MOLECULAR PHARMACOLOGY, 14, 185-198

Glucocorticoids Selectively Decrease the Synthesis of Hydroxylated Collagen Peptides

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(Received July 11, 1977)
(Accepted Augut 24, 1977)

SUMMARY

NEWMAN, ROBERT A. & CUTRONEO, KENNETH R. (1978) Glucocorticoids selectively decrease the synthesis of hydroxylated collagen peptides. *Mol. Pharmacol.*, 14, 185-198.

Multiple daily injections of triamcinolone diacetate to newborn rats result in decreased body and skin weight gain, which is related to a specific decrease of collagen polypeptide synthesis. The effects of glucocorticoid administration on protein synthesis of the dermis was determined by measurement of the incorporation of labeled proline. The percentage decrease in collagen synthesis in triamcinolone-treated animals was greater than that of non-collagen protein synthesis at all doses examined, indicating a selective effect of glucocorticoids on collagen synthesis. This selective decrease of collagen synthesis was greatest after multiple injections at higher doses of the steroid. DNA synthesis was also suppressed after multiple injections of steroid. Steroid administration resulted in a decrease in prolyl hydroxylase activity (EC 1.14.11.2) in a dose- and time-dependent manner. Lysyl hydroxylase activity (EC 1.14.11.4) was decreased to the same extent as prolyl hydroxylase activity, while glucose 6-phosphate dehydrogenase activity (EC 1.1.1.49) was unchanged and tyrosine aminotransferase activity (EC 2.6.1.5) was slightly elevated. Collagenase digestion of nascent polypeptide chains released from dermal polysomes demonstrated a selective, dose-dependent decrease in collagen polypeptide synthesis. Furthermore, collagen nascent chain synthesis was decreased to the same extent as hydroxyproline formation, indicating that glucocorticoids do not cause the synthesis and subsequent accumulation of underhydroxylated collagen.

INTRODUCTION

In young growing animals and children, glucocorticoids have a profound inhibitory effect on normal growth and developmental processes (1-3). Despite the importance of growth inhibition, the mechanism whereby this occurs remains unknown. Although glucocorticoids have been shown to induce the syntheses of several liver enzymes (4), these steroids also depress the hepatic synthesis of DNA (5) and protein (6). These antianabolic effects are also evident in skin (7-10) and other peripheral tissues, where DNA and protein syntheses are decreased following glucocorticoid administration (11).

The administration of glucocorticoids re-

This study was supported by United States Public Health Service Research Grant AM 16216. A preliminary report of this work has appeared [(1977) *Fed. Proc.* 36, 752].

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0026-895X/78/0141-0185$02.00/0

sults in altered connective tissue metabolism. The excretion of hydroxyproline in urine is decreased both in young rats (9, 12) and in children (13) following glucocorticoid therapy, which suggests a decrease in collagen turnover. Administration of glucocorticoids has also been shown to decrease the content of acid-soluble collagen in skin (9, 10). Several investigations have demonstrated glucocorticoid-mediated decreases in the incorporation of labeled amino acids into collagen in skin (7–10), bone (14), and granuloma tissues (15–17). The decrease in newly synthesized proteins, at least in skin tissue, is not associated with an alteration of precursor pool specific activity (7).

The decreased rate of collagen synthesis in skin (8–10), bone (13), and granuloma (16–19) tissues after a single injection of glucocorticoid was initially thought to result from nonselective inhibition of protein synthesis, since hydroxyproline formation was decreased to approximately the same extent as total proteinaceous proline incorporation. However, following multiple injections of triamcinolone diacetate to newborn rats, hydroxyproline formation was decreased in whole skin to a much greater extent than total proline incorporation (7). Since prolyl hydroxylase activity was concomitantly reduced, this finding suggested that glucocorticoids selectively decreased either the rate of collagen polypeptide synthesis and/or the rate of prolyl hydroxylation.

In the present study the rate of collagen synthesis relative to non-collagen synthesis was quantitated in dermis at both the tissue and ribosomal nascent chain levels. The activity of prolyl hydroxylase as well as the degree of proline hydroxylation of collagen nascent chains was determined. The results indicate that glucocorticoids selectively decrease collagen polypeptide synthesis in rat dermis, which may account for the selective decrease in newly synthesized acid-soluble collagen previously reported (9, 10). While prolyl hydroxylase activity is decreased in a dose- and time-dependent manner by triamcinolone diacetate, there is no concomitant decrease in the extent of hydroxylation of nascent collagen polypeptides.

MATERIALS AND METHODS

Sprague-Dawley rats (1 day old) were used throughout this study. Animals were given one to three intraperitoneal injections of either triamcinolone diacetate, tetrahydrocortisol, or 0.9% (w/v) sodium chloride. Powdered triamcinolone diacetate was kindly supplied by Dr. E. W. Cantrall of Lederle Laboratories; tetrahydrocortisol, calf thymus DNA, Triton X-100, and β-aminopropionitrile fumarate were obtained from Sigma; and protease-free bacterial collagenase was obtained from Advance Biofactures Corporation (Lynbrook, N. Y.). [2,3-^{3}H]Proline (20 Ci/mmole), [4-^{3}H]proline (27 Ci/mmole), [4,5-^{3}H]lysine (60 Ci/mmole), and [*G*-^{3}H]hydroxyproline (5 Ci/mmole) were purchased from New England Nuclear. [5-^{3}H]Proline (29 Ci/mmole) and [*methyl*-^{3}H]thymidine (50 Ci/mmole) were purchased from Amersham/Searle. Dowex 50W-X8 (200–400 mesh) was obtained from Bio-Rad, and acid-soluble calf skin collagen was obtained from Calbiochem.

Dermis preparation. Animals were killed by decapitation, and their skins were removed for the determination of dry skin weight. The fascia and musculature were removed by dissection. The tissue was dried at 70° for 24 hr and then weighed. For other procedures whole skins, excluding that on limbs and tail, were dissected free from the carcasses and rinsed in 200 mM KCl–0.05 M Tris-HCl (pH 7.5). The subcutaneous layer was scraped free, and the dermis was dissected from the epidermis and weighed. The removal of epidermis and subcutaneous layers was periodically accessed by histological examination.

