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RADIOACTIVITY OF HYDROXYPROLINE FROM URINE AND COLLAGENS OF NORMAL AND CORTISONE-TREATED RATS*

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Abstract—Twelve weanling male rats each received 15μc of L-proline-^{14}C in 3 equal and consecutive daily i.p. injections. Fourteen days after the third isotope injection, 4 of the animals were sacrificed (0-day control). Four of the remaining rats each received 9 daily injections of 10 mg cortisone acetate/kg body wt. and then were sacrificed with the other animals (9-day control) 24 hr after the final steroid injection.

Salt-soluble skin collagen represented a significantly lower fraction of the total skin collagen in the cortisone-treated rats than in the 9-day control animals. The sp. act. of hydroxyproline from salt-soluble skin collagen, mature skin collagen, and total femur collagen from the cortisone-treated rats were each significantly greater than those of the 9-day control animals.

The mean urinary hydroxyproline excretion of the steroid-treated rats was significantly less after 4 days of treatment until termination of the experiment. Slightly greater urinary hydroxyproline specific activities of the cortisone-treated rats reflected the higher sp. act. of the collagen hydroxyproline pool of the steroid-treated rats. No significant differences were observed between the 24-hr urinary hydroxyproline radioactivities of control and cortisone-treated rats.

The above observations, in conjunction with additional data given in this report and the findings of earlier experiments, were interpreted as providing further evidence in agreement with anti-anabolic changes in collagen metabolism (decreased collagen synthesis) resulting from the administration of pharmacologic quantities of cortisone to rats which would normally undergo rapid body growth. Although the data from the various experiments indicate that the steroid had an anticatabolic effect on femur collagen (decreased collagen degradation), urinary hydroxyproline excretions and radioactivities were inconclusive for the demonstration of an anticatabolic effect of the hormone on the total body pool of collagen.

THE CHRONIC administration of pharmacologic quantities of cortisone and related steroids to rats results in a decrease in the quantity of dermal collagen.[1–4] No significant change in quantity and concentration of femur collagen, concomitant with the decreased amount of dermal collagen, was observed in cortisone-treated rats.[5, 6] Diverse explanations have been given for the alterations of collagen metabolism in animals treated with cortisone and its analogs. Data from different laboratories[6–9] were interpreted as indicating that various glucocorticoids decreased synthesis of collagen (anti-anabolic effect on collagen metabolism). Other investigators suggested that the changes may be explained by stabilization of the subcellular organelles, the

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lysosomes,[10] or by release of enhancement of the activity of tissue collagenases.[11, 12]

In this paper we are reporting additional information concerning collagen metabolism of cortisone-treated rats obtained from the evaluation of the quantity and sp. act. of urinary and tissue hydroxyproline after administration of radioactive-labeled proline. The excretion of urinary hydroxyproline has been reported to be lower in at least some ages of cortisone-treated rats.[6, 7] Radioactivity from proline-^{14}C incorporated into salt-soluble collagen hydroxyproline decreases rapidly, and 4 weeks after injection of the isotope, the predominant urinary hydroxyproline radioactivity is from mature collagen.[13] If cortisone treatment of rats which have previously received labeled proline is not begun until after the sp. act. of the salt-soluble collagen hydroxyproline has decreased to a quantity much lower than that of the mature collagen hydroxyproline, an evaluation of any differences from control values in the sp. act. of the urinary hydroxyproline may indicate whether the decrease in the quantity of urinary hydroxyproline results principally from a change in the contribution of the salt-soluble or the mature collagen hydroxyproline to the total urinary hydroxyproline. Harris and Sjoerdsma used this technique to demonstrate that parathyroid extract acts directly on mature collagen.[14]

METHODS

Twelve weanling male rats (26 days old, 80 g average body wt.) each received 15μc of L-proline-^{14}C (200 μc/μmole, uniformly labeled; Schwarz Bioresearch, Inc.) in 3 equal and consecutive daily i.p. injections. Fourteen days after the third isotope injection, the rats were divided into 3 weight-matched groups of 4 animals. The animals in one group (0-day control) were immediately subjected to light ether anaesthesia. Blood was removed from each rat by direct cardiac puncture (citrate anticoagulant) and the plasma was separated. The 4 animals were sacrificed by excess ether and shaved with electric clippers. The entire skin except for face, feet and tail was removed, cleaned of adhering noncutaneous tissue, and pulverized as previously described.[15] The right femur was dissected from each animal and after removal of adhering soft tissue, extracted for 24 hr in a Soxhlet extractor with 1:1 (v/v) alcohol–ether and dried to constant weight at 110°.

