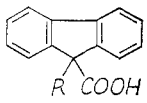
poses, as their melting points may not always be reproducible (*1*).



The compounds listed in the tables were prepared in the course of other work (*1*, *3*), a few being described in the literature. All new compounds have given satisfactory analytical figures for carbon and hydrogen. Their description and a discussion of the theoretical aspects of the anion (I) will be published elsewhere (*2*, *3*).

This method of characterizing alkyl halides should prove of value because fluorene-9-carboxylic acid is readily prepared from either benzilic acid or fluorene, and is easily esterified by methanolic hydrogen chloride; the alkylations are rapid, give good yields, and result in a large increase of molecular weight. As little as 50 mg. of the ester may be successfully alkylated. The alkylations succeed with chlorides as well as with bromides and iodides. Tables I, II, and III illustrate the wide variation possible in the alkyl radical. Hydrolysis of the alkylation product, which need not be isolated, to the 9-alkylfluorene-9-carboxylic acid and measurement of the equivalent weight enable the size of the alkyl group to be estimated.

ACKNOWLEDGMENT

The author is indebted to K. Clarke for his interest and criticism of the manuscript. The award to the author of an I. C. I. Fellowship by The University, Hull, is acknowledged.

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RECEIVED for review August 28, 1959. Accepted January 4, 1960.

A Modified Method for Hydroxyproline Determination

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▶ **The chromogen produced by the reaction of the oxidation product of hydroxyproline with *p*-dimethylaminobenzaldehyde has been stabilized by increasing the concentration of *n*-propyl alcohol in the reaction mixture to 45.5 volume %. To eliminate nonspecific interference, differential spectrophotometry at 500 and 560 mμ has been employed, and a suitable correction formula devised. The improved reaction and measurement conditions permit precise and accurate microdetermination of hydroxyproline in complex materials, such as tissue hydrolyzates.**

CURRENT analytical methods for hydroxyproline determination are, for the most part, based on Lang's work (*5*), the method of Neuman and Logan (*8*, *9*) being most widely used. Several modifications of this method have been developed (*2*, *4*, *6*, *7*). The general lack of precision and accuracy in these methods is attributable to instability of the specific chromogenic compound under the conditions at present used, impurity of *p*-dimethylaminobenzaldehyde (*p*-DAB), incomplete removal of the oxidizing agent (hydrogen peroxide), and loss of reaction specificity in the presence of various tissue components. In the method described, these sources of errors were eliminated.

EXPERIMENTAL

Reagents. L-Hydroxyproline (Nutritional Biochemicals Corp., Cleveland, Ohio) was purified by precipitation from methanol–ethyl alcohol (*1*).

p-Dimethylaminobenzaldehyde (*p*-DAB) (Eastman Kodak Co., Rochester, N. Y.) was dissolved in ethyl alcohol (100 grams of crude material per 200 ml. of ethyl alcohol) by heating at 70° C. Charcoal was added with continued heating and stirring for 5 minutes and the solution was filtered by suction. More water than was needed to precipitate the *p*-DAB was added to the solution; then the precipitate was washed on a Büchner funnel with water and dried in vacuo. The procedure was repeated until a white product, giving a colorless solution with propyl alcohol, was obtained. A 5% solution in *n*-propyl alcohol was prepared.

Hydrogen peroxide (Superoxol Merck) was standardized by titration with potassium permanganate. A 4% solution was prepared.

Recommended Procedure. Place 1.0 ml. of a solution containing 1 to 10 γ of hydroxyproline in a suction tube (Fisher Scientific Co., Catalog No. 14-941).

Add 0.2 ml. of 0.05*M* $CuSO_4 \cdot 5H_2O$, 0.5 ml. of 2.5*N* NaOH, and 0.5 ml. of 4.0% H_2O_2. After shaking, let it stand for 5 minutes at room temperature.

Place in a 70° C. water bath for 10 minutes. After the first 5 minutes, apply vacuum and agitate the tube for about 30 seconds to complete removal of the H_2O_2.

