are bound together by linkages almost certainly covalent, which have a stability beyond the ordinary for nucleic acids. The evidence as it presently stands provides substantial support that the insoluble residue is constituted from natural polymers of various kinds, held together by covalent crosslinks of extraordinary stability.

The findings on the composition of soluble Fraction V-4, which contains most of the radioactivity of the insoluble residue, strongly suggest that RNA is the component carrying the radioactivity. Although the evidence is not yet compelling, we cannot but note that various species of RNA are involved in very active metabolism and are more exposed to a variety of potential crosslinking agents than is DNA. DNA, in contrast, is most of the time protected from potential crosslinking agents by its intimate association with nucleohistones, commensurate with its function as a stable information-storage molecule (17).

Various kinds of crosslinkages between RNA, DNA and protein are conceivable and numerous possibilities of this sort have been presented [Bjorksten (15, 16)]. Of the possible crosslinking combinations between these 3 polymers, a covalent crosslink between the two strands of a DNA

molecule would appear to result in irreparable damage to the DNA. Repair mechanisms are known by which an organism can excise the damaged portion in a single strand of DNA and use the intact companion strand as a template in reconstructing the original structure (18). But a crosslinkage joining both strands at the same point is irreparable because neither strand can then serve as template for repair. The chromosome then cannot complete its replication because the DNA strands cannot separate. The cell will die in the next mitosis, or in some rare instances, mutate. This appears a highly probable mechanism among all those suggested so far, for the progressive cell death observed in aging [Bjorksten (16, 19)].

Fraction V-4, containing most of the radioactivity of the insoluble residue, is clearly of great interest and will provide one of the foci of our continuing investigations.

This investigation provides molecular evidence of crosslinkage as an important factor in the gerogenic insoluble fraction, because:

1. The insoluble gerogenic fraction contains elements of RNA, DNA and proteins in substantial quantities.
2. Even after hydrolytic solubilization with strong acids at high temperatures, the soluble fractions are of high molecular weight.
3. The linkages in the insoluble gerogenic fraction appear to be more resistant to acid hydrolysis than do most of the linkages in normal biological polymers.

*Acknowledgments*

We are indebted to The Upjohn Company for the donation of fractions from organs of a radioactive rat, by-products of Upjohn's project with Bjorksten Research Laboratories, Inc.; to the Glenn Foundation for Medical Research, and the Marcus and Bertha Coler Foundation, for donations of $2,900 and $1,000 to defray a part of the expenses of this work. We acknowledge with thanks the valuable help of Dr. Roswell Boutwell in preparing animals.

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Crosslinked Network

The five amino acids in which almost all of the nitrogenously bound paranatally administered tritium was found, were those which have a reactive group at the end of an extended side-chain. The most abundantly present, lysine, is the one which has the most reactive group on the most extended chain. These findings are consistent with the crosslinkage theory of aging, and have no apparent relation to other theories advanced.

This is one of the reasons, supported by other evidence, that we continue to believe that crosslinkage is a primary cause of aging.

They were further confirmed and amplified in a subsequent paper which gave evidence that the gerogenic insoluble residue is composed of a highly crosslinked network of at least RNA, DNA and protein, which is stabilized by covalent crosslinkages of unusual stability. Formation of these stable crosslinked structures would unavoidably interfere with the function of certain critical molecules of RNA, DNA or other polymers, leading to impaired cell function and death.

Having thus reconfirmed the actuality of the crosslinkage concept in aging we considered this phase closed and proceeded to look at overall strategy for age prolongation.

## Life Span Potential Estimates and Strategy Formulation

In order to decide on a strategy for any game, or any business venture, it is necessary to know the maximum potential gain. Only when the potential winnings are known, can we judge what risks we can afford, and move intelligently.

At the time most of our work was done, the only scientifically based estimate available was that of Henry Simms (J. Gertonol. 1: 13-26 (1946))who felt that the highest attainable life expectancy would be reached if we could maintain through life the same resistance to traumata that was found at the age when life expectancy was maximal. On this basis he calculated a theoretical maximum of 800 years. This was recently updated by Donald Carpenter (Rejuvenation 8: 33-49 (1980)), who used considerably more selective methods, taking in account several variables. Carpenter arrived at a projected mean lifespan of 2,400 years for men and 3,800-4,300 years for women. Our study of this question follows.

# LIMITS OF HUMAN LIFE EXTENSION AS INDICATED BY A STATISTICAL APPROACH

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Johan Bjorksten
Sept. 8 - 1980

**Summary**

Previous studies by H. Simms and by D. Carpenter have estimated the theoretically possible human life span, based on the assumption that a complete control of age dependent deterioration could be achieved, if the mortality throughout life could be kept constant at its optimum rate around age 10, and that this is within the realm of possibilities.
Their estimates of the potential mean human longevity thus arrived at ranges from 800 - 4300 years.

The present study considers that the life extending effect of the growth stage should not be a part of these calculations, because the cessation of growth is unavoidable in humans as in other land based organisms. This study is, therefore, based on the death probabilities under otherwise favorable conditions after growth has substantially ceased, at ages about 17 - 19. Under these conditions we arrive at ultimately attainable longevity mean values in the ranges of 640 - 940 for men and 2220 - 3450 for women.

No further speculation is attempted. It is noteworthy, however, that the presently commonly envisaged targets of attempted life extension are a very small part of the ultimate potentials.



**Details**

In planning strategies for research on the extension of healthy life, it is useful to know what the theoretical limits of the individual life span might be.

Simms, in 1946, approached this question by calculating the mean average life expectancy if the mortality rate in the most favorable age groups at the present in the USA were continued indefinitely. This, he felt, would eliminate the influence of aging and show how much longer an average human could live if the effects of aging were removed.

Simms thus arrived at a mean life expectancy at birth of 800 years (1).

Carpenter (2) reviewed the data of Simms, using as his basis the 1977 statistics for USA (3) and for Finland (4). The latter was viewed as a developed country with good statistics available, with modern medical facilities and more uniform life style and less complex population than the USA. The limit values for the mean human life, if the aging rate was assumed to be constant at the level of the 10 - 12 year old, the corresponding life expectancies were calculated to be :

| | Males | Females |
|---|---|---|
| U.S.A. | 2400 years | 3800 years |
| Finland | 2600 years | 4300 years |

The applicable mathematics were reviewed by Rennenkampff (5).

The life expectancy is found to be :

$$\int_0^{\infty} \mu t e^{-\mu t}\, dt = \frac{1}{\mu}$$

where $\mu$ is the death rate and t the continuous time. This is also the expected survival time at any age, since $\mu$ is independent of t*.

Applicable mathematics have been discussed in further detail by Kolari (6). On this basis, if we followed Simms, we would use the most favorable mortality figures for males and females. These were in 1977 in Finland 0.05 ‰

for 12 year old females and 0.19 for 10 year old males. However, it seems statistically sounder not to use the value of 0.05 because this deviates entirely from the age curve, the preceding values for 9, 10 and 11 year old females being 0.17, 0.25, 0.14, then the 0.05, followed by 0.11, 0.13, 0.18 at 13, 14 and 15 years. (Average 0.147). For the males, these figures are, including 3 years before and following the lowest value of 0.19 at 11 years : 0.37, 0.52, 0.41, 0.19, 0.32, 0.29, 0.31 (Average 0.344). The total number of children on which these statistics are based was 260,692 boys and 256,328 girls (7).

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*

If $\mu$ is the death rate, that is the probability of dying within unit time or proportion of those dying in unit time to those living, which is the same, then the probability of surviving time t will be :

$$\lim_{n \to \infty} e\left(1 - \frac{\mu t}{n}\right)^{n} e^{-\mu t}$$

This is the basis for the above equation.

Applicable mathematics have been discussed by R. Kolari (6).

Carpenter (2) segregates from the total, those deaths which are clearly not due to aging, such as accidents, suicides and other kinds of violence, and arrives thus at corrected mean longevity projections of 930 years for males and 2400 for females.

I have not made these adjustments, as the line between a traffic accident on a city street and a traffic accident which leads a blood clot to a coronary artery can be hairfine, and a «hit» by a crosslinking agent is, indeed, also an accident, although it takes place on a molecular scale.

On the other hand, it would appear that the protective effect of growth should be segregated. Rapidly dividing cells do not age, because the synthesis of new cytoplasm prevents increases in **the concentration** of crosslinked or otherwise insolubilized and irremovable molecules. This, therefore, does not become critical until after growth has ceased, as it necessarily must do in all land based organisms.

Figure 1 shows specifically the death of each year group for the 24 years ending in 1977. All are calculated as number of death per 10,000 persons. The number of individuals of each group varied between 27,161 (girls, 4 years old) and 41,765 (men, 21 years old). This clarifies the somewhat erratic death results in the years having the lowest mortality. The figure 0.05 ‰ of the 12 year old girls in 1977 means that of the 36,429 girls in Finland of that age only 2 died in 1977. Any lethal pure chance event could thus have changed the calculated mortality in that year 50 %. The actual variances are apparent from Fig. 1. so that the reader can judge the degree of reliability.

No attempts are made to correct for future conditions in population trends, guesses as to war or peace, increases in radiation level or total return to a primitive agricultural mode of life. Taking conditions as they are now, but basing calculations on the death probabilities **after cessation of growth** (37,889 females at 17 years ; 39,626 males at 18, in Finland in 1977), we arrive at death probabilities of 0.42 and 1.10, respectively. Using these values and assumptions, we arrive at mean ultimately possible life expectancies of **2380 years for women and 909 years for men.** For the United States the corresponding values would be moderately lower, but more difficult to define precisely due to the greater complexity of population as well as of ambient factors.

Nor are the Finnish values entirely fixed. I chose to graph the 1977 data, because more population data was complete for that year, but the 1978 death probability figures have also become available. For the three years in which growth appears to cease, the corresponding death probabilities have been tabulated from the statistics of R. Kolari (8) and the corresponding potential longevities calculated as shown above, and included in the following table.



TABLE 1
Death Probabilities and Corresponding Potential
Mean Life Expectancies for Males and Females Aged 17 - 19

| | | ♂ | | ♀ | | |
|---|---|---|---|---|---|---|
| Year | Age | $\mu$ | mean longevity years | $\mu$ | mean longevity years | longevity ratio ♀ / ♂ |
| 1978 | 17 | 1.17 | 850 | 0.27 | 3450 | 4.06 |
| | 18 | 1.06 | 940 | 0.45 | 2222 | 2.36 |
| | 19 | 1.57 | 640 | 0.40 | 2500 | 3.91 |
| 1977 | ♀ 17, ♂ 18 | 1.10 | 909 | 0.42 | 2380 | 2.62 |

While the statistics for recent years show great general similarities, the values for any particular year will vary. The reasons for these variations may, in part, be pure chance particulary in the lowest mortality years where the numbers of death are too low for good reproducibility.
In part they are undoubtedly influenced by the various factors Carpenter enumerates, in part also from differences between years in temperatures and other weather conditions, and in foods available, as well as environmental contaminations.

This will provide challenges for many researchers far into the future. While the data here presented show a considerable latitude in range of estimates they none-the-less permit us now to draw certain conclusions. It appears an inescapable fact that **evolution has sacrificed over half of the potential lifespan of the human male in order to maximize his efficiency as a fighter and a hunter.**

Consequently, it is expectable that the gap in longevity between male and female is going to widen as research progresses. It also seems indicated that more data should be obtained on to what extent female hormones could be practically used for male life extension in the post-reproductive part of life. The known effect of estrogens in reducing arterio-atherosclerotic changes is probably only a small part of much larger total effect. For example, the placenta hormone production has been brought into focus in connection with production of nerve growth hormones and general increase of resistance. Both of these are likely to be factors in longevity.

The time required for actually realizing the potentials indicated is too uncertain to justify any present speculation. To us, now, the important observation is that goals now commonly envisaged are very short of ultimate potentialities regardless of which of the above estimates will be closest to the actual future findings.

In this context, we might consider the problems prior to Oerstedt, Faraday and Edison, who gave us electric light, and Lebon, who envisaged gas light. If someone at an earlier date had given huge sums for the development of better lights, the funds would surely have been channeled to «feet on the ground» proposals for making better oil lamps, candles and torches. Certainly, anyone talking then of harnessing the powers of lightning, or of magnets for this purposes would have received a minimum of funds, if any.

We may now stand at the threshold of a similar awakening in aging research. To make the recognition of new approaches possible, it may be well not to be too modest in setting our goals, but to give thought to the potential that, according to calculations based on reliable statistics, proven mathematics and reasonable assumptions, is still before us.

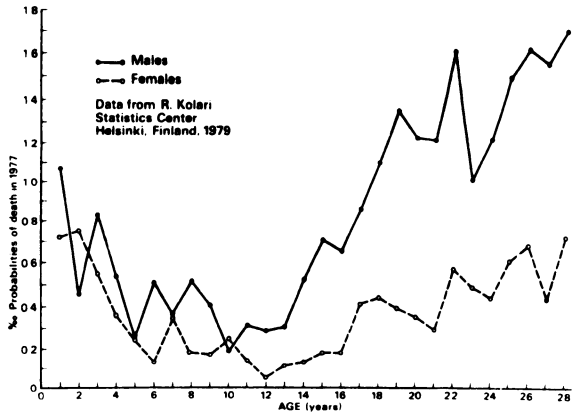


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Figure 1. CHANGES IN MORTALITY RATES BETWEEN AGES 1 and 25.

These changing rates of death risk at the ages when resistance to traumata are the highest enable us to estimate the potential life span, should this range of change be brought under control. Ref's. (4,6-8).

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## Short Term Strategy

From these calculations it appears that we are still a long distance from realizing the probable potential. Therefore, it is well worthwhile to actively seek a breakthrough.

The strategy we use, therefore, is to follow the guidelines given by the cross-linkage theory toward its ultimate goal, while still giving some time to shorter term studies of such special situations as might otherwise be neglected and which, if neglected, could cause our death or mental decline before the goal of the major breakthrough can be attained. One such instance will be discussed later (pages 160-166).

## The Enzyme Approach

If crosslinked aggregates are a major factor in aging, the removal of such aggregates should be beneficial. Any removing means should be compatible with life, and should have a relatively small molecular size in order to be able to penetrate the dense structures and overcome steric hindrances.

The best candidates, therefore, should be found among enzymes, from those microbes which can thrive on media consisting of highly crosslinked proteins or peptides as sole nitrogen sources. A search was instituted on this basis.

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# Study of Low Molecular Weight Proteolytic Enzymes

by *Johan Bjorksten, Elliott R. Weyer, and Stephen M. Ashman*

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# Study of Low Molecular Weight Proteolytic Enzymes

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*Summary*

A search was made for microorganisms capable of dissolving insoluble macromolecular fractions from aged mammals. From such microorganisms we obtained proteolytic enzymes which pass intact ultrafilters and dialysis membranes nominally cutting off at mol. wt. 10,000 and are retained by membranes cutting off at 1000 mol. wt. Such enzymes have been found in 3 taxonomically identified and several isolated but unidentified micro-organisms.

At least three of these enzymes differ in activation – inactivation behavior. Molecular weights determined by molecular sieve methods have given values for one of these enzymes of $5700 \pm 20$ % and for others in the possible range $5000 - 8000$.

Proteolytic properties have been determined by the Congocoll method, caseinolysis, and by the extent of liberation of tritium from the insoluble fraction of muscle tissue of aged rats who had received tritiated tyrosin at birth.

*General Approach*

In the study of insoluble, apparently crosslinked macromolecular fractions in organs of old humans and animals difficult to hydrolyze with any enzyme, the observation was made that some of these fractions could be fairly easily broken down by certain living organisms, or with macerates from these organisms, but not to any comparable degree by any available hydrolytic enzyme, or enzyme combination.

On the other hand, a very slow but progressive decomposition could be attained even with ordinary proteolytic enzymes if these were applied in huge concentration (say equal in weight to the substrate) and the solutions renewed daily. An explanation for this observation is that to achieve hydrolysis in these instances we need first of all to overcome steric hindrances. The slight degree of efficacy of the ordinary hydrolases is consistent with the view that these can attack the conglomerate molecules only from the outer surface, while enzymes capable of efficient attack can better penetrate aggregates and act on a much larger total surface.

Such a view is consistent with the crosslinkage theory of aging (1, 2). If the gerogenic high molecular substances have indeed been formed by cumulative random crosslinkages over a lifetime, it is to be expected that compact, sterically hindered agglomerate structures will result, which cannot be easily dissolved by highly specific hydrolases, but rather will yield to enzymes of less specific hydrolytic activity which are capable of overcoming the steric hindrances and of penetrating into the compact structures involved. This means that enzymes capable of hydrolyzing such structures would have to be sufficiently small to be capable of entering through the meshes even in very tightly knit conglomerate molecules.

We therefore embarked on a search for such very small enzymes. This quest can be divided into the following phases:

1. Separation of gerogenic complexes of high resistance to enzymic hydrolysis.
2. Searching for organisms capable of breaking down such complexes.
3. Searching for organisms capable of breaking down artificially heavily crosslinked macromolecular conglomerates.
4. Isolating the active hydrolases from such organisms.
5. Separating the lowest molecular weight fractions of these enzymes.
6. Studying the effect of such low molecular enzymes on the gerogenic fractions.

1. *Separation of gerogenic complexes from the human brain.*

The procedure of isolation followed, in its general outline, that employed in a previous study of the insoluble fractions of old human heart tissue(3). 1040 g of cortex from an 80 year old human brain was dispersed in 10 times its volume of acetone, to which an equal volume of water was added, and the remaining solids filtered off and washed 3 times with a smaller volume of 50 % acetone. After drying, the remaining solids were extracted with a 2:1 by volume mixture of chloroform and methanol, using a Soxhlet extractor overnight. To make certain that the extraction was complete, the residue was dried, pulverized and re-extracted in the same manner for another night. The remaining material, 90 grams, air dry, now free from water solubles, fats and phospholipids, was dispersed in water, and digested with agitation at pH 9 – 11 by the addition of 300 ml. n/10 phosphate buffer and 6 mg of "Pronase" (a protease mixture containing at least 4 protein hydrolases, from *Streptomyces griseus*) for 24 hours at 37°C. As the pH declined during the hydrolysis, it was readjusted repeatedly by addition of sodium hydroxide to bring the alkalinity back to the desired range of

pH 9 – 11. "Pronase" was added every 24 hours, while microbial contamination was suppressed by means of a few drops of toluene.

The material was then separated by centrifugation and washed with water on the centrifuge until the clear supernatant, after centrifugation, evaporated on a watch glass without showing traces of solids. The resultant material, about 15 % of the starting material, was insoluble in 8*M* urea, phenol, acetamide, formamide, strong detergent solutions with or without additions of urea or guanidinium chloride, dimethyl sulfoxide, dichloroethane, hexane, and chloroform-methanol in proportion 2:1. It was also insoluble in 8*M* urea and 6*M* guanidine chloride with addition of 20 % of thioglycolic acid or equivalent reducing agents, and insoluble in alkyl aryl sulfonate detergents, and in various combinations of the above. Insolubility in anhydrous hydrofluoric acid further indicated its crosslinked condition(4). This generally insoluble residue could however be apparently dissolved by anhydrous hydrazine at room temperatures.

Similar insoluble fractions could be prepared also from old human heart, muscles, and liver; and from heart, muscles, liver, kidney and brain from senile rats.

We initially used the insoluble material from human brains because we obtained the highest yields of insolubles from that organ, and because human brains are large and relatively easily available in comparison with brains from other species of similar longevity.

2. *Searching for organisms capable of breaking down insoluble fraction from aged human brain.*

R. Dubos originated the method of searching for organisms capable of breaking down substances, by exposing the medium containing the substance in question as an exclusive source of a nutrient needed by the organisms. In a variant of this technique, we dispersed one gram of the insoluble gerogenic fraction prepared as just stated, in 100 ml of 1.5 % agar gel, which was then poured into Petri dishes. These were inoculated with suspensions of media rich in bacteria, such as rich garden soil, sand contaminated with proteinaceous substances, sewage of various kinds, putrefying organic material, or were exposed to air at various localities.

After incubation for 1 – 7 days at 37°C, circular halos were sometimes noted on the agar, indicating solubilization of the

dispersed insoluble particles. Organisms causing such dissolutions were subcultured and studied. These organisms proved capable of attacking and dissolving the enzyme-and-solvent-resistant fraction from aged human brain.

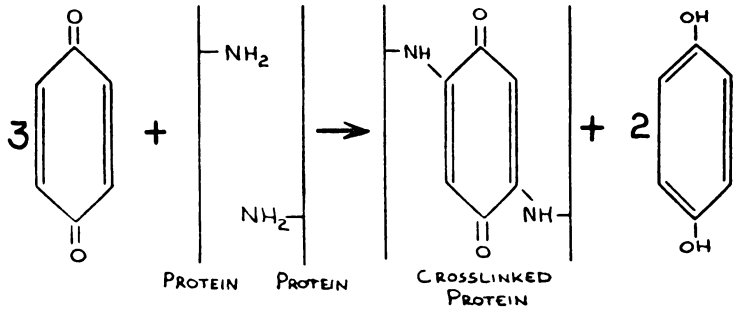
Several species were found which possessed the halo forming properties in varying degrees, and with the same wide difference between strains. Many of these were not identified taxonomically, but among those which were may be mentioned *Streptomyces coelicolor* and *Streptomyces annulatus*, and *Bac. cereus*. Considerable variation in halo-forming ability existed between strains of the same species. These were often greater than the differences between different species.

It would have been interesting to pause here to define the various organisms, optimize the enzyme production and specificities of each, determine whether the differences in halo size depended on molecular weight or on quantity secreted, study influence of matrix media, pH, temperature gaseous ambient, activators, inactivators and enzyme induction physiology for each organism. However, our purpose was beyond this and we therefore did only the minimum of work necessary to provide adequate tools for the next step.

It appeared interesting to determine whether these organisms were also capable of breaking down very heavily cross-linked insoluble artificially prepared protein agglomerates. To this end we added 50 grams of para-benzoquinone (Eastman) to 150 grams of Grayslake pigskin gelatin, (315 Bloom) dissolved in 1 liter of 0.5 molar phosphate buffer, pH 7.7. After 32 days at room temperature the firm dark gel was broken up in a Waring Blender, dialyzed against tap water for five days, washed, filtered on a Buchner funnel and centrifuged many times with water to remove excess quinone over the following two weeks. Still impure, the same was left to stand with 500 ml of 3A alcohol (ethyl alcohol denatured with approximately 5 % methyl alcohol) for the next two years. Finally the sample was Soxhlet extracted with the same solvent for 48 hours, removed, dried and stored at room temperature for an additional five years and again re-extracted on Soxhlet as above, prior to use. This crosslinkage treatment, of more than 7 years duration, should have allowed adequate time for the crosslinkage agent present in excess even after the two year standing with alcohol, to penetrate and to act in most of the possible sites of the large molecules.

We have chosen for the prime example a preparation made with p-benzoquinone as the crosslinking agent, because of the

hydrothermal resistance of the quinone bonds. In this case the major crosslinking reaction is(5):




Ciferri, *et al*, in their careful quantitative studies of the crosslinking reactions (6) (7) have shown that quinone linkages are incompar ably more stable than the aldehyde linkages and even more stable than chromium linkages. While the quinone reaction is of a single type, there are five competing reactions in the aldehyde crosslinkage. Although the initial reaction with lower aldehydes is impressively rapid, the resultant crosslinked products do not compare in stability with the quinone tannages. Furthermore, the work done with old hearts tends to emphasize the possibility of quinone tannages as a factor in long term effects (3).

A second crosslinked product was made with aluminum tanned material because of the prevalence of aluminum among those metal ions which increase dramatically in concentration with age (8), and because of the findings of Zinsser, Butt and Leonard (9) of X-ray diffraction evidence in human aorta of crosslinkages which are consistent with the dimensions of aluminum oxide linkages.

6.6 grams of aluminum acetate (24 % basic solution – U.S. Vanadium Corp.) was added to 66.7 grams of casein dissolved in 450 ml of water to which 9 ml of a 1 molar phosphate buffer (pH8.0) and 33 ml of 1 N NaOH solution had been added. After 22 days at 53°C the sample was blended with four times its weight of water, centrifuged and the precipitate transferred to a Soxhlet extraction thimble where water extraction was carried out for 66 hours. Finally the sample was extracted with acetone several times and air dried prior to use as a substrate.

The following tabulation compares the halo formation of a series of organisms as applied to the insoluble fraction made

from human brain, with those made from the first mentioned of the above preparations by following the identical preparative scheme:

| Organisms designation | Halo Width on Synthetic insoluble fraction | Halo Width on insoluble fraction from human brain |
|---|---|---|
| 1. | 8 mm | 4 mm |
| 2. | 4 | 2 |
| 3. | 2 | 2 |
| 4. | 4 | 4 |
| 5. | 8 | 4 |
| 6. | 4 | 2 |
| 7. | 2 | 4 |
| 8. | 12 | 4 |
| 9. | 4 | 2 |
| 10. | 2 | 2 |
| 11. | 8 | 2 |

These figures are not completely comparable, as they relate to separate tests, and observation was made at the time the halos seemed well enough developed for observation – not necessarily at identical stages of development. On the whole, however, they are indicative of a close parallel between the synthetic and natural insoluble fractions. No amount of waiting would have enabled the other organisms to match the spectacular 8 – 12 mm halos of organisms 5, 8 or 11 on artificially crosslinked gelatin. On the other hand, the inconsistency of organism 7, the only organism which shows a larger halo on natural than on synthetic hardcore, might well be due to the fact that these were run separately, under somewhat different conditions.

It is thus seen that with this one uncertain exception, the organisms active on the human brain insolubles were at least as active on the artificially crosslinked proteins, and in all but possibly one instance, were more active.

It would have been interesting to pause here to determine the sites and frequencies of the crosslinkages, the degree of uniformity of the peptide bonds, the amino acid sequences in the artificially crosslinked media before and after each preparative step. However, our purpose was beyond this and we therefore did only the minimum amount of work necessary to provide the tool needed for the isolation of gerolytic organisms.

*Isolation of low molecular weight proteolytic hydrolases*

A 14 l. Brunswick Fermentor was charged with 10 l. of a nutrient broth composed of 5 g Bacto Peptone, 3 g yeast extract powder and 1 g beta-D-glucose per liter of broth (tap water).

The pH was adjusted to 8.0. It was then inoculated with 240 ml of a shake flask culture of a strain of *Bac. cereus.* which had formed a pronounced halo on an insoluble brain fraction suspension in 1.5 % agar when tested as described above. Fermentation was carried out at 33°C with agitation at 350 rpm and an airflow at 2000 ml/min., 5 ml of 5 % "Alkaterge C" solution in corn oil was used to suppress foaming. After 76 hours, the bacteria were separated with a Sharples supercentrifuge. The cell-free solution was subjected to ultrafiltration on an Amicon PM-10 membrane, which provides a barrier for molecules nominally larger than mol. wt. 10,000 (mfd. by Amicon Corp., Lexington, Mass.). The filter was checked with horse heart Cytochrome n.c.(MW – 12,400) after this filtration, thus confirming that the filter remained in good condition during the operation.

The resultant cell free solution was checked for protease activity by the Congocoll assay method of Nelson, Ciaccio and Hess(10) in a slightly modified form: pulverized Congocoll hide powder dyed with Congo Red was screened through an 80 mesh sieve to eliminate coarser lumps. 20 mg of this fine powder was added to 1/2 ml of the ultrafiltrate, together with 1/2 ml of a 0.10 molar solution of tris (hydroxymethyl) aminomethane adjusted to pH 7.2 with hydrochloric acid. It was then digested 1/2 hour at 34°C with shaking. The material was then filtered clear, and the filtrate absorbance at 495 nm determined. This ultrafiltrate showed $A_{495} = 0.87$/ml. A control test was made with the same ultrafiltrate after immersion in boiling water for 5 minutes. This showed a value of $A_{495} = 0.02$/ml., showing substantial inactivation by rapid heating to the boiling point. This confirms that the effect measured was indeed enzymatic.

An additional 10 ml of ultrafiltrate having nominal mol. wt. between 1,000 and 10,000 showed an initial activity of $A_{495} = 7.5$/ml indicative of the concentration effected by removal of considerable water by ultrafiltration through the second membrane. Surprisingly, on standing at room temperature this activity increased slightly:

| | *0 day* | *1 day* | *4 days* | *5 days* | *6 days* |
|---|---|---|---|---|---|
| $A_{495}$/ml | 7.5 | 8.5 | 8.4 | 14.8 | 10.8 |

This effect has been noted several times. It has not yet been established whether it is due to release of an activator, removal of an inactivator, or to formation of active material by combination of components. It also appears possible that the enzymes may be initially formed as large molecules and that

some of these are auto – digested giving rise to fragments which retain enzymically active groups in molecules much smaller

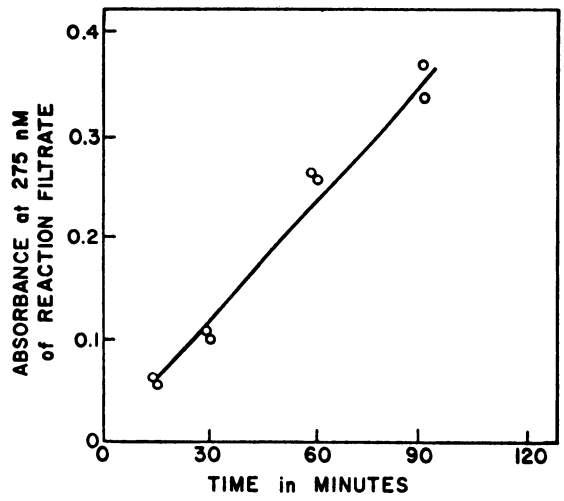



*Fig. 1.* Caseinolytic assay of Ultrafiltrate of nominal mol. wt. 1000– 10,000, from organism 17.

than the original, and that these can then penetrate further into the aggregate, giving rise to still smaller active enzyme molecules until finally activity begins to decline.

Another halo-forming organism, "14", showed a similar increase of activity over the initial. This organism has not yet been taxonomically identified. It is air borne, showed in Czapek-Dox nutrient solution a white translucent growth with occasional white, cottony aerial hyphae. On potato culture it developed a viscid, slimy mucous sticky tan layer over the surface. This organism was grown in submerged fermenter culture, under the following conditions.

Broth: 12 000 ml containing per liter 2.0 g $K_2HPO_4$, 1.0 g $H_3PO_4$, 0.20 g $MgSO_4 \cdot 7H_2O$, 7 g casein, tap water to make 1 liter.

| | |
|---|---|
| Starting pH | 7.1 |
| Temperature | 34°C |
| Agitation | 400 rpm |
| Air flow | 2000 ml/min. |
| Antifoam added | 50 ml total, Alkaterge C 5 % in corn oil |
| Time | 137 hours |
| Final pH | 8.1 |

After centrifugal separation of the cell mass, ultrafiltration was carried out (PM-10 filter) the membrane was checked with Cytochrome n.c. to make certain it was undamaged, and the proteolytic activity determined as above with Congocoll.

The ultrafiltrate through Amicon filter passing molecules nominally smaller than 10,000 mol. wt. showed the following proteolytic activities:

| | *Initial* | *42 hours* | *119 hours;* |
|---|---|---|---|
| $A_{495}$/ml | 0.22 | 2.44 | 0.59 |

Here is seen another example of an apparent increase in protease activity on standing, followed by a decline.

*Separation of low molecular weight enzymes by dialysis*

Four different organisms, "8", "12", "13", and "14" were grown in 250 ml shake flasks, using Peoria Broth until abundant growth had been established. The broths were Seitz filtered separately (not ultrafiltered). The cell-free filtrates were then combined and kept 3 days at room temperature to permit interaction.

They were then dialyzed in a tube of regenerated cellulose which did not pass hen eggwhite lysozyme (M.W. = 14,400), so that a bag containing 5 ml of sterile, enzyme-free Peoria Broth was inserted in the flask, and shaking continued 3 days.

The exterior fluid gave, in a few minutes, the characteristic deep orange with Congocoll. The sample from the inner bag similarly tested released after 30 min. incubation a faint color from the Congocoll. At the end of 24 hours this color had deepened until it almost equalled that on the undialyzed side. The bag was then re-tested with lysozyme in acetate buffer at pH 4.5 and found impermeable to this enzyme.

The findings of enzyme dialysis under these conditions were confirmed with two specimens of regenerated cellulose tubing. Both gave the same positive results using the crude enzyme mixture stated above: all four Seitz filtrates incubated together, also all possible combinations of three of these, and further with six of the possible nine combinations of two-at-a-time. In all cases the same dialysis tubes were used with lysozyme with no evidence of porosity to that molecule, also subcultures confirmed the sterility of the diffusate.

The use of ultrafiltration proved more convenient as well as more dependable and rugged for separation of enzymes having smaller molecules than those previously known – therefore in subsequent preparation work main reliance was placed on ultrafiltration.

*Casein hydrolysis assay*

In order not to depend wholly on the collagenolytic assay methods, we also applied the method of casein hydrolysis.

The casein assays were made by Hagihara's modification of the Anson and Kunitz casein hydrolysis method(12). We employed for this the low molecularweight enzyme solution obtained by ultrafiltration of the fermentor broth obtained as described above, through "Diaflo" membranes PM10 and UM2 respectively, thus taking the cut between nominal molecular weights 1,000 and 10,000. A linear rate of production of trichloracetic acid soluble digestion products was found, as shown in Figure 1.

Ultrafiltrate from Organism 17 nominal mol. wt. between 1,000 and 10,000

| | Casein Method 30 min. incubation $A_{275}/ml$ | Congocoll Method 30 min. incubation $A_{495}/ml$ |
|---|---|---|
| Ultrafiltration fraction 1000–10 000 | 1.00 | 5.86 |
| Ultrafiltrate before concentration on UM2 membrane | 0.27 | 1.80 |

*Assays with tritiated insoluble protein residue*

To prepare the tritiated assay substrate, a pregnant Sprague-Dawley rat was given orally in her drinking water 8 millicuries of tritiated tyrosin as follows: 2 mc seven days before parturition, 2 mc daily the first and second days, and 1 mc daily the fifth and sixth days following parturition. The litter born thus received tritium through the body of their mother, either in the last days of her pregnancy, or during lactation. They never received any other radioactive material than this.

A male rat from this litter died of pneumonia at age 480 days. The muscles were removed as completely as possible, combined, and all matter that could be dissolved or enzymatically hydrolysed using commercially available enzymes, was removed. Some of the tritium administered at birth still remained in the insoluble residue. This tritium is assumed to be in structures resistant to attack by the catabolic enzymes of the rat.

The muscle tissue separated as above was homogenized in a Virtis disintegrator, extracted exhaustively with water, precipitated after extracting with 10 times its volume of acetone, the precipitate extracted 24 hours by the Soxhlet method using as solvent a mixture of chloroform and methanol (2:1) so that

water solubles, fats and phospholipids were effectively removed. The residue was then dried and re-pulverized, and the extraction resumed for an additional 5 hours.

After drying the residue was then hydrolysed with "Pronase". 1.20 g (air dried) of rat muscle residue was digested with 60 mg of "Pronase" at 35°C for 68 hours. Every 12 hours pH was re-adjusted to pH 9 and an additional 30 mg of the enzyme added. After 68 hours the digestion appeared complete in that radioactivity in the supernatant fluid showed no further increase.

This residue was washed thoroughly on the centrifuge, to remove remaining enzyme, dried and pulverized. A 20 mg sample was combusted in oxygen according to the Thomas-Ogg method (14). The resultant water, which contained all the tritium in the sample, was added to a scintillation counting liquid, 1/2 ml to 15 ml of "cocktail", and the radioactivity was determined on a Beckman 1000 scintillation counter. 440 CPM were found in the 20 mg sample.

50 mg of the above described tritiated protein residue, already exhaustively hydrolyzed by "Pronase" was digested with 2 ml of ultrafiltrate from a strain of *Bac. cereus*. The 50 mg of tritiated insoluble residue contained a tritium activity of 1,100 cpm. An aliquot of supernatant was removed and counted at the time intervals shown below. After the 47 hour sample had been taken, a fresh 3 ml of the enzyme ultrafiltrate was added, and the digestion continued. The progressive liberation of previously very firmly bound radioactivity is apparent from the following table.

Digestion Time

| | Fresh enzyme ultrafiltrate added, new count started | | | | | |
|---|---|---|---|---|---|---|
| | 1 hr | 6 hrs. | 23 hrs. | 47 hrs. | 70 hrs. | 143 hrs. |
| CPM per ml | 28 | 46 | 168 | 606 | 40 | 66 |
| CPM for total supernatant | 56 | 69 | 168 | 606 | 120 | 165 |

It is thus seen that this low molecular weight enzyme was capable of dissolving over half of the radioactivity fixed in insoluble residues from the animal and not liberated by exhaustive "Pronase" digestion.

In order to determine whether the radioactivity liberated was really dissolved in the water phase, and not merely suspended in the colloidal state, it was subjected to ultracentrifugation for 2 hours at 50,000 g. There was no sedimentation of radioactivity and no visible sign of sedimentation.

To check the molecular weight with a somewhat different procedure and a low molecular weight enzyme prepared from a different organism, we proceeded as follows: A cell-free fermentor broth of a mutant strain of *Bac. cereus.*, which had a Congocoll assay value of $A_{495}$ = 30/ml, purified by presipitations and ultrafiltrations so as to obtain an enzyme concentrate which had an activity at its optimum, pH 7.2, of 320 $A_{495}$/ml.

We applied the method of Whitaker (11) for molecular weight determination for globular proteins. The freshly prepared

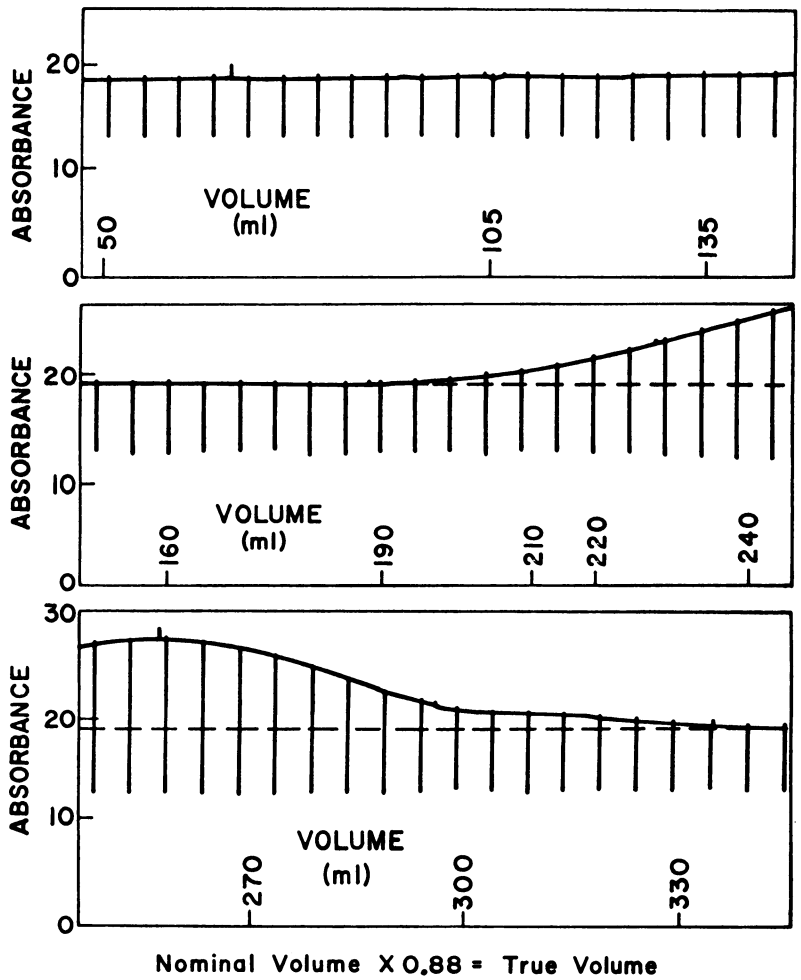



*Fig. 2.* Enzyme preparation Congocoll activity 320/ml, filtered twice through Amicon filter nominally holding back mol. wts. above 10,000. Passage through Biogel-P10 column.

ultrafiltrate was passed twice through Amicon filters with nominal cutoff at 10,000 mol.wt., and then concentrated by ultrafiltering through Amicon filters with nominal cutoff at 1,000. This material showed an activity of $A_{495}$ = 320/ml by the Congocoll method. A chromatographic column 2.2 × 70 cm was packed with "Biogel P-10", 200–400 mesh (a crosslinked polyacrylamide gel with resolving power over the molecular weight range 1,500 to 20,000). The gel had been washed free of fines and equilibrated 24 hours with 0.1 M Tris-HCl buffer pH 8.1. Samples were applied in a 1.0 ml volume and the column operated at room temperature at a flow rate of 50 ml/hr. The effluent absorbance at 280 nm was recorded continuously and 5.0 ml fractions were collected. The results of several successive (calibration and sample) runs on the same column are tabulated below. Figures 2 and 3 show the experimental records of absorbance vs. effluent volume for runs 3 and 5 respectively.

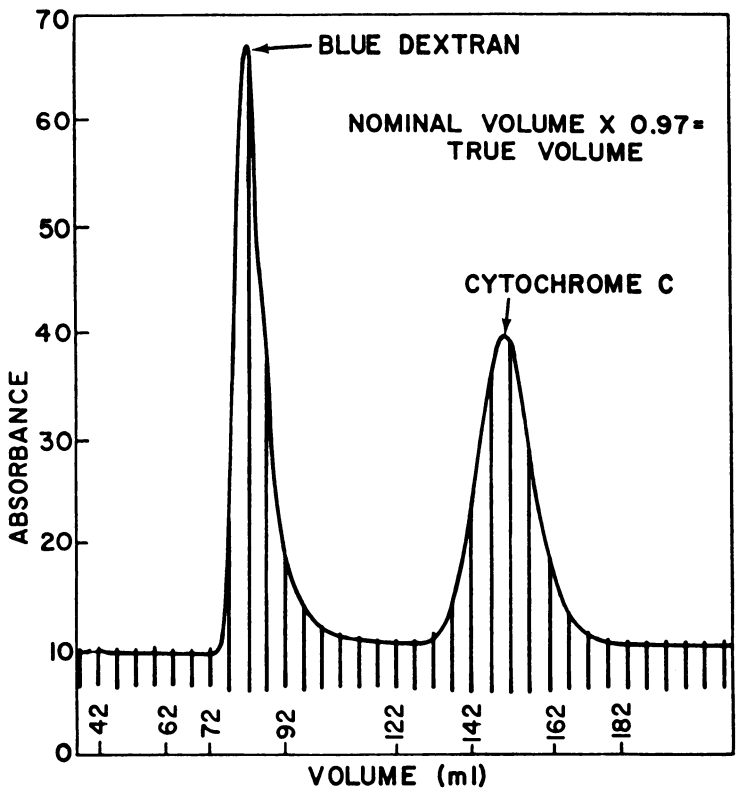



*Fig. 3.* Calibration (in part) for column of Fig. 2.

| No. of runs in actual sequence | Blue Dextran $M = 2 \times 10^6$ | Cyto-chrome c $M = 12{,}400$ | Pancreatic Trypsin Inhibitor, $M = 6{,}500$ | *Bac. cereus* Ultrafiltrate Conc. |
|---|---|---|---|---|
| 1 | 15 mg; 76 ml | ..... | ..... | ..... |
| 2 | ..... | 6 mg; 148 ml | 11 mg; 211 ml ± 5 ml | ..... |
| 3 | ..... | ..... | ..... | 10 mg; 238 ml ± 5 ml |
| 4 | 0.7 mg; 78 ml | ..... | ..... | ..... |
| 5 | 3.2 mg; 76 ml | 7 mg; 143 ml | 6.5 mg; 235 ± 10 ml. | ..... |

From the above data a linear plot was constructed relating $V/V_0$ to $\log_{10}M$, where V is the elution volume at the peak of a component, $V_0$ is the void volume (determined with blue dextran), and M is the molecular weight of a component. This plot is shown is Figure 4 with the region of uncertainty lying between the two lines. These results yield a molecular weight of between 6,700 and 4,600, or a mean probable weight of 5,700 ± 20 %. The passage of the material through the column is accompanied by inactivation, possibly by separation of a co-factor, or by change of extent of polymerization of the enzyme.

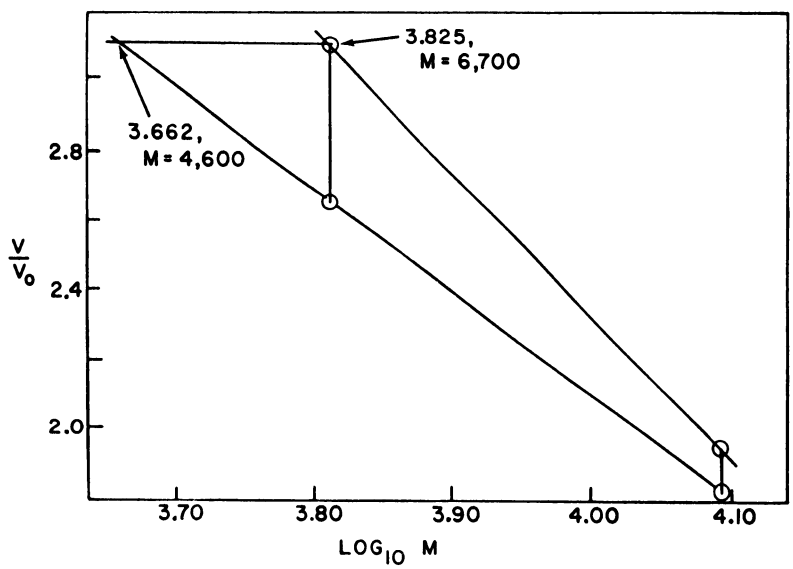



*Fig. 4.* Whitaker plot for mol. wt. range estimation from data of Fig. 2 and 3.

*Characterization of three proteases of molecular weight < 10,000 with respect to inhibitors and activators*

The assay procedure used was the Congocoll method described above. The table below shows additions made, and activities obtained expressed as percent of the control assays on the ultrafiltrates.

| Partially purified protease of mol. wt. less than 10,000 | Standard assay + cysteine $1.7 \times 10^{-3} M$ | Standard assay + $CaCl_2$ $7 \times 10^{-3} M$ | Standard assay + phenyl methyl sulfonyl fluoride $1.6 \times 10^{-4} M$ | Standard assay + p hydroxy mercuri benzoate, $3.2 \times 10^{-4} M$ | Standard assay + EDTA $1.0 \times 10^{-3} M$ | Standard assay solution | Enzyme solution heated 5 min. at 100°, before assay |
|---|---|---|---|---|---|---|---|
| Organism 8 Ultrafiltrate | 55 % (80 % when rechecked)* | 92 % | 88 % | 160 % (173 % when rechecked) | 31 % | 100 % | 0 % |
| Organism 12 Ultrafiltrate | 27 % (72 % when rechecked)* | 57 %** | 100 % | 101 % | 9 % | 100 % | 0 % |
| Organism 13 Ultrafiltrate | 108 % | 132 % | 95 % | 126 % | 97 % | 100 % | 0 % |

* Some oxidation (formation of insoluble cystine) occurred in the cysteine stocksolution overnight — this may account for the observed lessened inhibition.

** This contained a fine precipitate, probably insoluble calcium phosphate, since a substantial concentration of inorganic phosphate is present in the B-broth growth media.

