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CHANGE WITH AGE OF UV ABSORBANCE AND FLUORESCENCE OF COLLAGEN AND ACCUMULATION OF ∈-HEXOSYLLYSINE IN COLLAGEN FROM WISTAR RATS LIVING ON DIFFERENT FOOD RESTRICTION REGIMES

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SUMMARY

Accumulation of glycation products (as revealed by the thiobarbituric test and hexosyllysine assay) and the pigmented products (350 nm UV absorbance and $370_{ex}/440_{em}$ nm fluoresence) in aortal and skin collagen was investigated under the conditions of different nutritional regimes. Four groups of animals were tested: (1) ad libitum fed controls, (2) animals which were food restricted throughout their whole life (50% food intake), (3) animals fed ad libitum during their first year of life and then food restricted and (4) animals food restricted when young and fed ad libitum from the age of 1 year onwards. It was shown that all food-restricted animals showed lower levels of glycation and pigmentation products in collagen preparations from skin and aorta. The lowest accumulation was observed in group 4 which exhibited the longest 50% survival (29.4 months, as compared with 18.3 months in normally-fed controls). Of particular interest is the fact that in this group the decreased rate of accumulation of the glycated and pigmented products was preserved even after 1 year of life, i.e., when the animals had a free access to food. Though not directly supporting the glycation theory of aging (Cerami, 1985), our data are indicative of the involvement of glucose metabolism in the ageing process. Correlation between the levels of glycated and pigmented products in aortal and skin collagen as well as the correlation between the rate of accumulation of these products and 50% survival was impossible to establish. Nevertheless, each time that food restriction was imposed on the animals it always resulted in decreased accumulation of glycated and pigmented products and increased 50% survival. Possible mechanisms for this process are discussed.

Key words: Food restriction; Glycation; Collagen; Aging

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INTRODUCTION

It has been repeatedly shown that food restriction in rodents retards the ageing process [9,16]. The evidence is based on the fact that food restriction not only increases life-span but also retards a number of age-associated biochemical and physiological changes. Recently Cerami [2] presented a hypothesis that non-enzymatic glycation of tissue proteins affected by glucose may be visualized as a mediator of aging. Though the concept is attractive, there is lack of direct evidence. A recent study [10] showed that in food-restricted rats (aged up to 6 months), blood glucose level dropped to 88.7% on average resulting in a 36% decrease in hemoglobin glycation. On the other hand, the same authors emphasize that such a reduction in plasma glucose concentration does not reflect decreased carbohydrate intake (as well as other nutrients) per unit body mass in this manner in food-restricted rodents. McCarter et al. [11] have shown that the flux of glucose through the metabolic system per unit of lean body mass is as great for food-restricted as for ad libitum-fed rats. Consequently it was proposed by Masoro [9] that the sustained reduction in plasma glucose concentration in food-restricted rats must reflect homeostatic readjustment of glucose metabolism.

In a previous communication [12] we have demonstrated that insoluble collagen from old animals and humans exhibits an increased absorption at 350 nm and an increased fluorescence at 370/440 nm. Simultaneously increased collagen glycation (thiobarbituric acid test, hexosyllysine assay) was found in old animals. The increase differs considerably when comparing collagen preparations from aorta and skin. Based on the assumption of constant blood glucose level during the life-span it appears feasible to conclude that the degree of non-enzymatic glycation reflects the time-period for which the protein is exposed to the action of sugars; this period due to increased cross-linking is likely to be extended in older animals. The UV absorbing chromophore (350 nm) as well as the fluorophore (370/440 nm) are assumed to be products of Maillard reaction in which non-enzymatically glycated lysine residues of collagen are involved, but direct evidence in this respect is missing so far.

When changes in non-enzymatic collagen glycation are followed in 2- and 24-month-old rats and in parabiotic animals of the same age, not only increased collagen glycation of old animals can be detected but also aortal and skin collagen of young animals is rapidly non-enzymatically glycated in the common milieu created in parabiotic animals. The proportion of non-enzymatically incorporated glucose approaches in the young counterparts the level found in old individuals [5].

