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The Effect of Aging upon Collagen Synthesis and Reutilization

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THE EFFECT OF AGING UPON COLLAGEN SYNTHESIS AND REUTILIZATION

by

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Notable difficulty was encountered in attempting to find a suitable expression of gratitude toward the vast number of faculty members and friends who have given so freely of their time and energy. Ordinary limits here imposed by custom and human memory prevent listing by name all of the individuals to whom I am so indebted. Realizing this I wish to take the present opportunity to thank these individuals en mass and to express my heartfelt appreciation to each one of them. More direct expressions of this debt of gratitude have been long overdue. Acknowledgement of this debt would not be completely honest without the public expression of gratitude for the patience and understanding demonstrated by these individuals and by my wife and children.

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INTRODUCTION AND REVIEW

GENERAL EFFECTS OF AGING

After defining aging as a change in living systems due to the passage of time, Strehler (1962) asks how does one establish 1) the order of events in a time sequence and 2) the direction of time change. In short he appears to question the order of events and their pattern of occurrence. These changes are considered in the individual as a gradual process separate from disease (Comfort, 1951) and should be seen universally within the species. Multiple functional changes have been reported in specific organ systems during the aging process.

Visible changes in the skin are a generally noted indication of aging in man (Olansky, 1961). Old skin tends to show a decreased elasticity and an increased tendency toward neoplastic changes. The thickness of the epidermis is decreased as are the size of the epithelial pegs and dermal papillae. Healing occurs at a slower rate in "old skin".

The digestive system shows signs of aging in the normal patterns of tooth substance loss and diminished sensitivity to taste and smell. Secretion of gastric hydrochloric acid and digestive juices tend to decrease (Polland, 1933). Further gross effects of aging in the gastro-intestinal system are reviewed extensively by Ivy (1942).

The circulatory system shows a decrease in cardiac output (Strehler, 1959) and a change in the walls of the blood vessels (Landowne, 1958; Woerner, 1959). The extent to which these changes represent aging rather superimposed disease is questionable.

Aging processes can be seen to have an effect upon the nervous system. The speed of maximum conduction of a propagated nerve impulse decreases with age (Norris et al., 1953). Sensory functions, usually considered secondary to nervous system function, are seen to decrease with advancing age. This is reflected in the loss of hearing (Weiss, 1959; Beasley, 1940) decreased visual acuity (Friedenwald, 1942), loss of accomodation (Duane, 1931; Bernstein, 1945) and a decreased rate of performance of integrated nervous system functions (Jerome, 1959; Willoughby, 1929).

Respiratory system studies show decreased maximum breathing rate and vital capacity (Norris et al., 1955, 1956) combined with a decreased basal metabolic rate.

COLLAGEN STRUCTURE

Collagen, essentially an extracellular protein, represents the major protein of the extracellular substance of all vertebrates and most of the invertebrate phyla. The wide occurrence and distribution of collagen in the mammal serves to point out that the function of collagen may vary in different tissues (Piez and Likins, 1960). Collagen is a major component of skin, tendons, ligaments, and the calcified matrix of bone, dentin, and cementum.

Analysis of the amino acid composition of collagen (Eastoe and Leach, 1958) facilitated by the use of ion exchange chromatography (Moore and

Stein, 1951; Piez and Eagle, 1958; Spackman et al., 1958) reveals a characteristically high content of glycine, proline, and hydroxyproline along with the presence of hydroxylysine. In addition collagen has an unusually low concentration of aromatic and sulphur containing amino acids. Collagen from various tissues appear to be identical in amino acid composition except for the concentration of lysine and hydroxylysine (Piez and Likins, 1960). Mammalian collagens are characteristically dissolved rapidly by pepsin and collagenase but show poor solubility by trypsin and chymotrypsin.

The current concept of the intramolecular organization of this protein has been described by Rich and Crick (1958). In most instances the collagen fibrils have periodic patterns of 600 to 700 Angstroms but this typical structure is not a necessary feature of collagen (Jakus, 1956). The tropocollagen macromolecule, an ordered, specific arrangement of collagen molecules and not the collagen fibril, is the basic structural unit of the collagenous tissue and matrix (Gross et al., 1954). This molecule is currently viewed as a three-stranded helix.

