Effects of Maternal Dietary Restriction on Hydroxyproline Levels in Urine and Tissues of the Young (37279)

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Dietary restriction in rats during gestation and lactation results in permanent growth-stunting of the progeny, despite *ad libitum* feeding an adequate stock diet after weaning (1). The cause of growth-stunting is unknown. The stunted animals have greater feed requirements for growth and maintenance and excrete more urinary nitrogen than the controls (2). The increase of urinary nitrogen, urea and total amino acids in the progeny of dietetically restricted mothers suggested that the growth stunting may be related to certain defects in protein metabolism. Since collagen accounts for about one-third of total body protein and since urinary hydroxyproline is a reflection of collagen metabolism, this study involves the effect of maternal dietary restriction on hydroxyproline excretion of the offspring.

Experimental Material and Methods. Preparation of animals. Rats of the McCollum strain were used in all experiments. The method of maternal dietary restriction has been described in detail eleswhere (2). The following comparisons reported are between groups of male and female rats born to mothers on *ad libitum* feeding (referred to nonrestricted progeny) and groups (referred to restricted progeny) born to mothers which were fed daily amount equal to 50% of the intake of the nonrestricted mothers during gestation and lactation. After weaning, 28 days of age, all progeny were housed individually in stainless steel cages. Fresh water and diet were offered *ad libitum* in all experiments.

Diet. Purina Laboratory Chow, used throughout the study, contained not less than 23% protein and 3.78% fat. Vitamins, minerals and trace elements were present in amounts adequate to support normal reproduction and growth of the young.

Urine analysis. At various ages, rats were transferred to metabolic cages and fed Purina Laboratory Chow *ad libitum* for about 1 wk. Then 48-hr urine samples were collected under toluene. The total volume, including cage washings was made to 100 ml with distilled water and analyzed for creatinine and total hydroxyproline.

Hydroxyproline assay in various tissues. To determine hydroxyproline contents in various tissues, 6 nonrestricted females and 6 restricted females were used. A portion of dorsal skin, aorta, tail tendon, liver and lung was removed, cleaned, weighed and stored at —4° until analysis. In addition, femur and xiphoid were also removed and separated carefully from muscle and adhering subcutaneous tissue.

Analytical methods. Total creatinine in urine was determined by alkaline picrate method of Hare (3). Samples of urine, skin, aorta, femur and other tissues were hydrolyzed with 6 *N* HCl at 102° for 24 hr. Hydroxyproline was determined in the hydrolyzed samples by the method of Kivirikko, Laitinen and Prockop (4).

Isotope studies[1]*. Collection and assay of expired* $^{14}CO_2$. The progeny were fasted for 16 hr and then received an intramuscular injection of L-proline-U-^{14}C (5 μCi/100 g body wt) and placed immediately in individual glass metabolism cages for 1 to 6 hr. The expired $^{14}CO_2$ was collected and the radioactivity was counted according to the procedure described previously (5).

[1] Obtained from New England Nuclear Corp., Boston, MA, L-Proline-U-^{14}C (sp act 254.4 mCi/μmole).

Preparation of tissues for determination of radioactive protein. A portion of liver, pancreas, kidney, brain, spleen and gastrocnemius muscle was homogenized with ice-cold water to make a 5% homogenate. One milliliter of tissue homogenate was precipitated by adding 10% ice-cold trichloroacetic acid and further purified by the procedure described previously (6). When the protein precipitated was still wet, 4.0 ml of 0.4 *N* KOH solution were added and the mixture was incubated for 30 min at 40°. An aliquot was used for the determinations of protein radioactivity and protein content. Radioactive protein was measured by mixing 0.5 ml of Hyamine hydroxide with an equal volume of the KOH sample and shaking until clear. To this mixture, 15 ml scintillation fluid was added and the samples were counted. Tissue protein concentration was estimated by the method of Lowry *et al.* (7) with bovine serum albumin as standard.

Counting procedures in tissue protein. All radioactivity was measured by liquid scintillation spectrometry.[2] Diotol, composed of 4.6 g 2,5-diphenyloxazole (PPO), 0.091 g 1,4-bis(2-(5-phenyloxazoylbenzene)) (POPOP), 73 g naphthalene, 210 ml methanol, 350 ml dioxane and 350 ml toluene, was used as scintillation fluid. All samples were corrected for decay and for quenching by the internal standard techniques of Herberg (8). All values for tissue protein radioactivity were expressed as disintegrations per minute per milligram of protein (dpm/mg protein).

[2] Packard, Series 314A, Packard Instrument Co., Inc., Downers Grove, IL.

