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Anti-inflammatory Steroids and Collagen Metabolism: Glucocorticoid-Mediated Alterations of Prolyl Hydroxylase Activity and Collagen Synthesis

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SUMMARY

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Several daily injections of triamcinolone diacetate to newborn rats resulted in a decrease of prolyl hydroxylase activity in skin. After labeling of glucocorticoid-treated animals with radioactive proline, hydroxyproline formation was decreased to a greater extent than total proline incorporation, indicating a specific effect on collagen biosynthesis. In contrast, 12 hr after a single injection of steroid, when enzyme activity was only slightly decreased, the depressed level of hydroxyproline formation was almost totally accounted for by the decrease of total proline incorporation. The specific activity of the proline precursor pool was unchanged in animals receiving one or three injections of drug as compared to controls. After 3 days of steroid treatment the amounts of deoxyribonucleic acid, protein, and proteinaceous hydroxyproline per skin were decreased to the same extent as skin weight. Our data indicate that the general antianabolic effect of glucocorticoids on protein synthesis is succeeded by a specific effect on collagen synthesis. The decrease of prolyl hydroxylase which is realized only after multiple injections of glucocorticoids is associated with this specific effect on hydroxyproline formation. These data suggest that the decrease of prolyl hydroxylase following treatment with glucocorticoids is involved in the molecular mechanism of action of this class of drugs on collagen biosynthesis.

INTRODUCTION

Administration of triamcinolone diacetate to newborn rats results in a decrease of prolyl hydroxylase in various tissues (1). Recent studies have indicated that prolyl hydroxylase activity is not affected at times after steroid treatment when incorporation of proline into proteins is markedly decreased (2–4). In these studies administration of glucocorticoids resulted in decreased incorporation of radioactive proline into protein and decreased hydroxyproline formation in bone (3) and granuloma

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tissues (2) at a time when prolyl hydroxylase was unaffected. These observations suggested that a general decrease of protein synthesis following steroid treatment resulted in a decreased synthesis of collagen polypeptide, thereby accounting for the total antianabolic effect of this class of drugs on collagen metabolism.

In the present study our data indicate that after multiple injections of triamcinolone, when prolyl hydroxylase activity is decreased in skin, hydroxyproline formation is decreased to a level not accounted for by the decrease in total proline incorporation, thus indicating a specific effect on collagen synthesis. The results suggest that the decrease of prolyl hydroxylase activity following multiple injections of anti-inflammatory steroids is involved in the molecular action of this class of drugs on collagen biosynthesis.

MATERIALS AND METHODS

Sprague-Dawley rats (1 day old) were used throughout this study. Animals were given either one or three daily intraperitoneal injections of triamcinolone diacetate (50 mg/kg). Animals receiving a single injection of steroid were killed 12 hr afterward. Animals receiving multiple daily injections were killed 24 hr after the last injection. Powdered triamcinolone diacetate was kindly supplied by Dr. J. M. Smith of Lederle Laboratories. A suspension of this drug was prepared in 0.9% NaCl. Control animals received 0.9% NaCl. [4-^{3}H]Proline (15 Ci/nmole) was purchased from Schwarz/Mann. [^{14}C]Proline (200 mCi/mmole) was obtained from New England Nuclear Corporation. Samples were counted in a liquid scintillation mixture as described (1).

Prolyl hydroxylase activity. At death the animals were decapitated and whole skins, excluding that on limbs and tails, were dissected free and weighed on a Mettler balance. The skin was then homogenized with a Polytron ST homogenizer in 5 ml of 0.25 M sucrose. An aliquot of the homogenate was centrifuged at 16,000 × g for 20 min, and the resulting supernatant was assayed for protein by the method of Lowry *et al.* (5) and for enzyme activity by the method of Hutton *et al.* (6) as described (1).

DNA content. Two ml of 0.5 N NaOH was added to 0.5 ml of the original homogenate. The sample was heated in a 70° water bath for 1 hr. An aliquot of the sample solution was then added to an equal volume of 1 N HCl. The sample was centrifuged at 10,000 × g for 10 min, and the resulting supernatant was assayed for diphenylamine-reactive material by the method of Burton (7).