The results shown in the above Table show patterns of response to potential inhibitors and activators that are typical of proteolytic enzymes. They also show that the three enzymes examined are all distinctly different from one another. This can be seen more easily in the following abbreviated summary of the agents that affect each enzyme. The code for effects is (− − −), strong inhibition; (− −), definite inhibitio;n (−), weak inhibition; (+ + +), strong activation; (+ +), definite activation; (+), weak activation; (0), no effect.

*Organism 8 Ultrafiltrate MW 10,000*

Boil (– – –), cysteine (– –), pHMB (+ +), EDTA (– –)
*Organism 12 Ultrafiltrate MW 10,000*
Boil (– – –), cysteine (– –), pHMB (0). EDTA (– – –)
*Organism 13 Ultrafiltrate MW 10,000*
Boil (– – –), cysteine (0), pHMB (+), EDTA (0)

All of the low molecular enzymes so far observed were optimally active in the pH range 6.5 to 8.0.

*Variability of enzyme content between strains of Bac. cereus*

Cultures of several strains of *Bac. cereus* were grown in shake flasks 24 and 48 hours, under simulation of the conditions used in low molecular weight protease fermentor run. The variability both of total enzyme content, and of the ratio of low molecular weight protease to total protease, is apparent in the following table.

Congocoll assay of the ultrafiltrates mol. wt. below 10,000
$A_{495}$ values by Congocoll method

| Shake flask incubation time: | 24 hrs | | 48 hrs. | |
|---|---|---|---|---|
| | Crude broth | Ultrafiltrate mol. wt. below 10,000 | Crude Broth | Ultra-filtrate |
| Strain No. 1 | 0 | 0.04 | 0.01 | |
| 2 | 7.0 | 2.92 | 9.8 | 0.42 |
| 3 | 0.12 | 0.08 | 0.08 | |
| 4 | 0 | 0 | 0.03 | |
| 5 | 3.0 | 0.66 | 1.7 | 0.10 |
| 6 | 1.26 | 0.14 | 0.18 | |
| 7 | 1.36 | 0.46= | 1.0 | 0.09 |
| 8 | 9.9 | 1.9 | 22.6 | 2.6 |

*Discussion*

The low molecular weight proteases now found in three defined and a considerable number of taxonomically still undefined organisms may provide at least a partial explanation for the well known ability of many microorganisms to attack substrates which are resistant to generally recognized enzymes. This is particularly true of highly crosslinked aggregates. It is well recognized that crosslinkages provide severe steric hindrances. This is basic for the leather industry, the rubber industry and a large segment of polymer industry, and appears to be a significant factor in the basic chemistry of aging. Smaller enzyme molecules can obviously penetrate where larger molecules are sterically hindered.

The fact that we have already established differences between at least three protein hydrolases in the molecular weight range substantially below 10,000 indicates that we may be dealing with a large family of enzymes previously unrecognized because of their limited stability, their tendency to polymerize, and because they are usually accompanied by larger molecular weight enzymes of similar activity from which they must be separated to be recognized.

It appears probable that this observation will be found to apply to many other classes of enzymes, including lipases, amylases, nucleases, and others.

The hydrolytic enzymes here reported are viewed as early representatives of a family of substances. It may be appropriate to call these *gerolytic* enzymes. It would be fortuitous indeed if the present examples were not soon superseded by still more effective and more easily prepared members of the same broad class of enzymes. We will direct our search toward enzymes characterized mainly by molecular weights low enough to penetrate cage structures, and compatibility with living systems. The enzymes in hand are being studied further as they represent the best means now apparent to act upon gerogenic insolubles under physiological conditions.

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# The Search for Microenzymes: The Enzyme of Bacillus cereus

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## *Abstract*

Microenzymes are defined as enzymes, including subunit forms with molecular weight less than 10,000. Microenzymes were initially detected on the basis of their ability to penetrate thru an Amicon UM-10 membrane which effectively excludes substances of molecular weight greater than 10,000, and which did in practice exclude over 99 % of Cytochrome C (MW 12,400). A single exogenous microenzyme was found to exist in equilibrium with oligomers, with oligomers predominating, in a strain of Bacillus cereus (BRL-70).

Calcium ion was found to stabilize and to activate the enzyme. Lead ion also served this role. Other divalent ions were less effective stabilizers of the enzyme. Monovalent and aluminum ions had little or no stabilizing effect. The enzyme was extremely unstable below pH 5.0, had peak activity about pH 6.5 and retained considerable activity even after prolonged storage at pH 10.0.

Gel filtration gave an enzyme activity peak at a molecular weight somewhat greater than Cytochrome C (MW 12,400) but with activity tailing out to the region corresponding to molecular weight 2,500. Treatment with anhydrides caused the enzyme activity to peak at a higher molecular weight. Sodium dodecyl sulfate treated enzyme in acrylamide gel electrophoresis gave bands around 5,000–5,500, 8,000–8,200, 10,700 and 16,000. These relate to each other in the ratio of 2:3:4:6, implying a monomeric weight of about 2,700 for this microenzyme.

## *Introduction*

Microenzymes are defined as enzymes, including monomer or subunit forms, with molecular weights below 10,000.[1] The search for microenzymes was initiated at this laboratory as a result of aging research which indicated a possible therapeutic utility for such enzymes.[2]

In the aging process, amorphous "hyalin" materials accumulate in and around cells and gradually impede the functionality of the cells and consequently of the body. These hyalin materials are mostly proteinaceous, and appear to be highly cross-linked, dense, insoluble, and resistant to dissolution by the body's enzymes.[3] The insolubility and high density of the materials suggests that inability to penetrate the hyalin matrix

may explain why normal body enzymes do not eliminate this material.

Since these materials *are* broken down naturally after death, enzymes for achieving such dissolution clearly do exist. If the high density of these hyalin materials prevents their dissolution by the body's enzymes, then a very low molecular weight proteolytic enzyme – a microenzyme – seems called for. The search for such enzymes in our laboratory has lately centered around spore-forming microorganisms whose spores also have extremely dense walls, which they obviously must at least partially dissolve in order to germinate.

Evidence for the existence of low molecular weight enzymes was reported earlier.[2] One such enzyme was found to dissolve hyalin materials in kidney in vitro.[4] Subsequent evidence has indicated that the enzyme most intensely studied – that from a strain of Bacillus cereus (BRL-70) – exists in equilibrium with oligomers of molecular weights between 10,000 and 25,000. The effects of pH and ionic composition on this enzyme are reported here, as are ultrafiltration and electrophoresis studies, plus other efforts to establish the molecular weight of the monomeric enzyme.

## *Methods*

### *Production of enzyme solution*

A strain of Bacillus cereus (BRL-70) was inoculated into a 500 ml shaker flask with 100 ml of broth containing 0.5 g of bacteriological peptone (Nutritional Biochemicals Co., Cleveland, Ohio), 0.3 g of yeast extract (also Nutritional Biochemicals Co.), and 0.5 g of glucose, pH $7.0 \pm 0.2$, 34°C, (SAR Broth). Sixteen hours later the culture was inoculated into a 14 liter New Brunswick fermentor containing 10 L of broth at 34°C with 150 g of bacteriological peptone, 90 g of yeast extract, and 50 g of glucose in unsoftened tap water, adjusted to pH $7.0 \pm 0.2$. Growth was monitored by turbidity, pH measurements, and finally for proteolytic activity by Congocoll assay.[5] A 20 % glucose solution was added periodically until the total glucose added was 450 g.

After 74 hours, the broth, at pH 7.8, contained proteolytic activity of over 1,000 Congocoll units per ml. A sample of cell-free broth was filtered thru an Amicon UM-10 membrane designed to retain materials of molecular weight greater than 10,000. Approximately 6 units of proteolytic activity per ml passed thru the membrane. The microbial cells were removed from the rest of the broth by passage thru a Sharples super-centrifuge and the broth was then frozen until used. Over 300

units or 30 % of the activity passed thru the UM-10 membrane when the broth was subsequently thawed and filtered. High percentages of activity have passed thru intact UM-10 membranes on several occasions but we have not as yet been able to achieve this consistently.

### *Assays for proteolytic activity*

Proteolytic activity was routinely determined by the Congocoll method of Nelson et al.[5] This method was found reliable in this work, except where variability was sometimes observed at very high enzyme concentrations. For extremely low enzyme concentrations, the caseinolysis method of Williams and Chase was used.[6]

After it became apparent that calcium ion protected enzyme activity, $1 \times 10^{-4}$ M $CaCl_2$ was incorporated in the pH 7.2 Tris buffer in which the enzyme and Congocoll were incubated. A Congocoll unit, as used in this study, is defined as an absorbance of 1.0 at 495 nm produced by 1.0 ml of enzyme solution reacting with 20 mg. of Congocoll for 30 minutes at 30°C.

### *Ultrafiltration*

Enzyme samples were filtered thru Amicon UM-10 membranes (Amicon Co., Lexington, Mass.) with nitrogen at 40 – 50 psi pressure at 4°C. These membranes are designed to retain substances with molecular weights greater than 10,000, and did routinely retain over 99 % (generally over 99.5 %) of an aqueous solution of Cytochrome C (M. W. 12,400; Calbiochem, A grade, equine heart).

Studies on the effect of calcium ion concentration and pH on penetration of the enzyme thru the membrane were carried out. Salts of lithium, sodium, magnesium, calcium, barium, strontium, and zinc were added at 0.05 M concentration to calcium-free enzyme solutions in 0.01 M N-ethyl morpholine (NEMO) at pH 7.0 (zinc at pH 5.7), and left to equilibrate for four days at 4°C. The solutions were then diluted 50× with 0.01 M NEMO, pH 7.0, and were immediately passed thru a UM-10 membrane. The total activities of these solutions and the activities which passed thru the UM-10 membrane were then determined.

Enzyme samples were frequently concentrated over UM-2 membranes which are designed to retain substances with molecular weights over 1,000. Solutions in which the enzyme was dissolved were also changed by washing in the new solution over the UM-2 membrane.

### *Gel Filtration*

Most gel filtration studies were run with Biogel P-10. A number of runs were also made with Sephadex G-50 as the fractionating material. Initially, filtration was with a pH 7.65 solution of 0.02 M N-ethyl morpholine (NEMO) containing 0.1 M NaCl and 0.001 M $CaCl_2$, but in most experiments the solutions were adjusted to pH 7.0. Other filtrations were carried out between pH 5.5 and 9.5 using 0.02 M Tris buffer, usually with maleate or glycine. Unless specifically excluded, $CaCl_2$ was routinely incorporated as a stabilizer in these solutions at 0.0001 M to 0.001 M.

Enzyme previously passed thru a UM-10 membrane was washed free of calcium ion with 0.02 M NEMO and 0.1 M NaCl. The solution was mixed with 0.3 M salt solutions to achieve a 0.1 M concentration of LiCl, NaCl (only), KCl, $MgCl_2$, $BaCl_2$, $Sr(NO_3)_2$, or $ZnCl_2$. Solutions containing 0.01 M lead acetate or aluminum nitrate were also prepared. The samples were equilibrated for at least one week at 4°C, and were then fractionated on a Biogel P-10 column with solutions at pH 7.0 containing 0.1 M NaCl, 0.02 M NEMO and 0.001 M concentrations of the particular salt tested. Since the Zn and Al equilibrated enzyme samples had essentially no activity when they came off the Biogel column, a $CaCl_2$ equilibrated enzyme of comparable potency was later fractionated thru the $ZnCl_2$ or $Al(NO_3)_3$ equilibrated columns.

Standards for molecular weight determination included Cytochrome C (M.W. 12,400), Vitamin B-12 (M.W. 1,355), a blue dextran of M.W. $10^6$ and sucrose (M.W. 342). Pancreatic trypsin inhibitor (Worthington Biochem Co., Freehold, N.J.), one of the few compounds available with molecular weight about 6,000, appeared to have a contaminant (trypsin?) which made it unusable as a standard. A yellow dextran of M.W. 20,000 supplied by Pharmacia (Uppsala, Sweden) was used in several instances.

### *Electrophoresis*

The preparative electrophoresis method of Whitehead et al [7] was used initially as a technique for purification of the micro-enzyme. Electrophoretic mobility and enzyme purity were evaluated with this technique using Sephadex G-25 as the support medium. The enzyme moved toward the cathode at pH 5.5 and 7.0 and toward the anode at pH 9.0, so these poles were placed at the bottom of the column. After electrophoresis the samples were eluted from the column with the same buffer.

Gel electrophoresis using the method of Weber and Osborn [8] was utilized to determine the molecular weight of the micro-

enzyme. A 4X concentration of their cross-linker (methylene bisacrylamide) was used to achieve density high enough to fractionate the low molecular weight components.

### *Dispersement Studies*

Treatment of oligomeric proteins with succinic and other anhydrides has been reported to disperse these proteins to monomeric form.[9,10] Several anhydrides were therefore used in attempts to disperse and stabilize the monomeric or subunit form of the microenzyme. Molecular weight distributions of the resulting substances were evaluated by gel filtration.

Treatment of proteins with sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (2ME) was also reported to disperse proteins into their subunits.[11] Enzyme solutions treated with these materials were fractionated by gel filtration as well as electrophoretically.

## *Results*

### *Initial Studies*

The initial criterion for the detection of microenzymes was ultrafiltration thru an Amicon UM-10 membrane, which reputedly retains substances of molecular weight greater than 10,000; and which did in practice routinely retain 99+ % of Cytochrome C (M.W. 12,400).

It gradually became clear, however, that the enzyme is not a minor component in a solution containing a large amount of high molecular weight enzyme; but rather that the enzyme exists in a monomer-oligomer relationship with an equilibrium normally greatly favoring the oligomer. Thus a Biogel P-10 fractionation of an enzyme which had previously passed thru a UM-10 membrane, showed the enzyme activity centered at a molecular weight above Cytochrome C, but its activity tailed out to the low molecular weight side as shown in Figure 1. This characteristic pattern occurred routinely in these studies. Unfiltered enzyme was found to give an identical activity curve, indicating that we were dealing with a single enzyme system, so subsequently unfiltered systems were normally used.

Fractionation on Sephadex G-50 also gave a distribution curve where the enzyme eluted substantially before Cytochrome C (Figure 2), but again the enzyme exhibited the characteristic tailing.

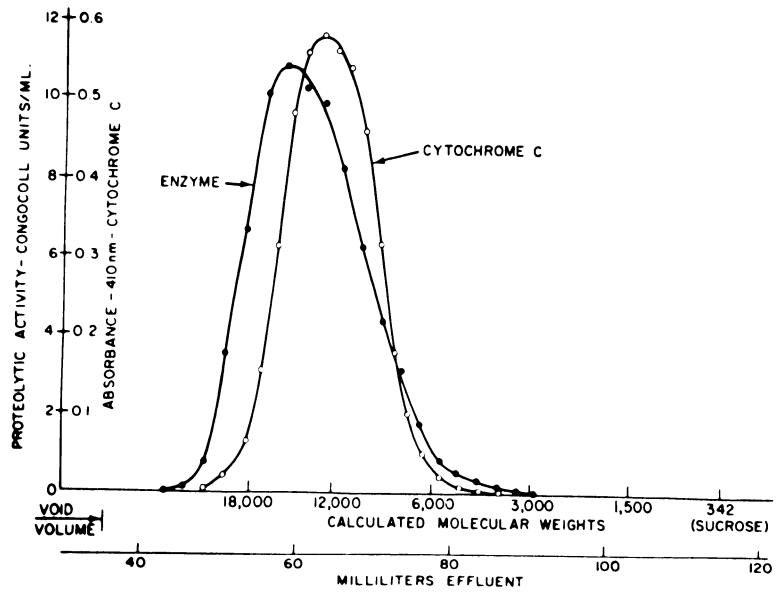


*Fig. 1.* Activity distribution of enzyme after fractionation on Biogel P-10 column. Cytochrome C distribution curve was added to show the tailing of enzyme activity to the lower molecular weight region.

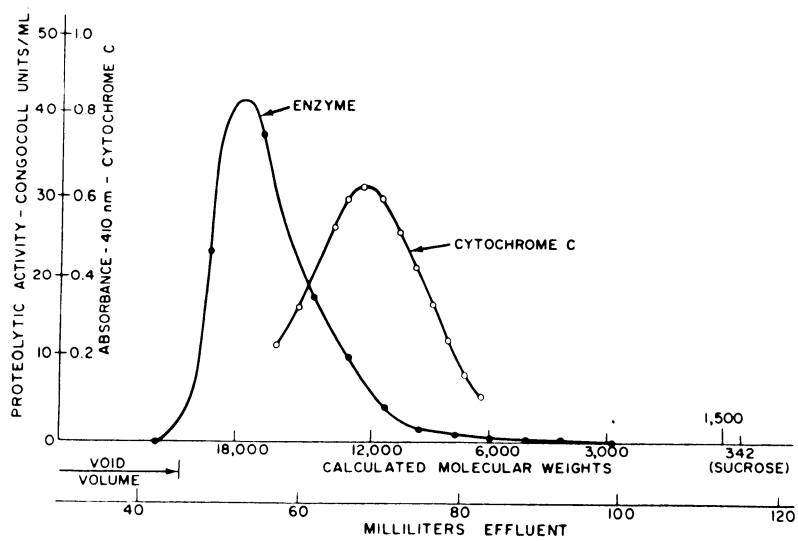


*Fig. 2.* Activity distribution of enzyme after fractionation on Sephadex G-50 column. Cytochrome C distribution curve was again added to show the tailing of enzyme activity.

### *Evidence for a single enzyme*

Electrophoretic studies at pH 5.5, 7.0, and 9.5 showed that the enzyme migrated toward the cathode slowly at pH 5.5 and very slowly at pH 7.0; and moved slowly toward the anode at pH 9.5 (Figure 3). As in the gel filtration, only one enzyme peak was observed, plus the usual tailing which occurred also when a sample was passed thru the Sephadex G-25 supporting medium without prior electrophoresis. Since Sephadex G-25 fractionates only up to about M.W. 5,000, the tailing suggests that the monomeric enzyme may have a molecular weight less than 5,000. In conjunction with the gel filtration data the presence of only one electrophoretic peak at three differing pH's makes it highly unlikely that there is more than one proteolytic enzyme system present.

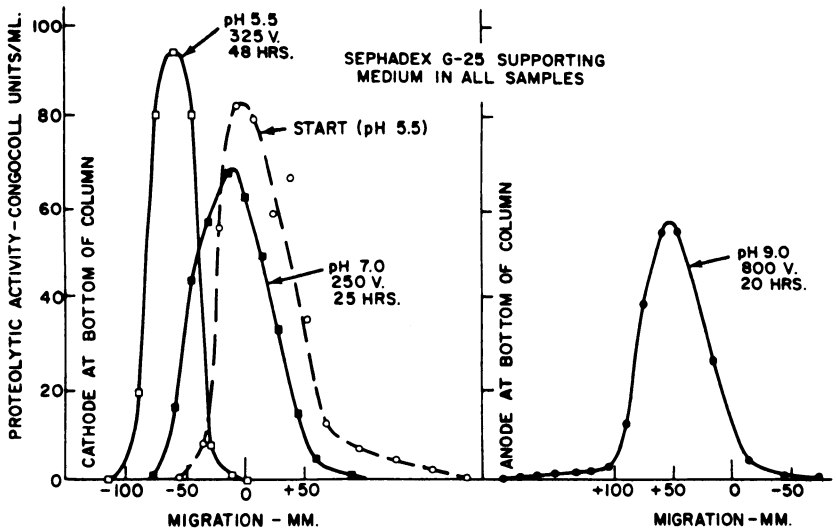


*Fig. 3.* Electrophoretic mobility of enzyme at pH 5.5, 7.0, and 9.5. Samples were placed at the top of a Sephadex G-25 column at the start of electrophoresis; and were eluted from the column after electrophoresis was completed.

Evidence for the presence of only one enzyme contrasts with the results of Furukawa et al [12] who reported three exogenous proteolytic enzymes produced by their strain of Bacillus cereus. However these were alkaline proteases, whereas ours is a neutral protease. Feder et al [13] reported only one "neutral protease" in their strain of Bacillus cereus. Their enzyme was also stabilized by calcium and had a maximum activity around pH 7.0 – 7.2. These properties resemble those of our enzyme. They reported

the presence of 1 mole of zinc per 63,000 g of enzyme, thus indicating a minimum molecular weight of 63,000.

Aronson et al [14] also found only one principal protease in broth from their Bacillus strains. This was an extracellular enzyme activated by calcium ion.

### *Tests of inhibitors and preservatives*

Crude enzyme solutions were mixed with a number of enzyme inhibitors and potential preservatives. The activities of the solutions were determined after 20 hours at 4°C. Table I shows that many of the solutions had activities greater than the control. Only sodium fluoride, ferric chloride and cobaltous chloride appeared to inhibit the enzyme activity. In another series stored for 78 days at room temperature, cobaltous chloride treated enzyme gave the lowest activity. Thimerosal appeared to be most effective for protecting the enzyme activity, so it was incorporated at 0.001 – 0.01 % in our solutions.

*Table I.* Effects of inhibitors and preservatives on proteolytic activity of enzyme solutions.

| Substance | Proteolytic Activity, Congocoll Units/ml | |
|---|---|---|
| | 20 Hours | 78 Days |
| Cadmium chloride, 0.001 M | 14.2 | |
| Cupric chloride, 0.001 M | 19.2 | 11.2 |
| Cobaltous chloride, 0.001 M | 10.8 | 3.0 |
| Cysteine, 0.001 M | 15.6 | |
| Ferric chloride, 0.001 M | 10.8 | |
| Plumbous nitrate, 0.001 M | 13.6 | |
| Manganous chloride, 0.001 M | 16.0 | 15.1 |
| Mercuric chloride, 0.001 M | 14.6 | |
| Sodium azide, 0.001 M | 13.2 | |
| Sodium cyanide, 0.01 M | 16.6 | 13.3 |
| Sodium fluoride, 0.001 M | 8.4 | |
| Sodium salicylate, 0.001 M | 17.2 | 14.7 |
| Sodium benzoate, 0.1 % | 16.6 | 11.2 |
| Thymerosal, 0.01 % | 16.8 | 19.6 |
| Thymol, 0.05 % | 17.4 | 13.7 |
| Control | 13.0 | |

### *Calcium ion and pH effects*

*Calcium concentration* – Enzyme containing solutions were stored in 0.01 M Tris solutions containing several levels of $CaCl_2$ for 6 days. Total enzyme activity was then assayed. Table II shows that a concentration of $3 \times 10^{-5}$ M $CaCl_2$ or higher substantially stabilized the enzyme.

*Table II.* Effects of calcium ion concentration on enzyme stability in 0.01 M Tris solutions, pH 7.2, after storage for 6 days at 4°C

| $[Ca^{+2}]$ | Proteolytic Activity, Congocoll Units/ml |
|---|---|
| 0 | 9.0 |
| $1 \times 10^{-5}$ | 11.0 |
| $3 \times 10^{-5}$ | 21.1 |
| $1 \times 10^{-4}$ | 16.5 |
| $3 \times 10^{-4}$ | 22.2 |
| $1 \times 10^{-3}$ | 22.5 |

A further study at several pH's indicated that high calcium ion either activated the enzyme or deactivated an enzyme inhibitor, since the activity at 0.01 M $CaCl_2$ was substantially higher than the original enzyme activity (Table III). The enzyme was found to be less stable in the absence of calcium ion, particularly at higher pH.

*Table III.* Proteolytic activity of enzyme solutions after storage for three weeks at 4°C.

| Sample | Proteolytic Activity, Congocoll Units/ml | | |
|---|---|---|---|
| Original solution | 150 | | |
| | 0 $Ca^{+2}$ | $10^{-3}$ $Ca^{+2}$ | $10^{-2}$ $Ca^{+2}$ |
| pH 7.1 | 117 | 157 | 216 |
| pH 7.6 | 67 | 157 | 229 |
| pH 8.2 | 52 | 150 | 183 |

However, at very high calcium concentrations (i.e. 0.1 M or higher) enzyme activity decreased, though this may be an effect of the calcium on the substrates collagen and casein.

*Enzyme Dilution Effects* – Ultrafilterability, or passage thru a UM-10 membrane, was reduced by calcium ion; but dilution of the enzyme greatly enhanced its ultrafilterability, even with calcium ion present (Tavle IV). Such a shift toward the monomeric form is expected for monomer-oligomer equilibria upon dilution. Therefore, this behavior suggests a monomer-oligomer equilibrium.

*Table IV.* Effects of Calcium ion and dilution on ultrafilterability of micro-enzyme.

| Enzyme Solution | Proteolytic Activity, Congocoll Units/ml | | |
|---|---|---|---|
| | Original Enzyme | Ultrafiltrate | |
| | | Orig. Conc. | 1/100 Dilution |
| 0.02 M NaCl | 150 | 0.27 | 11.1 |
| 0.001 M $CaCl_2$ in 0.1 M NaCl and 0.02 M NEMO | 150 | 0.13 | 2.1 |

*pH and Temperature Effects* – Enzyme samples buffered at pH 3.5 thru 10.0 were assayed after storage for one week at 4°C. Table V – A shows that samples stored below pH 5.0 had lost all activity. Most of the activity was retained in the rest of the samples. Optimum storage pH was at pH 6.5 or 7.0. This is also the optimum pH for enzyme activity (Table V – B).

*Table V.* Effects of pH on enzyme activity.

A. Identical enzyme samples stored at different pH's. Assayed at pH 7.2

| pH (Storage) | 3.5–4.5 | 5.0 | 5.5 | 6.0 | 6.5 | 7.0 | 7.5 | 8.0 | 9.0 | 10.0 |
|---|---|---|---|---|---|---|---|---|---|---|
| Proteolytic Activity, Congocoll Units/ml | 0 | 139 | 159 | 162 | 184 | 177 | 161 | 159 | 148 | 119 |

B. Identical enzyme samples assayed at different pH's.

| pH (Assay) | 4.5 | 5.0 | 6.0 | 6.5 | 7.0 | 8.0 | 9.0 | 10.0 |
|---|---|---|---|---|---|---|---|---|
| Proteolytic Activity, Congocoll Units/ml | 75 | 83 | 145 | 160 | 135 | 90 | 24 | 7 |

Congocoll assays of the enzyme, run at 5°C intervals, showed that the enzyme activity increased to a temperature of 50°C (Figure 4) in solution containing $10^{-4}$ M $CaCl_2$.

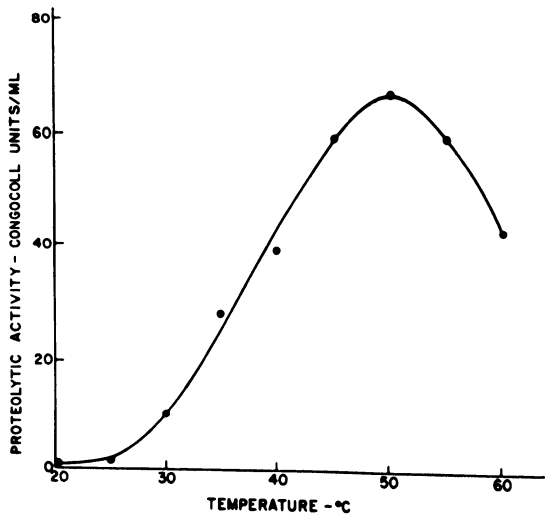


*Fig. 4.* Activity of enzyme at different temperatures as determined by a Congocoll assay requiring a 30-minute incubation.

*EDTA* – A 100 ml enzyme sample, washed free of calcium ion with 0.1 M NaCl and 0.002 M NEMO solution at pH 7.0, was placed in an ultrafiltration cell over a UM-10 membrane. Ninety mls of the solution was filtered thru the membrane under 40 psi nitrogen pressure and the sample was rediluted with the same solution except that 0.0001 M EDTA was added. Ninety mls was filtered thru again. Ninety ml samples with increasing concentrations of EDTA were subsequently added and filtered thru the membrane. The proteolytic activity passing thru the UM-10 membrane increased substantially and then decreased as the EDTA concentration was increased (Table VI). The

*Table VI.* Effects of increasing concentrations of EDTA on penetration of microenzyme thru UM-10 ultrafilter.

| EDTA Concentration | Proteolytic Activity, Congocoll Units/ml | |
|---|---|---|
| | Thru Membrane | Above Membrane at start |
| None | 0.2 | 975 |
| 0.0001 M | 2.0 | 870 |
| 0.0002 M | 5.9 | 700 |
| 0.0003 M | 0.6 | – |
| 0.00036 M | 0.1 | – |
| $10^{-6}$ M $CaCl_2$ | 0.1 | 660 |

proteolytic activity above the membrane also decreased somewhat, but not proportionally. This behavior appears to corroborate other evidence which indicates the presence of inactive higher molecular weight oligomers under conditions where calcium concentration is suboptimal. Thus, enzyme samples stored in solutions devoid of calcium ion for several weeks and then fractionated on a Biogel P-10 column with an effluent containing $CaCl_2$ consistently showed an increase in enzyme activity two weeks after fractionation (Figure 5). The increased activity usually peaked around the 15,000 and 20,000 molecular weight regions. This indicates a molecular weight around 5,000 or a fraction of this for the monomeric enzyme.

Figure 6 shows that the enzymatic activity was greatly reduced merely by fractionating the enzyme on a Biogel column in the absence of calcium ion. Addition of $5 \times 10^{-4}$ M EDTA to chelate ions released by the enzyme almost totally eliminated any residual enzyme activity. This indicates that calcium ion is essential for the stability and the activity of the enzyme. Also it suggests that the enzyme has a strong chelating effect on calcium ion.

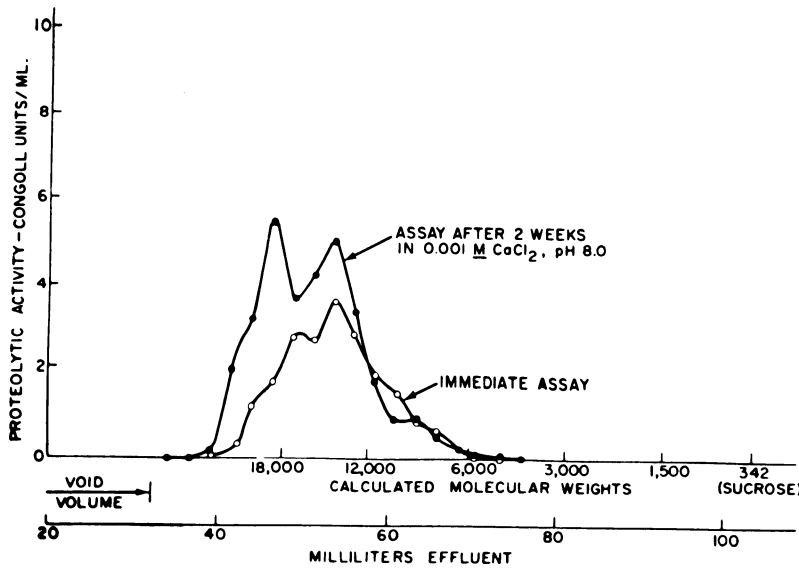


*Fig. 5.* Activity distribution of enzyme previously stored without calcium ion, immediately after fractionation on Biogel P-10 column with solution containing $10^{-3}$ M $CaCl_2$; and increased activity of fractionated samples after storage for two weeks in the calcium containing solution.

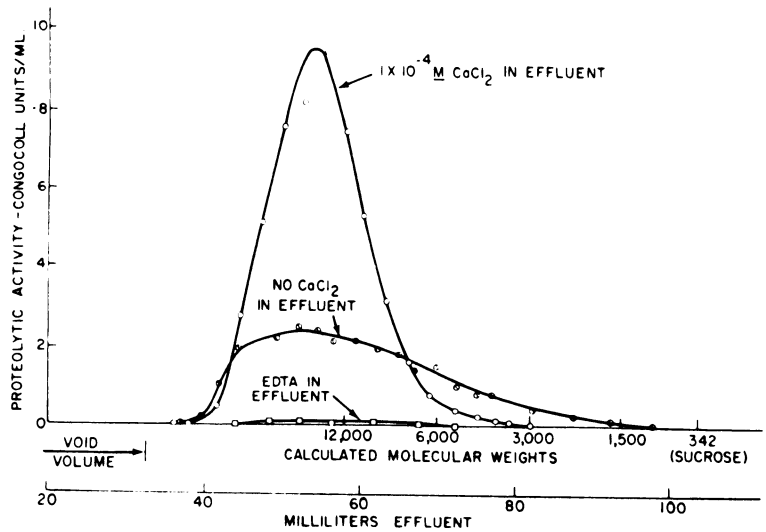


*Fig. 6.* Distribution and total activities of enzyme samples fractionated on Biogel P-10 in the presence of calcium ion, in the absence of calcium ion, and with EDTA to chelate any calcium ion present.

### *Effects of other metallic ions*

The proteolytic activities of enzyme solutions stored in the presence of a number of different cations are shown in Table VII. A comparison with the percentage activity which passed thru the UM-10 membrane, also shown in the table, indicates an inverse correlation between total activity and amount of enzyme passing thru the membrane. This implies that the monomeric form of the enzyme is less stable than the oligomer. Sodium and calcium stand out as exceptional in that they permitted a substantial amount of activity to pass thru the membrane but still retained most of their total activity. Barium and strontium had this effect to a lesser degree.

*Table VII.* Proteolytic activity and ultrafilterability of enzyme solutions after storage for 4 days at 4°C in 0.05 molar solutions of the specified salt. The solutions were buffered with 0.01 M N-ethyl morpholine at pH 7.0 (zinc at pH 5.7)

| | Proteolytic Activity, Congocoll Units/ml | | | | | | |
|---|---|---|---|---|---|---|---|
| Salt | | | | | | | |
| Total Proteolytic Activity | LiCl 6.5 | NaCl 11.5 | $MgCl_2$ 5.5 | $CaCl_2$ 12.8 | $BaCl_2$ 8.7 | $Sr(NO_3)_2$ 11.9 | $ZnCl_2$ 15.8 |
| Activity Thru UM-10 Membrane | 0.22 | 0.71 | 0.34 | 0.33 | 0.53 | 0.08 | 0.03 |
| % Thru Membrane | 3.4 | 6.2 | 6.2 | 2.6 | 6.1 | 0.7 | 0.2 |

The total proteolytic activities of enzyme solutions equilibrated with salts of several cations for two weeks are shown in Table VIII. Again, as was seen in Table VII, sodium ion preserved the activity better than did the other monovalent cations. Aluminum and zinc containing solutions had lost almost all of their activity. These two samples contained rather large precipitates. Calcium and strontium had retained most of their activity. Activity retained and molecular weight of the equilibrating ion did not appear to be correlated.

*Table VIII.* Proteolytic activities of enzyme solutions after storage for 2 weeks at 4°C in 0.1 M salt solutions also containing 0.07 M NaCl.

| Salt | Congocoll Units/ml | Salt | Congocoll Units/ml |
|---|---|---|---|
| $AlCl_3$ | 9 | | |
| $MgCl_2$ | 540 | $ZnCl_2$ | 21 |
| $CaCl_2$ | 1,380 | LiCl | 150 |
| $BaCl_2$ | 405 | NaCl (only), 0.1 M | 534 |
| $Sr(NO_3)_2$ | 1,200 | KCl | 195 |

The proteolytic activities and activity distributions of the above enzyme solutions and also of a sample equilibrated with lead acetate are shown in Figure 7 after fractionation on Biogel

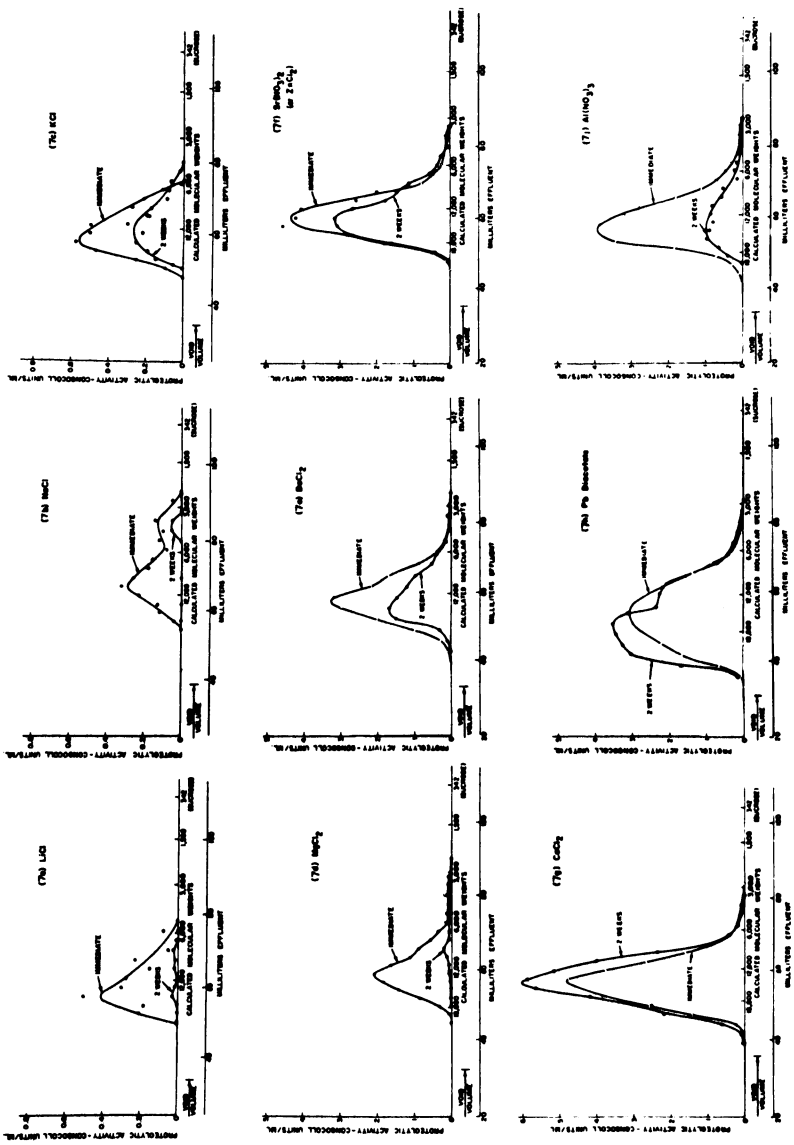

*Fig. 7.* Activity distribution of enzyme fractionated in the presence of a number of differing monovalent, divalent and trivalent ions. Activities were assayed immediately and then two weeks after fractionation.

| | | | |
|---|---|---|---|
| 7A | LiCl | 7F | $Sr(NO_3)_2$ or $(ZnCl_2)$ |
| 7B | NaCl | 7G | $CaCl_2$ |
| 7C | KCl | 7H | $Pb(OAC)_2$ |
| 7D | $MgCl_2$ | 7J | $Al(NO_3)_3$ |
| 7E | $BaCl_2$ | | |

P-10. A second curve in each graph shows the activities of the same sample following a subsequent two week storage at 4°C. The activities of the samples equilibrated with monovalent ions was so weak that an expanded scale was required to effectively show the activities. The maximum lithium (7A) and potassium (7C) enzyme activities eluted at around molecular weight 15,000, but the activity tailed out to the region at which substances of 5,000 molecular weight elute; sodium (7B) enzyme activity eluted at about molecular weight 10,000 and extended to the region corresponding to molecular weight 2,500.

The divalent cations gave greater activity, and the activity increased with molecular weight of the cation (7D to 7F). The decrease in activity two weeks after fractionation was also proportionately less with the higher molecular weight divalent ions. Zinc gave results essentially identical to strontium (7F) and so was not shown. Calcium was a clear exception (7G). It not only gave exceptionally high initial activity, but it also usually produced an increase in activity after two weeks. Lead also gave an increase in activity two weeks after fractionation (7H). The lead-enzyme distribution curve was much broader than for the other cations, due to activity beginning at a higher MW. This may indicate a greater tendency for higher molecular weight oligomers.

These results indicate that calcium and probably lead have a protective as well as an activating effect on the enzyme. On the other hand, the trivalent aluminum ion (7J) had less protective effect than did the higher molecular weight divalent ions. The results support the earlier ultrafiltration studies (Table VII) which showed an increased total activity with increasing mass for divalent ions. Divalent ions again tended to give greater stability than did monovalent ions.

### *Molecular weight studies*

*Double Biogel run* – The variable rates at which the enzyme moved thru the UM-10 membrane as well as the regular tailing out of the enzyme activity – often to the 2,500 molecular weight region – indicated that we were dealing with a microenzyme in equilibrium with its oligomers, with the oligomers predominating.

Early evidence had suggested a molecular weight of 5,700 ± 20 % for the enzyme.[3] To check this a sample was passed thru Biogel P-10 and a second sample at the 5,000 – 6,000 molecular weight region was promptly repassed thru the same column. Figure 8 shows that the profiles of both samples were very similar. This indicates that the equilibration rate of the enzyme

was rather rapid. The absence of any second peak in the second pass also further confirms the presence of only one enzyme system.

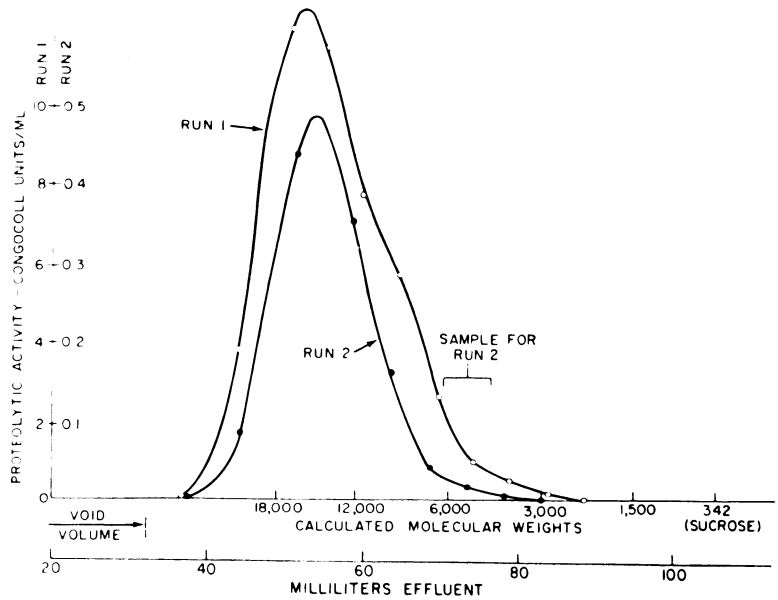


*Fig. 8.* Activity distribution of enzyme fractionated on Biogel P-10 column and activity distribution of the 5,000—6,000 molecular weight region from above sample after it was again fractionated on the Biogel P-10 column (Run 2).

*Treatment with anhydrides* — Reaction of aggregated proteins with succinic and other anhydrides, reported to favor dispersal of protein subunits, instead appeared to favor aggregation. Figure 9 shows the effects of treatment with citraconic anhydride. The enzyme activity peaked at a higher than usual molecular weight, 20,000 or higher, and had much less intense tailing toward lower molecular weights. The activity increased substantially after incubation for two weeks at pH 6.0 and 40°C. Similar high molecular weight fractions occurred after treatment with other anhydrides, but the activity was not increased by subsequent incubation.

*Treatment with sodium dodecyl sulfate* — An enzyme sample treated with 1 % sodium dodecyl sulfate (SDS) in the presence of 2-mercaptoethanol (2 ME) was fractionated on a Biogel P-10 column with pH 7.0 buffer containing 0.1 % SDS, 0.1 % 2 ME, 0.02 M NEMO and 0.1 M NaCl. A comparable run was made with Cytochrome C previously treated with SDS and 2 ME.

The SDS-Cytochrome C peaked almost at the void volume

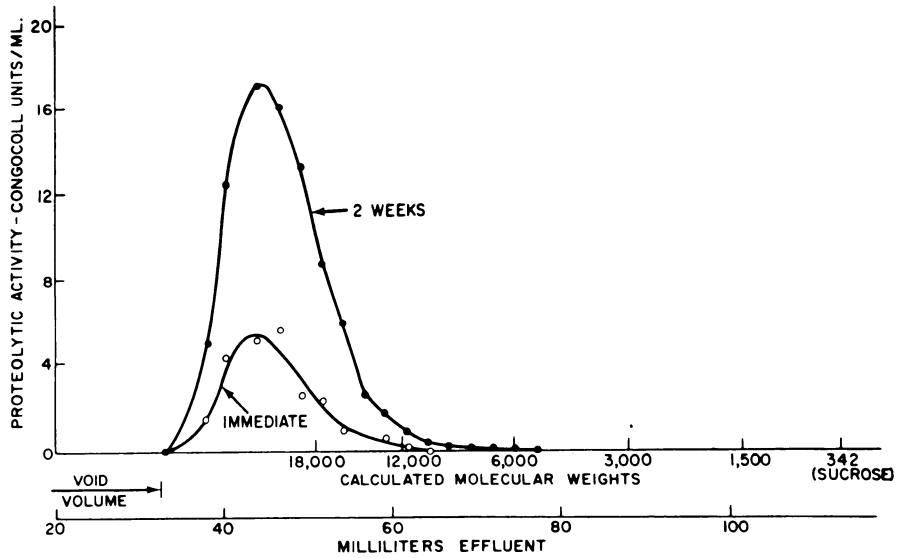


*Fig. 9.* Activity distribution of enzyme fractionated on Biogel P-10 column following reaction with citraconic anhydride. Sample fractions were assayed immediately after reaction and fractionation, and again after two weeks incubation at 40°C and pH 6.0.

on Biogel P-10, whereas normally it peaks midway in the effluent (Figure 10). The SDS-enzyme peaked with the SDS-Cytochrome C, but showed a quite substantial tailing. These results indicate that the effective molecular volumes of proteins are greatly increased by SDS treatment, probably due to attachment of large numbers of SDS molecules. The tailing of the enzyme also supports the evidence for a low molecular weight monomer in equilibrium with oligomer.

*SDS-enzyme on acrylamide gel* – An acrylamide gel with 4X the concentration of cross-linker used by Weber and Osborn [8] finally succeeded in fractionating a concentrated microenzyme solution.

The bands for insulin and for the microenzyme were quite diffuse, whereas the bands for Cytochrome C and other markers were quite sharp. This is interpreted by us as resulting from a tendency for the insulin and microenzyme to oligomerize even in the presence of SDS. The front of the diffuse band was therefore considered to correspond to the distance migrated by that particular fraction. On this basis, in comparison with Cytochrome C (MW 12,400) and insulin (MW 5,700, dimer MW 11,400), the enzyme gave three bands corresponding to MW 5,500, 8,200 and 16,000 (Figure 11).

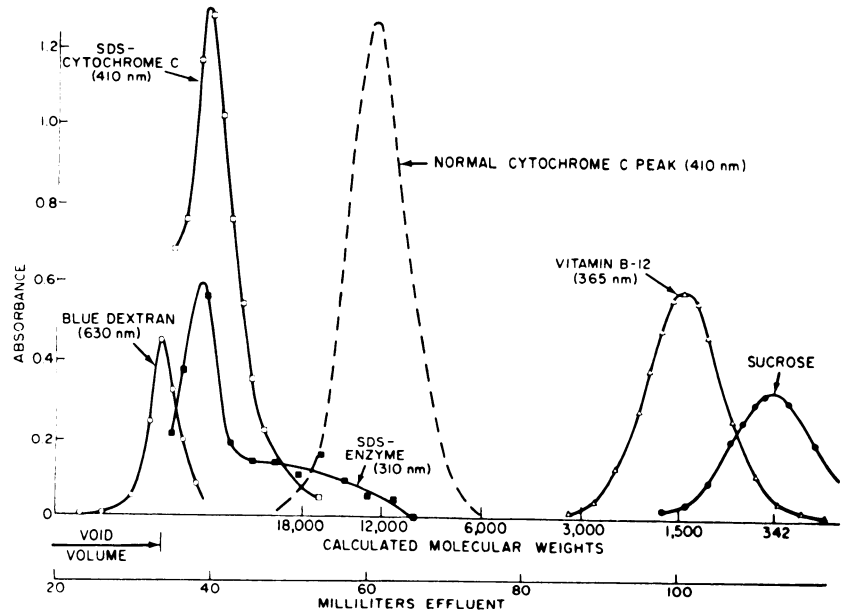


*Fig. 10.* Distribution of SDS-treated enzyme and of SDS-treated Cytochrome C after fractionation on Biogel P-10. The normal curve for Cytochrome C is also shown as are the curves for the blue dextran, Vitamin B-12 and sucrose.