Four of the remaining animals received 9 daily intramuscular injections of 10 mg cortisone acetate/kg of initial body wt. Twenty-four hr after the final cortisone injection, the 4 cortisone-treated rats and the 4 other animals (9-day control) were sacrificed. Blood, skin, and femur samples from each animal were processed by the techniques given above. A urine sample was collected from a control and a cortisone-treated rat for each 12 hr throughout the experiment in a sequence such that a specimen was obtained from each rat every second day.

The hydroxyproline content of an aliquot of each pulverized fresh skin was determined by the method of Prockop and Udenfriend.[16] Collagen content of the skin samples was calculated by multiplying hydroxyproline content by 7·46.[17] A quantity of each pulverized fresh skin, which contained 0·50 g of collagen as given by the above analyses, was extracted with continual shaking for 24 hr at 4° with 50 ml of 0·45 M NaCl. The extraction fluid was separated from the skin residues by filtration through coarse filter paper (S and S no. 589, black ribbon). Hydroxyproline in the extraction media was determined and the amount of collagen extracted (salt-soluble collagen) was calculated by the methods given above.

The radioactivity of the hydroxyproline in the salt-soluble skin collagen, the skin collagen remaining after salt extraction (mature collagen), and the total femur collagen was obtained by the procedure of Prockop *et al.*[18] with an additional extraction of the final toluene solution with 1·5 M semicarbazide in pH 4·0 phosphate buffer, as suggested by Harris and Sjoerdsma.[14] One ml phosphor solution (15 g of 2,5-diphenyloxazole* and 50 mg of 1,4-*bis* 2-(4-methyl-5-phenyloxazolyl) benzene* per 1000 ml of toluene) was added to 10 ml of the final toluene extract and the samples were counted in a Packard Tri-Carb liquid scintillation counter. The radioactivity of all tissue hydroxyproline samples was at least 40 cpm above background (20 cpm). All samples were counted to a minimum of 10,000 counts above the background radioactivity. Radioactivities of the hydroxyproline from the various collagens are expressed as cpm/μmole hydroxyproline.

Urinary hydroxyproline was determined by the Prockop and Udenfriend method.[16] The urinary hydroxyproline excretion is given as μg total hydroxyproline excreted/24 hr/rat. Since no diurnal variation was observed in the urinary hydroxyproline excretion, the 12-hr excretion values were multiplied by 2 to give 24-hr quantities. Urinary creatinine was quantitated by the alkaline picrate technique[19] and is expressed as mg excreted/24 hr. The creatinine excretion and visual inspection of the urine collection apparatus were used to judge adequacy of sample collection. No specimens were eliminated from the present experiment on the basis of the above criteria. The ratio of μg hydroxyproline to μg creatinine excreted/24 hr was calculated for each urine specimen.

Urinary hydroxyproline radioactivity was estimated by the same methodology as applied to the hydroxyproline from the various collagen samples. The minimum radioactivity of the urinary hydroxyproline samples was 20 cpm above background. The radioactivity of the urinary hydroxyproline is reported as cpm/μmole hydroxyproline. Radioactivity excreted in urinary hydroxyproline/24 hr was calculated from the 24-hr urinary hydroxyproline excretion and the specific activity of the urinary hydroxyproline.

The plasma content of hydroxyproline-containing protein was obtained by the technique of LeRoy *et al.*[20] Analytical results are expressed as μg protein hydroxyproline/ml plasma.

