*HYDROCORTISONE-INDUCED INHIBITION OF PROTEIN SYNTHESIS AND URIDINE INCORPORATION IN ISOLATED BONE CELLS IN VITRO**

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Although much is known about the hormonal regulation of bone metabolism, the mechanism whereby adrenal corticosteroids in excess retard bone growth and produce osteoporosis has not been established. Hydrocortisone *in vitro* inhibits the accumulation of acid mucopolysaccharides in cultures of embryonic bone,[1-3] the sulfation of cartilage,[4, 5] and the incorporation of radioactive amino acids into collagen of cartilage and bone,[5, 6] suggesting direct interference with the formation of collagen-rich organic matrix. However, bone and cartilage are not ideal for studying short-term hormonal effects *in vitro*, because dense connective tissue is interposed between incubation medium and cells. For this reason, we have examined the direct effects of glucocorticoids on the metabolism of cells dispersed from rat calvaria and maintained in primary culture, where they are capable of forming collagen and sulfated acid mucopolysaccharides.[7] Hydrocortisone in physiologic concentrations was found to inhibit collagen and noncollagen protein synthesis and to deplete the cells of RNA within five hours. Incorporation studies suggested decreased RNA synthesis, but small increases in RNA breakdown could not be excluded. These effects were not associated with a significant alteration in DNA metabolism.

Materials and Methods.—(a) *Isolation of bone cells and preparation of primary cultures:* Bone cells were dispersed enzymatically from the calvaria of 20-day-old rat fetuses and cultured on the bottom of 25-ml Erlenmeyer flasks as previously described.[8] Cells were maintained at 37°C in Minimum Essential Medium (Eagle's) containing 15% calf serum, 2 mM glutamine, 100 units each/ml penicillin and streptomycin, and 0.3 mM ascorbic acid. All studies were performed using cells that had already spread and proliferated to form a confluent layer, since collagen synthesis is maximal and net DNA accumulation minimal at this stage.[9] Immediately before study, cells were refed with identical medium, except that calf serum was replaced by 0.5% bovine serum albumin, providing a defined environment. Hydrocortisone (California Corporation for Biochemical Research) was dissolved in absolute alcohol and added to the medium so that the final alcohol concentration was 0.4%; control cultures contained the same concentration of alcohol. Hydrocortisone hemisuccinate (Upjohn) was dissolved in medium directly.

(b) *Isolation of free amino acids, collagen, and noncollagen protein from cell layers:* Collagen synthesis was studied by following the incorporation of uniformly labeled L-proline-C^{14} into peptide hydroxyproline. This method makes use of the fact that hydroxyproline in peptide linkage is unique to collagen in mammals, and is derived almost totally from free proline.[10] Cells were incubated aerobically and with ascorbic acid, conditions previously found to produce maximum proline hydroxylation.[11] After incubation, free amino acids and newly synthesized soluble collagen were extracted from the cell layers with cold water, and insoluble, more highly aggregated collagen with hot perchloric acid as described previously.[11] Cells were first washed rapidly with ice-cold, nonradioactive medium, frozen and thawed rapidly over CO_2, and then extracted twice with water at 4°C for 30 min. Free amino acids were separated from cold water-soluble peptides by descending chromatography on Whatman 3 MM paper using an n-butanol:acetic acid:water (4:1:2) solvent system. After chromatography, papers were dried, and appropriate areas cut out, placed in Bray's solution,[12] and counted in a Packard Tri-Carb liquid scintillation spectrometer. Over-all recovery of radioactivity from the chromatograms was estimated with known radioactive

standards. In some experiments, the cold water-soluble proteins were isolated by dialysis and the radioactivity of individual amino acids was estimated after hydrolysis and chromatographic separation.[11] Cold water-insoluble proteins, including collagen, were extracted from the residue twice with 10% perchloric acid at 70°C for 20 min, these extracts were combined, and an aliquot was hydrolyzed and assayed for proline and hydroxyproline radioactivity.[9] In these experiments, DNA was also extracted with hot perchloric acid and was estimated colorimetrically.[13] In order to correct for minor differences in the number of cells per culture, data are expressed per microgram of DNA.