Similar trends as with non-enzymatic glycation were found with the fluorescent product (370/440 nm) present in both categories of collagen preparations. This fluorescence was considerably increased in the young counterpart of the parabiotic couple 6 weeks after operation [5].

The above in vivo alterations of collagen can be observed also in vitro. Tanaka et al. [17] demonstrated that collagen preparations incubated in vitro with another

aldehydic sugar, ribose, exhibit not only a higher proportion of bound sugar residues but exhibit a considerably higher proportion of polymerized collagen α-chains in comparison with the controls. Non-enzymatic glycation was shown to result in decreased solubility of the protein and changes in mechanical properties similar to aging. In vitro glycation was shown to result in collagen polymerization through yet unidentified intermediates of the glycation reaction. Because collagens modified in this way exhibit a higher denaturation temperature it can be concluded that the tertiary structure is stabilized through glycation.

Non-enzymatic glycation is started by a reducing sugar reacting through its aldehyde group with a primary amino group of a protein i.e., either an *N*-terminal amino group or an $\in$-amino group of a lysine residue. The beginning of such a reaction is quite similar with that resulting in lysinonorleucine. Next comes the Amadori rearrangement followed by the formation of coloured and/or fluorescent condensation products, which appear as favourable candidates for the presumptive cross-link formations. The reactions involved are unfortunately not quite elucidated in spite of the effort put into this field by food chemists (non-enzymatic browning and the so called Maillard reaction (for review see Ref. 7)). Therefore, without going into the chemical aspects of the problem, one is limited to assaying hexoses bound through the aldimine or assaying one of the presumptive products, hexosyllysine.

With regard to the glycation hypothesis of aging there are several questions to be answered, the main being whether restricted food intake may result in a difference in the extent of generation of advanced glycation end products (AGEs) in long-lived proteins such as crystallin or collagen. The aging process could be influenced by glucose other than through the glycation pathway. Glycation changes could be merely be viewed coincidental rather than representing one of the causes of the aging process.

In the present communication we summarize data obtained with laboratory rats having limited food access either during the first or second year of their life or which were food restricted through their whole life with regard to life expectancy.

MATERIALS AND METHODS

Animals

Wistar rats were kept in separate cages each populated with a single animal. Undernourished animals were given exactly one half of the amount of food consumed by controls. The experimental animals were divided into four groups. Control animals were fed ad libitum throughout their whole life (group No. 1) contrary to animals in group No. 2 which were food restricted throughout their whole life. Group No. 3 animals were fully fed when young (until 1 year old) and then food restricted. In group No. 4 the regime was reversed, animals were food restricted when young (until 1 year old) and then fed normally afterwards. Throughout the paper the term 'food restricted' is used to denote 'food restricted to 50% of the ad libitum intake'.

Within the group two sets of animals were formed. The first set comprising 25 animals in each group was used for measuring the survival curves, the second set of animals (35 in each group) was used for the determination of HMF, hexosyllysine and absorbance and fluorescence properties of collagen.

Tissue investigated

Segments of thoracic aorta (2—4 cm long) or samples of dorsla skin (4 × 4 cm) were excised, freed from extraneous tissues, flushed with isotonic saline and defatted by successive immersions in acetone and ether for 18 h, dried for 24 h in vacuo and weighed.

Solubilization of collagen by autoclaving

Collagen was separated by autoclaving each aortal specimen in 5 ml distilled water for 18 h at 103.5 N/m^2 and decanting the extract. The amount of collagen in each extract was determined from its hydroxyproline content following hydrolysis in 6 mol/l HCl in sealed tubes at 105 °C for 16 h. The quantity of collagen was estimated by multiplying the hydroxyproline content by 7.46 (Neuman et al., 1950).

