# A Modified Method for Determination of Hydroxyproline.* (20386)

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During the course of investigations on the effects of thermal exposure upon rat skin and tail-tendon(1), we had occasion to utilize the method of Neuman and Logan for the determination of hydroxyproline(2) as a measure of the collagen content(3). In the application of this method considerable difficulty was experienced in obtaining reproducible, linear standard curves. Frequently, the standard curves would display the form of an exponential function, making the selection of suitable aliquots of an unknown troublesome. Consequently, the following study was undertaken in an effort to establish conditions which would consistently result in a linear proportionality between light absorption and known concentrations of hydroxyproline.

*Experimental.* The colorimetric method of Neuman and Logan(2) for the determination of hydroxyproline consists of the following steps: a) oxidation of the hydroxyproline with peroxide in the presence of alkali and copper sulfate; b) removal of the excess peroxide by heating at 80°C for 5 minutes; and c) color development with p-dimethylaminobenzaldehyde† in acid solution. If the excess peroxide is not removed, the color intensity is considerably decreased. It was felt that the erratic results obtained in this laboratory might be related to the step involving removal of the excess peroxide. However, variations in temperature between 70° and 80°C, and in the heating periods from two to ten minutes failed to yield a more consistent response. If the heating step was replaced by the addition of small amounts of ferrous sulfate to remove peroxide, a linear standard curve was obtained in the range of zero to 25 μg of hydroxyproline. In our procedure 0.1 ml of M/20 ferrous sulfate, containing 0.5 ml concentrated sulfuric acid per 100 ml, is added to each tube and shaken for 6 minutes. The concentration of the added ferrous sulfate solution could be varied from M/100 to M/10 with no change in color intensity. A qualitative test for peroxide, dependent on complex formation with dilute dichromate in acid solution, showed that the ferrous sulfate was efficacious in removing the excess peroxide after oxidation of the hydroxyproline.

In Fig. 1, the relationship between optical density at 550 mμ (determined in the Coleman Universal Spectrophotometer) and concentration of hydroxyproline is shown as determined by our modified procedure.

Prior to applying our modified method to the determination of hydroxyproline in collagenous tissue, we have found it expedient to make several changes in manipulative meth-

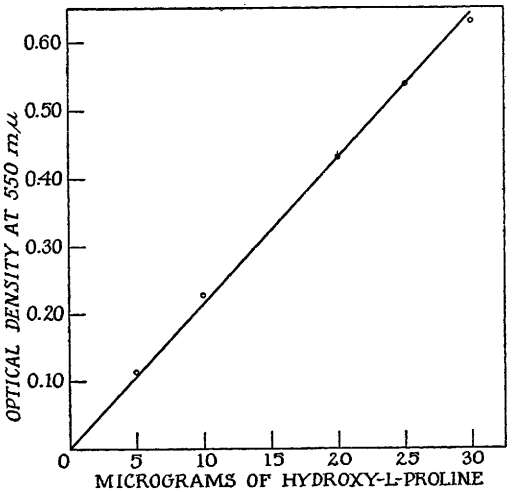


FIG. 1. Relation between color intensity and concentration of hydroxy-L-proline.

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† We have found that several commercial samples of p-dimethylamino-benzaldehyde have proved refractory to purification by the method of Adams and Coleman(4) even when repeated 3 times and followed by crystallization from warm aqueous ethanol. Recrystallization from other solvents was also ineffectual. It was subsequently found that a product from the Matheson Co. could be used without purification.

odology. These included: a) the use of 2 ml aliquots of standard hydroxyproline solutions or of unknown rather than one ml aliquots; b) the addition of the copper sulfate and sodium hydroxide solutions in a single step (reagents mixed immediately before use) rather than separately; c) the addition of the color reagent jointly with the sulfuric acid; and d) the increase in the normality of the sulfuric acid from 3N to 4N to compensate for the increase in volume occasioned by doubling the volume of the test solution employed. An investigation of the reagent concentrations used in Neuman's and Logan's method indicated that they were present in optimal concentration for maximal color yield and, with the exception of changing the concentration of p-dimethylaminobenzaldehyde from 5 to 4%, the original reagent concentrations were not altered.

*Results.* When our modified method was compared with the method of Neuman and Logan, it was found that both methods gave the same results for the hydroxyproline content of gelatin, rat skin and rat tail tendon. Experiments in which known quantities of hydroxyproline were added to defatted rat skin and tail-tendon before acid hydrolysis yielded recoveries of 98 to 105% by both procedures. The modified procedure, however, was the more advantageous to employ since standard solutions of hydroxyproline consistently gave reproducible color intensities. This permitted the selection of aliquots from an acid-hydrolyzed tissue (in which the hydroxyproline content was approximately known) which produced color intensities lying in the more sensitive portion of the optical density scale. This was of considerable aid in the routine assay of collagenous tissue.

It should be pointed out that we have found the method not to be applicable for the determination of hydroxyproline in the presence of high concentrations of tyrosine. Neuman and Logan(3) make a correction for the tyrosine content by using its per cent color contribution (1.5% as much as hydroxyproline) after an independent determination for tyrosine. We have confirmed this value utilizing their method and have found that by our modified method, tyrosine contributes only 0.5% as much color as does hydroxyproline. However, when known mixtures of hydroxyproline and tyrosine were analyzed by either procedure, it was found that quantitative recovery of the hydroxyproline was not obtained. It was subsequently found that the light absorption of a tyrosine-hydroxyproline solution at 550 m$\mu$ was less than the summation of their individual light absorptions. Thus, a tyrosine correction will tend to give too low a value for the hydroxyproline content. In view of these considerations, it becomes important to remove high tyrosine-containing proteins from those tissues containing small amounts of collagen before hydroxyproline determinations are applied. Neuman and Logan suggest the use of a preliminary urea extraction to accomplish this purpose.

*Summary.* The method of Neuman and Logan for the determination of hydroxyproline has been modified by the application of ferrous sulfate to remove peroxide. This modification has yielded a consistent, linear standard curve and markedly reduced the color contribution of tyrosine.

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