RADIOACTIVE ISOTOPES

The early use of isotopes as tracers in chemical and physical processes has been described by Hevesy (1948) who reports investigators using the naturally occurring isotopes of lead and bismuth as early as 1920. This early work was primarily utilized in chemical and physical systems rather than experimental biological investigations. Although he reports later work using radium D and E as tracers for lead and bismuth distribution in animals, the way for the great expansion of the use of isotopes as tracers was opened by the discovery of artificial radioactivity by Joliot

and Curie in 1934. The nuclear energy program of World War II made possible the production of large quantities of radioactive isotopes of various elements.

Radioactive isotopes are useful as tracers due to the chemical properties of all isotopes of the same element being identical. The isotopes of a single element are identified by their physical properties which are independent of the isotopes' chemical properties. The chemical properties of an element are dependent upon the number and arrangement of the extra-nuclear, planetory electrons while the isotopes differ not in extra-nuclear structure but in intra-nuclear structure. It should be noted that the change in mass would affect theoretical considerations of reaction rates within a chemical system.

The use of tritiated organic compounds as tracer elements in biological systems is readily undertaken with the currently available tagged molecules. (Sachs, 1955) Amino acids may be tagged (or labeled) with tritium by means of a catalytic exchange (Wilzbach, 1957). This process results in a labeled compound with high specific radioactivity (Wolfgang et al., 1955). Subsequent recovery and repurification of the labeled compound is accomplished by means of electrophoretic or chromatographic separation. In the specific instance of proline the tritium labeling is confined to the ring structure in a general, random pattern.

MEASUREMENT OF RADIOACTIVITY

The four basic means of detecting and measuring radioactive emmissions are: 1) photographic emulsions, 2) ionization chambers, 3) Geiger counters, and 4) scintillation counters.

The use of photographic films, papers and emulsions as a means of detecting alpha, beta and gamma radiations result in a pattern of the distribution of the radioactive source or a measure of differential absorption of the radiation. Quantitative use of film and similar emulsions is of relatively limited application. (Doniach and Pelc, 1950; Ficq, 1960; Gross et al., 1951; Hertz, 1951; Lajtha and Oliver, 1959).

Ionization of gases by radioactive emmissions form the basis for ionization chamber function. The electrical conductivity of an enclosed gas is dependent upon the number of ions formed within the enclosed space. The energy input which results in the formation of ions within the previously non-ionized gas can then be determined from the product of the number of ions formed (as reflected by the conductivity increase) and the energy required to produce these ions. In practice this system is not 100% efficient and must, therefore, be corrected for the efficiency of the system. For any radioactive source the energy output can be expressed as the nimber of disintegrations occurring per unit time.

The Geiger-Muller counter may be viewed as an ionization chamber with concentric electrodes operating as a gas filled diode below the potential of continuous discharge. It is a triggering device in which a voltage pulse is produced by a discharge initiated by a particle detector and provides a measure of the number of ionizating encounters that have taken place within the tube per unit time.

The function of the scintillation counter depends upon the production of fluorescence in a phosphor by the absorption of ionizing radiation or

similar energy input (Harrison, as reported by Germann et al., 1954). When electrons drop into vacancies in the electron shell left by the production of ion pairs energy is liberated in the form of visible light. Early scintillation studies were conducted by direct observation os this emitted light. Present scintillation counting systems provide a means of counting the light flashes or scintillations. The scintillation counter produces a pulse with an amplitude which is proportional to the energy input and therefore provides the particle-counting and detection of the Geiger tube with the energy-measuring of the ionization chamber.

The last major discovery observed in the classical scintillation method (by direct visual observation of the scintillations) was the observation of the first man-made disintegration of an element with artificially accelerated particles. This was performed by Cockcroft and Walton in 1932 during the experimental disintegration of lithium with accelerated protons. Increased use and development of scintillation studies occurred following the development of highly sensitive, rapidly responding counting systems. Two major types of scintillation counters are presently available: 1) the photon-tube scintillation counter and 2) the photomultiplier tube scintillation counter.

Photon-tube counters consist of a system in which the scintillation phosphor and the photoelectric detector are separate units (Krebs, 1955). Changes in system design have resulted in commercially available scintillation counting systems with high area sensitivity and short resolving times (Mandeville et al., 1950; Porter, 1953).

Photomultiplier-tube counting systems, first built in 1944 by Curran and Baker, have combined the classical method with a photomultiplier for detection of the scintillations.

Further distinctions in scintillation counters can be made on the basis of the physical state of the phosphor (or phosphors) used in the system. (Raben and Bloemberger, 1951) The phosphor may be in crystal form of may be present in solution with the radioactive source. The liquid solution scintillation system, in which the phosphors are in solution with the radioactive source, has assumed an important role in the counting of low energy beta particles. Ease of operation and the large relative range of sample size which may be used are reasons for regarding the liquid scintillation counting as a method of choice in biological experiments.