Preparation of collagen digests

Insoluble collagen from skin specimens was prepared as described previously in detail (Deyl and Adam, 1976). Briefly, neutral salt soluble and acid soluble fractions were extracted and the remaining material was lyophilized. Collagen remaining after these extraction procedures was considered insoluble and represented over 80% of original skin collagen. In aortal specimens, insoluble collagen was prepared by the method of Faris et al. [6] in which elastase is used to remove the bulk of aortal elastin. The recovery of collagen by this method was better than 85%.

The insoluble fraction of skin or aorta collagen was washed twice with distilled water and centrifuged at 75 000 × *g* for 20 min at 4 °C. The pellet was suspended in 20 ml of 1.0 mol/l NaCl and sonicated until a fine suspension was obtained. Five hundred microliters were taken to determine the total amount of collagen present in the sample. A volume equivalent to 10 mg of collagen was then centrifuged at 20 000 × *g* for 20 min at 4 °C. The pellet was resuspended in 1.0 ml of 0.01 mol/l $CaCl_2$/ 0.02 mol/l Tris—HCl (pH 7.55) containing 0.05% toluene to prevent bacterial growth. To each sample 0.5 ml of a solution containing 1 mg purified collagenase (type CLS PA, Worthington, Millipore) in the above specified Tris—HCl buffer was added and then were incubated at 37 °C with shaking for 24 h. An insoluble pellet accounting for less than 5% of total collagen was removed by centrifugation. Aliquots (50 μl) of the supernatant were taken to determine the amount of digested collagen. The remaining supernatant was used for spectroscopic and fluorescence measurement and for the determination of Amadori products.

TBA (2-thiobarbituric acid) test [14]

The 2-thiobarbituric acid test for determining the presence of 5-hydroxymethyl-

furfural (HMF) was performed by mixing 1 ml of the collagen extract (obtained by autoclaving) with 0.5 ml 0.3 mol/l oxalic acid and heating for 1 h in a boiling water bath. After the samples were cooled to room temperature, 0.5 ml of 40% trichloroacetic acid was added and the resulting precipitate removed by filtration. After the addition of 0.5 ml of 0.05 mol/l TBA, the solution was incubated at 40 °C for 30 min and then the absorbance at 443 nm was measured and evaluated according to the standard curve.

Assay of the Amadori product (∈-hexosyllysine)

Amadori products after borohydride reduction were assayed by affinity chromatography according to Brownlee et al. [1] using the Glyco.gel Test Kit (Pierce, Rockford, IL). An amount equivalent to 200 μg of collagen (28 μg of hydroxyproline) was pipetted into a 10-ml test tube and brought to 0.5 ml by addition of buffer H from the kit. Then the borohydride reduction was performed with approximately 200 M excess borohydride over the total number of amino groups per collagen α-chain. To each tube was added 50 μl of 0.001 mol/l NaOH containing 256 μCi of [^{3}H]$NaBH_4$ (ICN, Covina, CA, spec. act. 50 mCi/nmol). Reduction was carried out for 10 min at room temperature, 50 min at 4 °C and brought to an end with 50 μl of 6 mol/l HCl after addition of 50 μl of *n*-pentanol. The solution was diluted four times with 1 ml of distilled water and evaporated with a Speed Vac (Savant Hicksville, NY) concentrator to diminish the contents of volatile components. All steps described above were performed under a hood to prevent contamination of the lab with radioactivity. The dry residue was acid hydrolyzed with 6 mol/l HCl for 12 h at 110 °C and afterwards the HCl was evaporated. The evaporation sequence was repeated five times to decrease the background radioactivity. The residue was dissolved in 0.5 ml of 0.25 mol/l ammonium acetate (Pierce buffer) and loaded onto a Glyco.gel minicolumn (Pierce). The column was washed with 20 ml of buffer and the Amadori products were eluted with 5 ml of 0.2 mol/l sorbitol. Three milliliters of the eluate were mixed with 17 ml of Hydrofluor (National Diagnostics, Sommerville, NY) and tritium activity was counted with a Beckman LS liquid scintillation counter. The calibration line was obtained with borohydride reduced and acid hydrolyzed samples of α-formyl-∈-fructosyllysine prepared in the laboratory. Non-specific incorporation of tritium into collagen as estimated by the radioactivity not retained on the column was 92.3—95.7% with the samples studied. The background activity was 34% of total counts incorporated into our samples (25% with the samples obtained from diabetic animals); though quite high it was constant during the preliminary tests (CV = 9.6; N = 6).