Prolyl and lysyl hydroxylase activities. Dermal tissue was suspended in 4 volumes of 0.25 M sucrose, 10 μM dithiothreitol, 10 μM EDTA, and 0.05 M Tris-HCl (pH 7.5), homogenized for 30 sec at full speed with the Polytron ST system, made 0.1% (v/v) with Triton X-100, and homogenized again for 15 sec. The homogenate was then centrifuged at 20,000 × *g* for 20 min, and the resulting supernatant was assayed for enzyme activities. Protein content of the supernatant was determined by the method

of Lowry *et al.* (20), using bovine serum albumin as standard.

Prolyl hydroxylase activity was assayed with [4-^{3}H]proline substrate by a modification of the method of Hutton *et al.* (21) as described (22), except that the assay mixture contained 0.2 mg/ml of catalase and 10 mg/ml of bovine serum albumin. Lysyl hydroxylase activity was assayed with [4,5-^{3}H]lysine substrate as described (23).

Glucose 6-phosphate dehydrogenase and tyrosine aminotransferase activities. Dermis was isolated as described above and homogenized in 3 volumes of 150 mM KCl and 1 mM EDTA (pH 7.0) for 30 sec, using the Polytron ST system. The homogenate was centrifuged at 30,000 × *g* for 30 min, and the resulting supernatant was either used directly for determination of tyrosine aminotransferase activity or diluted 1:4 (v/v) with buffer for the glucose 6-phosphate dehydrogenase assay. Tyrosine aminotransferase activity was assayed by the method of Diamondstone (24) as modified by Granner and Tomkins (25). Glucose 6-phosphate dehydrogenase activity was determined by a modification of the method of Meyer and Kunin (26). Aliquots of the 30,000 × *g* supernatant were incubated at 25° for 2 min in the presence of 80 mM Tris-HCl (pH 7.6) and 0.5 mM NADP. Glucose 6-phosphate was then added to the reaction mixture to a final concentration of 0.5 mM, and the change in absorbance at 340 nm was monitored with time. Both tyrosine aminotransferase and glucose 6-phosphate dehydrogenase activities are expressed as enzyme units, 1 enzyme unit being defined as 1 μmole of product formed per minute per milligram of supernatant protein.

DNA synthesis. Animals were injectd intraperitoneally with 3 μCi/g of [*methyl*-^{3}H]thymidine. Dermis was isolated as described above, rinsed, and homogenized with the Polytron ST system for 45 sec in 10 ml of a solution containing 2 mM EDTA and 1.5 mM Tris-HCl (pH 7.5). DNA was isolated from the homogenate by the Schmidt-Thannhauser extraction procedure (27), using perchloric acid as suggested by Ogur and Rosen (28). DNA content was determined according to the diphenylamine procedure of Burton (29) as modified by Giles and Myers (30), using calf thymus DNA as standard. Radioactivity incorporated into DNA was determined by counting an aliquot of this extract in a Triton scintillation mixture.

Collagen and non-collagen protein synthesis. Animals received 5 μCi/g of [5-^{3}H]proline intraperitoneally 30 min before death. Dermis was isolated as described above and homogenized in 6 volumes of 0.6 M NaCl–0.05 M Tris-HCl (pH 7.5) for 45 sec, using the Polytron ST system. The samples were placed in a boiling water bath for 10 min and then homogenized for 30 sec. Aliquots were removed for determination of protein and DNA contents. Another aliquot was assayed for collagenase-digestible radioactivity as described (23). Maximum collagenase digestion was obtained at 1 hr. The amount of collagenase-digestible product was not increased by doubling the amount of collagenase. Collagenase digestion solubilized greater than 85% of the proteinaceous [^{3}H]-hydroxyproline, which was isolated by Dowex cation-exchange chromatography as previously described (7).

Standard skin collagen preparation. The efficiency with which different collagenase preparations were able to digest labeled skin collagen was monitored throughout this study by determining the percentage solubilization of a [2,3-^{3}H]proline-labeled standard collagen preparation. The collagenase preparations used throughout these studies solubilized 85–90% of the standard collagen preparation. To prepare the standard collagen preparation, 2-day-old rats were injected intraperitoneally for 3 consecutive days with β-aminopropionitrile, 10 mg/rat. One hour after the last β-aminopropionitrile injection the animals were injected intraperitoneally with [2,3-^{3}H]proline (50 μCi/rat) and then killed 8 hr later. The skins were removed from the carcasses, minced, and homogenized in 4 volumes of 0.6 M NaCl–0.05 M Tris-HCl (pH 7.5) with the Polytron ST system. The homogenate was centrifuged at 160,000 × *g* for 2 hr, using a Beckman 42.1 rotor. The resulting supernatant was strained through glass wool, saturated to 30% with $(NH_4)SO_4$, stirred

for 2 hr, and centrifuged at 30,000 × *g* for 60 min. The pellet was suspended in 25 ml of the Tris–NaCl buffer and extensively dialyzed against three changes of the buffer at 4° overnight. The sample was made 20% (w/v) with respect to sodium chloride, stirred overnight, and centrifuged at 30,000 × *g* for 90 min. The resulting pellet was dissolved and dialyzed against the Tris–NaCl buffer and stored in aliquots at −60°. An aliquot of the collagen was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis by the method of Weber and Osborn (31). Comparison with an acrylamide gel of commercially obtained acid-soluble calf skin collagen showed the sample to contain predominately α_1 and α_2 chains.

