Connective Tissue V. Effect of Topical Trypsin upon Dermal Chemical Response to Local Inflammation and Necrosis.*‡ (27002)

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Interest in the enzymatic debridement of wounds reaches back to John Hunter, who is reported to have employed pancreatic juice for this purpose(1). Later, Morris claimed that pancreatic extracts would clear the eschar of a burn within 4 hours(2).

Connell and Rousselot(3) reported that trypsin was useful as a debriding agent, and Wilde and Derry(4) indicated that trypsin was of use in debridement of burns. Reiser *et al* have discussed in detail the tryptic debridement of necrotic tissue(5,6).

Baetzner's original description of the antiphlogistic action of parenteral trypsin(7) has been developed by Innerfield(8) on the basis of the destruction of the proteinaceous barriers to capillary permeability, and recent experimental work(9) has shown that injection of trypsin into bruised tissue increased the rate of tissue repair.

This paper describes the chemistry of the response of rat skin to local croton-oil induced inflammation, and compares these results with the chemistry of the inflammatory response in lesions which have been topically treated with trypsin.

Materials and Methods. One hundred and twenty male Sprague-Dawley rats (300-330g) were subjected to intradermal injection, into a previously shaven area of abdominal skin (right side), of 0.4ml of a dilution of the chemical irritant croton oil made up to a concentration of 75% irritant in an inert carrier of peanut oil. After eschar formation (3 days), the resulting necrotic lesions of one group of animals were sprayed topically with a solution containing 1 mg/ml of crystalline trypsin in isotonic saline daily for 20 days. The other group was subjected to daily topical applications of saline alone. Separate control studies indicated that the daily topical application of trypsin to shaven areas of intact rat skin was without effect upon the dermal chemistry while the application of saline alone to the necrotic lesion did not result in significant alterations in the patterns of the chemical response of the skin to similar concentrations of croton oil.

At various times after injury, 8 animals from each group were sacrificed by etherization and exsanguination and both the necrotic lesion and about 1.5 g of uninjured abdominal skin distal from the site of injury were removed, dissected free of adhering hair, fat, fascia and muscle, weighed and stored in the frozen state. Tissue samples obtained before and after eschar sloughing were lyophilized, and their water content determined by weight loss.

The necrotic lesions, and aliquots of the uninjured skin from each animal, were hydrolysed for 8 hours at 100°C in 10ml/g of 4N HCl. Triplicate samples of these hydrolysates were removed and analysed in triplicate for nitrogen(10), hydroxyproline(11), and hexosamine(12). These results were translated via a standard curve into μmoles or mmoles per g of fresh, wet, cleaned rat skin. The mean and standard deviation of the 9 determinations for each analysis are reported in the tables.

The cleaned and shaven uninjured abdominal skin remaining (ca. 1.0 g) was pooled into 2 groups of 4 animals each, and 3.5 g of skin from each pooled sample of tissue was extracted sequentially in the cold with isotonic saline, 0.5M NaCl and 0.5M citrate buffer (pH 3.6) as has been described previously(10,13). Triplicate samples of each extract after centrifugation were similarly hydrolysed, along with one ml of sera from each animal, in 4N HCl, and their nitrogen, hydroxyproline and hexosamine content determined. The mean and standard deviation of each set of determinations are presented below. These extracts have been shown previously to extract quantitatively the ground substance, neutral soluble and acid soluble collagens respectively(13).

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Experimental and Results. Grossly, the croton oil injury produced an ulcerous lesion which was lightly outlined by erythema. After 3 days, both the trypsin treated and the control groups of animals formed an eschar within a period of 24 hours. Isolation and contraction of the eschar to the point of sloughing occurred after 13 days in the untreated control group and after 10 days in the topically trypsin treated animals. Thirteen days after sloughing, the tissue remained pink, soft and hairless in the trypsin treated animals, while the skin of the injured area in the control group had become re-covered with hair, and although it still tore easily, was not noticeably different from normal. Mean water content determined by lyophilization and standard deviation of the trypsin treated lesion 2, 4, and 14 days after injury was 65±2, 60±1 and 58±1% respectively, while for the untreated lesion it was 60±1, 58±1 and 55±1% for the same days after injury. Uninjured tissue showed no changes in water content.

