9. Krebs, H. A., Ruffo, A., Johnson, M., Eggleston, L. V., Hems, R., *Biochem. J.*, 1953, v54, 107.

10. Krebs, H. A., Hems, R., *Biochim. Biophys. Acta*, 1953, v12, 172.

11. Krebs, H. A., Eggleston, L. V., Terner, C., *Biochem. J.*, 1951, v48, 530.

12. Umbreit, W. W., Burris, R. H., Stauffer, J. F., *Manometric Techniques and Tissue Metabolism*, Minneapolis, 1949, Burgess Publishing Co.

13. Palladina, L. I., Gudina, A. M., *Ukrain. Biokhim. Zhur.*, 1958, v30, 1958.

14. Fonnesu, A., Severi, C., *J. Biophys. Biochem. Cytol.*, 1956, v2, 293.

15. Greenbaum, A. L., Greenwood, F. C., Harkness, R. D., *J. Physiol.*, 1954, v125, 251.

16. Allard, C., DeLamirande, G., Cantero, A., *Cancer Research*, 1952, v12, 580.

17. Hecht, L. I., Potter, Van R., *ibid.*, 1958, v18, 186.

18. Levin, E., Albert, S., Johnson, R. M., *Arch. Biochem. Biophys.*, 1956, v64, 272.

19. Clerici, E., Guidotti, G., Sambo, G., *Sperimentale, Arch. Biol.*, 1958, v108, 283.

20. Ciaranfi, E., Fonnesu, A., Guidotti, G., *Atti Soc. It. Patol.*, 1957, v5, 521.

21. Sorof, S., Claus, B., Cohen, P., *Cancer Research*, 1951, v11, 873.

22. Guidotti, G., Clerici, E., *Experientia*, 1958, v14, 341.

23. Clerici, E., Guidotti, G., Bazzano, E., *Tumori*, 1958, v44, 285.

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Effect of Dietary Proline and Hydroxyproline on Tensile Strength of Healing Wounds.* (26117)

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The deleterious effects of ascorbic acid deficiency and protein depletion on collagen synthesis in healing wounds have been well established. Analysis of tissue from the wounds of scorbutic animals reveals abnormally high levels of the amino acid-proline and abnormally low levels of hydroxyproline (2). The reversal of the normal hydroxyproline-proline ratio in scurvy and the finding of low hydroxyproline levels in protein depletion states have suggested that an inability to hydroxylate proline may be an important factor in the retarded synthesis of collagen in both conditions(11). Abnormalities in the ground substance, such as a reduction in the acid mucopolysaccharides, may also be responsible for the failure to develop normal tensile strength which requires, in addition to collagen synthesis, that newly created tropocollagen particles be organized into purposeful patterns. However, if there were not serious fundamental defects in the intracellular synthesis of the collagen molecule, one would expect to find normal proline-hydroxyproline ratios in the wound tissues because the amino acid composition of tropocollagen is essentially the same as that of polymerized or mature collagen(6).

We have postulated that, if there is a defect in synthesis of collagen by fibroblasts in scorbutic or protein depleted animals (presumably at the hydroxylation of proline stage), tensile strength of their wounds might be favorably influenced by supplementing their diets with pure synthetic hydroxyproline. Previous investigations of the metabolism of hydroxyproline by Stetten(3), Green and Lowther(9), and Wolf and Berger(10) strongly suggest that free hydroxyproline probably would not be utilized in production of collagen during healing. In normal animals there is very little incorporation of dietary hydroxyproline in the general protein pool; nearly all of N^{15} labeled hydroxyproline can be recovered in the urine and stools (Stetten(9)). However, there is an extremely low turnover of the amino acids in a normal animal's collagen (Neuberger and Slack(8)), therefore Stetten's experiments do not necessarily mean that an animal which was ac-