Isolation of dermal polysomes. Animals received intraperitoneally 10 μCi/g of [5-^{3}H]proline 30 min or 3 hr prior to death. The skins were removed, rinsed, and soaked in 200 mM KCl, 1 mM DTT,[3] 0.3 mM EDTA, 250 mM RNase-free sucrose, 10 mM $MgCl_2$, 10 units/ml of heparin, 10 μg/ml of polyvinylsulfate, and 0.05 M Tris-HCl (pH 7.5). Three volumes of buffer were added to 12 g of minced dermis isolated as described above, and the resulting solution was made 0.25% (w/v) with respect to sodium deoxycholate and 0.5% (w/v) with respect to Triton X-100. The sample was homogenized for 45 sec with the Polytron ST system and then centrifuged at 10,000 × *g* for 30 min. The supernatant was layered over a 6-ml cushion of 1.5 M RNase-free sucrose made up in homogenizing buffer, and the sample was centrifuged at 136,000 × *g* for 4.5 hr in a Beckman 42.1 rotor. Each polysomal pellet was rinsed with 1 ml of buffer and resuspended in 3 ml of 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 0.25 M RNase-free sucrose adjusted to pH 7.0 with 1 N KOH. The sample was clarified by centrifugation at 8000 × *g* for 10 min. The supernatant was layered over another 1.5 M sucrose cushion, and the sample was centrifuged at 136,000 × *g* for 4.5 hr. The resulting pellet was resuspended in 2 ml of the sucrose–$MgCl_2$–EDTA–DTT solution and stored at −60° for less than 1 week. The polysomes were pelleted twice through a sucrose cushion to remove nonspecifically associated proteinaceous material. No radioactive proteinaceous material was released when these double-cushion polysomes were centrifuged through a 15–30% (w/v) sucrose gradient (Fig. 6). This latter finding indicated that the ribosomes were free of associated proteinaceous material.

Release and isolation of ribosomal nascent chains. Nascent chains were released by incubating the polysomes in a wheat germ lysate system prepared by a modification of the method of Roberts and Paterson (32). Six grams of wheat germ (General Mills) were mixed with 6 g of acid-washed glass beads and 28 ml of 0.1 M KCl, 1 mM magnesium acetate, 2.0 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM DTT, and 0.02 M HEPES buffer (pH 7.6). The mixture was ground at 4° and centrifuged at 30,000 × *g* for 10 min. The supernatant (S-30) was applied to a Sephadex G-25 column (2.5 × 40 cm) equilibrated with 0.12 M KCl, 5 mM magnesium acetate, 1.2 mM DTT, and 0.02 M HEPES (pH 7.6). The most turbid fractions in the void volume were collected, and stored at −60°. Protein concentration of the S-30 preparation was determined by the method of Lowry *et al.* (20).

To release nascent peptides, 40 A_{260} units (1 A_{260} unit ≅ 50 μg of RNA) of polysomes were added to a mixture containing 10 mM puromycin, 155 mM KCl, 4.6 mM magnesium acetate, 2 mM DTT, 0.8 mM ATP, 16.7 μM GTP, 6.7 mM creatine phosphate, 33 μg/ml of creatine phosphokinase, 17 μM 20 unlabeled amino acids, 20 mM HEPES (pH 7.6), and 2 mg of S-30 protein made to a final volume of 5.75 ml with deionized water. After incubation at 25° for 60 min, the reaction mixture was centrifuged at 170,000 × *g* for 2 hr in a Beckman 65 rotor. The nascent chain supernatant solution was concentrated by ultrafiltration with an Amicon PM30 system. Proline incorporation into collagen and non-collagen nascent chains was determined by collagenase digestion as previously described (33).

[3] The abbreviations used are: DTT, dithiothreitol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

RNA content of the polysomal preparations was determined by the orcinol method (34).

To determine the nascent nature of the radioactive peptides released in the lysate system, 5–8 A_{260} units of polysomes which had been incubated with puromycin as described above were placed on 30 ml of a 15–30% (w/v) sucrose gradient containing 5 mM $MgCl_2$, 1 mM DTT, and 10 mM Tris-HCl (pH 7.5). The gradient samples were centrifuged at 82,000 × g for 2.5 hr in a Beckman SW 27 rotor and were analyzed using an Isco gradient fractionator by continuously monitoring A_{254} and collecting 0.6-ml fractions. A 0.5-ml aliquot of each fraction was counted in a Triton scintillation mixture.

Determination of extent of hydroxylation of collagen nascent polypeptide chains. To determine the amount of hydroxyproline formed per collagen nascent chain, animals received 10 μCi/g of [5-^{3}H]proline intraperitoneally 3 hr before death. Dermal tissue was isolated and polysomes were prepared as described above. Ten to fifteen A_{260} units of polysomes were combined with cofactors and collagenase in 1 ml as described (23). Samples were incubated at 37° for 120 min, 1 ml of 3.8 M Tris (pH 10) was added, and the samples were incubated for an additional 20 min. The samples were placed on ice, precipitated with 2 ml of 50% trichloracetic acid, and filtered through Millipore filters. Dried filters were counted in a toluene scintillation mixture. For determination of radioactive hydroxyproline formed, 30–40 A_{260} units of dermal polysomes were combined with an equal volume of concentrated HCl, and the sample was hydrolyzed for 18 hr, evaporated, resuspended in 2 ml of deionized water, applied to a Dowex cation-exchange column, and eluted as previously described (7).

Preparation of ribosomal subunit markers. Ribosomal subunits were prepared by a modification of the method of Holder and Lingrel (35). Thirty-five A_{260} units of dermal polysomes were suspended in a final volume of 3 ml with 2.2 mM sodium pyrophosphate and 0.1 M Tris-HCl (pH 7.8). The sample was incubated for 20 min at room temperature and then layered on 30 ml of a 10–40% (w/v) linear sucrose gradient containing 1 mM DDT and 10 mM Tris-HCl (pH 7.5). The samples were centrifuged in a Beckman SW 27 rotor for 14 hr at 81,000 × g. Gradients were analyzed by monitoring A_{254} and pooling the appropriate fractions. After lyophilization, the 40 S and 60 S subunit preparations were separately dissolved in 6 ml of distilled water and then dialyzed against water. Aliquots of each subunit preparation were stored frozen.

RESULTS

Multiple injections of glucocorticoid resulted in a dose-dependent decrease in neonatal dry skin weight that closely paralleled the inhibition of body weight gain. Maximum growth-inhibitory effects of body and skin weight gain were obtained at doses between 5 and 10 mg/kg of steroid (Fig. 1).