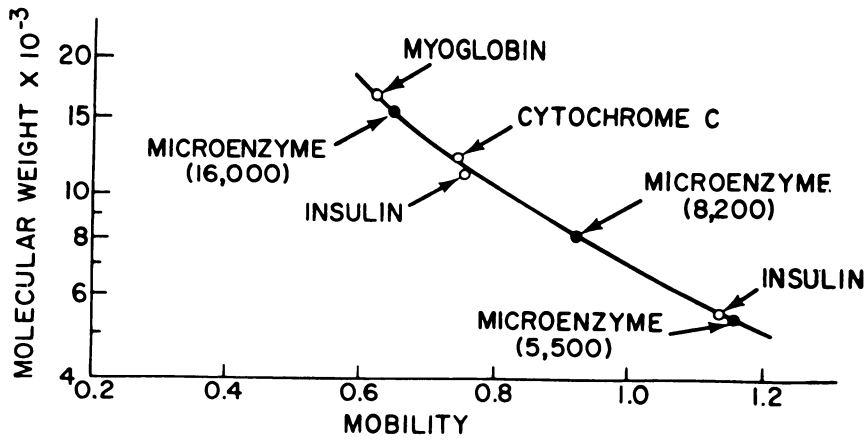


*Fig. 11.* Determination of molecular weights of *Bacillus cereus* microenzyme by plotting of mobility of enzyme fractions in comparison with substances of known molecular weight.

Other acrylamide gel runs gave values of 5,400 and 5,000 for the smallest component; and 8,000 and 10,700 for the next larger component. The 16,000 MW band did not stand out in these latter samples. The values obtained in these runs approximate ratios of 2:3:4:6; suggesting that the smallest component is a dimer and thus that the monomer probably has a molecular weight around 2,700.

This method is the only one of a multitude of methods used by us that has yielded reasonably convincing evidence for the molecular weight of the monomer of this enzyme. A number of methods have given results which could best be interpreted as revealing an enzyme with molecular weight near 5,000 or multiples thereof. If the molecule was actually a dimer, then these results seem in accord with the present observations.

*Further Biogel studies* – Alcohol was incorporated in the effluent of the Biogel P-10 column to lower the polarity of the solution in the hope that this would slow the equilibration rate and permit detection of the microenzyme monomer. When 10 % alcohol was incorporated into the effluent on the Biogel column, two rather unclear peaks at 3,000 and 6,000 molecular weights were observed (Figure 12). However, the enzyme activity also extended out to beyond the sucrose peak. At 33 % alcohol, the enzyme was evidently adsorbed, since none of the activity passed thru the column. This suggests that adsorption may have been responsible for much of the retardation in the 10 % alcohol fraction. However, the peaks around 6,000 and 3,000 have occurred consistently in several runs, and so may be real. Certainly they are consistent with the acrylamide gel values.

*DEAE purification* – A massive brown-colored impurity in the enzyme solution was observed to move during electrophoresis toward the anode even at pH 5.5. Removal of this impurity with DEAE cellulose or DEAE Sephadex was therefore tried and found to be successful. Subsequent to this purification step, UV absorption peaks at 220 nm and 278 nm were obtained with the enzyme solution, indicating that this enzyme may well be a polypeptide.

Passage of the enzyme thru these ion-exchangers also increased the activity of the enzyme to about 140 % of its prepassage level; but there are some indications that stability has been adversely affected.

## *Discussion*

Viewing the molecular weight determinations in detail, ultracentrifugation is not accurate below Mol, wt. 10,000. We could only conclude that we had active substances below this value. In the last decade gel filtration has been developed as a promis-

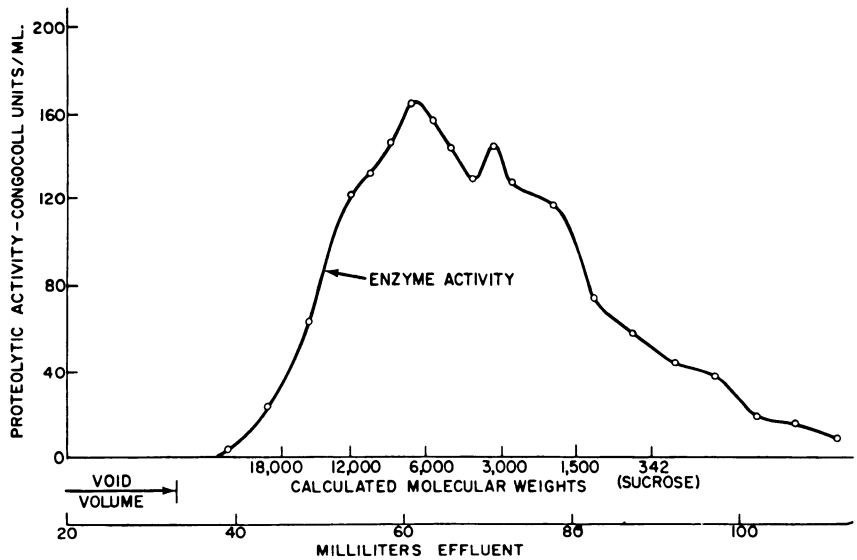


*Fig. 12.* Comparison of mobilities of *Bacillus cereus* microenzyme of insulin and of Cytochrome C on polyacrylamide gel with 4X concentration of cross-linker. Disc electrophoretic columns are shown following development, with marks beside the columns to show the extent of the diffuse bands.

ing method. Adsorption does occur with some proteins, however, in which case too low molecular weight values result. For this reason we used two different gels, the dextran derived Sephadex G-50 and the polyacrylamide, Biogel P-10, both of a degree of crosslinkage appropriate for the 2,000 – 20,000 mol. wt. determinations.

In both gels enzyme activity tailed out into low molecular weight regions. While this tailing out does not define the molecular weight, it does indicate that material of low molecular weight was present.

Proof of the existence of a low molecular weight moiety was clearly provided by the results of extensive ultrafiltration studies. These showed that significant amounts of the enzyme passed through a membrane that effectively excludes materials with molecular weight above 10,000, and that penetration of the enzyme through the membrane could be substantially influenced by changes in concentration, metallic ions, and EDTA; while these did not similarly affect Cytochrome C which was used to confirm the integrity of the membrane.

Penetration of a substantial part of the proteolytic activity thru these membranes is clear proof that the enzyme monomer has a molecular weight less than 10,000.

Continually there have been indications that the monomer of this enzyme has a molecular weight of 5,000 to 6,000 or some fraction of this. There are yet to be confirmed indications of values as low as 1,500. Our results show that we are dealing with an enzyme system involving several oligomers.

In the SDS-2 ME polyacrylamide gel electrophoresis (Figure 11) both the enzyme and the insulin bands showed diffuseness, which is a characteristic of systems subject to rapid shifts between monomer and oligomers. Thus, it appears that the SDS-2 ME treatment did not completely disperse either of these two systems. The diffuseness reduces the precision of the molecular weight determinations, but still leaves no doubt about their exceptionally low values.

*Acknowledgment*

The present work was greatly aided by a donation in memory of Mr. Morris P. Neal.

We thank Bjorksten Research Laboratories, Inc. for the permission to use its equipment, as well as some data obtained in the course of a technical study. We are indebted to Dr. Donald Wetlaufer of the University of Minnesota and to Dr. David E. Green of the University of Wisconsin for valuable advice; and to Messrs. Stephen M. Ashman, Edward J. Rock, and George Burrowbridge for valuable assistance in the analytical work.

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Molecular Size Limitations

The first of the above two papers shows the general approach in the search for enzymic means for removing densely crosslinked aggregates. The second paper shows the observations and some of the relations more precisely, particularly in Figure 7, p. 130.

A subsequent paper by Holmquist (Biochem. 16: 4591-4594 (1977) reported that the initial molecular weight of the BRL-70 enzyme is 34,000. However, he pointed out that substantial amounts of lower molecular fragments routinely exist. Our lowest molecular weight fraction (Figure 7B of Schenk et al.) is 3,000-6,000 Daltons and is evidently the result of this fragmentation.

From the standpoint of our objective, the important point is that we here have at least a measurable trace of an enzyme fraction capable of penetrating dense structures to a degree corresponding to lower molecular weight than any enzyme previously used toward this end.

On the basis of 5 years of work with these and other related enzymes, we cannot foresee enzymes or precursors of molecular weight below 1,500. Our smallest active fragment appeared to have molecular weight 3,000 Daltons and might conceivably be constituted of two monomers, joined by the bivalent ion necessary for activation.

Therefore, we did not tarry longer, but proceeded to study other systems of still lower molecular weight; such as the free hydroxyl radical.

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# Pathways to the Decisive Extension of the Human Specific Lifespan*

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**ABSTRACT: Three approaches to reversal or removal of gerogenic aggregations of macromolecules have shown promise. Of these the *enzyme approach* is the most gentle, and can be made specific. Aside from this, the lower the molecular weight of an enzyme, the better chance it will have to be immunologically tolerated as well as replicated synthetically in whole or in part. The *chelating approach* provides a powerful means for removing a single class of unwanted, random crosslinkages, i.e., those due to extraneous polyvalent metals such as lead, cadmium and aluminum. The *free hydroxyl radical approach* is the most penetrant and most versatile means for removing otherwise insoluble aggregates, but its very lack of specificity will demand great foresight in control and use. Together, these three methods, when properly applied, might bring some principal objectives of gerontology within closer range.**

The following steps are required to effect a breakthrough for extending the specific maximum lifespan attainable by humans, with concurrent improvement in health and vitality:

1) To define the objective.

2) To define the principal adverse mechanism.

3) On the basis of 1) and 2), to define the targets.

4) To find ways to attack these targets effectively.

5) To demonstrate results.

After 36 years of work it is beginning to appear that steps 1-4 are now behind us and step 5 well under way, held up only by lack of funding.

## 1. *Definition of objective*

At present the life expectance of a man at age 60 is less than 2 years more than it was 188 years ago, in 1789. A positive change is overdue, and would appear to be a worthy prime objective. The number of years remaining to the average white man at age 60 (1) is represented in Figure 1.

## 2. *Principal adverse mechanism*

The principal adverse mechanism is crosslinkage, because no other mechanism can cause as much damage with as small an input as a random, unwanted crosslinkage at the wrong time and place. The components and conditions for this process are unavoidably present in all living organisms (2-13).

## 3. *The targets*

Specific targets for correction are represented in Figure 2, A and B, showing crosslinking of two strands of DNA and various crosslinkages in the course of a lifetime.

## 4. *Ways to attack the targets*

These are:

A. To develop a lytic enzyme of small molecular weight which can reach the desired sites: 1) to overcome the steric hindrances which block the access of larger enzymes, so that enzymatic removal of gerogenic crosslinked aggregates becomes possible (4, 14); and 2) to shorten the time necessary for excision repair of DNA (10, 15). The effects of such an enzyme that dissolves the "insoluble" are shown in Figure 3.

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* Address on acceptance of the Modern Pioneer Award of the American Institute of Chemists, March 19, 1977.

We were materially aided by donations from the Paul Glenn Foundation, Dr. V. Blinoff and Dr. Stanley Buckman. Dr. R. Schenk carried out the experimental work represented in Figure 4, using tritiated materials prepared by Bjorksten and Ashman.

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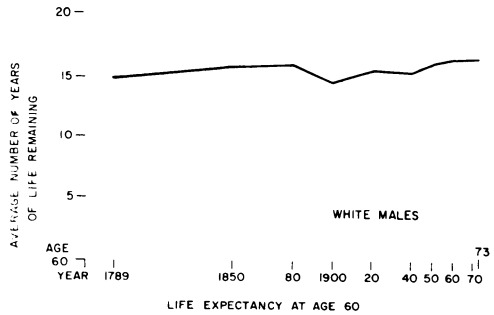


*Fig. 1.* Number of years remaining to the average white man at age 60. (Prepared from data obtained from the Metropolitan Life Insurance Company and the U.S. Bureau of Vital Statistics.)

B. To select and test chelation agents and techniques for removing metal-based crosslinkages (17). Although limited to metal-containing bonds, this may be important, particularly in countering senile mental disintegration (18, 19).

C. To use free hydroxyl radicals (OH) for depolymerizing the gerogenic aggregates (Fig. 2A) and possibly effect gradual renewals in DNA molecules before the crosslinking agent (Fig. 2B) has connected to the second strand.

## REDUCTIVE-OXIDATIVE DEPOLYMERIZATION

Pathways A and B (enzymes and chelation), have been covered in detail in previous publications. Here, we shall discuss chiefly reductive-oxidative depolymerization, which apparently has not before been stressed in this context.

Like many others interested in aging, we felt the need for a quick assay method to determine in a few hours whether or not any given treatment was effective in eliminating the gerogenic insoluble materials. For this purpose we prepared assay animals, for example, as follows:

A pregnant rat received 40 millicuries of tritiated acetate at the time of giving birth; the offspring thus received tritium from their mother, and no more of it during their lifetime. Their tritium was initially excreted very fast, but finally excretion leveled out so that after about two years very little was excreted, while the residual radioactivity remained firmly locked into immobile compounds (20, 21). Anything that would remobilize these compounds would immediately be apparent from a sudden peak in the radioactivity of the urine of the animals. This litter served its purpose, but the mother, which had the highest tritium content of them all, died after 23 months. We isolated from her liver an insoluble fraction by repeated extractions with water (acidic and alkaline), acetone, methanol-chloroform, repeated digestion with a huge excess of

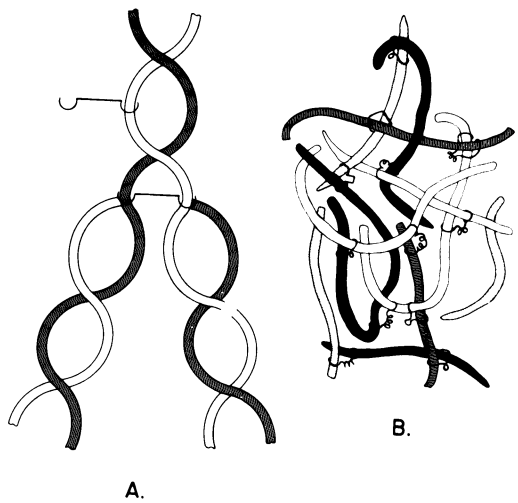


*Fig. 2.* **A.** Two strands of DNA have become cross-linked at the corresponding sites. This cannot be repaired, because the same site is involved in both strands. The cell will die or mutate in the next mitosis (10, 11).

**B.** In the course of a lifetime, numerous large molecules will randomly become tied up by crosslinkages so tightly that repair enzymes are excluded. They will form nets or cages which impede transport within the cell and reduce the space available for normal functions (10, 11).

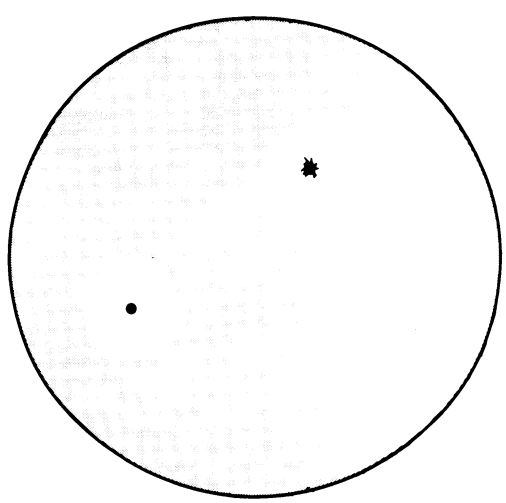

*Fig. 3.* Petri dish containing transparent agar gel in which is suspended an aggregate of Figure 2B material, isolated from old human brains. Two bacterial colonies are shown, from which enzyme is diffusing. This enzyme has dissolved the "insoluble" in a halo-like zone surrounding the colonies (10, 16).

"Pronase" (a proteolytic enzyme-aggregate), plus a final solvent; and then exposed the aggregate to *free hydroxyl radicals* from oxidizing ferrous salt, which was being continually reduced back by .01 mol. ascorbic acid and so used over and over. This method of depolymerization by oxidative-reductive processes was first indicated by Skanse and Sundblad for the specific application to hyaluronic acid (22); it was explored in great detail and applied to carbohydrates broadly by Pigman and co-workers (23-28). It was *re*discovered by Orr in the context of depolymerizing the enzyme catalase (29), and was found by Robinson, Richheimer, Westall et al (30-32) to be active on proteins, particularly transferrin, an encephalitogenic protein, and a pentapeptide. Richheimer and Robinson (32) showed that this involved direct breakage of peptide linkages.

The study of this reaction had been carried out with a minimum of cross-references between the groups. The similarity of findings, nonetheless, indicated the identity of the reaction. It appeared that this distinctly nonspecific reaction should have a chance to release the gerogenic frozen metabolic pool (17), so we tried it on the radio-tagged insolubles from the described 40-millicurie mother rat, with the result shown in Figure 4.

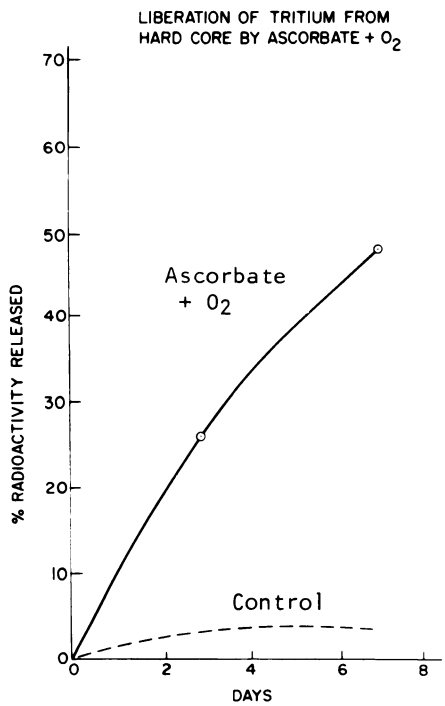


*Fig. 4.* Liberation of tritium from hard core by ascorbate + $O_2$. (Experiment on radio-tagged mother rat.)

This figure shows that a rapid liberation of the radioactivity, which had defied other means for release, immediately took place.

When it comes to penetration of dense gerogenic aggregates, we could hardly wish for a smaller, more mobile and penetrant agent than the free hydroxyl radical, which is readily generated and used under physiologic conditions, by a ferrous salt and any enediol containing the sequence (33, 34):

```
|
C.OH
‖
C.OH
|
C=O
```

What it lacks in specificity it makes up in mobility.

Each of the three outlined pathways has its limitations and advantages. Together they represent an arsenal which, properly used, should enable us to extend decisively the specific lifespan of rats and, upon completion of the requisite testing, perhaps also of man.

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Modulated Reaction Rates

In the lower molecular weight enzymes, and the free hydroxyl radicals, we have systems which in some combination might enable us to break down even highly crosslinked gerogenic substances, under conditions compatible with human life. The former of these has the specificity common to enzymes; the latter is highly penetrant, but will destroy anything in its way. None the less, the human lymphocytes seem to have mastered this means for destroying foreign bodies. We can accept such destruction when we can modulate the concentration, thus keeping the reaction slow enough to permit the organisms' synthetic and repair mechanisms to replace anything needed that has been destroyed along with the targets.

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# The Crosslinkage theory of aging.

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## *Background and Definitions*

The obvious changes – anatomical and psychological – which occur in aging must necessarily have developed from changes on a cellular level; these changes in turn must have originated on a molecular level. Early evidence revealing that fact was provided by Northrop and Loeb in 1916 – 17. They showed that the mortality rate of the banana fly Drosophila has the temperature coefficient of a chemical reaction. Therefore, if we want to start the exploration of aging from its origin, our first question is: "Which chemical reaction initiates the sequence of events that ends in functional impairment, degeneration, senility and death?".

In seeking the answer to this question, we must pay primary attention to those molecules which above others are carriers of vital processes. This leads us to the proteins and nucleic acids. Next, we must determine the most frequent way in which these molecules are damaged. The scanning of all types of reactions, which could be in question, very rapidly singles out one of these: crosslinking. The leverage of this type of reaction is overwhelmingly great. One single small molecule which is capable of reacting to join two giant molecules will immediately double the molecular weight of the aggregate, totally changing the solubility, swellability, permeability, indeed all of the biologically critical properties of the aggregate. In fact, one single crosslink for every 30,000 component monomer units is enough to change entirely the solubility behavior of the molecules. Therefore, crosslinkage could quite well be that very first molecular change which initiates the sequence of events leading to senility and death. Incidentally, the time-viscosity curve in a crosslinkage reaction has been shown to match closely the time-mortality curve for humans.

The crosslinkage theory states that the principal initiating reaction in aging is the linking together of two or more macromolecules in any manner that can to be reversed in the organism in question. Such linking together can be caused by a small bifunctional molecule accidentally contacting each of two macromolecules, or caused by a direct side to side contact between reactive sites on the macromolecules themselves. The bond can be covalent, or a hydrogen bond, or perhaps even some form of bond not yet discovered, or all of these, so long as the bond is strong enough not to be breakable by the resources of the organism in question. For reasons apparent below, the oleophilic bond, collagen derived bonds and sulfur bonds appear to be eliminated as major causes of aging in the system most closely studied to date.

Once two macromolecules are linked at one point, the molecular motion will cause them to make further contacts with each other, thereby increasing the probability of additional crosslinkages being formed between them. Additional macromolecules may join the aggregate. As this progresses, the cells become increasingly burdened with an accumulation of malfunctioning or inert large molecules which cannot be removed. In addition, irreplaceable nucleic acid molecules may be damaged leading to mutations or, more frequently, to cell death. Such cell death is critical, because some vital cells (such as nerve and large muscle cells) do not divide and thus cannot be replaced. As brain cells die, or are impaired in functionality, mental abilities will decrease. As glandular cells are clogged by non-removable crosslinked aggregates, the secretion of hormones will decline, causing other deleterious changes throughout the interdependent systems of the body.

Sometimes the crosslinkage theory has been connected with the waste product theory. However, these two theories have little in common. The crosslinked molecules are not clinkers, but important functional molecules which are immobilized by crosslinking accidents. Such accidents are random. They are not an inherent part of a normal metabolic process, as are the waste products. In waste product theory, aging is caused by normal metabolic process waste products which choke the system. In crosslinkage theory, the gerogenic process affects proteins, nucleic acids and possibly other vital macromolecules, which gradually lose their function and

1. create a deficiency of one or more irreplaceable molecules;
2. while inert in the cell, interpose a network which impedes intracellular transport;
3. progressively reduce the volume of space available for vital molecules within the organism.

*Supporting Evidence*

The crosslinkage theory was first presented in 1942 [1] on the basis of observations made on collagenous protein extracted from pigskin with aqueous extractants at low temperatures (High Bloom pigskin gelatins). R. S. LaBella, F. Verzár, R. R. Kohn, S. Bakerman, H. Elden, Arthur Veis, K. A. Piez and many others have since studied collagen in great detail. There is now a superabundance of evidence of crosslinkage progressing with age, and of the obvious effects of this process. E. Heiskanen has recently summarized the growing literature in this area.[2] It has been fully proven by many authors and beyond possibility of rational doubt that, in collagen, crosslinkages increase with age both intra- and inter-molecularly.

In the body, all of the proteins and nucleic acids as well as possibly other large molecules having reactive groups are exposed to the same crosslinking reactants as is collagen. Furthermore, there is the proximity to each other, so that random crosslinking between them is a certainty. Although the ease of working with collagen has caused a somewhat wasteful concentration of research on this particular protein, sufficient work has now been done with other large molecules to show to the satisfaction of many researchers that crosslinkage with aging is common to all of them. Medvedeva has found progressive insolubilization of proteins with aging in humans; Alexander, Swarcbort and Stacey have shown the feasibility of covalent crosslinkages in DNA in vitro; von Hahn and Verzár have shown crosslinkage with age in nucleic acids; and Bjorksten, Andrews, Bailey and Trenk for aggregate proteins in rat liver.

However, many researchers do not accept the existing evidence as sufficient to prove that crosslinkage is the general underlying reaction which dominates the onset of the aging syndrome. Despite their reluctance to agree, the accumulation of evidence appears overwhelming. For example, a substantial number of investigations have been published which show that the progression of aging is typically accompanied by loss of elasticity of all tissues, increase in molecular weight of collagens, reduction of solubility and swellability, increase of brittleness in osseous and other tissues, and increase in oleophilicity of at least arterial walls and collagenous tissues. Pertinent literature has been listed in previous publications which the reader may find of interest.[3 12]

To the polymer chemist this evidence is compelling, and can only mean that crosslinkage is taking place on a broad scale and to an extent sufficient to account entirely for the observed progressive disruption of all life processes with aging.

On the other hand, the medically oriented researchers, with some exceptions, consider this type of evidence as too indirect, and not clearly enough connected to those specific symptoms with which each of them is particularly familiar. They are more impressed with the in vivo experiments of Milch and co-workers [4] who showed (with test animals) that the administration of known crosslinkers normally present as intermediates in human metabolism causes lesions apparently identical with those caused by degenerative disease in humans; with the finding of H. H. Zinsser Jr. and co-workers [5] showing, by X-ray diffraction on human aorta, that spacings between large molecules in aggregates coincide with the spacings for crosslinkage by those metal oxides which are observed to accumulate in these tissues with progressing age; with the finding of Alexander and Connell [6 11] that the life span of mice is greatly reduced by administration of bifunctional crosslinking agents but not by equally mutagenic noncrosslinking substances; and with the classical experiments of Maria Rudzinska [7] who in the unicellular Tokophrya showed the progressive precipitation with age of proteinaceous substance showing remarkable similarities under the electron microscope with precipitates found by Sulkin in human organs.

The molecular biologist or biochemist tends to discount this latter evidence as not identifying clearly enough the entire chain of events between birth and death. He is more impressed by the experiments in which radioactive tracer substances were administered to small mammals prior to birth. The tracers were fed to their pregnant mothers, and then were identified in steady state positions after 600 – 800 days in the offspring.[9] The occurrence of a steady state has been identified by cessation of excretion of tracer and, in some cases, by the absence of any radioactivity in tumors formed in the animal subsequent to the attainment of the metabolic steady state by the radioactive molecules remaining in the organism.

For example, the most rapidly metabolizing major organ is the liver. While much larger amounts of radioactivity tend to accumulate in other organs, the liver presents the most accelerated test condition, and was therefore the first organ investigated in some detail, from a radioactive rat which died 809 days after its birth. The mother of this rat received 8 millicuries of tritium at the time of parturition. Thus, the entire amount of radioactivity of this rat was received from its mother, and remained in the animal 809 days, long enough to establish a steady condition state.

The liver of this animal was extracted to remove water, acetone and chloroform-methanol solubles; the largely nitrogenous residue was hydrolyzed at body temperature. Five water soluble peptides resulted; these were then hydrolyzed with

acid at 110°C and found to contain in the aggregate the following isotope-tagged amino acids only: lysine 30 %, arginine 20 %, aspartic acid 20 %, glutamic acid 17 %, serine 10 %. The remaining 3 % of radioactivity resided in an ether-soluble fraction, and was definitely not any fatty acid between $C_{10}$ and $C_{26}$.

After the above mentioned hydrolysis at room temperature a smaller fraction remained unaffected and still insoluble in water, urea, acetamide, dimethyl sulfoxide, detergents and so forth. This fraction was also hydrolyzed with strong acid at 110°C, and appeared to contain the same radioactive amino acids as the soluble peptides above. The much lower radioactivity of this fraction necessarily reduces our certainty of its composition.

A separate, milder hydrolysis of a portion of the same insoluble fraction showed the apparent presence of 0.17 % of ribose, when analyzed by the colorimetric method of Dische and Schwartz.

The molecular biologist is also impressed by the fact that the foregoing amino acids, in which the tritium administered at birth remains lodged, after attainments of steady state, all have polar side chains. They are thus readily capable of forming covalent cross linkages under physiological conditions.

The additional finding of 0.17 % of ribose in the hydrolysate of a very refractory radioactive molecular aggregate after steady state was attained suggests that ribonucleic acid molecules participate in crosslinkage processes about in the proportion of their accessibility and reactivity.

## *Objections*

Against the impressively mounting evidence in favor of crosslinkage being the principal gerogenic reaction, only two objections have been raised. These are:

1. In 1964, Verzár wrote: "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 non-renewed substances age. Most cells being continually renewed are not aging." [8]

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.

The first of these objections was anticipated and answered in 1942 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." [1] 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 nondividing 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 discarded.

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:

### *Aldehydes*

Formaldehyde is the most active of the aldehyde type crosslinking agents. Because of the speed of its reaction with all proteins or amino compounds, it will not accumulate as such in the organism. However, it has been found in vivo as a result at least of the following metabolic reactions:

1. Dimethyl glycine + 1/2 $O_2$ → Sarcosine + formaldehyde
2. Sarcosine + FAD → glycine + formaldehyde + $FADH_2$
3. N-methyl-l-amino acids + $O_2$ → l-amino acids + formaldehyde
4. Erythrulose-1-phosphate → dihydroxyacetone phosphate + formaldehyde
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 methyl-deoxycytidylate

Acetaldehyde, which is somewhat less active than formaldehyde, has been found in the blood stream. In the bibliography on the formation of acetaldehyde in metabolism, Pansini,

Fersini and Ripa quote 149 references of which many show the occurrence of acetaldehyde in vivo.

Almost equal in rapidity in crosslinking proteins and nucleic acids are other aldehydes: the water-soluble monomeric $C_1$ to $C_3$ aliphatic monoaldehydes and the $C_2$ to $C_5$ di-aldehydes, including several known metabolites such as glyoxal, glyceraldehyde, and pyruvaldehyde. Lipid oxidation leads to the formation of many aldehyde crosslinking agents including acrolein. In addition, the lipid oxidation derived crosslinkers include peroxides and epoxides.

The crosslinking (tanning) efficacy of the oxidation products of unsaturated oils was shown as "well known" by Isaacs in 1915. It can be dramatically demonstrated by emulsifying 1 to 2 % of an unsaturated oil such as safflower oil, or such as methyl linoleate in a gelatin gel melting around 53°C. If such a gel is exposed to ultraviolet radiation from the sun or from a mercury lamp for a couple hours and then placed in the dark, no change in the melting point will be immediately apparent, but the melting point will steadily increase in the following weeks until in about 4 weeks it may exceed the decomposition temperature of the gelatin. Thus the crosslinkages causing this increase in melting point do not seem likely to have been formed by any unstable free radicals or the like, but rather by relatively stable reaction products formed at the time of the irradiation and gradually reacting with the gelatin to effect the crosslinkages.

The crosslinking agents formed from unsaturated fatty acids are particularly significant because of the site of their formation. Most of the unsaturated fatty acids are constituents of the phospholipids in cell membranes. These same cell membranes are the sites of the nucleic acids and of all enzymes in the cell. A crosslinking agent formed in this environment has the best possible chance of striking a nucleic acid molecule, locking the two strands together so that they cannot part in a subsequent division. The two strands remain bound together covalently. This will result sometimes in a mutation, most often however in the death of a cell on mitosis.

When a crosslinkage involves both strands of DNA, this damage is irreparable. The known mechanisms for removal and repair of damaged nucleotides in DNA depend on removal of elements from a damaged chain in the double helix, and using the other chain as template for reconstruction. Such mechanisms should function well for damage to a single chain, and might even be able to reverse many intermolecular crosslinkages where only a single chain of the DNA helix is involved. However, where both chains of a double helix happen to become linked together at the corresponding points, there will remain no template that can be used. The non-repairability which results

from this kind of damage seems particularly critical for the non-dividing brain and large muscle cells, which must last through life.

*Sulfur crosslinkages*

Oeriu has postulated the significance of disulfide linkages in senescence. His postulate is not without support; sulfur crosslinkages have been the subject of a vast amount of study in the fields of elastomer technology, particularly because they are the basis of the vulcanization process discovered by Charles Goodyear in 1839, which provided the keystone for modern elastomer technology.

It is notable however that the sulfur bearing essential amino acids (methionine and cysteine) are not among those which so far have been found fixed in the body from birth to death in the rat.[9]

*Alkylating and Acylating Agents.*

A chief reason why radiomimetic agents and mustard gases 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 had the time to penetrate into the cells. The alkylating and acylating agents are generally sufficiently slow-acting to be able to penetrate the cell membranes, yet sufficiently fast acting to cause rapid results. Bifunctional agents of this sort which might possibly be expected to occur in normal metabolic reactions include co-enzyme A or adenosine related esters suds as, for example, succinyl-, citryl- or malonyl diesters, diacetyl glutamate and possibly some polymers of acetyl glutamate, acyl adenylates and peptides with more than one mono methyl substituted amino group. Alexander and Connell report that bifunctional mutagens (thus crosslinkers) shorten the life span of mice very substantially; the monofunctional mutagen used for comparison did not shorten the lifespan appreciably in spite of its having the same mutagenic effect. This has been cited repeatedly as evidence that crosslinkage within cells shortens life, while mutagenicity per se does not.

The percentage of total amino acid tritium found 600 – 800 days after administration, when a steady state was established, was slightly less than 10 % lodged in serine, the only hydroxyl bearing isotope carrying amino acid found present. On a molecular basis, however, this is considerably higher because serine has the lowest molecular weight of the 5 amino acids involved. This appreciable fixation of serine in the frozen metabolic pool gives added importance to alkylating and acylating agents as gerogenic crosslinkers.

*Quinones*

The quinone induced crosslinkages have the highest hydrothermal stability, not even exceeded by that of the chromium linkages. These quinone crosslinkages have been implicated in senile cataracts, particularly by Uyama, Ogino and co-workers and have been found present in aged hearts. In fact, quinones are present in every living cell as an indispensible link in the oxidation chain. Catechol derivatives are oxidized to orthoquinones by peroxidase, and adrenaline is biologically oxidized to adrenochrome. Green and Richter isolated this very reactive orthoquinone in crystalline form.

*Integrally formed crosslinkages*

We also have to consider the crosslinkages which are formed as integral parts of the reacting molecules, without any external reactants. Piez and LaBella have particularly studied this type. Piez has shown that specific lysine residues can be converted to aldehydes which react to crosslink collagen. LaBella and Paul have shown that oxidation of constituent tyrosine residues can form reactive quinoid structure capable of binding adjacent functional groups covalently.

*Free radicals induced by ionizing radiation*

Free radicals have long been known to be powerful crosslinking agents and are used as such routinely in the plastics industry to cause crosslinkage, thereby increasing the temperature stability of the plastics. A part of the crosslinkages in the body may be due to cosmic radiation, naturally occurring radioactive isotope of potassium, and incidental contact with fallout, X-ray equipment, diagnostically used radioisotopes, and luminous dials in watches or other instruments. These are special cases of crosslinkage. Inasmuch as the average human in a lifetime is exposed to about 200 Roentgens, and approximately 4000 Roentgens of long term exposure are required to cause changes equivalent to senility, it is apparent that the radiation induced radicals under the environmental conditions still prevailing could only cause a small fraction of the total crosslinkage in the body.

*Antibodies*

Walford, who pioneered the auto-immunity theory 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.[13]

### *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 their slower activity, these have remained largely unexplored as crosslinkers.

They include polybasic acids and their esters. Citric acid, an abundantly available metabolite, has been used industrially to crosslink polyamide resins, such as the soluble nylons.

Silicon could have similar effects, and any poly halo derivative that is to any extent hydrolyzable is a potential slow crosslinker. Some condensed hydrocarbons 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 lifetime exposure is considered.

### *Metallic crosslinking agents*

All polyvalent metals are potential crosslinkers. Living organisms are equipped to handle certain of these efficiently: iron, zinc, magnesium, cobalt, copper, chromium and calcium. But aside from these, polyvalent metals are capable of accumulating with age, most conspicuously in the circulatory system. When the damage done by a trace metal is slow enough not to become effective until after completion of the normal reproductive period, the evolutionary processes have not been concerned with adaptation, and the body has no defense. Trace metals which conspicuously accumulate with age include aluminium, silicon, lead, tin, cadmium and titanium. Schroeder states that all anti-hypertensive agents not acting on nerves – and thus presumably restoring lost elasticity – appear to be metal binding agents. This may be viewed in context with Zinsser and co-workers' demonstration of metal oxide crosslinkages in arterial tissue. A. Tyler found that the life of spermatozoa from widely differing species is extended several hundred percent by treatment with metal chelating agents which remove polyvalent metals.

From the above it is apparent that the organism normally is exposed to an abundance of crosslinking influences.

## Evaluation of the Crosslinkage Theory in the Light of Established Criteria

*1. Life Span*

Every theory on aging of which I am aware can explain the finite life span of multicellular animals. Furthermore, whatever factors influence anything concerning multicellular animals will be affected by the hereditary differences between these animals, so that whatever we choose to measure about those factors will fall within a certain range, specific to each species. This criterion is compatible also with the crosslinkage theory, as with all other theories.

*2. Distribution of Mortality*

The mortality distribution curve for humans has been very closely simulated by the melting point increase curve (a measure of crosslinkage) of a gelatin gel in the presence of an active crosslinking agent.

*3. Genetic Factors*

This is essentially a re-phrasing of criterion 1 and is likewise met by every theory of which I am aware. Nobody today disputes that subcellular, cellular, tissue, and organism characteristics depend on genetic factors. Thus, if any single one of these has any influence on longevity or on health, as many of them assuredly do have, then genetic factors will influence life expectancy. The crosslinkage theory also meets this "test".

*4. Aging and Growth*

The crosslinkage theory postulates that there is a progressive accumulation of immobile crosslinked macromolecules, which are essentially inert, yet cannot be excreted. Gradually, they crowd out other cell elements, to the point of reducing viability and ultimately causing death.

From this follows that if the organism grows sufficiently rapidly to add new viable material and cell space in at least the same proportion as crosslinkage takes place, then the *relative* percentage of crosslinked material does not increase, but remains at an innocuous level.

*5. Temperature Effects*

Using Drosophila, in 1917 John H. Northrop and Jacques Loeb showed that the temperature coefficient of aging is the temperature coefficient of a chemical reaction. Crosslinkage obviously is a chemical reaction and the temperature coefficient is the same as in aging.

### *6. Radiation*

Ionizing radiations have two chemical effects on biopolymers: chain fission and crosslinkage. Chain fission results in the formation of fragments, which are generally excretable and thus have no lasting effect except in the lower probability case that the fission could result in the elimination of an irreplaceable essential molecule. The principal effect of radiation with regard to actual or simulated aging is therefore crosslinkage, whether this is caused directly, or by the secondary formation of crosslinking agents such as aldehydes, free radicals, peroxides or epoxides.

For example, if an unsaturated fatty acid compound such as a linoleate is suspended in a gelatin gel and then exposed to sunlight, or to an ultra violet source, little change is observed immediately following the irradiation. However, the radiation has triggered a reaction or series of reactions which subsequently, in the dark, in a period of weeks or months dependent on the intensity of radiation used, will cause a progressive crosslinkage of the gel with consequent increases in molecular weight, melting point and decreases in solubility, swellability and water retention – all changes characteristic of aging.

The progress of these changes is greatly impeded by the addition of tocopherol or equivalent synthetic antioxidants. The addition of bisulfite, an aldehyde blocking agent, blocks the post radiation change at least in the case of insulin, indicating that the above effects are principally due to the secondary formation of aldehydes.

All of the above is logically expectable in view of the crosslinkage theory.

### *7. Differential Aging Among Organ Systems*

Even in a very simple case, like a horse-drawn vehicle, it would be a most unusual occurrence if aging caused all of its parts to fail at the same instant. This "One hoss shay effect" only occurs in imaginative poetry.

How much more fantastic, would it not then be, to postulate a theory which would require that various organs in the body would age at precisely the same rate, and thus fail in exactly the same instant? Indeed, no such theory exists. Every known theory meets the above criterion, the crosslinkage theory not excepted.

### *8. Reserve Capacities*

The macromolecular aggregates formed by irreversible crosslinkage in an aging organism fill progressively larger space in a volume which remains constant as soon as growth has ceased.

The space available in the cells for other, viable and functional molecules thus progressively contracts. The reserve capacities of the organism contract with it.

*9. Total Performance*

In a complex machine there are many more things that can go wrong than in a very simple machine. A motorboat malfunctions more often than a sailboat.

As the number and complication of organs increase, the possibilities and probabilities of deleterious changes multiply geometrically. This is true no matter what kind of theory is assumed. The crosslinkage theory is no exception.

*10. Complex Performance*

This is essentially a re-statement of criterion 9, above, and the same comments apply.

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In the decade that has passed since Shock proposed the above ten criteria for judging aging theories, it has become apparent that criteria 1, 2, 7, 9, and 10 are met by all theories now extant or likely ever to be conceived. Their utility as criteria is correspondingly limited.

On the other hand, advances in knowledge of aging phenomena have focussed attention on additional requirements:

*11. Starvation Effect*

Controlled low calorie feeding with maintenance of adequate essentials prolongs life both in youth and when applied after maturity. The change in longevity is much greater than could be attributed to increases in weight or processing load per se, and does not seem connected with any somatic mutation effect.

Applying the crosslinkage theory, it is easily visualized that a high caloric intake favors the accumulation of metabolic intermediates, of which several are powerful crosslinking agents, as explained below.

*12. That the diploid and haploid forms of the same species have life spans in the same order of length.*

This finding is entirely compatible with the crosslinkage theory, which builds on the formation of agglomerates of macromolecules, which cannot be broken down by body enzymes.

*13. That life span is shortened quite dramatically by bifunctional alkylating crosslinking agents, while it was not similarly shortened by a monofunctional alkylating agent having the same mutagenic effect.*

This finding by Alexander and Connell is expectable in view of the crosslinkage theory, since it shows that crosslinkage is correlatable to life span but that the genetic effect is not.

*14. Radiotracer administered at birth is lodged, after a steady state has been attained, largely in essential amino acids having a side chain terminated by a reactive group.*

This finding is compatible with the crosslinkage theory, since side chains are the most obvious and common components in crosslinkages.

*Effects Predictable by the Crosslinkage Theory*

On the basis of the crosslinkage theory, certain phenomena can be predicted:

1. High caloric intake should have a life-shortening effect. With a low caloric diet, oxidation proceeds rapidly to innocuous end products: carbon dioxide and water. On high caloric diet, when the food ingested exceeds what can be processed at the slowest steps of metabolism, the intermediate products prior to these "bottlenecks" will accumulate in the organism and some of them are bound to diffuse out into the system. Many of these intermediates are crosslinking agents, such as for example glyceryl aldehyde, acetaldehyde, methyl glyoxal, glyoxal and the like. These crosslinking agents will have a life-shortening effect.

2. It is predictable that intense solar radiation will cause accelerated aging of the skin as well as some systemic effects in the same direction. It has been demonstrated that ultraviolet irradiation of gelatin film containing even small amounts of unsaturated fatty acids or esters, will induce crosslinking effects which continue several weeks after the irradiation has ceased.

In a model system, crosslinking from lipid oxidation was greatly retarded by the presence of vitamin E, and it is thus predictable that vitamin E and such equivalent antioxidants as are physiologically acceptable will have an anti-aging effect and will also have a protective effect on skin exposed to strong irradiation.

This line of reasoning poses an interesting question. It is apparent from vital statistics that once a negro has reached

the age range of 65 – 70 years, his life expectancy is about a year better than that of a white person at the same age. Is this because skin pigmentation counteracts the formation of crosslinkage agents on exposure to solar radiation?

3. It is further predictable that, on aging, the immunological behavior of proteins will be changed, and auto-immunity may ensue. Inasmuch as crosslinkage radically changes the structure and solubility characteristics of proteins, it is expectable that their immunological behavior will be altered.

4. It is predictable that with aging the body proteins will gradually change from hydrophilic to oleophilic. This is because in crosslinkage the polar groups are primarily involved. These polar groups are the most reactive groups. Therefore, in crosslinkage they will be attached to each other so that both macromolecules involved will tend to turn their more hydrophilic sides inward, and their more oleophilic sides outward, thus becoming more oleophilic as crosslinkage proceeds. In view of this, it is predictable that increased infiltration, attraction and deposition of oleaginous matter will take place with aging.

5. Increased rigidity of molecules is a principal effect of crosslinkage. This is the same as decreased elasticity. Just as any carpenter or engineer knows that crossbracing stiffens mechanical constructions, so it is predictable that crosslinkage will result in a progressively reduced elasticity of all tissues, which in turn will increase the danger of micro fractures in muscles, glands, arterial endothelia, and elsewhere.

Some of the above phenomena have been amply documented, others insufficiently or not at all.

### *The Present Status of the Crosslinkage Theory*

The crosslinkage theory stands unique among the primary theories in meeting all the criteria listed above for a theory of aging. It stands simply and without strained assumptions. It is of course entirely compatible with the free radical theory which deals with a special case of crosslinkage, and with the diffusion, cybernetic, hormone, autoimmunity, progressive cell death, reticulocytic effect and enzyme deterioration theories, which deal with sequels of primary crosslinkage.

While the crosslinkage theory no doubt will be polished and supplemented in future years, we believe that its essential elements will continue to stand the test of time. We are doing no more work to prove the theory, but are following the guide lines it has provided toward applications, and exploring sequential component steps.

The trail is blazed. Within our grasp now lies the possibility of an extended productive future for the individual, with all the promise, the dangers and the fatefulness which this implies.

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*Johan Bjorksten*

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Short Term Goals - Chelation

Since this work will require years for completion, it seemed advisable to explore those potentialities for life extension which, applied immediately, could give us 10 - 20 years more in good health, which may be needed for completion of the major breakthrough. An important topic offering such potential when properly applied, appears to be the chelation removal of unwanted metal ions.

# POSSIBILITIES AND LIMITATIONS OF CHELATION AS A MEANS FOR LIFE EXTENSION*

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## SUMMARY

An overview is presented of functions and limitations of chelation, for removing undesired metals, including but not limited to acute poisons, chronic environmental poisons, bone-seeking radioisotopes, and cumulative poisons active in senile dementias and scleroses. The chelating techniques are applicable to all metals. Present trends include the development of injectible or oral chelation. Among promising developments are mentioned cholyl hydroxamic acid, which discharges metal thru the liver and digestive tract as well as by the kidney route, and other orally administerable chelators which on the basis of animal tests appear to have advantages over those now in use.

## INTRODUCTORY

The process of chelation is now used routinely, for treatment of lead poisoning, and for removal of radioactive bone-seeking elements such as plutonium.

**The extension of this therapy to the removal of calcium in scleroses, is being used clinically on a substantial scale, but is hotly debated.** Without taking any sides in this controversy, it appears timely to review the growing literature on the chelation agents available, and apparent trends in their development.

Chelation (a word derived from the Greek word «Chela» - a claw) is based on the simple fact that two or more attractive forces acting simultaneously on a metal atom are stronger than only one. A chelating agent is a molecule which contains at least two groups of polarity opposite to that of the atom it is wanted to remove, in such a sterical position as to fit the size of that atom, thus exerting a double or multiple pull on it. Where the atom is strongly anchored, more than two contact points may be needed. An exemple of a sixfold pull («hexadentate») which has attracted and locked in an aluminum atom (relevant in current research on Alzheimer's disease) is shown in Figure 1.