Spectroscopical measurements

Absorption at 250 nm was measured with a Zeiss PM6 spectrophotometer, fluorescence at $370_{ex}/440_{em}$ nm was measured with the Perkin-Elmer fluorimeter model 204.

Statistical evaluation

Slopes for the regression lines were calculated according to standard formula for linear regression.

RESULTS

In concert with our previous report [16] food-restricted animals (at any stage of their life) exhibited always a longer survival compared to those kept on a full access to food (Fig. 1). The differences are most distinct when comparing 50% survival of individual experimental groups. The best results with respect to animal survival were observed in animals which were food restricted during their first year of life. The shortest survival was found in animals fed normally throughout the whole period. The survival curves were not completed because of the relatively small number of animals used. Consequently differences in maximum survival under these conditions would not offer reliable data.

Data regarding the time dependence of the level of hydroxymethylfurfural (HMF) and hexosyllysine as well as absorbance at 350 nm and fluorescence at 370/440 nm in preparations of aortal and skin collagen are summarized in Figs. 2 and 3. It is clearly evident that for both investigated tissues the highest levels of all the magnitudes measured were formed in unrestricted (ad libitum fed) animals. Food restriction resulted shortly after the restricted regime was started in a decrease of glycation related products, 350 nm absorbance and 370/440 nm fluorescence no matter whether the animals involved were young or 1 year old and this change was observable for both tissues investigated.

As a rule values obtained for animals food restricted during the first year of life and ad libitum-fed thereafter were always the lowest. As shown in Table I, these animals exhibited the highest 50% survival (29.4 months as compared to 18.3

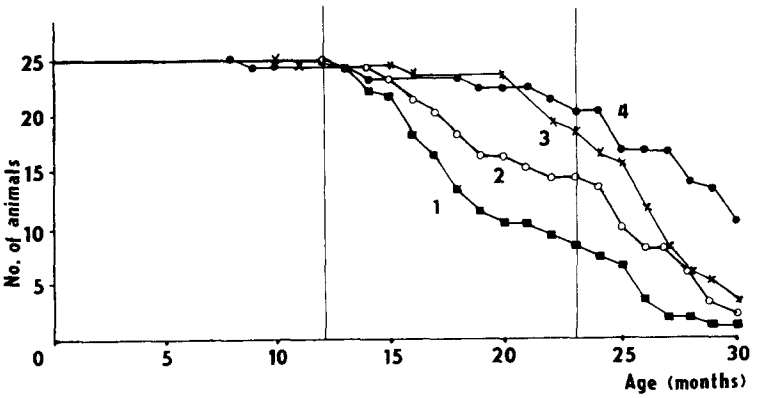

Fig. 1. Survival curves for rats kept on different dietary regimes (1 — fed normally, 2 — food restricted, 3 — normally fed for 1 year then food restricted, 4 — food restricted for 1 year then fed normally.

