Biochemical Changes in Thermally-Injured Cutaneous Tissue, Susceptibility to Proteolytic Enzymes, and Extractability of Collagen. (20387)

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A thorough knowledge of the basic biochemical changes in thermally-injured cutaneous tissue is a prerequisite to the complete understanding of the local and, perhaps, systemic reaction resulting from thermal injury. Since there exists a dearth of such information, this laboratory has undertaken to investigate these fundamental changes in the components of thermally-injured skin. It is planned to study a variety of burns, ranging from the relatively low-temperature, hot water burn to the "flash" burn which is produced by extremely short exposure to radiant energy of very high intensity. The present paper records the results of the first of these investigations dealing with the effects of a hydrothermal burn on certain properties of the protein components of skin.

Experimental. 1) *Animals.* Young adult, male albino rats of the Holtzman strain were utilized in all of the experiments described in this paper. The animals, weighing approximately 150 g when received at the laboratory, were fed the commercial dog food, "Friskies," and used within 4 months after arrival. 2) *Analytical procedures.* Total nitrogen determinations were done in duplicate by the nesslerization procedure of Miller(1) or by the micro-Kjeldahl method of Pregl. Free NH_2-N was determined by the manometric procedure of Van Slyke(2). Hydroxyproline was determined by a modification of the method of Neuman and Logan(3,4). 3. *Enzyme studies.* It has been noted frequently that the sensitivity of a protein to proteolysis reflects the degree of its denaturation. The susceptibility of skin to the proteolytic action of pepsin and trypsin was, therefore, utilized in the present studies as a criterion of the degree of protein denaturation resulting from thermal injury. Abdominal skin was utilized in all experiments. a) *In vitro thermal exposure.* The rats were anesthetized with sodium nembutal (5 mg per 100 g body weight), injected intraperitoneally, and the abdominal hair removed with an electric clipper and a razor blade. The animals were then decapitated and an area of abdominal skin (approximately 4 x 7 cm) removed and dissected free of underlying fatty and muscular tissue. This dissected skin was either used immediately or stored overnight at −30°C. In order to obtain sections of reproducible thickness, the skin was treated in the following manner. After momentary immersion in water, the skin was laid, epidermis down, on the cold stage of a specially designed sliding microtome.† After freezing, 15 μ sections were removed until the remaining tissue was 350 μ thick. This technic enabled us to obtain consistently a uniform sample of skin composed only of epidermal and dermal tissue as shown by histological examination. These skin sections were scissor-minced in the cold and homogenized in ice-cold distilled water, using the Potter-Elvehjem all-glass homogenizer. The resulting suspensions were sufficiently uniform and stable to permit the removal of reproducible samples. The dry matter content of these suspensions was determined by heating for 24 hours at 110°C. Suitable corrections were made for the weight of glass introduced into the homogenate by the grinding procedure. Aliquots of the skin suspensions (approximately 2 mg dry weight per ml) were heated in glass-stoppered test tubes with constant shaking in

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† This instrument was a modified motor-driven microtome equipped with a horizontal freezing stage (5 x 9 cm). With each horizontal cycle of the cold stage, it could also be advanced vertically in increments of one to 25 μ. The cold stage was cooled by a carbon dioxide expansion jet.