Thyroid study. From 4 mo old female rats, thyroid weights were recorded and circulating thyroxine concentration measured by the specific binding radioassay of Murphy and Jackan (9).

Statistical tests. The significance of the difference between the means of two groups of values was determined by Student's *t* test (10).

Results. Urinary excretion of hydroxyproline. When comparisons were made at 3 mo and again at 9 mo of age, the restricted male progeny excreted significantly more hydroxyproline in the 48-hr urine/unit of body weight than the nonrestricted ones (Table I). When hydroxyproline concentration was expressed as per 100 mg of urinary creatinine, the value also showed a significant increase in 3 mo old progeny from mothers maintained with restricted food intake during pregnancy and lactation.

The data shown in Table II demonstrate that the female progeny from underfed mothers had an increase of about 65% of urinary hydroxyproline over the control progeny. This significant difference was noted when the results were calculated on per unit of body weight or per unit of urinary creatinine.

Hydroxyproline concentrations in various tissues. The concentrations of hydroxyproline in various tissues were compared in 3.5 mo old female progeny (Table III). The tendon from rat tail had the highest amount of hydroxyproline expressed as per unit wet weight of tissue. This was followed by dorsal skin, femur, aorta, xiphoid, lung and liver. Maternal dietary restriction appeared to have little or no effect on the hydroxyproline contents

TABLE I. 48-hr Urinary Hydroxyproline in Male Rats.

Maternal diet	Age (mo.)	Body wt (g)	Hydroxyproline (μmoles/100 g body wt)	Ratio (hydroxyproline/creatinine) (μmoles/100 mg)
Nonrestricted	3	386 ± 18[a]	2.65 ± 0.47[b]	36 ± 9[b]
Restricted	3	285 ± 31	3.89 ± 0.50	49 ± 8
Nonrestricted	9	441 ± 29	2.17 ± 0.28[b]	40 ± 14
Restricted	9	354 ± 21	2.94 ± 0.39	45 ± 15

[a] Mean ± SD.
[b] $p < .05$.

TABLE II. 48-hr Urinary Hydroxyproline in Female Rats.

Maternal diet	Age (mo.)	Body wt (g)	Hydroxyproline (μmoles/100 g body wt)	Ratio (hydroxyproline/creatinine) (μmoles/100 mg)
Nonrestricted	1	132 ± 8[a]	6.5 ± 1.59[b]	168 ± 20[b]
Restricted	1	70 ± 13	10.9 ± 3.50	299 ± 97
Nonrestricted	2	196 ± 11	6.9 ± 0.60[b]	110 ± 11[b]
Restricted	2	146 ± 17	10.9 ± 2.11	164 ± 36
Nonrestricted	3.5	248 ± 27	6.5 ± 0.99[b]	113 ± 18[b]
Restricted	3.5	211 ± 21	8.7 ± 1.74	170 ± 49

[a] Mean ± SD.
[b] $p < .05$.

in the examined tissues of the progeny.

Conversion of proline-U-^{14}C into $^{14}CO_2$. Offspring from underfed mothers exhibited (Table IV) an increased conversion of proline to CO_2 1 hr after isotope administration. Further increases were noted with respect to the $^{14}CO_2$ collection with time. At the end of 6 hr the recovery of injected ^{14}C in the progeny from prenatal dietary restriction was about 40% greater than that of normal value. Similar findings were obtained when L-methionine-methyl-^{14}C was injected.

Incorporation of proline-U-^{14}C into tissue proteins. The incorporation of proline-U-^{14}C into tissue proteins in male rats was studied 24 hr after isotope injection. The results shown in Table V indicate that the specific activity present in the liver, pancreas, kidney, brain, muscle and spleen of the pups of underfed dams was approximately the same as in those of the pups from *ad libitum* fed mothers. This observation suggests all tested pups appeared to have equal capacity in the incorporation of labeled proline into tissue proteins.

TABLE III. Hydroxyproline Contents in Various Tissues (μmoles/100 mg Tissues).

Tissues	Nonrestricted	Restricted
Aorta	11.8 ± 0.47[a]	11.6 ± 0.50
Cartilage	10.0 ± 0.94	9.0 ± 0.65
Femur	11.8 ± 0.18	11.4 ± 0.72
Skin	25.0 ± 1.05	24.4 ± 1.07
Tendon	37.9 ± 5.85	39.5 ± 6.67
Lung	1.7 ± 0.51	1.9 ± 0.24
Liver	0.14 ± 0.01	0.15 ± 0.02

[a] Mean ± SD.

Thyroid weights and plasma T_4 content. The wet weight of thyroids expressed as mg per 100 g body weight (Table VI) in the female progeny from mothers fed a restricted diet was significantly higher than that in the female rats from mothers receiving an unrestricted diet. However, there was no significant difference observed in the concentration of circulating thyroxine (T_4) between two groups.