After cooling in ice, add 0.8 ml. of 8.0*N* H_2SO_4 and 2.5 ml. of 5.0% *p*-DAB in propyl alcohol. Shake vigorously.

Place in a 70° C. water bath for 40 minutes.

Cool and read the absorbance measured against a blank determination in a photometer at 560 mμ using a 1-cm. cell.

A standard curve was prepared employing this procedure with five replicate determinations of each point (Table I). In routine practice, duplicate determinations of the standard need be made at only two points to define the curve.

Effect of Solvent. The oxidation product of hydroxyproline and the red chromogen obtained by the condensation of this oxidation product with *p*-DAB are both decomposed during heating in acid (*10*, *12*). The decomposition of red chromogen results in a decrease of absorbance at 560 mμ (Figure 1). In aqueous systems the reaction proceeds rapidly, but the color yield is low and the decomposition of the red compound is rather rapid. Both increased concentration and lengthened alkyl chain of the alcohol (lower di-

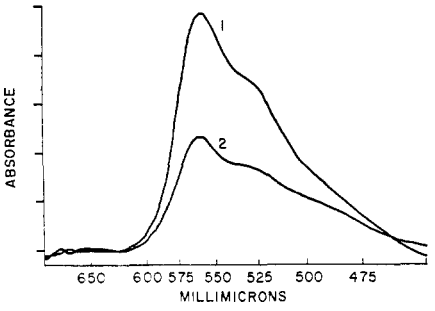

Figure 1. Decomposition of red chromogen

Absorption curves of chromogen produced by reaction of oxidation product of hydroxyproline with p-DAB under conditions of Neuman and Logan (8)

1. Heating for 15 min. at 70° C.
2. Heating for 45 min. at 70° C.

Absorption curves were prepared using Spectracord recording spectrophotometer

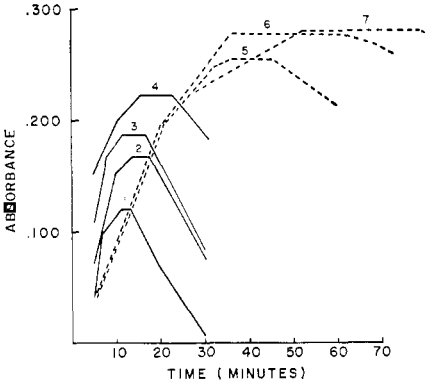

Figure 2. Effect of solvents on color stability and intensity

1. Completely aqueous system, p-DAB dissolved in sulfuric acid
2. Methanol, 20 volume %
3. Ethyl alcohol, 20 volume %
4. n-Propyl alcohol, 20 volume %
5. n-Propyl alcohol, 36 volume %
6. n-Propyl alcohol, 45.5 volume %
7. n-Propyl alcohol, 54.5 volume %

electric constant) used result in enhanced intensity and stability of the color produced. Optimal results are obtained with 45.5 volume % of *n*-propyl alcohol in the reaction mixture. Further increase in the concentration of the organic solvent or the use of higher alcohols may result in the separation of the organic and aqueous phases, particularly if the sample contains salts even in low concentration, and the color development is prolonged with very small increase in color intensity (Figure 2).

Effect of *p*-DAB. Because *p*-DAB decomposes on standing, careful purification has been carried out. Purity, rather than concentration, is the primary consideration. Nearly full color production is reached with addition of *p*-DAB at a concentration of 2% weight/volume (Figure 3), but the proposed 5% solution (*8*) is optimal for biological materials.

Effect of H_2O_2. While addition of 1% H_2O_2 gives near maximal color in a pure hydroxyproline solution (Figure 3), 4% H_2O_2 provides optimal results with tissue hydrolyzate, as impurities present therein catalyze a more rapid decomposition of the oxidizing agent. The effect of the residual H_2O_2 on color development was considered by several authors to be the main source of error (*4*, *6*). Heating of the reaction mixture was found inadequate for the removal of residual peroxide. Addition of $FeSO_4$ (*6*), on the other hand, may result in increased decomposition of benzaldehyde to perbenzoic acid. Removal of residual peroxide by suction in vacuo gave reproducible results. Traces of peroxide could not be detected by dichromate after vacuum treatment. Effects of varying the time of heating at 70° C. and varying the normality of the H_2SO_4 added are also shown in Figure 3.