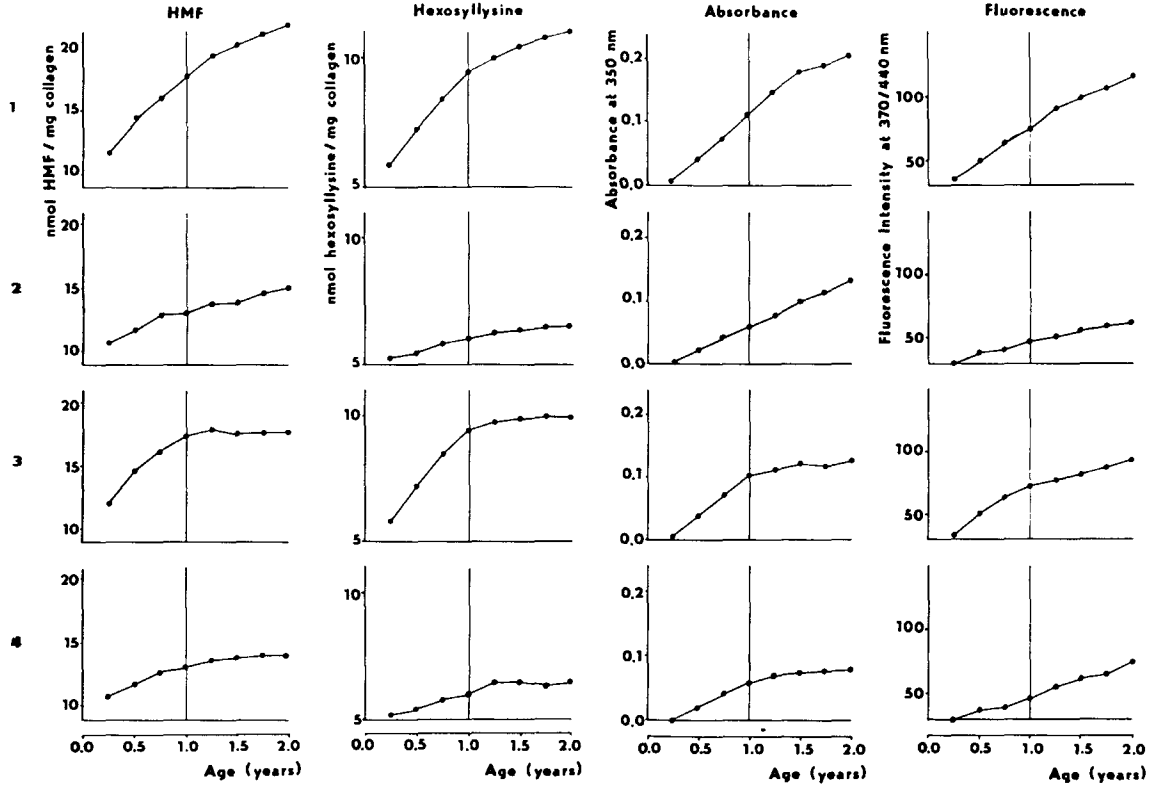

Fig. 2. Time dependence of the concentration of hydroxymethylfurfural (HMF) and hexosyllysine as well as time dependence of absorbance at 350 nm and fluorescence at $370_{ex}/440_{em}$ nm for aortal collagen: 1 — fed normally, 2 — food restricted, 3 — fed normally for 1 year then food restricted, 4 — food restricted for 1 year then fed normally. Each point represents the average value for three animals.