Dermis, a tissue rich in fibroblasts, was used to study the effects of glucocorticoid on collagen synthesis. Incubation of skins

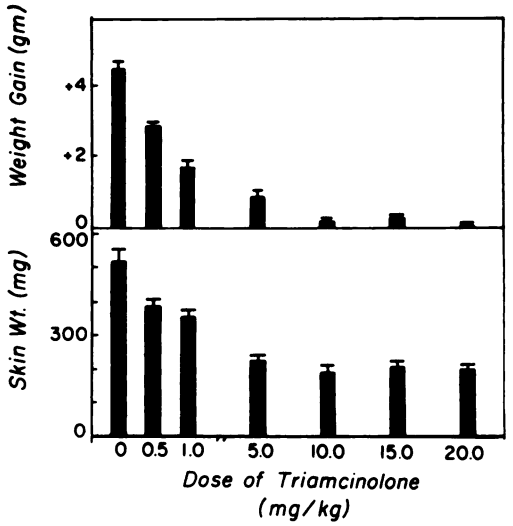

FIG. 1. *Dose response of animal weight gain and dry skin weight to triamcinolone diacetate*

Newborn rats received three daily intraperitoneal injections of triamcinolone diacetate (0.1 ml/10 g of rat). The dry skin weight was determined as described in the text. Values represent means ± standard errors of determinations from three to eight animals.

in a buffer containing 200 mM KCl greatly facilitated the physical separation of dermal from epidermal tissues. Histological examination of the dermis indicated this tissue to be free from contaminating cells of the epidermis (Fig. 2). In addition, der-

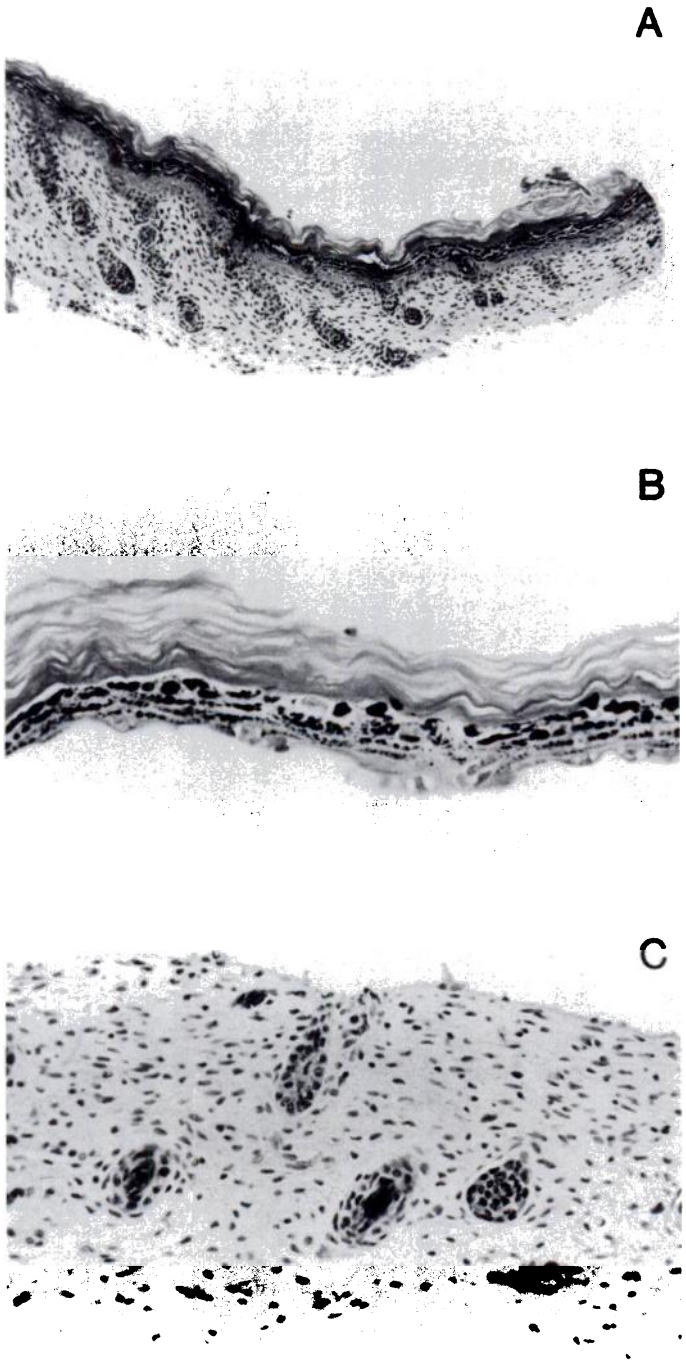

FIG. 2. *Photomicrographs of whole skin, epidermis, and dermis*
A. Whole skin (× 100). B. Epidermis (× 400). C. Dermis (× 250).

mal tissue contained 13 times more specific activity of prolyl hydroxylase than did the epidermis (Table 1).

The effects of glucocorticoid administration on dermal protein synthesis were determined by measurement of the incorporation of [5-^{3}H]proline. Incorporation of labeled proline into both collagen and non-collagen proteins of rat dermis was linear for up to 1 hr (Fig. 3). Subsequent measurement of tissue protein synthesis was made with a pulse time of 30 min. Although proline incorporation into both collagen and non-collagen proteins at a dose of 12 mg/kg of triamcinolone was less than the corresponding control values, there was a much more marked change in proline incorporation into collagen protein.

Animals were treated with daily injections of triamcinolone to determine the dose response of changes in protein synthesis. The percentage decrease in dermal collagen synthesis was greater than that of non-collagen protein synthesis at doses of 1 mg/kg and higher (Table 2). While this selective effect was noted even at a dose as low as 1 mg/kg, it was more evident at the higher doses. At 12 mg/kg, collagen synthesis was almost totally suppressed while non-collagen protein synthesis was decreased by 58%. The glucocorticoid-mediated selective inhibition of tissue collagen synthesis was also evident by the progressive dose-dependent decrease in the collagen to non-collagen ratio, therefore suggesting that an alteration of precursor pool specific activity did not account for the total decrease in collagen synthesis.

The effect of multiple glucocorticoid injections on labeled thymidine incorporation was determined. Incorporation *in vivo* of [^{3}H]thymidine was linear for up to 30 min (Fig. 4), and thus a pulse time of 20 min was used. Multiple injections of triamcinolone at doses as low as 0.5 mg/kg resulted in 20% inhibition of thymidine

TABLE 1

Localization of prolyl hydroxylase activity in dermis

Dermis and epidermis were isolated from skin, and the relative specific activities of prolyl hydroxylase for the two tissues were determined as described in the text. Values represent the means ± standard errors of enzyme activity from 8–11 animals.