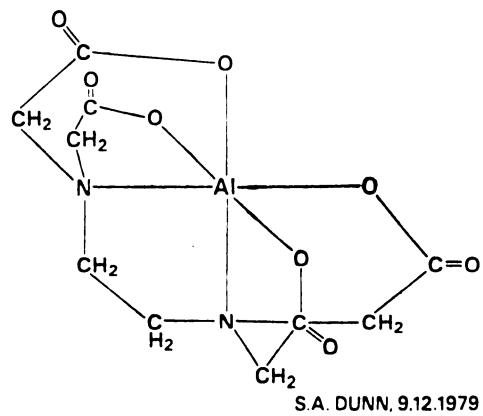


Fig. I shows the end result of a hexadentate chelation. The aluminum atom, which has a valence of III but a coordination number of 6, has here entered the 6-pronged grip of a single molecule of ethylene diamine tetraacetic acid (EDTA), one of the oldest, and most versatile chelators we know.

## METHODS AND PRECAUTIONS

The chelating substances most widely used and on which by far the most complete data have been assembled, are derivatives of ethylenediamin tetra acetic acid and its chemical siblings, the EDTA'S. The principal chelators in present use have been recovered from the excretions without loss and without any chemical changes affecting their basic structure. Accordingly, it may be assumed, that they cannot cause any lingering or unexpectable toxic effects as none of these substances remains in the body.

**A drawback is that these chelators will not only remove accumulated undesirable metals, but also those which are necessary for life or health.** These must be replaced during or immediately following the chelation treatment. The affinity, or attraction, of various metals is only somewhat selective for the members of the EDTA family. No chelating agent I know can be set to bind only one target metal, though some are selective for strontium,[1] plutonium,[2] mercury,[4] or cadmium.[5-6] The desiable or necessary metals, such as calcium, iron, cobalt,[7-8] zinc, magnesium to men-



* Presented at the Conference of the American Academy of Medical Preventics, Denver, Colorado, USA. Nov. 3 1979.

tion only a few[9] consume a part of the chelating capacity.
They also have to be returned to the system either as now is common practice, by adding some calcium gluconate solution to the last few mls of the infusion liquid, or as dietary supplements, in food, pills or capsules. [10-11] Any surplus of these replacements will also be attracted to the chelating agent used, further reducing its payload. The theoretical maximum removal capacity of the chelator is only partly used ; there is room for improvement.

The theoretical metal removal capacity of 3 grams disodium EDTA, the usual **daily** dose in infusion therapy, is 1.8 grams of mercury, or 1.8 of lead, 1.1 of plutonium, 1.0 of cadmium and 0.2 of aluminum in the most stable, hexadentate bonding.

Ideally, a chelating agent should reach the site of the target metal, pull out this metal preferentially and permit its removal, while leaving desired atoms untouched ; it should be easy to apply, safe, inexpensive, and permit utilization of its entire metal removal capacity. While striving to reach this target, we must meet present needs with the tools we now have.

## CHELATORS OF THE EDTA group

EDTA, the principal chelating agent in the past, [10,12] is poorly absorbed when taken orally. Tests indicate that only about 5 % of the quantity ingested enters the blood stream. It causes nausea when taken orally. One of my correspondents states that he has been able to effect improvement in calcium removal with EDTA, by taking it orally in very long dilutions on empty stomach, just before going to bed. His tests were not well monitored or quantitated, but may nonetheless contain a clue to an approach which might be kept in mind for future explorations.

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EDTA and most of its derivatives likewise do not pass readily thru other barriers in the organism, such as cell membranes, endothelias or the blood/brain barrier. In the treatment of lead poisoning for example, EDTA rapidly clears from the blood any lead that is circulating, but it can only indirectly remove intracellular lead, particularly that in the central nervous system.

Removal from tissues follows repeated treatment slowly, to the extent that some lead gradually diffuses back into the blood stream, where it can be reached by EDTA. Thus, treatment with most EDTA type chelators, although used widely and with more demonstrated practical success than any other form of chelation, still leaves something to be desired. Some recently developed not yet fully clinically tested related compounds may permit oral use, for example EDHPA,[13,14] as will be further discussed below.

Much effort has been given to the development of alternate methods, which in these regards may be more advantageous or flexible, or which may make possible combination therapies.

## INJECTIBLE CHELATORS

Among the injectible chelators, «DFO,» (Desferrioxamine) has the longest and best established clinical record.[15-19] **This chelator, produced by the mold Streptomyces pilosus,** has been used particularly to remove excess iron, which is associated with mass destruction of erythrocytes following massive blood transfusions. Deferoxamine, as it is now officiallly called in the USA, has been established as a clinical chelator, safe under medical supervision. Though it has its highest affinity for iron we must never forget that the use of **any** chelator now known also will **remove some necessary** polyvalent metals, which must thus be continually replaced.

The most usual dosage of deferoxamine is by injection of 500 mg. dissolved in any aqueous isotonic medium.

The success of deferoxamine led to further investigations of chelators. To date three substances are emerging, which appear to be at least in some regards superior to EDTA and DFO. Extensive studies of a large number of chemically plausible candidates in 1976[20] and 1978[20] by R.W. Grady and co-workers emphasized two such substances, namely **rhodotorulic acid, a naturally occurring iron chelator isolated from the supernatant of cultures of the wild yeast Rhodotorula pilimanae ;** and the synthetic cholylhydroxamic acid. Rhodotorulic acid is much less water soluble than deferoxamin, and thus is released very slowly following intramuscular injection, so that the blood level following an injection remains therapeutically active for several hours.

## ORALLY EFFECTIVE CHELATORS

R.W. Grady and co-workers had for an important object to find clues to orally administerable chelating agents, effective also on iron. Their search has centered on hydroxamic acid derivatives, which as a group seem to be tolerated and absorbed when taken orally. Cholyl hydroxamic acid is singled out by them on the basis of tests **with rats,** as superior to deferoxamine in efficacy and well worth clinical trials.

The excretion of chelated metals may thus occur along two different routes, thru the kidneys, or thru the liver, passing thru the biliary route and excreted in the feces. [5] Cholyl hydroxamic acid, is taking the liver route and is mainly excreted in the feces. This is also to a considerable extent true of the secondary aliphatic hydroxamic acids, Deferoxamine and Rhodotorulic acid.

Since feces are less convenient to monitor than urine, the existence of the two separate exit routes has to be considered in the organization of clinical tests with these substan-

**TABLE 1**

| Compound | Toxicity LD50 | Relative Iron Excretion in Feces | in Urine |
|---|---|---|---|
| Primary aliphatic hydroxamic acids | | | |
| Cholylhydroxamic acid | >1.6g/kg | 3 | 0 |
| Secondary aliphatic hydroxamic acids | | | |
| Desferrioxamine | >800mg/kg | 2 | 1 |
| Rhodotorulic acid | >800mg/kg | 3 | 2 |

"2" means 300-500 µg/kg/day
"3" means 500-700 µg/kg/day

Excerpts : R. W. Grady et al : J. Pharmacol and Exp Therapy 205 : 671, 1978.

ces. The highest efficacy seems to be found in those chelators, which are excreted by both routes, and this may warrant the inconvenience of having to monitor the feces.

Grady, et al. have found a total of five compounds, of quite different structures, that significantly increase the total amount of iron excreted by hypertransfused rats, when orally administered. These were 2, 3-dihydroxybenzoylglycine, 1-histidine, ellagic acid, tropolone and cholylhydroxamic acid.

C. G. Pitt, in a paper together with 13 named co-workers,[13] screened over 70 chelators, using hypertransfused mice. They found the **orally active** EDHPA (Ethylendiamine-N, N'-bis (2-hydroxyphenyl acetic acid)) to be considerably more effective than deferoxamine B, which was used as the standard [13] Cleton [14] had previously had favorable results with EDHPA, but these were not followed through at the time.

The following is a summary abstract from a comprehensive table, showing the most conspicuous of 49 compounds. The reference should be consulted for details on changes in spleen and liver, and data on many less promising substances tested.

**TABLE 2**

Excerpts C. G. PITT et al. J. Pharm. Exp. Ther. **208** ; 14, (1979)

| Compound and Chemical Family | Dose | Surviving Animals | Percent Iron Change vs. Control Urine |
|---|---|---|---|
| | mg/kg | | |
| Microbial hydroxamic acids | | | |
| Deferioxamine B (DFB) | 250 | 10 | + 400 |
| Rhodotorulic acid (deferri) | 300 | 10 | + 229 |
| Triacetylfusarinine C (deferri) | 300 | 10 | + 247 |
| Phenols | | | |
| EDHPA, dihydrochloride | 50 | | + 1439 |
| Ethylenediamine-N, N-bis (2-hydroxy-5-methylphenylacetic acid) | 100 | 9 | + 698 |
| Catechols | | | |
| N-(2.3-dihydroxybenzoyl)glycine | 100 | 10 | + 254 |

**Almost all plants contain more or less of naturally occurring iron chelators, designed by millions of years of evolution to specifically seize the usually scarce iron molecules from soil and waters.**

Some among these are of outstanding efficiency and extractable in reasonable yields. Deferoxamine, rhodotorulic acid, and triacetyl fusarinine C are the now most promising of these. They are more or less efficient for other metals as well. So far, none of these have yet been screened with aluminum.

## ALUMINUM CHELATION ?

**The need for aluminum removal is rapidly moving toward the center of gerontological attention, with the recent paper of Crapper, Karlik and Deboni, [22-25] the findings of Alfrey of aluminum accumulation paralleling severe mental disturbances in patients in chronic treatment on artificial kidney[26] the immunohistochemical relationship between Alzheimer fibrillary tangles and aluminum induced filaments[27] and the detection of focal accumulations of aluminum and silicon within neurofibrillary tangle bearing neurons of Alzheimer's disease.[28] Such widely differing diseases Parkinson's Disease [29] and Down's Syndrome[22. Fig. on p. 472] have also been associated with elevated aluminum content.**

Symptoms closely resembling those of human senile dementia can now be induced at will in test animals by minute quantities of a soluble aluminum salt injected once only into the Hippocampic ventricle, where aluminum has then been located firmly bound to the chromatin in pyramidal neurons. [22,24]

**Because of its great capacity for immobilization of large vital molecules by crosslinkage, and its widespread presence in nature, aluminum has been suspected to be a basic factor in aging,** long before Crapper, et al. brought the uncontrovertible proof.[30-32] T. M. Riddick strongly assailed the use of soluble aluminum compounds in antacids and in water «purification»[33] Evolution has endowed most of us with high capacity to reject the aluminum which is unavoidable in our daily nutrition. It now appears that a few nanograms may slowly work past these defenses each year, to reach damaging totals at some ages after 60. Tests with short lived animals cannot reveal these effects which take decades to become dangerous.

**Removal of aluminum may well become a key target for chelation in preventive gerontology.** Some ground work has been done toward this.[34]

Why should aluminum be so threatening ? The following hypothesis might be considered : The transaminases are enzymes highly essential in the brain metabolism. They, in turn, are depenent on pyridoxal (Vitamin $B_6$) with which they form Schiff bases as metabolic intermediates.

In vitro, the Schiff bases of alpha amino acids and pyridoxal are catalyzed by $Fe^{3+}$, $Cu^{2+}$, $Ni^{3+}$, $Co^{3+}$, $Zn^{2+}$ and $Al^{3+}$. Of these, ferric-, cupric-, nickelic, and cobaltic- ions can be readily manipulated by oxido reductive reactions, because these metals all have at least two oxidation stages. Zinc and Aluminum are the two mavericks. Of these, zinc is an essential element. Evolution has been forced to find ways to handle it. We may note, though, that zinc does have a critical toxic limit, and that zinc intoxication involves the central nervous system. Aluminum has no known metabolic utility. Wherever it is affixed, it cannot be dislodged by shifting it to another oxidation stage, because aluminum has only one stage. When it has occupied a key position, this is final inactivation for the molecule involved, so far as natural resources are concerned.



## EXERCISE AND CHELATION

Having considered chelation by infusion, injection and taken orally, it remains to add a few thoughts about chelation by agents generated within the body, and agents directed by electrolysis.

**Lactic acid is a chelating agent, somewhat comparable with citric acid in selectivity and potency.** Lactic acid is formed in muscular action of any kind. In exercise, the lactic acid percentage in blood is about doubled so long as important use of major muscles takes place,[35] as in brisk walking, or any other activity where **endurance** is stressed, rather than peak performance. Immediately on cessation, the lactic acid content drops back to normal levels. Lactic acid is not as effective as EDTA in speed, but given enough time to act, it seems comparable in total removal of chelatable metal.

**The favorable therapeutic results often obtained by muscular exercice [36-37] in cases of vascular impairment might well be due to this internal chelation even as much as to improvements in blood circulation.** This chelation would include removal of undesirable deposits of calcium and hopefully most of the aluminum, as well as of lead, mercury and cadmium. It may, however, still leave a final residue, $<1\%$ of the undesirable metal to be scavenged by an ultimate chelation using the most potent chelators.

## ELECTRICAL FOCUSING

The other method, still not developed, is the possibility of electrically focusing chelating ions, such as lactate ions. The use of electricity in quackery has delayed the legitimate development of valuable applications. Subcutaneously injected ionizable chelators can in some cases be carried by galvanic currents to a target point thus minimizing systemic effects and achieving higher local concentrations than would otherwise be tolerated. In vitro experiments show that, for example, lactate ions can be thus directed to definite targets and that lactate ions in vitro can dissolve absorbed aluminium. [31]

## LIFE EXTENSION BY CHELATION

So far, we have discussed chelation in terms of therapies specific to certain defined diseases and syndromes. However, I like to reserve the final words for a still broader outlook : The effect of chelation on the longevity of organisms.

**In the 50's, Lansing[38-39] showed that the life span of some very primitive organisms could be extended if chelating agents were added to their culture medium.** More recently, Sincock, et al. [40] have shown life extension up to 70 % of the Rotifer Mytilina brevispina var. redunca. More germane to **human** gerontology is perhaps the work of Dr. A. Tyler, whose work in the years 1953-1965 also embraced mammalian cells. Tyler tried to determine the reason for the short life span of sea urchin sperm cells in sea water. [41] He assumed that **nutrition** might be the limiting factor, so he added amino acids to the sea water in which they were swimming, and found indeed that with certain amino acids, the life span as measured by motility was appreciably prolonged. However, some of the amino acids were not effective, and the results did not fit any nutritional hypothesis. **Those amino acids which gave positive results were the potential**

**chelators.** So, to clinch the answer, Tyler added to his water a non-nutritional amino acid which was a strong chelator, namely EDTA. Immediately the effects became much greater — increases of up to 50 times the normal life span were recorded !

The effect of the chelator **is** due to the removal of deleterious ions. This was proven by Tyler's getting similar life prolongation with a synthetic sea water, compounded from meticulously purified components but with no chelating agent

These tests were repeated with sperm cells from higher animals, rooster and steer, with similar results. [42] The promising work was interrupted by Tyler's untimely death, and has not yet been resumed

## CONCLUSION

A poet wrote :

> «The best thing, I suppose
> that a man can do for his land
> is the work that lies under his nose
> with the tools that lie under his hand»
> [43].

**The tools under our hands now are those now established in therapy. These include also chelators, particularly EDTA derivatives applied by infusion, deferoxamin by injection and exercise as an applicable technique for generating a chelator, lactic acid, within the body.** However, further work is needed to follow, review, and when so indicated, **apply** the work now being done with the new chelators : cholyl hydroxamic acid, the hexadentate triacetyl fusaramine C, EDHPA, and others which will follow as research pushes on with increasing force, success and recognition.

Ultimately, we might aspire to reach for man, a life extension at least in line with the 75,9 % that Sincock, et al. achieved with EGTA for a Rotifer and Tyler exceeded for a particular kind om **mammalian** cell.

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# IN VITRO STUDY OF FOCUSED CHELATING TECHNIQUES FOR METAL-CONTAINING AGGREGATES IMPLICATED IN SENILE DEMENTIA *

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Dr. D. R. Crapper's findings reported in the abstracts for these meetings 1) bring into focus the question of possible causes of Alzheimer's disease and of senile dementia.

He leaves us still in some doubt, because the fibrils induced in cats are not neatly paired as those in Alzheimer's disease - Even if aluminum should be the cause in both cases, it would not be surprising if a syndrome which was artificially induced in a few weeks, showed a less ordered protein structure than that developed in 50 or 60 years.

However, leaving this aside, Dr. Kaneko, and co-workers 2) have given us another strong reason to suspect aluminum. Their finding of a 40 % decrease in water soluble protein would be fully explained by the presence of aluminum which is a known potent protein crosslinker, used as such in tanning industry. It is further a powerful anti-coagulant. Aluminum salts are deliberately added to water supplies in many cities to speed the coagulation of suspended colloidal matter and thus help clear the water. Added to a complex colloid as the brain it will certainly destroy the equilibrium and cause aggregation of solids ana exsudation of liquid. Thus, aluminum would cause shrinkage of the brain.

Could an addition of aluminum to our water supplies over a period of 50 - 60 years increase the passage of aluminum into the brain ? This question must now be asked.

Aluminum is 8.4 % of the earth's crust. It is present in almost all foods. Unavoidably we all ingest about 30 milligrams daily. Evolution must have given man a defense against it or we would not have survived. But none the less, in 50 - 60 **years** a few milligrams of aluminum might slip through the defenses and cause cerebral symptoms, like those we are now discussing.

Is it possible to remove aluminum accumulated in the brain ? Zinsser, Bjorksten, Bruck et al. published in 1962 3) a list of chelating agents then known as effective in extracting aluminum.

The list has widened since then. But chelating agents are like a 2-edged sword that cuts both ways. They could easily deplete the organism of needed trace elements. It would thus seem highly desirable to cause localized removal of undesired metal deposits, primarily calcium and aluminum thus avoiding the need for saturating the whole system with drugs which have known side effect.

The following Figures will illustrate some efforts in that direction.

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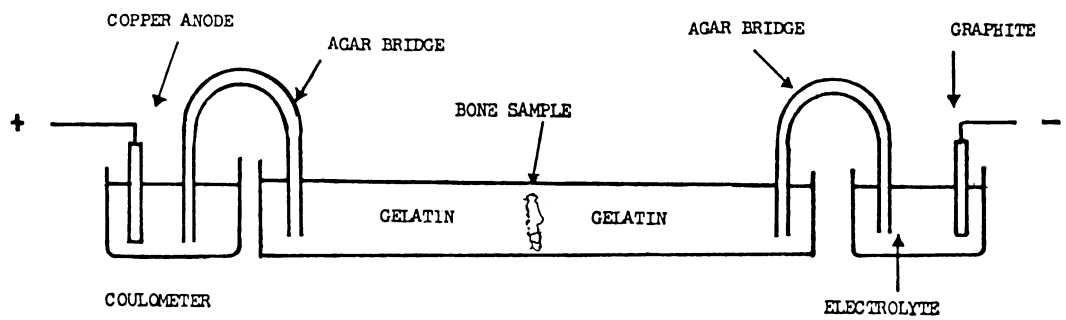
Fig. 1.

FLOW DIAGRAM FOR BONE ELECTROLYSIS




* Paper presented at the XIth International Congress of Gerontology Tokyo, Japan, Aug 20-25, 1978

Arrangement for in vitro tests with iontophoretic removal of chelatable metal.

A polystyrene drawer 3 x 7 cm was filled with a gel of 10 % gelatin, 50 % glycerine and electrolyte to make a conductivity approximating that of human tissues.

The current is supplied via bridges of conductive acrylamide gel from a set of graphite electrodes. This to minimize contamination.

In the center of this gel, we placed a fish bone, specifically backbone from a red snapper, defatted and cleaned. We applied current with a copper coulometer in series, to keep track of the exact amount of current passed.

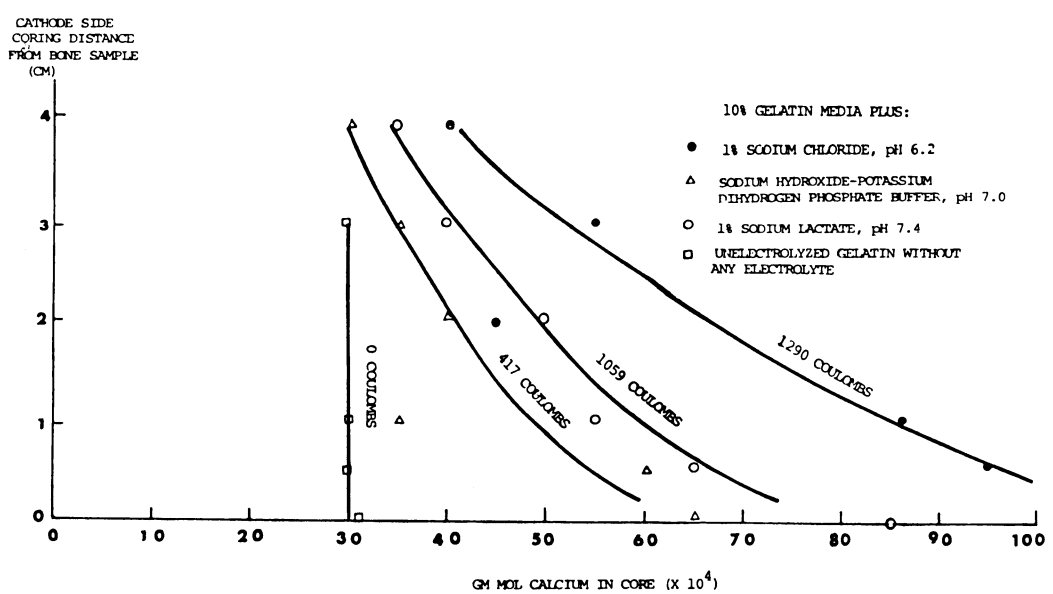
The total extent of bone substance removal under 3 conditions is apparent from table I and fig. 2.

Table 1

**SUMMARY OF OVERALL CELL OPERATION**

| Electrolyte | Total Calcium In Cell Gel (gm mol x $10^4$) | Copper loss from Coulometer Anode (gm) | Coulombs | Calcium Removal Efficiency (%) |
|---|---|---|---|---|
| 1 % NaC1 | 428 | 0.8964 | 1290 | 303 |
| 1 % Na lactate | 490 | 0.7218 | 1059 | 358 |
| Buffer Solution Phosphate Ph 7 | 166 | 0.2897 | 4117 | 36 |

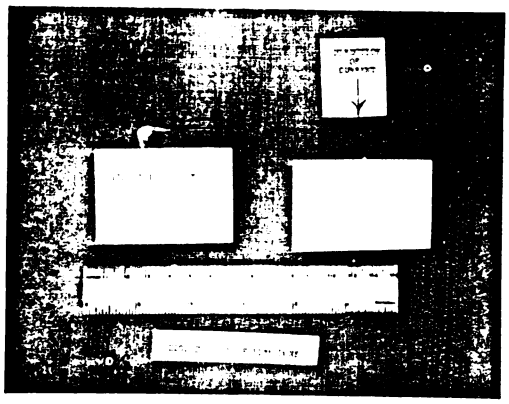
Fig. 2.




It is noteworthy that the lactate ion was more effective than even the chloride ion in removing calcium from bone.

To focus the current, we inserted polystyrene partitions with a hole, so as to limit the ion flow to a narrow stream. We placed a fan shaped fish bone from a flounder in the path of this beam and adjusted the current to 5mA at 20V. In 48 hours the bone substance had lost 12 % in weight, and a 4 mm deep circular notch had been formed in the edge of the bone, in the most direct path of the current.

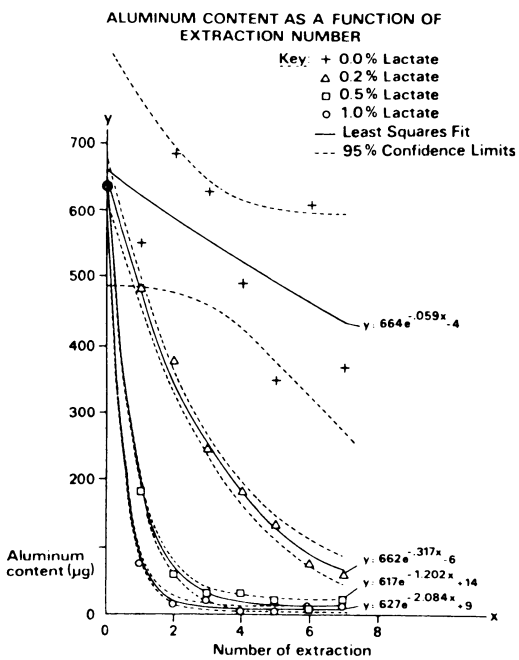
Fig. 3.



It is thus definitely possible to cause directed flow of ions to selectively remove even very firmly established **calcium** deposits, at physiologically acceptable conditions.

Moving then to aluminum, we tested first the possibility to remove aluminum by extraction with chelators as a preliminary, without as yet applying electricity. As test samples we used discs punched out from fresh hog aorta, immersed in Al-Solution and washed until clean. The following slide shows removal of the bound aluminum by lactate of different concentrations.

Fig. 4.




Lactate is not as rapid a chelator as EDTA, (ethylene diamine tetra acetic acid), but it has the advantage of known long term compatibility with human physiology. It is also a fairly mobile ion, and seems suitable for electric focusing.

An interesting question is whether not much of the beneficial effect of exercise is due to metal removal by the > 100 % elevation in lactic acid content of the circulating blood during exercise, which drops back in a matter of minutes immediately following the cessation of muscular exertion.

It is thus indicated, that ions, including chelating ions, have been positionally directed by moderate electric currents under physiologically practical conditions. This might provide means for removing calcium and aluminum accretions by topical means, gently, painlessly and gradually.

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## ACKNOWLEDGMENT

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Reprinted with Permission from REJUVENATION VI, No.1, October 1978, pp. 73-75. Editor in Chief, H. LeCompte, M.D.



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Antioxidants - Free Radical Scavengers

The first of the following two papers shows in Experiment 5 that after an initiating irradiation, a system of unsaturated fatty acid and proteinaceous gel slowly increases the rate of cross-linking (as measured by melting point) even in the dark, until in about a month the melting point becomes higher than the decomposition point. The second paper shows that in this process no free radicals can be shown present during the period when the crosslinking takes place. The crosslinking in this model of aging protein seems, therefore, not to depend on any free radical action, continued beyond the initiating irradiation.

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# A Chemical Model of Ultraviolet Radiation Effects on Skin Collagen

JOHAN BJORKSTEN and THOMAS COLLBRING*

THE detrimental effect of ultraviolet radiation on skin was recognized by Unna in 1894 (1) and has received much attention (2, 3). Much of the data are summarized by Cockerell, Freeman, and Knox (4) as follows:

"... It seems clear that the onset of collagen degeneration is independent of age, being determined simply by the cumulative amount of injury from ultraviolet light. ...The visible cutaneous changes usually interpreted as aging are apparently due for the most part, if not entirely, to sunlight."

The precise mechanism of this damage, however, has not been defined. An important factor in this process may be due to the known oxidation of unsaturated fats in the skin to form both peroxides and aldehydes, both of which are protein reactive cross-linking agents (5, 6). The association of cross-linkage with protein damage (7, 8, 9, 10), genetic damage (9, 11, 12, 13, 14), and aging (7, 15), led to a study of the effect of ultraviolet radiation on a simplified model system where a tenacious gelatin film containing unsaturated fats was exposed to radiation from a mercury vapor lamp.

## Experimental

As the gelatin component in the system, pigskin gelatin of high gel strength (275 Bloom) was used, because this gelatin is extracted from pigskins at the lowest temperature and thus has undergone the least changes in processing.

The following general procedure was used, modified in detail as stated in connection with the particular experiments, in order to prepare the desired model. The gelatin was allowed to swell for at least two hours in a 1:1 water-glycerin mixture at room temperature in order to open the gel structure. The gel was then placed in a water bath at temperature not exceeding 53°C.

A 90% aqueous glycerin solution (10 times the weight of the gelatin) was heated to 50°-53°C. and poured onto the swollen gelatin which had then also melted, and both were mixed rapidly. This stock gelatin gel was then used as the base and mixed with the fats to be tested, and in some cases mixed with other components. The details of the individual experiments are shown below:

EXPERIMENT 1

| | |
|---|---|
| Gelatin, pigskin, 275 Bloom | 50 grams |
| Glycerin (96%) | 50 grams |
| Water | 130 grams |

This mixture was allowed to swell for two hours at room temperature, and then for another two hours in a water bath at 53°C. This was added to a solution of 50 grams of water and 140 grams of glycerin, preheated to 53°C.

Aliquots were used for as follows:

1–Control, no addition.

2–Raw linseed oil (10% by weight of the total mix) was added and mixed thoroughly.

3–10% of safflower oil added, mixed as above.

4–10% of coconut oil (Emery 622 double distilled) was added, mixed as above.

After mixing, the respective samples were cast onto glass plates so that they flowed out and gelled to films 3 mm. thick. The thickness is not very critical, for similar melting point values were obtained with 20% thinner or thicker films. After these had hardened by standing overnight, circles of 10 mm. diameter were cut out with a cork borer and pierced by a stiff metal wire which was then suspended horizontally in a beaker filled above the level of the wire with mineral oil, and gently mechanically stirred with a bent glass rod as propeller. The oil bath was warmed slowly on an electric hot plate until the melting points were reached. At this temperature the discs of gel fused and fell off the wire.

Observation by microscope showed that the oil remained suspended in minute droplets which did not separate on the surface. Thus the melting points recorded were the true melting points of the gelatin gel, dependent on the degree of cross-linkage of the gelatin. Polymerization of oil could not have affected the melting point because the oil was present in discrete droplets. All determinations were made in duplicate.

Results were as follows:

| | Melting Point Start | Melting Point after 4 Hours Under UV |
|---|---|---|
| Control | 47 C | 52°C |
| Linseed oil | 50 | 56 |
| Safflower oil | 52 | 73 |
| Coconut oil | 48 | 52 |

It appeared that the unsaturated linseed and safflower oils induced more cross-linking under exposure to ultraviolet than the control or the saturated coconut oil.

In order to obtain a comparison over a longer period of time, we repeated the experiment with four hours' exposure to ultraviolet, and made melting point determinations at intervals over a period of time as follows:

EXPERIMENT 2

| | Melting Point in C after 4 Hours UV Radiation as Above, after Following Number of Days in Dark | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 7 | 8 | 9 | 10 | 11 |
| Above described gel with: | | | | | | | | | | |
| .35% Safflower oil | 54 | 55 | 57 | 56 | 56 | 55 | 55 | 56 | 57 | 58 |
| 10% Safflower oil | 85 | 86 | 88 | 86 | 90 | 95 | 98 | 98 | 98 | 97 |

In these experiments the oils were stirred into the gelatin by hand using a glass rod. It seemed well distributed and did not separate. However, the low increase in melting point when the 0.35% of safflower oil was used led us to conclude that a

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better subdivision of the oil should lead to a more rapid cross-linkage and higher light sensitivity of the system.

In all subsequent experiments the procedure used in preparing the gel was as follows:

Fifteen grams of pigskin gelatin (275 Bloom gel strength) were mixed with 15 grams of 96% glycerin and 39 grams of water and left to stand at room temperature until a gel had formed. This requires about two hours—when convenient, standing overnight is suitable. The main point is to pre-gel the gelatin to prevent the lumping which occurs when dry gelatin is added directly to hot water or glycerin.

A mixture of 132 grams of 96% glycerin and 15 grams of water was kept in a water bath at 53°C. The gelled gelatin was added with agitation into this glycerin-water mix, and promptly melted and mixed with it.

An aliquot of the gel is set aside as the control; the desired additions are mixed into it with a Waring Blendor for four minutes, with care that the temperature in mixing does not exceed 53°C or, at the utmost, for a few minutes, 54°C. If it approaches this point, the mixing may be done in two steps of two minutes mixing with a few minutes cooling at room temperature interposed.

Irradiation was done with a 500-watt mercury vapor lamp placed at a distance of 46-48 cm. from the samples.

EXPERIMENT 3

A gel, prepared as just stated, was made with the mixture of safflower oil, 0.17% calculated on the total weight of the gel. Aliquots were irradiated two, three and four hours to determine the most suitable radiation dosage.

| Time of Irradiation | Melting Point after Following Number of Days in Dark 0 | 1 | 4 | 5 | 21 |
|---|---|---|---|---|---|
| 2 hours | 54° | 54° | 55° | 56° | 55° |
| 3 | 58 | 57 | 71 | 66 | 81 |
| 4 | 74 | 74 | 96 | 87 | 91 |

Since the four-hour result was initially too high for ideal observation and somewhat irregular, three-hour ultraviolet exposure was chosen as the standard procedure.

Next a series of similar determinations was run to determine whether there would be the same progress of cross-linkage in gelatin in contact with chemically pure methyl linoleate, methyl linolenate, and linoleic acid (Hormel) after a three-hour irradiation in the same manner as in the preceding experiments.

EXPERIMENT 5

| | Hours UV Exposure | Melting Point the Following Number of Days in Dark after Exposure 1 | 4 | 5 | 6 | 7 | 8 | 11 | 12 | 15 | 29 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Gel with: | | | | | | | | | | | |
| No linoleate | 0 | 49 | 50 | 51 | 50 | 50 | 50 | 51 | 51 | 51 | 53 |
| 0.17% Methyl linoleate | 3 | 53 | 58 | 62 | 69 | 70 | 84 | 98 | 100 | 99 | 105 |
| 0.17% Methyl linoleate | 0 | 51 | 49 | 48 | 51 | — | 50 | 52 | 52 | 51 | 53 |

This confirms that the progressive cross-linkage evident from the increase in melting point was due to the presence of the unsaturated fatty acid ester and to its irradiation.

A similar experiment was made using methyl linolenate instead of methyl linoleate. The gel was prepared as above and poured onto glass to form a 3 mm. film. This film was divided into two portions—one-half was exposed as above under ultraviolet for three hours and ten minutes. The other half was not irradiated. Both were kept in the dark until melting points were determined as above.

EXPERIMENT 6

| | Exposure Under Mercury Vapor Lamp | Melting Point after Following Number of Days in Dark 3 | 4 | 5 | 6 | 7 | 10 | 11 | 14 | 19 |
|---|---|---|---|---|---|---|---|---|---|---|
| Gel with: | | | | | | | | | | |
| 0.17% Methyl linolenate | 0 hrs. | 50 | 52 | 53 | 51 | 50 | 53 | 53 | 54 | 52 |
| 0.17% Methyl linolenate | 3 hr. 10 min. | 54 | 55 | 55 | 55 | 58 | 59 | 63 | 64 | 86 |

It is noted that methyl linolenate after irradiation causes a similar continuous cross-linkage of the gelatin as expressed by a continuous increase of the melting point of the gel, and that no such increase occurs in another portion of the same mix kept in the dark without any irradiation.

The following experiment was made to determine whether diffuse daylight would affect the melting point of the methyl linoleate-containing gelatin films.

EXPERIMENT 7

| | Hours UV Exposure | Then in Dark | Melting Point after Following No. of Days in Diffuse Daylight 0 | 1 | 3 | 6 | 9 | 15 | 22 |
|---|---|---|---|---|---|---|---|---|---|
| Gel with: | | | | | | | | | |
| 0.17% Methyl linoleate | 3 | 6 days | 70 | 73 | 82 | 89 | — | 100 | 104 |
| 0.17% Methyl linoleate | 0 | 4 days | 51 | 50 | 52 | 52 | 51 | 55 | 56 |
| Control, no oil | 0 | 4 days | 48 | 50 | 53 | 51 | 50 | 55 | 56 |

EXPERIMENT 4

| | Melting Point after 3 Hours' Exposure to UV after Following Number of Days in Dark 1 | 2 | 3 | 5 | 6 | 7 | 8 | 9 | 10 | 13 | 14 | 16 | 22 | 29 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gel with: | | | | | | | | | | | | | | |
| 0.17% Methyl linoleate | 57 | 59 | 64 | — | 73 | 79 | 79 | 83 | 86 | 91 | 96 | — | 103 | 106 |
| 0.17% Linoleic acid | 56 | 56 | 56 | — | 60 | 67 | 73 | 73 | — | 96 | — | 96 | 102 | 102 |
| 0.17% Methyl linolenate | 57 | 57 | — | 60 | 63 | 63 | 63 | 67 | — | 67 | — | 65 | 73 | 76 |
| Control, no oil | 52 | — | — | 52 | 54 | 54 | — | 54 | 54 | — | 57 | — | — | — |

A repeat test with methyl linoleate was run with two controls: A, one in which no methyl linoleate was added, and B, with methyl linoleate but without irradiation.

The influence of diffuse daylight on the rate of cross-linkage under the conditions of this experiment is nil, or within the limits of experimental error.

EXPERIMENT 8

**Melting Point after Exposure in Sun**
**(Days not listed, the samples were in the dark)**

| Exposure in Sun: / Subsequent time lapse before next exposure | 1st Day 6 hours / 5 days | 2nd Day 5 hours / ½ day | 3rd Day 7 hours / ½ day | 4th Day 8 hours / 2 days | 5th Day 5 hours / ½ day | 6th Day 7 hours / 1 day | 7th Day 7 hours / ½ day |
|---|---|---|---|---|---|---|---|
| Gel with: | | | | | | | |
| 0.17% Methyl linoleate | 52° C | 59° C | 57° C | 60° C | 61° C | 64° C | 68° C |
| 0.17% Methyl linolenate | 54 | 55 | 72 | 98 | 105 | 113 | >115 |
| 0.17% Linoleic acid | 51 | 55 | 56 | 58 | 60 | 61 | 66 |

Experiments 1, 6, and 7 seemed to indicate that after ultraviolet irradiation methyl linoleate accelerated the cross-linking of the gelatin somewhat more rapidly than methyl linolenate.

An experiment with exposure to sunlight was made in order to see whether the same relationship would apply. These exposures were made on a flat roof during bright sunny summer days.

It thus appears that under the conditions of this experiment, methyl linolenate causes a more extensive cross-linkage in sunlight than methyl linoleate, while under the mercury lamp the order is reversed. However, there is a marked cross-linkage with both esters, both in sunlight and under the mercury lamp.

It was deemed desirable to have another control with the three-hour radiation but without any linoleate added. Since it was preferred not to complicate the melting point determinations by having too many specimens likely to melt at the same moment, another series was made with this control:

EXPERIMENT 9

| | Hours UV Exposure | Melting Point the Following No. of Days after Exposure 1 | 4 | 7 | 8 | 11 | 13 |
|---|---|---|---|---|---|---|---|
| Gel with: | | | | | | | |
| 0.17% Methyl linoleate | 3 | 59 | 55 | 67 | 70 | 67 | 77 |
| No methyl linoleate | 3 | 58 | 61 | 59 | 58 | 62 | 61 |
| 0.17% Methyl linoleate | 0 | 50 | 50 | 52 | 53 | 53 | 52 |

It is apparent that gel containing the methyl linoleate became cross-linked considerably faster than the oil-free aliquot while the nonirradiated sample remained essentially constant.

The same test was repeated with methyl linolenate and gave similar results:

EXPERIMENT 10

| | Hours UV Exposure | Melting Point the Following No. of Days after Exposure 1 | 4 | 6 | 7 | 8 | 11 | 13 |
|---|---|---|---|---|---|---|---|---|
| Gel with: | | | | | | | | |
| 0.17% Methyl linolenate | 3 | 70 | 67 | 72 | 68 | 86 | 73 | 86 |
| No oil added | 3 | 58 | 57 | 60 | 62 | 64 | 61 | 61 |
| 0.17% Methyl linolenate | 0 | 51 | 51 | 53 | 54 | 54 | 55 | 55 |

The same experiment was made using a typical saturated fatty acid (stearic):

EXPERIMENT 11

| | Hours UV Exposure | Melting Point the Following No. of Days after Exposure 1 | 4 | 6 | 7 | 8 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|
| Gel with: | | | | | | | | | |
| 0.17% Stearic acid | 3 | 54 | 52 | 58 | 55 | 55 | 57 | 57 | 60 |
| No oil added | 3 | 54 | 53 | 58 | 58 | 56 | 58 | 59 | 60 |
| 0.17% Stearic acid | 0 | 51 | 49 | 52 | 50 | 51 | 52 | 52 | 52 |

Stearic acid does not cause cross-linking of gelatin after UV radiation.

Since the technique had improved from the first experiments, a test with safflower oil was repeated under conditions similar to the last three experiments.

EXPERIMENT 12

| | Hours UV Exposure | Melting Point the Following No. of Days after Exposure 4 | 5 | 6 | 7 | 10 | 11 | 12 | 14 |
|---|---|---|---|---|---|---|---|---|---|
| Gel with: | | | | | | | | | |
| 0.17% Safflower oil | 3 | 75 | 83 | 78 | 78 | 76 | 72 | 83 | 90 |
| No oil added | 3 | 51 | 52 | 54 | 54 | 54 | 54 | 54 | 57 |
| 0.17% Safflower oil | 0 | 49 | 52 | 50 | 51 | 51 | 52 | 52 | 52 |

The same experiment was further repeated with linoleic acid (Hormel, pure).

EXPERIMENT 13

| | Hours UV Exposure | Melting Point the Following No. of Days after Exposure 4 | 5 | 6 | 7 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|
| Gel with: | | | | | | | | |
| 0.17% Linoleic acid | 3 | 56 | 62 | 58 | 60 | 59 | 65 | 67 |
| No oil | 3 | 52 | 56 | 53 | 54 | 59 | 55 | 57 |
| 0.17% Linoleic acid | 0 | 49 | 50 | 50 | 50 | 50 | 50 | 51 |

The fact that unsaturated chains on irradiation had induced cross-linkage of gelatin led us to test ubiquinone (coenzyme Q) which has a polyisoprene side chain. A concentration of 0.034% of ubiquinone was used. With this low concentration, no sign of cross-linkage of gelatin was observed beyond the mild effect evident in the irradiated control.

EXPERIMENT 14

| | Hours UV Exposure | Melting Point the Following No. of Days after Exposure 1 | 3 | 4 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|
| Gel with: | | | | | | | |
| 0.034% Coenzyme Q | 3 | 50 | 52 | 51 | 51 | 52 | 52 |
| No addition | 3 | 52 | 53 | 53 | 54 | 53 | 53 |
| 0.034% Coenzyme Q | 0 | 49 | 52 | 52 | 51 | 51 | 51 |

EXPERIMENT 15

| | Hours UV Exposure | Melting Point Following No. of Days after Exposure 3 | 4 | 5 |
|---|---|---|---|---|
| Gel with: | | | | |
| 0.17% Safflower oil + 0.00074% Tocopherol acetate | 3 | 57 | 62 | 67 |
| 0.17% Safflower oil + 0.00074% Tocopherol acetate | 0 | 50 | 50 | 51 |
| No addition | 3 | 55 | 59 | 61 |
| 0.17% Safflower oil, No antioxidant | 3 | 69 | 75 | 83 |

It appeared further of interest to know whether the presence of tocopherol (vitamin E) would retard the process leading to cross-linkage of gelatin following irradiation.

Under the conditions of this experiment the cross-linkage of gelatin by safflower oil was markedly retarded, though not entirely inhibited, by addition of 0.4% of vitamin E calculated on the safflower oil.

## Discussion

The above experiments show clearly and consistently that the melting point of gelatin gels increases when irradiated in the presence of an unsaturated oil, specifically, linseed oil, safflower oil, methyl linoleate, methyl linolenate, and linoleic acid, but not when irradiated in the absence of unsaturates, even in the presence of saturated oils (stearic acid, coconut oil).

Other things being equal, the only explanation for the increase in melting point under these conditions is that cross-linkage has taken place. This was confirmed by light scattering measurements in the liquid phase. Precise determination was not possible because an increasing portion of the irradiated material was becoming insoluble and thus not accessible for the light scattering measurements. Even though a larger portion of the higher melting point gels had become insoluble in the glycerin employed as solvent, the remaining solute showed an increase in molecular weight. This confirms that an increase in molecular weight had accompanied the increase in melting point.

Gelatin is chemically as close an analogue of collagen as it is possible for a water soluble substance to be. With the unsatured fats normally present in skin, it would appear that the irradiation of skin by ultraviolet will cause a cross-linkage of collagen. And since the same reactive groups occur in all proteins and nucleic acids, irradiation will also cause the cross-linkage of all other proteins and nucleic acids present in the skin. This can possibly account for the major portion of the "aging" symptoms observed in skin exposed to actinic radiation.

Experiments 9 to 13 show that cross-linkage at a much slower rate will occur on irradiation even in the absence of the unsaturated fats. Experiments 1 and 11 show that saturated fats (stearic acid and coconut oil) are inert in regard to generating cross-linkages on irradiation.

Regarding the nature of the chemical reaction which causes the cross-linkages, Tappel (15) stresses the peroxide formation, which was postulated by Fahrion (16) as a mechanism for oil tannage. Unquestionably this is a factor, though possibly not the principal one. On oxidation of unsaturated acids, aldehydes are formed which are more dramatically rapid cross-linkers than the fatty peroxides (5, 17).

After a thorough analysis of the extensive literature of oil oxidation in the presence of proteins to effect tannage, supplemented by additional experimental work, Gustavson concluded that two main reactions occur in oil tannage (5). The first is a reaction between acrolein and aldehydes generally with the basic groups of the proteins; secondly, peroxides of the oils or of the fatty acids interact with the nonionic protein groups, probably the numerous peptide bonds. This may well prove to apply also to the reactions initiating the aging of skin, as both are catalyzed by ultraviolet radiation.

When kraft paper is oiled with linseed oil, for example, to make backing for hectograph rolls, many thousands of square yards of oiled paper are hung in loops or "festoons" from ceiling to floor in a large room to dry. The atmosphere in this room is almost unbreathable due to what is unmistakably a high concentration of lower aldehydes in the air. To make it possible for maintenance people to work in the room even for a short time, trays with ammonium carbonate are placed in the room for removal of aldehyde (16). It is thus evident that formation of lower aldehydes occurs readily as a result of the oxidation of unsaturated oils under conditions sufficiently similar to those of exposed skin to be applicable.

It is notable that in the experiments here reported, a brief exposure to ultraviolet resulted in a sustained progressive cross-linkage of the gelatin. This shows that the cross-linkage was not an effect of the ultraviolet directly, but of a cross-linking agent which had been formed by irradiation and continued its effect over a long period of time. The progress of this cross-linkage shows characteristics of low molecular weight aldehyde cross-linkage. Cross-linkage based on cross-linking agents having the molecular weight of fatty peroxides would have shown more signs of leveling out within 10 to 15 days. As previously pointed out (7), lower aldehyde tannage behaves like a truly catalytic reaction. With a few parts per million of formaldehyde, a gelatin gel like the one in the above experiments shows progressive cross-linkage which has been followed more than two years. After the melting point of the gel has passed the decomposition point, the further cross-linkage was followed by measurements of mechanical properties, particularly elasticity, tensile strength, and in the third year, crumbling (17). A possible explanation for this phenomenon might be that the very mobile formaldehyde molecule readily reaches any points where two protein molecules are sufficiently near each other for formation of cross-linkage and ties them together. After the two molecules have thus been brought into a more lasting fixation near each other, the probability of further contacts between them has been vastly increased. This leads to opportunities for direct cross-linkage, for example by interaction of an amino group of one molecule with a carboxyl group of another protein molecule or a phosphate or a carboxyl group of a nucleic acid molecule, as well as greater exposure to mobile molecules of cross-linking agents. In this fashion a multiplicity of covalent bonds could become established.