a water bath maintained at the desired temperature. The time required to raise the temperature of the suspension to the desired point was predetermined. Thus, all exposure times recorded in this paper represent the period during which the suspension was actually *at* the stated temperature. In all cases, the tubes were cooled in an ice bath immediately after heating. Unheated suspensions served as controls. The *proteolytic effects* of pepsin and trypsin were determined by incubating the skin suspensions for varying times with the requisite enzymes and measuring the resultant increases in total TCA-‡soluble nitrogen or TCA-soluble free NH_2-N. The concentration of skin substrate employed did not permit the maximal rate of reaction under these assay conditions. This sub-optimal concentration of skin substrate was chosen in order to avoid saturation of the enzyme, and thus permit the detection of any increased reaction rate which might result from the action of heat. The reactions were conducted in 25 ml glass-stoppered Erlenmeyer flasks shaken continuously throughout the experiment in a water bath maintained at 35.0°C ± .05°. The various substances, excepting the enzyme solution, were added as indicated in Table I, placed in the water bath and incubated for 5 minutes. The similarly equilibrated enzyme solution was then added and the incubation continued. At the desired times, 3 ml aliquots were removed and added to 4 ml of 10% TCA. Suitable autolysis and zero time controls were utilized. In the autolysis controls, the enzyme solutions were replaced with an equal volume of water or 0.003 N HCl in the pepsin and trypsin experiments, respectively. In the zero time controls, 4 ml of 10% trichloracetic acid were added prior to the enzyme to unincubated flasks containing one-fourth of the indicated amounts of each component. (Table I). After standing for one-half hour at room temperature, the trichloracetic acid suspensions were filtered through Whatman No. 3 filter paper. Anson(5) has shown that this paper does not absorb protein split-products. One or 2 ml aliquots of each filtrate were used for the determination of total nitrogen. Free NH_2-N was determined on 3 ml aliquots. In these latter experiments the concentrations of the various substances were increased proportionally in order to provide sufficient quantities of free NH_2-N for analysis. b) *In situ thermal exposures.* The abdominal hair was removed from the anesthetized rat as described in the preceding section. The following day the rat was anesthetized with nembutal and a large proportion of the abdominal area injured thermally by exposure for varying periods of time to a large volume of rapidly circulating water whose temperature was maintained within 0.01°C. A constant temperature water bath with centrally located heaters and stirrer assembly served as the heat source. An additional stirrer directly under the rat provided further mixing. The anesthetized rat was placed in a transite box with an elliptical hole which permitted the protrusion of the abdomen and a small portion of the thoracic region. The rat was sufficiently limp so that the skin-board junction was water-tight, and no other seal was necessary. Thus, the abdominal area could be burned with no injury to the rest of the body surface. The rat was placed in position and the entire assembly lowered into the water bath to a depth which maintained the water level of the bath 3-4 mm above the bottom of the box. After a stated period of time, the apparatus was removed from the bath, the animal decapitated and the uniformly burned area excised as rapidly as possible. The fri-

TABLE I. Protocol of Enzyme Experiments.

Additions	Pepsin exp., ml	Trypsin exp., ml
Skin suspension*	4	4
Enzyme†	4	4
0.44N HCl	2	—
M/10 phosphate buffer (Sorenson, pH=7.6)	—	4
Water	2	—
Final vol	12	12
Final pH	1.4	7.6
Incubation temp.	35°C	35°C

* Approximately 2 mg dry wt/ml.

† Freshly prepared solutions of Worthington's crystalline pepsin and trypsin in 0.003N HCl in concentrations of 25 and 40 μg/ml respectively. Concentration of trypsin corrected for labelled impurity of 50% magnesium sulfate.

‡ TCA = trichloracetic acid.

TABLE II. Effects of *In vitro* and *In situ* Thermal Exposure upon Susceptibility of Skin to Pepsin and Trypsin.

		μg trichloracetic acid-soluble nitrogen/mg dry skin						
		In vitro exposure*				*In situ* exposure†		
Enzyme	Enzyme incubation (min.)	Unheated	50°C	60°C	100°C	Unheated	55°C	70°C
Pepsin	0	10				12	10	22
		8	13	40	64	12	11	24
		12	15	36	61			
		6						
		10		32				
	10‡	35				43	41	36
		41	32	39	22	45	45	39
		36	34	30	22			
		38						
		46		33				
Trypsin	0	12				13	13	20
		9		30		18	18	24
		9		22				
		9	11		46			
		12	15	35	57			
		5		33				
	10‡	19				24	20	47
		16		29		27	28	57
		20		32				
		21	15		35			
		12	25	38	34			
		17		31				

* Suspensions held at indicated temperature one min.
† Rats exposed to bath temp. of 55°C and 70°C for 3 and 2 min., respectively.
‡ Corrected for zero time and autolysis values.

able nature of this *in situ* heated skin prevented the use of the sliding microtome for sectioning as described above. These skins, therefore, were dissected and scraped very carefully by hand in order to free them of any underlying extraneous tissue. Histological examination showed the presence of only epidermal and dermal tissue. Control animals were treated in an identical fashion with the exception that the thermal episode was omitted. The skins excised from these rats were also dissected only by hand. The preparation of the homogenates and the subsequent enzymatic procedures were identical with those described above.

