A SPECTROPHOTOMETRIC METHOD FOR DETERMINATION OF CYSTEINE AND RELATED COMPOUNDS

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The voluminous literature on the methods of cysteine determination has been reviewed by several authors (1–3). Most of the proposed methods will not differentiate between cysteine and cysteine-containing peptides. The Sullivan method with its numerous modifications (4, 5), though widely used, has not yet, however, evolved to an entirely satisfactory procedure (3). The absorption of cysteine in the ultraviolet region of the spectrum lacks a characteristic peak and is of a relatively low intensity; therefore, its exploitation for analytical purposes (6, 7) seems to be of rather limited value. The sensitive spectrophotometric method, based on the increase in absorbency in the 250 nm region accompanying mercaptide formation with p-chloromercuribenzoate, cannot be specifically applied for cysteine estimation, as this reaction is shared by thiol compounds in general (8).

In the course of a study of the inhibitory effect of fluoropyruvic acid on the respiration of microorganisms¹ and mitochondrial preparations,² it was noted that thiol compounds are able to prevent the inhibitory action of fluoropyruvate. Further investigation revealed that, as a result of interaction between fluoropyruvate and thiol compounds, the —SH groups disappear and equivalent amounts of hydrofluoric acid are liberated. The absorption spectra of the reaction products show well defined peaks in the ultraviolet region. The utilization of this phenomenon for the determination of cysteine and related compounds forms the subject of the present communication.

**Materials and Methods**

The thiol compounds used were purchased from commercial sources, and their —SH content was checked by iodometric (2) or ferricyanide titrations (9). N-Acetylcysteine was a gift of Dr. H. J. Strecker (New York State Psychiatric Institute). Fluoropyruvic acid was synthesized according to the method of Blank et al. (10). Methyl bromopyruvate was a gift of Professor E. D. Bergmann of The Hebrew University, Jerusalem. Free

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bromopyruvic acid was obtained by shaking overnight at room temperature an acidified emulsion of the methyl ester in water. Inorganic fluorine was determined by titration with thorium nitrate with sodium alizarinesulfonate as indicator (11). The absorption spectra were studied in the Beckman spectrophotometer, model DU, with the use of silica cells of 1 cm. light path.

![Graph illustrating UV spectra](http://www.jbc.org/)

**Fig. 1.** A, the ultraviolet spectra of the reaction products of fluoropyruvic acid with cysteine, ○; mercaptoethylamine, ●; homocysteine, □. Values for homocysteine may be somewhat low, as the concentration of the solution has not been checked titrimetrically. B, N-Acetylcysteine, △; glutathione, ■; thiomalic acid, ▲; thioglycolic acid, ■. Experimental conditions as described in text under “Standard procedure.”

**Results**

Spectra—The ultraviolet absorption spectra of the reaction products of various mercaptans with fluoropyruvate are shown in Fig. 1, A and B. As can be seen, the peaks appear in the 265 to 275 m\(\mu\) region (type I spectrum) if the thiol compound participating in the reaction possesses no unsubstituted amino group in the \(\alpha\) or \(\beta\) positions relative to the \(-\text{SH}\) group (Fig. 1, B). On the other hand, cysteine and other related compounds give rise to the formation of derivatives characterized by a peak at 300 m\(\mu\) (type II spectrum, see Fig. 1, A). The molecular extinction coefficient is much higher for type II compounds (\(\epsilon_{390} = 5.2 \times 10^6\) cm.\(^2\) mole\(^{-1}\) for cysteine) than for the type I derivatives (\(\epsilon_{370} = 5.4 \times 10^5\) cm.\(^2\) mole\(^{-1}\) for glutathione). In a few experiments in which bromopyruvate was used instead of fluoropyruvate in the reaction with thiol compounds, essentially identical spectra were obtained.
Stoichiometry—When the concentration of the fluoropyruvic acid was kept constant and that of cysteine was gradually increased, the light extinction at 300 µm increased in a nearly exponential fashion, approaching a maximal value with a 2.5-fold excess of cysteine. The concentration of —SH groups as measured by the quantitative nitroprusside reaction (12) or iodometrically (2) decreased in a parallel manner. Concomitantly with this reaction, stoichiometric amounts of hydrofluoric acid were liberated (Fig. 2). Neutralization of the resulting acid was necessary in order to drive the reaction to completion.