Tissue	Prolyl hydroxylase activity
	dpm × 10^{-3}/mg supernatant protein
Dermis	37.7 ± 4.4
Epidermis	2.8 ± 0.4[a]

[a] Significantly different at $p \leq 0.05$.

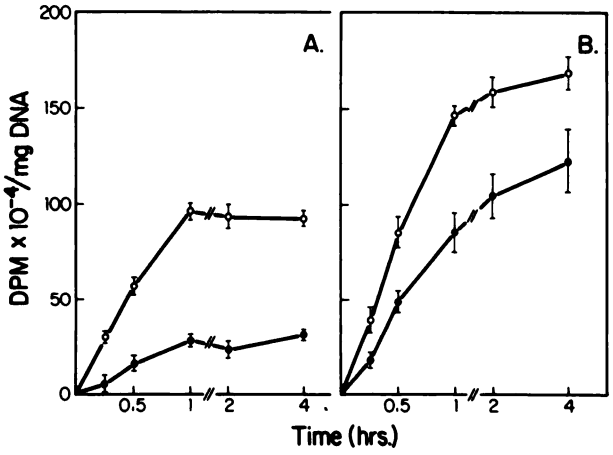

FIG. 3. *Incorporation of [^{3}H]proline into collagen and non-collagen protein of rat dermis*

One-day-old rats received three daily intraperitoneal injections of triamcinolone diacetate (12 mg/kg) and were killed 24 hr after the last injection. Thirty minutes prior to death the animals received [5-^{3}H]proline (5 μCi/g). Proline incorporation into collagen (A) and non-collagen protein (B) was determined by collagenase digestion as described in the text. The values represent the means ± standard errors of determinations from three to six control (○) or triamcinolone-treated (●) animals.

TABLE 2

Dose response of collagen, non-collagen protein, and DNA syntheses in rat dermis to triamcinolone diacetate

Values represent the means ± standard errors of determinations from four to eight animals. Steroid-treated animals received three daily intraperitoneal injections of triamcinolone diacetate at the doses indicated and were killed 24 hr after the last injection. All rats received intraperitoneally either 5 μCi/g of [5-^{3}H]proline for determination of collagen and non-collagen protein synthesis or 3 μCi/g of [*methyl*-^{3}H]thymidine for determination of DNA synthesis. Proline incorporation into collagen and non-collagen protein was determined by collagenase digestion as described in the text. The numbers in parentheses represent the percentage decrease from the control value.

Triamcinolone dose	Collagen (A)	Non-collagen protein (B)	A:B	[^{3}H]Thymidine incorporation
mg/kg	*dpm × 10^{-4}/mg DNA*			*dpm × 10^{-4}/mg DNA*
0	141.5 ± 18.5	195.3 ± 13.1	0.724	93.3 ± 9.7
0.5	134.4 ± 20.5	185.2 ± 26.2	0.726	74.1 ± 2.9[a] (20%)
1.0	122.3 ± 17.1 (14%)	182.3 ± 25.2 (7%)	0.670	53.8 ± 8.8[a] (42%)
2.0	95.9 ± 8.6[a] (32%)	173.2 ± 8.2[a] (11%)	0.554	44.9 ± 5.4[a] (52%)
4.0	64.3 ± 10.6[a] (54%)	133.3 ± 16.1[a] (32%)	0.482	34.5 ± 6.9[a] (63%)
7.0	40.0 ± 4.4[a] (72%)	129.3 ± 10.5[a] (34%)	0.309	28.9 ± 4.9[a] (69%)
12.0	17.0 ± 3.6[a] (88%)	82.7 ± 5.4[a] (58%)	0.206	12.4 ± 1.4[a] (88%)

[a] Significantly different from non-steroid-treated control at $p \leq 0.05$.

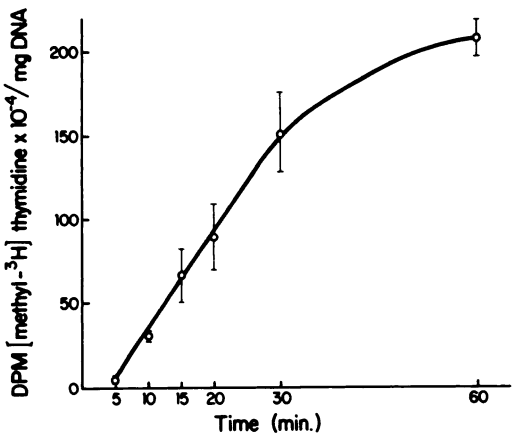

FIG. 4. *Incorporation of [^{3}H]thymidine into rat dermis*

Animals were injected with [^{3}H]thymidine (3 μCi/g) at the times indicated prior to death. DNA was extracted from dermal tissue, and its specific activity was determined as described in the text. Values represent the means ± standard errors of three to six animals.

TABLE 3

Dose response of dermal prolyl hydroxylase activity to triamcinolone diacetate

One-day-old rats were treated for 3 consecutive days with triamcinolone diacetate at the doses indicated and were killed 24 hr after the last injection. Prolyl hydroxylase activity in the 20,000 × *g* supernatant of dermal homogenates was determined as described in the text. The values represent the means ± standard errors of enzyme activity from five to eight animals. The numbers in parentheses indicate the percentage decrease of enzyme activity compared with the control value.

Triamcinolone dose	Prolyl hydroxylase activity
mg/kg	*dpm × 10^{-3}/mg supernatant protein*
0	154.5 ± 4.2
0.25	152.9 ± 6.0
0.5	131.9 ± 7.0[a] (15%)
1.0	113.9 ± 5.3[a] (26%)
2.0	101.2 ± 6.1[a] (35%)
4.0	87.7 ± 4.1[a] (43%)
7.0	68.3 ± 4.3[a] (56%)
12.0	65.8 ± 4.3[a] (57%)

[a] Significantly different from non-steroid-treated control at $p \leq 0.05$.

incorporation (Table 2). Higher doses of steroid produced greater reductions of DNA synthesis.

