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Effect of Cis-Hydroxyproline on Collagen and Other Proteins in Skin Wounds, Granuloma Tissue, and Liver of Mice and Rats[1]

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Acute and chronic pharmacologic effects of cis-hydroxyproline (cis-Hyp) were studied in mice and rats. Given as a single dose intraperitoneally, cis-Hyp is relatively non-toxic and the LD_{50} approaches that of *l*-proline.

Chronic administration of cis-Hyp (200 mg/kg/day) to mice did not affect breaking strength of dermal wounds or content and specific activity of noncollagenous protein in the liver. Although cis-Hyp produced a significant decrease in noncollagenous protein content of polyvinyl alcohol sponge induced granuloma tissue, collagen content and rate of synthesis in granuloma tissue and liver were increased significantly.

Rats given cis-Hyp (200 mg/kg/day subcutaneously in divided doses every 12 hr) and control animals demonstrated identical hepatic microsomal protein, cytochrome P_{450} and cytochrome b_5 content. Breaking strength of dermal wounds was increased significantly in treated animals. Ultrastructural analysis of rat liver failed to demonstrate any structural alterations of cellular organelles due to cis-hyp treatment.

We conclude that chronic *in vivo* administration of cis-Hyp does not inhibit collagen synthesis or accumulation in normal or repaired tissues and does not decrease the breaking strength of skin wounds. Other effects on protein metabolism could result from nonspecific, chronic toxicity of cis-Hyp.

In tissue culture and tissue slice incubation systems, divalent iron chelating agents (Hurych and Chvapil, 1965; Chvapil *et al.*, 1967; Chvapil and Hurych, 1968), an absence of ascorbic acid (Bates *et al*, 1972), proline analogs, or lysine analogs (for review see Grant and Prockop, 1972), inhibit synthesis of collagen selectively. Although the mechanism of action differs slightly in each case, these agents ultimately slow collagen synthesis by preventing hydroxylation of proline and lysine. Ferrous iron chelators and ascorbic acid deficiency inhibit peptidyl proline (lysine) hydroxylase (PPH) activity. Because PPH converts proline and lysine in collagenous polypeptides to hydroxyproline and hydroxylysine, the collagen synthesized is underhydroxylated. Proline and lysine analogs are incorporated into collagenous polypeptides *in vitro* but, for structural reasons, cannot be hydroxylated. Because underhydroxylated collagen is extruded by fibroblasts slowly (Takeuchi *et al.*, 1969; Blumenkrantz *et al.*, 1969, Rosenbloom *et al.*, 1968; Dehm and Prockop, 1971; Ramaley and Rosenbloom, 1971) and, if

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released into the tissues, is degraded by collagenase and tissue proteases more rapidly than normal collagen (Hurych *et al.*, 1967), collagen accumulates at a significantly slower rate in each instance.

Certain effects of proline analogs on animal models of fibrotic injury have suggested to several authors that the synthesis of collagen is inhibited selectively *in vivo* by these agents. Bora *et al.* (1972) demonstrated a significant decrease in breaking strength of peritendinous adhesions in rats receiving 3,4-dehydroproline (3,4-DHP); Daly *et al.* (1972, 1973) reported a decrease in bursting strength of colon wounds in animals treated with cis-hydroxyproline; Lane *et al.* (1972) observed a decrease in bursting strength of peritendinous adhesions in rats given cis-hydroxyproline. Although the authors suggest that 3,4-DHP and cis-hydroxyproline influence the physical properties of injured tissues by altering collagen synthesis, no direct measurements of collagen metabolism were performed. More important, alterations in physical properties observed could have occurred in several ways other than by selective inhibition of collagen synthesis.

Jimenez *et al.*, (1971) did demonstrate a significant reduction in collagen concentration and total collagen content of carrageenan induced granulomas in rats fed cis-hydroxyproline. The granulomas in treated animals, however, were much smaller than controls and measurements of non-collagenous protein synthesis were not reported. Although this study suggests that cis-hydroxyproline may have a specific effect on collagen synthesis *in vivo*, the observations are also consistent with a generalized inhibition of protein synthesis or an alteration in carrageenan toxicity.