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tively attempting to synthesize new collagen under the handicap of protein or scorbutic acid deficiency would not be able to by-pass the hydroxylation of proline and utilize free hydroxyproline for collagen synthesis. Green and Lowther(3) investigated the possibility of incorporating free N^{15} labeled hydroxyproline into collagen which was being formed by an *in vitro* tissue slice from a carrageenin granuloma. Their results showed that practically all of the hydroxyproline was produced by hydroxylation of bound proline and that almost none of the free labeled hydroxyproline was incorporated in the saline extractable or new collagen fraction. They showed, however, that fibroblasts' cell membranes were freely permeable to hydroxyproline, therefore we still wondered if free hydroxyproline could be utilized by fibroblasts in healing wounds.

Because Green and Lowther also showed that the hydroxyproline in newly formed collagen was derived from free labeled proline, we also thought it worthwhile to determine whether administration of proline to protein depleted rats would influence rate of collagen production.

Methods and materials. 1. Two hundred and twenty-four Sprague Dawley female rats weighing between 165 and 190 g were divided into 4 groups of 56 rats each. The first group was fed a standard rat chow. Group 2 was offered 15 g daily of a 5% casein protein depletion diet with full vitamin supplements. The rats in Group 3 received a 5% casein protein depletion diet to which .5% d-l hydroxyproline had been added. This amount of hydroxyproline was selected on the basis of experiments by Mitoma and Udenfriend which showed that approximately one-sixth of this amount of 4-keto-l-proline would double the blood level of free hydroxyproline for 4 hours(7). The rats in Group 4 received a 5% casein protein depletion diet to which 1% d-l synthetic proline had been added. This amount of proline approximately replaced the proline in the protein depleted diet to normal levels. All of the rats were given tap water *ad lib.*

Amount of food consumed each day was calculated by weighing the uneaten residuum. Rats in Groups 3 and 4 were pair fed with the rats in Group 2 on the following day. After a period of 6 days to allow the rats to become accustomed to their diets, they were anesthetized with ether and the hair over the back of the neck and shoulders was clipped. A 2 cm longitudinal incision was made through the skin and subcutaneous fat midway between the shoulders. The wounds were closed immediately with four #40 steel wire sutures.

Eight rats in each group were sacrificed at 2-day intervals between the 6th and 21st days after wounding. A 1 cm segment of the wound was excised and tensile strength of the scar tissue was determined by the method of Williamson(1). Results of the tensile strength determinations were recorded and expressed as number of grams of water which exerted a force on the wound sufficient to cause a sudden complete separation of the wound edges. Such an endpoint was quite definite in most of the wounds tested.

2. One hundred and fifty-eight female guinea pigs weighing between 250 and 300 g were divided into 3 groups of 56 each. Group 1 was fed a normal diet. Group 2 was fed an ascorbic acid deficient diet. Group 3 was fed an ascorbic acid deficient diet and also received a .5% hydroxyproline dietary supplement. The guinea pigs were wounded and tensile strength of the resulting scar tissue was determined as in 1.

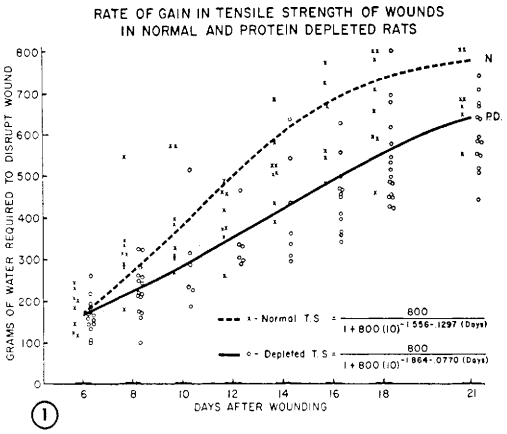
Results. Fig. 1 shows a highly significant 1% level reduction in rate of gain in tensile strength in wounds of depleted animals. The data also reveal that rats fed a 5% casein protein-depletion diet did not show significant reduction in the strength of their wounds during the pre-collagen (0-5 day) period and that ultimately the wounds become as strong as those of normal animals.