Histologically, both injuries showed a typical hemorrhagic pattern of ulceration and necrosis throughout both layers of the skin, resulting in a non-suppurating defect. The erythema was somewhat more noticeable in the trypsin treated lesions, and edema appeared slightly more profound than in the untreated lesions. A few days after eschar isolation, granulation tissue formed in both groups of rats. The granulation tissue was apparently maintained in the trypsin treated group, whereas the control lesion proceeded to heal via fibroplasia and epithelization. The area of skin distant from the local lesion demonstrated no significant histochemical change over the whole time course of the experiment in either group of animals.

The alterations with time after injury of the total dermal concentrations of hydroxyproline and hexosamine at site of croton oil injection with and without daily topical application of trypsin are compared in Table I with the analysis of skin from uninjured animals of similar body weight. These results indicate that there was a marked increase in the hydroxyproline and an increase in hexosamine concentration of the necrotic lesion. Nitrogen concentration of these lesions demonstrated no significant alteration with inflammation. These changes in dermal chemistry were prolonged when the wound was treated topically with trypsin. The application of this enzyme also decreased quantitatively the increment in the dermal concentration of hexosamine which occurred normally in response to local injury.

TABLE I. Hydroxyproline and hexosamine content (μmoles/g skin) of dermal necrotic lesions with and without topical trypsin treatment‡.

Days after Injury	Hydroxyproline (μmoles/g) control	Hydroxyproline (μmoles/g) trypsin	Hexosamine (μmoles/g) control	Hexosamine (μmoles/g) trypsin
0	240 ± 12	230 ± 12	9.3 ± .5	10.0 ± .5
2	*140 ± 7	*133 ± 7	*12.6 ± .6	*12.6 ± .6
3†	*152 ± 7	*126 ± 6	*15.3 ± .7	*12.8 ± .6
4	*180 ± 9	—	*17.3 ± .9	—
5	*100 ± 5	*127 ± 6	*24.0 ± 1.2	*17.3 ± .9
7	*100 ± 5	*105 ± 5	*23.5 ± 1.2	*14.0 ± .7
10	—	*125 ± 6	—	*15.3 ± .7
14	*113 ± 6	*115 ± 6	*21.3 ± 1.1	*18.6 ± .9
23	200 ± 20	*113 ± 5	10.6 ± .5	*14.0 ± .7

† First day of trypsin treatment.

‡ Dermal nitrogen content of both groups of lesions for all days fell within the range of 4.6 to 5.4mmoles/g and was not significantly different from control mean (day 0).

* Significantly different from control mean ($p<.05$).

Underlined mean differs significantly from that of untreated lesion ($p<.05$).

Concentration of hexosamine in the wound was unaffected by formation of the eschar in the untreated animals, while the amino sugar content of the trypsin treated wound did not change until the 5th day after injury.

The hexosamine, nitrogen and hydroxyproline concentrations in the sera from rats with croton oil induced dermal injuries with and without topical trypsin treatment are presented in Table II. The circulating concentration of nitrogen or hexosamine was not significantly affected by production of a dermal lesion. Elevations of both materials were found in the sera of those animals whose lesions were treated with trypsin. The results also suggested that topical trypsin treatment of the dermal lesions permits the hydroxyproline containing materials from the necrotic lesion to drain into the circulation.

The uninjured skin from rats distal to the site of local injury demonstrated a significant decrease in total dermal hydroxyproline con-

TABLE II. Effects of dermal necrosis upon analysis of sera of rats with and without trypsin applied topically to the lesion.

Days after Injury	Nitrogen (mmoles/ml)		Hydroxyproline (μmoles/ml)		Hexosamine (μmoles/ml)	
	control	trypsin	control	trypsin	control	trypsin
0	.65 ± .04	.60 ± .04	.36	.36	4.5	4.5
2	.71	*.71	.35	.35	4.5	4.5
3†	.68	*.79	.40	.34	4.3	*14.6
4	.63	—	.40	—	4.5	—
5	.67	*.74	*.48	*.64	4.8	*10.6
7	.63	*.79	*.50	*.52	4.0	* 9.1
10	—	*.81	—	*.73	—	* 7.9
14	.60	*.79	.42	*.58	4.1	* 9.1
23	.63	*.84	.40	.36	4.2	* 8.2

† First day of trypsin treatment.
* Significantly different ($p<.05$) from control mean (day 0).
__ Underlined mean differs significantly from that of untreated lesion ($p<.05$)

TABLE III. Hydroxyproline content (μmoles/g) of rat skin distant from total site of croton oil injury with and without topical trypsin treatment.