4. *Extraction studies.* During the course of the enzymatic experiments, it was noted that heating a suspension of defatted, saline-extracted skin powder at 60° for one minute increased markedly the amount of nitrogen soluble in TCA. This observation led to further investigations of the effects of heat upon the ease of extraction of various components from abdominal skin and tail tendon. Loss in weight resulting from successive treatments with 0.9% sodium chloride, water, 95% ethanol, ether and 0.1 N sodium hydroxide was determined. In some cases analyses were made of the collagen contents of the extracted residues. Both *in vitro* and *in situ* thermal exposures were conducted with skin. Studies with tendon involved *in vitro* heating only. a) *In vitro thermal exposures.* Abdominal skin sections prepared as described in the *in vitro* enzymatic studies and tendon fibers of the rat tail were scissor-minced in the cold and homogenized in ice-cold distilled water. Ten ml aliquots of these suspensions (10-20 mg dry weight per ml) were centrifuged for 30 minutes in the International Centrifuge, Size 2, at 1000 g in 15 ml tared tubes, the residues resuspended in 10 ml of 0.9% sodium chloride and heated in a water bath with constant stirring. The tubes were cooled in an ice bath immediately after heating. Unheated suspensions served as controls. These saline suspensions were shaken in a water bath at 35°C for one hour and then centrifuged at 1000 g for 30 minutes. The residues were washed twice with 10 ml of distilled water, ice-cold 95% ethanol and ether at the centrifuge. The final residues

TABLE III. Effects of *In vitro* and *In situ* Thermal Exposure upon Extractability of Skin and Tendon Components.*

		% loss of original dry wt by extraction with:		
	°C	Water + saline + alcohol + ether	Same + 0.1N NaOH	% collagen on dry wt basis
		Skin		
In vitro†	Unheated	47 ± .9 (14)	66 ± .9 (14)	29 ± .8 (6)
	60	73 ± .8 (10)	89 ± 1.2 (10)	6.8 ± .1 (4)
	100	81 ± 1.2 (4)	93 ± 1.4 (4)	3.2 (2)
In situ‡	Unheated	26, 29, 29	46, 49, 49	44, 46
	50	29	49	46
	55	28	51	44
	60	41, 34, 40, 48	53, 53, 66, 75	42, 43, 33
	65	54	76	20
	70	58, 59	79, 85	16, 10
		Tendon		
In vitro†	Unheated	7.0 ± .7 (6)	34 ± .2 (4)	64 ± 1.0 (4)
	60	85 ± 1.8 (6)	95 ± 1.2 (4)	3.5 ± .2 (4)
	100	97 (2)	98 (2)	2.0 (2)

* In *in vitro* exposures, suspensions kept at stated temperatures for one min. All *in situ* exposures made for 3 min. with exception of 70° exposure which lasted for 2 min.

† Mean values ± stand. error are presented. No. of samples given in parentheses.

‡ Individual values are presented.

were dried *in vacuo* to constant weight over P_2O_5 at room temperature and weighed. The calculated percentage weight losses at this stage are given in column 2 of Table III. The residues were then extracted repeatedly with 10 ml of 0.1 N NaOH at room temperature according to the procedure of Lowry *et al.*(6). After neutralization, subsequent washings with water, alcohol and ether, and drying were conducted as described above and the residues weighed. The calculated percentage losses at this stage are given in column 3 of Table III. The collagen contents of the resulting residues were determined by suspending them in 5 ml of distilled water and autoclaving at 120 lb pressure for 8 hours(6). The residues remaining after centrifugation were washed with water, alcohol and ether, and dried in the usual manner. Since autoclaving converts collagen into soluble gelatin, the loss in weight due to autoclaving represents the collagen content of the extracted residues (Column 4 of Table III). In a few experiments, the elastin content of skin and tendon was estimated by suspending the dried, autoclaved residue in 10 ml of 0.1 N NaOH and heating in a boiling water bath for 30 minutes(6). After centrifuging, the residues were washed with water, alcohol and ether and dried as usual. This residue was considered to be elastin. b) *In situ thermal exposure.* The abdominal area was burned and skin sections prepared as described in the *in situ* enzymatic studies. Skin obtained in this manner from both burned and unburned rats was treated exactly as described in the preceding section, with the exception that the saline suspensions were not subjected to any further heating.