Solutions that have been employed for liquid scintillation counting include: 1) p-terphenyl in toluene (Renolds et al., 1950), 2) 2,5 diphenyloxazole in toluene (Hayes et al., 1952) and 3) p-terphenyl in various mixtures of dioxane and water (Farmer and Bernstein, 1952).

AIMS OF PRESENT STUDY

The tropocollagen molecule is composed of three polypeptide chains each approximately 3000 Angstroms long and 15 Angstroms wide. This macromolecule contains approximately 1000 amino acid units of which glycine, proline, and hydroxyproline contribute a major share. Glycine constitutes about 30% (w/w) of the collagen macromolecule while proline and hydroxyproline together provide about 25% (w/w) of the total molecular weight. This study provides information about the effect of aging upon the rate of collagen formation and turnover by utilizing radioisotopic studies of

tritiated proline uptake and turnover. The uptake and retention of injected tritiated proline as reflected by its concentration in tissue samples was determined in mice of varying ages.

EXPERIMENTAL PROCEDURE

Male mice of a CF-1 strain were used throughout this study. These mice were obtained commercially and housed in large, plastic bottomed colony type cages. A balanced diet of Purina Laboratory Chow and water was provided for the mice without limit and was available to the animals at all times.

Four age groups of mice were utilized in this study: 1) three week (21 day) old mice, 2) four week (28 day) old mice, 3) eight week old mice and 4) eight month old mice. Each age group consisted of eight mice which received tritiated proline intraperitoneally and were sacrificed in two subgroups of four mice each. One subgroup of four mice was sacrificed one hour post-injection while the second subgroup was sacrificed four hours post-injection. In addition to the afore-mentioned groups two groups of ten mice each were studied for longer periods of time post-injection. These groups of mice were three weeks and eight months old, respectively. The mice in these groups were sacrificed in subgroups of two mice each at intervals of one week beginning with the first subgroup one week post-injection and continuing at weekly intervals until the last subgroup was sacrificed at five weeks post-injection.

Tritiated proline (l-proline, tritiated, purchased from Volk Radiochemical, Skokie, Illinois) was prepared in an aqueous solution with an activity of 300 microcuries per milliliter. The animals were weighed and received 6.5 microcuries per gram body weight of the tritiated proline administered intraperitoneally. To insure accurate measurement of the tritiated proline all injections were performed with tuberculin syringes

and the solutions measured to the one hundredth of a milliliter.

The animals were sacrificed by placing them in a large jar which contained a cotton pad saturated with chloroform. As soon as the mice stopped moving about they were removed from the jar and decapitated. Following decapitation the mouse head was split sagittally and placed into a 10% solution of buffered neutral formalin. In addition to the sectioned head other tissue samples were gathered and stored in the same fashion. All specimens were fixed in formalin for one week or more before being subjected to decalcification. Demineralization of the specimens was accomplished by means of formic acid decalcification using citric acid or ion exchange resin to prevent saturation of the formic acid solution by the eluted minerals. Following demineralization of the specimens, which was confirmed by roentgenographic examination of the skeletal segments present, the tissues were washed in running tap water for six hours. The specimens were then transferred to a 10% formalin solution for further storage prior to laboratory examination. Samples of skin from the superior margin of the sagittally split mouse head and sections of the gastrocnemius muscle were removed for the determination of tritium content by means of liquid scintillation counting.

Samples of tissue were prepared for liquid scintillation counting by first treating them with 10% trichloracetic acid. This was followed by washing the tissue with a solution composed of 50% (v/v) ethanol and 50% (v/v) diethyl ether. A final wash of diethyl ether was then employed. The tissues were air dried and weighed at this point. All samples were

weighed on the same Mettler analytical balance and the tissue weights recorded for subsequent use in the calculation of tritium activity. Each sample was placed into a 20 milliliter glass, screw top vial which was made of glass having a low radioactive potassium (K^{40}) content. The samples were then dissolved in one-quarter milliliter of a one molar solution of hyamine hydroxide in methanol. This process was aided by heating the vials to temperatures ranging from 58°C to 60°C for periods of 36 to 72 hours.