Days after Injury	Without Trypsin	With Trypsin
0	240 ± 12	240 ± 12
2	*170 ± 8	*170 ± 8
3†	*176 ± 9	*202 ± 10
4	*173 ± 8	—
5	*171 ± 8	*202 ± 10
7	*173 ± 8	*204 ± 10
10	—	*214 ± 11
14	*203 ± 10	*202 ± 10
23	240 ± 14	*200 ± 10

† First day of trypsin treatment.
* Significantly different ($p<.05$) from control mean (day 0).
__ Underlined mean differs significantly from that of untreated lesion.

centration (Table III). These changes in hydroxyproline were not associated with significant alterations in the dermal concentration of either hexosamine or nitrogen, however. Trypsin treatment of the local lesion both prolonged this "distal dermal collagen response" to local inflammation and decreased these losses of hydroxyproline from the uninjured skin.

The uninjured skin distant from the site of local injury was extracted sequentially in the cold as described previously. The hydroxyproline content of the 0.15 and 0.50M saline extracts obtained from the uninjured skin of the control group of animals is compared with the results using the skin from trypsin treated animals in Table IV. These results indicate that

TABLE IV. Saline soluble hydroxyproline concentration (μmoles/g) of rat skin distant from site of croton oil injury with and without topical trypsin treatment.

Days after Injury	Extract .15M NaCl control	.15M NaCl trypsin	.50M NaCl control	.50M NaCl trypsin
0	4.6 ± .5	4.5 ± .5	19.3 ± 1	19.0 ± 1
2	4.4 ± .5	4.8 ± .5	* 9.3 ± .5	* 8.8 ± .4
3†	4.5 ± .5	4.7 ± .5	* 9.0 ± .5	*10.6 ± .5
4	4.4 ± .5	—	* 9.3 ± .5	—
5	4.5 ± .5	5.6 ± .5	* 9.0 ± .5	* 9.0 ± .5
7	5.1 ± .5	5.1 ± .5	* 8.0 ± .4	*10.0 ± .8
10	—	5.3 ± .5	—	*14.0 ± 1
14	5.3 ± .5	5.3 ± .5	* 9.1 ± .5	*13.3 ± 1
23	5.4 ± .5	5.1 ± .5	* 9.0 ± .5	*12.0 ± 1

† First day of trypsin treatment.
* Significantly different ($p<.05$) from control mean (day 0).
__ Underlined mean differs significantly from that of untreated lesion.

the isotonic saline soluble imino acid content of uninjured tissue was not affected by either local inflammation or topical trypsin treatment of the local lesion. The 0.5M saline soluble hydroxyproline concentration of these tissues decreased with distant local injury, however, and this decrease was statistically less profound when the local lesion was sprayed with trypsin.

Table V shows that the concentration of citrate soluble hydroxyproline in uninjured skin distal to the site of local injury increased some 4-fold in concentration while topical trypsin treatment of the local lesion resulted in a 7-fold increment in this material. After treatment of the lesion for 20 days with trypsin, the dermal concentration of citrate soluble hydroxyproline remained elevated above normal.

Subtraction of the sum of all 3 soluble fractions (0.15 and 0.50M NaCl and citrate) from total hydroxyproline content of the tissue permits calculation of the concentration of the insoluble hydroxyproline (Table V). From these data it may be seen that most of the de-

TABLE V. Citrate soluble and insoluble hydroxyproline concentration (μmoles/g) of rat skin distant from site of croton oil injury with and without topical trypsin treatment.

Days after Injury	Citrate soluble		Insoluble Residue	
	control	trypsin	control	trypsin
0	8 ± .6	8 ± .6	200 ± 20	195 ± 18
2	*25 ± 1.2	*25 ± 1.2	*132 ± 13	*132 ± 13
3†	*30 ± 1.5	*29 ± 1.5	*134 ± 13	*160 ± 16
4	*31 ± 1.6	—	*130 ± 13	—
5	*33 ± 1.7	*59 ± 3.0	*129 ± 13	*156 ± 16
7	*33 ± 1.7	*57 ± 2.6	*128 ± 13	*132 ± 13
10	--	*57 ± 2.6	—	*140 ± 13
14	*34 ± 1.7	*48 ± 2.3	*154 ± 16	*144 ± 14
23	*18 ± .9	*38 ± 2	200 ± 20	*130 ± 11

† First day of trypsin treatment.
* Significantly different ($p<.05$) from control mean (day 0).
— Underlined mean differs significantly from that of untreated lesion.

crease in hydroxyproline content of uninjured skin distal to the site of injury was accounted for on the basis of a loss in hydoxyproline content of the insoluble residue of the tissue. Topically applied trypsin was without significant effect upon the concentration of this insoluble material.