RESULTS

Fourteen days after the third isotope injection, the average body weights of the 3 groups of experimental animals were not significantly different; however, the animals which then received cortisone acetate failed to gain additional weight. The body weights of the steroid-treated animals were significantly less ($P < 0{\cdot}01$) on sacrifice 9 days later than were those of the 9-day control rats (Table 1). In contrast to the lower body weights of the cortisone-treated rats, the dry fat-free weights of the femurs of the hormone-treated rats were at least equal to those of the 9-day control rats (Table 1). These body weights and dry fat-free femur weights of the cortisone-treated animals correspond to those previously observed in rats of a similar age treated with the same quantity of cortisone acetate.[1, 5, 6]

The fraction of the total skin collagen represented by salt-soluble skin collagen was significantly less ($P < 0{\cdot}01$) in the cortisone-treated rats than in control animals of

* Obtained from Nuclear-Chicago Corp.

the same age (Table 1). The amount of collagen per total skin of rats of various ages is reduced by administration of the quantities of cortisone acetate given in the present experiment.[1] Thus, the amount of salt-soluble collagen per total skin of the cortisone-treated rats is lower in comparison to control animals than is indicated by that fraction of the dermal collagen which is salt-soluble. The significant decrease ($P < 0.01$) with age in the fraction of the collagen extracted from the skin of the control rats by the salt solution corroborates prior data.[21, 22] Earlier studies showed that the collagen content of the dry fat-free femurs from rats treated for 1–21 days with the quantity of cortisone acetate utilized in the present experiment is not significantly changed from control values.[5, 6]

TABLE 1. BODY WEIGHTS, DRY FAT-FREE FEMUR WEIGHTS, SALT-SOLUBLE COLLAGEN CONTENTS OF SKIN, AND HYDROXYPROLINE-CONTAINING PROTEIN CONTENTS OF PLASMA OF CONTROL AND CORTISONE-TREATED RATS*

	Experimental group		
	O-Day control	9-Day control	Cortisone-treated
Body weight (g)	206 ± 2	246 ± 4	198 ± 10†
Femur weight (mg)	257 ± 2	317 ± 22	353 ± 6
Salt-soluble skin collagen (% total skin collagen)	11·9 ± 0·1	8·0 ± 0·4	5·3 ± 0·1†
Hydroxyproline-containing protein (μg hydroxyproline/ml plasma)		9·6 ± 0·5‡	8·4 ± 0·5

* Data are given as the mean ± S.E.

† Significantly different from 9-day control ($P < 0.01$).

‡ 0-day control, 9-day control no significant difference; value given is pooled sample from both groups.

Fourteen days after the final injection of labeled proline, the specific activity of the dermal mature collagen hydroxyproline was more than 6 times as great as that of the salt-soluble collagen hydroxyproline (Table 2). At the same time, the specific activity of the total femur collagen hydroxyproline exceeded that of either the skin salt-soluble or mature collagen hydroxyproline (Table 2). The sp. act. of the hydroxyproline from all 3 collagens of the cortisone-treated rats was significantly greater ($P < 0.01$ and 0·05) than that of the 9-day control animals (Table 2). The hydroxy-

TABLE 2. RADIOACTIVITIES OF HYDROXYPROLINE (CPM/μMOLE) FROM VARIOUS COLLAGENS OF CONTROL AND CORTISONE-TREATED RATS*

	Experimental group		
	O-Day control	9-day dontrol	Cortisone-treated
Salt-soluble skin collagen	22 ± 1	9 ± 1	16 ± 2†
Mature skin collagen	145 ± 6	77 ± 6	92 ± 9‡
Total femur collagen	177 ± 18	94 ± 7	124 ± 10‡

* Data are given as the mean ± S.R.

† Significantly different from 9-day control ($P < 0.01$).

‡ Significantly different from 9-day control ($P < 0.05$).

proline sp. act. of the collagens from the cortisone-treated rats decreased, however, to values significantly less ($P < 0{\cdot}01$) than those of the 0-day control animals.