When the cross-linkage has thus been multiplied and formed, the somewhat hydrothermally unstable aldehyde bridge is ruptured and the aldehyde molecule freed to move on to a different cross-linking site. In this fashion a very small amount of a low molecular weight aldehyde, given enough time, can cause cross-linkage of an almost unlimited quantity of protein or nucleic acid material. In contradistinction, for example, metal or quinone cross-linking is not progressive so that the reaction ceases when the cross-linking agent has reacted once. It is believed that this would also be true of fatty peroxides, because of the low diffusivity of these molecules. *In vivo*, of course, the aldehyde would be oxidized further or otherwise metabolized within a quite limited time. The experiment with addition of tocopherol confirms that an oxidation reaction is involved.

If the aging effect of ultraviolet exposure of skin is due in any significant degree to products of light-activated oxidation of unsaturated fats in analogy with the model systems of the above experiments, then certain predictions can be made. Individuals or test animals which have a high percentage of unsaturated fat in the skin should be more susceptible to ultraviolet-induced aging than individuals or animals having either a low fat content or mainly saturated fats in the skin; and persons or animals with a high level of vitamin E in the skin should be more resistant to skin aging stimulated by actinic radiation than persons or animals deficient in this vitamin.

## Summary

As a model system to study effects of ultraviolet radiation films were cast of a gelatin-water-glycerin gel to which, in

some cases, were added emulsified unsaturated and saturated fats. These films were then exposed to shortwave ultraviolet radiation and to sunlight. Cross-linkage of the gelatin was measured by determination of changes in melting point of the gel. The cross-linkage was extensive when both radiation and unsaturated fats were present simultaneously. It progressed long after the initiating radiation had been discontinued. The antioxidant vitamin E (tocopherol) markedly inhibited this phenomenon, which is interpreted as evidence that the cross-linkage was caused by an oxidation product formed by shortwave radiation of unsaturated fats. Possible mechanisms are discussed.

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# Cross-Linking of Collagen in the Presence of Oxidizing Lipid

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## ABSTRACT

Gelatin films containing unsaturated lipid have been exposed to ultraviolet and visible irradiation. No sign of paramagnetism could be detected in the films, although the gelatin was undergoing cross-linking reactions. The addition of nitroxyl-forming radical scavengers decreased the rate of cross-linking, as did addition of ascorbic acid to the reacting mixture. Nitroxyls could not be detected in the gels, however. The conclusion is drawn that the main reaction in the cross-linking reaction of collagen is a condensation of amino groups and extrinsic or intrinsic carbonyl groups. The extrinsic aldehydes are formed in the autoxidation of unsaturated lipid.

An object of the present study is to increase our knowledge of the causative mechanisms of the fully demonstrated premature aging of human skin which has been excessively exposed to solar radiation.

On one hand, it has been postulated by Harman (1) and others that free radicals are primary causative factors in the aging process and that these are formed in the process of lipid oxidation (2,3).

On the other hand, doubt has been raised regarding the necessity for free radicals in this reaction. The free radicals observed by Roubal (3) would seem expectable from the enzyme reactions connected with the material used.

The reaction between oxidizing fat and protein was known in the hectograph industry prior to 1915 (4). Fahrion (5,6) postulated that the reactive groups involved in the cross-linkage reaction or "tannage" with oxidizing lipids are peroxide groups formed by the action of oxygen in air on the unsaturated fatty acids of the oil (7).

The formation of acrolein and other pungent compounds in the tanning process has been known to generations of chamois tanners (8). Procter (9), suggested that acrolein was the principal tanning agent. Indeed, acrolein is an effective cross-linkage agent. Salway (10) showed that acrolein is formed by the oxidation of free unsaturated fatty acids from linseed oil. Balfe, in his review (11), stated that acrolein is the major tanning agent in oil tanning. On this basis, Kuntzel and Nungesser (12) reinvestigated the matter. Cod liver oil, exposed to air oxidation in a Mackay tester, gave acrolein yields of 0.7-1.5% of the oil being oxidized under conditions corresponding to oil tanning. When hide powder was added to the oxidizing oil, no free acrolein could be found and the hide powder became cross-linked.

Farmer (13), has shown that fatty hydroperoxides alone can effect a cross-linkage in proteins. On the other hand, the cross-linkage of proteins in proteinaceous products containing unsaturated lipids has been ascribed to effects of free radicals by Desai and Tappel (14).

Okamura and Shirai (15) studied oil tanning by incubating preoxidized cod oil in hide powders. They conclude that oil tanning follows a complex course including the combination of peroxides of cod oil with hide. No electron spin resonance (ESR) investigation was undertaken, however.

In view of the fact that both aldehydes and peroxides can induce cross-linkage and damaging reactions in proteins, it was of interest whether free radicals can be observed with ESR spectroscopy while a demonstrable accelerated protein cross-linkage by oxidizing unsaturated lipid is in progress.

## EXPERIMENTAL PROCEDURES

Gelatin films containing 0, 0.17, 1, 5, and 10% of lipid, respectively, were prepared (16). As the gelatin component, pigskin gelatin (275 Bloom) was used. The lipid used was a commercially available corn oil containing 86% (w/v) unsaturated fat and free from added antioxidants. The antioxidant ascorbic acid (Pharmaceutical grade F) was dissolved in a small volume of water prior to addition. The ascorbic acid content of the gel was 0.00074%. The radical scavenger *tert.*-nitrosobutane was prepared as described (17), and the radical scavenger 2,4,6-tri-*tert.*-butyl nitrosobenzene was prepared by the procedure outlined (18). The scavenger was ground in a mortar together with the dry gelatin before the swelling procedure. The final concentration of the scavenger was 0.001 and 0.1%, respectively. The gels were stored before and after irradiation as previously described (16).

The gels were irradiated in a Rayonet Photochemical chamber reactor model RRR-100. The

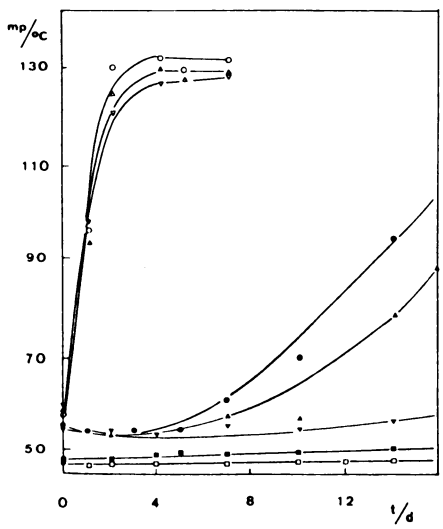


FIG. 1. Crosslinking of lipid containing collagen. Solid figures refer to samples irradiated 5 hr at 360 nm; empty figures refer to samples irradiated 60 min at 253 nm □, ■ no oil added, ▽, ▼ 1 % oil added, △, ▲ 5 % oil added, and ○, ● 10 % oil added.

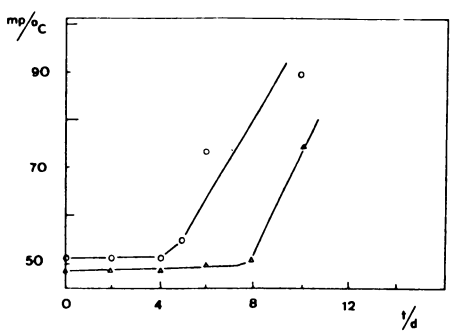


FIG. 2. Crosslinking of lipid containing collagen in presence of 7.4 x $10^{-4}$ % ascorbic acid. Irradiation 60 min at 253 nm., △ 5 % oil added, ○ 10 % oil added.

gelatin films were cooled during the irradiation. The films were exposed to both daylight (360 nm) and UV-light (263 nm) in different experiments. The experimental values represent the average of at least five measurements.

The cross-linking of the collagen was followed by measuring melting points from time to time. The melting points were determined in mineral oil as has been described by Bjorksten and Collbring (16).

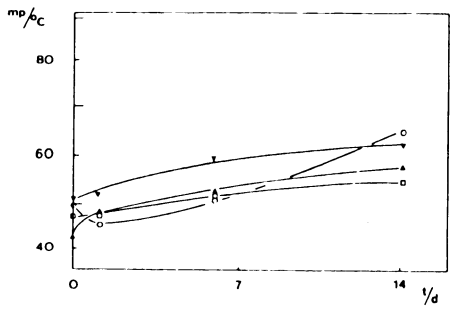


FIG. 3. Crosslinking of lipid containing collagen in presence of 7.4 x $10^{-4}$ % 2,4,6-tri-*tert.*-butylnitroso-benzene and 5 % oil (▼), 0.1 % 2,4,6-tri-*tert.*-butyl-nitroso-benzene and 5 % oil (▲), 0.1 % 2,4,6-tri-*tert.*-butyl-benzene (○), and no added compounds (□).

ESR measurements were performed on a Varian X-band E-3 spectrometer at temperatures ranging from +100 – -150 C with variation of all the measuring parameters. The gels were checked for paramagnetism before and after irradiation, and at time intervals equal to time between melting point measurements.

## RESULTS AND DISCUSSION

The change in melting points was taken as a measure of the rate of cross-linking in the lipid-containing gels (16). The results of these determinations are collected in Figures 1, 2, and 3.

As judged by the change in melting point, there is no mechanistic difference between the reactions initiated at 263 nm and 360 nm, respectively. This finding could be explained as follows: The photochemically active chromophore (in the lipid or in the collagen) has its absorption maximum in the ultraviolet region, with some absorption probability also in the visible region, due to tailing. The difference is seen in the velocity of the aging reaction of the gels (Fig. 1). After 60 min irradiation at 263 nm, the rise in melting point is from about 50 C to about 100 C in one day, whereas 5 hr irradiation at 360 nm hardly affects the melting point in one day. By using the short wavelength light for the irradiation of the gels, we hoped to reach a higher and measurable concentration of free radicals in the gels. This was not found, however, and every effort to detect an ESR signal in the system of lipid-containing gelatin was unsuccessful.

Experiments have been reported (16) in which the effect of an added antioxidant on the melting point of the gel was tested. It was shown that tocopherol efficiently retarded the

melting point raising reaction. Accordingly, the effect of ascorbic acid was tested. The results are collected in Figure 2. The concentration of ascorbic acid was kept sufficiently low, $< 10^{-3}$%, not to affect the pH of the gels. The addition of ascorbic acid quite evidently slows down the melting point raising reaction, after 60 min UV irradiation the melting point remains unaltered for 5 days.

The gels containing lipid and ascorbic acid were tested for paramagnetism at intervals equal to intervals between melting point determinations. No ESR signal could be detected.

Radical scavengers forming relatively stable paramagnetic products in systems where radicals are present have been developed. Among these nitroxyl-forming nitroso compounds or nitrones are commonly in use (22). The effect of the scavengers *tert.*-nitrosobutane (2-methyl-2-nitrosopropane) (17) and 2,4,6-tri-*tert.*-butylnitrosobenzene (18) on the reactions in the lipid-containing gels was tested.

In a preliminary experiment *tert.*-nitrosobutane was chosen as a scavenger. The choice was due to the high solubility of *tert.*-nitrosobutane in hydrophilic systems. The scavenger was carefully mixed in the gels after the irradiation to avoid its known photochemical degradation (19). The signal detected from the gels after this treatment was, however, shown to be due to di-*tert.*-butylnitroxyl.

Recently the use of the photochemically stable scavenger 2,4,6-tri-*tert.*-butylnitrosobenzene was reported (18) (note, however, ref. 20). The effect of this scavenger on the melting point raising reactions in the gels was similar to that of the antioxidants, tocopherol and ascorbic acid, but lasted over a longer period. The melting points of the gels remained unchanged within experimental error for 14 days. No sign of paramagnetism could be detected. The results of the measurements are collected in Figure 3.

The molecular reactions in the cross-linkage of collagen containing unsaturated lipid remain somewhat doubtful. Probably radical and condensation reactions take place in complicated sequences. It is possible that the irradiation, or an initially formed alkoxy- or alkylperoxy radical causes hydrogen abstraction in the peptide chain thereby causing intramolecular or intermolecular cross-linking between peptide chains. Reactions of these types are reported in the literature (21,22).

On the other hand, it has been pointed out that the normal sequence of events in the intramolecular cross-linkage of collagen appears to be: collagen molecules, which contain carbonyl groups, self-assemble into fibers which then become cross-linked because of the reactions that occur between the carbonyl groups and amino groups of adjoining amino acids. When aldehydes react with proteins, Schiff bases are commonly formed. Extrinsic, as well as intrinsic, aldehydes may cause this reaction (23). We, therefore, conclude that even if a free radical reaction might occur in the cross-linking reaction, the condensation between carbonyl compounds and amino groups is far more important in the cross-linking of autoxidizing lipid containing collagen. The effect of ascorbic acid or tocopherol to the lipid containing collagen as well as the radical scavengers *tert.*-nitrosobutane and 2,4,6-tri-*tert.*-butylnitrosobenzene could be the inhibition of the autoxidation of the lipid.

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Harnessing Free Radicals

This, and the papers presented on the immediate past pages indicate that free radicals play a definite part in the total aging syndrome, though not necessarily beyond the initiating step. On the other hand, free radicals when harnessed, can play a major part in the defense system of cells and/or tissues as shown in the next two papers.

Oxidation Reduction Depolymerization ("ORD") systems have been discussed and pertinent literature cited on pages
It appears that these systems can be developed to remove otherwise progressively damaging crosslinked aggregates.

# THE THEORETICAL BASE FOR THE ANTI-INFECTIVE FUNCTION OF ASCORBIC ACID

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Our knowledge of a principal biologic function of ascorbic acid stems from four lines of research. Originally far apart, these have but recently converged. It seems appropriate to trace these lines of work separately, before examining their confluence. They are :

I. The work on depolymerizing hyaluronic acid, later starches and celluloses, still later even some synthetic resin emulsions, by the use of ascorbic acid, a peroxide and a metallic catalyst.

II. The work on detoxifying encephalogenic protein coupled with data on inactivating enzymes depolymerizing polypeptides and proteins by the use of ascorbic acid, a peroxide and a metallic catalyst.

III. The work on the use of ascorbic acid in combating some infections.

IV. The work on establishing the mechanism whereby lymphocytes kill and obliterate bacteria and attack bodies recognized as extraneous or detrimental.

These are discussed below :

## I. The Oxidation-Reduction Depolymerization Reaction Applied to Carbohydrates

The depolymerization of hyaluronic acid was a desirable therapeutic goal, comprising efforts to facilitate subcutaneous injections by removing a principal impediment to spreading. Snake venom, by evolution designed to spread on injection, contains hyaluronidase - it was desired to achieve a similar effect.

B. Skanse and L. Sundblad[1] (1943) used an oxido-reductive depolymerization with ascorbic acid to depolymerize hyaluronic acid and chondroitin sulphuric acid.

W. Daubenmerkl, working also on improved spreading of injected material, broadened the range of reducing agents, with his observation that the depolymerization of hyaluronic acid could be effected by every enol-diene tried, which had a carbonyl group proximate to the double bond.[2,3,4]

W. Pigman and his co-workers studied in depth the reaction systems involved theoretically and practically, with a broad range of carbohydrates. They coined the term «ORD» reaction to describe the oxido-reductive depolymerization.[5] Their work was centered on ascorbic acid as the consumed reductant, either iron or copper[7] as the catalyst, and a broad range of carbohydrates, including starches, and cellulose, as the large molecular substance to be de-polymerized.[6,8,9].

The hydroxyl free radical was specifically implicated, and it was shown that depolymerization of the carbohydrates could be induced in separate electrode compartments, one holding the autoxidant (ascorbic acid) and the other holding the carbohydrate[10] :

$$O_2 + H^+ + e \rightarrow HO_2$$
$$HO^\circ + H^+ + e \rightarrow H_2O_2$$
$$H_2O_2 + H^+ + e \rightarrow H_2O + OH^\circ$$
$$OH^\circ + H^+ + e \rightarrow H_2O$$

The extensive work of Pigman et al. extended even to some methacrylate emulsions, which were also depolymerized.
This line of work is thoroughly reviewed in 1977 by A. Herp and T. Rickards.[11]

## II. Oxido-Reductive Depolymerization Applied to Nitrogenous Compounds and Found to Break Peptide Linkages

In the meanwhile, in 1967, C.W.M. Orr,[12] working with ascorbic acid found that the enzyme catalase was depolymerized following incubation with ascorbate particularly in the presence of copper as a catalyst. This was the first reference to an oxido-reductive depolymerization of a nitrogenous polymeric substance by means of ascorbic acid and a metallic oxido-reduction catalyst.

This concept was expanded in 1973 when Robinson et al.[13] in a study of human transferrin, a low molecular protein, found that ammonia was given off quite fast in an oxido reductive system, when ascorbic acid was present. In following this lead, F.C. Westall, M. Thompson and A.B. Robinson 1976 expanded it to study of the encephalitogenic protein[14] thoroughly explored by E. Roboz and co-workers. Westall et al. found this too to be depolymerized in aerobic ascorbic acid solutions.

Richheimer and Robinson[15] studied in 1977 the reaction mechanism, involving free radicals and clearly proved that the reaction involved

breakage of peptide linkages, and showed that this was the principal source of the ammonia evident during and after the reaction. Their conclusion, checked in several ways, was that the reaction was based on the formation of free hydroxyl radicals formed by a variant of the Fenton reaction, which in turn can be viewed as a variant of the Haber-Weiss reaction.

### III. Ascorbic Acid as Therapeutic Agent for Respiratory Infections

While the work I and II progressed along separate, often parallel paths, the application of ascorbic acid to increase resistance to infections proceeded further in the irregular patterns established since centuries. This has been described at intervals, those interested in history are referred to the excellent book of I. Stone, [18] and to the summarizing book by L. Pauling. [19]

The necessity for fresh vegetables to avoid scurvy was recognized by the Dutch as early as 1652, when Jan van Riebeek founded Cape Town so as to provide a harbor where the ships of the Dutch East India Co. could receive fresh vegetables in order to reduce scurvy. Without this, losses in scurvy on the long runs to Java would have been prohibitive. In 1703 after Russia had taken the Swedish fortress of Nyenskans at the site where Leningrad now stands, the Russian General Schaumburg and Governor Alexander Menschikoff, in dealing generously with Swedish prisoners of war coupled their release with a request that the Swedish Admiral please let pass through his blockade such Dutch ships as carried no contraband, and particularly stressed their anxiety to receive lemons and oranges. [20]

James Lind, of the British Navy proved the specific efficacy of citrus juice as a cure for scurvy by a controlled experiment in 1753, but it was not until 50 years later that the Admiralty adopted this measure, barely in time to make this a major contributor to Admiral Nelsons victories in the naval part of the Napoleonic wars around 1800.



None the less, 30,000 men died in the American Civil War from scurvy alone. As soon as it seemed that a firm base for widespread acceptance of citrus juice - later Vitamin C or ascorbic acid therapy was secure, someone somewhere, would run into trouble by gross misapplication. Opposition would get new wind in its sails and the cycle would repeat in some other setting.

The advent of bottle feeding of babies with consequent inactivation of Vitamin C by sterilizing led to the death of a vast number of babies.

Negative results with West Indian citrus strains or with boiled citrus marmelades led to disbelief; in fact in the race for the South Pole Scott did not bring along any citrus, and this cost him the race and his life. In World War One, based on observations in prison camps, the theory won credence that scurvy was an infectuos disease, transmitted by lice. Since a dose as low as 100 mg/day sufficed to cure even a bad case of scurvy, it was felt by a majority of the medical profession that Vitamin C was a specific for scurvy, and that the needed dose was no more than 100 mg.

In the meanwhile, the observations of many that the resistance to respiratory infections was increased, led to new studies, and in 1961-63 Ritzel made a well-planned, thorough, double blind, statistically validated study, [21,22] which conclusively proved that a very substantial reduction of respiratory infections resulted from a dose of 1 gram/day. This study was conclusive as to anti-infective efficacy and should have been accepted as such. It has been amply confirmed, as summarized most recently by Pauling. [19,23]

The failure of such acceptance to materialize appears to have been due to the inability of its critics to read the original German text, and to the lack of necessary detail in the English abstract.

A debate still continues in the literature and at meetings. Those opposing dosages in excess of what is needed to avoid scurvy keep on reporting tests based on too small quantities and/or improper spacing of dosages. Even the least founded negative report receives the same amount of publicity as the most thoroughly documented positive reports, so that those not having the time or the knowledge to analyse the growing pile of data objectively, become confused.

Among the positive reports, next after Ritzels conclusive study, are the studies of C. W. M. Wilson and his co-workers in Dublin, who have traced in detail the movements and transformations of newly ingested Vitamin C, in health and disease, and its great dependence on stresses, challenging, and hormonal balance. [24,25,26,27]

### IV. The Mechanism of the Lymphocyte Attack on Foreign Organisms or Molecules by «Respiratory Burst»

The fourth of the approaches separately and parallelly pursued, which leads to better understanding of underlying mechanisms, is the study of oxygen dependent killing and/or lysis by phagocytes.

In 1933 C. W. Baldridge and R. W. Gerard reported a powerfully enhanced oxygen consumption in phagocytoses. [28] Nungester and Ames found in 1948 that the phagocytes cease to function in scurvy. Ericsson and Lundbeck 1955 noted that highly bactericidal substances are formed when ascorbic acid and peroxide interact in the presence of $Cu^{++}$. [29]

The Baldridge report was confirmed by others. [31,32,34]

Sbarra and Karnovsky confirmed the enhanced oxygen consumption even in a nitrogen atmosphere, thus independently of mitochondrial respiration [70] Iyer, Islam and Quastel found that hydrogen peroxide was present around the stimulated phagocytes [71]

Babior, in two excellent papers [72] summarizes and clarifies the work focussed on the Respiratory Burst citing the several reactions proposed including the various forms of activated oxygen : $O^{-}_{2}$, $OH^{\circ}$, $^{1}O_{2}$, $XO^{-}$

This immediately leads the thought to the Haber-Weiss reaction [73] : $O_2^{-} + H_2O_2 \rightarrow OH^{\circ} + O_2$ which generates free hydroxyl radicals $OH^{\circ}$ by the catalytic action of iron salts, and to the Fenton reaction, [76] wich has played a considerable part in the theoretical considerations of groups I as well as II above.

The free hydroxyl radical has been recognized as an active agent in the Fenton reaction both by the groups working with carbohydrates (I above) and with proteins (II above) and it was demonstrated in 1977, [36, pp 397-398] that this system indeed breaks up the most refractory highly crosslinked compounds, which had remained immobil in the metabolism of a rat about two years beyond the time when these received a single massive injection of tritiated tyrosin or tritiated acetate and thus supplied the lytic force required for removal from the organism of gerogenic accretions. [37]

This even without knowledge then of the concurrent work from the different viewpoint of IV but with benefit of knowledge of the anti-infective potency of ascorbic acid, made it extremely probable that the bactericidal and lytic actions of lymphocytes are due to the generation of free hydroxyl radicals by a modification of the Haber-Weiss-Fenton reactions in which free hydroxyl radicals are generated oxido-reductively by oxidation of $Fe^{++}$ to $Fe^{+++}$ and coupled to regeneration of $Fe^{++}$ by ascorbic acid, which **is thus essential for the phagocytic action of the lymphocytes.**

This was clinched when H. Tauber and B.M. Babior in 1977 proved that free hydroxyl radicals are produced by human neutrophils. [38] S.J. Weiss P.K. Rustagi and A.F. Lo Buglio proved the same with human granulocytes. [39]

## Conclusion and Projection

Taking the Babior, Weiss and co-workers studies of the «respiratory burst» together with the definition of the same mechanism and the same effect along three different paths, with different media, in conjunction with Ritzels proof that ascorbic acid indeed greatly aids the organism in controlling infections and C.B.M. Wilson's, Loh's and their co-workers detailed investigations of the rate of consumption of ascorbic acid under conditions of lymphocyte activation, there is now no doubt left as to this being a principal function of ascorbic acid. In addition to its well-known vitamin functions it is the specialized **nutrient** which in case of lymphocyte stress is necessary to furnish the driving force for continuous production of free hydroxyl radicals - the principal weapon the lymphocytes use in eliminating undesirable organisms or molecules

The recognition of this fact enables us to draw the following conclusions :

1. Ascorbic acid is necessary ammunition for lymphocytes in fighting infections and other morbidity involving removal of organic matter.
2. Any estimate of the optimal requirement of ascorbic acid, which is based only on vitamin effects with regard to prevention of scurvy, or on any other single vitamin effect, grossly underestimates the actual optimum requirements in an organism fighting infection or immunologically recognizable abnormalities, including cancers.
3. The observations of actual consumption of ascorbic acid already proven in organisms fighting common colds, will be found to apply with equal force to any other condition which calls for phagocytosis by lymphocytes. This has so far been confirmed only in vitro with infantile paralysis virus, and with some cancers. It will apply generally because of the generality of the defensive mechanism described.
4. For full utilization of this ammunition it is necessary that the supply of catalytic metal be adequate. This certainly includes iron, partly also copper and possibly manganese.
5. This new understanding of the function of ascorbic acid in the lymphocyte metabolism, may sharpen the cutting edge of the immunologic therapies generally. [40,41]

April, 26, 1979,

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Antioxidants and Longevity

The field of antioxidants is far flung indeed. Its highest development has been achieved in the rubber industry. It is important also in perfume, paint and food industries, the pharmaceutical industry and academic research. A good summary of antioxidant chemistry generally is presented by K. U. Ingold (Chem. Reviews 61: 563-589 (1961).

The more narrow focus on antioxidants and their relation to longevity is presented in the three publications which follow.

# THE PRIMARY CAUSE OF THE HIGH MYOCARDIAC DEATH RATE IN EASTERN FINLAND DEFINED AS SELENIUM DEFICIENCY

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Life itself depends on unstable systems. The principal driving force is oxidation at normal body temperatures. Therefore, the living systems must unavoidably depend on many substances which are so easily oxidisable that some spontaneous, unscheduled auto-oxidation is unavoidable. [I1]

Such substances are for example, linoleic acid as well as other unsaturated fats, phosphatides, the isoprenoid chains in ubiquinones, the carotinoids including retinoid substances, and others. These will on exposure to any oxygen source at body temperatures undergo unwanted oxidations. These are a drain on oxygen, and will also form toxic substances among which the lower aldehydes are the most thoroughly documented. [P1, B2, B3, B4 pp 415 - 417, B5 p. 28, M1, M2, F2, F3, B6, R2] That acrolein and other pungent aldehydes are formed in autoxidation of unsaturated fatty oils, causing tannage (= crosslinkage) of proteins has been known to generations of chamois tanners. [G1, P3, S1, B1]

Peroxides can also function as crosslinkers, which implies long term potential hazards, although direct toxicity seems slight. [F1, F2, F3] Free radicals were used by Charlesby industrially in 1953 to crosslink high polymers [C1]. In protein and aging contexts they were stressed by Harman. [H4] No evidence of free radicals could be found[S2] during the crosslinking in vitro of a model system of gelatin and unsaturated lipids.

Light, which further promotes crosslinkages and polymerization is necessary for the natural synthesis of Vitamin D, which all mammals need, but the higher life expectancy of black persons above age 65 [U1] indicates the formation by light of deleterious systemically diffusible substance, which shortens overall longevity. In nature the unwanted oxidations are repressed by a system of antioxidants, of which the tocopherols (Vitamins E) and selenium are the cornerstones [T3, H1, H2, H7, B8, F7, F8, S9, C3, N2, R4] Ascorbic acid, lecithin, some phenolics such as tannic and gallic acid are useful synergists [H1, H3]. The ability of an antioxydant to substantially increase the anoxia tolerance of rats was demonstrated in 1945.[H5]

McCay, Sperling and Barnes (1943) [M3] showed that Vitamin E administered in the form of wheat germ oil, prolonged the life expectancy of female rats 21 %. This was statistically significant. The male rats showed a life prolongation of only 4 % which was not sufficient for conclusions, but the duration of their virility, as measured by sperm motility, was increased 500 %. Hickman states : [H2]

«As I view the longevity problem, it contains as a central question : How much can we have of the preservative factors without depressing active metabolism, making the organism as a whole lethargic», and Kaunitz [K2] is quoted as saying in the discussion following the Vitamin E Symposium in 1949 that it does not require much intelligence to realize that the antioxidants will extend life span by increasing control of oxidations.

This fact was rediscovered and re-labeled by Harman and Tappel in years following [H4, T1]. Machlin and co-workers [M4, M5] showed that this and related effects could be produced by all the anti-oxidants tried.

While the above work was done with tocopherols, similar effects were attained with selenium, which as an antioxidant on a weight basis is at least ten times more potent than the tocopherols. [R1, S5]. In countries where green plants are not an established part of the diet for the entire year, selenium carries the major part of the antioxidant burden, [M7, R1]. Tocopherol substantially increases tolerance to oxygen deprivation in rats.[H5] Analogous tests might provide a fairly rapid way of assessing the biological efficacy of various complex antioxidant systems.

The selenium content of soils and plants, even though not optimal, seems sufficient to avert flagrant deficiency diseases in most of the countries where the worlds agricultural production is centered : The American continents, [N1] Central Europe, much of the Soviet area [T2, S5], and Japan [Y1]. If any of the major powers had been as deprived as Finland or New Zealand, the awakening to the importance of Selenium would have taken place long ago.

The optimal percentage of selenium in human diets is still unknown. It will however exceed natural levels almost anywhere. The best data now relate to reindeer and racing horses. [K19, K9]. It has been estimated that levels of up to 1 - 2 ppm of nutrients might be useful for humans to counteract carcinogenesis. For rats selenite does not appear to be carcinogenic even in extreme dosages [S6], while selenate

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has been reported as carcinogenic in rats in dosage larger than any suggested nutritional or medical use. [C7]

The fact that selenical feed additives do not pose any carcinogen hazard for humans has been officially accepted [F9].

In those warm countries, where green vegetables are available a large part of the year, tocopherols are contained in the diet to an extent sufficient to mitigate some deficiencies in selenium. Research has established patterns of significantly reduced incidence of cardiac failures in locations where the selenium content is high. [S8, K9 pp 11-13]. High selenium content [Y1] could well be one of the several contributing factors which have made Japan the country with at present the highest longevity in the world. In myocardiac failure in humans, the area directly involved has shown a depressed selenium content in comparison with surrounding areas.

In addition to its importance as an antioxidant which it shares with the Vitamins E and C, it has a second function, as detoxicant for several deleterious metals with which it forms non-reactive compounds. [H6, M7, F4, G2], in particular cadmium [P4], mercury lead, silver and arsenic, as $Aso_2$.

The work of A. Tyler, [T4, T5] points to the potential of inactivation of deleterious trace metals for the prolongation of the life spans of sperm cells (judged by motility) including cattle and poultry. Most regrettably, this important work has not been continued after Tyler's untimely death. Tyler obtained up to 50 times increased life spans of sperm cells with all non-toxic chelating agents at his disposal. It would be interesting to know if this result could be matched using selenium as the metal inactivator since this may act on a principle different from chelation.

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In deep sea fish, selenium is found to increase at the same rate as mercury. This could be interpreted as evidence that only those fish survive otherwise lethal concentrations of mercury, which have coupled selenium with mercury to form non-reactive compounds. [G3, H7]. Similar effects have been found with copper [H7] and arsenic [H8].

Possible scavenging of aluminum with selenium has not been explored. In the light of the most recent work of Crapper, Karlik and de Boni [C2] further knowledge on any means to control aluminum long term effects would seem important.

The detoxicating effects could be one reason why selenium cannot be wholly replaced by other antioxidants. Another reason is that it is an essential component of the vital enzyme glutathione oxidase, which contains at least 2, probably 4 selenium atoms per enzyme molecule. [R3, H9]

The lowest selenium content in water, soil and crops is found in Finland, and in New Zealand. Finland also has the highest cardiac infarct rate in the world, followed by New Zealand.

To study this convincingly in humans, it would be necessary to take two large groups of humans, at least several hundred in each, controlling in detail their entire life pattern including diet, exercise, sleep, sex, alcohol intake and exposure to pollutants, such as tobacco and other sources of carbon monoxide, lead, mercury, soluble forms of aluminium, to mention only a few key points. All potentially important factors and clinical histories, including anomalies at birth and causes of death should be recorded and statistically analyzed. So far as possible, these patterns should be kept constant at least for about 1/3 of the normal life span, thus at least 20 years for each group.

Inasmuch as such tests are almost prohibitively expensive to arrange for humans, and time consuming, but are available for domestic animals, it is natural that knowledge of nutritional effects on health for cattle is ahead of our corresponding knowledge of humans.

The best opportunities for constructive observation of the relation between selenium deficiency and cardiac infarct exist in Finland, because there the cardiac infarct rate is the world's highest and the base selenium content in soil and water the worlds lowest (1/20 to 1/30 of the general levels elsewhere) so that it approaches a natural condition of dramatic deficiency of selenium. To provide many thousands of cattle reared on an equally selenium deficient diet would elsewhere require great expenditures.

New Zealand has a comparably low selenium content, but there the year round availability of green vegetables assures a reasonable supply of tocopherols, which at least in part supplies anti-oxidant needs. This could explain why New Zealand does have a somewhat lower incidence of cardiac infarct than Finland.

Since experimenting with humans on the necessary scale is impractical in Finland, and facilities for studies of selenium deprived cattle near ideal, the responsibility of utilizing the unique experimental conditions there with large domestic animals was shouldered by the veterinary profession.

From the above short, and therefore necessarily incomplete abstract, we have seen that numerous scholars in many countries have occupied themselves with selenium in nutrition. The overpowering realization of its importance, and its concern with humans have been sparked by Dr. P. Kurkela. Working with the reindeer in Lapland he was intimately familiar with every detail of their nutrition and behaviour in their natural habitat where they have abundant access to selenium.

The following table is condensed from the original paper [K12] wich contains full details and statistical confirmation $p < O_{.01}$.

Table 1 :

| | Selenium content of meat, on dry base in parts of Se per million |
|---|---|
| Reindeer, half wild, range animals | 2.2 ± 0.51 |
| Reindeer, transferred to Central Finland, Fenced | 0.34 ± 0.2[2] |
| Cows, from farms in Central Finland | 0.48 ± 0.41 |
| Cows, from Lapland | 0.43 ± 0.17 |

The high selenium value of reindeer in Lapland is due to a diet principally consisting of lichens Cladonia alpestris and Cladonia rangifer, which accumulate selenium.

The low selenium content of cows in Lapland is beceause the cows do not eat lichens.

The cows are however somewhat adapted to low selenium, but the reindeer are not. When now world demand for reindeer meat as a delicacy and for reindeer horns has created strong economic pressures for expansion, strong efforts were made to introduce reindeer in Central Finland. These ended in disaster.

The reindeer succumbed massively to syndromes known elsewhere in milder form, but practically unknown in Lapland. The lichens could not be exported from Lapland ; only a reindeer can collect them economically.

After the problem of Central Finnish nutrition had been worked out by selenium addition to the fodder, and Dr. Kurkela had a healthy herd of reindeer south of Lapland, attention could be directed to problems of cattle, swine and dairy operations in selenium poor areas. Selenium added to the feeds produced substantial improvement in muscular integrity and general health of the animals. The survival rate at all ages was improved. These improvements were most dramatic in the areas poorest in selenium.

In these regions humans were dying in their forties or fifties in myocardiac disease, with the highest incidence of myocardiac infarct in the world. The connection was inescapable to Dr. Kurkela, and he pointed it out in the cautious language of a veterinarian whose professional field does not include humans, p 19 of K9. However, even for a person not coming from Lapland, it was strange to see farmers in Finland commonly giving selenium supplement to their cattle with excellent results, but not to their own families.

The following tables speak for themselves :

Table 2 :

The Selenium Content mg/kg of Cereals in Typical Samples from Several Countries

| | |
|---|---|
| Rye Argentina | 0.071 |
| Rye Turkey | 0.059 |
| Rye USA | 0.080 |
| Rye Sweden | 0.006 |
| Rye Finland | 0.004 |
| Wheat Canada | 1.300 |
| Wheat USA | 0.368 - 0.703 |
| Wheat Argentina | 0.520 |
| Wheat Sweden | 0.007 - 0.022 |
| Wheat Finland | 0.004 - 0.085 |

(Sources : Kurkela, 1977, Lindberg, 1968, Oksanen & Sandholm 1970)

Muscle Se values agree with Map of Regional Se in Crops, A1, K3

Table 3 :

Swine, Breeders

Number born per sow/year ; averages from 10,000 breeders from the low selenium areas of Southern Ostrobotnia and Eastern Finland. Age of the breeders :in the range 3/4 to 5 years. The groups are matched.

**Group A :** Added 0.1 mg of 100 % Se in the form of selenite per 1000 g of feed.

**Group B :** Matched control group, same feed - nothing added.

Offspring per sow/year

| | No born alive | surviving 2 months | surviving 6 months |
|---|---|---|---|
| Group A. | 25 | 20 | 19,4 |
| Group B. | 18 | 9 | 4.5 |

Source, Kurkela ; P. 1979 K13

Table 4 :

Cows

Averages from 10,000 milking cows, 1calf/year for veal.

From low selenium area (Southern Ostrobotnia and Eastern Finland)

Group A. Selenium addition same as in Table 3.

Group B. Matched control group- same base feed as A, nothing added.

Average number of calfs per 100 cows

| | Born alive | Surviving 2 months | Surviving 6 months |
|---|---|---|---|
| Group A | 75 | 67 | 65 |
| Group B (control) | 74 | 54 | 44 |

Source : Kurkela, P. 1979 K13

Human myocardic Infarcts

Table 5 compares 2 pairs of matched counties, differing markedly only in selenium content of soil and water. The match between Alahärmä and Ylihärmä is particularly close, because

these are neighboring counties, having medical facilities in common. Cardiac infarct cases from both counties are diagnosed by the same staff and treated in the same hospital. The social structure of these counties are nearly identical. Isokyrö and Evijärvi are also counties differing in selenium content, but otherwise very similar. None of the commonly considered factors, tobacco- cholesterol- lipemia- stress- shows anything that could account for the great differences in infarct incidence.

Table 5 :

| | Population | Number of inhabitants per which one infarct occurred in 1977 | |
|---|---|---|---|
| | | In total population | In working-age population (15-64 years) |
| Group A<br>Counties having Selenium content in water supplies > 0.100 ppm | | | |
| Alahärmä | 7,000 | 725 | 1,673 |
| Isokyrö | 8,000 | 920 | 1,785 |
| Group B<br>Counties having Selenium content in water supplies < 0.05 ppm | | | |
| Ylihärmä | 6,000 | 222 | 224 |
| Evijärvi | 6,000 | 198 | 224 |

Sources : Infarct frequency : Savola, R. [S10]
Selenium content :Kurkela, P. [K13]

Data from larger units of comparison show the same basic differences, Lapland against S.E. Finland, or Vaasan Lääni against Northern Carelia show about a 1 : 2 ratio of infarcts across the board, with selenium content the only consistently present explanation. In the entire Northern Carelia the variance between the countries approximates 150 %. Taking averages, for men in the age range 30-64 the yearly infarct rate is one infarct for 81-91 persons. This is 1 ½ to 2 times greater than a corresponding averge in South West Finland. Helsinki or Lapland. This again conforms to the difference in selenium content in food and water supplies. No other explanation has been found.

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# OPTIMIZATION OF THE ANTIOXIDANT SYSTEM IN A DIETARY SUPPLEMENT

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## FOREWORD

Remarkable synergistic effects in the field of antioxidants were discussed by the pioneers in Vitamin E therapy and in the use of antioxidants in the food industry. Quoting from Hickman's 1949 paper on practical application of physiological antioxidants:[1]

> "It was Professor Mattill and his various pupils who showed us that an unprotected oil that might go rancid in an hour (in the Swift Tester) could be, for instance, protected for eight (8) hours with a-tocopherol, for fifty (50) hours with a-tocopherol plus phospholipid for two hundred (200) hours with a-tocopherol, phospholipid and ascorbic acid and for three hundred (300) hours with all the above plus tannins. Thus, was offered an insight into how nature stabilized vegetable fats for relative vast periods with meager supplies of antioxidants---"

We have been unable to find this precise combination in the literature, but as Mattill was the leading antioxidant researcher of his time and Hickman a highly reputed pioneer in antioxidant technology, we are giving weight to the above quote as a personal

[1]Hickman, K.C.D., "Practical Application of Physiological Antioxidants", pp. 114-123, Proceedings of Josiah Macy Conference on Physiological Antioxidants, New York, 1949.

communication from Mattill to Hickman. It seemed timely, therefore, to explore with more precise methods, the synergistic effects indicated by Mattill and Hickman in a system where an excess of phospholipids furnishes a medium for interaction and in the presence of a multiplicity of biological reagents available to living cells.

Particular importance is given here to the presence of phospholipid, because this component is present in all known lipoproteins and essential to the protection in transitu of sensitive biological materials. Selenium has been added as an extra component.

Therefore, to determine whether these continue to be synergistic or become antagonistic in the presence of other biologically desirable chemicals is the purpose of this investigation. A further purpose is to "fine tune" the dietary supplement formulation for optimum performance of the antioxidants at minimum concentrations in conjunction with unsaturated lipids.

## INTRODUCTION

Measurement of the assimilation of oxygen by the samples in a Warburg Respirometer is being used as a screening method. The best combination of antioxidants from each series run is used as a control in the next series so that a model goal for constant improvement is always present. The test conditions:

Manometer fluid: Tris-chlorethyl phosphate (sp. gr. 1.425)
Reaction flask capacities: 15 ml
Sample size: 2.0 g
Water bath temperature: 33°C
Atmosphere: Pure Oxygen
Agitation: 110 oscillations per minute

All lipids are catalyzed with $5 \times 10^{-6}$ M ferrous phthalcyanine.

Multiple tests were made on the same sample to determine reproducibility in the procedure. One series compared different runs in the same station on the Warburg and another, different stations on the same run. Pressure drops against time were plotted and zero pressures taken as failure times. See Figures 1 and 2.

Times to Zero Pressure (Hrs)

| | Station to Station | (Deviation) | Run to Run | (Deviation) |
|---|---|---|---|---|
| 1. | 40 | -0.50 | 46 | 0.67 |
| 2. | 43 | 2.50 | 43 | -2.33 |
| 3. | 43 | 3.50 | 47 | 1.67 |
| 4. | 36 | -4.50 | - | |
| | Ave. 40.50 | | Ave. 45.33 | |

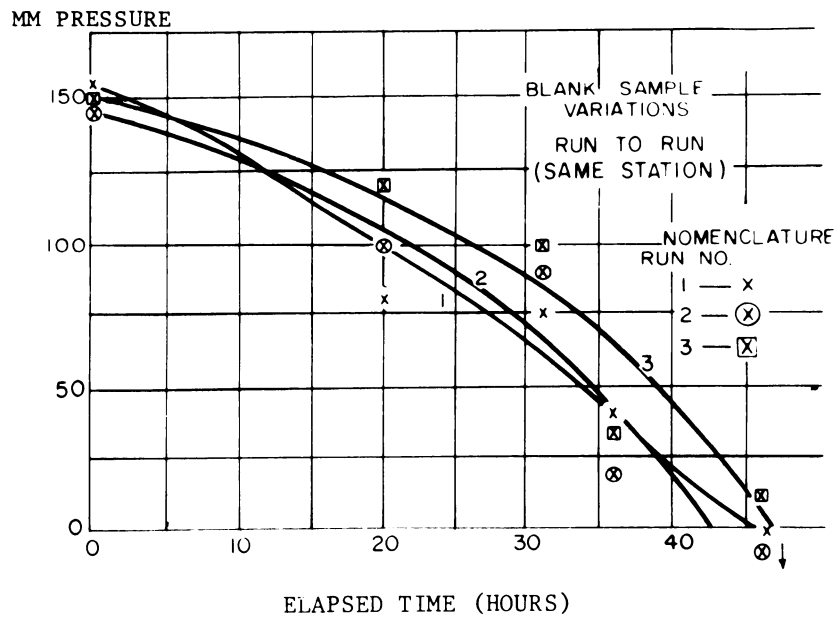


Figure 1. Comparison of different runs in the same station

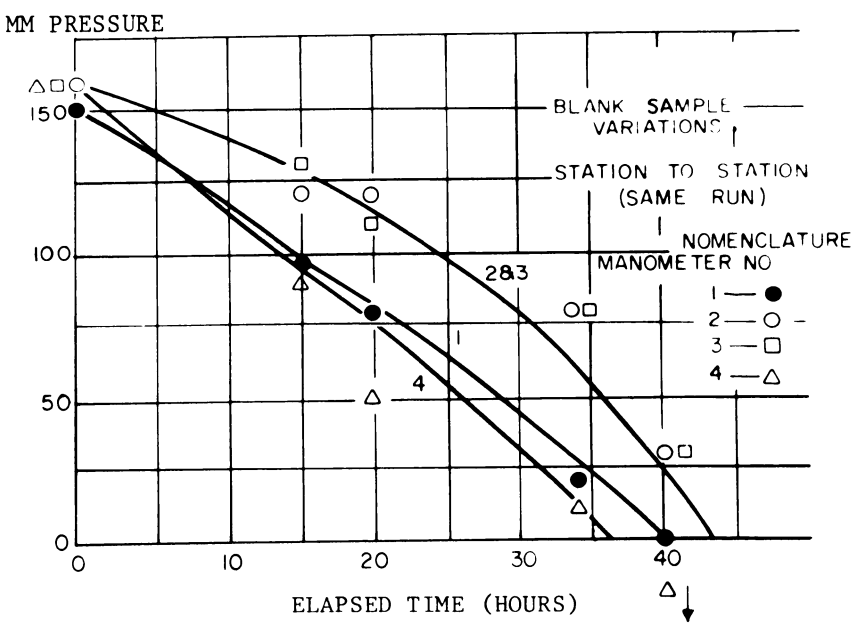


Figure 2. Comparison of different stations on the same run

The composition of the dietary supplement being used at present is:

Formulation GR 1358

| | Parts By Weight |
|---|---|
| Lecithin | 11,857.00 |
| Dextrose | 7,937.50 |
| Inositol hexanicotinate* | 1,184.90 |
| Vitamin E, 400 IU (commercial mixed tocopherols) | 473.36 |
| Ascorbic Acid | 295.47 |
| Vitamin A, 25,000 IU | 49.40 |
| Riboflavin | 13.84 |
| Pyrodoxine HCl | 16.70 |
| Thiamine $HNO_3$ | 13.74 |
| Calcium pantothenate | 24.70 |
| Vitamin B-12 | 4.94 |
| Folic acid | 4.94 |
| Biotin | 3.95 |
| Rutin | 19.67 |
| Butylated hydroxyanisole | 49.40 |
| Sodium hydrogen selenite | 0.40 |
| Water | 50.15 |
| TOTAL | 22,000.00 |

## ANTIOXIDANT SYSTEM IN A DIETARY SUPPLEMENT

The antioxidants content is:

| | |
|---|---|
| Ascorbic acid | 1.34% |
| Vitamin E | 2.15% |
| Butylated hydroxyanisole | 0.22% |
| Sodium Selenite | 0.0018% |

*Hydrolyzes to 1,000 parts niacin and 263 parts inositol.