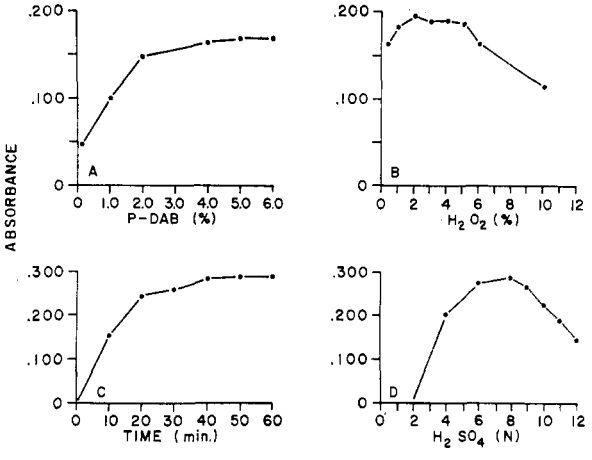

Figure 3. Effect of varying reagent concentrations and heating time

A. Variation of p-DAB concentration
B. Variation of H_2O_2 concentration
C. Variation of heating time
D. Variation of H_2SO_4 normality

APPLICATION OF METHOD TO BIOLOGICAL MATERIAL

Specificity of Reaction. Tissue hydrolyzate components often interfere with the specificity of the reaction (*2*, *9*). In the determination of the hydroxyproline content of rat liver protein, for example, of which hydroxyproline comprises only about 0.1%, the absorption spectrum (Figure 4) indicates the presence of nonspecific chromogenic material. This interfering absorbance constitutes about 20 to 25% (23.7 ± 3.0%, calculated from 10 individual cases) of the total absorbance at 560 mμ. The material responsible for this interference is extractable by various organic solvents (chloroform, ether, benzene), while the specific chromogen remains in the aqueous phase.

Correction for this interference may be made by differential spectrophotometric measurement at 560 and 500 mμ or by extractive removal of nonspecific chromogenic material with chloroform, followed by measurement of absorbance of the aqueous phase at 560 mμ. While both procedures give essentially similar results, the instability of the red chromogen in the aqueous

Table I. Accuracy and Reproducibility of Method at Various Levels of Hydroxyproline

Hydroxyproline, γ		Std.	Absorb-
Added	Found[a]	Dev., %	ance[b]
1.00	1.00 ± 0.052	5.20	0.028
4.00	4.00 ± 0.024	0.70	0.112
5.00	5.00 ± 0.025	0.50	0.140
10.00	10.00 ± 0.036	0.37	0.280

[a] ± std. dev.
[b] From curve. Readings taken in Beckman DU spectrophotometer using 1-cm. cell.

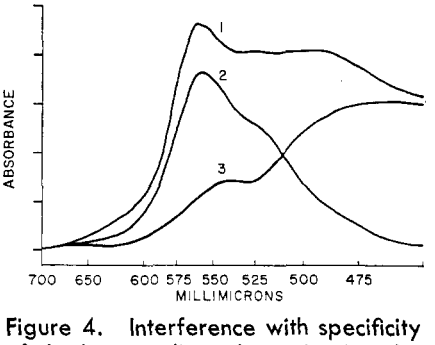

Figure 4. Interference with specificity of hydroxyproline determination by tissue hydrolyzate components

1. Absorption spectrum of chromogenic material after hydroxyproline determination in hydrolyzed rat liver protein
2. Absorption spectrum of hydroxyproline specific chromogen remaining in aqueous phase after extraction of hydrolyzed rat liver protein with ether
3. Absorption spectrum of interfering material present in organic phase after extraction of hydrolyzed rat liver protein with ether

phase makes use of the former preferable.