(c) *Isolation of RNA and DNA from cell layers:* RNA metabolism was studied by following the incorporation of uridine-2-^{14}C into the tissue pool of free uridine, uridine nucleotides, and RNA. DNA metabolism was measured by the incorporation of thymidine-2-C^{14} into DNA. RNA and DNA were extracted from the cell layers by a modification of the method of Schmidt and Thannhauser,[14, 15] extracting RNA with 0.3 *N* KOH for 18 hr at 37°C. Aliquots of the RNA and DNA fractions were dissolved directly in Bray's solution[12] and counted. Total RNA content was estimated by the orcinol method[16] using ribose standards, and DNA by the method of Ceriotti[13] using calf thymus DNA standards. The radioactivity of the total free nucleoside-nucleotide pool extracted from the cell layer was estimated by direct counting of the initial cold 2% perchloric acid extract. The radioactivity of uridine and uridine nucleotides in this fraction was determined after chromatographic separation on ECTEOLA cellulose paper (Whatman) using a 0.15 *M* NaCl solvent system. Aliquots were cochromatographed with known nucleoside and nucleotide standards, and spots were identified with an ultraviolet scan (254 mμ). Consecutive strips were cut out from the region of the standards and counted directly in Bray's solution.

Results and Discussion.—Effects of hydrocortisone on collagen and noncollagen protein synthesis: Hydrocortisone and hydrocortisone hemisuccinate inhibited the incorporation of proline-C^{14} into collagen in five hours, as indicated by marked decreases in peptide hydroxyproline radioactivity (Table 1). Labeling of two forms of collagen, cold water-soluble and -insoluble, was inhibited to the same degree, suggesting that collagen maturation was unaffected. Hydrocortisone also appeared to inhibit the incorporation of proline-C^{14} into noncollagen, proline-containing protein. This fraction is approximated by proline radioactivity in excess of hydroxyproline since both amino acids are derived from free proline[10] and their molar ratio in collagen is approximately one.[17] Because of the marked excess of proline radioactivity appearing in protein (Table 1), inhibition of collagen synthesis alone would not explain the equivalent reduction in peptide proline and hydroxyproline radioactivity.

Possible effects of hydrocortisone on protein breakdown were examined with

TABLE 1

HYDROCORTISONE-INHIBITED INCORPORATION OF PROLINE-C^{14} INTO PEPTIDE PROLINE AND HYDROXYPROLINE IN ISOLATED BONE CELLS

	DNA (μg/culture)	Radioactivity (cpm/μg DNA) — Cold Water-Soluble Protein: Proline	Hydroxyproline	Insoluble Protein: Proline	Hydroxyproline
Expt. I					
Control	42.8	229	21	252	123
Hydrocortisone	42.7	101 (−56%)‡	9 (−57%)‡	123 (−51%)*	58 (−53%)*
Expt. II					
Control	42.7	200	17	250	132
Hydrocortisone Hemisuccinate	41.8	106 (−47%)*	6 (−65%)*	159 (−36%)‡	64 (−52%)†

Primary cell cultures were incubated with 10^{-5} *M* hydrocortisone or hydrocortisone hemisuccinate for 5 hr. L-proline-U-C^{14} (New England Nuclear Corporation, 186 mc/mm) was added to treated and control cultures for the final 2 hr of incubation in a final concentration of 0.0022 mM. After incubation, cultures were sacrificed and prepared as described in the text. In this and all subsequent tables, each value represents the mean of three or four separate cultures.

* Differs significantly from control (p = <0.01).

† p = <0.02.

