Effect of Cortisone on Metabolism of Cystine in Regenerating Wound Tissue.* (31080)

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The rate of wound tissue formation is inhibited by glucocorticoids(1-5). These steroids appear to have no effect on the tensile strength of wound tissue until collagen production becomes significant(4-6). The administration of large amounts of glucocorticoids is thought to inhibit collagen formation by altering protein metabolism; the effect of lower levels on the collagen content of wound tissue is believed to be a reflection of appetite reduction(2). This latter effect on the tensile strength of wound tissue may be produced also by restricting the protein intake or otherwise limiting the availability of methionine and cystine(7).

Cystine has been shown to be the critical factor in the formation of wound tissue(7-9), even though collagen, the principal protein in this tissue, contains no cystine(10). In wound tissue, the synthesis of collagen is paralleled by deposition of cellular proteins containing relatively large amounts of cystine(8). It has been suggested, therefore, that the role of this amino acid in wound tissue may be involved in the mechanism by which collagen, or its intracellular precursor, is formed.

To determine whether glucocorticoids interfere with the formation of the collagen-producing mechanism or with its function, an investigation into the rate of incorporation of labeled cystine into cellular proteins of wound tissue was undertaken. The rate of formation of cellular proteins in granulation tissue can be distinguished from the rate of metabolic utilization of these proteins by following the rate of uptake and turnover of cystine in these proteins.

Experimental. The effect of cortisone on wound tissue formation was studied in virgin female albino rats weighing 180 ± 7 g at start of the experiment. Weighed paired animals were maintained on a 4% casein diet (10 g/day)(12), beginning 3 days before the animals were wounded and continuing until the wound tissue was collected. The rats consumed all the food presented daily. At the same time, half of the paired animals were given 1.0 mg/day of cortisone acetate, subcutaneously. The other animal in each pair was injected with an equal volume of solution in which the cortisone had been suspended (20% ethanol, 80% 0.15 *M* NaCl). The wound was produced by excising a piece of skin 4 cm in diameter from the scapular region, according to the procedure previously described(12).

Nine days after wounding, each rat was injected subcutaneously with 20 μcuries of L-cystine-S^{35} dissolved in 0.15 *M* NaCl. At intervals of 30, 60, 90 and 150 minutes thereafter, granulation tissue from several rats in each group was collected(12). Each sample of wound tissue was immediately frozen at −70°C in an air-tight polypropylene bag and stored at −18°C until the analytical procedures could be carried out.

To determine the amount of collagen in each sample, a weighed aliquot of wound tissue from each sample was hydrolyzed in 6 *N* HCl for 4 hours. This period of hydrolysis was found to be adequate for maximal recovery of hydroxyproline in the sample. After filtration, aliquots of the hydrolysates were analyzed for hydroxyproline(13-15) and for tyrosine(16).

The residual frozen tissue sample was homogenized at 4°C in 5 volumes of 0.2 *M* carbonate-bicarbonate buffer (pH = 9.7) for 15 minutes. An aliquot of each sample of homogenized tissue was dialyzed against 150-200 volumes of 0.03 *M* Na_2SO_3, containing 10^{-3} *M* cupric ion, for 4 hours at room temperature with agitation. Another aliquot was dialyzed similarly against 150-200 volumes of 0.03 *M* Na_2CO_3. Subsequently, excess sulfite and carbonate were removed from the dialy-

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TABLE I. Effect of Cortisone on Body Weight of Wounded Rats.

	No. of rats	Days after wounding		
		-3	0	-9
		g		
Untreated	20	180 ± 7	182 ± 6	167 ± 5
Cortisone treated	20	180 ± 7	177 ± 6	158 ± 8
p		—	.05	.001

sands by dialysis against 100 volumes of distilled water for an hour(17). The tyrosine content of each dialysand was determined (16); the S^{35} activity in each dialysand was measured in a gas-flow counting system.

Results and discussion. As reported earlier (7), the effect of the low protein diet and wounding resulted in a significant loss of body weight (Table I). Treatment with cortisone produced an additional weight loss in the experimental animals, over and above that resulting from the low protein diet and wounding. To some extent, the excess catabolism of protein associated with the loss of weight might serve as an additional source of amino acids for the production of wound tissue protein.