The fluoropyruvate excess needed to bind all the thiol compound present was found to be generally larger with mercaptans lacking an unsubstituted amino group than with those related to cysteine.
Effect of pH—The velocity of the reaction between fluoropyruvic acid and mercaptans was found to be affected by variation of the pH. A more detailed study of the pH effect was made in the case of cysteine and glutathione. By using a large excess of fluoropyruvic acid the course of the reaction was found to be of first order. By plotting the velocity constants of this pseudomonomolecular reaction against the pH, a sigmoid curve with a pK of approximately 7.30 was obtained for both cysteine and glutathione. Maximal velocities were reached at pH 8 (Fig. 3).

The reaction product between cysteine and fluoropyruvic acid showed no spectral changes in the range of pH 5 to 10.4. With increasing acidity, however, the peak of the type I spectrum (cysteine-fluoropyruvic acid) at 300 mμ was gradually displaced to the 270 mμ wave-length. This conversion of the type I spectrum into type II was complete in 1 M HCl solutions (Fig. 4). The transformation was reversible. With the glutathione-fluoropyruvic acid compound no shift in the peak (265 mμ) was observed under similar conditions, but its molecular extinction increased considerably in strongly acid solution.

Stability—The stability of the cysteine-fluoropyruvic acid compound was measured at different pH values at 28° by following the decrease of absorbency at 300 mμ. As can be seen from Fig. 5, the stability decreased with rising alkalinity. At pH 8.5 there was a 50 per cent decrease in absorbency after 2.5 hours of incubation.
FIG. 3. Effect of pH on the velocity of the reaction between fluoropyruvic acid and thiol compounds (cysteine, O; GSH, •). The experimental conditions were as described under the section on standard procedure, but the pH of the buffer was varied. 0.5 M phosphate buffer was used in the pH range between 5.8 and 7.0 and 0.5 M Tris buffer in the range between pH 7.0 and 9.0.

FIG. 4. Ultraviolet spectrum of the cysteine-fluoropyruvic acid compound (●, ■, △) and of the GSH-fluoropyruvic acid compound (O, □, △) at various pH values. ●, O, in 1 M HCl; ■, □, in 0.1 M HCl; △, △, in Tris buffer, pH 8. The concentration of the reactants was 10 times higher than that indicated under the standard procedure. The reaction products obtained in 0.1 M NaHCO₃ were diluted ten times with the respective acid or buffer solutions.
Interference—A comparison of type I and type II spectra shows that mercaptans without the unsubstituted amino group, if present in moderate excess, will interfere but little with the determination of cysteine. If greater accuracy is required and the nature of the interfering thiol compound is known, the corrected value for cysteine can be found by recording the extinction at two different wave-lengths (e.g. at 300 and 270 m\(\mu\)) and calculating the concentration of the two compounds from two equations.

Amino acids other than homologues of cysteine (homocysteine) do not interfere. Thus cysteine added to a 1 per cent solution of casein hydrolysate could be quantitatively recovered (Fig. 6).

High concentration of salts (1 m NaCl or 1 m Na\(_2\)SO\(_4\)) which might be present in neutralized protein hydrolysates had no effect on the determination. Reducing agents, however, such as KCN and Na\(_2\)S\(_2\)O\(_4\) had to be excluded.

The cysteine-fluoropyruvic acid compound exhibited the same extinction values in phosphate, tris(hydroxymethyl)aminomethane (Tris), or NaHCO\(_3\)-Na\(_2\)CO\(_3\) buffers of equal pH.

If the light extinction of the test solution at 300 m\(\mu\) is too high to make the cysteine determination impracticable, the cysteine-fluoropyruvic acid compound can be easily extracted with organic solvents (ether, ethyl acetate) after acidification of the aqueous solution with HCl to about pH 1.
Standard Procedure for Determination of Cysteine

Reagents—

Fluoropyruvic acid. An aqueous solution of 0.02 M fluoropyruvic acid was used. When kept in the frozen state, it was stable for at least 3 months. At neutral pH at room temperature, it deteriorated slowly.

Standard cysteine solution. A stock solution of 0.1 M cysteine in 0.1 M HCl was stored in the deep freeze and was renewed weekly. The stock solution was diluted with 0.1 M HCl to the required strength.

Buffer. 0.5 M Tris buffer, pH 8, was used.

Procedure—To obtain the standard curve 0.2 ml. of 0.02 M fluoropyruvic acid, varying amounts (0.1 ml. to 1.0 ml.) of 0.001 M cysteine, and water to 2.5 ml. were pipetted into a Beckman silica cell (Cell 1). Two control cells were used. Cell 2 contained 0.2 ml. of the fluoropyruvic acid solution and water to complete the volume to 2.5 ml., and Cell 3 contained 2.5 ml. of water. As the light extinction of cysteine at 300 μm, in the concentration range used, is negligible, the reading of Cell 1 against Cell 2 at this wavelength should be close to zero. The reaction was started by adding 0.5 ml. of the Tris buffer to each of the three cells.