‡ p = <0.05.

pulse-chase experiments. Cell cultures were initially exposed to incubation medium containing high specific activity proline-C^{14} for 18 hours, thus labeling a broad spectrum of cell protein. Cultures were then divided into three groups. All were washed and incubated in medium containing a high concentration of nonradioactive proline (2.2 mM). One group was incubated for 15 minutes without hydrocortisone, the other groups for five hours, with or without hydrocortisone. After incubation, cultures were sacrificed and the radioactivity was determined in protein, in free proline derived from the tissue, and in the incubation medium (Table 2). Comparison between 15-minute and five-hour control cultures revealed a net decrease in peptide radioactivity and an accumulation of radioactivity in the incubation medium. Hydrocortisone treatment failed to alter the distribution of radioactivity among these fractions, indicating no marked increase in the rate of protein breakdown, although the data do not exclude small changes.

The results suggest that hydrocortisone did not inhibit protein labeling by increasing the dilution of proline-C^{14} with unlabeled proline, either in cells or in the medium. Increased proteolysis, one major cause of such an effect, could not be demonstrated. Moreover, hydrocortisone repeatedly increased the amount of radioactive free proline in the tissue (Table 3), a finding not consistent with isotope dilution. In additional experiments, the effects of hydrocortisone on proline incorporation were examined in the presence of increasing concentrations of proline (Table 3), thereby blunting any effect of isotope dilution on proline-C^{14} incorporation. Each increment (0.0022–2.2 mM) enhanced the incorporation of exogenous proline into cells and into protein. Although the percentage changes in these parameters produced by hydrocortisone decreased, the net inhibition of protein

TABLE 2

FAILURE OF HYDROCORTISONE TO INCREASE PROTEIN BREAKDOWN

	Radioactivity (cpm/μg DNA)			
	Medium	Free proline	Water-soluble protein	Insoluble protein
Control (15 min)	56	30	689	408
Control (5 hr)	323	23	671	342
Hydrocortisone (5 hr)	315	25	670	328

Cell cultures were pulse-labeled for 18 hr with L-proline-U-C^{14} (New England Nuclear Corporation, 186 mc/mm) in a concentration of 0.0022 mM. Cells were then washed and incubated for 15 min (control) or 5 hr (control and hydrocortisone) in medium containing unlabeled L-proline (2.2 mM). Hydrocortisone (10^{-5} *M*) was added to some cultures during the final 5 hr of incubation. Cells were processed as described in the text. Aliquots of the incubation medium were counted in Bray's solution directly.

TABLE 3

EFFECT OF PROLINE CONCENTRATION ON HYDROCORTISONE-INDUCED CHANGES IN AMINO ACID AND PROTEIN METABOLISM

		Protein			Free Proline		
Proline concentration (mM)	Treatment	Cpm/μg DNA	Decrease (%)	Net decrease (*p* moles/μg DNA)	Cpm/μg DNA	Increase (%)	Net increase (*p* moles/μg DNA)
	Control	1025			486		
0.0022			55	1.1		43	0.42
	Hydrocortisone	465*			694*		
	Control	690			706		
0.22			47	65		36	57
	Hydrocortisone	365*			962†		
	Control	375			645		
2.2			21	160		5	70
	Hydrocortisone	295			680		

Conditions of the experiment were as described under Table 1. Net changes were calculated from the initial specific activity of proline in the incubation medium.

* Differs significantly from control ($p = <0.01$).

† $p = <0.05$.

labeling caused by hydrocortisone increased, indicating that isotope dilution was not an important factor.

Since neither increased protein breakdown nor isotope dilution could be demonstrated, decreased incorporation of proline-C^{14} into protein is best explained by an inhibition of protein synthesis. That radioactive free proline accumulated in the tissues during hydrocortisone treatment further suggests a block in the utilization of intracellular amino acids for peptide formation.

Effects of hydrocortisone on DNA metabolism: In the present experiments, cells were studied during a period of slow proliferation when DNA accumulation was small. Under these conditions, incorporation of thymidine into DNA did not change significantly after five hours of treatment with 10^{-5} *M* hydrocortisone (Table 4). In addition, hydrocortisone treatment did not decrease significantly the total amount of DNA per culture in any experiment. These findings suggest that inhibition of protein synthesis was independent of changes in DNA metabolism.