Total tissue hydroxyproline content. Trichloracetic acid (10%, w/v) was added to the remaining homogenate. The sample was centrifuged at 10,000 × g for 5 min. The resulting pellet was resuspended in 5% (w/v) trichloracetic acid and recentrifuged. This was repeated twice. Then 10 ml of 6 N HCl were added to the tissue pellet, and the sample was hydrolyzed for 18 hr in an autoclave. Norit A (500 mg/sample) was added to the hydrolysate, and the sample was filtered through Whatman No. 1 filter paper. The sample was evaporated and resuspended in 4 ml of deionized water, and an aliquot of this sample was assayed for ninhydrin-reactive material (8). Two drops of phenol red were added, and the sample was neutralized with 4 N KOH and then applied to a 20 × 1 cm Dowex 50W-X8 (200–400 mesh) cation-exchange column. The sample was washed onto the column with 50 ml of deionized water and eluted with 1 N HCl. The first 40 ml were collected and evaporated to dryness. The sample was resuspended in 1 ml of deionized water and assayed for hydroxyproline by the method of Prockop and Udenfriend (9). For calibration of the Dowex column the elution of both ^{3}H- and ^{14}C-labeled proline and hydroxyproline was determined. The purity of these radioactive amino acids was checked by descending paper chromatography on Whatman No. 1 paper with butanol–acetic acid–water (63:27:10) as solvent. The radioactivity applied to the paper ran with the R_F of either pure hydroxyproline or pure proline. Furthermore, the radioactive samples from tissues eluting in either the proline or hydroxyproline region after Dowex chromatography were routinely concen-

trated and chromatographed in descending fashion as another check on the Dowex cation-exchange chromatographic method. We also routinely applied radioactive tissue samples containing 5 mg of pure hydroxyproline to the Dowex column. The column was then eluted with 1 N HCl. An aliquot of each fraction was counted. To the remaining aliquot were added an equal volume of 1 N NaOH, 2 ml of isopropyl alcohol, and 1 ml of 0.02 M Chloramine-T solution, in the order given. The samples were incubated at room temperature for 20 min, followed by addition of 2 ml of Ehrlich's reagent (9). After 40 min at room temperature the fractions were read at A_{560}. The colorimetrically detectable hydroxyproline was found only in those fractions containing labeled hydroxyproline.

Total incorporation and hydroxyproline formation. Either 30, 60, or 90 min prior to death each animal was injected intraperitoneally with 5 μCi of [^{14}C]proline. Tissue homogenates were prepared and processed for Dowex cation-exchange chromatography of the trichloracetic acid-insoluble material as previously described. The radioactivity of the sample and the amount of ninhydrin-reactive material were determined prior to Dowex chromatography for the quantitation of total incorporation. [^{14}C]Hydroxyproline was separated from [^{14}C]proline by collecting 1-ml fractions and pooling the radioactive hydroxyproline peak. The pooled fraction was evaporated to dryness and redissolved in 4 ml of deionized water. An aliquot was counted for radioactivity, and another aliquot was chemically assayed for hydroxyproline content (9).

Measurements of free proline pool. The amounts of radioactive proline and total proline of the trichloracetic acid pool in skin from control and drug-treated animals were determined. The trichloracetic acid-soluble fraction was evaporated, redissolved in 10 ml of 6 N HCl, and hydrolyzed for 18 hr in an autoclave. After the addition of Norit A and filtering through Whatman No. 1 paper the sample was evaporated, redissolved in 4 ml of water, neutralized, and applied to a 20 × 1 cm Dowex cation-exchange column. One-milliliter fractions were collected, and those containing proline, which was eluted between fractions 40 and 80, were pooled, evaporated, and redissolved in 4 ml of water. One aliquot was assayed for proline by the method of Troll and Lindsley (10), and another aliquot was counted. Essentially all of the labeled proline in the trichloracetic acid-soluble pool was in the free form as determined by DEAE-Sephadex chromatography by the method of Carnegie (11). Of the total amount of radioactivity in the trichloracetic acid-soluble fractions, 95% was [^{14}C]proline and 5% was [^{14}C]hydroxyproline as determined by Dowex cation-exchange chromatography.

