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Distribution of Sulfur in Regenerating Wound Tissue. (23705)

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Methionine and cystine supplementation have been shown to be equally effective in accelerating healing rate of wounds in rats fed low and high protein diets(1,2). Methionine and cystine + cysteine (hereafter referred to as cystine) content of wound tissue was reported to exceed that of corresponding unwounded skin tissue 4 to 31 days after injury (3). Attempts to detect sulfur-containing compounds other than protein-bound amino acids have revealed no non-protein-bound wound sulfur(3). It was tentatively concluded that increased demand for methionine or cystine following injury is due to requirement of regenerating tissue for these amino acids, *per se*, as structural units of proteins (or, in the case of methionine, also as precursor of cystine). Since regenerating wound tissue contains considerable quantity of sulfate-containing mucopolysaccharides(4,5) the sulfur of which may be a product of *in vivo* metabolism of methionine and cystine(6), the presence of mucopolysaccharide sulfate in wound tissue was studied. Glutathione has been increased at site of rapid cell division(7) such as accompanies wound tissue regeneration. Non-protein fractions of wound tissue were studied with alterations in content of the peptide. Finally, levels of free cystine and methionine, and amount of S^{35} recently injected as S^{35}-L-methionine present in all forms discussed was studied. Special micro-technics were employed to detect minute amounts of methionine, cystine, and sulfate in protein and non-protein fractions of wound tissue. Rate of S^{35} uptake following administration of S^{35}-L-methionine was also studied, radioactive tracers serving as the most sensitive available method for detection of the various compounds.

Methods. Sixteen individually caged, female, albino rats (180 ± 15 g) of Sprague-Dawley strain, receiving distilled water *ad lib.*, were maintained on a protein-free diet (3) throughout. After 5-day acclimation period, each animal received intraperitoneally 5.05×10^6 cpm of S^{35}-L-methionine (Abbott) in 0.25 ml saline. Two days later, they were anesthetized with pentobarbital and a standard 4 cm circular skin wound placed on the back directly below scapula(3). Wound tissues were removed from 8 animals on 4 and 6 days after injury, the animals then sacrified by decapitation. Tissues were carefully removed, weighed, and frozen at −20°. The frozen tissues were ground in mortar with sand and transferred to centrifuge tubes by washing with approximately 10 ml of saline. Ten ml of aqueous 10% trichloroacetic acid solution (TCA) was added to precipitate wound protein, the tubes then being centrifuged at 2000 rpm at 5° for 10 minutes. The precipitate was washed once with 5 ml of 5% TCA and supernatant fluids combined, filtered through Whatman No. 2 paper and diluted to 35 ml. The protein fraction was hydrolyzed 20 hours in 12 ml of 20% HCl - 50% HCOOH mixture(8), then diluted to 25 ml and filtered. S^{35} radioactivity present as mucopolysaccharide sulfate was determined by direct precipitation from acid hydrolyzate as $BaSO_4$, after addition of carrier sulfate. Sulfate-S^{35} in supernatant fluid was determined similarly. Total S^{35} radioactivity was determined on aliquots of supernatant fluid

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TABLE I. Results of Chemical Analysis of Various Sulfur-Containing Compounds in Fractionated Wound Tissues. Results are expressed on the basis of mg sulfur/g of wet tissue.

Days after wounding	Mucopolysaccharide	Methionine	Cystine
	Wound hydrolysate		
4	.171 ± .033†	.470 ± .115	.548 ± .061
6	.186 ± .012	.374 ± .057	.532 ± .063
	Wound supernatant fluid		
4	0	.0054*	.0606 ± .0096
6	0	.0048*	.0592 ± .0051

* Samples were pooled prior to analyses.
† Stand. dev. of mean.