Effects of hydrocortisone on the incorporation of uridine-2-C^{14} into RNA and the tissue accumulation of labeled uridine and uridine nucleotides: Since RNA is required for amino acid activation and peptide formation, the possibility that hydrocortisone inhibited protein synthesis by depleting bone cells of RNA was examined. Hydrocortisone treatment produced small but consistent decreases in total RNA per culture and in RNA/DNA ratios within five hours (Table 5). Hydrocortisone also inhibited the incorporation of uridine-2-C^{14} into RNA (Table 5) and into the tissue cold PCA-soluble fraction (Table 6). Chromatography of this fraction

TABLE 4

FAILURE OF HYDROCORTISONE TO DECREASE THE INCORPORATION OF THYMIDINE-2-C^{14} INTO DNA

Treatment	DNA	
	μG/culture	Cpm/μg DNA
Expt. I		
Control	49.2	125
Hydrocortisone	55.4	100
Expt. II		
Control	53.8	222
Hydrocortisone	51.8	216

Conditions of these experiments were the same as those described in Table 1, except that thymidine-2-C^{14} (New England Nuclear Corporation, 23 mc/mm, 0.016 mM) was substituted for proline-C^{14}. In experiment I, cultures received labeled thymidine for the final hour of incubation, and in experiment II, for the final 2 hr.

TABLE 5

HYDROCORTISONE-INHIBITED INCORPORATION OF URIDINE-2-C^{14} INTO RNA

	RNA (μg/culture)	RNA/DNA (× 10^2)	RNA radioactivity (cpm/μg DNA)
Expt. I			
Control	16.8	32	176
Hydrocortisone	16.0†	29†	82 (−53%)*
Expt. II			
Control	17.6	32	325
Hydrocortisone	15.9*	29*	147 (−55%)*
Expt. III			
Control	17.2	32	326
Hydrocortisone	16.6	29*	141 (−57%)*

Cell cultures were incubated with 10^{-5} *M* hydrocortisone for 5 hr. Uridine-2-C^{14} (New England Nuclear Corporation, 30 mc/mm) was added to treated and control cultures for the final 1 hr (experiment I) or 2 hr (experiments II and III) of incubation in a concentration of 0.017 mM.

* Differs significantly from control ($p = <0.01$).

† $p = <0.05$.

TABLE 6

EFFECT OF URIDINE CONCENTRATION ON HYDROCORTISONE-INDUCED CHANGES IN URIDINE METABOLISM

Concentration (mM)	Treatment	Cold PCA Fraction: Cpm/μg DNA	Cold PCA Fraction: Decrease (%)	Cold PCA Fraction: Net decrease (p moles/μg DNA)	RNA Fraction: Cpm/μg DNA	RNA Fraction: Decrease (%)	RNA Fraction: Net decrease (p moles/μg DNA)
	Control	1002			196		
0.0017			57	8.5		59	1.9
	Hydrocortisone	428*			80*		
	Control	324			66		
0.017			43	21		52	5.4
	Hydrocortisone	186*			32*		
	Control	110			8.8		
0.17			25	43		44	6.2
	Hydrocortisone	82*			4.8*		
	Control	57			2.76		
1.7			11	93		21	8.8
	Hydrocortisone	51			2.19†		

General conditions were similar to those described under Table 5. Uridine concentrations were increased by adding unlabeled uridine so that the amount of radioactive uridine remained constant. Net changes were calculated from the initial specific activity of uridine in the incubation medium.

* Differs significantly from control ($p = <0.01$).

† $p = <0.02$.

TABLE 7

HYDROCORTISONE-INHIBITED ACCUMULATION OF COLD PCA-SOLUBLE URIDINE AND URIDINE NUCLEOTIDES

	Radioactivity (cpm/μg DNA): Cold PCA fraction	Radioactivity (cpm/μg DNA): RNA fraction
Expt. I		
Control	1046	277
Hydrocortisone	652 (−38%)†	145 (−48%)
Expt. II		
Control		
Actinomycin D	531	<10
Hydrocortisone		
Actinomycin D	410 (−23%)*	<10

Cell cultures were incubated for a total of 5 hr with or without 10^{-5} *M* hydrocortisone. Cultures in experiment II were treated with actinomycin D, 1 μg/ml, for the final $1^1/_2$ hr of incubation. All cultures received uridine-2-C^{14} (30 mc/mm, 0.017 mM) for the final hr.