Daily injection of triamcinolone resulted in a dose-dependent decrease in prolyl hydroxylase activity (Table 3). The maximum decrease in prolyl hydroxylase activity was obtained with doses of 7 mg/kg or more. The activities of lysyl hydroxylase, glucose 6-phosphate dehydrogenase, and tyrosine aminotransferase were also determined (Table 4). After multiple injections of 12 mg/kg, lysyl hydroxylase activity was also decreased by 61%. However, glucose 6-phosphate dehydrogenase activity

was unchanged, while tyrosine aminotransferase activity was slightly elevated above the control value. Furthermore, the active form of glucocorticoid was required to produce a decrease in dermal prolyl hydroxylase activity. Multiple injections of tetrahydrocortisol, a major metabolite of cortisol, had no effect on enzymatic activity (data not shown).

The temporal response of collagen and non-collagen protein synthesis to daily injections of either 2 or 12 mg/kg of triamcinolone is shown in Table 5. Significant decreases in both collagen and non-collagen syntheses were observed as early as 6 hr after the initial drug administration at both dose levels. At all time intervals collagen synthesis was decreased to a

TABLE 4

Effect of triamcinolone diacetate on glucose 6-phosphate dehydrogenase, tyrosine aminotransferase, and lysyl hydroxylase activities of rat dermis

One-day-old rats received intraperitoneally three daily injections of triamcinolone diacetate (12 mg/kg) and were killed 24 hr after the last injection. Values represent the means ± standard errors of four to eight determinations. The numbers in parentheses represent the percentage of the control value. EU represents the micromoles of product formed per minute at pH 7.6 and 37° for tyrosine aminotransferase activity and at 25° for glucose 6-phosphate dehydrogenase activity.

Group	Glucose 6-phosphate dehydrogenase	Tyrosine aminotransferase	Lysyl hydroxylase
	EU × 10^2/mg protein	*EU × 10^3/mg protein*	*dpm × 10^{-3}/mg protein*
Control	6.6 ± 0.3	2.6 ± 0.1	3.6 ± 0.3
Triamcinolone	7.2 ± 0.5	3.5 ± 0.1[a] (135%)	1.4 ± 0.1[a] (61%)

[a] Significantly different from control at $p \leqq 0.05$.

TABLE 5

Temporal response of collagen and non-collagen protein syntheses in rat dermis to triamcinolone diacetate

One-day-old rats received daily intraperitoneal injections of triamcinolone diacetate (2 or 12 mg/kg) and were killed at the times indicated. Each animal received intraperitoneally 5 μCi/g of [5-^{3}H]proline 30 min prior to death. Proline incorporation into collagen and non-collagen protein was determined by collagenase digestion of rat dermis as described in the text. The values represent the means ± standard errors of determinations from 3–12 animals. The numbers in parentheses represent the percentage decrease from the control value.

Time after initial triamcinolone injection	Triamcinolone dose	Collagen (A)	Non-collagen protein (B)	A:B
	mg/kg	*dpm × 10^{-4}/mg DNA*		
0	0	91.6 ± 7.5	124.5 ± 7.0	0.736
6	0	72.8 ± 4.2	108.2 ± 9.5	0.673
	2	39.0 ± 4.5[a] (46%)	72.6 ± 5.7[a] (33%)	0.537
	12	40.9 ± 8.6[a] (44%)	80.1 ± 16.5 (26%)	0.511
12	0	58.2 ± 4.5	103.4 ± 4.0	0.563
	2	29.4 ± 4.8[a] (49%)	56.4 ± 14.4[a] (45%)	0.521
	12	15.6 ± 1.4[a] (73%)	43.2 ± 4.9[a] (58%)	0.361
30	0	54.1 ± 5.2	91.9 ± 5.0	0.589
	2	33.6 ± 4.8[a] (38%)	75.9 ± 7.8 (17%)	0.443
	12	8.1 ± 1.3[a] (85%)	33.0 ± 4.2[a] (64%)	0.245
54	0	54.2 ± 5.2	90.6 ± 7.8	0.598
	2	31.5 ± 12.0[a] (42%)	63.3 ± 7.5[a] (30%)	0.498
	12	6.5 ± 0.4[a] (88%)	32.7 ± 3.0[a] (64%)	0.199
78	0	51.4 ± 6.4	87.9 ± 8.6	0.585
	2	11.4 ± 0.6[a] (78%)	30.6 ± 2.7[a] (65%)	0.373
	12	3.5 ± 0.4[a] (93%)	22.3 ± 2.7[a] (75%)	0.157

[a] Significantly different from respective non-steroid-treated control at $p \leqq 0.05$.

greater extent than was non-collagen protein synthesis.

The temporal response of prolyl hydroxylase activity to triamcinolone administration is shown in Fig. 5. Three hours after a single injection at either 2 or 12 mg/kg, prolyl hydroxylase activity was slightly elevated. Enzyme activity at both doses then returned to the control level at 6 hr. Multiple injections of triamcinolone resulted in decreased prolyl hydroxylase activity. Enzyme activity was decreased to a greater extent following multiple injections of 12 mg/kg than of 2 mg/kg. However, the maximum steroid-induced decrease in prolyl hydroxylase activity did not equal the maximum percentage decrease in tissue collagen synthesis at either 2 or 12 mg/kg (Table 5).

To determine whether the observed changes in the amounts of proteinaceous labeled proline in dermal tissue resulted from an actual decrease in peptide synthesis, dermal polysomes were isolated from rats which had received radioactive proline. The distribution of radioactivity throughout the sucrose gradient followed the A_{254} profile (Fig. 6A). The quantity of radioactivity associated with the A_{254} profile of triamcinolone-treated tissue poly-

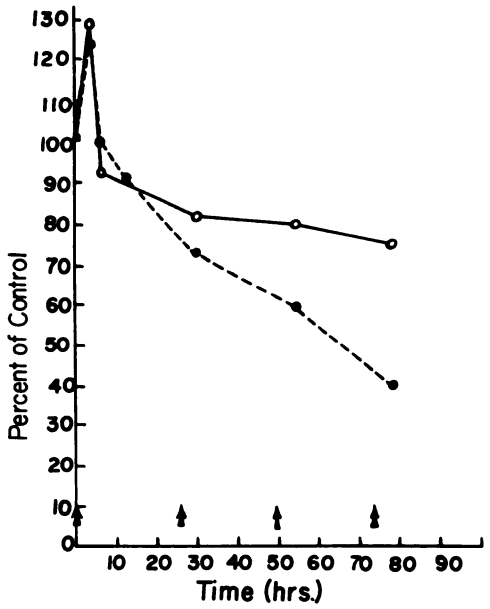

FIG. 5. *Temporal response of dermal prolyl hydroxylase activity to triamcinolone diacetate*

One-day-old rats were injected with 2 mg/kg (○) or 12 mg/kg (●) of triamcinolone diacetate at the times indicated by the arrows. Prolyl hydroxylase activity in the 20,000 × *g* supernatants of dermal homogenates was determined as described in the text. The values represent the mean percentage of control values from three to five samples.