In a recent study on the toxicity and metabolic effects of 3,4-DHP administered acutely and chronically to mice, we were unable to demonstrate a specific inhibitory effect on collagen synthesis or accumulation in reactive granuloma tissue, skin wounds or liver. Bursting strength of incised dermal wounds was increased rather than decreased under the influence of 3,4-DHP. Moreover, the analog produced significant toxic effects in several metabolic systems (Madden *et al.*, 1973; Chvapil *et al.*, 1973).

This communication summarizes our studies on the *in vivo* effects of cis-hydroxyproline in mice and rats. Using the dose of cis-hydroxyproline cited by other authors, we could not demonstrate a decrease in rate of collagen synthesis or collagen content in granuloma tissue or liver. Cis-hydroxyproline did not decrease the burst strength of incised dermal wounds.

MATERIALS AND METHODS

Experiment 1: Acute Toxicity

The toxicity of cis-hydroxyproline and *l*-proline (Calbiochem, Los Angeles, California) was compared. Each compound was injected intra-peritoneally in a neutral saline solution into adult male mice, CD-1 strain, body weight 39 ± 3 g. Death rate was followed for 24 hr after single injections. The LD_{50} was calculated according to Thompson and Weil (1952) and Weil (1952).

Experiment 2: Chronic effect of cis-hydroxyproline on the biochemistry of Ivalon sponge granulomas and liver, and on the burst strength of skin wounds in mice

Under chloral hydrate anesthesia, 5 cm midline dorsal skin incisions were made in 20 male CD-1 strain mice (21 ± 2.0 g) closed with 5, 5–0 interrupted

nylon sutures. Through a separate, distant dorsal incision, two 3 × 3 × 10 mm blocks of sterile polyvinyl alcohol sponge (Ivalon) were implanted subcutaneously.

Twenty-four hours following surgery, animals were randomly divided into two groups. Ten mice received cis-hydroxyproline, 200 mg/kg body weight daily in two divided doses intraperitoneally. The second group of 10 mice received an equal volume of saline and served as controls. All animals were fed standard laboratory chow ad lib and killed 14 days following surgery. One hour prior to sacrifice, all mice were injected intra-peritoneally with ^{3}H-proline (New England Nuclear, Boston, Massachusetts), 50 μCi/100 gm.

Experiment 3: Chronic effects of cis-hydroxyproline on the biochemistry of liver and the breaking strength of skin wounds in rats

Under chloral hydrate anesthesia, 5 cm midline dorsal skin incisions were made in 20 male Sprague-Dawley rats (145 ± 10 g) and closed with 5, 5–0 interrupted nylon sutures. Forty-eight hours following surgery, the animals were randomly divided into two groups. The first group received 200 mg cis-hydroxyproline/kg/day subcutaneously in two divided doses for 12 days. Control animals were injected with saline alone. Animals were sacrificed 14 days following surgery, during their 12th day of treatment.

Biochemical Analysis

Liver. After decapitation, the liver of each animal was perfused *in situ* with 20 ml of ice cold saline, removed and homogenized in 5 volumes of cold Tris–KCl buffer (0.05 *M*, pH 7.4). Mitochondrial and microsomal fractions were prepared from aliquots of liver homogenate using differential centrifugation. Total protein was measured in whole liver homogenate as well as in the microsomal fraction using Lowry's method (Lowry *et al.*, 1951). Chromogen P_{450} and b_5 were measured in the microsomal fraction using the technique of Smuckler (Smuckler *et al.*, 1967). Collagen was extracted repeatedly from an aliquot of whole liver homogenate using hot 0.3 *M* trichloroacetic acid. The specific activity of hydroxyproline was measured in the supernatent using the Juva–Prockop method (1966). The specific activity of non-collagenous protein was determined by measuring the ^{3}H-proline and total protein in the trichloroacetic acid precipitate. The specific activity of microsomal protein was determined by measuring the radioactivity in a dialyzed aliquot of the microsomal fraction.

Granuloma Tissue. The implanted polyvinyl sponges were exposed and the fibrous capsule formed around the sponges carefully dissected and isolated. Newly formed fibrous tissue was homogenized in liquid nitrogen and extracted repeatedly with hot trichloroacetic acid. Pooled extracts were dialyzed and hydrolyzed with 6 *N* HCl. Specific activity of hydroxyproline in the hydrolyzed sample was measured using the Juva and Prockop method (1966). The trichloroacetic acid precipitate was washed with ethanol:ether (3:1) and ethanol. The specific activity of protein was determined by measuring the radioactivity and total protein in the washed precipitate.