No increase or decrease in the urinary hydroxyproline excretion of the control rats was evident during the 9 days of sample collection (Fig. 1). The mean hydroxyproline excretions of the 9-day control animals, determined with 4 or 5 samples from each rat, were similar (1306 ± 87,* 1400 ± 47, 1468 ± 130 and 1383 ± 97 μg/24 hr) However, the mean of the 10 urinary hydroxyproline determinations (1390 ± 30 μg/24 hr) on samples obtained from the control animals at the 4-day and at subsequent experimental periods was significantly greater ($P < 0{\cdot}01$) than that of 10 samples

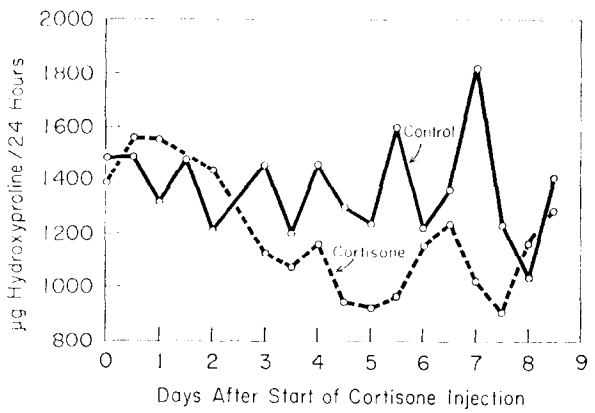

FIG. 1. Urinary hydroxyproline excretion of control and cortisone-treated rats.

(1090 ± 40 μg/24 hr) obtained from the cortisone-treated rats during the same experimental period. The urinary creatinine excretion (Table 3) of the cortisone-treated rats was also significantly less ($P < 0{\cdot}02$) from 4 days to termination of the experiment (mean of 10 samples each from control and cortisone-treated rats, $11{\cdot}8 \pm 0{\cdot}4$ and $9{\cdot}9 \pm 0{\cdot}5$ mg creatinine/24 hr respectively). Because the urinary excretion of both hydroxyproline and creatinine decreased in the cortisone-treated rats, the ratio of μg hydroxyproline to μg creatinine in the urine (Table 3) was not significantly different in control and cortisone-treated rats (mean ratio for control and cortisone-treated rats from 4 days to completion of experiment, $0{\cdot}120 \pm 0{\cdot}005$ and $0{\cdot}111 \pm 0{\cdot}005$ respectively).

The sp. act. of the urinary hydroxyproline from both the control and cortisone-treated rats decreased at the later experimental periods to about $\frac{1}{2}$ of that found initially (Fig. 2). Urinary hydroxyproline sp. act. of the cortisone-treated rats averaged 107 per cent of the values obtained with the control rats at corresponding experimental periods. The slightly higher sp. act. of the urinary hydroxyproline from the cortisone-treated rats correlate with the greater sp. act. of their collagen hydroxyproline pool (Table 2). At the termination of the experiment, the radioactivity in the 24-hr urinary hydroxyproline of both the control and cortisone-treated rats had also decreased to approximately $\frac{1}{2}$ of the initial values (Table 3). No significant differences were observed between the 24-hr urinary hydroxyproline radioactivities of the control

* Mean $\pm$ S.E.M.

TABLE 3. URINARY CREATININE, URINARY HYDROXYPROLINE TO CREATININE RATIOS AND 24-HR URINARY HYDROXYPROLINE RADIOACTIVITY OF CONTROL AND CORTISONE-TREATED RATS