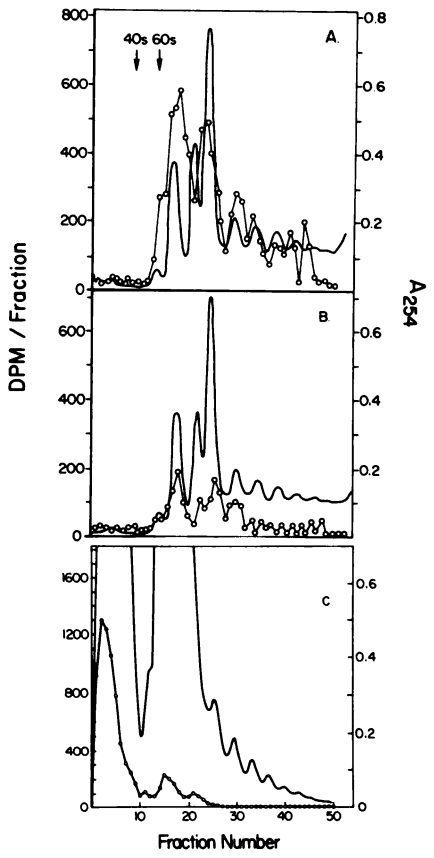

FIG. 6. *Sucrose gradient profiles of ribosome-associated radioactivity*

Newborn rats received three daily intraperitoneal injections of triamcinolone diacetate. Polysomes were isolated and chromatographed on a 15–30% sucrose gradient as described in the text. The radioactivity of the gradient fractions was then determined. A. Sucrose gradient A_{254} profile of 8 A_{260} units (approximately 400 μg of RNA) of control polysomes. B. Sucrose gradient A_{254} profile of 8 A_{260} units of dermal polysomes isolated from triamcinolone-treated rats. C. Sucrose gradient A_{254} profile of 8 A_{260} units of control dermal polysomes chromatographed after incubation at 25° for 30 min in the presence of puromycin in the wheat germ lysate system as described in the text. ——, A_{254}; ○, radioactivity.

somes was markedly reduced (Fig. 6B). Steroid treatment did not significantly alter the polysomal profile, suggesting polyribosomal integrity. The nascent nature of the labeled polysomal peptides was determined by release from the ribosomes by incubation in the wheat germ lysate system containing puromycin. As shown in Fig. 6C, essentially all the radioactivity formerly associated with specific polysomal A_{254} peaks was released to the top of the gradient.

Animals were treated with multiple injections of 1, 4, or 12 mg/kg of triamcinolone diacetate and then received radioactive proline 30 min before death. Polysomes were isolated, and the labeled nascent peptides were released and digested with bacterial collagenase. At all doses examined, collagen peptide synthesis was selectively inhibited to approximately twice the extent observed for non-collagen peptide synthesis (Table 6). Thus glucocorticoids caused a selective antianabolic response evident at the ribosomal nascent chain level. At a dose of 12 mg/kg, the percentage decrease in both collagen and non-collagen peptide synthesis was less than that observed for dermal tissue homogenates. This may indicate some catabolism of both species of tissue protein at this relatively high dose.

The degree of hydroxylation of nascent collagen polypeptide chains was determined by measuring radioactive hydroxyproline formed per collagenase-digestible nascent peptide (Table 7). No change in the degree of hydroxylation of nascent chains was observed in any of the triamcinolone-treated groups, since collagen polypeptide synthesis was decreased to the same extent as hydroxyproline formation.

DISCUSSION

The administration of pharmacological doses of anti-inflammatory steroids produces profound effects on collagen metabolism in both normal and inflamed connective tissues (7, 9, 14–19). Although investigators generally are in agreement that the anti-inflammatory steroid-mediated alteration of collagen metabolism is mainly antianabolic in nature, the mechanisms by which glucocorticoids exert their effects at the cellular and molecular levels remain unknown. Based on parallel changes of prolyl hydroxylase activity and collagen synthesis in both normal and diseased tissues, it has been suggested that this enzyme may be either rate-limiting or at least involved in a regulatory mechanism coordinate with that of collagen synthesis (36, 37). Following multiple injections of glucocorticoids to rats, both prolyl hydroxylase activity and hydroxyproline formation are decreased coordinately (7). Therefore in the present study the relationship between glucocorticoid-mediated changes in prolyl hydroxylase activity and collagen peptide synthesis was further examined.

Dermal protein synthesis is markedly altered by administration of triamcino-

TABLE 6

Dose response of collagen and non-collagen nascent polypeptide syntheses to triamcinolone diacetate

One-day-old rats received three daily intraperitoneal injections of triamcinolone diacetate at the doses indicated and were killed 24 hr after the last injection. All animals received 10 μCi/g of [5-^{3}H]proline 30 min before death. Nascent polypeptide chains were released from ribosomes with 10 mM puromycin in a wheat germ lysate system as described in the text. The amount of labeled proline incorporated into collagen and non-collagen nascent chains was determined by collagenase digestion. Values represent the means ± standard errors of determinations from three to five animals. The numbers in parentheses represent the percentage decrease from the control value.

Triamcinolone dose	Collagen (A)	Non-collagen protein (B)	A:B
mg/kg	*dpm × 10^{-3}/mg RNA*		
0	47.5 ± 8.1	63.4 ± 11.8	0.749
1	38.7 ± 2.0 (19%)	64.3 ± 13.3	0.601
4	18.2 ± 1.5[a] (62%)	44.1 ± 1.5[a] (30%)	0.413
12	10.4 ± 0.8[a] (78%)	36.8 ± 2.4[a] (42%)	0.283

[a] Significantly different from non-steroid-treated control at $p \leq 0.05$.