Preparation of Material for Analysis. Tissue for analysis is conveniently purified by the method of Schneider (*11*), excluding the hot trichloroacetic acid extraction which would remove all hydroxyproline-containing compounds (*3*). Hydrolysis may be carried out with 3.0*N* HCl in the autoclave (18 pounds, 8 hours). To eliminate the interference of high salt concentration (3.0*N* or above) with color development, the largest portion of the HCl should be removed. This is usually done in vacuo in the presence of NaOH and P_2O_5. The resultant sirup may then be redissolved, neutralized, and diluted to a convenient volume with water.

Analysis and Calculation. The absorbance, A, of the color is read at two wave lengths, 560 and 500 mμ. The corrected absorbance, X, is obtained by the application of the following equation:

$$X = A^{560}_{unk.} - \left\{[A^{500}_{unk.} - (A^{560}_{unk.} \times R)] \times \frac{1}{Q - R}\right\} \quad (1)$$

where

$$R = \frac{A^{500} \text{ of standard hydroxyproline}}{A^{560} \text{ of standard hydroxyproline}} \quad (2)$$

$$Q = \frac{A^{560} \text{ of standard hydroxyproline}}{A^{500} \text{ of standard hydroxyproline}} \quad (3)$$

Recovery experiments yielded satisfactory results, the standard deviation from the mean being 2.8% for five samplings.

Standard Ratio, *R*. Equation 1 is strictly applicable if the value of R is constant throughout the calibration curve. This is true only under ideal conditions. The R value was determined for each point of the calibration curve, and the mean value and standard deviation were 0.4000 ± 0.0074. Because the R value was used twice in Equation 1 and in contrasted manner, small differences in R do not affect the final result significantly. $R \pm 2\sigma$ gives a difference of only 0.7%. A significant variation in the R value may result from decomposition of *p*-DAB, and can easily be eliminated by recrystallization of this compound.

ACKNOWLEDGMENT

The authors express their appreciation to Hans Popper for his advice and encouragement and criticism of this study.

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RECEIVED for review December 29, 1958. Accepted January 21, 1960. Work supported by the Research and Development Division, Office of the Surgeon General, Department of the Army, under Contract No. DA-49-007-MD-290, and by Grant No. C-3342 (C1), Division of Research Grants, United States Public Health Service, National Institutes of Health.

Determination of Microgram Quantities of Sulfate in Organic Linkages

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▶ Chloric acid may be used for the digestion procedure in the turbidimetric determination of micro quantities of organic sulfate. Numerous samples may be run simultaneously with a minimum of manipulation. No quantitative transfer is required for the final development of turbidity.

THE necessity in this laboratory to estimate the sulfate content of mucopolysaccharide led to the development of a wet digestion procedure combined with the turbidimetric sulfate determination described by Toennies and Bakay (*1*). This method is suitable for the simultaneous manipulation of over 20 samples containing from 10 to 300 γ of sulfur in the +6 oxidation state.

Table I. Precision of Sulfate Determination on Digested Standard Ammonium Sulfate Samples

Sample	Sulfur, γ Standard	Sulfur, γ Recovered
1	100	101
2	100	103
3	100	103
4	100	103
5	100	104
6	100	98
7	100	98
Av.		101
Std. dev.		±2.3
8	200	199
9	200	192
10	200	199
Av.		197
Std. dev.		±4.1

SPECIAL REAGENTS

Ethyl Alcohol–Dipropylene Glycol Solution (ED Solution) (*1*). Mix 450 ml. of absolute alcohol and 550 ml. of dipropylene glycol (reagent grade or practical grade redistilled).

Barium Chloride Reagent. Prepare 100 ml. of 1.34*M* barium chloride in deionized water. After 3 hours (or longer) of standing, filter through a fine sintered-glass filter. This solution may be used indefinitely. Dilute 6 ml. of the 1.34*M* solution to 120 ml. with deionized water and add 80 ml. of ED mixture. After 2 to 3 hours of aging, the reagent is ready and may be used for the next 40 hours (*1*).

Chloric Acid. Prepare a 25% chloric acid solution from potassium chlorate and perchloric acid, as described (*2*).

Standard Sulfate Solution. Dissolve exactly 0.412 gram of anhydrous ammonium sulfate in deionized water