After the tissue samples were dissolved the vials were cooled to room temperature and ten milliliters of scintillator solution were added to each vial containing dissolved tissue and to a vial containing only hyamine hydroxide which served as a background blank. The scintillator solution was composed of: 1) 2,5 diphenyloxazole (PPO) 1.5 grams, 2) p-bis-(2,5 phenyloxazole) benzene (POPOP) 50 milligrams and 3) toluene 500 milliliters. The samples were then stored in the dark at 4°C for one day until counted. Counting was done at 4°C for a ten minute period with a Packard Tri-Carb liquid scintillation counter. All samples were counted four times. After completion of the counting 15 microcuries of tritium were added to the blank and to representative samples of tissue as an internal standard. These samples were then stored in the dark at 4°C and recounted one day later.

The data thus collected was then expressed as counts per minute per milligram of tissue and corrected for tissue quenching effect. Further calculations based upon the data derived from the use of internal standards allow the calculation of counting efficiency and conversion of counts per minute to actual disintegration rate per minute (Hayes, 1956; Davidson and Feigelson, 1957).

SUMMARY OF INJECTION AND SACRIFICE SCHEDULE INDICATING THE NUMBER OF MICE UTILIZED

length of time postinjection until sacrifice	age of mice at time of injection of tritiated proline			
	21 days	28 days	8 weeks	8 months
1 hour	4 mice	4 mice	4 mice	4 mice
4 hours	4 mice	4 mice	4 mice	4 mice
1 week	2 mice			2 mice
2 weeks	2 mice			2 mice
3 weeks	2 mice			2 mice
4 weeks	2 mice			2 mice
5 weeks	2 mice			2 mice

Figure 1

DATA AND DISCUSSION

The raw data collected from the liquid scintillation counter consisted of 10 minute counts uncorrected for background. This data was averaged using four separately determined values for each sample and further processed so as to obtain an expression of counts per minute per milligram tissue. Figures 2 and 3 present this data and the accompanying standard deviations for both the skin specimens (figure 2)and the muscle specimens (figure 3).

Use of internal standards permitted the calculation of the quenching effect of tissue present in solution. The quenching was first calculated as counts quenched per milligram tissue and then expressed as a percentage of the count obtained for the unquenched internal standard (figure 4). The average count per minute per milligram tissue for each sample was then corrected for quenching by adding to the original value the percentage of the value which represented the quenching effect. Negative quenching values would be subtracted from the original averaged counts per minute per milligram tissue.

The quenching effect of the skin specimens was calculated to be 5.6% while the muscle tissue samples were determined to have quenched the counts by -6.3%. This value for the negative quenching effect of muscle is a mean which represents a range of 1.2%.

It was difficult to explain the negative quenching effect of the muscle tissue and the first assumption was that the phosphors had been activated by the absorption of ultra violet radiation. Repeating the

AVERAGED SCINTILLATION COUNTS FROM SKIN SPECIMENS

age of mouse at injection	length of time postinjection until sacrifice	counts/minute/milligram	(standard deviation)
3 weeks	1 hour	991	(31)
	4 hours	1870	(43)
	1 week	504	(22)
	2 weeks	203	(14)
	3 weeks	58.7	(7.6)
	4 weeks	135	(12)
	5 weeks	166	(13)
4 weeks	1 hour	271	(16)
	4 hours	2630	(51)
8 weeks	1 hour	606	(25)
	4 hours	598	(24)
8 months	1 hour	83.4	(9.1)
	4 hours	1220	(35)
	1 week	237	(15)
	2 weeks	127	(11)
	3 weeks	26.9	(5.2)
	4 weeks	57.3	(7.6)
	5 weeks	59.5	(7.7)

Figure 2

AVERAGED SCINTILLATION COUNTS FROM MUSCLE SPECIMENS

age of mouse at injection	length of time postinjection until sacrifice	counts/minute/milligram	(standard deviation)
3 weeks	1 hour	2000	(45)
	4 hours	6610	(81)
	1 week	920	(30)
	2 weeks	514	(23)
	3 weeks	84.2	(9.2)
	4 weeks	86.7	(9.3)
	5 weeks	59.9	(7.7)
4 weeks	1 hour	872	(30)
	4 hours	687	(26)
8 weeks	1 hour	280	(17)
	4 hours	633	(25)
8 months	1 hour	211	(15)
	4 hours	289	(17)
	1 week	42.9	(6.5)
	2 weeks	285	(17)
	3 weeks	35.2	(5.9)
	4 weeks	236	(15)
	5 weeks	92.3	(9.6)