In Table II is shown the effect of cortisone on the formation of both collagen and cellular proteins. In agreement with the findings of others(2,4,20), it can be seen that cortisone produced a significant depression in the rate of formation of collagen. The synthesis of non-collagenous protein, however, does not appear to be significantly affected by administration of the glucocorticoid.

The technique described above has been used to distinguish cystine bound into protein by 2 different types of linkages(17-19). Sulfhydryl and disulfide groups react with sulfite ion, in the presence of cupric ion as a catalyst, according to the following scheme (11):

$$R\text{–}S\text{–}S\text{–}R + SO_3^{=} \rightleftharpoons R\text{–}S\text{–}SO_3^{-} + R\text{–}S^{-}$$
$$R\text{–}S^{-} + SO_3^{=} + 2\ Cu^{++} \rightleftharpoons R\text{–}S\text{–}SO_3^{-} + 2\ Cu^{++}$$

When these reactions are carried out in a dialysis membrane, the resulting thiosulfate derivatives may be separated on the basis of size. Remaining in the dialysand are the thiosulfate derivatives of cystine which are attached to the protein by peptide bonds. The rate of formation of the non-collagenous proteins may be determined by measuring the amount of incorporation of peptide-bound cystine-S^{35} per gram of tissue at several intervals after administration of the labeled cystine(17). The data in Fig. 1 show that cortisone causes a depression in the rate of incorporation of peptide-bound cystine-S^{35} in the wound tissue. This may be interpreted to mean that cortisone reduced the rate of formation of at least some of the cystine-containing proteins in the wound tissue. Since there is no significant change in the amount of tyrosine in the cellular proteins (Table II), it seems probable that the cystine-rich proteins whose synthesis is inhibited by the cortisone represents only a small fraction of the total cellular protein in the wound tissue.

Dialysis of the S^{35}-labeled tissue protein against Na_2CO_3 has been shown to have no effect on the peptide or disulfide bonds involving cystine(17,19). The dialysand from the Na_2CO_3, therefore, contains both the peptide-bound and the disulfide-bound cystine. The amount of cystine-S^{35} attached to the protein by the latter type of bond may be calculated from the difference in the two dialysands. The turnover of this cystine fraction may be taken as a measure of the activity of enzymes which are activated or inhibited by sulfhydryl groups. The data plotted in Fig. 2 appear to indicate that cortisone inhibits the turnover rate of the disulfide-bound cystine. This must mean that sulfhydryl activated enzymes in the wound tissue are being utilized at a more rapid rate in the control animals than in those which had received cortisone.

This conceptualization is supported by the

TABLE II. Effect of Cortisone on Protein Content of Wound Tissue.

	Cortisone-treated	Untreated	p
	mg/g wound tissue		
Hydroxyproline	5.94 ± .66	7.32 ± .88	.05
Tyrosine	5.50 ± .60	5.81 ± .47	.6
Collagen*	44.2 ± 5.0	54.6 ±6.6	.05
Cellular proteins†	106 ±12	111 ±9	.6

* Calculated from tissue hydroxyproline content of 13.4%.

† Calculated on basis of tyrosine content of cellular proteins being 5%, corrected for tyrosine in the collagen.

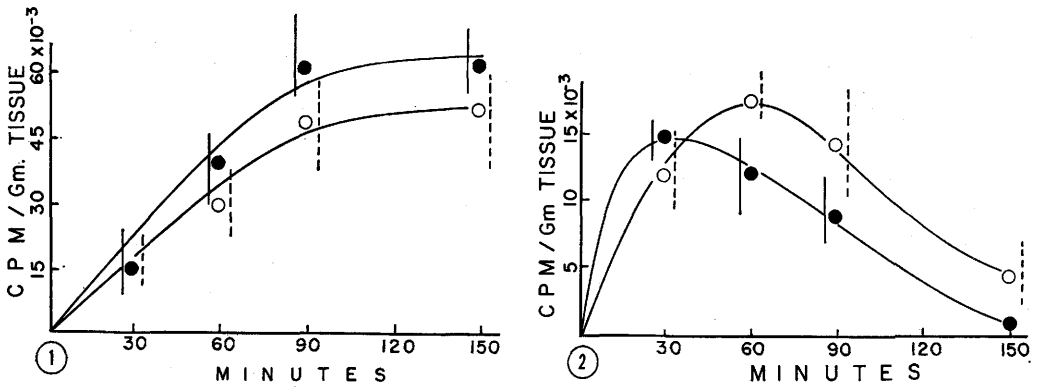

FIG. 1. Rate of formation of wound tissue proteins on 9th day after wounding, measured in terms of counts per min of S^{35} per mg tissue incorporated into peptide-bound cystine against time after administration of 20 μcuries of L-cystine-S^{35}. Each point represents analyses of 4-5 samples of wound tissue. Open circles show data from cortisone treated rats; solid circles, untreated control animals. Solid vertical lines indicate range of analytical values in wound tissue samples from untreated controls; broken vertical lines, from cortisone treated animals.