When the increase in light absorbency at 300 μm in Cell 1 was measured against the control cell containing fluoropyruvate (Cell 2) in 30 second intervals, maximal extinction was found to be reached in about 2 minutes.
Therefore, in routine determinations the complete reaction mixture was allowed to stand for 5 minutes before measurements were made.

The procedure used for cysteine determination in solutions of unknown strength was analogous to that described for obtaining the standard curve.

Under the standard conditions of the assay a linear relationship was obtained between the concentration of cysteine and the light extinction at 300 m\(\mu\). The values plotted in Fig. 6 represent averages from four experiments. Deviations between parallel experiments were within the limit of 2 per cent.

**Determination of Glutathione**—Reduced glutathione (GSH) can be determined by measuring the extinction of the compound formed with fluoropyruvate at its peak (265 m\(\mu\)) by the same procedure as described in the previous section for the determination of cysteine. In this case, too, a linear relationship between GSH concentration and light extinction was found to exist within a wide range of concentrations. As, however, the molecular extinction of the GSH compound (\(\lambda_{265} = 5.6 \times 10^5 \text{ cm}^2 \text{ mole}^{-1}\))
is nearly 10 times lower than that of the analogous cysteine derivative, the sensitivity of the method can be considerably increased by converting the GSH to cysteinylglycine prior to the reaction with fluoropyruvic acid. This can be accomplished in a quantitative manner by heating the GSH solution with 1 M phosphoric acid (5). Since the amino group of the cysteine in the cysteinylglycine moiety is free, its reaction product with fluoropyruvic acid shows a type I spectrum similar to that obtained with cysteine. The cysteinylglycine-fluoropyruvate compound was found to be much less stable than the analogous compound with cysteine; the spectrophotometric reading, therefore, should not be delayed for more than 5 minutes after the start of the reaction. A typical determination of GSH by this method is exemplified by Fig. 7. From Fig. 7 it can also be seen that, in a mixture of cysteine and hydrolyzed GSH, the extinction due to the reaction with fluoropyruvic acid, as measured experimentally, is equal to the calculated sum of the respective extinctions of the two components.

**DISCUSSION**

The high molecular extinction of the reaction products between thiol compounds possessing an unsubstituted amino group in the α or β position and fluoropyruvic acid allows accurate determination of cysteine and related compounds at concentrations as low as 0.02 μmole per ml. This sensitivity is matched only by Boyer's p-chloromercuribenzoate method (8). The wave-length (300 μm) used for measurement is well out of the region of the absorption maxima of proteins, purines, and pyrimidines.

As the concentration of the thiol compound is estimated from the increase in absorbency at 300 μm as a result of its interaction with fluoropyruvic acid, the light extinction of the test solution will usually not interfere with the measurement.

The method described does not permit a distinction between cysteine, homocysteine, and mercaptoethylamine; it may be useful, however, in differentiating between cysteine and glutathione.

The chemical reactions leading to the formation of compounds of type I and type II spectra (λmax 295 to 300 μm and 265 to 275 μm, respectively) have not yet been satisfactorily elucidated. The available data (disappearance of the sulfhydryl group and liberation of an equivalent amount of HF) suggest that the primary reaction consists in an alkylation of the thiol compound by fluoropyruvic (or bromopyruvic) acid according to the following formula: \[ \text{RCH}_2\text{SH} + \text{FCH}_2\text{OCOOH} \rightarrow \text{RCH}_2\text{SCH}_2\text{OCOOH} + \text{HF}. \] The mechanism underlying the auxochromic effect (bathochromic shift and increase in molecular extinction), correlated with the presence of a free amino group in the thiol compound, is not clear. Further work on the isolation and characterization of the reaction products is needed in order to clarify these points.
DETERMINATION OF CYSTEINE

SUMMARY

1. During the interaction between thiol compounds and fluoropyruvic acid a compound is formed exhibiting a characteristic spectrum in the ultraviolet zone with a peak in the wave-length region between 265 and 275 μμ.

2. With thiol compounds possessing an unsubstituted amino group in the ω or β position in relation to the thiol group, the peak is shifted to the 300 μμ wave-length and the molecular extinction increases nearly ten times.

3. A spectrophotometric method based on the above phenomenon, suitable for determination of mercaptoethylamine, cysteine, homocysteine, and related compounds, is described.

4. Reduced glutathione can also be determined by the same method after its hydrolysis to cysteinylglycine.

BIBLIOGRAPHY

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