Figure 3

CALCULATION OF QUENCHING EFFECT

$$\text{quenching} = \frac{[(B+IS)-B] \quad [(T+IS)-T]}{Twt} \times \frac{100}{IS} \quad \text{per cent}$$

B	background blank count
IS	internal standard count
T	tissue sample count
(B+IS)	background blank with internal standard added
(T+IS)	tissue sample with internal standard added
Twt	tissue weight

sample calculation of quenching effect of skin specimen

$$\text{quenching} = \frac{(680842 - 668) - (602354 - 5968)}{2.2} \times \frac{100}{680174} \quad \text{per cent}$$

$$= 5.6\%$$

B	668 counts per minute
T	5968
(B+IS)	680842
(T+IS)	602354
Twt	2.2 mg

Figure 4

counting procedure after storage of the samples in the dark at 4° Celsius for one day resulted in essentially unchanged counts. This increase in observed scintillations in samples containing disolved muscle tissue must, therefore, be due to components of the muscle tissue present in solution.

The counts per minute per milligram tissue corrected for quenching effects are shown in figures 5 and 6. Figure 5 lists the corrected values for the skin specimens while the corrected values for the muscle specimens are given in figure 6. This data is also presented in the graphic form (figures 7 and 8) for ease of visual comparison.

From the data thus collected it appears that the uptake and retention of tritiated proline varies with the age of the animal at the time of administration of the proline. This variation is not only a function of age but is also a complex function of the composition, gross and molecular, of the specific tissue sampled in our study under the conditions of our experimental procedures. A cursory examination of the data available at the present time indicates that the replacement rate of proline decreases with increasing age, in general.

It is strongly felt that this data indicates more extensive experiments on mice or other animals should be contemplated. Further review of the experimental findings with reference to future contemplated studies has revealed that the length of time postinjection until sacrifice of the animal has a bearing upon the utilization and turnover of the injected material so that future work must be planned to minimize this effect. The use of animals with a longer life span would aid in this respect.

AVERAGED SCINTILLATION COUNTS CORRECTED FOR QUENCHING: SKIN SPECIMENS

age of mouse at injection	length of time postinjection until sacrifice	counts/minute/milligram	(standard deviation)
3 weeks	1 hour	1047	(32)
	4 hours	1980	(43)
	1 week	532	(22)
	2 weeks	215	(14)
	3 weeks	62.0	(7.6)
	4 weeks	211	(12)
	5 weeks	176	(13)
4 weeks	1 hour	286	(16)
	4 hours	2774	(51)
8 weeks	1 hour	640	(25)
	4 hours	632	(24)
8 months	1 hour	88.0	(9.1)
	4 hours	1290	(35)
	1 week	251	(15)
	2 weeks	134	(11)
	3 weeks	28.4	(5.2)
	4 weeks	60.5	(7.6)
	5 weeks	62.8	(7.7)

Figure 5

AVERAGED SCINTILLATION COUNTS CORRECTED FOR QUENCHING: MUSCLE SPECIMENS

age of mouse at injection	length of time postinjection until sacrifice	counts/minute/milligram	(standard deviation)
3 weeks	1 hour	1880	(45)
	4 hours	6192	(81)
	1 week	862	(30)
	2 weeks	481	(23)
	3 weeks	78.9	(9.2)
	4 weeks	81.2	(9.3)
	5 weeks	56.1	(7.7)
4 weeks	1 hour	817	(30)
	4 hours	644	(26)
8 weeks	1 hour	262	(17)
	4 hours	593	(25)
8 months	1 hour	198	(15)
	4 hours	280	(17)
	1 week	40.2	(6.6)
	2 weeks	267	(17)
	3 weeks	33.0	(5.9)
	4 weeks	221	(15)
	5 weeks	86.4	(9.6)