RESULTS

The effects of either a single or daily multiple injections of glucocorticoid on the wet weight of skin and the amounts of DNA, protein, and hydroxyproline per skin are presented in Table 1. After multiple injections of glucocorticoid the values for control animals were approximately 2-fold greater than those for steroid-treated animals (Table 1). Since the wet weight of the total skin was elevated to the same extent as total DNA, protein, and hydroxyproline, these differences are due to general antianabolic effects of the glucocorticoid on growth. The amount of proteinaceous hydroxyproline per unit of protein was not significantly different from the control value after three injections of glucocorticoid.

Recently administraton of triamcinolone diacetate to newborn rats has been shown to decrease prolyl hydroxylase in various tissues (1). The effect of administration of triamcinolone diacetate after either a single or multiple injections on prolyl hydroxylase activity is summarized in Table 2. Twelve hours after one injection of anti-inflammatory steroid, enzyme activity was slightly decreased. However, after three injections of anti-inflammatory steroid, prolyl hydroxylase was decreased by 35%.

In order to determine whether the decreased level of prolyl hydroxylase following repeated injections of glucocorticoid was associated with a specific effect of this

TABLE 1

Effect of administration of triamcinolone on skin weight and content of DNA, protein, and proteinaceous hydroxyproline

Values represent the means ± standard errors of 6–17 animals. Animals receiving a single injection of triamcinolone diacetate (50 mg/kg intraperitoneally) were killed 12 hr later, while animals receiving multiple injections were killed 24 hr after the last injection. The amount of hydroxyproline was determined on the basis of the ninhydrin-reactive material of whole skin, using leucine as a standard.

Treatment	Skin wet weight	DNA	Protein	Hydroxyproline	
	g	*mg/skin*	*mg/skin*	*μmoles/skin*	*μmoles/mmole Leu Eq*
1 injection					
Control	0.64 ± 0.05	4.1 ± 0.4	53.4 ± 4.0	2.1 ± 0.2	24.9 ± 1.7
Triamcinolone	0.65 ± 0.03	4.1 ± 0.3	56.8 ± 2.1	2.3 ± 0.1	23.4 ± 1.2
3 injections					
Control	1.45 ± 0.09	9.0 ± 0.4	116.9 ± 4.5	5.1 ± 0.5	22.8 ± 1.7
Triamcinolone	0.71 ± 0.05[a]	4.2 ± 0.2[a]	62.0 ± 4.0[a]	2.6 ± 0.3[a]	26.1 ± 1.6

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

TABLE 2

Prolyl hydroxylase activity in skin after single or multiple injections of triamcinolone diacetate

The values represent the means ± standard errors of enzyme activity from six animals. One-day-old rats were treated daily with triamcinolone diacetate (50 mg/kg intraperitoneally). The groups receiving a single injection were killed 12 hr later. Those groups receiving multiple injections were killed 24 hr after the last injection. No comparison can be made of the levels of enzyme activity between experiments 1 and 2, since different substrate preparations were used to assay for enzyme activity.

Expt.	No. of injections	Prolyl hydroxylase activity	
		Control	Triamcinolone
		dpm × 10^{-2}/mg protein	
1	1	40.4 ± 5.8	36.8 ± 2.0
	3	44.7 ± 2.5	29.3 ± 1.8[a] (35%)
2	1	25.1 ± 2.3	23.6 ± 2.6
	3	25.4 ± 1.4	15.6 ± 1.5[a] (38%)

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

class of drugs on collagen synthesis, hydroxyproline formation was determined. Animals received 5 μCi of [^{14}C]proline either 30, 60, or 90 min prior to death (Fig. 1). The amount of total proline incorporated into protein as either proline or hydroxyproline and the specific activity of hydroxyproline were determined. Both total incorporation and hydroxyproline formation were linear for at least 60 min.