Ultrastructural Analysis of Liver

Two control and two experimental rats were anesthetized with chloral hydrate. The liver of each animal was perfused *in situ* through the portal vein with 20 ml

of cold (4°C) dilute solution of formaldehyde-glutaraldehyde buffered with 0.2 *M* sodium cacodylate at pH 7.3. Procaine hydrochloride (0.1%) was added to this preliminary washout solution to prevent vasoconstriction. Liver was then perfused with 20 ml of formaldehyde-glutaraldehyde fixative (Karnovski, 1965). A portion of the triangular lobe of each liver was removed, minced and immersed in cold fixative for one hour. Post-fixation was performed in 2% cacodylate buffered OsO_4. Tissues were prepared conventionally for electron microscopy using Epon-Araldite as the final embedment (Anderson and Ellis, 1965). Sections were stained in uranyl acetate (5% in absolute ethanol) and lead acetate (Venable and Coggeshall, 1965). Observations were made with a Philips EM-300 electron microscope at original magnifications of 3,300–20,000×.

Physical Property Measurements

Strips of skin 0.5 cm wide were cut from the neck of each animal with the wound transversely oriented at the center. Breaking strength was measured using an Instron Tensile Tester, Model TM equipped with the C-cell and pneumatic clamps, cross-head speed 12 cm/min. Three determinations were made on each wound and measurements averaged. Individual measurements were within 10% of the means.

Statistical Methods

Means, standard deviations, and standard errors were calculated for each group. Student's *t*-test was used to determine significance between test and control groups. Data are presented as mean ± S.E.

RESULTS

Acute Toxicity

Mice receiving the highest single dose of cis-hydroxyproline (81 mg/mouse) survived 24 hr without difficulty. Obviously, cis-hydroxyproline is relatively nontoxic and, because of the expense of the compound, we did not carry the toxicity experiments further. The toxicity of cis-hydroxyproline approaches the toxicity of *l*-proline. LD_{50} for 30 g body weight of a mouse equals 160 mg *l*-proline.

Chronic Effects in Mice (Table 1)

Liver. Cis-hydroxyproline had no effect on body weight or liver weight. Total protein and specific activity of non-collagenous protein in whole liver homogenates were the same in control and treated animals. Collagenous hydroxyproline, however, was significantly higher in the cis-hydroxyproline-treated group. Microsomal total protein and specific activity were reduced significantly in the cis-hydroxyproline-treated animals.

Granuloma Tissue. The cis-hydroxyproline-treated mice showed a significant increase in collagen concentration and total collagen content in the Ivalon sponge granuloma. The specific activity of hydroxyproline, however, did not differ from controls. The concentration of noncollagenous protein was significantly decreased

TABLE I

EFFECT OF CHRONIC ADMINISTRATION OF cis-HYDROXYPROLINE ON THE BIOLOGY AND BIOCHEMISTRY OF THE LIVER, GRANULOMA TISSUE, AND SKIN WOUND IN THE MICE

Parameter studied	Control (10)	cis-Hyp (10)	P
Initial body weight (g)	21.85 ± 0.40	21.55 ± 0.37	—
Terminal body weight (g)	29.3 ± 0.61	30.95 ± 0.54	—
Liver			
Liver weight (g)	1.820 ± 0.063	1.928 ± 0.038	—
Liver microsomes			
Protein mg/g	10.24 ± 0.44	8.43 ± 0.39	0.01
^{3}H Pro-DPM/mg protein	1802 ± 1.0	1206 ± 21	0.001
Total noncollagenous proteins			
Protein mg/g	78.5 ± 2.60	85.5 ± 3.02	—
^{3}H Pro-DPM/mg protein	12,103 ± 436	11,346 ± 270	—
Total collagen			
Hyp μmol/g	0.37 ± 0.02	0.43 ± 0.01	0.01
^{3}H Hyp-DPM/μmol Hyp	4180 ± 234	3775 ± 172	—
Sponge Granuloma			
Wet weight (g/sponge)	0.665 ± 0.037	0.683 ± 0.026	—
Collagen			
Hyp μmole/sponge	3.44 ± 0.22	4.29 ± 0.19	0.01
Hyp μmole/g	5.07 ± 0.135	6.185 ± 0.194	0.001
^{3}H Hyp-DPM/μmole Hyp	1812 ± 86.7	1638 ± 74.0	—
Noncollagenous proteins			
Protein mg/g	28.7 ± 0.62	23.2 ± 1.04	0.001
^{3}H Pro-DPM/mg protein	2081 ± 96.0	2031 ± 80.8	—
Wound Skin			
Breaking strength (g/0.5 cm)	228.3 ± 9.98	240.2 ± 13.45	—

The results are presented as Mean ± S.P.M. P value refers to the significance as calculated by Student's t-test. Number of animals is given in parenthesis.

in the treated mice, although the specific activity was comparable to control values.