Figure 6

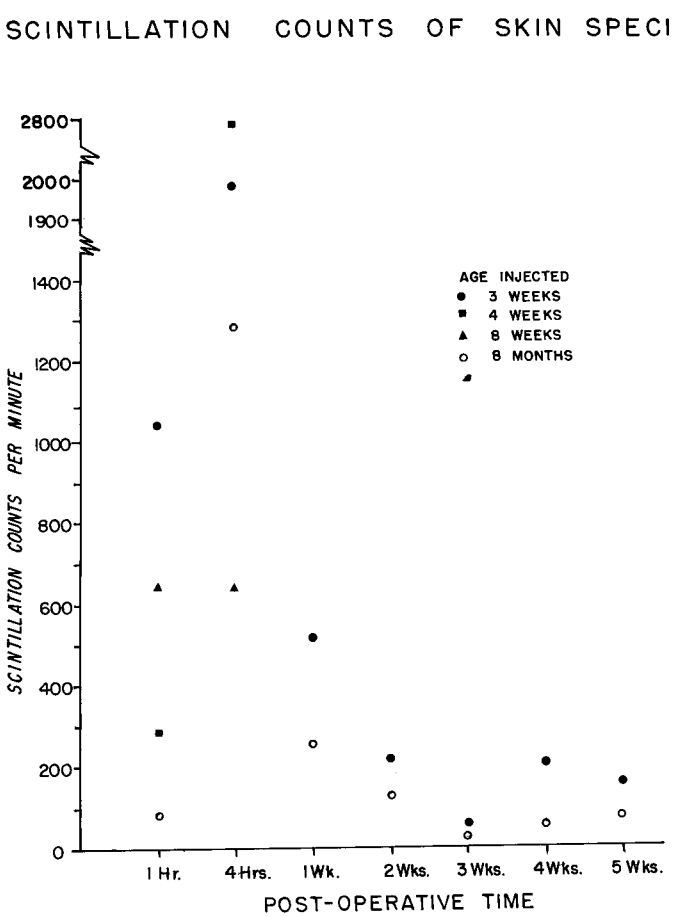
SCINTILLATION COUNTS OF SKIN SPECIMENS
SCINTILLATION COUNTS PER MINUTE
2800
2000
1900
1400
1200
1000
800
600
400
200
0
AGE INJECTED
3 WEEKS
4 WEEKS
8 WEEKS
8 MONTHS
1 Hr.
4 Hrs.
1 Wk.
2 Wks.
3 Wks.
4 Wks.
5 Wks.
POST-OPERATIVE TIME

Figure 7

SCINTILLATION COUNTS OF MUSCLE SPECIMENS

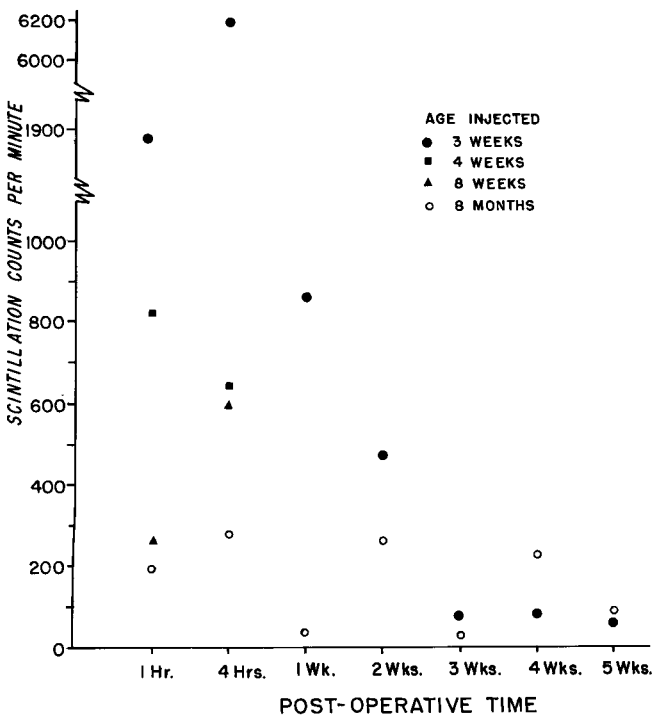

Figure 8

SUMMARY

Examination of the data obtained from the determination of tritiated proline uptake and loss as revealed by the tritium content of the tissues utilized in this study appear to indicate that the age of the mouse at the time of administration of the tritiated proline and the length of time post-administration alter the tritiated proline content of the various tissues. This variation is interpreted as a reflection of the rate of collagen formation and replacement. Patterns of rate differences require further gross and histological studies to firmly establish ratios and sites of activity. The studies represented here would indicate that the formation and replacement of collagen in the mouse decreases with increasing age of the animal. The change of rate of collagen formation and replacement does not appear to be constant when viewed in different tissues. It is expected that histological studies will reveal in more detail the sites of changing rates of formation and replacement of collagen and allow the establishment of reproducible patterns demonstrating the effects of aging upon collagen formation and replacement.

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APPROVAL SHEET

The thesis submitted by Dr. Allen Fred Goldberg has been read and approved by three members of the Department of Oral Biology.

The final copies have been examined by the Director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

Apr. 12, 1965
Date

E. W. Kapp, Ph.D
Signature of Adviser