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Effect of Diphenylhydantoin on Proline and Hydroxyproline Excretion in the Rat. (30563)

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Increased total dermal collagen deposition was induced in the young rat by diphenylhydantoin (DPH) administration in experiments performed by Houck *et al*(1). Increase in less soluble (acid soluble and insoluble) collagen was responsible for the observed increase in total collagen. Simultaneous decrease of more soluble (salt soluble) collagen occurred. DPH thus has the reverse effect from that induced in experimental lathyrism in young rats in which dermal salt soluble collagen was increased; however, total dermal collagen remained constant(2).

Peptide bound hydroxyproline excretion has been shown to reflect the metabolic degradation of collagen, the major site of which is the soluble fraction of collagen(3). Because an increase in bound hydroxyprolinuria accompanied the delayed appearance of increased soluble collagen pool in lathyritic rats(2), it seemed of interest to determine whether the excretion of hydroxyproline (HP) and its precursor proline were altered in DPH treated rats.

Methods. Eight-week-old male Sprague-Dawley (Holtzman) rats weighing 220-260 g were treated with either diphenylhydantoin, 15 mg, or propylene glycol-ethanol vehicle, 0.2 ml, daily by intraperitoneal injection for one week. The rats were weighed daily and fed Purina Lab Chow containing trace amounts of HP and 1.57% proline. Twenty-four hour urine collections were carried out every other day, yielding 4 collections per rat. On the morning of the eighth day, the animals were exsanguinated one-half hour after the usual intraperitoneal injection plus pentobarbital.

Plasma and urinary hydroxyproline levels were determined upon hydrolysed and unhydrolysed samples by the method of Prockop and Udenfriend(4). Proline determinations on similar specimens were made by an adaptation of the methods of Troll and Lindsley (5) and of Messer(6). Briefly, 1 ml of diluted urine, plasma filtrate or hydrolysate, the latter brought to pH 7.0 with potassium hydroxide, was added to 1 ml glacial acetic acid containing glycine 10 μm/ml. Thereafter, 1 ml ninhydrin solution, freshly prepared and containing 20 mg ninhydrin per ml in a mixture of 3 parts glacial acetic acid to 2 parts 6 M phosphoric acid, was added. The covered tubes were then heated in a boiling water bath for 30 minutes. After cooling the tubes in cold water, the color was extracted into toluene, 3 ml. The phases were separated by centrifugation and the supernatant read at 515 μ in a Coleman Junior Spectrophotometer. Standards were similarly treated. Concentrated HCl was added to blanks and standards when values for hydrolysates were determined, since the necessary neutralization step caused mild quenching of color. Proline recoveries averaged 96% in unhydrolysed urine, 92% in hydrolysed urine, and 93% in hydrolysed plasma.

Urine creatinines were determined by the method of Peters(7). The data for urinary values were calculated by analysis of variance. Data on plasma levels were treated by the student's T test.

Results. Growth: DPH therapy retarded growth of rats during the experimental period. The mean weight gain of the treated group was 16 g compared to 38 g in the vehicle-treated group. During the week prior to the experiment, the mean gain of all the

TABLE I. Excretion Rates.

		Control	Treated	F	Degrees freedom	n_2	P
Proline*	Free	5.2 ± .18	4.2 ± .18	12.74	1	6	.025–.02
	Total	13.1 ± .38	11.1 ± .30	13.75	1	6	.01 – .001
	Bound	7.9 ± .38	6.9 ± .24	4.73	1	6	.1 – .05
Hydroxyproline*	Free	.79 ± .07	.60 ± .05	21.3	1	6	.01 – .001
	Total	4.99 ± .13	3.98 ± .10	40.5	1	6	.001
	Bound	3.99 ± .14	3.36 ± .10	14.2	1	6	.01 – .001
Creatinine†		5.19 ± .11	4.85 ± .17	5.80	1	6	.1 – .05

* Expressed as μmoles/100 g body wt/24 hr, mean ± 1 S.E.
† Expressed as mg/100 g body wt/24 hr, mean ± 1 S.E.

rats was 35 g. Food intake in the DPH treated group was less than that of controls.

Proline excretion: Proline excretion per unit of body weight for control and treated groups appears in Table I. Both free and total proline excretion rates were significantly depressed by DPH therapy while the bound fraction was not. Approximately 40% of the total proline excretion occurred in the free form. The free proline daily excretion rate in this experiment was higher than that published for 4-month-old animals(8).