* Differs significantly from control ($p = <0.02$).

† $p = <0.05$.

TABLE 8

THE EFFECT OF HYDROCORTISONE ON RNA BREAKDOWN

Treatment	Chase interval	Radioactivity (cpm/μg DNA): Medium	Radioactivity (cpm/μg DNA): Cold PCA fraction	Radioactivity (cpm/μg DNA): RNA fraction
Expt. I				
Control	18	1830	410	1263
Hydrocortisone	18	2100	463	1248
Expt. II				
Control	1	710	902	656
Hydrocortisone	1	800	992	604
Expt. III				
Control				
Actinomycin D	18	400	235	993
Hydrocortisone				
Actinomycin D	18	360	249	1039

All cultures were pulse-labeled with uridine-2-C^{14}, 30 mc/mm, 0.0017 mM, for 4 hr (experiment I) or 2 hr (experiments II and III). At the end of this pulse period, cultures were washed and incubated in a chase medium containing unlabeled uridine, 0.017 mM, for either 1 or 18 hr, as indicated in the table. Cultures were then incubated for an additional 5 hr with or without hydrocortisone. The concentration of actinomycin D was 1 μg/ml in experiment III.

revealed an equivalent reduction in the radioactivity of free uridine and uridine nucleotides. When incorporation of uridine into RNA was inhibited simultaneously with actinomycin D (Table 7), hydrocortisone still decreased the radioactivity of free uridine and uridine nucleotides, indicating an independent effect of hydrocortisone on uridine accumulation. Further support for this concept was obtained by examining the effects of hydrocortisone on uridine incorporation at increased extracellular uridine concentrations (Table 6). The percentage inhibition of free nucleoside and nucleotide accumulation gradually decreased with each successive increase in uridine concentration and was not significant at the highest uridine concentration studied (1.7 mM). In contrast, the percentage inhibition of RNA labeling was still significant. The increase in net inhibition of RNA labeling obtained with increasing uridine concentrations (Table 6) suggests that decreased uridine incorporation was not caused by isotope dilution.

These results indicate two possible effects of hydrocortisone; inhibiting free uridine uptake and blocking the subsequent incorporation of uridine and uridine nucleotides into RNA. The importance of the first effect in modifying RNA synthesis is unknown, since nucleotides are synthesized by endogenous pathways at low extracellular nucleoside concentrations.[18] Furthermore, the hydrocortisone-induced block in uridine accumulation was largely overcome by high uridine concentrations. Although not established, the second effect of hydrocortisone would provide a mechanism for inhibiting RNA synthesis irrespective of changes in nucleoside or nucleotide metabolism.

Effects of hydrocortisone on RNA breakdown: RNA was pulse-labeled by brief exposure of cells to incubation medium containing high-specific-activity uridine-2-C^{14}. Cells were then washed and incubated in medium containing unlabeled uridine. Hydrocortisone treatment was begun either 18 hours or 1 hour after the pulse period, to examine possible selective effects on stable RNA (ribosomal RNA) and labile RNA (including messenger RNA and other species). In neither case was RNA radioactivity decreased significantly by hydrocortisone (Table 8), but slightly more radioactivity appeared in the incubation media and in the cold PCA-soluble fractions. In order to determine whether these changes represented a failure of reutilization of radioactive uridine and uridine nucleotides for RNA synthesis or increased RNA breakdown, a similar experiment was performed with the exception that incorporation of nucleosides and nucleotides into RNA was blocked with actinomycin D (Table 8). Under these conditions, hydrocortisone failed to increase the amount of cold PCA-soluble or medium radioactivity, indicating no marked increase in RNA breakdown. Since the data would not reveal small changes, increased RNA breakdown might still explain the slight reduction in total tissue RNA caused by hydrocortisone.