Wound Strength. The breaking strengths of dermal wounds in control and cis-hydroxyproline treatment groups were identical.

Chronic Effects in Rats (Table 2)

Liver. Although liver weight was not affected by cis-hydroxyproline administration, treated animals failed to gain body weight during the 12-day treatment

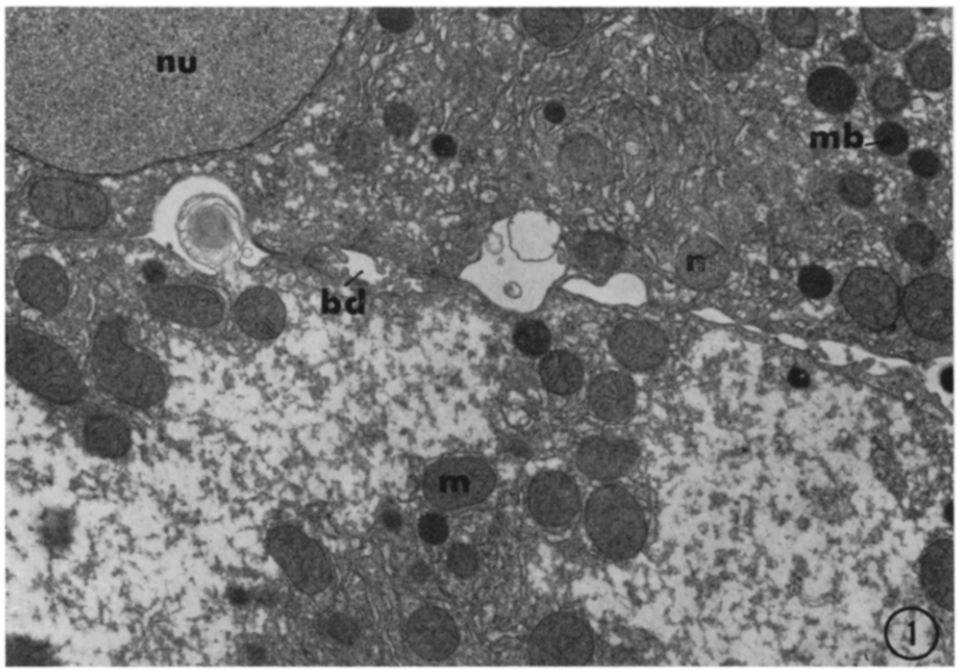

FIG. 1. Electron micrograph of adjacent hepatocytes from control rat. Upper cell shows rich ergastoplasm including numerous mitochondria (m) and microbodies (mb). Lower cell shows glycogen depletion with rough endoplasmic reticulum localized to clusters of mitochondria. bd, bile duct; nu, nucleus. ×9,200.

interval at the same rate as the controls. Neither microsomal protein, cytochrome P_{450}, nor cytochrome b_5 was affected by cis-hydroxyproline administration.

Wound Strength. The breaking strength of skin wounds was significantly increased by cis-hydroxyproline treatment.

TABLE II

EFFECT OF CHRONIC ADMINISTRATION OF cis-HYDROXYPROLINE ON MECHANICAL PROPERTIES OF SKIN WOUND, AND SOME INDICATORS OF LIVER CHEMISTRY IN THE RATS

Parameter studied	Controls (10)	cis-Hyp (6)	*P*
Initial body weight (g)	144.5 ± 1.8	144.2 ± 2.5	—
Final body weight (g)	303.4 ± 5.2	232.3 ± 6.2	0.001
Skin Wound			
Breaking strength (g)	431.9 ± 36.0	654.0 ± 46.7	0.001
Liver			
Liver wet weight	11.55 ± 0.75	11.28 ± 0.26	—
Microsomal protein mg/g	12.90 ± 0.31	13.02 ± 0.23	—
Cytochrome P_{450} (ΔOD/mg prot.)	3.45 ± 0.20	3.41 ± 0.15	—
Cytochrome b_5	1.31 ± 0.06	1.30 ± 0.5	—

The results are presented as $\bar{X}$ ± S.E.M. *P* value refers to the significance as calculated by Student's *t*-test.