Despite these changes in dermal hydroxyproline concentration with inflammation there were no significant alterations from normal in either the nitrogen or hexosamine content of the various fractions described above.

Discussion. Collagen contains 13.6% hydroxyproline or about 1 μmole/mg and 18.6% nitrogen or 13.3 μmoles/mg(14). Since this imino acid is characteristic of collagen(15), the mg of collagen per g of shaven dermis may be obtained from the μmoles of hydroxyproline contained in the tissue. Multiplication of these values by 13.3 permits concentration of collagenous nitrogen to be calculated. In view of the essential constancy of the nitrogen content of the lesion with inflammation, the marked decrease in dermal collagen must be compensated for with respect to nitrogen by increases in either non-collagenous protein or various non-protein nitrogen containing materials. Topically applied trypsin was without significant effect upon this local reaction to inflammation.

Associated with the decrease in dermal hydroxyproline, or collagen, with local inflammation was an increase in hexosamine concentration of the lesion. This increase was lessened when the wound was exposed daily to trypsin. By the third day after injury, the untreated lesion contained 65% more hexosamine than the normal skin. Trypsin treated lesions contained only 27% more amino sugar than the control. These increases occurred in the face of an increase in water content of the tissue of less than 12%. In light of the magnitude of the alterations in dermal concentration of collagen and hexosamine with local inflammation, the changes in tissue water content are not sufficient to explain either the increase in the hexosamine or decrease in collagen content of the lesion.

With local trypsin treatment, both the elevation in dermal hexosamine and non-collagenous nitrogen and the decrease in lesion collagen were prolonged beyond those demonstrated by the untreated lesion.

Despite the decreased time required for sloughing of the eschar, the local trypsin treated lesion therefore did not heal chemically as quickly as the untreated control. This finding supports the claim of Castigliano and Rominger(16) that trypsin destroys and digests marginally viable cells in injured tissues, and thereby inhibits healing. It should be emphasized, however, that the trypsin was applied even after the eschar sloughed, when this treatment would not be normally continued. These adverse chemical findings with topical trypsin thereby may not then be equivalent with the routine clinical application of this enzyme.

Menkin(17) indicates that the biological continuity of the skin is destroyed during inflammation by fibrin and other proteins plugging both the lymphatics and the capillaries. These proteins flood the injured tissue as a consequence of the initial increase in capillary permeability(8). As expected, the consequence of this restoration of the biological continuity of the injured tissue would be the appearance of wound products in the circulation. Table II suggests that dermal hydroxyproline, hexosamine and nitrogen drain from the lesion into the sera with topical application of trypsin.

Less than 10% of the nitrogen content of the isotonic saline extract was soluble in 20% trichloracetic acid. Therefore most of the

nitrogen found in uninjured skin was not non-protein-nitrogen. Since 20-30% of the dermal collagen was lost from the uninjured skin with local inflammation while total nitrogen content of the tissue was not altered, non-collagenous protein must be replacing the disappearing collagen. This "distant dermal collagen response" to local inflammation must therefore be specific for collagen rather than a general catabolic decrease of all proteins.

If the loss in dermal collagen from skin distant from site of injury was in response to the systemic distribution of either the products of local inflammation or some inflammatory factors released from white blood cells, then the local application of trypsin, which results in wound products entering the circulation, would be expected to increase this response. Since the distant response was actually decreased with topical trypsin, both possibilities would seem unlikely. The insoluble nature of the lipid irritant renders equally untenable the theory that this distant response occurs as reaction to the systemic distribution of either the irritant itself or to some contaminants of the croton oil. Two other major explanations of the etiology of the distant response i.e. 1) a neurally potentiated organ response to local injury and 2) a response to the adrenal hormones provided in reaction to the trauma produced by local injury, occur to the author. It is doubted that topical trypsin would inhibit, even slightly, the nerve transmission of local injury stimuli to the rest of the skin, particularly since the sizes of both the trypsin treated and the control lesion were similar. The trauma produced by the enzyme treated lesion might be minimized by the increased flow of blood elements through the dermal lesion, however. It should be noted that sham adrenalectomy, croton oil and alkali induced injury and small doses of cortisol result in very similar changes in dermal concentration and distribution of hydroxyproline(18).