	Control				Cortisone-treated			
Days on experiment	Animal no.	Cr* (mg/24hr)	μg Hypro/ μg cr	Hypro (cpm/24hr)	Animal no.	Cr (mg/24hr)	μg Hypro/ μg cr	Hypro (cpm/24hr)
0	5	12·4	0·120	1318	11	13·2	0·105	1478
½	6	11·9	0·125	1717	12	10·6	0·147	1547
1	7	11·0	0·121	1667	9	10·9	0·140	2004
1½	8	11·8	0·126	†	10	13·2		
2	5	10.3	0·118	1204	11	11·4	0·126	1522
2½	6	11·3			12	9·9		
3	7	11·1	0·132	1438	9	8·7	0·130	1364
3½	8	10·3	0·117	1081	10	8·7	0·122	1075
4	5	10·3	0·142		11	10·5	0·113	
4½	6	9·8	0·133	894	12	10·0	0·095	647
5	7	11·1	0·111	1086	9	7·4	0·125	802
5½	8	11·0	0·145		10	8·2	0·118	
6	5	12·7	0·095	660	11	12·1	0·097	866
6½	6	13·3	0·104		12	10·1	0·123	
7	7	12·2	0·150		9	7·9	0·129	
7½	8	10·6	0·117	804	10	10·7	0·085	700
8	5	11·6	0·099	707	11	10·4	0·113	637
8½	6	14·1	0·101		12	11·7	0·111	

* Cr = creatinine, hypro = hydroxyproline.

† Missing values were lost due to analytical error.

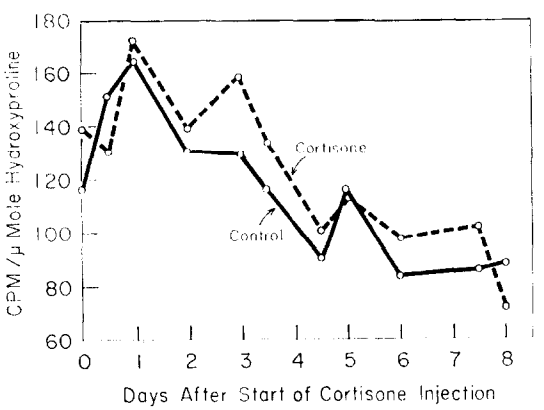

FIG. 2. Specific radioactivity of urinary hydroxyproline from control and cortisone-treated rats.

and cortisone-treated rats. At the time of start and finish of cortisone treatment, the sp. act. of hydroxyproline from the urine of both control and cortisone-treated rats was greater than that from soluble skin collagen but less than that from mature skin collagen or total femur collagen (Table 2 and Fig. 2).

The hydroxyproline-containing protein of the plasma from the cortisone-treated rats was insignificantly decreased from the amount found in control animals (Table 1). Possible relationships between the hydroxyproline-containing protein of the plasma

and soluble collagens, hydroxyproline-containing substances of the urine, and other collagen metabolites are not yet established.

DISCUSSION

The interpretation of changes in the quantity and specific activity of urinary hydroxyproline is complex because of the diverse tissue sources of urinary hydroxyproline and because of differences in the metabolism of collagen from different tissues. Incorporation and retention of labeled amino acids by collagen from the skin and the bones, the tissues which contain the major portion of the body collagen, are different.[5, 23] In the present investigation, the aliquots of dermal collagen are representative of the entire collagen pool of the skin, but femur collagen may not be typical of all calcified tissue. However, the data given in this report are in agreement with various changes in collagen metabolism resulting from the administration of pharmacologic quantities of cortisone.

It has been established that urinary hydroxyproline excretion and salt-soluble collagen content of the tissues are less when the rate of collagen synthesis decreases.[24] The data of the present study therefore are in agreement with a slower rate of collagen formation, with a consequent decrease in urinary hydroxyproline excretion, after cortisone administration. Previous data, consistent with an anti-anabolic effect on collagen metabolism after cortisone treatment, included a decrease in the incorporation of labeled glycine into salt-soluble and mature skin collagen and total femur collagen of rats previously treated with the steroid.[6]

The earlier experiments demonstrated a lower incorporation of glycine-2-^{14}C by femur collagen of young adult male rats when administered after 4 and 7 days of cortisone treatment.[6] The quantity of collagen per femur is not significantly changed from control values through at least 21 days of cortisone treatment as given in the present study.[5, 6] A slower decrease in the sp. act. of labeled hydroxyproline in femur collagen of cortisone-treated young adult rats was observed in the present investigation. The data from the above experiments suggest that synthesis of femur collagen is decreased by administration of pharmacologic quantities of cortisone. However, the degradation of femur collagen must also be less since synthesis of the protein is decreased without a change in its accumulation.