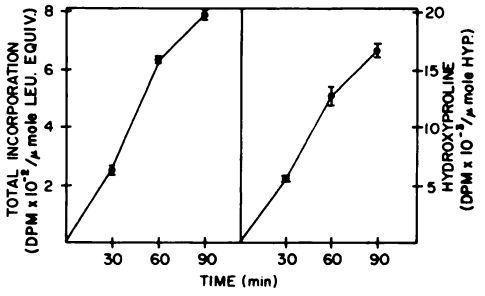

FIG. 1. *Time course of incorporation of [^{14}C]proline into proteins and formation of peptidyl-[^{14}C]hydroxyproline in skin of newborn rats.*

Newborn rats each received 5 μCi of [^{14}C]proline (200 mCi/mmole). At the times indicated the animals were killed, two skins per sample were homogenized, and total incorporation into proteins and the specific activity of proteinaceous [^{14}C]hydroxyproline were determined as described in the text. Values are the means ± standard errors of two or three samples.

Subsequently animals receiving a single or multiple injections of triamcinolone diacetate were pulsed with 5 μCi of [^{14}C]proline 30 min prior to death. Total proline incorporation after one injection of drug was decreased by 70% (Table 3). Hydroxyproline formation was decreased by 82%. Since there were approximately 25 μmoles of hydroxyproline per millimole of total amino acids (Table 1), and assuming a hydroxyproline to proline ratio of 0.8, approximately 51% of the total radioactivity was in collagen peptides as either pro-

TABLE 3

Triamcinolone-mediated decrease of total proline incorporation and hydroxyproline formation after either one or three injections of drug

The values represent the means ± standard errors of six to eight animals. Newborn rats were treated daily with triamcinolone diacetate (50 mg/kg intraperitoneally). The groups receiving a single injection were killed 12 hr later. Those groups receiving multiple injections were killed 24 hr after the last injection. Thirty minutes prior to death all animals were given 5 μCi of [^{14}C]proline (200 mCi/mmole) per rat. The amount of proline incorporation into protein and the amount of radioactive hydroxyproline formed were determined as described in the text. Control and drug-treated animals were litter mates. The differences observed in total proline incorporated and hydroxyproline formed by controls are accounted for by differences in the ages of these animals.

Treatment	Total incorporation	Hydroxyproline formation
	dpm/μmole Leu Eq	*dpm* × 10^{-3}/*μmole Hyp*
1 injection		
Control	557 ± 53	5.1 ± 0.4
Triamcinolone	165 ± 7[a] (70%)	0.9 ± 0.05 (82%)
3 injections		
Control	243 ± 24	2.2 ± 0.3
Triamcinolone	153 ± 16[a] (37%)	0.5 ± 0.04 (77%)

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

line or hydroxyproline residues. Since hydroxyproline formation represents both incorporation of proline into collagen peptides and subsequent hydroxylation of certain prolyl residues, the decreased level of hydroxyproline formation after a single injection of steroid was almost totally accounted for by the decrease in total proline incorporation. However, after three daily injections of triamcinolone diacetate, total proline incorporation was decreased by only 37% while the specific activity of proteinaceous hydroxyproline was decreased by 77%. After three daily injections of steroid, when prolyl hydroxylase was depressed, hydroxyproline formation was decreased to a level hardly accounted for by the decrease in total proline incorporation. In other experiments, in which animals treated with three injections were given labeled proline 90 min or 12 hr prior to death, the decreased level of proteinaceous hydroxyproline formation was not accounted for by the decrease observed in total proline incorporation (Table 4), which indicated a specific effect on collagen biosynthesis. The decrease observed in hydroxyproline formation represented either a decrease of proline incorporation specifically into collagen polypeptides and/or a decrease in the hydroxylation step. This specific effect on collagen synthesis was associated with a decreased level of prolyl hydroxylase.

The differences in total incorporation between 1- and 4-day-old control animals receiving labeled proline 30 min prior to death were accounted for by differences observed in the specific activity of the precursor proline pools (Table 5). No differences were observed in the specific activity of the precursor pool after one or three injections of triamcinolone when compared to the appropriate control value. These data indicate that the decreased level of proline incorporation observed after steroid treatment (Table 3) represented a true decrease of protein synthesis. The amount of labeled proline in the trichloracetic acid-soluble fraction of tissue homogenates in animals receiving one injection was the same as in controls (Table 5). However, in animals receiving three injections of drug, an elevated level of labeled proline in the acid-soluble fraction was observed. The increased amount of radioactive proline in the acid-soluble fraction did not represent an increase in uptake, since the total amount of labeled proline in this tissue—that is, both the amount of radioactivity in the precursor pool and that incorporated into proteins—was the same for controls

and animals receiving three daily injections of triamcinolone diacetate. The total content of proline in the trichloracetic acid-soluble fraction was the same in animals receiving one injection and was elevated in animals receiving three daily injections. The concomitant alterations of proline content and the amount of radioactive proline in the acid-soluble fraction of animals receiving three steroid injections resulted in no net change in the specific activity of the precursor proline pool.