EXPERIMENTAL DETAILS

Before oxidation tests were started with the supplement, evaluations of the antioxidants in its absence under the specified test conditions were made:

| Additives to Iron Catalyzed Linoleic Acid (Percentages) | Failure Time (Hours) |
|---|---|
| Vitamin E (0.25) + Sodium selenite (0.025) | 21 |
| Vitamin E (0.1) | 40 |
| Vitamin E (0.25) + lecithin (0.5) | 44 |
| Vitamin E (0.1) + lecithin (0.25) | 64 |
| Vitamin E (0.1) + ascorbic acid (0.1) | 70 |
| Vitamin E (0.1) + sodium selenite (0.025) | 70 |
| Vitamin E (0.1) + sodium selenite (0.025) + ascorbic acid (0.1) | 98 |
| Lecithin (0.25) + sodium selenite (0.025) | 46 |
| Lecithin (0.25) + ascorbic acid (0.1) | 70 |
| Sodium selenite (0.025) + ascorbic acid (0.1) | 59 |
| Ascorbic acid (0.1) | 70 |

A dilution to 10% of the dietary supplement with linoleic acid, which gave antioxidant concentrations approximately in the same range as the above tests, had a 150 hour failure time. This indicated that an entity of the other ingredients is strongly synergistic, and this synergism increases sharply with increasing concentration. Further tests at lower and higher loadings showed that the oxidation

resistance increases with supplement as indicated in Figure 3.

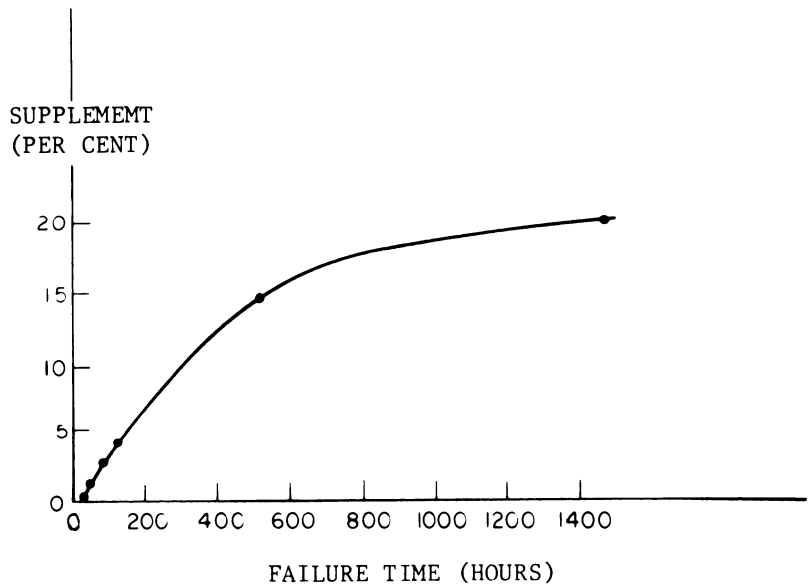


Figure 3. Increase of oxidation resistance with dietary supplement

The suggested daily intake of the formulation GR 1358 is 20 grams daily. In a conservative diet, this may be more than 20% of the unsaturated fats ingested the same day, so that the maximal protection figure of over 1,400 hours might apply.

| Percent Supplement in unsat'd fatty Acid | Failure Time (Hrs) |
|---|---|
| 0 | 12 |
| 1 | 24 |
| 2.5 | 48 |
| 5 | 110 |
| 10 | 150 |
| 15 | 560 |
| 20 | over 1,400 |

## CONCLUSION

The synergism of the blend of Mattill's antioxidants continues to be exhibited in the multiplicity of other ingredients present in the dietary supplement.

In protecting unsaturated fatty acids from spontaneous, non-enzymatic oxidation, a high degree of synergism exists not only by alpha tocopherol, ascorbic acid and lecithin as reported by Mattill and Hickman and with sodium selenite, but also between those anti-oxidants and the multiplicity of supplement ingredients in combination with each other.

## ACKNOWLEDGEMENTS

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DRIEMAANDELIJKS



# THE PLACE OF VITAMIN E IN THE QUEST FOR LONGEVITY

BY JOHAN BJORKSTEN, BJORKSTEN RESEARCH FOUNDATION, MADISON, WISCONSIN

**« As I view the longevity problem, it contains as a central question : How much can we have of the preservative factors without depressing active metabolism, making the organism as a whole lethargic ? »**
... K. C. D. HICKMAN, 1949.

CONTENTS

# THE PLACE OF VITAMIN E IN THE QUEST FOR LONGEVITY

BY JOHAN BJORKSTEN, BJORKSTEN RESEARCH FOUNDATION

## FOREWORD

An enormous amount of work has been done on Vitamin E since its discovery and study by Evans and co-workers (1922-1927). The conclusions as to dosages, and even utility, are poles apart.

This is largely because of preconceived opinions which interfere with judgment and objectivity.

I confess to a bias, but this is not related to Vitamin E : I am convinced that the long-term, major, permanent breakthrough in the preservation of vitality can only be gained by utilizing enzymes to cause lysis of the insoluble compounds which are formed in the course of advancing years. What can be hoped for of any vitamin therapy is only a delaying action. This, however, may be applicable now, and may give some of us the added years necessary to complete the larger and infinitely more tedious task of following the enzyme route to a happy conclusion.

I have written this review as an effort to determine from the welter of conflicting data, claims, and counterclaims just how much might be accomplished, and how Vitamin E can best be used to add some active and enjoyable years to the present human life span.

In order to keep this paper concise, it has been necessary to be brief in dealing with areas and findings which either have been adequately analyzed already, or which do not add directly to our knowledge of **longevity** effects, or which can best be treated as separate subjects in other papers.

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For example, I have only touched on syndromes such as encephalomalacia, muscular dystrophies, exudative diatheses, etc., as these are not closely enough connected with human longevity. The syndrome information has recently been updated and well summarized by Diehl (1974 pp. 334-342).

Marks has analyzed over 2,000 papers published before 1962 ; I am not repeating his unbiased and factual discussion excepting when modified or supported by later work directly connectable with longevity.

Selenium is an important as well as economical antioxidant, though its function only partly overlaps with that of Vitamin E. To deal with selenium at all adequately would expand the present article unduly ; also longevity data on selenium are not yet well developed.

## SUMMARY

Vitamin E is essential for well being, with optimum intake indicated to be in the range of 200-300 IU per day, proven at least in the case of intermittent claudication and peripheral sclerosis.

The much lower estimates of the official agencies of the USA and the USSR are based on determinations of the quantities required to avoid obvious deficiencies. Tests to determine optimum longevity are scarce and incomplete.

The much higher estimates of vitamin enthusiasts and the « orthomolecular » group of scientists are based on extrapolations, not taking sufficiently in account that the oxidation retardant properties of the tocopherols in the higher dosages will begin to impede desirable or necessary oxidations so that an intermediate dosage will be optimal.

Relevant literature is reviewed, with emphasis on specific clinical observations.

## INTRODUCTION

The broad acceptance of Vitamin E in therapy has been retarded for several reasons :

1. Due to the averaging action of the substantial storage capacity of tissues for Vitamin E, any effect will develop in a slow, undramatic way, which does not command attention, like scurvy, pellagra or beri-beri.

2. The claims made by enthusiasts, and the counterclaims, have led to exaggerations on both sides, which have too often obscured the real importance of Vitamin E.

3. Animal tests with longevity cannot be conducted at anywhere near the cost and time schedules which toxicologists or nutritionists are accustomed to. The cost of bringing an animal to senility increases logarithmically with time, as many animals die from various causes, the resistance declines as age progresses, and any carelessness could introduce artifacts.
   This has not been properly appreciated by the funding agencies who look with disfavor at requests for funding for animal tests at several times the projected costs per animal to which they are accustomed.

Accordingly, we do not have the guidance of well executed animal tests bearing on longevity with adequate facilities, but have to depend largely on clinical tests with humans. And here too we are handicapped by the fact that a physician seldom will consider giving a placebo to a group of patients when their lives are at stake and the question of malpractice might be raised.

An attempt will be made in the following to infer, from such data as are available, the extent to which Vitamin E may be used to favorably affect human longevity.

## THE OVERVIEW

To better understand the multiple and seemingly conflicting functions of Vitamin E which have a bearing on longevity, let us descend for a moment down on the evolutionary scale, and contemplate an unknown, very early life form, precursor to the monecellular green alga **Scenedesmus obliquus** (Fink et al., 1958) or the green chlorophyll-producing protozoon **Ochromonas malhamensis** (Green et al., 1959).

As an accidental mutation in the manipulation of the still relatively unaccustomed photosynthesis, this tiny organism in one of the shallow warm lakes millions of years ago stumbled onto making a microscopic quantity of Vitamin E. This gave it supremacy over other organisms in that it alone could control unwanted oxidations and thereby gain four crucial abilities :

1. To survive on lower oxygen concentrations than others ;
2. To get along with less protein than others ;
3. To minimize the formation of deleterious products from unwanted oxidations ;
4. To use biochemical pathways less accessible to others because of the oxidative instability of some of the intermediate products (Hickman, Harris, 1946).

The first of these points alone suffices to explain why the possessor of tocopherol was the survivor in the competition for oxygen in the last stages of the crowding of organisms in the primordial shallow warm seas eons ago ; and why it was the first organism capable of existing on land at a time when the earth's atmosphere contained but a minimum of oxygen, as is shown by the prevalence of iron in the reduced, bivalent state in the corresponding sedimentary layers. Today we cannot check this point with plants, because no green plant has survived which cannot make tocopherol for its own needs (Diehl 1974, p. 347 ; Green et al., 1959). However, Hove, Hickman and Harris (1945) showed impressively that a rat which receives tocopherol outlives a rat without it decisively under conditions of progressively diminishing oxygen supply.

The likelihood of multiple functions of reactive molecules present in an organism is aptly illustrated by Hickman's screwdriver analogy (Hickman, 1949-a, p. 102) :

« You buy a screwdriver for your house to drive a particular screw, but you extend its use to all kinds of screws. Eventually you cannot lay your hand upon it because it has strayed to your wife's sewing machine and later to the kitchen for opening a soup can, and finally it is bent by opening a jammed window. In short, wherever it can be employed, or even mis-used, there it will be put to service. And so it is with biochemically potent substances. The functions of each will be as many as its utility and versatility warrant. We tend to forget this essential fact, and when we have found one obvious function of a new metabolic agent such as a vitamin or enzyme we are apt to say : 'Well that's that' and look no further. »

This has been eminently true of Vitamin E. The complexity of its variants and functions has delayed understanding so greatly that at the present, 25 years after Hickman wrote the above, an enormous amount of argumentation has taken place, and millions of dollars used uneconomically to cover points which Hickman and his co-workers had made clear. Their documentation was quite sufficient to have made it possible to push on to more important tasks than seemingly interminable quibbling about points already clarified and sufficiently proven. The intelligent overall direction so much needed is still not in evidence.

## UNIQUENESS OF VITAMIN E

Vitamin E has two characteristics which set it apart from other vitamins, with possible partial exception of Vitamin A :

1. It is a requisite for all tissues and therefore is contained in these in so large a total quantity, that day-to-day, or even month-to-month fluctuations in intake are slow to become apparent and slow to disappear (Karl Mason, 1939-1945 ; Engel 1949 ; Quaife et al., 1949 ; Dju et al., 1958).
2. Vitamin E, though fat soluble, also functions as a water soluble vitamin in the form of a lipoprotein, in synergism with lecithin and other phospholipids (Hickman, Harris, 1946, p. 477).

No single dramatically apparent deficiency disease facilitates testing. The best tests now are based on variations of enzyme activities, a sophisticated approach not available in the early years (Gallo-Torres, 1972 ; Hyldgaard-Jensen et al., 1969 ; Boyd, 1968). Prior to these developments time lags up to a couple years could be necessary to determine deficiencies ; the deprivation symptoms are slow to develop and to disappear. The reason for this is that Vitamin E is present in the human body in much greater quantities than any other vitamin. Quaife and Dju (1948) reported that the total tissue mass of an adult female contained an average of 8130 mg and an adult male 3440 mg. Thus the tissues hold a store which is slowly depleted, and equally slowly re-built. If the supply falls, say, 10 mg/day short of what is needed to avoid severe symptoms, a person may not develop marked deficiency symptoms for periods as long as a year, before the deficiency becomes obvious. When the intake level is increased, even dramatically, the cure can be long delayed because of averaging with the substantial store retained by the tissues. This has been confirmed repeatedly, including Dinning and Day's 1957 extensive work with Rhesus monkeys, which ties in with the clinical results.

This slowness of dramatic response to changes delayed the discovery of Vitamin E, and is still delaying the full recognition of its importance. Mattill and Conklin reported already in 1920 that rats fed only on milk grew slowly, and did not reproduce. If Mattill had recognized the long time period necessary for a positive response, he would have found before Evans (1922) that continuation

of the wheat germ feeding which he and his collaborators used, would have brought about a complete cure (Mattill, 1924). Evans et al. isolated the substance responsible for sterility in milk fed rats and showed it to be a new fat-soluble vitamin. In 1936 Evans et al. named this « Tocopherol » from the Greek « tokos », birth, and « pherein », to carry.

The sterility in rats caused by lack of Vitamin E is due to resorption of the fetus beginning the 8th day of pregnancy. There is no difficulty with the conception or with the early development of the fetus (Evans and Burr,1927). Mason et al. (1947) ascribe this resorption to primary damage to the blood vessels, due to Vitamin E deficiency, and this view was essentially supported by Adamstone. The development of immunology may lead to a different interpretation. Some sort of antigen barrier exists between maternal and fetal tissues (Billingham, 1964). Pericellular sialomucins may present an electrochemical barrier to immunologically competent cells (Currie and Bagshawe, 1974). In the absence of Vitamin E, the desired negative charges may not be maintained so that the protective layer fails te function (Krul, 1974). Exploration of the various immunologic aspects is proceeding (Walford, 1969 ; Tengerdy, Heinzerling, 1972 ; Adler, 1974).

Apart from the antioxidant-based properties stressed above, Vitamin E has many unrelated functions. These are apparent from the enzyme assay methods referred to on page 8. Porphyrin and heme synthe ses have been shown to be Vitamin E related activities totally separated from the anti-oxidant effects (Nair et al., 1971, 1972 ; Green et al., 1959 ; Cawthorne et al. 1968 ; Green, 1972 ; Diehl, 1974, p. 367).

The chemical structure of alpha tocopherol was correctly deduced by Fernholz (1938) and proven by Karrer et al. by synthesis in the same year.

## 40 LONGEVITY RELATED EFFECTS OF VITAMIN E

The literature is replete with references to specific syndromes or symptoms associated with Vitamin E deficiency, but when we come to longevity, there is a scarcity of data.

Longevity tests, by their very nature, are time-consuming and expensive. It is ever so much more convenient to test only for some one characteristic, such as any one of the syndromes, changes in the level of an enzyme or of the content or excretion of some one substance.

Yet, while in the aggregate this type of data may point in one or another direction and furnish material for the theorizing, actual longevity tests are the only solid base to build on when longevity is our target.

To be meaningful in this context, animal tests should meet these conditions :

1. The control animals in the study must live as long as a healthy strain of the animals under optimal conditions. This means at least 3 years mean longevity for rats and more than 2 years for mice.
2. The animals must have exercise facilities. Animals in narrow cages without exercise facilities live in artificial conditions, and longevity results using such animals are clouded.
3. The causes of death must be reported and show the same general distribution of causes of death as occur in a sampling of the random deaths under average conditions.

To illustrate points 2 and 3, it is well known that if rodents are caged without adequate opportunity to use their front teeth, these will grow to the point that the animal cannot chew. It will then die prematurely from starvation.

If someone were ignorant enough to take this as an accelerated test for aging, he might find that the addition of hard wooden pieces to the diet would increase the longevity at least 300 % ! He could even go further and theorize that this is due to the entrapment of free radicals in the wooden pieces, or in products formed from these in chewing.

Without opportunity to exercise, the large muscles of the rat atrophy dramatically over a life time of 3 to 5 years. Other more subtle changes are likely to occur, which are artifacts more difficult to detect but no less damaging. The requirements above would add cost to the tests, but are a safeguard against erroneous conclusions which can lead to far greater losses in money, which is regrettable, and in time, which is inexcusable. Control and analysis of the causes of death would provide a safeguard against conclusions based on effects actually due to some single cause, which might not be age-related.

None of the reports of longevity effects of Vitamin E on mammals meets the above standards.

Two studies seem sufficiently indicative to be recognized in the present context, the McCay et al. (1943) study which shows the extension of life by administering Vitamin E, and the Tappel study (1973) which shows a reduction of lifespan following quite high dosages.

McCay et al. administered wheat germ oil, which for male rats increased sperm motility from 150 days to $>$ 750 days, but only extended life span for the males 4 % which McCay views as not significant. The females, on the other hand, gained 21 % in longevity.

McCay's observation on increased spermatogenicity is supported by Adamstone's finding (1941) that castrated cockerels require less testosterone when Vitamin E supply is high.

Hickman concluded (1949-a, p. 101) : « As I view the longevity problem, it contains as a central question, ' How much can we have of the preservative factors without depressing active metabolism, making the organism as a whole lethargic ? ' » Vitamin E was foremost of the preservative factors to which Hickman refers.

This touches the core of the longevity problem in two ways :

1. It shows the error of those tests for Vitamin E requirements which are based on establishing a maintenance dose and then backing away from it by reducing input of Vitamin E until the deficiency symptoms appear. Such methods were used in establishing the officially recommended dosages of Vitamin E both in the USA and the USSR, and were used even by Jager in 1973. The proper test would be to establish a safe maximum level, and then backing away from it until the longevity of the animals declines, thus determining by direct test the optimum dosage for longevity.

2. The problem of the balance between preservation and lethargy was focused by Hickman (1949-b, p. 115 and 116) as follows :

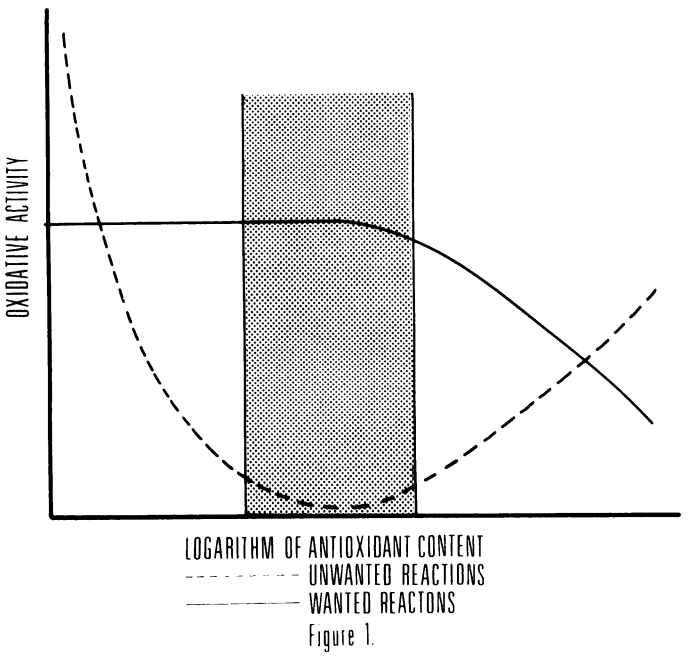


Figure 1.

« The problem for the nutritionist and medical man is to recognize the middle region, between the vertical lines of figure 1. »

As this is a multiple factor problem, the most direct approach is to use the longevity assay as criterion. This is a slow procedure, but it could have been carried through for a fraction of the scientific man hours which have been largely dissipated on tangential diversions.

The wanted reactions (Hickman and Harris, 1946, p. 505) are « preservation of labile substances from accidental oxidation and repression of formation of ketones, aldehydes and acids by random reactions. Anaerobic bacteria and certain viruses are encouraged. «

Unwanted effects of Vitamin E include the inhibition of the oxidation reactions which destroy carcinogens, such as 3-4 benzopyrene (Hickman, Harris, 1946, pp. 506-507) and retardation of the oxidation of aldehydes and other potential cross-linkers to innocuous products. Further, a greatly excessive amount of antioxidant may « make the organism as a whole lethargic » by extending the oxidation retardation to some of the vital energy producing processes (Hickman, 1946).

In addition, poliomyelitis virus (Thiele's virus) was shown to survive passage to the stools only in the presence of extra tocopherols (Hickman 1949-a, p. 120). That such protection of pathogenic viruses is a more general phenomenon, appears plausible, but is not known.

Even alpha-tocopherol itself may be subject to random oxidations, such as the formation of dimers and trimers by oxidative condensation in quantities not consistent with participation in quantitatively important pathways. (Strauch, et al., 1969).

Dinning (1962) deduces from work with radioactive formate and thymidin that DNA synthesis is sharply increased in Vitamin E deficient monkeys and rabbits. Since this important synthesis is thus sensitive to tocopherol inhibition, should we suspect that an excessive amount of Vitamin E might slow down the DNA synthesis to a point where necessary replacement or repair functions would be seriously impaired ? This has not yet been checked adequately.

To the above, I should like to add :

As a wanted function : To suppress random oxidation of lipids to form aldehydes and other oxidation products which react with proteins or nucleic acids to generate immobile crosslinked aggregates (Fahrion, 1891, 1903 ; Procter, 1904 ; Bjorksten, 1941 ; Milch et al., 1963 ; Milch, 1964). And as an unwanted function : The suppression of oxidation reactions capable of rendering inno-

cuous the aldehydes formed, and the oxidative degradation of such mildly crosslinked substances as are not yet sterically hindered but would become inaccessible if crosslinkage density were to progress further (Bjorksten, 1974).

## CLINICALLY BASED LONGEVITY RELATED OBSERVATIONS

Lacking more dependable animal based data on longevity, we must turn to the clinical material. This is extensive and diverse.

Marks (1962) made an excellent effort to winnow clarity from over 2,000 papers on therapeutic effects of Vitamin E. The literature has increased substantially since then. While much potentially valuable detail data has been gathered, the overview has suffered from the duplication. A vast number of publications, not all of the highest quality, has beclouded the main issues.

The habit of some authors to omit references to work more than 10 years old has encouraged needless duplication and has intensified the fog which the activities of many casual contributors has spread over this field.

In reviewing longevity related information on Vitamin E therapy which can be gleaned from clinical work to date, we must separate proven facts from inferences which are not completely proven but yet have a high degree of credibility and in the aggregate must be given weight.

John Marks (1962) in his critical appraisal of the therapeutic utility of alpha tocopherol, accepts the evidence of increased life expectancy in patients with severe sclerotic disturbances of the circulation in the lowel extremities (Intermittent Claudication). He refers in particular to Boyd et al. (1949) who worked with claudication patients receiving 400 mg/day of synthetic dl-alpha tocopherol for at least 3 months. The results were encouraging enough and confirmed by others (Marks l.c. p. 578). Livingston and Jones (1958) undertook a double blind control study on tocopherol in intermittent claudication. The daily dose was 600 mg and treatment was continued 40 weeks. In a group of 40 including the matched controls, 13 showed improvement on treatment while only 3 of those receiving placebo were improved. By the Fisher and Yates modification of the $x^2$ test, this is significant ($P < 0.001$).
Marks reviews four controlled trials on claudication in which alpha tocopherol gave positive results when used in doses of 400 mg/day or more for at least 12 weeks, and one which showed no improvement. The last mentioned (Hamilton et al., 1953), however, received the Vitamin E in the form of wheat germ oil, in which the estimation of alpha tocopherol present is open to question. The nitrosation method for estimating Vitamin E has subsequently been proven unreliable. Ward (1961) found that a germ oil which tested 667 microgram/gram by the old method, had a true value of only 56 micrograms/gram.



Having shown a definite beneficial effect in a sclerosis related condition, it was natural that attention was directed to the obvious question : Is the life expectancy of the claudication patients also affected by the tocopherol treatment ? It seems reasonable to assume that the sclerosis in many of these cases would extend to vital organs.

The results of reports reviewed by Marks are :

Boyd, Bloor (1959) and Bloor (1961) treated 1508 patients in the Manchester Clinic between 1947 and 1953, and 1476 could be tracked. They observed some improvement of the walking distance in intermittent claudication after a daily dose of 200 mg of alpha tocopherol, but significantly better effect after at least 3 months of treatment with 400 mg daily.

They show 88 % survival after 3 years and 76 % after 5 years, while with other therapies the best reported results, Richards (1957) was 71.7 %, alive after 5 years and the worst, Hines and Baker (1940) with 45.5 % alive after 3 years. Six additional studies cited by Marks gave a fairly even scatter between these latter values, thus all were well below the survival rates of those with Vitamin E therapy.

However, the above survival data had the weakness of being based on data from different institutions, without placebos. At the time of Marks' survey no studies using placebos had been made.

Such a study was finally undertaken in 1968 by Haeger, who used as placebo ordinary multivitamin preparations not containing Vitamin E. Initially the dosage was 600 mg of Vitamin E daily, but as it was proved that the same serum concentration of tocopherol was reached for a similar period of time with 300 mg the latter dose was subsequently administered in all cases. The minimum of observation was 2 years ; in 69.2 % of all cases the period of observation exceeded 3 years.

During the course of the investigation 28 patients died, leaving 199 patients for the complete follow-up study. The total survival rate was 88 %. In all groups not receiving tocopherol 19 patients of 123, or 15.5 % died, and in the tocopherol group the death rate was 9 out of 104 patients, or 8.7 %. Evaluating his data very cautiously, Haeger did not consider this difference sufficient yet for a firm conclusion, though it appears at least indicative.

Statistically unquestionable results were obtained with regard to improvement in walking distance until onset of pain, where the difference was significant at the 1 % level, and in the amputation rate.

The groups were matched as well as possible, using angiographic criteria, 39 patients received vasodilators, 44 antiprothrombin, 42 multivitamins excluding Vitamin E, and 104 received 300 mg/day alpha tocopherol. Altogether 12 legs had to be amputated due to intractable pain and/or gangrene. Of these 4 cases were in the vasodilator group, 2 in the antiprothrombin group, 5 in the multivitamin group and only 1 in the tocopherol group, although this group was larger than any two of the others together. The respective percentages were 1.1 % in the tocopherol group and 10.6 %

in all of other groups combined. The difference is significant at the 1 % level.

The efficacy of tocopherol in this type of sclerosis is established beyond doubt. The effect on overall longevity seems probable. In situations involving human lives in studies requiring many years, it is difficult to find human subjects willing to expose themselves to the limitations of a placebo, with life at stake, and the physicians might risk malpractice suits if they persisted in administering placebo up to the point of death in the face of strong evidence indicating the efficacy of a certain therapy.

The reason for the effects discussed above, could be oxygen sparing effects, or pharmacodynamic effects on the circulation.

## OXYGEN SPARING EFFECTS

The proven oxygen sparing effect of Vitamin E, was clearly demonstrated by Hove, Hickman and Harris (1945). These authors exposed rats and guinea pigs to anoxia, as the air pressure was reduced gradually to 185 mm. mercury. The reduction was carried out over a time span of 15 minutes, after which low pressure was maintained. Natural mixed tocopherols given orally to adult rats resulted in a survival 89 % higher than that of the controls on a Vitamin E-free, low fat diet. The effectiveness of Vitamin E was decreased to 31 % when the diet contained fat on the 12 % level.

Injection of large single doses of d-alpha tocopheryl phosphate (80 mg, 18 hours before the decompression) increased the survival time of guinea pigs 330 % over the controls, while the effect on rats of the single large injection was, if anything, slightly negative. The guinea pig controls had been receiving rabbit chow, of a Vitamin E content adequate for maintenance. This oxygen sparing effect of Vitamin E was confirmed under different conditions and different techniques by Telford et al., 1954 ; Degenhardt, 1959 ; Prokap, 1960 ; Kamimura et al., 1962 ; Alexeev and Tkachenko, 1965. It should be useful not only in therapy, but also for skin divers.

A similar oxygen sparing effect has been obtained by the use of other antioxidants, but d-alpha tocopherol is unique among these in that it is not separated from the substances it is protecting in the various transit steps in the organism (Hickman, 1949-b). It is noteworthy that **delta** tocopherol has the highest in vitro antioxidant potency in the entire Vitamin E complex (Olcott, Emerson, 1937) although its biopotency is the lowest, 0.02 I.U./mg (Jager, 1973). This might be explained by differences in the in vivo transit (Hickman, 1949).

The favorable effect of Vitamin E in sclerotics suffering from peripheral circulatory problems is thus understandable. Even if Vitamin E had no effect on the circulation, the oxygen sparing effect in the muscles is considerable, so that the patient can get along with less oxygen.

This principle is general. Where anoxia is the immediate hazard, death can be postponed by an augmented supply of Vitamin E. With guinea pigs this could be done with a single large injection ; for rats only with a gradual build-up by increasing the oral intake (Hove et al., 1945). For humans, we do not yet have the comparable information. If they react as guinea pigs do, a single injection of d-alpha tocopheryl phosphate might be useful as an emergency measure when oxygen is not available.

Relatively high Vitamin E dosage not only reduces oxygen requirements, but also protects against hyperbaric oxygen (Taylor, 1956). This latter effect is probably due to the suppression of non enzymic reactions which led to the formation of toxic oxidation products, including particularly aldehydes, and quinones, secondarily peroxides, free radicals etc. The suppression of unwanted random oxidation is a common denominator in Vitamin E action in hyperbaric as in hypobaric oxygen.

2. However, this may not be the whole story. Roth et al. (1968) have shown that a high dosage of Vitamin E effectively counteracts the age-dependent reduction in liquid exchange between capillaries and tissues. This points to a concurrent pharmacodynamic effect on the capillary blood supply, or possibly to reduced adherence between blood corpuscles (Knisely, 1965 ; Riddick, 1968).

## PROTEIN SPARING EFFECTS

The protein sparing effects of Vitamin E could be predicted on the basis of the oxygen sparing effect. With a limited protein supply, it is obviously advantageous to eliminate unwanted random reactions. The sparing effect was confirmed in many studies (Dam, 1944 ; Victor and Pappenheimer, 1945 ; McCoord et al., 1947 ; Sos et al., 1950).

The unwanted random reactions not only take their toll of protein, but also lead to the formation of highly toxic aldehydes (Procter, 1904 ; Salway, 1919 ; Küntzel and Nungesser, 1951). They also lead to the formation of peroxides. (Fahrion, 1891, 1903 ; Pedersen and Glavind, 1952 ; Witting and co-workers, 1964-1972). Both aldehydes and peroxides contribute to the crosslinkage of proteins (Gustavson, 1956, p. 296 ; Okamura and Shirai, 1974). It is to be remembered, however, that many of the aldehydes thus formed are highly toxic and have been shown to simulate degenerative disease on injection in mammals (Milch et al., 1963 ; Milch, 1964), while doses of lipid peroxides up to 1000 times the lethal dosage calculated from the radiation hypothesis produced neither damage nor Vitamin E deficiency (Glavind, 1967). This shifts the emphasis to the aldehydes formed by oxidation of unsaturated fats and advanced as important factors in aging by Bjorksten (1941, 1942, 1974).

Inasmuch as protein is a limiting factor in the food supply of a great part of humanity, the protein-sparing efficacy of Vitamin E may yet gain considerable humanitarian and economic significance and increase the average longevity particularly where low protein diet is the rule.

Detailed mechanisms of the complex reactions involved in the protein sparing have been reviewed by Diehl (1974), who has also contributed to our

knowledge in this area (Diehl, Diehl et al., 1962-74). A discrepancy exists between the increasing amino acid accumulation in Vitamin E deficient muscles, and the simultaneously reduced capacity of these muscles to maintain a sufficient creatine concentration. A possible explanation might be that molecules are thus withdrawn, and the cell strives to replace these. This activates mechanisms for replenishing amino acids, which, however, cannot be used because of crosslinkage induced blockages of the synthetic mechanism.

## LIPOFUSCINS

Lipofuscins were first observed by Eisig (1887).

Heidenreich and Siebert (1955, p. 122) define « Lipofuscin » as any pigmentous fluorescent material accumulating in tissues. Thus the term « Lipofuscin » includes the practically identical « Ceroid » and « Vitamin E deficiency pigments ».

The analytical findings on these are compatible with the view that the insoluble parts of these pigments are highly crosslinked aggregations, the compositions of which vary in detail but which in the main are proteinaceous conglomerations with inclusion of lipids, nucleic acids, in fact, anything macromolecular and reactive that can be tied to anything else by means of crosslinking agents of any kind from any source.

We are concerned principally with these insoluble parts of the lipofuscin granules. The « amyloids » are viewed as essentially precipitates formed by immunologic crosslinking between large molecules as suggested by Walford (1964). The lipofuscins owe at least much of their color to beta carotene and heme compounds (Björkerud, 1964). It is noteworthy that a gelatin-glycerin gel applied in .04" layer to a linseed oil coated sheet of paper, during two years exposure to the atmosphere will gradually develop a similar brownish color, concurrently with the insolubilization and embrittlement of the film.



Much of the crosslinkage is caused by aldehydes and other products resulting from oxidation of unsaturated lipids in the presence of amino group containing macromolecules, as described by Isaacs (1951), discussed in the context of aging by Bjorksten (1941, 1942, 1951, etc., and summarized in 1974). Tappel (1962), Desai, Roubal and co-workers, interpreted this in the light of free radical reactions (Harman et al., 1966 ; Harman, 1968, 1971). The free radicals are powerful crosslinking agents (Charlesby, 1953).

Of the various crosslinkers formed by random oxidation reactions, the aldehydes appear to be the most important. Probably all of the possible reactions occur concurrently (Gustavson, 1956).

In vitro, the crosslinkage which results from oxidation under irradiation of unsaturated lipids in the presence of amino or/and amido groups containing macromolecules continues to progress after discontinuation of irradiation. Electron spin methods sensitive to $10^{-14}$ fail to show any signal of paramagnetism in the samples (Sundho'm et al. 1975) The electron resonance signals detected from frozen fish tissues (Roubal 1970) are probably due to enzyme generated free radicals. Jebsen (1974) shows that a formaldehyde generating enzyme action takes place in fish tissues. (cod.).

The lipofuscin pigments fluoresce, and have therefore attracted attention out of proportion to their real significance. The important property is not the fluorescence, but rather the insolubility and the resistance to enzyme action. The insoluble lipofuscin components are one of the many crosslinked, relatively refractory aggregates. The majority of such crosslinked aggregates, however, do not fluoresce, but may be identified by solubility differences (Medvedeva, 1939 ; Bjorksten et al., 1960, 1962) or still more conclusively, by electron microscopy (Acharya, 1975). This invisible majority of crosslinked aggregates is a factor in aging ((Bjorksten, 1941, 1942, 1974). The formation of crosslinkages, to the extent that these are caused by lipid oxidation products, will obviously be retarded by an adequate supply of antioxidants.

## HEMOLYSIS

Horwitt (1960) maintained a group of neurological patients 8 years on a daily Vitamin E intake of only 3 mg/day and found increased hemolysis index and shortened life span of the erythrocytes. The life spans of the patients were not reported.

A macrocytic anemia, very similar to the one reported by Dinning and Day in monkeys (1957, 1958) was observed in a Jordanian fugitive camp. Vitamin E therapy proved of value (Majaj et al., 1963). In this case other deficiencies, as well as protein deficiency, were present ; however, the therapeutic efficiency of Vitamin E was clearly established along with the other corrective steps.

## LATERAL SCLEROSIS AND MUSCULAR ATROPHY

By 1946 the possible implication of Vitamin E was surmised by the medical profession in certain rheumatic conditions and muscular diseases. One of these was amyotrophic lateral sclerosis in which the normal nerve tissue of the anterior horn cells in the lateral column of the spinal cord becomes replaced with scar tissue. Another was progressive muscular atrophy, which is analogous to the experimentally produced dystrophies in the rat and rabbit. Partial cures and temporary remissions have been reported for both diseases when Vitamin E is given with thiamin and riboflavin, but the majority of evidence is that these diseases know no remedy (Wechsler, 1940 ; Ferrebee et al., 1941). This has been attributed to the irreversible damage to the cell structure, confirming the histopathologists, that tissue acutely damaged by lack of Vitamin E is not amenable to repair by the vitamin. From a chemical standpoint, this was as expected, because Vitamin E deficiency greatly increases the formation of low molecular aldehydes by random oxidation of unsaturated lipids. The aldehydes react with the amino groups in proteins and nucleic acid in five different ways (Gustavson, 1956), forming crosslinked aggregates which, if the reaction has gone far enough, become irreversibly

enzyme resistant and thus impossible for the organism to remove.

**HYPERBARIC PRESSURE - CHEMICAL STRESSES**

A high level of Vitamin E increases the tolerance of mammalian organisms not only against the consequences of lack, as well as of superabundance of oxygen, but also protects against other stresses. Resistance against high and low temperatures are increased (Wiswell, 1962). Also, it increases resistance against carbontetrachloride and orthocresyl esters (Hove, 1955).

These protective effects may or may not be due to control of oxidation. On the other hand, the protective action against liver damage in rats is ascribed to an increased availability of methyl groups needed for this detoxification (Levander, 1970). These effects, not yet confirmed with humans, appear likely to be of a nature sufficiently general to affect life expectations in other contexts as well, as contaminants become more widespread and more « sophisticated ». Menzel et al. (1972) showed that Vitamin E substantially enhances resistance to atmospheric contaminants, including ozone and nitrogen oxides.

**VITAMIN E IN PREGNANCY, AND IN PREMATURE OR NEWLY BORN BABIES**

The newly born baby has less than one-fifth the blood plasma content of Vitamin E that his mother had at the time of his birth, although he has double his mother's plasma content of Vitamin C and at least half the level of Vitamin A (Straumfjord and Quaife, 1946). Evidently the fairly bulky tocopherol molecule does not pass through the placenta readily. The mother's plasma content of Vitamin E increases before giving birth, apparently to compensate for this at least in part. No effort seems to have been made to correct this by the simultaneous administration of lecithin as a carrier. Infants at birth have on the average only 0.35 mg of Vitamin E per 100 ml plasma, prematures almost none.

The relevant facts have been analyzed by Hickman (1946, pp. 507-508), who although not a medical man felt compelled to suggest that « For the adult human we speak of a maintenance dose, but for the fetus we must provide a dose sufficient for manufacture, maintenance, and endowment. The child must be created and launched in life with sufficient reserve to tide over the limited dietary resources of the first few months.

« If the mother is in a borderline condition of health and nutriture, and if the endogenous supply of progesterone is suboptimal, Vitamin E supplement may make just the difference between abortion and a healthy child. In any event, ingestion of plenty of Vitamin E will favor adequate reserve in the child, and will increase both the fat- and the oil-soluble vitamins in the mother's milk, again benefiting the child. »

Several syndromes in premature or newborn babies have yielded to treatment with Vitamin E.

Premature babies, who received a nutrition high in unsatured fatty acids developed a syndrome characterized by low plasma tocopherol, edema and hematologic and dermatologic abnormalities (Hassan et al., 1966). All of these symptoms disappeared rapidly when Vitamin E was administered.

Newborn babies suffering from scleroderma neonatorum, a previously mostly fatal disease characterized by hardening and edema of the subcutaneous lipid carrying tissues, were cured by administration of tocopherol (Gerloczy, 1949). The tissues of babies afflicted with this disease have extremely low tocopherol levels. Several authors recommend prophylactic administration of alpha tocopherol to all prematurely born babies, since their tocopherol level invariably is very low in relation to that of normal births (Beckmann, 1958 ; Nitowski et al., 1962 ; Dyggve and Probst, 1963). Such administration favorably influences the teratogenic effects of hypoxia in well controlled animal experiments (Degenhardt, 1959).

**VIEWPOINTS ON THE DOSAGE OF VITAMIN E**

The problem of proper dosage of Vitamin E is to find the balance at which the beneficial effects of the preservative factors are utilized as fully as possible without yet slowing normal or desirable oxidation processes.

The range between the desirable and the excessive antioxidative action of Vitamins E has not yet been fixed, but on the basis of published data we can with a high degree of confidence state the range which appears safe and beneficial, and beyond which it seems disadvantageous to venture.

The National Research Council of the U.S.A. has established a recommended range of 10 - 30 mg per day for adults. The Pharmacology Committee of the USSR Ministry of Health officially recommends a dosage of 5 - 10 mg/day for adults (1967). These are only the ranges necessary to avert obvious deficiency symptoms, but by no means the optimal range for longevity. Increased requirements for Vitamin E result from :

1. Anything increasing the oxygen demand systemically or locally, or reducing the available oxygen supply ;
2. Anything creating an increased demand for protein, or need for a protein sparing effect for any reason ;
3. Chemical stresses, particularly those related to alkyl halides, or to effects of ozone or nitrogen oxides.

A commonly occurring increased oxidation demand is imposed by increased intake of unsaturated fatty acids. This factor, though significant, has been overestimated because most authors on this subject neglected to ensure that the diet used to limit Vitamin E intake was still adequate with regard to essential fatty acids. Deficiency of these essential membrane components places a biological premium on Vitamin E with the result that the Vitamin E response curve is abnormally steep until an adequate level of essential fatty acids is

supplied. Taking this in account, Jager (1973) has determined that a fat consumption of 30-40 cal % dietary fat should contain 100 mg d-alpha tocopherol or equivalent per kg fat to exceed the critical deficiency minimum for Vitamin E. This requirement can rise to 200 mg if the fat contains more than 25 % linoleic acid.

Independent of these findings, the presence of Vitamin E in all tissues as well as the high level of storage, points to broad needs of widely varied nature, so that an entirely adequate intake should be secured.

In seeking to determine a surely sufficient, but not needlessly wasteful dosage for these needs, the papers by Haeger (1968, 1973) seem to provide a well documented basis. Haeger's work is careful and statistically sound. It shows indisputable benefits in improvement in the circulation on the legs and in reduction of amputations. He attained this with dosages of 300 mg of dl-tocopherol per day for a minimum period of 3-4 months. A higher dosage did not increase the blood plasma level of Vitamin E, nor show any further advantage.

Boyd and Bloor (1959) also find only a moderate improvement of the walking distance in intermittent claudification after a daily dose of 200 mg of alpha tocopherol, but significantly better effect after at least 3 months of treatment with 400 mg daily. Larsson and Haeger found that in intermittent claudication a dosage of 300 mg was as favorable as one of 400 or 600, and Haeger has based his subsequent work on a dosage of 300 mg/day. Hoffer (1972) states that he has used 800 mg daily for several years, and 1000 mg doses have been used without report of hypervitaminosis. The extreme is Hillman's (1957) report of a healthy male taking 2000-4000 milligrams dl-tocophera daily without other evident disadvantage than some creatinuria.

Hickman (1946, p. 519) considers 200 mg/day of d-alpha tocopherol a safe maximum dosage. This corresponds in potency to 240-300 mg dl-tocopherol dependent on assay method which checks remarkably well with Haeger's findings. In 1973 Haeger used 300 mg/day d-alpha tocopheryl acetate, with good results.

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In animal work, Degenhardt (1959) determined with care and accuracy the effect of dl-alpha tocopherol on birth injuries in rabbits exposed to hypoxia. A single application of hypoxia 5 hours at 255 mm Hg air pressure in the 9th day of pregnancy caused reproducible and readily defined and quantitated deformations of the backbone, which were maximally reduced by administration of 20 mg dl-tocopherol daily during the entire gravidity. This corresponds to 3-4 mg per kilogram body weight for humans, thus 210-280 milligrams/day for a 70 kg human, at least for the first 30 days of pregnancy. Thus, the same order of magnitude, of 200-300 mg is indicated by Boyd's and Haeger's clinical work, by Hickman's extensive experience, and by the animal work of Degenhardt. I have found no contradiction of these findings in the copious literature. As for the **upper limits** of the dosage range, strong evidence supports the conclusion that beyond a certain level, Vitamin E is exerting maximum practical retardation on those deleterious reactions which should be retarded, and that at a certain higher level it will begin gradually to extend the retardation even to the point of interfering with normal metabolism :

Hillman (1957) reports of a healthy, 41-year-old physician who ingested 296 grams of dl-alpha tocopherol in 93 days as follows : 2 grams daily for 37 days, then 4 grams daily for 55 days and 2 grams one additional day. He used Ephyrol acetate (Hoffmann-LaRoche) 80 days and Aquasol E (U. S. Vitamin) 13 days, usually in 2 daily doses 8 hours apart.

During this period there was no change in his activities, and no intercurrent infection.

His serum level of plasma tocopherol rose to 2.26 ± 0.86 mg/100 ml, which was twice the control level.

A significant transient creatinuria resulted, but there was no change in excretion of creatinine or of ketosteroids, and no change in exercise tolerance, electrocardiogram, ballistocardiogram, serum cholesterol, liver function or blood coagulation. A muscle biopsy showed no deviation from normal. Hillman concluded that the creatinuria may be an early manifestation of an adverse metabolic effect of this very high dosage.

R. Beckman et al. (1963) administered 300 mg, 600 mg of alpha tocopherol (Ephyrol) to women 3 hours before parturition as a single dose orally. The babies were tested for capillary resistance by the method of Küchmeister and Schärfe (1950). Only 14 % petechiae were found in the 300 mg group, against 52 % in the control and 31 % in the 600 mg group. Increase of the dose to 600 mg to the mothers was therefore not recommended.

Harris, Kaley and Hickman (1944) determined the optimum dosage of natural mixed tocopherols in sparing protein in rats, by determining the difference in weight gain with a standard diet plus protein supplement in Vitamin E deficient animals, and in animals receiving increasing quantities of the vitamin. The following table shows weight gain over controls over a period of 35 days :

| Tocopherol supplement per rat | Average difference from controls |
|---|---|
| 0 | — 16.1 |
| .025 | — 6.2 |
| .15 | 11.8 |
| .50 | 28.7 |
| 1.5 | 23.5 |
| 5.0 | 9.6 |

It is thus seen that a vitamin concentration beyond the optimum leads to decreased growth response.

The Vitamin E preparation used was e molecular distillate of unsaturated vegetable oils. It contained 40 % of an equal mixture of d alpha and d gamma tocopherols, with a small, unknown quantity of beta tocopherol. Analogous results were obtained by Miles et al. (1949).

Gray, Chisholm and C. H. Lee Peng (1960) measu-

red DNA content, comparing a group of rats receiving 0.5 mg each of Vitamin E with a group receiving 100 mg per animal. The second group showed a significant increase in DNA, in the liver. Since the DNA content is nearly constant in all cells of the same organ and species they conclude that the extreme dosage caused hyperplasia in the young animals.

R. Huber and E. Schröder (1962) showed that dl alpha tocopherol acetate protects against damage from whole body x-ray irradiation of mice, but that the effective range is quite narrow. This is illustrated by the following data :

**Survival time after 30 days of total body irradiation, 500 r, AB mice, males, weighing 25 grams. 30 animals in each group.**

| Protective | Survival time in days |
|---|---|
| Alpha tocopheryl acetate 10 mg 24 hours before, 5 mg 10-20 minutes after irradiation | |
| 15 mg | 7 days |
| 5 | 9 |
| 2.5 | 16 |
| Physiol. NaCl | 7 |

With mice of another strain, C57bl-6, the optimum dosage for protection was 1 mg, with survival time of 24 days while at 3 mg and control the survival time was 8 days. With a third strain of white mice the tocopherol was ineffectual.

de Duve and co-workers (1963) showed that the stability of lysosome membranes is reduced by high concentrations of alpha tocopherol acetate.

That increases beyond the dosages needed ultimately can shorten life has been indicated by Tappel 1973, p. 419). His Fig. 6 shows that for rats a large overdosage of Vitamin E shortened the life expectancy, comparing with controls receiving a sufficient, but much lower amount of Vitamin E.

By addition of some selenium, even Tappel's peak percentage of Vitamin E could be tolerated by rats without much loss of longevity, but no increase above the controls was shown and the use of selenium introduces a new complex and still controversial element (Diplock et al., 1971 ; Scott, 1971 ; Chen, 1973 ; Levander et al., 1973).

While any one of these findings of high level retardation possibly might be disputed, their totality appears convincing in confirming that the beneficial effect of Vitamin E can be reduced when an inordinate amount is taken. Thus, an upper limit of the dosage should be observed not only for reasons of economy but also for optimal results. It would seem neither necessary nor advisable to exceed 400-500 I.U. per day.