Effects of hydrocortisone concentration and duration of treatment on altered protein and RNA metabolism: In experiments performed to determine the time course of hydrocortisone effects, decreases in proline and uridine incorporation appeared in three hours (Fig. 1). Moreover, inhibition of uridine and proline incorporation was parallel over a range of hydrocortisone concentrations from 10^{-5} to 10^{-8} M (Fig. 2). Small but significant effects appeared at 10^{-8} M, which is lower than the physiologic concentrations of free glucocorticoids in rat and human plasma.[19] Similar time and concentration relationships do not exclude a primary effect on

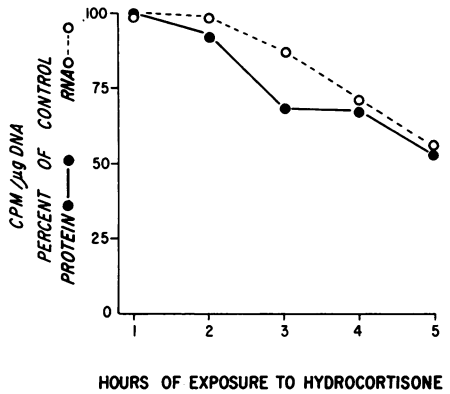

FIG. 1.—Cultures were either untreated or they received hydrocortisone (10^{-5} *M*) 1–5 hr before sacrifice. Proline-U-C^{14} or uridine-2-C^{14} was added to all cultures 1 hr before sacrifice. Each point represents the mean of three or four separate cultures.

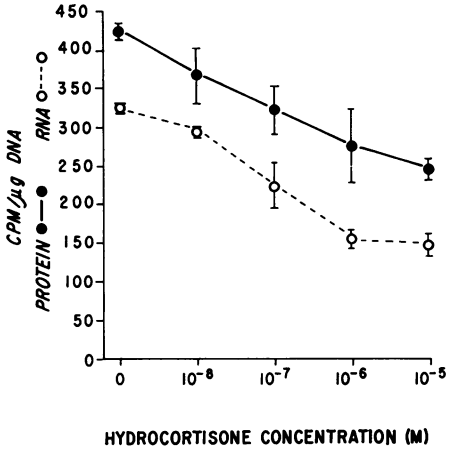

FIG. 2.—Conditions of these experiments were the same as described in Table 1, except for the variations in hydrocortisone concentration. Protein radioactivity represents the 2-hr incorporation of proline-U-C^{14} into both water-soluble and water-insoluble peptides. RNA radioactivity the 2-hr incorporation of uridine-2-C^{14}. Each point represents the mean of three separate cultures, bracketed by two standard errors of the mean.

protein or RNA metabolism. However, the slow and simultaneous appearance of both effects suggest that they may result from a separate and earlier change, as yet unidentified, induced in bone cell metabolism by hydrocortisone.

Summary.—Hydrocortisone (10^{-5} to 10^{-8} *M*) was found to inhibit the incorporation of proline C^{14} into collagen and noncollagen protein by bone cells in primary culture. Decreased protein labeling was accompanied by an accumulation of radioactive free proline in the tissue, suggesting a block in the utilization of intracellular amino acids for protein synthesis. No marked increase in the rate of protein breakdown could be demonstrated. Hydrocortisone also inhibited the uptake of uridine-2-C^{14} into the tissue pools of free uridine and uridine nucleotides, and its incorporation into RNA. Decreased pool and RNA labeling were dissociated with actinomycin D and at high extracellular uridine concentrations. There was a 5–10 per cent decrease in total tissue RNA content, which, together with decreased radioactivity, suggests an inhibition of RNA synthesis. However, an increase in RNA breakdown, too small to be detected with pulse-chase experiments, cannot be excluded. These changes were not accompanied by significant alterations in thymidine incorporation or in the amount of DNA per culture. The slow and simultaneous appearance of hydrocortisone-induced changes in protein and RNA metabolism indicates that they may both be caused by a separate and earlier effect on bone cell metabolism.

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