DISCUSSION

Administration of pharmacological doses of anti-inflammatory steroids produces marked alterations in collagen metabolism of both normal and inflamed tissues (12–15). These effects ultimately result in a decrease of the tensile strength of tissues (16, 17) and are believed to be due to a decrease in the collagen biosynthetic process. Our data indicate that glucocorticoid treatment of newborn rats results in general antianabolic effects on metabolism of skin. The decrease in hydroxyproline formation 12 hr after administration of drug, when no decrease in prolyl hydroxylase was observed, is totally accounted for by the decrease in total proline incorpora-

TABLE 4

Comparison of total proline incorporation into skin proteins and hydroxyproline formation in newborn rats treated with multiple injections of triamcinolone diacetate

The values represent the means ± standard errors of six to eight animals. Newborn rats were treated for 3 consecutive days with triamcinolone diacetate (50 mg/kg intraperitoneally). The animals were killed 24 hr after the last injection. Either 90 min or 12 hr prior to death the animals received 5 μCi of [^{14}C]proline (200 mCi/mmole) per rat. The amount of proline incorporated into protein and the amount of radioactive hydroxyproline formed were determined as described in the text.

Pulsing time	Total incorporation		Hydroxyproline formation	
	Control	Triamcinolone	Control	Triamcinolone
hr	*dpm/μmole Leu Eq*		*dpm × 10^{-3}/μmole Hyp*	
1.5	859 ± 66	532 ± 32[a] (38%)	14.6 ± 2.1	3.0 ± 0.2[a] (79%)
12.0	1150 ± 64	857 ± 76[a] (25%)	13.8 ± 0.7	6.4 ± 0.8[a] (54%)

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

TABLE 5

Specific activity of free proline pool in animals receiving either one or three injections of triamcinolone diacetate

The values represent the means ± standard errors of six animals. Newborn rats were treated daily with triamcinolone diacetate (50 mg/kg intraperitoneally). The groups receiving a single injection were killed 12 hr later. The groups receiving multiple injections were killed 24 hr after the last injection. Thirty minutes prior to death each animal received 5 μCi of [^{14}C]proline (200 mCi/mmole). The trichloracetic acid-soluble fraction was prepared as described in the text. Differences in control values may be accounted for by developmental changes after birth.

Treatment	Radioactive proline	Total proline	Specific activity
	dpm × 10^{-4}/mg DNA	*nmoles × 10^{-2}/mg DNA*	*dpm × 10^{-4}/μmole Pro*
1 injection			
Control	8.5 ± 1.0[a]	3.4 ± 0.4	25.7 ± 2.0[a]
Triamcinolone	7.7 ± 0.5	3.5 ± 0.2	22.2 ± 1.2
3 injections			
Control	3.7 ± 0.2[a]	2.9 ± 0.4	13.1 ± 1.1[a]
Triamcinolone	5.3 ± 0.1[b]	4.0 ± 0.2[b]	13.4 ± 0.9

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

[b] Significantly different from control at $p \leq 0.05$.

tion. However, after repeated injections of anti-inflammatory steroid, when prolyl hydroxylase was decreased, proteinaceous hydroxyproline formation was decreased beyond the level accounted for by the decrease of proline incorporation, which indicates a specific effect on the collagen biosynthetic process. The observed decreases of both collagen and non-collagen protein synthesis in skin are not the result of steroid alteration of the specific activity of the acid-soluble proline pool.