The extraction of the uninjured skin distal to the site of local injury indicates that inflammation results in a generalized decrease of about 60% in the neutral soluble collagen content of the tissue, and an increase of 400% in the citrate soluble collagen. Trypsin, applied topically to the local lesion, results in the former material decreasing only 45%, while the latter collagen increases 700% above the normal control value. The enzyme treatment did not affect the decrease in concentration of insoluble collagen, however, except that this decrease was prolonged beyond that demonstrated by the enzymatically untreated animals.

Since the increases in dermal content of citrate soluble collagen are never enough quantitatively to account for the loss in insoluble collagen, it is unlikely that this latter type of collagen is being converted into soluble collagen. Further, the circulating concentration of hydroxyproline is not profoundly elevated unless the local lesion is subjected to topical trypsin treatment. Therefore it also appears unlikely that the break-down products of insoluble collagen from the uninjured skin drain into the blood.

Summary and Conclusions. The effects of daily topical application of trypsin upon the dermal chemistry changes produced by croton oil induced local inflammation at the site of injury as well as distant from the site of injury, were compared with enzymatically untreated irritant injected control rats. Locally, trypsin treatment resulted in an inhibition of the increase in lesion hexosamine normally encountered in response to chemically induced inflammation. The collagen content of the lesion decreased independently of topical trypsin application. Although the eschar sloughed 10 days after injury with trypsin treatment, as opposed to the 13 days required by the controls, continued topical trypsin treatment inhibited the chemical healing of the lesion.

The serum nitrogen, hexosamine and hydroxyproline were significantly increased with trypsin treatment; evidence for the increased drainage of wound products into the circulation via proteolytically unblocked capillaries and lymphatics.

Local inflammation resulted in profound and specific decreases in concentration of collagen in uninjured skin distal to site of inflammation. Topical application of trypsin to the lesion appeared to partially inhibit this decrease in dermal collagen distant from the site of local injury. Topical trypsin application increased the amount of citrate soluble collagen found in uninjured skin distant from the site of local injury, but both groups of animals demonstrated a similar and profound loss in

insoluble collagen. This dermal collagen loss was prolonged with continued trypsin treatment of the local lesion.

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Salivary Gland Function and Electrolyte Composition in Potassium-Deficient Rats.* (27003)

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Previous investigations(1-6) have shown that dietary deprivation of potassium, in addition to altering electrolyte and water content of specific tissues, may also lead to changes in their function. Alteration in function has been observed in contractile(1-4) and some secretory tissues(5,6). This investigation was undertaken to determine the effects of dietary deprivation of potassium on function and on tissue electrolyte content of another secretory system, the salivary glands, with a view of correlating possible changes in function (altered composition or volume of secretion) with gland electrolyte changes.

Methods. Long-Evans rats, 6 weeks to 4 months of age, weighing 100 to 200 g, were used in these experiments. Experimental animals were placed on a potassium-deficient diet (Nutritional Biochemicals Corp.) composed of cornstarch, 64.2%; casein, 30%; butterfat, 3.5%; calcium carbonate, 1.3%; sodium chloride, 1.0%; and complete vitamin supplements. Potassium content, determined on an ashed sample, was 0.54 mM/100 g of diet. Animals drank tap water (potassium less than .04 mM per liter) or distilled water. Control animals were fed a standard laboratory diet. After 45 days on the diet, animals were fasted 24 hours, lightly anesthetized with Nembutal, and the left parotid, submaxillary, and sublingual glands were removed, individually weighed on a torsion balance, and subsequently dried in Vycor crucibles to determine tissue water content (expressed on a dry weight basis). The duct from one of the remaining salivary glands (in some cases, all 3) was freed from surrounding tissue and cut. Pilocarpine nitrate (2.2 mg) was injected subcutaneously and ensuing secretion was collected, either directly in a small tared vessel(7) or, by micropipette placed at the duct orifice. After saliva collection, the secreting gland was removed for weighing and water and electrolyte analysis. Gland electrolytes were determined on samples dry-ashed (525°C) in Vycor crucibles and are expressed as milliequivalents per kg wet weight (meq/kg

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