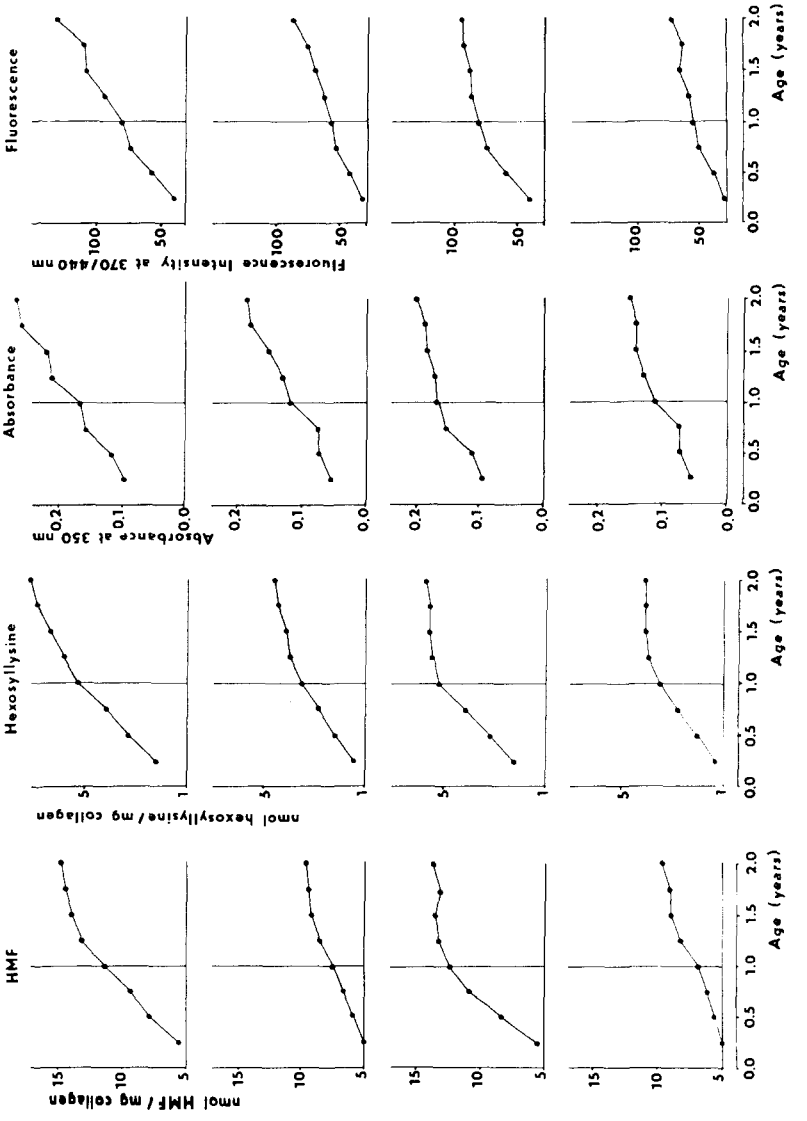

Fig. 3. Time dependence of the concentration of hydroxymethylfurfural (HMF) and hexosyllysine as well as time dependence of absorbance at 350 nm and fluorescence at $370_{ex}/440_{em}$ nm for rat skin collagen: 1 — fed normally, 2 — food restricted, 3 — fed normally for 1 year then food restricted, 4 — food restricted for 1 year then fed normally. Each point represents the average value for three animals.

TABLE I

FINAL VALUES FOR HYDROXYMETHYLFURFURAL (HMF), AMADORI PRODUCT AND RELATIVE FLUORESCENCE IN AORTAL AND SKIN COLLAGEN AS COMPARED TO 50% SURVIVAL (AVERAGE ± S.D.)

Regime	*50% Survival (months)*	*HMF nmol HMF (mg collagen)$^{-1}$*	*Amadori product nmol hexosyllysine (mg collagen)$^{-1}$*	*Relative fluorescence*	*No. of animals*
1	18.3	A 21.42 ± 2.07	A 11.02 ± 0.98	A 115 ± 12	7
		S 14.64 ± 0.98	S 7.01 ± 0.94	S 132 ± 14	
2	24.2	A 15.28 ± 1.78	A 6.59 ± 0.96	A 62 ± 11	6
		S 9.48 ± 1.46	S 4.62 ± 0.68	S 87 ± 14	
3	26.1	A 17.82 ± 2.48	A 9.83 ± 0.87	A 91 ± 13	6
		S 13.69 ± 1.92	S 5.92 ± 0.99	S 78 ± 12	
4	29.4	A 14.27 ± 2.15	A 6.59 ± 0.91	A 75 ± 12	9
		S 5.98 ± 1.26	S 4.27 ± 0.94	S 76 ± 14	

A — aortal, S — skin preparations. Regime 1 — both years fed normally; 2 — both years food restricted; 3 —first year fed normally than food restricted; 4 — first year food restricted than fed normally.

months in the normally-fed group). It should be noted that the animals which were 50% food restricted during the first year of their life consumed approximately 3.5 times the amount of food when fed ad libitum compared to animals of the same age fed normally during their whole life. However, no correlation between the achieved 50% survival and the final levels of the glycation and pigmentation products could be established within the three categories of food-restricted animals. Another view is shown when comparing the slopes of the regression lines (least squares method) for the changes with age of the levels of the glycation and pigmented products (Table II). As expected, the slopes are steepest for the animals fed normally throughout their whole life regardless of which parameter is followed. Fasting leads to a drop in the slope and slower accumulation of glycation and pigmentation products. In animals that are fed normally during the first year and food-restricted during the second year the slope drops below that of animals food restricted throughout their whole life except for the fluorescence parameter in skin collagen. This may be interpreted in such a way that some of the modified molecules are metabolized under the conditions of sudden food limitation.