and protein hydrolyzate by precipitation as $BaSO_4$ after oxidation with an HNO_3-$HClO_4$ mixture(9) and addition of carrier sulfate. Cystine radioactivity was determined after precipitation of the cuprous mercaptide(10). Methionine radioactivity was taken as the difference: total hydrolyzate radioactivity—(cystine radioactivity + mucopolysaccharide radioactivity). All radioactivity measurements were made at infinite thickness with a Tracerlab Windowless Flow Counter. Free sulfate in supernatant fluid and mucopolysaccharide sulfate in the acid hydrolyzate were determined turbidimetrically(11). Methionine in protein hydrolyzates was determined colorimetrically by Horn's modification (12) of the Sullivan-McCarthy method, while Folin-Winterstein colorimetric method involving reduction of phospho-18-tungstic acid was employed to determine cystine(13). These colorimetric methods were adapted to microscale for determinations on supernatant fluid fraction. For methionine assay 0.5 ml distilled H_2O, 0.05 ml 5 N NaOH, 0.01 ml sodium nitroprusside, 0.10 ml of 3% glycine and 0.10 ml of 85% phosphoric acid were employed. The microcystine method involved 1.3 ml distilled H_2O, 1.3 ml saturated sodium bicarbonate, 0.4 ml phospho-18-tungstic acid (1:1, aqueous), and 0.2 ml 10% sodium sulfite. Both methods gave linear absorption curves in 0.01-0.10 mg range of amino acid. A group of 36 animals, maintained under previously described conditions, were injected with 3.88×10^6 cpm doses of S^{35}-L-methionine, given 3 cm circular skin wounds, and 6 animals sacrificed by decapitation at scheduled times after wounding. Radioactivity and chemical measurements were performed on unfractionated wound tissues after acid hydrolysis, to ascertain presence of radioactivity as a function of time.

Results and Discussion. Results of chemical analyses (Table I) indicate that, on fourth and sixth days after wounding, covering interval of maximal metabolic activity, as indicated by previous work(3), protein-bound cystine contains the largest amount of sulfur in the wound, protein-bound methionine 20% less, and mucopolysaccharides approximately 50% as much sulfur as does methionine. No free sulfate was detectable in the non-protein fraction, either radioactively or chemically. The non-protein fraction contains approximately 1/10th as much cystine and 1/100th as much methionine as the protein fraction.

These data indicate that, while comparatively little sulfur is present in wound tissue in the supernatant fluid fraction, a considerable amount is present as mucopolysaccharides. As data in Table II indicate, the specific activity† of wound protein-bound cystine and methionine is much higher than that of mucopolysaccharide sulfur. This indicates that, while considerable sulfur is present in wounds as mucopolysaccharide sulfate, relatively very little of sulfur recently introduced into the body as S^{35}-L-methionine has been incorporated into wound tissue mucopolysaccharide.

TABLE II. Specific Activity of S^{35} in Various Fractions of Wound Tissue. Each rat received 5.05×10^6 cpm of S^{35}-L-methionine intraper. 48 hr prior to wounding.

Days after wounding	Mucopolysaccharide $SO_4^=$	Methionine-S^{35}	Cystine-S^{35}
	Wound hydrolysate		
4	1.30 ± .14*	10.20 ± 1.64	12.82 ± 1.06
6	1.29 ± .21	32.40 ± 3.86	10.82 ± .93
	Wound supernatant fluid		
4	0	75.74 ± 13.3	5.11 ± .59
6	0	95.08 ± 15.5	4.12 ± .25

* Stand. dev. of mean.

† Specific activity =

$$\frac{(\text{cpm/aliquot})(\text{wt of animal (g)})(10)}{(\text{mg amino acid S/aliquot})(\text{dose in cpm})}$$

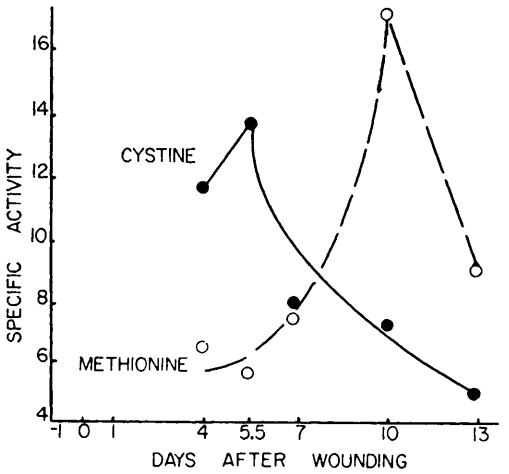

FIG. 1. Specific activity† of wound methionine and cystine as a function of time. Each point represents average of determinations on 6 rats. The more labile proteins of all animals were pre-labeled by injection of 3.88×10^6 cpm of S^{35}-L-methionine 2 days prior to wounding.

Variation of specific activity of wound methionine and cystine with time are shown in Fig. 1. Apparently rate of uptake of cystine-S^{35} by wound tissue, under conditions described, is more rapid than that of methionine, the specific activity of the former reaching a maximum at 5½ days post-injury while the latter reaches a peak at 10 days, thereafter dropping rapidly. The data in Table II from a separate experiment involving larger wounds, are consistent with these findings, protein-bound methionine specific activity rising between 4th and 6th days, while data for protein-bound cystine are considerably lower and approximately equal on 4th and 6th days.