Results and discussion. Enzyme studies. It is apparent from Table II that the proteolytic activity of pepsin upon *unheated* suspensions of rat skin was more pronounced than that of trypsin. Since skin proteins consist mainly of collagen, these observations are in general agreement with other reports demonstrating the marked action of pepsin upon native collagen(7,8). The skin from which these suspensions were prepared contained a total of 90 μg of nitrogen per mg of dry weight. The pronounced action of heating *per se* upon the TCA-solubility of skin nitrogen is shown by the large increases in TCA-soluble nitrogen in the zero time flasks of suspensions heated *in vitro* at 60° or 100°C. In contrast to its effect on peptic activity, heating at 60° or 100°C resulted in significant increases in the proteolytic action of trypsin. This finding of an increased susceptibility of heated skin to the action of trypsin is in conformity with the observations of others that

heat denatured collagen is more readily attacked by trypsin than is undenatured collagen(9,10). Experiments utilizing the changes in TCA-soluble free NH_2-N as a measure of enzymatic activity corroborated the above-described effects of heat upon the susceptibility of skin suspensions to peptic and tryptic digestion. The effects of pepsin and trypsin upon *in situ* heated skin in general paralleled those noted in the *in vitro* experiments (Table II). Thus, *in situ* exposure at 55°C produced no change in the susceptibility of the skin suspensions to peptic or tryptic digestion, whereas *in situ* exposure at 70°C resulted in a marked increase in tryptic digestion. *Extraction studies.* The increased extractability of rat skin resulting from *in vitro* exposures at 60°C and 100°C is shown in Table III. Determinations of the collagen contents of original and extracted skins by the hydroxyproline method demonstrated that the increased weight losses resulting from these thermal exposures were due to the removal of collagen. It is also apparent that the effect of heat upon the extractability of rat tail tendon was very pronounced. In the experiments cited, the percentage weight losses were computed from the weights of residues obtained by centrifugation at 1000 g. Centrifugation at 26,000 g in the Spinco centrifuge did not affect the amount of sedimentable material from tendon. In contrast, ⅓ of the skin substance rendered extractable in saline by heat was sedimented at 26,000 g. Using the hydroxyproline method on autoclaved tissue filtrates(11), it was determined that collagen constitutes 42 and 87% of unheated whole dry skin and tendon, respectively. These values are in contrast with 29 and 64% obtained by Lowry's procedure(6) which involves determination of the weight losses resulting from autoclaving the alkali-extracted residues (column 4, Table III). The lower values obtained by Lowry's procedure were found to be due to the loss in hydroxyproline-containing compounds accruing from the extraction steps preceding autoclaving. In general, the effects of *in situ* heating resembled those observed in the *in vitro* experiments. A change in extractability first became evident at 60°C and increased as the thermal episode became more severe (Table III). This increased extractability was paralleled by losses in collagen (Table III). In order to avoid the secondary complications of *in situ* heating, *e.g.,* edema formation, in some experiments the abdominal area was exposed *in situ after* the animal was sacrificed by decapitation. The effects of heat remained unaltered. The thermistor method described in the accompanying paper(12) was utilized to determine subcutaneous temperatures attained during *in situ* exposure. Subcutaneous temperatures ranging from 2-4 degrees lower than the bath temperatures were maintained for one minute or longer during the thermal episode. Elastin constituted 4% of the dry weight of skin and its quantity was unaffected by heat.

Summary. 1. The effects of *in vitro* (50-100°C) and *in situ* (55° and 70°C) hydrothermal injury upon susceptibility of the abdominal skin of rats to peptic and tryptic digestion were determined. The action of pepsin upon unheated skin was considerably greater than that of trypsin, whereas heating, both *in vitro* and *in situ,* increased only the susceptibility toward trypsin. 2. Similar hydrothermal exposures produced changes in rat skin and tail tendon collagens which rendered them extractable by normal saline.

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