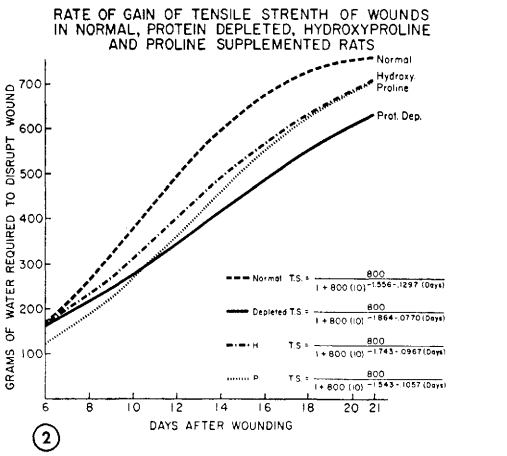
Fig. 2 compares rates of healing of normal, protein depleted, protein depleted-hydroxyproline supplemented, and protein depleted-proline supplemented rats.

Apparently, the rate of gain of tensile strength in the wounds of rats receiving hydroxyproline supplement is not statistically significant. Neither proline nor hydroxypro-

line influenced rate of gain of tensile strength in protein depleted rats.

Fig. 3 compares rates of gain of tensile strength in the wounds of normal guinea pigs, scorbutic guinea pigs, and scorbutic pigs receiving a .5% hydroxyproline dietary supplement. It was difficult to keep scorbutic guinea pigs of this size alive longer than 3 weeks—therefore the number of determinations is smaller than in Fig. 1. Hydroxyproline supplement did not aid healing in the scorbutic guinea pigs.





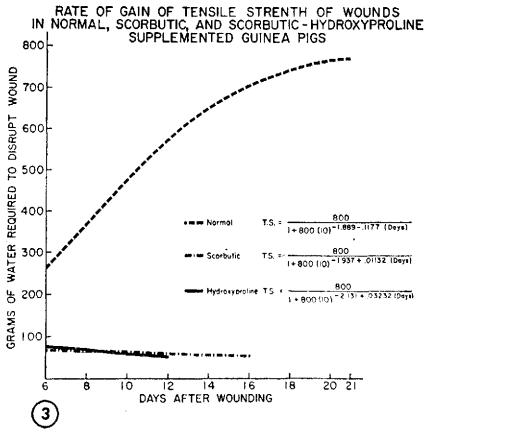


FIG. 1-3.

Discussion. Stetten's dietary experiments with intact rats led her to conclude that, "The hydroxyproline of proteins is not derived to any appreciable extent from dietary hydroxyproline, but rather from the oxidation of proline which is already bound, presumably in peptide linkage(3)." Green and Lowther, working with *in vitro* tissue slices of carrageenin granuloma, reported that free hydroxyproline was not incorporated into new collagen fibers(9). Depletion and ascorbic acid deficiency reveal that the results of Stetten and Green also apply to the wounded animal, and that impaired wound healing in pathological states cannot be overcome by administration of dietary hydroxyproline. It is interesting to note, however, that abnormally high levels of serum hydroxyproline did not cause a reduction in tensile strength of healing wounds; apparently there was no interference with formation of intermolecular hydrogen bonds between the hydroxyl group of the hydroxyproline and the keto group of the peptide link within the collagen fiber. Competitive inhibition might have been anticipated at this point as suggested by Gross and Kirk(4).

Summary. Protein depleted rats were given dietary supplements of .5% synthetic d-l hydroxyproline and 1% synthetic d-l proline. Guinea pigs on an ascorbic acid deficient diet were given a dietary supplement of .5% d-l hydroxyproline. The animals were wounded by a standard technic and the tensile strength of their healing wounds was tested at 48-hour intervals between the 6th and 21st postoperative days. Neither hydroxyproline nor proline exerted a significant effect upon the rate of gain of tensile strength in the wounds of protein deficient rats or scorbutic guinea pigs.