## THE RELATIVE POTENCIES OF THE TOCOPHEROLS AND TOKOTRIENOLS

The natural d-alpha tocopherol and the synthetic dl-alpha tocopherol are equal in antioxidant potency in vitro, but in the biological tests for control of sterility in rats, the d-alpha tocopherol is 35 % more potent (Harris, Ludwig, 1949). By chick liver storage method, d-alpha was found 1.21 times more potent than the dl form (Quaife and Dju, 1949). Pure l-alpha tocopherol has on the biological test only 20 % of the activity of the d-alpha form. If we take the international standard of 100 units for 100 mg of dl-alpha tocopherol, then the quantity of pure d-alpha form (50 mg) contained in it has a potency of 61 I. units per mg and the 50 mg of l-tocopherol, 27 units. In intravenous injection the d-form is about 5 times more potent biologically on the sterility test than the l-form, so the difference does not depend on differences in resorption.

On the other hand, the effect of the dl-form is essentially the sum of that of each of the d-form and the l-form. The l-form does not have any retardant properties nor does it disturb the function of the d-form in any way. A reason for the lower activity of the l-form may be in part that it is broken down or excreted more rapidly than the d-form (Fitch and Diehl, 1965).

It is noteworthy that the antioxidant properties of the tocopherols in vitro are quite the reverse of their biological potency tested on sterility or on muscular dystrophy or on hemolysis, to take a couple other Vitamin E effects used for bio-assays (Olcott, Emerson, 1937). The in vitro antioxidant activity of the tokotrienoles is equal to that of d-alpha tocopherol, while their biological efficiencies are much lower (Olcott, 1966).

Jager (1973) in his dissertation summarizing recent investigations, reports the following relative potencies for the natural d-forms of alpha to delta tocopherols.

| | Biopotency (I.U./mg) |
|---|---|
| d-alpha tocopherol | 1.5 |
| d-beta » | 0.4 |
| d-gamma » | 0.2 |
| d-delta » | 0.02 |
| dl-alpha tocopherol acetate | 1.0 |

d-delta with the lowest biopotency, has the highest antioxidant properties (Olcott, Emerson, 1937). Matterson and Pudelkiewicz (1974) find a ratio of 1.00 to 1.21 for dl-alpha to d-alpha tocopherol in chick liver assay.

The acetates have 9 % lower biopotencies than the corresponding free tocopherols as listed above (Brubacher and Weiser, 1967). Because of their much higher storage stability they are rightly used in almost all practical vitamin preparations. This is entirely proper because the acetates readily split in the body to release tocopherol and acetic acid.

## COMPATIBILITIES AND SYNERGISMS

No specific, potent anti-vitamin E exists, as, for example, avidin is an antagonist to biotin. The tocopherols are sensitive to oxidation, though much less so than Vitamin C, but their acetates or propionates are much more stable in storage, while they remain available to the organism.

Vitamin E is destroyed by iron salts and should therefore not be administered in the same prescription as iron, for example, in anemias, or even given at the same time (Hickman, 1949-b, p. 121). Iron is toxic to animals deficient in Vitamin E (Goldberg, Smith, 1960 ; Tollerz, Lanner, 1964).

The antioxidant efficiency of Vitamin E was increased 500 % in vitro by the addition of lecithin, and an additional 400 % by the further addition of Vitamin C, and additionally by phenolics such as tannic or gallic acids (Hickman, 1949-b, p. 117).

The efficiency of these synergists in the organism has not been as sharply defined, but is nonetheless recognized as substantial. Vitamin C supports the antioxidative properties, and lecithin assists in the dispersion and transport of Vitamin E (Hickman, 1949-b) particularly in the water phase and through membranes. The transport in the bloodstream is carried by lipoproteins, and these contain phospholipids « lecithins » in all fractions (Putnam, 1965).

## CONCLUSION

It may never be known whether the genesis of tocopherols occurred as suggested in the beginning of this paper, as a development at the time when photosynthesis of carbohydrates was new to terrestrian life, or whether it took place concurrent with the creation of the first self-propagating molecule at a time of violent cosmic bombardment. I favor the former assumption because there are many simpler stabilizers which would have been adequate for stabilization of complex molecules. The superiority of the tocopherols which explains their evolutionary selection consists, firstly, of their selectivity in transfer processes, and secondly, in the biological fine tuning made possible by a choice between a multiplicity of very similar molecules capable of performing a function similarly yet not completely identically. Both of these presuppose the existence of a fairly well developed cell structure.



As for dosage, it is possible that a dosage even as low as the 10-30 mg officially suggested can satisfy optimal requirements if maintained steadily from birth, together with a diet uniformly low in unsaturated lipids.

However, as a practical matter, it has been clearly shown by Haeger, in conjunction with Boyd and Bloor, that to correct a suboptimal condition in about 3 months, a dosage in the range of 200-300 mg/day of alpha tocopherol is required, and that 400 mg/day for sereval years causes no observable disadvantage.

While these results were obtained in clinical study mainly of patients with intermittent claudication, it is reasonable to assume that they have a more general validity. Particularly Haeger's observations on mortality now extending over a period of 13 years indicate that this is indeed the case.

It is predictable that the officially recommended dosages in time will take cognizance of these findings.

The total effect of Vitamin E on mammalian longevity will depend principally on the effect on the circulatory system. In small mammals the addition of large quantities of Vitamin E beyond generally adequate rations, affected life expectancy unfavorably, when at all (Tappel, 1973).

On long-lived organisms, such as humans, the preserving effect of Vitamin E becomes more noticeable. On the basis of the results discussed above an increase of life expectancy in the order of about 3 - 5 years and possibly up to 10 - 15 years in individual instances might be attainable by optimizing the supply of Vitamin E. A major part of this increase will be due to increased protection of the circulatory system.

An effect on the cellular level was reported in October 1974. Packer and Smith found that human lung cells in free culture, with Vitamin E added to the culture medium, kept on dividing beyond 120 times without sign of tapering off, while controls in the same medium died after about 50 cell divisions, as Hayflick has shown is generally true of human cells in free culture.

It seems relevant to recall that Tyler (1953 and 1965) showed a similarly great life extension of free sperm cells of diverse species, from sea urchins to mammals, when the polyvalent metals in the ambient medium had been removed by means of ethylene diamine tetracetic acid, (« EDTA ») or any one of a number of other chelating agents. The common denominator here could be that in both cases crosslinkers are brought under control : Vitamin E inhibits the formation of aldehydes and peroxides by oxidation of unsaturated fatty acid radicals invariably present as components of phospholipids in the cell membranes which are the sites of the genetic matter, while the chelating agents remove polyvalent heavy metals, likewise powerful crosslinkers for proteins as for nucleic acids.

The life extending effect of Vitamin E in mammals is thus of a much lower order of magnitude than what has been achieved by Packer and Smith (1974) and by Tyler (1953, 1965) in free culture. This indicates that the phenomenon which limits longevity is not primarily dependent on the genetic matter, but on events in the cytoplasm. This conclusion has previously been expressed by Hayflick (1974) and by Bjorksten (1974).

Observations to date are consistent with the assumption that a network of crosslinked macromolecules is gradually formed in the cytoplasm and that this progressively restricts the intracellular transport, to the point of death (Bjorksten, 1974, p. 49, Fig. 4) unless the cells are in free culture so that the cytoplasm can continue to increase in total volume as fast as crosslinking takes place, thereby keeping the crosslinkage percentage below a critical level.

For reversal, and major gains in life expectancy, we shall require enzymes capable of penetrating these structures and breaking the molecular backbones between the crosslinkages. In the meanwhile, however, Vitamin E can delay the net-forming process though only to the extent this is

due to inhibitable oxidative formation of cross-linking agents. This could produce a life extension of several healthy years.

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**IN THE YEAR 2000 :**

**"Elderly People will be young and stay so, till their breath of life snaps. One has to realise that a comparison between the elderly people of tomorrow and the old people of today will become impossible.**
**Not only because one will live for a 100 years, or even more, but because being old will be completely different from now :**

**Grandmothers and grandfathers, the old age, the gray haired people will, also in name, belong to the past. Teenagers and twens will in their following life-phases continue to feel and behave as they did before. (Prof. Polak in "The future of growing old", Agon Elseviers, Brussels-Amsterdam).**

Longevity Limiting Factors

Mankind is designed to remain functional for the time necessary to produce its offspring, and to give it protection, tuition and some guidance for a start in adult life. This is, on the average, 60 - 70 years.

In an organism as complex as man many things can go wrong, but a few of these account for the major number of deaths at various successive ages.

First of these come the diseases due to mismangement and abuse of the circulatory systems, which becomes apparent in the 60 - 70 range.

Second at present are the cancers, a group of diseases manifested by uncontrolled cellular growth.

Both of these have been so multiply studied, that the problem is more to make sense of the data we have than to get more data. I shall therefore not discuss these here. They need pruning of the data we have and intelligently focusing of their application, much more than still more discussion or even new data.

Therefore I shall go directly to the third major danger: the destruction of the central nervous sytem. This has a design time of about 100 years plus minus 20, and thus will become a principal cause of death as circulatory disease and cancer are yielding to control and prevention on the basis already at hand. While central nervous damage may have as many variants as its predecessors, the most central of these causes are discussed in the following papers.

# ALUMINUM IN DEGENERATIVE DISEASE

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Aluminum is a reactive metal, so it may seem surprising that it has found no place or useful function in any pathway of animal metabolism. Almost all of the other reactive, abundantly available metals have found such uses.

It may be of interest therefore to review the interaction of aluminum with biological systems, particularly since some of these are becoming important in various degenerative diseases, and possibly in some aging syndromes. The total avoidance by evolution of using aluminum for any metabolic function ominously raises questions, which beg an answer. Aluminum, constituting 8.4 % of the Earth's crust, is very widely present in normal human nutrition. Normally, almost every person will unavoidably ingest 30 - 50 mg daily of aluminum.

*NEUROTOXICOLOGICAL EVIDENCE*
*ANIMAL EXPERIMENTATION*

In a series of brilliant studies Dr. D. R. McLachlan (formerly D. R. Crapper) and his co-workers (C10 - C21, D1, D2) caused a replication in cats of the entire sequence of symptoms of Alzheimer disease by a single injection of 100 nanomoles of aluminum chloride into the hippocampic ventricle. (C13-14). No change was observed in the next week, but about the 9th day the cats began to fail to remember where they had seen food hidden. About 10 days later their short range memory was completely gone.

Concurrently, the coordination of movement began to fail. The cat no longer could land from a jump with both forepaws simultaneously and adjust the alignment of the rear paws while still in the air. At this time, the electroencephalograph curves became abnormal. Deterioration continued - Epilepsiform seizures and periods of rage occurred. Usually failures of the central nervous system led to death about 3 months from the time of the one and only injection.

In the few instances when careful nursing could save the life of the animal, the neurological damage persisted - the harm done was permanent. The similarities to Alzheimer Disease and Senile Dementia were striking.

*EXTENSIONS TO CLINICAL FINDINGS*

These conclusions were confirmed and broadened by histochemical and microscopical studies. (C20,C2,D2). The aluminum was found preferentially absorbed by the chromatin in the pyramidal neurons in the hippocampic area. LaPresle et al. (L2), Cartier (C2), Duckett, S. and Galle P. (G2) showed the presence of aluminum also in Parkinsons Disease, Crapper et al. in Downs Syndrome (C16), though in the latter case additional data will be needed for final certainty.

Perl and Brody (P9) confirmed that aluminum is associated with the nuclei of neurons in neurofibrillary degeneration in senile brains, quite similarly to Alzheimer's dementia. Immunologic tests have confirmed the chemical similarity between abnormal tubular intracellular growths in Alzheimer neurons and similar tubules induced in rats by administration of aluminium. (G2)

Important corroboration was obtained by A.C. Alfrey and co-workers (A1-A7,B22). An endemic neurologic syndrome occurred in a group of uremic patients on dialysis with an «artificial kidney». This disease typically terminated in death, in 3-6 months. It was originally ascribed to an aluminum hydroxide gel, given to these patients to depress the serum phospate levels. (A5) However, this may have been only the «top of the iceberg». Reports of the same dialysis syndrome came from many institutions, in many countries. (A10, B1, C1, C3, F1, F2, G4, H2, L4, M12, N1, O2, P5, R3, S2, S3, W2). The European Dialysis and Transplant Association inquired in 1977 about the frequency of dialysis encephalopathy, and received reports of 153 cases from 17 countries. (H1, E4).

The people receiving chronic treatments on the artificial kidney are typically exposed to about 180 liters of water 2-3 times every week. This exposure is directly through the arterial bed. Any substance dissolved in this water bypasses not only the gastro-intestinal barrier, but also the kidneys which normally remove much of the aluminum which gets through the gastro-intestinal barrier. (A2, A4, A5). Normally, we excrete about 15 micrograms of aluminum daily in our urine. This must, however, be viewed in the perspective that aluminum is present in almost all foods so that we normally ingest 30-50 milligrams daily (S11) and that, judging from McLachlan-Crapper's work, very few milligrams reaching the hippocampic area of the brain in a time span of about 60 years would be extremely harmful.

That the neurological damage due to alumi-

num cannot be reversed by presently known methods was indicated by Crapper, who by careful nursing succeeded in saving the lives of a few cats, which had received the usually fatal single injection of aluminum chloride in the hippocampic ventricle. The neurological symptoms of these cats proved completely irreversible even after three years of careful nursing. (C14).

Clinically, this is confirmed by the failure of successful renal transplantation to reverse the dialysis associated encephalopathic syndrome. (M11).

The kidney excretion of Aluminum in 60 years would amount to about 300 milligram, enough to be potentially critical.

Appreciable amounts of aluminum are also excreted in hair and nails, and can be excreted in the feces.

Bone appears to be a major storage organ (or dumping place) for excess aluminum (A4, B5, B22, C5, C8, L5, P2, P3, P5, R1, R4).

When aluminum stored in the bones exceeds certain limits, it appears to cause embrittlement of the bones, and spontaneous fractures. (P5, P1, P7). This is not surprising, since aluminum is a crosslinking agent for collagen, (G5) particularly if polyhydroxy compounds are also present (S10, O3) and thus when not immediately engaged by the phosphate groups, is bound to embrittle the collagen matrix of the bone structure.

In the uremic patient, without the capability of excreting excess aluminum through the urine, the aluminum burden from even normal intake will markedly increase. Although the quantity is small in micrograms per day, over a lifetime this reflects in a major alteration in body aluminum.

**12**

Until the present studies, the dialysate in Denver was prepared by tap water containing 120 - 240 micrograms of aluminum. The difference in resultant plasma levels between this and aluminum-free dialysate supported the contention that if there was aluminum present in the water, the patients' serum aluminum would increase substantially. (A2).

Plasma aluminum is about 7 micrograms per liter. This is not correlated with the transport of aluminum in plasma, neither does parathyroid hormone nor Vitamin D effect any appreciable change in bone aluminum in 3 months, which suggests that absorption of aluminum is not affected by these agents. (B1, A4, A19).

It has become evident that most centers which have had severe outbreaks of dialysis encephalopathy, have had dialysate markedly contaminated with aluminum. (A2, D4). I shall here only cite one typical instance in greater detail.

In Växjö, Sweden, the aluminum content in incoming water and dialysis liquid in the period March until May, 1978 was in the range of 100 - 400 µg per liter. During the period May, 1977 - June, 1978 9 patients with uremia were dialyzed twice weekly for a total of 7.3 months average. One of the patients died after one month from uremia. Four patients did not develop any clinical signs of dialysisencephalopathy. The remaining four patients developed neurologic symptoms 6 ½ - 11 months from admission and within 5 ½ - 10 months from the beginning of treatment. They died 3 - 6 months later. Three of these fatal cases were typical. The fourth patient who had multiple symptoms (nephrosclerosis, coronary insufficiency, unilateral cyst.) was considered atypical. However, 2 EEG examinations with 1 month interval showed a slowed basal activity, and, with frontal dominance, numerous bilateral, rhythmic delta episodes with high amplitude and occasional sharp waves.

Apparatus for purification of dialysis water (reverse osmosis) subsequently installed, has functioned satisfactorily as of this time (15 months after installation). The aluminum content has consistently remained below the detection limit of present analysis methods ($< 10$ µg per liter). Patients in home dialysis treatment also received reverse osmosis aggregates if aluminum could be found in their water supplies or if, as is the case in Växjö, the city water works use aluminum sulfate treatment of the water. During the time June 1978 - September 1979 11 patients were dialysed at the dialysis department in Växjö for 1 - 15 months (average 7.7). One patient died from malign hypertonicity after less than 2 months. No symptoms of dialysisencephalopathy have been observed in any of the patients in Växjö, nor in the home dialysis patients. (H1).

In low serum levels, there is a proportionality between fluctuations of aluminum contents of serum and of bone, (P3, R1, A2) but in high levels, the excess which cannot be excreted accumulates preferentially in brain and in liver. (A1, A2). Correlation between tissue stores of aluminum in bone vs. brain and liver was fairly good in persons dying from other causes, but in persons dying in the neurological syndrome there was no good direct correlation between these. This suggests that when the buildup of aluminum exceeded the bones ability to sequester this metal, the aluminum was shunted to brain and liver. (A1, A2). The deposits in the liver are turned over and ultimately excreted, but the deposit in the brain remains. Virtually for any time on dialysis, the patients with the neurological syndrome had peak brain aluminum levels. (Fig. 1, below).

A group in Holland found a relation between contaminated dialysates and dialysis dementia. This group used an aluminum heater to warm the dialyisate. When their heating element was removed, they eradicated the disease. (A2). Riddick presents cardiographic

evidence of a connection between premature ventricular contraction and aluminum. (pp. 304, 305 of ref. R2).

In England an association was established between aluminum content in water and neurological disease in patients on home dialysis, using various local water supplies. Those who developed neurological disease all had aluminum in their water while patients who were free from this disease did not have aluminum in their water. (A2, P7, E2).

It should be mentioned, however, that in a *very small* percentage of dialysis patients, a similar neurologic disease developed when they were being maintained with dialysate that was *not* aluminum contaminated. (A2). This might be due to an extraneous aluminum source, or to reaction with some other rare substance, possibly certain organic polyfunctional reactants. In any event the role of aluminum in human neurologic disease is firmly established, and can be closely duplicated at will by injection into brain ventricles of animals of minute quantities of aluminum. (C14). Brain gray matter aluminum values in a group of uremic patients on dialysis who died of a neurologic syndrome was 25ppm as compared with 6.5ppm in dialysis patients who died of other causes and 2.2ppm in non-dialysis controls. (A2). The accumulation of aluminum in bones is apparently a factor in senile osteopathy. (C9, P2, W1).

As a consequence of recent cognizance of these matters, the new values adopted in the USA for short term exposure limit based on time weighted averages in 8 hour working days require for the first time in 1979 a limit for inhalation in air of 2 mg/m$^3$ max. for soluble aluminum salts and aluminum alkyls. (A12).

*HISTORICAL*

While the above work concluded and clinched the role of aluminum as causative factor in non-ischemic encephalopathies, the knowledge of its neurotoxic properties and the suspicion of its role in the aging syndromes are not new.

The neurotoxic action of aluminum salts was observed by L. Spira, (S6) who also summarizes early literature.

Harvey W. Wiley, MD, Director of the precursor of the USA Food and Drug Administration 1906-1912 and a pioneer in preventative food and environmental science, wrote in the closing chapter of his book «History of a Crime Against the Food Law» : «From the earliest days of food regulation, the use of alum (aluminum sulphate) in foods has been condemned. It is universally acknowledged as a poison and deleterious substance in all countries». (W4). More recent data are available. (W5).

HW Sharps and CF Church stated in 1937 that intracranially injected aluminum sulfate or lactate caused widespread nerve cell changes of an type known as «toxic degeneration». Kopeloff JM, Kopeloff N and Barrera SE found that convulsive seizures could be produced in animals by aluminum hydroxide paste. However, histopathological findings were not distinctive. (K4). At the Baltimore convention of the Gerontological Society in 1955, J. Bjorksten and H. Gottlieb mentioned aluminum among those crosslinking substances which could be expected to cause age dependent degeneration. (B12). This was supported by Zinsser et al. in 1957 (Z1) and by Bjorksten in 1958 (B7). Zinsser, Bjorksten and 8 coauthors furnished X-ray spectrographic indications of aluminum bonds in aging human aorta, and listed chelating agents then considered for removal of aluminum. (Z2, pp. 481-2, Tables 8 & 9).

In 1965 Klatzo et al. made the fortuitous observation that the use of aluminum in an immunochemical procedure of «Holz Adjuvant», intracerebrally produced a severe convulsive state with striking neurological changes throughout the central nervous system. (K3). Further study pinpointed aluminum phosphate as the causative substance. There was a latency period of 9 - 14 days before the onset of the symptoms. Only the neurons seemed affected.

RD Terry, NK Gonatas and M Weiss (T1) reported indications of cross-linkages, as expressed by branching in human intraneural filaments. This was further elaborated by Terry and Penna, who state : (page 208 of (T2)). The crossbranching which is rather prominent in the experimental tangles may indicate a state of abnormal gelatin with crosslinks established between adjacent fibrils. This would increase cytoplasmic viscosity and perhaps impede flow of cytoplasmic contents.» This checked well with the earlier concepts and X-ray spectographic data.

Riddick (R2) stressed in 1968 the coagulating properties of aluminum and strongly attacked the use of soluble aluminum compounds in medicine, its use in baking powders and particularly in water treatments to precipitate colloidal turbidity in the water and probably also disturbing the colloidal equilibrium of the brain by affecting the zeta potential. If we have a certain coagulating power from 3000 ppm of sodium ion in an aqueous medium, we can double it by adding as small a quantity as 200 ppm of calcium or of only 5 ppm of aluminum. (R2 pp. 274,275). These enormous changes in stability can occur without noticeable changes in specific conductance, which is only slightly affected. Thus an insignificant change in specific conductivity which would escape present routine controls, could hide an overwhelming change in colloid stability if the added ion were aluminum.



*PROPERTIES OF ALUMINUM RELEVANT TO AGING GENERALLY*

Reasons which could explain why aluminum is

uniquely adapted to affect the central nervous system, and possibly also, aorta, the sinoatrial node, the atrioventricular node and the Purkinje fibers (the «pacemakers») of the heart, the heart muscle itself and other sensitive organs :

A. Aluminum is a powerful flocculant and as such causes shrinkages of colloids. The human brain contains large amounts of colloidal gels as essential constituents. Abnormal coagulation of these will cause shrinkage, and thereby severing of interneuronal connections, similar to those known to occur in aging. (A13, B19).

B. Crosslinkings involving large vital molecules (A16-18, H4). In no other broad type of chemical reactions can as great changes of biochemical behavior of large vital molecules be caused, as by crosslinkage. (B8, B13-16, B27). The kind and positions of unwanted crosslinkages are specific in carcinogenesis, but in aging largely random and unspecific. If two persons are cuffed together, their motions will be hampered whether handcuffs or footcuffs are used, and regardless of what metal or strong plastic is used in the construction of the chains. Deleterious effects of crosslinking are :

1. Forming tangled molecular chains or nets which progressively impede intracellular transport.
2. Causing loss of elasticity of all tissues, thereby increasing susceptibility for rupture.
3. Increasing the lipophilicity of proteins causing crosslinkages preferentially of hydrophilic areas.
4. Converting essential molecules into inert aggregates.
5. Inactivating vital molecules by creating steric hindrances.
6. Any one of these above factors can have secondary affects on accuracy of mitotic processes, protein and other synthesis, or disturbance of any enzyme.

C. Inhibitor of transaminations. The transaminases, largely pyridoxal dependent, are widely distributed in the brain.

Much work remains to be done to completely unravel the intricate patterns and interplays of brain function but reactions involving transamination are amply implicated. These prominently entail the intermediate steps of oxidation and/or decomposition of Schiff bases involving pyridoxal and aminoacids. Such interaction is catalyzed in vitro by the metal ions $Fe^{++}$, $Co^{++}$, $Ni^{++}$, $Cu^{++}$, $Zn^{++}$ and $Al^{+++}$ (W3). Of these, the four first are readily reducable to their lower valence stages, which is an elegant way to effect release from an intermediate compound. The great significance of the changes in oxidation stage in the brain has recently been further stressed by the work of Jöbsis, Sylvia and co-workers. (J1-J3, S2, S3).

Of the metals which can catalyze pyridoxal-Schiff base reactions, only zinc and aluminum have a single oxidation stage. Zinc, however, is needed in normal metabolism, (C6, A9) for example, as a constituent of carboxy anhydrase, an essential blood enzyme, also in uricase and in renal phosphatase. It is also a frequent component of insulin. The organisms thus has been forced to develop systems for handling zinc. We may note, however, that zinc is toxic at relatively low concentrations and that the symptoms of zinc intoxication are neurological. (B20).

Aluminum remains the maverick. It has no known metabolic function. It cannot be reduced, and it has valence 3, coordination No. 6. Thus, it can form extremely firm attachments, which do not permit removal by reduction of oxidation stage or any other biologic means. Most likely, this is the reason why evolution has avoided assigning any metabolic function to aluminum, in spite of its ubiquitous and abundant presence.

Further, the aluminum ion is one of the smallest ions known. Small dimensions and high charge combine to maximize the electric charges in the immediate proximity of the ion. (G3).

Analytical data from many sources have shed light on these aspects. Such data include water analyses, (A8) studies of toxicities of substances used in dialysis (K7, M8, M9) and relevant data on human organs (Table 1 of A1). (B4, B21, B25, L3, M1, M6, M7, M10, K1, K2, S9, T3).

*DISCUSSION*

The mammalian body has a digestive system which rejects all but traces of the aluminum daily passing through. But even a very good defense permits some traces to pass. Aluminum penetrates more easily with advancing age, when continuing exposure to numerous crosslinkers normally and unavoidably present (B14, B17) has damaged membranes regardless of what proportion of these agents are free radicals (D5, D6, H3, P8), what proportion compounds formed by an initiating spurt of free radicals (S12, B28) and effecting the critical reactions irrespective of these (M14) and what proportion other crosslinking reactants normally present in human metabolism (B14, B17).

The defenses against these were not designed by evolution to last longer than the 60 years or so required for the average human to have children and help them get a start in life. Pathologically or genetically weakened membranes permitting absorbance or transport of aluminum might explain the occurrence of

elevated cortical aluminum in some cases of Downs Syndrome and of Parkinson Disease in younger individuals. (C16). However, more data will be needed for any general conclusions regarding these.

Where aluminum is engaged with all of its 3 valences in covalent union with a substrate, it is difficult to dislodge. Having a catalytic effect similar to that of $Fe^{+++}$, $Co^{+++}$, $Ni^{+++}$ and $Cu^{++}$ it might well fit into the same positions as these in the substrates' receptor structures. Where a reduction to a lower oxidation stage, such as ferric- to ferrous-, can release any of these other metals, aluminum, being non-reducible would remain fixed, thus blocking the critical site permanently.

The study of distribution of aluminum in the tissues and organs of dialysis patients, uremic non-dialysis patients, and controls is summarized in Fig. 1. The scenario which emerges from this and other studies is in essence that aluminum in the wrong places at the wrong time is a hazardous element.

Because of its ubiquity in nature, it cannot be evaded. Therefore, evolution has been forced to provide every surviving life form with means to reduce its aluminum retention to the point that accumulation will be slow enough to permit it to have, protect, rear and teach its youngs to the extent beneficial for the survival of its kind. For man, this is in the range of 60-70 years.

According to older data (Z2, K8), aluminum in the human organism slowly increases until about 55 years, and then begins to drop. Before drawing conclusions, I should like to see more determinations made with modern methods, covering the aluminum concentrations in heart and brain at ages from 50 years up. Present data might indicate that those who have retained aluminum in excess of the 55 year top level do not survive the following 5 years.

*SUMMARY AND CONCLUSION*

A remarkable similarity in course exists between the animals injected with aluminum as reported by McLachlan-Crapper and his coworkers in animal experimentation, and in the already voluminous literature on dialysis encephalopathy on the one hand, and the slower, yet otherwise seemingly identical course of Alzheimer Disease and of senile dementia on the other. This similarity extends to clinical systems as well as to histochemical, immunological and anatomical observations. Particularly convincing is the accumulation of aluminum in vital organs, notably the cerebral cortex and heart, also in aging. The finding of Kaneko and co-workers (K6) of a 40 % decrease in soluble protein indicates a corresponding relative increase in the insoluble fraction. This is consistent with the flocculating and crosslinking effects of aluminum.

From the solid data of recent years, we may conclude that most of the 30-50 mg daily that we unavoidably ingest with almost any diet, traverses the digestive tract almost without absorption. The small quantity that passes, is partly absorbed by the bone structure, with a minimum of bad effects, although very high levels lead to bone fragility. Of the aluminum still remaining in circulation a major part is removed by the kidneys, which thus constitute an important third line of defense. If this is by-passed, as it is in dialysis, the brain-blood barrier and the neurons' cellular membranes constitute last lines of defense. These alone, however, do not suffice to protect brain and heart, which show an accelerated rate of aluminum absorption when the storage capacity of the bones is exceeded. (A1, Table 1). Thus, aluminum is accumulating progressively until neural- or other disturbances kill its host.

When cancer and scleroses have been controlled, the accumulation of aluminum may become the next principal cause of human mortality.

Johan Bjorksten,
october 1, 1980.

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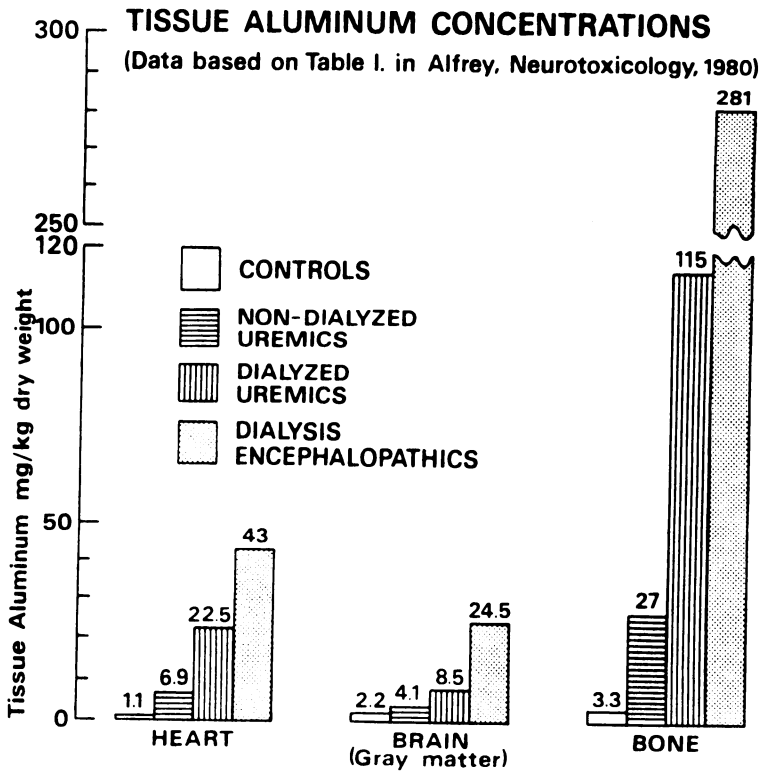
FIGURE 1



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# EXTRACTION OF ALUMINUM FROM AORTA TISSUES BY CHELATING AGENTS AND LACTIC ACID

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*Condensed Title :* Extraction of Aluminum from Aorta

*ABSTRACT*

*Development of methods for the reduction of aluminum accumulation levels in the human body was the objective of this study.*

*Aluminum adsorbed to aorta surfaces in vitro was extracted with organic acids and synthetic chelating agents. Lactic acid removed most of the aluminum at concentrations achievable in the blood during strenuous exercise. EDTA and other chelating agents rapidly removed the aluminum at levels approximating those used in clinical practice.*

Index terms : Aluminum, Aorta, Chelation, EDTA, Exercise, Lactic Acid.

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Although aluminum has no known value in human nutrition, due to its great abundance (nearly 8 % of the earth's crust), humans ingest about 10 to 100 mg daily. In spite of this, very little aluminum is normally absorbed. Blood concentrations average 0.11 mg/liter (Ulmer, 1976). Consequently, aluminum has generally been regarded as a non-toxic substance.

However, in 1957 Zinsser and co-workers (Zinsser et al., 1957) found progressive amounts of aluminum deposited with age in the aorta. Zinsser et al. (1962) postulated, on the basis of the demonstrated ability of aluminum to cross-link or «tan» proteins, that this accumulating aluminum creates a substantial hazard from a health standpoint. Earlier authors (Odier, 1925, Spira, 1922) had also reported that aluminum was toxic for some people. But the problem did not appear to be very widespread, so little effort has been expended to study it.

Further evidence of aluminum toxicity has more recently been found with kidney dialysis patients where «a relentlessly progressive form of dementia in chronic-dialysis patients» (Anon., 1976) has been reported when an aluminum hydroxide antacid has been routinely administered to decrease phosphate absorption. Aluminum has been found to accumulate in body tissues and particularly in the brain grey matter (Alfrey, 1976).

Crapper and co-workers (Crapper et al., 1973 ; Crapper, 1976, Crapper et al., 1978) have demonstrated that symptoms extremely similar to senile human dementia but with a compressed time-span can be induced in cats by a single injection of nanogram quantities of aluminum chloride into the brain cavities.

These results by Crapper are compelling. He confirmed them by physiological, histochemical and electroencephalographic evidence. The results are further strengthened by the quite recent finding of French researchers (Duckett and Galle, 1976 ; LaPresle et al, 1975) of high levels of aluminum in human brain autopsy materials where the cause of death was connected with neurological disorders.

This accumulating evidence reveals that aluminum can, under certain conditions, be quite toxic to some persons, with a major focus on the brain grey matter and the aorta.

The trivalency of aluminum together with its

well-known ability to «tan» leather (Reed, 1972), suggest that aluminum behaves in the body as a cross-linking agent, tying together two or more large molecules and thereby impeding or destroying their functionality. At the same time solubility would be reduced — thus impairing the body's ability to repair or remove the impeded molecules.

The damage from the accumulation of aluminum can be quite serious — often terminating in death (Alfrey, 1976). Consequently it seems important to develop methods to counter this toxicity even if only a small percentage of the population may be affected. Methods of reduction in the accumulation of aluminum will be important ; but when these fail, methods for subsequent removal are also urgently needed.

In an earlier paper, Bjorksten reported a crude but intriguing method for determining the effectiveness of removal of heavy metals from hog aorta, utilizing deposition of a cholesterol emulsion (Bjorksten, 1952 ; Bjorksten and Gottlieb, 1954). He found that EDTA and some proteolytic enzymes reduced the metal-induced deposition of the cholesterol emulsion. EDTA has also been used to reduce heavy metal toxicity. However, side effects can produce serious problems (Lamar, 1966).

In the first part of this study we sought to determine if there were common organic acids with sufficient chelating properties to extract aluminum from hog aorta. The cholesterol deposition method was initially used to compare the acids, but was not sufficiently sensitive. A number of dyes and stains were then tested including aurin tricarboxylic acid, which was finally modified to produce effective differential staining of the aorta.

A number of tests were conducted using the aurin tricarboxylic acid stain to compare the aluminum removing capabilities of a number of common organic acids. Subsequently an AOAC colorimetric method for aluminum was modified and adapted to provide more quantitative results (AOAC, 1970).

Although citric, oxalic and malic acids appeared to be more potent aluminum extractors, lactic acid was chosen for further study because it is generated by exercising muscles and can build up and accumulate in the blood to concentrations much greater than the other organic acids — as high as 0.1 % (w/v) or even higher (Hawk et al., 1954). Also lactic has been reported to remove the trivalent, cross-linked chromium ion from shoe leather (Roddy and Lollar, 1955).

## *METHODS*

### AORTA PREPARATION

Aorta of 5-6 month old hogs, secured from a local packing plant, were freed of external connective tissue and fat and were cut into 20 mm diameter disks. The disks were immersed in normal saline solution containing a microbial growth inhibitor and stored at 4° C until used. Para-Cresol, 0,5 % proved to be the most effective growth inhibitor, and it was incorporated in all of the solutions except the aluminum sulfate solution.

### LACTATE SOLUTIONS

Lactate solutions were prepared by neutralizing 50 ml of 85 % lactic acid solution (1 g lactic acid/ml) with sodium hydroxide and diluting to 500 mls. This 10 % lactic acid solution was diluted 10 times to produce the 1 % lactate solution.

### ALUMINUM STAINING OF DISKS

Disks were shaken with 5 % aluminum sulfate ($Al_2$ $(SO4)_3$ . $7H_2O$) solution for 30 minutes and then rinsed three times with distilled water.

The disks were then shaken with the chelators or acid salts (5 % concentration pH7.4 ± 0.1) overnight (rotary shaker 240 rpm). They were rinsed three times with distilled water and were shaken in 0.5 % ammonium aurin tricarboxylate (aluminum), adjusted to pH7.7 with ammonia, 10 ml. per disk, for 90 minutes. They were rinsed three times in distilled water, and were rated from 0 (blank) to 6 (high aluminum).

### COLORIMETRIC ALUMINUM DETERMINATION

The AOAC colorimetric assay for aluminum was not used initially because cloudiness was regularly encountered in the assay solutions. We finally found we could eliminate the cloudiness by shaking the disks in normal saline solution three times for one hour. This removed the interferring substance, which appeared to be traces of blood.

The disks were then shaken with 0.1 % aluminum sulfate solution (10 ml/disk) normally for 30 minutes, and were rinsed three times with normal saline solution.

Disks were shaken with 10 ml of the extracting solution per disk (chelator or acid salt) for 1 to 16 days. The extracting solutions were normally replaced each time one or more disks were removed for aluminum assay.

### ASSAY METHODS

One or two disks, depending on anticipated aluminum concentration, were removed and rinsed three times with normal saline solution and placed in a 125 ml Erlynmeyer flask. To this was added 20 ml of 2*N* $NH_4OH$. The flask was stoppered and shaken for two hours. The disks were then rinsed off and removed from the flask. The solution was adjusted to pH7.0 ± 0.1 with glacial acetic acid. To this solution was added 2.0 ml of thioglycollic acid solution, 0.5 ml of antifoam solution and 10 mls of aluminon solution. The solution was heated 20 minutes in a boiling water bath,

filtered, cooled and diluted to 100 mls. The absorbance was read at 540 nm. on the spectrophotometer. A standard curve was prepared by putting 0,5, 10, 20, 60 and 80 micrograms of Al into 10 mls of solution containing a washed aorta disk, adding 20 mls of ammonia solution and proceeding with the assay as above.

*RESULTS*

INITIAL STUDIES

A few preliminary trials with the cholesterol emulsion deposition technique showed that the method could give only limited and qualitative results. Since aluminum salts are used as mordants to fix many dyes, it seemed reasonable that a dye could be used to detect the aluminum. Tests of a number of dyes indicated however that only the aluminum reagent, ammonium aurin tricarboxylate, (called aluminon) was sufficiently sensitive to detect the level of aluminum present on the aorta disks.

Aluminum sulfate (5 %) treated aorta disks were extracted with a series of organic acid salts at approximately 5 % concentration, pH7.4, for 19.5 hours on the rotary shaker, and were subsequently stained with the aluminon solution. Table 1 shows that EDTA, citrate and oxalate removed the aluminum most effectively. The tea and tannic acid stained the aorta so that the dye color was not visible. Lactate, which is produced by exercising muscle cells, was moderately effective in extrating the aluminum. Aspirin (acetyl salicylate) was the least effective of the acids. Since lactate can be produced in exercising muscles at concentrations of 0.1 % or higher ; we chose to look at this acid further even though it was not as effective as citrate or oxalate.

LACTATE STUDY 1

Fresh aorta disks shaken for one hour with 0.1 % aluminum sulfate, were extracted with 1.0 %, 0,5 %, 0.1 % and 0.05 % lactate for 2 and 20 hours. Table 2 shows all concentrations of the lactate extracted a substantial amount of the aluminum from the disks, with greater effectiveness as the concentration was increased.

LACTATE STUDY 2

As the staining studies progressed, methods to more effectively quantify the aluminum assay were being sought. A colorimetric method involving ammonia extraction of the aluminum from the aorta was finally developed. A certain amount of day to day variability plagued the method and was never really satisfactorily resolved. However, even with this, the method gave us greater accuracy than any other method we examined.

Table 3 shows that the 1 % lactate very quickly extracted the aluminum, whereas the 0.2 % lactate had extracted only about half of the aluminum after 16 days. In this study the lactate solutions were replaced after 4, 8 and 14 days. As can be noted, the aluminum content of the aorta disks remained unchanged between days 1 and 2 indicating that the lactate had equilibrated with the aluminum by the end of one day. This was found to be a consistent pattern ; consequently with the later studies, the data was plotted against number of extractions rather than number of days.

CONTINUOUS EXTRACTION WITH 0.1 % LACTATE

High levels of lactate were found to extract essentially all of the aluminum from the aorta whereas physiologically achievable levels did not do so in the time span of these studies. A continuous extraction system was therefore set up. Nine disks were placed in 90 ml of 0.1 % lactate solution in a flask. The system was designed to add and remove 1.0 ml of the lactate solution per minute, while being stirred gently with a magnetic stirrer.

Figure 1 shows that by 328 hours the maximum extraction, approximalety 90 % of the original aluminum, had been achieved and extraction for an additional 408 hours did not reduce the amount of aluminum present on the aorta disks. It appears that a small amount of the aluminum is bound more tightly to the chelating centers of the aorta tissue and so was not extracted by the 0.1 % lactate.

ALUMINUM EXTRACTION FROM FORMALDEHYDE «AGED» TISSUES

Since aluminum accumulates over an extended time period, other cross-linkages may trap the aluminum and make it inaccessible for extraction. To test this, aluminum treated aorta disks were shaken for three hours in 0.1 % formaldehyde at 37°C. Subsequently they were extracted with seven 0.5 % lactate solutions for at least 24 hours each. Figure 2 showed that the formaldehyde treatment had no effect on the rate of extraction of aluminum by the lactate. In both treatments 95 % of the aluminum had been removed by the seven extractions.

EXTRACTION OF ALUMINUM BY CHELATING AGENTS

Since physiologically normal levels of lactate removed only about 90 % of the bound aluminum from the aorta disks, other chelators with greater affinity for aluminum were evaluated. This greater affinity created problems for the assay since the chelated aluminum did not react with the aluminon reagent. A number of metal ions were tested and it was found that 10 mg of $ZnCl_2$ added to the assay solution was effective in freeing the aluminum for reaction with the aluminon reagent.

*EDTA and DTPA*

Ethylene diamine tetraacetic acid (EDTA) and diethylenetriamine pentaacetic acid (DTPA)

were tested at 0.05 % and 0.01 % for extracting aluminum from the aorta disks. Figure 3 shows that the two chelating agents were very similar in their removal of the aluminum. The 0.01 % solutions did not remove as much aluminum during the first extraction, which was shortened to four hours. Beyond this, all four of the extractions produced very similar results. After seven extractions, about 99 % of the aluminum had been removed.

DESFERAL

Desferal mesylate (Ciba-Geigy Corporation), mesylate ester of desferoxamine (Neilands, 1973), was compared to EDTA. Hog aorta were no longer available, so beef aorta were used. These are coarser, more porous and variable in thickness and weight than were the hog aorta. Figure 4 shows that the 0.01 % Desferal did not extract the aluminum as rapidly as did the 0.01 % EDTA. Even so it had completed removal of the aluminum after five extractions. In this same study 0.2 % lactate was found to remove the aluminum at a much slower rate than had been observed with hog aorta. In addition, 0.5 % procaine was found to remove aluminum at about the same rate as did the 0.2 % lactate. Procaine is used as a treatment to retard aging. (Aslan et al., 1965) and it was of interest to see if it functions as an aluminum chelator.

DISCUSSION

Methods for removal of accumulating aluminum ions from body tissues, especially aorta and brain gray matter, should prove beneficial in retarding aluminum cross-link induced aging, including the aluminum-induced deterioration of the brain.

Since cross-linkage by aluminum ions or any other cross-linking agent is a slow process, a relatively slow rate of removal of the aluminum is acceptable, and indeed may be desirable so as to avoid undesirable side effects.

In this study, lactate, a product of vigorous physical exercise, has been shown to reduce aluminum accumulations in vitro by 90 % or more. Three chelating agents, EDTA, DTPA and desferoxamine have been shown to effectively and rapidly chelate and extract aluminum accumulations. In a few days they remove the aluminum to extremely low, even undetectable levels.

EDTA has been used for some time (Lamar, 1966), perfused at the rate of 4 g. per day for 7 day periods, to remove calcium deposits in human vascular tissues. Given our results showing the rapidity of removal of aluminum from the aorta disks, at the weight equivalent of 7 grams of EDTA for a 70 kg person, it seems reasonable to presume that this treatment would also substantially reduce the body load of aluminum.

The potential benefit of lactate should not, however, be ignored. Certainly reduction of the body load of aluminum by 90 % would be highly beneficial.

A number of workers, even as far back as 1803 (Corsbruch and Ebermaier) have claimed that exercise improves health and longevity (Prout, 1972). At least a part of the improvement is attributed to a reduction in coronary diseases (Poffenbarger and Hale).

In the latter report in particular, «repeated bursts of high energy output» were found to be particularly beneficial for longevity. It is exactly such conditions that induce the body to produce and excrete lactic acid into the blood stream. This supports the view that a significant part of the benefits of exercise may be the removal of aluminum and perhaps other toxic metals from aorta, brain and the body tissues by chelate extraction with lactic acid.

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*TABLE 1.*

*Rating of Aluminum Sulfate Treated Aorta Disks Stained with Aluminon after Extraction with Chelating Solutions for 19.5 Hours.*

| Chelating Solution | Aluminon Rating[a] |
|---|---|
| EDTA | 1 |
| Sodium Salicylate | 4 |
| Sodium Malate | 2 |
| Sodium Lactate | 3 |
| Sodium Tartarate | 3 |
| Sodium Ascorbate | 4 |
| Sodium Acetyl Salicylate (aspirin) | 5 |
| Control, no aluminum | 0 |
| Control, aluminum | 6 |
| Saturated Tea, pH7.4 | Dark brown |
| Saturated Tannic Acid pH7.4 | Dark brown |
| Sodium Citrate | 1 |
| Sodium Oxalate | 1 |
| Sodium Orotate | 4 |

NOTE :

a. Rating System
0 - yellow, no aluminum detected
6 - deep red, maximum aluminum present

*TABLE 2.*

*Rating of Aluminum Sulfate Treated Aorta Disks Stained with Aluminon after Extraction with Lactate Solution.*

| | Hours Immersed | |
|---|---|---|
| | 2 | 20 |
| Treatment | Rating[a] | |
| 1 % Na Lactate | 1.0 | 0.25 |
| 0.5 % Na Lactate | 2.5 | 1.0 |
| 0.1 % Na Lactate | 3.5 | 1.5 |
| 0.05 % Na Lactate | 4.0 | 3.0 |
| 0.00 % Lactate | 5.0 | — |
| No Aluminum Treatment | 0.0 | — |

NOTE :

a. Rating System
0 - yellow, no aluminum detected
6 - deep red, maximum aluminum present

*TABLE 3.*

*Aluminum Present in Hog Aorta Disks after Extraction with Lactate.*

| | Micrograms Aluminum per Disk | | | |
|---|---|---|---|---|
| Treatment | Days 1 | 2[a] | 8[a] | 16 |
| A1 + 0 Lactate | 108 | 114 | 96 | 103 |
| A1 + 0.2 % Lactate | 77 | 86 | 59 | 41 |
| A1 + 0.5 % Lactate | 31 | 32 | 4 | 2 |
| A1 + 1.0 % Lactate | 11 | 11 | 2 | 0 |

a. Lactate extracting solution replaced, days 4, 8 and 14.