The most interesting finding is, however, that the slopes for all the product accumulations in animals food restricted during their first year of life retain their low values even during the second year when the animals are fed normally. In fact, the slopes in this case have a lower value (except for the fluorescent product accumulation in aorta and HMF in skin) than in animals that were food restricted during the whole life. This seems indicative of the fact that during the fasting period not only accumulation of the glycation and pigmented products was slowed down in connec-

TABLE II

SLOPE OF THE REGRESSION LINES FOR THE HYDROXYMETHYLFURFURAL (HMF), AMADORI PRODUCT AND FLUORESCENCE AT 370/450 nm

Regime	Aortal collagen			Skin collagen		
	HMF	Amadori product	Fluorescence	HMF	Amadori product	Fluorescence
1	7.19	4.71	56.00	7.51	3.89	54.40
	2.92	1.34	30.81	2.13	1.61	44.80
2	3.07	1.04	22.40	3.19	2.75	33.20
	1.82	0.26	15.20	1.27	1.08	31.20
3	7.19	4.71	56.00	7.51	3.89	54.40
	0.50	0.14	18.80	0.34	0.23	13.20
4	3.07	1.04	22.40	3.19	2.75	33.20
	0.65	0.06	24.40	1.65	0.29	16.80

Regime 1 — both years fed normally; 2 — both years food restricted; 3 — first year fed normally than food restricted; 4 — first year food restricted than fed normally. For each of the four regimes distinct values are given for the first year (first line) and the second year of their life (second line).

tion with a limited food intake, but also metabolic pathways preventing their accumulation under the conditions of normal feeding were put into operation.

DISCUSSION

The data present evidence for decreased collagen glycation and formation of pigmented and/or fluorescent products in aortal and skin collagen under the conditions of restricted feeding. This decrease in accumulation of the glycated and pigmented products can be introduced even at a later stage of development, e.g., in adult (1-year-old) animals. Since the rate of accumulation of these products in animals fed normally throughout the first year and food restricted thereafter is (except for the fluorescent product in aorta) lower than for animals food restricted during the whole period it may be visualized that under conditions of sudden food insufficiency part of the modified proteins can be metabolized. The final concentration reflects then an equilibrium between the formation rate of these products (which is ruled by the availability of glucose and chemical reaction kinetics only) and the metabolic rate for the modified proteins.

Animals food restricted during the first year of life and fully fed thereafter showed a lower accumulation rate of the glycated and pigmented products compared with animals fed normally for their whole life as well as compared with animals that were food restricted during their whole life (except for the fluorescent product in aortal collagen and HMF in skin). It may be assumed that during the first year of life under the conditions of food limitation metabolic pathways allowing bet-

ter food exploitation are put into gear. These however, are not switched off after a wealth of food is available. Consequently, accumulation of the glycation and pigmented products would be slowed down even under ad libitum feeding (the way of formation of the modified proteins is ruled by chemical kinetics only).

Data of Dalderup and Visser [3] and Shafrir and Adler [15] show that increased content of sucrose in the diet (but not polymeric sugars [8]) does reduce the lifespan in laboratory rats and spiny mouse. Interestingly from our data it is impossible to formulate a detailed relation between animal 50% survival and the concentration or rate of accumulation of the glycated and/or pigmented products. However, in all situations when the animals exhibited a prolonged 50% survival they also exhibited a lower rate of accumulation of the collagen glycation and pigmentation products as compared to ad libitum-fed controls.

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