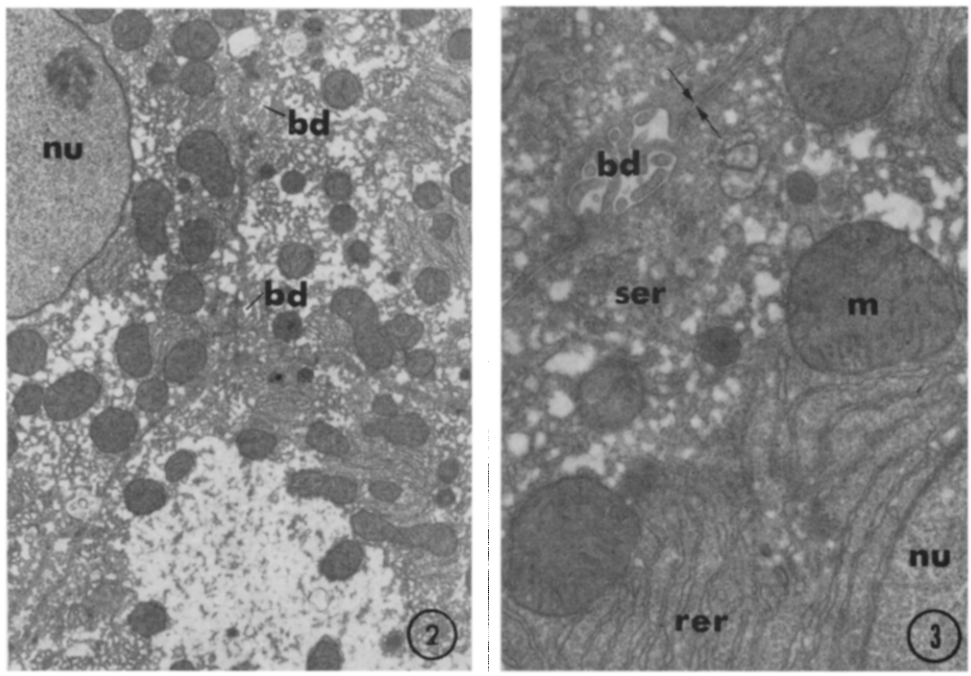

FIG. 2. Electron micrograph of three adjacent hepatocytes from rat treated with 200 mg/kg/day cis-hydroxyproline. This tissue is ultrastructurally indistinguishable from controls. nu, nucleus; bd, bile duct. ×6,400.

FIG. 3. Higher magnification electron micrograph of adjacent hepatocytes from rat treated with 200 mg/kg/day cis-hydroxyproline. Mitochondria (m) exhibit dense matrices and well-developed cristae. Rough (rer) and smooth (ser) endoplasmic reticulum are composed of continuous Lipo-protein membranes. Adjacent cell plasmalemmae (arrows) diverge to form a bile duct (bd); nu, nucleus. ×16,000.

Ultrastructural Analysis of Rat Liver

Electron microscopic observations of hepatocytes from control rats showed normal morphological characteristics (Fig. 1). Round nuclei, surrounded by rich granular and agranular endoplasmic reticulum, with numerous spherical and elongated mitochondria were present. Polyribosomes, lysosomes, microbodies, and other organelles considered to be normal constituents of hepatocytes were observed. Liver cells from rats treated with cis-hydroxyproline exhibited no fine structural alterations (Figs. 2 and 3). All cellular organelles appeared to be within the limits of normal variation and were ultrastructurally indistinguishable from those seen in controls.

DISCUSSION

The data from our experiments fail to support the contention that administration of cis-hydroxyproline to intact rats or mice produces a specific inhibition of collagen synthesis. In the mouse, chronic administration of cis-hydroxyproline reduced the amount and rate of synthesis of liver microsomal protein significantly. Although the analog had no effect on the rate of hepatic collagen synthesis, total collagen content and collagen concentration in the liver increased

significantly. These data are in conflict with those presented recently by Rojkind (1973) who showed a striking inhibition of liver fibrosis by 1-azetidine-2-carboxylic acid. The dose used by Rojkind was inhibitory to microsomal drug-oxidizing system to the same extent as was 3,4-dehydroproline in our experiments (Chvapil, Madden, and Peacock, unpublished results). Thus, the protective effect of this proline analog would be related rather to the metabolism of this hepatotoxin than to its direct effect on collagen hydroxylation and metabolism.