Specific activity of the immediate precursor of the protein-bound amino acids, during period when specific activity is rising, must be higher than that of the material into which it is incorporated. While evidence indicates that this is true for the immediate precursor of protein methionine—free wound methionine—the specific activity of non-protein cystine is lower than that of the protein-bound form, on 4th and 6th days post-injury. That the specific activity of wound supernatant mercaptide-precipitable compounds on both days is lower than that of protein-bound cystine may suggest many explanations, among them, either (a) all material in wound supernatant precipitable as cuprous mercaptide is not the immediate precursor of wound protein-bound cystine (this would be true if a large amount of material present were glutathione, the cysteine of which was supplied from sources different from those of highly radioactive wound cystine) or (b) wound tissue contains enzymes capable of converting methionine to cystine.

Our data substantiate the previous conclusion(3) that recently injected S^{35}-L-methionine is incorporated into wound tissue almost exclusively as protein-bound cystine and methionine, at least during first week of healing. Presence of elevated amounts of mucopolysaccharides and glutathione in wound tissue is indicated, but their low S^{35} contents indicate that little S^{35} from recently injected S^{35}-L-methionine has gone into formation of these compounds.

The seemingly anomalous results obtained here (Fig. 1) compared with previous publications(3) can be reconciled as follows. Under conditions of these experiments tissues were pre-labeled prior to injury and thus methionine-containing proteins would have to undergo catabolism before this amino acid was incorporated into wound protein. Therefore data in Fig. 1 would be expected to show a slow methionine incorporation over a long time; on the other hand, where one administered methionine to previously wounded animal, catabolism of tissue protein is not immediately involved in incorporation of labeled amino acid into wound tissue. Thus one would expect, and it was indeed found(3) that methionine was immediately taken up by wound tissue and rapidly turned over.

Summary. 1. Following pre-labeling of labile body proteins by injection of S-^{35}-L-methionine and subsequent wounding, regenerating tissue was removed at 4 and 6 day intervals, fractionated, and assayed chemically and radioactively for bound and non-protein bound cystine and methionine, and for free and mucopolysaccharide sulfate. 2. Specific activity of unfractionated wound cystine and methionine was studied at intervals over 3 weeks. 3. The data indicate that, for the first 6 days post-injury, S^{35} administered as

S^{35}-L-methionine 2 days prior to wounding is incorporated into wound tissue in large measure as protein-bound methionine and cystine. 4. During the interval studied, administered methionine-S^{35} is not a precursor for wound tissue mucopolysaccharide sulfate.

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Sensitivity of the Common Bile Duct to Acid Peptic Digestion.* (23706)

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Narrowing of the terminal biliary papilla as it enters the duodenal lumen has been observed in more than 50% of patients operated upon for gallstones(1). The causes for such narrowing are not apparent. The purpose of this study is to evaluate the sensitivity of the mucosa of the common bile duct to injury by acid-peptic juices, as the biliary component of the papilla does project into the duodenal lumen and could there be subjected to periodic submergence in gastric juice. If the bile duct epithelium proved sensitive to acid-peptic digestion, it is possible that erosive injury may play a role in bringing about the narrowing of the papilla observed so frequently in patients with gallstones. The recurrent stricture following choledochoduodenostomy could also find an explanation in such an occurrence. These considerations suggested the need to test the sensitivity of the mucosa of the common bile duct to acid-peptic digestion by perfusion with human gastric juice, with acid-pepsin mixtures, and with pancreatic juice and bile mixtures.

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Method. Adult cats were anesthetized with approximately 26 mg/kg body weight of sodium pentobarbital given intraperitoneally. Simultaneous perfusions of the esophagus and common bile duct were carried out. The sensitivity of the esophageal mucosa to injury by acid-peptic juice is well known(2) and concomitant perfusion of that organ could serve as a useful control. The gastric juice employed in the perfusion had been obtained from fasting patients by an inlying gastric tube attached to suction overnight for an 8-hour period from 11:00 p.m. to 7:00 a.m. Patients with peptic ulcer, cholelithiasis, as well as those with normal gastrointestinal tracts were studied. Gastric secretions were collected on ice and either analyzed immediately or frozen and stored below 0°C until used. The volume was measured and determinations of pH, free acid and pepsin were made. The pH was measured with the Northrup & Leeds glass electrode, free HCl was determined by titration with 0.1 N NaOH, and pepsin was determined by the hemoglobin substrate method of Anson(3). The gastric juice was adjusted to pH 1.6-1.7 at which acidity, optimal peptic activity is achieved. Commercial pepsin and acid solu-