TABLE 7

Effect of triamcinolone diacetate on hydroxyproline formation in collagen nascent polypeptides

One-day-old rats received three daily intraperitoneal injections of triamcinolone diacetate at the doses indicated and were killed 24 hr after the last injection. All animals received (5-^{3}H)proline (10 μCi/g) 3 hr before death. The amount of labeled proline incorporated into ribosomal collagen peptides and the amount of hydroxyproline formed were determined as described in the text. The numbers in parentheses represent the percentage decrease from the control value.

Triamcinolone dose	Hydroxyproline (A)	Collagen (B)	A:B
mg/kg	*dpm* $\times 10^{-2}$*/mg RNA*		
0	18.6	33.7	0.552
1	14.4 (23%)	26.7 (21%)	0.539
2	11.9 (36%)	22.6 (33%)	0.526
4	10.5 (43%)	18.3 (46%)	0.574
12	3.3 (82%)	5.5 (84%)	0.600

lone. Multiple injections of steroid produce dose-dependent, selective inhibition of tissue collagen synthesis relative to non-collagen protein synthesis. Furthermore, a significant decrease in collagen and non-collagen protein syntheses (7) is evident with doses as low as 2 mg/kg. Consequently, even relatively low doses of glucocorticoid may selectively decrease the content of collagen in tissues. After a single injection of triamcinolone at either 2 or 12 mg/kg, proline incorporation into tissue proteins is significantly depressed. As early as 6 hr after drug administration, collagen synthesis is selectively inhibited relative to non-collagen protein. Continued daily injections of triamcinolone result in further dose-dependent, selective inhibition of collagen synthesis.

In order to determine whether the observed glucocorticoid-mediated changes in tissue protein synthesis resulted from corresponding changes in nascent chain synthesis, polysomes were isolated from the dermis of rats that had received radioactive proline. Multiple injections of triamcinolone produced a significant reduction in the quantity of radioactive peptide chains associated with polyribosomes. However, the polysomal profile of the steroid-treated sample remained largely unaltered, indicating the integrity of polyribosomal structure. This finding is in good agreement with the report of Gould and Manner (38), who observed that the distribution of polysomes from chick embryos was not greatly affected even by relatively high doses of hydrocortisone. However, a more direct relationship existed between the dose of steroid and the uptake of labeled proline into collagen as well as non-collagen proteins. The decrease of proline incorporation into collagen nascent chains is probably not the result of steroid alterations of the specific activity of the precursor pool, since neither a single nor multiple injections of triamcinolone at doses as high as 50 mg/kg affected the specific activity of the acid-soluble proline pool in skin (7). Under these circumstances, however, hydroxyproline synthesis was significantly decreased.

After multiple injections of steroid, a decrease in prolyl hydroxylase activity in skin was observed (7), which was paralleled by a decrease in the amount of enzyme protein (39). In the present study multiple injections of triamcinolone produced a dose-dependent decrease in dermal prolyl hydroxylase activity, with the maximum effect occurring at doses greater than 7 mg/kg. At a dose of 12 mg/kg, this alteration of prolyl hydroxylase activity was paralleled by an almost identical change in dermal lysyl hydroxylase activity. These changes in dermal collagen hydroxylase activities are not due to a non-specific decrease in total tissue enzymatic activity, since neither glucose 6-phosphate dehydrogenase nor tyrosine aminotransferase activity was reduced.

Examination of the temporal response of prolyl hydroxylase to triamcinolone administration indicated an early rise in enzymatic activity. Three hours after the initial steroid injection, prolyl hydroxylase activity was slightly elevated over control values. The reason for this early elevation of enzyme activity is not known. After multiple injections of steroid prolyl hydroxylase activity decreased.

During the biosynthesis of collagen, the hydroxylation of proline residues occurs before helix formation of the peptide

chains (40–44). Since the extent of hydroxylation of proline residues of collagen is directly related to the structural stability of the molecule, an inhibited hydroxylating system could possibly influence the quality and quantity of newly synthesized collagen. In a previous study (7), multiple injections of triamcinolone resulted in a decrease in hydroxyproline formation in neonatal rat skin, which was greater than the decrease in total proline incorporation into tissue proteins. This finding suggested that underhydroxylated collagen may be synthesized in the skin of glucocorticoid-treated animals. Accordingly, the degree of hydroxylation of collagen nascent polypeptides was determined. No evidence for the presence of underhydroxylated collagen polypeptides was found. The degree of inhibition of hydroxyproline formation was identical with the decrease in ribosomal nascent chain synthesis. It would appear that during glucocorticoid treatment alteration of the activity of enzymes involved in translational modification reactions is not involved in the regulation of collagen polypeptide synthesis. Alternatively, the regulation of collagen polypeptide synthesis probably occurs at the transcriptional or translational level.

The dramatic decrease in DNA and protein synthesis, especially collagen formation, may be responsible for many of the therapeutic and toxic effects of glucocorticoid therapy in animals undergoing rapid growth and reparative or inflammatory processes. Our data indicate that the response of connective tissue to pharmacological doses of glucocorticoid is related to a selective decrease in collagen nascent chain synthesis. It is therefore suggested that this selective effect of glucocorticoid to decrease tissue collagen synthesis may be an important mechanism of action of this class of drugs on connective tissue metabolism. Although after multiple injections of glucocorticoid the decrease in prolyl hydroxylase activity does not result in the synthesis of underhydroxylated collagen, enzyme activity and collagen synthesis are both decreased. The triamcinolone-mediated decreases in collagen synthesis and prolyl and lysyl hydroxylase activities suggest coordinate regulation of several key steps in collagen biosynthesis at the translational or transcriptional level.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Brian Little for his consultation in evaluating the histological sections, and Dr. Cassius Bordolon for his consultation on preparing the wheat germ lysate system. We also acknowledge the expert technical assistance of Ms. Elizabeth Tuthill, Ms. Gloria Hoff, Mr. Brian McNelis, and Mr. James Evans.

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