Proline blood levels: These values appear in Table II. Only free proline values were significantly depressed during DPH therapy. Values of free serum proline in 4-month-old rats(8) were in the same range as those for 2-month-old rats. Between 1 and 2% of total serum proline existed as free amino acid.

Free proline clearances were similar in both groups when calculated from the last 24-hour collection prior to sacrifice.

Hydroxyproline excretion: Hydroxyproline excretion rates appear in Table I. The differences between free, total, and bound HP excretion in treated and control animals were significant. The total HP excretion was similar to that reported for 6- and 8-week-old rats of the same strain(4,9,10). The free 24-hour excretion was higher than that of 6-week-old animals(4,9). Eighty per cent of the total HP was bound in the present control group whereas Prockop *et al*(4,9) reported an average figure of 92%.

Hydroxyproline serum values: These values appear in Table II. Free serum HP was significantly depressed during DPH therapy. The values were slightly higher than those of Bates *et al*(11) for control animals, as were the total values.

Creatinine excretions: The values are incorporated into Table I. The 24-hour urine collections were reasonably complete. There was no significant difference of total creatinine excretion between animals from day to day or between therapeutic groups.

Discussion. Depressed free proline levels in DPH treated rats may reflect decreased dietary intake of proline or its precursors with resultant fall in plasma level and urinary output. Alternatively, the decreases could reflect accelerated conversion of proline into other amino acids as hydroxyproline, citrulline, ornithine, or glycine. The fall in free plasma proline in treated animals cannot be attributed to increased renal clearance.

The proline liberated from plasma by hydrolysis is over 60 times more than is present

TABLE II. Plasma Values.

		Control	Treated	P
Proline*	Free	.42 ± .03	.31 ± .02	.025–.02
	Total	32.25 ± .71	28.13 ± 2.35	.2 – .1
	Bound	31.83 ± .70	27.80 ± 2.36	.2 – .1
Hydroxyproline*	Free	.100 ± .003	.079 ± .003	.005–.001
	Total	.277 ± .020	.283 ± .020	insignificant
	Bound	.177 ± .024	.204 ± .007	"

* Expressed as μmoles/ml plasma, mean ± 1 S.E.

as free amino acid. It may therefore reasonably be presumed that most bound plasma proline is present in substances retained by the glomerular filter. Supporting this assumption is the relatively small amount of proline liberated by hydrolysis of urine. Further, the rate of excretion of bound proline is only 1.5 times that of free proline.

The bound proline excreted in urine includes that found in HP-proline peptides(12). Equimolar concentrations of proline and HP are found in roughly 78% of the total HP-peptides in human urine(12). Thus, it was of interest to note a mean 2:1 ratio of urinary bound proline to bound HP in the present groups of rats. The significance and origin of non-HP peptide proline is unknown but may be related to collagen metabolism.

The 25% decrease in free HP serum concentration upon therapy was greater than that observed by Bates *et al*(11) following parathyroidectomy in rats on normal diet. The fall may be due to nutritional deprivation alone. Decreased urinary free HP in the treated rats was probably caused by diminished food intake. It has been documented that dietary HP appears as free HP in the urine(13), that little or no conversion of endogenous bound HP to free HP occurs *in vivo*(9).

In contrast, bound HP excretion is probably independent of dietary variation(13); its rate of excretion is constant even in young guinea pigs starved for 48 hours(10) and reflects metabolic turnover of collagen(3). In the DPH treated rats in the present experiment, bound HP excretion decreased promptly during the first day on the drug and remained fairly constant during the week's therapy while a small weight gain ensued.

If it be true that increased soluble collagen pool is associated with increased bound HP excretion as appears to be the case in experimental lathyrism(2), then the decrease in bound HP output in the present experiment is probably related to the decreased soluble collagen pool known to occur in DPH treated rats. Scorbutic guinea pigs also demonstrate decreased bound HP excretion(10) and concentration of dermal salt soluble collagen(14).

Although bound HP urinary excretion is constant in young guinea pigs during a 48-hour fast(10), Gross has shown depletion of dermal salt soluble collagen during severe caloric restriction in 2 days in the same species(15). This raises the question of whether nutritional deprivation alone may be responsible for decreased salt soluble collagen pool in DPH rats although simultaneous increase in total collagen content makes this unlikely. Paired feeding of experimental groups accompanied by direct analysis of dermal collagen fractions should help resolve any controversy.

Summary. Urinary excretion of bound hydroxyproline (HP) was significantly decreased in young growing rats treated with diphenylhydantoin, a drug known to increase insoluble collagen. The results suggest the diminished bound HP excretion is related to a decreased pool of soluble collagen.

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