The decrease of hydroxyproline formation may result from a decrease in the prolyl hydroxylation step and/or a specific decrease in collagen polypeptide synthesis. Since the decrease of 35–40% in prolyl hydroxylase seen after 3 days of drug treatment is not enough to account for the 77% decrease in hydroxyproline formation, at least half the decrease of hydroxyproline synthesis in drug-treated animals must be due to a decrease in collagen polypeptide synthesis. The decrease of enzyme may either result in synthesis of underhydroxylated collagen or be associated with a further decrease of collagen polypeptide synthesis. One must also entertain the thought that steroid treatment may result in aberrant proline incorporation into collagen, so that normal hydroxylation may not occur.

Recently the decreased synthesis of proteinaceous hydroxyproline following steroid treatment was attributed to a decrease in general protein synthesis. Administration of hydrocortisone and fluorinated anti-inflammatory steroids to organ cultures of chick embryo tibiae (3) and granuloma tissue *in vivo* (2) resulted in a decrease of incorporation of radioactive proline into proteins and subsequent conversion to hydroxyproline at a time when prolyl hydroxylase activity was unaltered. We have found that addition of a wide range of concentrations of drug to either organ or cell culture systems failed to elicit a response in prolyl hydroxylase.[3] However, in the whole animal a temporal decrease in the level of prolyl hydroxylase activity following steroid treatment is observed. The data reported here indicate that after 2 or 3 days of treatment decreases of both enzyme and hydroxyproline formation are observed. Since proteinaceous hydroxyproline formation is not totally accounted for by the decrease in total proline incorporation, a specific effect on collagen biosynthesis is indicated.

Although the decrease of prolyl hydroxylase following steroid treatment is secondary to a decrease in incorporation of amino acids into total skin, it may be specifically involved in the molecular action of these anti-inflammatory steroids on collagen biosynthesis. The decreased level of prolyl hydroxylase could result in the synthesis of underhydroxylated collagen, which might account for the abnormal physical properties of skin after chronic treatment with anti-inflammatory steroids. Alternatively, the decreased level of prolyl hydroxylase may result in a coordinate decrease of collagen polypeptide synthesis. An observable decrease in enzyme amount would be a function of the rate of enzyme degradation. Thus, to detect a coordinate effect of the steroid on both collagen polypeptide synthesis and prolyl hydroxylase shortly after drug administration would require that the synthetic rate of the enzyme be measured.

[3] Unpublished observations.

REFERENCES

1. Cutroneo, K. R., Stassen, F. L. H. & Cardinale, G. J. (1975) *Mol. Pharmacol.*, 11, 44–51.
2. Nakagawa, H., Fukuhara, M. & Tsurufuji, S. (1971) *Biochem. Pharmacol.*, 20, 2253–2261.
3. Uitto, J. & Mustakallio, K. K. (1971) *Biochem. Pharmacol.*, 20, 2495–2503.
4. Nakagawa, H. & Tsurufuji, S. (1972) *Biochem. Pharmacol.*, 21, 1884–1886.
5. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, 193, 265–275.
6. Hutton, J. J., Tappel, A. L. & Udenfriend, S. (1966) *Anal. Biochem.*, 16, 384–394.
7. Burton, K. (1956) *Biochem. J.*, 62, 315–323.
8. Moore, S. & Stein, W. H. (1954) *J. Biol. Chem.*, 211, 907–913.
9. Prockop, D. J. & Udenfriend, S. (1960) *Anal. Biochem.*, 1, 228–239.
10. Troll, W. & Lindsley, J. (1955) *J. Biol. Chem.*, 215, 655–660.
11. Carnegie, P. R. (1961) *Nature*, 192, 658–659.
12. Bavetta, L. A., Bekhor, I., Shah, R., O'Day, P.

& Nimni, M. E. (1962) *Endocrinology*, 71, 221–226.
13. Kivirikko, K. I. & Laitinen, O. (1965) *Acta Physiol. Scand.*, 64, 356–360.
14. Smith, Q. T. & Allison, D. J. (1965) *Endocrinology*, 77, 785–791.
15. Fukuhara, M. & Tsurufuji, S. (1969) *Biochem. Pharmacol.*, 18, 2409–2414.
16. Vogel, H. G. (1970) *Acta Endocrinol.*, 64, 295–303.
17. Ehrlich, H. P. & Tarver, H. (1971) *Proc. Soc. Exp. Biol. Med.*, 137, 936–938.