1. Charney, J., Williamson, M. B., Bernhard, F. W., *Science*, 1947, v105, 396.

2. Gould, B. S., Goldman, H., Stolman, J. M., *Ann. N. Y. Acad. Sci.*, 1960, v85, 385.

3. Green, N. M., Lowther, D. A., *Biochem. J.*, 1959, v71, 55.

4. Gross, J., Kirk, D., *J. Biol. Chem.*, 1958, v233, 355.

5. Gustavson, K. H., *The chemistry and reactivity of collagen*, New York, Academic Press, 1956, 33.

6. Jackson, D. S., Leach, A. A., Jacobs, S., *Biochim. Biophys. Acta*, 1958, v27, 418.

7. Mitoma, C., Smith, T. E., DaCosta, F. M., Udenfriend, S., Patchett, A., Witkop, B., *Science*, 1959, v129, 95.

8. Neuberger, A., Slack, H. G. B., *Biochem. J.*, 1953, v53, 47.

9. Stetten, M. R., *J. Biol. Chem.*, 1949, v181, 31.

10. Wolf, G., Berger, C. A., *ibid.*, 1958, v230, 240.

11. Udupa, K. N., Woessner, J. F., Dunphy, J. E., *Surg. Gynec. and Obst.*, 1956, v102, 639.

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Inhibitory Effect of Bicarbonate on ATP Levels and Cholesterol Biosynthesis in Liver Homogenates.* (26118)

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Rat liver homogenate preincubated with protamine loses the capacity to convert mevalonic acid (MVA) to non-saponifiable material (NSF, largely cholesterol)(1). This loss of synthetic capacity is associated with the absence of ATP in these homogenates. ATP is present and conversion of MVA to NSF occurs if the homogenates are supplemented prior to incubation with RNA, DNA or polyethylene sulfonate. Thus it would appear that there is a requirement for a relatively non-specific polyanion that is essential for maintenance of ATP levels in liver homogenates. During studies on the specificity of protamine as an inhibitor of respiration or of oxidative phosphorylation it was observed in a number of experiments that protamine that had been trypsinized in sodium bicarbonate solution was no longer deleterious but the sodium bicarbonate solution in which protamine had been trypsinized was quite inhibitory. More extended study has now shown that bicarbonate is a potent inhibitor of the aerobic system concerned with maintenance of ATP levels in homogenates and that trypsinized protamine, amino acids of the urea cycle, or canavanine, for reason to be discussed later, reverse, at least over a narrow range, this inhibition. In this connection the studies of Miller and Evans who showed that bicarbonate is an inhibitor of cytochrome oxidase are pertinent(2).

Methods and materials. The biosynthetic experiments involved preincubation of 5 ml aliquots of a 200 xg supernatant fraction of rat liver homogenate prepared as previously described(3,4,5) with 6 ml amounts of solution containing 1 mg ATP, 10 mg DPN, 100 mg sodium succinate, various amounts of sodium bicarbonate, and other supplements to be studied. Each flask was aerated with a stream of oxygen and preincubated with agitation for 30 min at 37°. Following preincubation each flask was opened and 1 ml MVA-2-C^{14} solution added, the contents aerated and the flasks then reincubated for an additional 3 hr. After final incubation, homogenates were saponified, extracted with petroleum ether, the ether extracts dried with sodium sulfate, filtered, evaporated, taken up in scintillation mixture, and counted. In experiments involving a determination of ATP content of homogenates at time of MVA addition following preincubation the contents of paired flasks were treated with an equal volume of cold 4% perchloric acid. The precipitate that formed was removed by centrifugation and the supernatant solutions were freed of excess perchloric acid by careful neutralization with cold 10% potassium hydroxide.

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