In polyvinyl alcohol sponge induced granuloma tissue, our results differed significantly from the data reported by Jimenez and Prockop (1971). While Jimenez and Prockop showed a significant decrease in total collagen content and collagen concentration in the carrageenan granuloma, our data demonstrate a significant increase in both parameters. In addition, noncollagenous protein content and concentration were decreased significantly in sponge granuloma. The inconsistencies in these results could be due to failure to incorporate the analog, differences in species, differences in routes of administration, presence or absence of proline in the diet, or differences in the granuloma models. Although we did not perform total amino acid analyses on our proteins, Jimenez and Prockop demonstrated such a high incorporation rate (14 residuals/α chain) that lack of incorporation of the proline analog studied seems an unlikely explanation. Because we failed to demonstrate specific alterations in hepatic collagen synthesis or breaking strength of wounds in rats using identical animal weights, route of administration, and dose of cis-hydroxyproline, the next two possibilities seem unlikely. Our previous study of 3,4-DHP demonstrated that pool size of free proline, at least in the liver, is not influenced by the presence or absence of proline in the diet; therefore, diet does not seem a critical factor (Madden *et al.*, 1973). Differences in the granuloma model studied seem the most likely cause of the divergent data.

Our previous difficulties with the carrageenan granuloma model (Chvapil and Cmuchalova, 1961; Cmuchalova and Chvapil, 1963) prompted us to select the more stable Ivalon sponge model for study. Polyvinyl sponge granulomas have a predictable consistent life history which will allow one to test drug effects on collagen synthesis (Viljanto, 1964). The carrageenan model appears to be less consistent and to be a poor choice for such studies (Stassen and Kuyper, 1972).

The increase in collagen content and decrease in noncollagenous protein observed in the sponge granuloma suggests a mild nonspecific toxic effect of cis-hydroxyproline in the mouse. Nonspecific tissue injury stimulates the activity of fibrogenic cells (Castor *et al.*, 1973). A mild toxic effect of cis-hydroxyproline in mice is also supported by the changes in liver microsomal protein and the changes in total hepatic collagen concentration. No such toxic effect was demonstrated in rats; hepatic collagenous and noncollagenous proteins were unaffected by cis-hydroxyproline treatment. The ultrastructural studies of rat liver were consistent with cis-Hyp's nontoxic nature. Organelles known to be responsible for protein synthesis showed no structural changes following cis-hydroxyproline treatment.

Because the data from mice did not support the concept that cis-hydroxyproline interferes specifically with collagen metabolism *in vivo*, we examined the effects of cis-hydroxyproline using the species, route of administration, and

dose cited by other authors as effective in altering mesenchymal tissue metabolism. The only significant effects we were able to demonstrate in rats were a reduction in body weight and a significant increase in the breaking strength of the dermal incisions. Our failure to demonstrate a reduction in burst strength of dermal wounds in both rats and mice differs from the effects of cis-hydroxyproline on the burst strength of colon wounds and peritendinous adhesions (Daly *et al.*, 1972; Bora *et al.*, 1972) reported by other authors. Again, the discrepancies noted may be due to differences in the models studied. Our previous experience indicates that the mechanical strength of skin wounds represents a reliable screening test to evaluate the effects of anticollagenous agents on wound healing. 2,2′-dipyridyl (Whitson and Peacock, 1969), β-aminopropionitrile, both systemically (Peacock and Madden, 1966) or locally (Speer *et al.*, 1973), and *d*-penicillamine (Geever *et al.*, 1967; Haney *et al.*, 1973) produce a significant decrease in burst strength of dermal wounds. Because several of these agents have been shown subsequently to have significant effects on the pathophysiology of fibrotic disease in animals (Davis *et al.*, 1972; Madden *et al.*, 1973; Craver *et al.*, 1968) and man (Peacock and Madden, 1969), alterations in burst strength of dermal wounds seems a reliable method of testing the antifibrotic properties of pharmacologic agents. The increase in breaking strength of dermal wounds in rats receiving 1-3,4-DHP and cis-hydroxyproline remains unexplained.

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