Urinary hydroxyproline is a product from the metabolism of both soluble and mature collagens of all tissues. If the collagens contain radioactive hydroxyproline, and the sp. act. of the hydroxyproline from soluble collagen is much lower than that from the mature collagen, a lesser urinary hydroxyproline excretion from only a decrease in the contribution of soluble collagen hydroxyproline would result in urinary hydroxyproline with an increased sp. act. A smaller urinary excretion of hydroxyproline from mature collagen, without a corresponding decrease in the amount of hydroxyproline from soluble collagen, would result in a lower sp. act. of the urinary hydroxyproline. The excretion of urinary hydroxyproline was significantly decreased and the average sp. act. of the urinary hydroxyproline was slightly greater in the cortisone-treated rats studied in the present esperiment. The higher sp. act. of the urinary hydroxyproline from the steroid-treated rats was no greater than may be accounted for by the lesser decrease in the sp. act. of the hydroxyproline from the collagens of the cortisone-treated rats. The data given in this paper therefore do not indicate a large change in the relative contribution of soluble and mature

collagen to the urinary hydroxyproline of the cortisone-treated rats. The magnitude of the changes observed in the cortisone-treated rats is, however, not great enough to provide conclusive evidence that the contribution of both soluble and mature collagen to the urinary hydroxyproline is decreased.

Because of the rapid decrease in the sp. act. of the soluble collagen hydroxyproline, the major portion of the radioactivity in the urinary hydroxyproline analyzed in the present study is derived from the degradation of mature collagen. Since no significant difference was observed between the 24-hr urinary hydroxyproline radioactivity of the control and cortisone-treated rats, it might be concluded that the degradation of mature collagen was the same in the control and cortisone-treated rats. This conclusion may not be valid since the collagen hydroxyproline radioactivity of the cortisone-treated rats was diluted less by formation of new collagen, and thus the collagen subject to degradation had a higher sp. act. in the cortisone-treated rats. The radioactivity in the hydroxyproline excreted per 24 hr, however, does not indicate a large change in the degradation of the total body pool of collagen. Most of the results and conclusions discussed above are in agreement with the report of Kivirikko *et al.*[7] in which it was concluded, from experiments with a design similar to that of the present experiments, that pharmacologic doses of cortisone have an anti-anabolic effect on the synthesis of soluble collagen but no effect on the degradation of insoluble collagen fibers. Although the results of the present report do not provide conclusive evidence for an anti-catabolic effect (decreased degradation) on the total body pool of collagen, evidence is provided for an anti-catabolic change in the metabolism of one source of collagen the femur.

It has been suggested that collagenolytic enzymes are released or activated *in vivo* by cortisone administration.[11, 12] A marked decrease in insoluble skin collagen 2 hr after administration of a single dose of cortisol was reported.[11] If the administration of corticosteroids to young rats results in the rapid degradation of insoluble collagen, an increase in the quantity of urinary hydroxyproline and urinary hydroxyproline radioactivity would be expected in the steroid-treated rats under the conditions of the present report and in the earlier work of Kivirikko *et al.*[7] The failure to observe any catabolic changes in the urinary hydroxyproline analyses argues against any released or activated proteolytic activity having an action *in vivo* on the total body pool of collagen. With the investigational conditions of the present report, cortisone must be administered for at least 4 days before a change in the quantity of insoluble skin collagen is observed.

Body weight and creatinine excretion were also decreased by the amount of cortisone given in the present study. The amount of steroid given thus had multiple effects upon the animal. The changes noted in collagen metabolism of the cortisone-treated rats may therefore be at least partially an indirect effect of the hormone. Additional studies are required to determine whether the changes in collagen metabolism that follow administration of pharmacologic quantities of cortisone result from a direct or an indirect action of the steroid.

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