TABLE IV. Conversion of L-Proline-U-^{14}C Into $^{14}CO_2$.

Maternal diet	Body wt (g)	Injected dose in 6 hr (%)
Nonrestricted	159 ± 24[a]	16.9 ± 4.12[b]
Restricted	78 ± 14	23.9 ± 4.22

[a] Mean ± SD.
[b] $p < .05$.

Discussion. The growth-stunting of progeny from mothers maintained with restricted dietary intake during gestation and lactation has been considered a result of an impairment in protein metabolism (2). This plausible explanation was based upon the observation of low feed efficiency and increased urinary ammonia, urea and total amino acids nitrogen in these animals. This view is now supported by the present report indicating that urinary hydroxyproline excretion was also increased in progeny from underfed mothers. Since hydroxyproline is almost ex-

TABLE V. 24-hr Incorporation of Proline-U-^{14}C Into Tissue Proteins.

	Nonrestricted	Restricted
	Sp act dpm/mg protein	
Liver	222 ± 16[a]	217 ± 41
Pancreas	204 ± 5	173 ± 43
Kidney	236 ± 19	224 ± 27
Brain	20 ± 3	26 ± 8
Muscle	350 ± 91	334 ± 101
Spleen	144 ± 20	150 ± 29

[a] Mean ± SD.

clusively in collagen and is not reutilized for the synthesis of this protein, the increase of urinary hydroxyproline would indicate an alteration in collagen metabolism. This defect could be due to changes in the rate of collagen synthesis, changes in the rate of conversion of one form of the collagen to another, or changes in the rate at which any of the forms is degraded (11). It has been suggested (12) that a greater proportion of urinary hydroxyproline in growing animals and children is derived from the soluble collagen pool with a higher turnover rate. Thus, the offsprings from underfed mothers may have an increased rate of soluble collagen turnover.

Although data (Table III) failed to show any significant difference in total hydroxyproline contents in various tissues, the effect of maternal dietary restriction on the amount of this amino acid in different types of collagen remains to be investigated.

Increased rates of hydroxyproline excretion has been observed in hyperthyroidism (13) and in patients after administration of thyroid hormone (14). Conversely, decreased levels of hydroxyproline excretion have been reported in patients with hypothyroidism (15) and in animals with experimental hypothyroidism (16). The finding of an increased weight of thyroid in the progeny from restricted fed mother suggests a possible relationship between maternal feed intake and thyroid function of the offspring. In this connection, it is worthwhile to mention that pituitary weight and growth hormone activity were reduced in the progeny of rats whose dietary intake was restricted during gestation and lactation (17).

Since proline is the precursor of hydroxyproline and since proline-^{14}C incorporation into tissue protein-^{14}C was unaffected it is reasonable that under the experimental conditions, the progeny from underfed mothers are still capable of synthesizing proteins. The observed increase in proline oxidation, hydroxyprolinuria and reported aminoaciduria by others (2) may be therefore interpreted as a result of an increase in the rate of protein catabolism. Additional studies concerning the enzyme system(s) in protein catabolism are in progress to determine the validity of the interpretation.

Summary. Studies to determine the effect of maternal dietary restriction during gestation and lactation on hydroxyproline excretion of the progeny were undertaken. Results indicate that urinary hydroxyproline excretion was consistently and significantly increased in the male and female progeny of dietary restricted mothers than those of *ad libitum* fed ones. Similar findings were obtained when the results were expressed either as amounts of hydroxyproline per unit of body weight or the ratio of hydroxyproline per milligram of creatinine. Whether this enhancement is due to changes in the pool size of any of the forms of collagen or changes in

TABLE VI. Thyroid Weight and Plasma T_4 Content.

Maternal diet	Sex	Body wt (g)	Thyroid wt (mg)	Thyroid wt (mg/100 g body wt)	Plasma T_4 (μg/100 ml)
Nonrestricted	F	244 ± 21[ab]	15.3 ± 1.79	6.27 ± 0.59[c]	7.41 ± 1.77
Restricted	F	211 ± 20	16.8 ± 3.07	7.93 ± 0.88	6.80 ± 1.13

[a] Mean ± SD.
[b] $p < .05$.
[c] $p < .01$.

the rate of conversion of one form of collagen to another is not clear. Additional experiments revealed that a significant increase in $^{14}CO_2$ production was noted in the offspring from dietary restricted mothers after intramuscular injection of protein-U-^{14}C. However, the *in vivo* incorporation of proline-^{14}C into tissue proteins appeared to be unaffected by maternal dietary restriction.

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