FIG. 2. Rate of turnover of cystine associated with wound tissue proteins by disulfide bonds only, on 9th day after wounding, measured in terms of counts per min of S^{35} per mg tissue against time after administration of 20 μcuries of L-cystine-S^{35}. Symbols as in Fig. 1.

evidence showing that the disulfide-bound cystine becomes associated with the protein by means of the following exchange reaction (22,23):

Protein-SH + Cys-S-S-Cys → Protein-S-S-Cys + Cys-SH

The turnover of the disulfide-bound cystine may then be considered to be a reflection of the turnover of the free sulfhydryl groups in the wound tissue protein. Preliminary experiments(21) appear to indicate that the labeled disulfide-bound cystine is removed from the protein by reaction with the reduced glutathione in wound tissue by means of one, or both, of the following reactions:

Protein-S-S-Cys + G-SH → Protein-SH + G-S-S-Cys

Protein-S-S-Cys + G-SH → Protein-S-S-G + Cys-SH

The formation of protein in the microsomes and in the protein synthesizing mechanism of the cellular nuclei of wound tissue has shown them to be the principal sites of turnover of disulfide-bound cystine(19). The inhibitory effect of cortisone suggests a similar correlation, *i.e.*, between the decreased turnover of disulfide-bound cystine (Fig. 2), the synthesis of cystine-rich proteins (Fig. 1) and that of the precursor molecules of tropocollagen (Table II). However, the possibility that these relationships are not directly associated must be borne in mind until further corroborative evidence becomes available.

Summary. 1. Administration of 1.0 mg/day of cortisone to wounded female rats over a period of 12 days resulted in a weight loss significantly greater than that due to wounding and low protein diet alone. 2. Cortisone inhibited the formation of collagen. On the basis of tyrosine content, the sterol had no effect on the synthesis of total cellular proteins. 3. The rate of formation of cystine-rich proteins in the wound tissue was inhibited by the cortisone treatment. 4. The turnover of disulfide-bound cystine attached to the protein of the wound tissue cells was reduced in rats receiving cortisone, indicating a decreased utilization of cystine (or sulfhydryl) activated enzymes.

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Unresponsiveness of the Adult Toad to Thyroxine Administration.* (31081)

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An *in vitro* effect of thyroxine on vasopressin sensitive structures of the toad has been reported(1). We have confirmed the effect of thyroxine in water transfer and active sodium transport across the isolated toad bladder, and further demonstrated that the effects of thyroxine and vasopressin on water transfer but not on sodium transport, were synergistic (2).

Reports in the literature are contradictory as to the effects of thyroxine administration to the intact adult toad. Enzymes related to oxidative metabolic pathways, known to be altered in the thyroxinized mammal, have been reported to have normal activity in the adult toad after repeated injections of thyroxine(3).

In thyroxine treated mammals an increase in oxygen consumption has regularly been found in intact animals, tissue slices and tissue homogenates(3), whereas similar studies in the toad have been reported as showing either an increase in oxygen consumption(4, 5,6) or no demonstrable change(7). In the isolated bladder of normal toads Thornburn and Matty(8) have reported increased oxygen consumption after adding thyroxine *in vitro,* an observation which we have confirmed. Reports on the effect of thyroxine on water uptake and transport of electrolytes through the skin of the adult amphibian are also contradictory(9,10,11).

Our interest in the interrelation of thyroxine and vasopressin stimulated us to study the effects of thyroxine in *Bufo spinolosus,* the species in which our former studies were made.

In addition to studies on the action of thyroxine on vasopressin sensitive structures of toads given daily injections of thyroxine, determinations were made of the oxygen consumption by the isolated bladder and liver homogenates obtained from the same animals; and also of the cystathionase and glutamic pyruvic transaminase activity of these liver homogenates. The activity of these enzymes, unrelated to oxidative metabolism, has

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