*FIGURE LEGENDS*

Figure 1. Continuous extraction of aluminum - treated hog aorta disks with 0.1 % lactate for 31 days.

Figure 2. Extraction of aluminum - treated hog aorta disks, unaged and formaldehyde aged, by 0.5 % lactate.

Figure 3. Extraction of aluminum - treated hog aorta siks by EDTA and DTPA.

Figure 4. Extraction of aluminum - treated beef aorta disks by several extracting solutions.

*SUMMARY*

Aluminum toxicity is becoming recognized as a serious long term chronic problem, with increasing evidence implicating aluminum accumulation in the brain as a cause of senile dementia.

Methods for removal of aluminum in body tissues was the objective of this study. Aluminum adsorbed to hog aorta tissues in vitro was extracted with several organic acids having chelating properties, and with several synthetic chelating agents.

EDTA and the other synthetic chelators tested rapidly removed the aluminum at levels approximating those used in clinical practice. Lactic acid removed most of the aluminum at concentrations achievable in the blood during strenuous exercise. This may prove to be an added extra benefit for exercise.

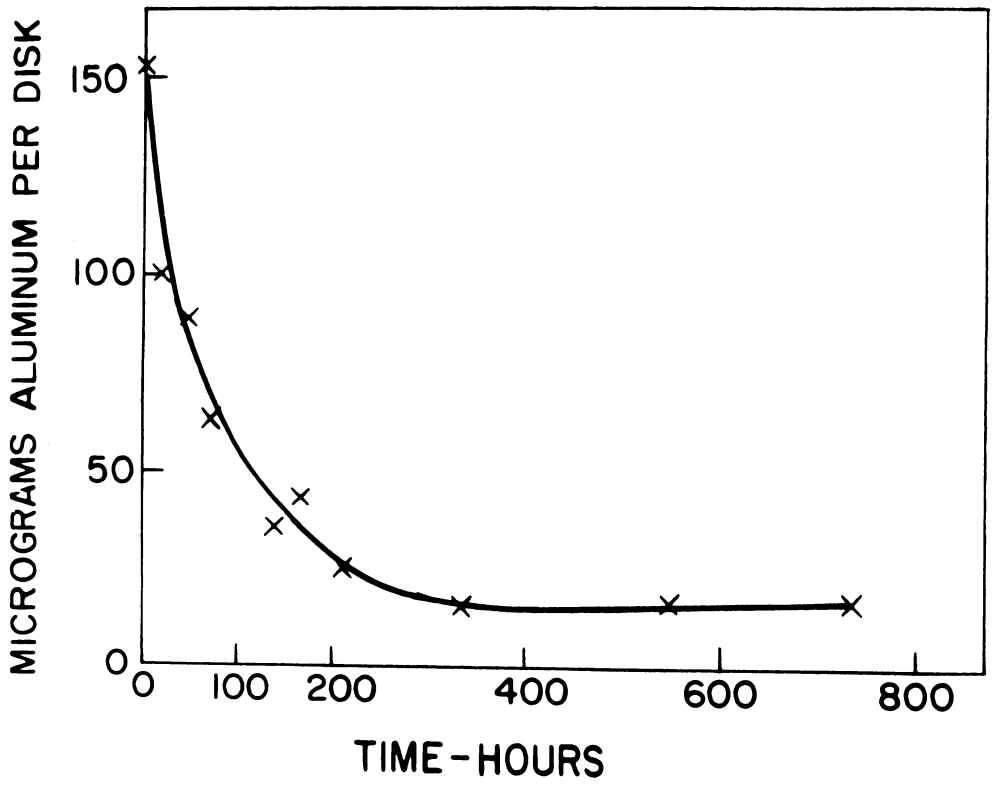


FIGURE 1. Schenk et al. Extraction of Aluminium from Aorta

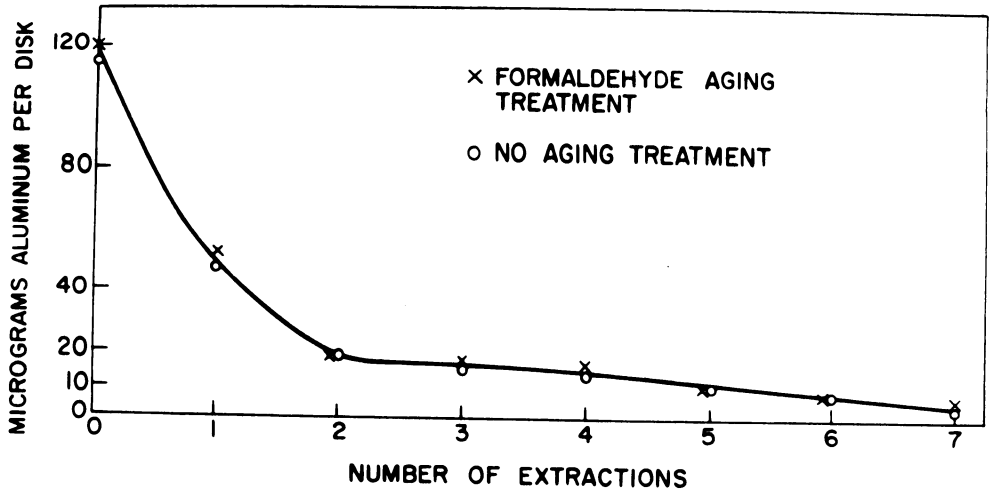


FIGURE 2. Schen et al. Extraction of Aluminium from Aorta.

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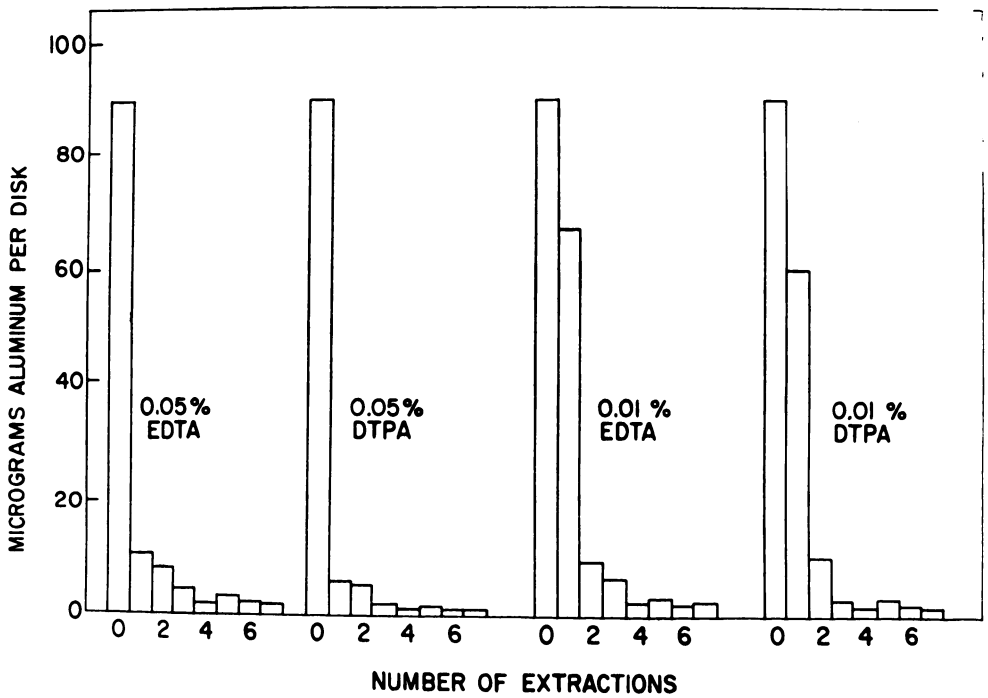


FIGURE 3. Schenk et al. Extraction of Aluminium from Aorta.

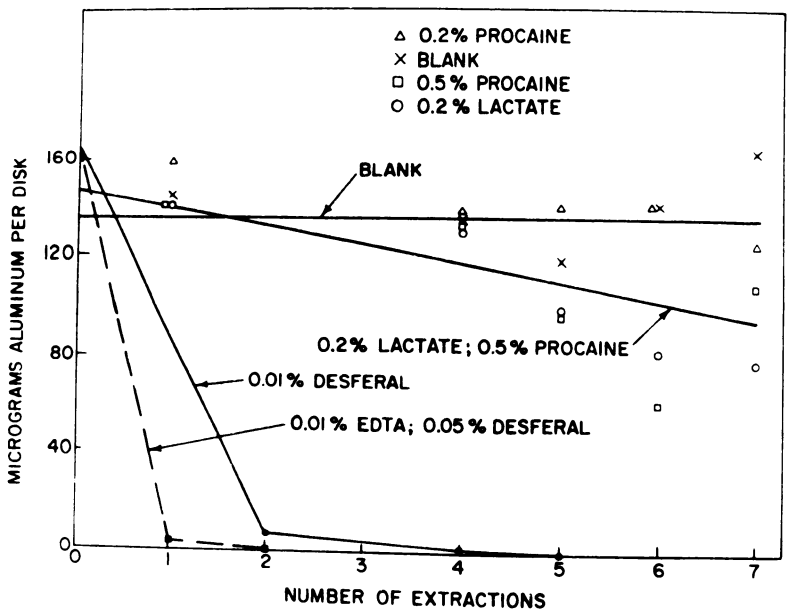


FIGURE 4. Schenk et al. Extraction of Aluminium from Aorta.

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Data reproduced below from a recently published article by Dr. R. Crapper, S. Quillkat, S. S. Krishnan, A. J. Dalton and U. Deboni (from Table I, Acta Neuropathol (Berl) 50, 19-24 (1980) confirm the increase of aluminum accumulation with age.

| Group | Age Range | Number of Brains | Number of Prep. | Al/DNA |
|---|---|---|---|---|
| Control 1 | 2 - 69 | 12 | 22 | 674 ± 395 |
| Control 2 | 53 - 69 | 5 | 7 | 716 ± 295 |
| Intermediate | 62 - 88 | 5 | 10 | 1,183 ± 949 |
| Alzheimer | 53 - 95 | 12 | 25 | 1,487 ± 1,179 |
| Pre-senile Alzheimer | 53 - 69 | 6 | 11 | 1,401 ± 721 |

These data, so far as they go, indicate that the intermediate age group has perhaps advanced half way to the insanity values.

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# THE CROSSLINKAGE THEORY OF AGING AS A PREDICTIVE INDICATOR

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## SUMMARY

**The theory that the progressive insolubilization and immobilization of proteins, nucleic acids and other large moleculas is a principal underlying cause of age dependent degeneration, was proposed in 1941-42.**

It has subsequently been rediscovered at least 4 times and has withstood the test of time. Certain predictions made on the basis of the crosslinkage theory have been fulfilled, others are gaining ground under debate, still others remain open.

Among the former groups are the predictions that :

1. **Aluminum** would be found to be an important factor in aging

2. It will prove essential to avoid overloading any one metabolic pathway so as to cause back-up of intermediate products - in other words, **many small meals are preferable to few large,** the quantity and composition of the meals being the same

3. The superior longevity of colored races from age 65 on is largely due to avoidance of systemic effects of radiation, **in other words, applying an UV screen every morning to exposed parts of the skin throughout life will result in an extended life past 65 also for non-pigmented persons**

4) The constant exposure of arterial endothelia to circulating **crosslinking agents** unavoidably present in the blood stream, will result in a loss of elasticity, and hardening. This secondarily leads to stressed parts of the endothelia forming microcracks or otherwise becoming permeable

5. Habitual exposure of unprotected eyes to **fluorescent** light will result in increased incidence and earlier appearance of senile cataracts

6. The incidence of Alzheimer disease will be found reduced in those who persistently and daily under many years have practiced an endurance type of **muscular activity,** such as walking, swimming, skiing, rowing.

## INTRODUCTION

Evolution has provided for every mammal a specific life span, long enought to keep most individuals of its species in functional health until they have bred their offspring and given it protection and an informed start in life. That this «Design Life» of species is genetically based, is apparent from the work of Hart and Setlow.[1,2] We may thus conclude that the design life of man is about 60 years. After this time, evolution is no longer concerned with us. Since man is not a hazard to his offspring, there is no reason for a mandatory genetically determined upper limit to our lives, **we are free to add or detract from the total life by maintenance and artificial renewal, or lack thereof.**

Thus, evolution has provided defenses against the progressive accumulation of injurious substances in the body, up to the specific design life of each species. After this the slow cumulative accretions and resultant injuries will reach damaging proportions, and finally lead to degeneration on a broad front unless we intercede.

This is the general scenario. Accordingly, for humans total accretions and damage are by our design controlled so as to be tolerable up to about age 60

For a mouse they are tolerable up to about age one, for a rat, on the average to about age three, for a rabbit or guinea pig somewhat longer. Evolution protects these small mammals up to age 10 at the most. Accordingly, key molecular events that require between 10 and 60 years to become fatal are largely missed in studies with these small mammals — yet, such events remain dangerous to humans and principal factors in human degenerative disease. This limits the applicability to humans of research done with shorter lived mammals. It stresses the need for more incisive work **with humans** in the age range 50 - 60, before, in still later years, secondary effects overshadow and obscure the initiating phases.



These destructive molecular events may be fairly straightforward, as is the overall loss of elasticity due to **cross linkage,**[3,4] and its sequels. They may also be quite complex with many variants, as for example is arterial stenosis which unless countered progresses at a rate of on the average 1.4 % per year from age 40 onward.[5] These, and a host of

other detrimental molecular events co-act to cause «degenerative» ailings after our design life of about 60.

Analysis will highlight common traits in this degeneration. It has already permitted predictions, of which many have been confirmed by events.

It is self evident that any accumulation of insoluble, irremovable substances if unchecked, will ultimately become destructive to any organism.

It follows that degeneration ensues from any molecular event, which in a reaction time of up to about 60 years gives rise to even a minute percentage of such an insoluble, irremovable product, which will have accumulated to an extent which later contributes measurably to the progressive deterioration of life. In the aggregate such deterioration explains the genesis and progress of the seemingly inexorable advance of aging, along a broad front.

Too often, in weighing the probability of a reaction even an experienced reseacher thinks : «I have kept these ingredients together at accelerated conditions for a week and nothing happens. This reaction can be disregarded.» Such a conclusion is premature until we have allowed it to stand at least 20 years before analyzing. An autocatalytic reaction, which up to 20 years proceeds at a hardly perceptible rate, could already in 60 years be an important damaging factor. A safer assumption, is : **Any** molecular event which is theoretically possible under conditions compatible with life, will in 60 years actually come to pass. **Any such molecular event which theoretically can produce even a vanishingly small yield of an insoluble, irremovable substance will in 60 years actually produce such substance in a quantity capable of causing major, progressive damage.**

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This time allowance brings to our view a vast number of possible reactions. It may be assumed that given this liberal time allowance every one of these reactions will actually occur and will in a human life time, make its contribution to the accretion of insoluble, nonremovable destructive residues or changes somewhere in the organism. A corollary is that anyone who on sound theoretical grounds postulates the formation of any insoluble, biologically irremovable compound in the body, and sets out to find it, will actually succeed, assuming that he has the requisite instrumentation and the persistence and patience necessary.

**Among the large number of possible molecular events, the category of crosslinked macromolecules is dominant.**[6] In no other molecular event can as small a molecule cause so great a change in physical, chemical and biological properties. Potential reactants are abundant in every living cell. [7-11] Simplest of these is ozone, ubiquitous in the atmosphere, thoroughly explored and documented in rubber industry for its crosslinking action on automobile tires, [12] but of course equally reactive not only with the long chain of the vitamin A molecule, **but with every unsaturated compound in human metabolism.**

A single molecule as small as formaldehyde, can crosslink ADNA. [13] If the strands of DNA crosslinked, the cell can seldom survive the next mitosis. [14] In fact, all polyfunctional reactive organic molecules including numerous metabolic intermediates, given enough time, can crosslink any protein or nucleic acid to any other reactive molecule or to itself to form a loop, or it could unite two strands irreversibly or to another giant molecule in an inexhaustible multiplicity of variations [15-18]

Many metals, generally found in small quantities in human blood [19] can be similarly reactive. **Cadmium and Aluminum, which accumulate with age, are typical exampes.** [20-22] In comparison to the enormous scope and variety of the crosslinkage products, the other potentially gerogenic reactions become secondary.

When one crosslinkage has been established between two giant molecules, these are fixed in the proximity of each other, and the probability of additional crosslinkages between them them is thereby logarithmically enhanced. [23] **The logarithmic characteristics of the mortality curve** [24] **are thus explained.**

The crosslinking theory of aging was conceived in 1941, [25] and stated in 1942 as follow : **«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 molecule. In either event, the result is that cumulative tanning of body proteins which we know as old age».** [26]

In the 40 years which have passed since the first thoughts along these lines, many predictions could be made, some of which have materialized while other still await resolution.

## PREDICTIONS

1. **An underlying cause of senile psychic disintegration.**
   That the ubiquitous crosslinking element **aluminum** plays a role in aging was suggested in 1955, at the Baltimore convention of the Gerontological Society. [27] It was quoted, supplemented and expanded in 1957 by Zinsser et al. [28] and followed up by subsequent papers. [29,30] Attempts to secure funds for experimental work failed at the time. T. M. Riddick strongly criticized the use of soluble Aluminum compounds

in baking powders, antacids and water «purification». [31]

In a series of brilliant research papers [32-41] McLachlan - Crapper and his collaborators **have conclusively proven the role of aluminum as causative factor in senile human dementia.** The role of aluminum in this, as well as in other brain syndromes has been confirmed by others. [42-48]

The crosslinker based prediction of aluminum as a general factor in disease of the circulatory system still remains unconfirmed.

However, indications exist of heart and aorta involvement [30] and the crosslinkage theory predicts a general, slow accumulation which ultimately will reach critical levels in the circulatory system as well; when the present dominating causes have been brought under control.

2. **The significance of avoiding overloading of any step in the metabolic pathways.** That caloric limitation is conducive to increased longevity was conclusively shown by McCay. [49,50] As the food intake exceeds the immediate processability in any intermediate step in any pathway, the intermediate product will back up and temporarily accumulate so that its time of existence «in transitu» is increased manifold. [51]

When this has happened, **some** diffusion of the backed-up product is unavoidable, and will have systemic effects inactivating vital molecules by crosslinking, [8,10,52-55] which may cause mutagenicity. This however is not essential for their activity[56] which mainly depends on forming insoluble, irremovable aggregates which impede intracellular transport and progressively reduce functional space within the cell. [57]

Among such crosslinking intermediates may be mentioned formaldehyde, [25,58] acetaldehyde, [59,60] glyoxal, malon aldehyde, glyceraldehyde, glutaraldehyde, [61,62] dibasic acids and their esters, [63,64] intermediate quinones [65,66] and bifunctional alkylating agents. [67] The slow acting but nonetheless cumulative crosslinking substances are still more numerous, including for example polyoxy acids, di- or polycarbonic acids already referred to, most poly halo compounds and kynurenic acid, which is a quinoline polyoxy carboxylic acid. Since many of these are metabolic intermediates which could back up, accumulate, and diffuse into general circulation particularly in case of overfeeding at any one meal, life expectancy will be more favorable for individuals having many small meals, than for those having fewer, larger meals, the composition and caloric intake being the same.

3. **Skin pigmentation - a factor in aging. Persons having pigmented skin have a longer life expectancy than persons lacking this protection.** [68,69]

It is accepted by dermatologists that skin exposed to strong light ages faster than skin not so exposed. [70-73] It was proven by Bjorksten and Collbring [74] and confirmed by Sundholm et al. [75] that the initiation of the crosslinkage process which presumably starts this chain of events, in the case of **unsaturated** fatty acids is a matter of minutes, and that the crosslinking itself proceeds steadily and slowly, usually during a time interval of many days or weeks, even in the dark. The revelant energetics have been discussed. [76] The findings of F. Sundholm and co-workers strongly indicate that the actual crosslinking process in this system depends upon photochemical **initiation** only. It then proceeds in the dark, and apparently in the absence of any free radicals as none could be detected during the time period following irradiation, when the crosslinkages were formed. This finding has general biological validity, because **unsaturated fatty acid radicals** are present as parts of the ubiquitous phospholipids. This is illustrated by Table I

**TABLE 1**

Composition of the lipid fractions of the 3 principal types of lipoproteins (after Van Gent) (77)

| Lipoprotein type | Phospho-lipid % | Choles-terol % | Trigly-ceride % |
|---|---|---|---|
| High density | 53 | 27 | 7 |
| Low density | 29 | 45 | 7 |
| Very low density | 18 | 22 | 50 |

Inasmuch as cholesterol and its esters and phospholipid easily combine in ratio 1 : 2 it can be understood that HDL lipoprotein can collect cholesterol for transport from the arterial wall, while LDL and particularly VLDL lipoprotein will dump their excess cargo of cholesterol and triglycerides at the first receptive point they find. (77-79) Since multiple crosslinkages generally cause protein molecules to orient themselves with the more hydrophilic sides facing each other, the opposite, exposed protein surfaces become more lipophilic, and thus provide the logical points of deposit. [14,15,78-80]

**The effects of radiation may well become systemic.** Since the primary effect of radiation is not in itself crosslinkage, but only the initiation of a slow progressive effect, it is expectable that a primary irradiation product might diffuse, reach the circulation, and thus have systemic effect. This might **explain why black persons have a dramatically higher life expectancy than whites after the age of 65.**

In younger age groups this effect may be

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overshadowed, but after the age of 65 it becomes apparant in the life expectancies, progressively widening the gap at least from 65 - age 84. [68,69]

**4. Osteoporosis**

Inasmuch as the bone structure is largely dependent on collagenic inner structure, [81] and this is highly sensitive to crosslinking, it is predictable that the consequent shift from hydrophilicity to hydrophobicity [14,82,83] will lead to impairment of its adhesive union with the bone hydroxyapatite. [84-86] The failure of this union in turn will be reflected in loss of elasticity and impact resistance of the bone, and in the consequent reduction of total bound calcium. Meema HE and Meema S have shown that **the female sex hormones confer a substantially raised protection against these influences.** [87]

**5. Cancer**

The cancer problem is too multiple and complex for predictions here. **It is noteworthy, however, that carcinogens, as well as cytotoxins, generally either are crosslinkers, or have metabolites which are.** [88-91] Without wishing to enter any discussion about cancer, a negative point may be mentioned as possibly relevant. Since the cytotoxins are crosslinkers, they will accelerate a basic aging process. Since cancer incidence is logarithmically age dependent, it may be inferred that cytotoxic agents will increase the probability of new cancers and reduce the resistance to any metastases that may occur.

As to possible alternatives, the immunologic system has marshalled the forces of certain free radicals for controlled destruction of undesirable products it can be made to recognize. [92]

From this standpoint, the recent paper by Tallberg et al. seems noteworthy. [93]

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**6. Senile Cataract**

The eye is a particularly suitable focal point for studies of age dependant changes, because of the ease of optical observation. The etiology of senile cataract has been analyzed and discussed by JG Bellows and RT Bellows [94] who have considered its relation to **progressive crosslinkage.** Uyama, Ogino and Ichihara [65,66] have isolated quinone related compounds of high crosslinking capacity from cataracts, and from the urine of patients so afflicted.

**7. Immunology**

No further predictions are required, as it is already firmly established that immune reactions leading to precipitation or deposition of insolubles **are in fact crosslinking processes.** [95-99] That uncontrolled or excessive immune reactions lead to «amyloid» or hyalin deposits is established.

**Thymus atrophy** is an important cause of decline of the immunologic defenses. [100, 101] The most likely extrinsic cause of this atrophy is a corresponding regulatory breakdown in the hypothalamus - pineal, pituitary - neuroendocrine axis in relation to thymus. [103-104] This in turn might well have its roots in the interference with intracellular transports due to developing net - structures of macromolecules, interlinked by progressive **crosslinkage.** (p. 49. Fig. 4 of (14)) ; ((15) pp. 553-554)

**8. Exercise**

That certain exercise is beneficial for health is no longer doubted. [105-112] This has mostly been acribed to improvements in circulation and in muscle tonus.

It is an established fact that the lactic acid or lactate content of blood is about doubled during the time of moderate muscular exertion, and declines to the normal level abruptly, in a few minutes upon cessation of the muscular action. [113] **Since lactic acid is a fair-to-good chelating agent** [114] **it may help remove from the system potentially crosslinking aluminum, cadmium, mercury, lead, arsenic and excess iron, among others, thereby increasing longevity if depletion of the needed chelatable metals manganese, cobalt, zinc, iron and perhaps molybdenum is counteracted by supplemental medication or a couple quarts daily of skim milk.**

The time x mass product of circulating elevated lactic acid is more dependent on the **duration** of muscular exertion than on its **intensity.** Therefore it is predictable that the beneficial effects of exercise on longevity will be found to be highest in endurance type activities such as walking, jogging, rowing, skiing and swimming, where an elevated lactic acid content of blood is maintained regularly and for a considerable time. That this in indeed the case is evidenced by Rönnemaa et al. [115] and the further references they cite.

**9. Athero - arterioscleroses**

Space precludes dealing with this subject at the length it would deserve.

**Blood normally contains a multitude of crosslinking agents, as does the organism generally.** [116-122] In addition, fats oxidized non-enzymatically particularly when carried in arterial blood, will give rise to lower aldehydes, which are powerful crosslinkers. [123-126]

Crosslinking has the primary effect of reducing elasticity, by imparting to the crosslinked material rigidity and hardness which eventually may lead to ruptures or intercellular leakage [58,116,128] of the endothelium. As already mentioned, [14,15] crosslinking also alters the protein surface properties from hydrophilic to oleophilic. This may lead to subendothelial lipid accumulation, which together with cell proliferation and ingrowth also may rupture the endothelium or otherwise secondarily initiate atherae [80,129,130]

In 1955 Dr. H. Gottlieb suggested that the first visible plaque initiating event might be

a microrupture of the endothelium in circulatory organs at points of maximal hydrostatic pressure. [131] We attempted to verify this and succeeded in many cases, but not sufficiently regularly and clearly to justify publication at the time. Being limited in time and equipment with a $ 5000.00/year grant from the Air Force as the only outside support, we could not persist in this study. (p 7 of (132))

It was established by Friedman et al., [133] Fishman et al. [134] and Constantinides and Wiggers [135] that the first observable change in the initiation of arterial plaques, is an increase of permability of the endothelium. This matter is still being pursued by many large groups with increasingly sophisticated equipment. In view of the obvious continuous exposure of the intima to circulating crosslinking agents, it appears certain that this, as well as every other of the theoretically plausible mechanisms postulated will be proven to actually take place within the human life span.

**10. Longevity**

**Those organisms which live interminably** are those wich can add continually to their mass so as to maintain a constant ratio with the insoluble irremovable aggregates which are unavoidably formed. These include bacteria, malignant cells, perhaps some water supported fishes reptiles and possibly giant squids which can grow in size without having to meet the problem of supporting a steadily increasing bulk and weight.

**To challenge the exclusivity of this polycentenary company, man would have to find artificial means for dissolving or removing those gerogenic aggregations which cannot be removed or assimilated by man's natural resources.** (p 357 of ref (7) ; (8)

It is predicted that when such a life span is attained by man, it will be by the application of at least two of the following three approaches : [136]

1. Chelation, to control and break down metal crosslinked macromolecular aggregates. (pp 480.481 of (30))
2. Low molecular enzymes, enzyme fragments or precursors to break down specific types of organic aggregates.
3. Free hydroxy radicals which are destructive to :
   a. Hyaluronic acid [137-142]
   b. Carbohydrates, including starch and cellulose [143]
   c. An enzyme [144]
   d. Proteins and polypeptides [146-148]
   e. Synthetic polymers [145]
   f. Gerogenic, nitrogenous insoluble aggregate [136]

In view of the above, and of CWM Wilson's studies on the **ascorbic acid** equilibration between tissues and lymphocytes in inflammatory situations [149-151] and of Babior's studies of the sudden energy bursts in microbial killing by phagocytes [152] and the 133 references in that area assembled by Babior, it is indicated **that the free hydroxyl radicals are a principal vehicle for destroying organic foreign bodies in the human organisms.** [136]

**However, they cannot alone cope with metallic crosslinkages, and completely unshackled they would be too destructive.** We need to work out methods for removing some of the present physiological residues and yet retain a modulatable control of the therapy. A combination of all of the 3 above approaches and possibly still others, may be the way to move toward a breakthrough in the major extension of healthful life.

## CONCLUSION

Many other theories have come and gone in the 40 years which have passed since the crosslinkage theory of aging took form. **The crosslinkage theory is unique in meeting all of the criteria for a valid aging theory** - and in standing the test of time together with those other theories which build on crosslinkage as a basic premise, such as the cybernetic theory, [153] the free radical theory, [154] the immunologic theory [155-157] and the diffusion theory. [158]

**In the past 40 years the crosslinkage theory has been re-discovered at least four times.** [158-161] **It covers the known facts and has made possible predictions, some of which have already been confirmed.**

May 1980
Johan Bjorksten

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**Our Journal urges all the people of the world to send any ideas, suggestions and recommendations (of any form they choose) concerning the way of possible artificial influencing the aging processes and the way of the achieving of the rapid and effective solving the problem of the artificial posponing of the natural (specific) limits of the onset of senility and death and of an appropriate life-prolongation of humans.**

**Paying the authors of such recommendations is not provided for.**

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Cross-Linking Related Dehydration

H. Malcolm Hodkinson deals with the predictability of causes of death in an article entitled "Value of Admission Protocol in Elderly Patients" J. Amer. Geriatrics Soc. 29, No. 5, 1981. On page 209 of that article, Dr. Hodgkinson states that in his geriatric patients a strong relation exists between urea concentration and life expectancy. This is ascribed not only to kidney problems but particularly to the failure of tissues to retain water. Dr. Hodkinson comments that urea concentration is an important indication of "dehydration, which is commonly an accompaniment of severe illness in old age".

Dehydration is a principal result of progressive crosslinkage of tissue proteins (J. Am. Geriatrics Soc. 10 (1962) p. 127).

This is one more significant observation which supports the concept of the insidious, slow action of the great number of cross-linking agents normally present in the organism.

# THE MULTIPLICITY OF CAUSES OF DEATH AS A DETERMINANT OF LONGEVITY

## Introduction

In seeking to fathom the path nature might have taken to achieve a certain metabolic result it is sometimes useful to ask oneself: "If I were called upon to get this end result how would I go about it?" So in this spirit we may ask ourselves: "If I were to produce a control mechanism designed to terminate the specific human life span after a certain age, how would I go about it?"

In searching for an answer, I recalled a story I had heard in the past. I was told that Alfonso X, King of Castilia and Leon (1311-1350) was often asked for advise by other rulers. Because of his good counsels he was surnamed The Wise. On one occasion he made the remark: "If God had asked me for advice before creating the world, I could have suggested to Him several useful simplifications."

And so, like Alfonso X, my answer would be: "Look for simplicity". The way of evolution and of nature is to favor simple solutions over the complex. However, before attempting to formulate a conclusion, we may explore some plausible alternatives.

The words of Oliver Wendell Holmes come to mind: "I would not give a fig for simplicity before complexity, but I would give my life

for simplicity after complexity". At least a hope of finding such simplicity is now at hand.

I. Is there a pre-determined time of death?

The ATP (Adenosine Tri Phosphate) energetics seem to meet the criterion of maximum simplicity. The extreme interconnection of the energy control systems favors a multiple-choice situation. It is not well suited for overall control or for the development of clear cut, single purpose situations. Because of their broad scope, the ATP systems deserve careful consideration.

An opening wedge resulted from Peter Mitchell's two monumental discoveries: Vector transmission ion flow, and charge separation during electron flow or hydrolysis of ATP[M1]. This was followed in the mid seventies by the recognition and subsequent application of the pairing principle in the grand design of bioenergetics by David E. Green and his co-workers[G1,G2,B1]. The pairing principle is a key to the simplicity after complexity in that it limits the otherwise seemingly infinite possibilities to a few logical paths, which a mind can hold and manipulate.

To exemplify, the coupling of reactions is necessary because it is the only way to ensure split second timing of reaction sequences in relation to each other. Like a modern gearshift, the sequence of contacts is mechanically secured, so is the sequence and relative timing of reactions in a coupled sequence. Therefore, as D. E. Green points out, the cytochrome system is so constituted that all of its

components must be present at the same time, or the system will not work. Nothing is then left to chance. The couplings obey simple numerical rules which govern the formation and utilization ATP. Elimination of any component would have fatal consequences. It is a kind of all or nothing phenomenon.

The question has been raised: which component is the weakest link? An answer to that question is elusive, because in this cytochrome-ATP system failure of any component would be fatal. Where no one point of the system shows a clear cut advantage, it seems less likely that evolutional forces could have operated with the precision necessary for development of a specific life-length control system.

Phosphofructokinase - a possible pivot

Phosphofructokinase decline likewise seems a possible cause of death. In the principal energy producing pathway from carbohydrates to carbon dioxide, energy and water, phosphofructokinase occupies a strategic position (Figure 1). Further down the chain branching becomes accentuated, and multiple choices occur. But a bind at the phosphofructokinase step could stop the energy flow when carbohydrates are the food ingested[M2,L1,P1,P2,P3,P4].

Phosphofructokinase activity does decline with aging. Therefore this enzyme system seems to have all the qualifications for a central mechanism for causing death at a controlled point - only no such deaths have been observed. This could be taken as evidence that evolution does not bother to kill the individual organisms at a certain age -

because they die and get out of the way in any event due to natural time dependent wearing out or errors, thus obviating the need for special provisions for killing.

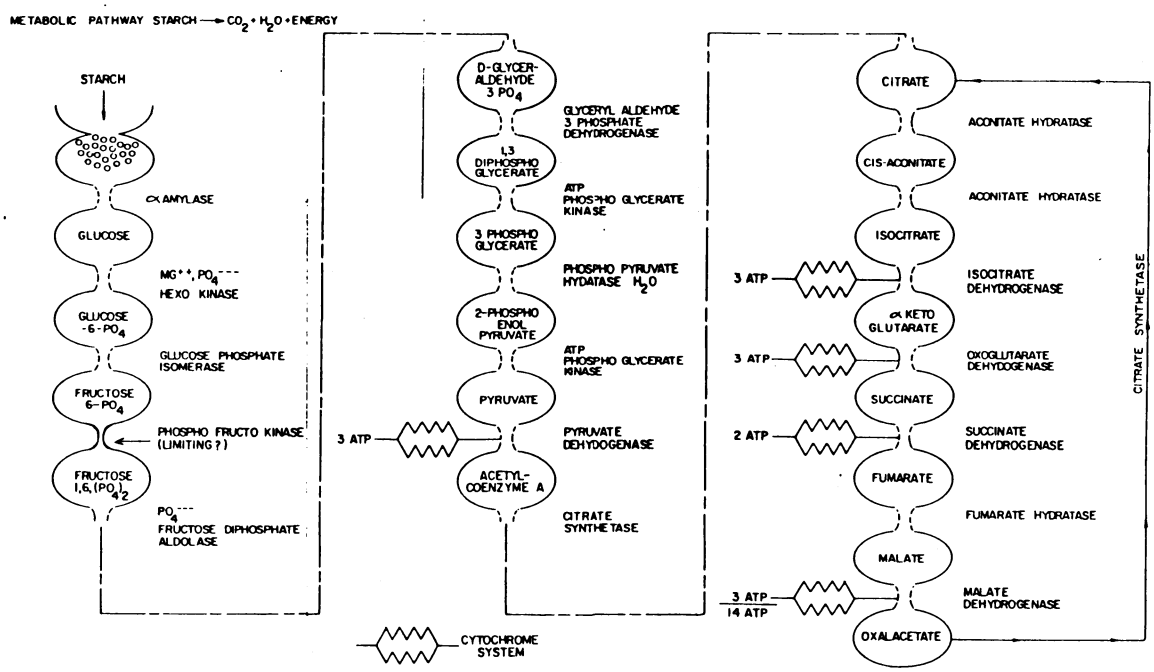


Figure 1. Metabolic Pathway of Carbohydrate Metabolism Showing Location of Potentially Limiting Phosphofructokinase

This concept is entirely compatible with the views of R. C. Adelman, who stresses the age dependent changes in hormones and energy adaptations[A1,A2].

L. V. Komarov in the Soviet literature expresses the belief that death is genetically programmed unless an earlier random death intervenes[K1].

The possibility also exists that phosphofructokinase actuated deaths may occur now and then, but not often enough to have attracted notice.

II. Multiple unconnected death causes

Every clinician knows that death can have at least as many immediate causes as there are organ systems in the human body. These are clearly very different from each other. Their common denominator is the energy needs. But as yet no clear evidence of a specific longevity regulator has been found. Perhaps there is none. Rather we have the slow, gradual loss of activity predicted by the irreversible cross-linking of macromolecules.

Another question of great theoretical significance is: Is there any system which operates independently of ATP or energy supply and which would suffice to terminate all higher life independently within a finite time limit?

Aluminum

One such substance could be aluminum. Aluminum constitutes 8.4% of earth's crust and is unavoidably present in almost everything we

eat.

The accumulation of aluminum has been shown to take place at a steady rate and to cause death under Alzheimer syndrome-like symptoms [A3,C1,U1] if allowed to continue long enough. This would suffice to account for 100% mortality in a timespan not substantially in excess of 120 years, if every other form of death were counteracted. An underlying reason for this is two-fold:

1. Aluminum is a very strong flocculant, it causes flocculation or coagulation of colloidal suspensions. The human brain is essentially a colloidal suspension where the ionic equilibrium is greatly disturbed by the presence of aluminum. Aluminum has 600 times the coagulant power of sodium chloride[R1].

2. Aluminum is a powerful crosslinking agent and as such leads to the attachment and immobilization of needed proteins[O1]. The critical absorption of aluminum is based on this. Additional reasons have been discussed in detail (pages 214-222).

Harvey W. Wiley, M.D., Director of the Precursor of the United States Office of Drug Administration from 1906 to 1912, and pioneer in health and pollution prevention, wrote: "From the earliest days of food regulation, the use of aluminum sulphate in the food industries had been condemned. It is recognized in all countries as a poison and deleterious substance[W1]".

For detailed discussion reference is made to the many clinical papers of Crapper and Alfrey and their co-workers (see e.g. ref. C1

and A3 cited above) and the recent summarizing paper by Bjorksten included in this book (page 214).

Dr. Wiley was thus keenly aware of the hazards of water soluble aluminum, but did not connect aluminum with the aging syndrome. This was first done by Bjorksten who presented it at the Baltimore meeting of the Gerontological Society in 1955 and published it three years later[B2]. H. H. Zinsser, Jr. who was present at the 1955 meeting added data in 1957. The collaboration between Zinsser and Bjorksten resulted in a paper by Zinsser, Bjorksten and their co-workers, (pages 45-69[Z1]). That study included X-ray spectrographic evidence of aluminum in aging.

Funds for an extended study of aluminum in aging were refused, and the matter was dropped for lack of funds. The breakthrough came by the brilliant work of D. R. Crapper-McLachlan and the painstaking, careful and crucial studies of Alfrey, which opened this field. This work is discussed and referred to in the article on pages 214-218 of this book.

This article cites and discusses 152 relevant papers which leave no reasonable doubt about the serious problems posed by aluminum. From Alfrey's work with human patients in conjunction with other work, it can be inferred that aluminum is practically unavoidable and will accumulate in about 80 - 100 years, which should give severe neurological symptoms, and which can be extrapolated to the conclusion that in the course of normal life, the intraneural aluminum will reach lethal concentrations if allowed to continue substantially longer.

It is easy to extract the major part of tissue aluminum, but so far nobody has succeeded in complete removal of intranuclear aluminum from living aged tissue. It appears that chelation alone will not free hexadentately bound intracellular aluminum.

When sclerotic and circulatory diseases and malignancies are all cured or prevented, aluminum could become the next major life terminating scourge.

The organics

Equally important even though more difficult to come to grips with, is the large field of insoluble, irremovable organic polymeric substances. Of these the best recognized are the hyalins, amyloids and lipofuscins, end products of progressive crosslinkage with anything co-reactive that happens to come within reactive reach.

Any reaction product which can be formed in a lifetime and which cannot be removed can be expected to occur and to contribute to aging. Any compound which theoretically can be formed in a reaction time up to 100 years and more, and which the organism cannot in any way remove, will slowly accumulate and impede intracellular transport. The number of chemical entities thus formed in a lifetime staggers the imagination. Few of these products have been defined. Anyone who sets out to find any one particular compound theoretically possible in these aggregations will actually succeed if he has infinite time, infinite patience and good microanalytical facilities. None of these compounds is alone critical. Together they set a limit for human life.

Outlook

For the organic aggregates, there is good hope.

Many authors cited above (pages 139-142) have broken down in vitro such representative polymers as proteins, hyaluronic acid, starches, acrylic resins, and even residues from exhaustive multi-enzyme extraction of gerogenic insolubles. This was done using free hydroxyl radicals generated under conditions compatible with life. Apparently human lymphocytes are able to use these free radicals to kill invaders and break down insolubles without detriment to themselves.

If this destructive force can be handled even as well as our lymphocytes seem to be able to do, we should be a long way toward control of gerogenic organics.

It is much easier to follow aluminum analytically and data on humans are now plentiful. The reversal of the process has not succeeded so far. Aluminum is unique in the firmness of its bonds when hexadentately fixed in the nuclei of a cell. Possibly a combination of many techniques might lead to at least partial extraction. Prevention seems impossible because of the ubiquity of this element.

It will not be easy to clear this hurdle which now seems to limit human lifespan. Howevever, seeing how much the concentrated will and resources of humanity has accomplished in a few decades, we must be optimistic. And so, as you see, we have waded through an ocean of great complication and detail, where many of our colleagues

are still floating around. We have come through this, I believe, to the Simplicity After Complexity for which Oliver Wendell Holmes said he would give his life. I have given most of mine. But before summing up I want to see the solution to the crosslinked polymer problem and the separate and distinct aluminum problem, so longevity will have a decent stretch of clear sailing.

Johan A. Bjorksten
May 15, 1981

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# THE SUM OF IT ALL

## Premises

This is the scenario of the aging process as I see it 40 years after my first gerontological paper.

There is no programmed death. Rather the factors causing death are numerous. Underlying this is LeCompte's Law: The rate of aging is inversely proportional to the number of deficiencies and to their severity[1]. By deficiency I mean anything that would benefit health if present to a higher degree or in larger quantity.

Of the present biggest killers, scleroses and heart disease can be largely prevented by diet, and malignancies by avoidance of known carcinogens and increasing the margins of tolerance by high Vitamin A and C intake.

Since these are problems with relatively well known solutions we will bypass them for now, and given particular attention to problems looming large without adequate solution. Behind these present principal killing diseases now loom the hazards of aluminum and unscheduled random crosslinking.

## The two primary unsolved hazards

The aluminum hazard is easily quantitated and proven by well known inorganic methods. It cannot be prevented because aluminum is present virtually everywhere. Approximately 8.4% of the earth's crust is aluminum. Given enough time, it will reach fatal levels.

Recent research appears to show conclusively that much of the senile neurologic disturbances diagnosed loosely as neurological "sclerosis" or as "atrophy of the brain" are caused by aluminum probably through crosslinkage and immobilization of the protein-lipid matrix of the brain cells.

The role of aluminum in osteoid formation in osteoporosis has been confirmed by independent investigators[2,3]. The bones appear to be the body's main line of defense in protecting the brain from the damaging effects of aluminum, but they themselves suffer from this increased aluminum. This progressive destructive potency of aluminum has been documented above (pages 214-230).

Crosslinkage

The other principal hazard is uncontrolled random crosslinkages (page 9). These also gain on us and reach fatal concentrations in 80 years $\pm$ 25%. Slowly accumulating progressive crosslinking seems fully as great a hazard as aluminum, and it functions as independently of aluminum as sclerosis is independent of cancer.

It could be that in a foreseeable time atherosclerosis and malignancies will go the way of small pox and plague and that aluminosis and crosslinkage will aspire to their place as the major cause of age related deaths. Unfortunately much of the crosslinking effects have been overlooked because of analytical difficulties.

Anything capable of reacting with two or more polar groups is a potential crosslinking agent. These agents are capable of reacting with any protein or with each other and with any combination of these,

also with polyfunctional cellulose, with other carbohydrates, and indeed with anything at all that has two strongly polar groups. Hundreds of thousands of combinations thus exist of components for the aggregates which accumulate on aging. This enormous multipicity explains why these aggregates, e.g. hyalins, amyloids and lipofuscins are all of variable composition. They are formed by chance, random contacts between bi- or polyreactive substances; as a result no two are alike. And, given a time of 60 years and more for these reactions to take place, a very large number of different polymer aggregates will result. So anyone who looks for a particular product and who has the needed facilities, patience and analytical skill can succeed in finding whatever structure he is looking for.

The amyloids, hyalins,lipofuscins, etc., are merely "tops of icebergs". Before these become conspicuous there have been precursors. These precursors are still submicroscopic, but they are already capable of interfering with intracellular transport.

The important thing in lipofuscin is not the fluorescence which attracts the eye. Before the fluorescence has developed, there have been structures capable of resisting the body defenses and of growing by addition of new bi- or polyfunctional reactants. Thus the first step is the crosslinking or the electronic activation which often but not always precedes it.

Free radicals

Free radical reactions are a special case of crosslinking as well as of fission. However, fission reaction products have

inherently lower molecular weight than the starting materials and therefore of subordinate, if any, interest in gerontology. The crosslinking products in contradistinction prominently include a great multitude of irremovable insoluble products and of their precursors.

The many other ways in which such polymer intermediates are formed have been discussed (pages 144-153). For example, when an unsaturated fatty acid is exposed to free radicals it is induced to polymerize. However, a similarly progressive polymerization is induced by the addition of formaldehyde or other low molecular aldehydes. Formaldehyde is formed in the body in at least 8 metabolic reactions. Any leakage of this aldehyde, or of any one of a large number of alternative crosslinking agents also leads to a similar aging reactions. Free radicals are a special case of crosslinking: important - yes, unique - no.

Derivatives of the primary processes

The other aging processes are second, third or higher order derivatives of crosslinking. These include the metabolic disturbances, the glandular irregularities, the hormone effects, the decline of the immunologic systems and the reduced response to activators of various kinds.

Preventive possibilities

How can we use this knowledge? To prevent crosslinkages is impossible because they are necessary parts of all life. It seems

possible, however, to slow the rate at which crosslinkages occur and to break down the crosslinked aggregates after they are formed and in this way rejuvenate the body. The means for this are potent and not discriminating (pages 180-183). The immune system of our bodies possesses means to generate free hydroxy radicals which can break down any organic substance on which it has been tried, including age generated insolubles. However, we have not yet been able to make the process selective - it will destroy also the normal and necessary tissues or cells present. The path which seems open to us is to use free radicals to break down everything, but so slowly that the normal tissues can be rebuilt, while abnormal aggregates stay destroyed.

With aluminum, this may not work and our efforts may require the combination of several power sources, both chemical and physical, to surmount the energy thresholds which now bar the complete removal of aluminum from neurons.

It will take time and much work to overcome all of the obstacles, which are now clearly visible and understood. However, every forward step along this path will bring us closer to maximum longevity in good health.

Johan Bjorksten
July 1,1981

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# AUTHOR INDEX

Dr. Donald Glaser,

With pleasant recollection of
our conversation in Williamsburg
at the convention ~3 years ago -

Stan Spiegelstein