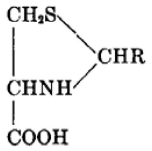
THE EFFECT OF PYRUVIC ACID ON THE ESTIMATION OF CYSTINE AND CYSTEINE

By M. X. SULLIVAN and W. C. HESS

(*From the Chemo-Medical Research Institute, Georgetown University, Washington*)

(Received for publication, August 24, 1937)

In a study of the reaction of aldehydes and ketones with thiol acids Schubert (1, 2) found that cysteine reacts with various aldehydes to form condensation products with the loss of water. The probable structure of the complex formed he gives as



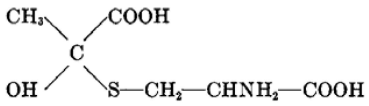
Independently, Ratner and Clarke (3) found that formaldehyde reacts with cysteine to form thiazolidinecarboxylic acid and that formaldehyde and aminoethyl mercaptan give thiazolidine, and described the compounds in detail.

Since aldehydes such as formaldehyde, glyoxal, and methylglyoxal and keto acids such as pyruvic acid are possible metabolic products of proteins, carbohydrates, and fats, and as such might occur more or less in biological solutions, blood and urine, considerable study has been made in this laboratory on their effect on the determination of cystine and cysteine.

In previous work (4, 5) it was shown that in cystine or cysteine determination the various aldehydes have little effect in low molar ratios and dilute solutions. With increasing concentration of cysteine and aldehyde and decreasing acidity, on the other hand, new compounds no longer reacting like cysteine are formed. These compounds, derivatives of thiazolidinecarboxylic acid, described by Schubert and by Ratner and Clarke, do not yield

cysteine readily either by the action of zinc and hydrochloric acid or by dilute acid hydrolysis and are negative in the various reactions for cysteine.

In his early work Schubert (2) considers that cysteine and pyruvic acid, on the other hand, make a simple addition compound with a probable structure of



but in more recent work (personal communication) he questions his early conclusions and considers that the complex between cysteine and pyruvic acid may also be a thiazolidine derivative. Without prejudice as to the nature of the pyruvic acid-cysteine complex, in the present paper we deal with the effect of pyruvic acid on cystine and cysteine determinations by various methods and also with the possibility of liberating cysteine from the complex by simple means.

EXPERIMENTAL

Pyruvic Acid and Cystine—Solutions of cystine and pyruvic acid in 0.1 N HCl were made so that 5 cc. contained 1 mg. of cystine and increasing amounts of pyruvic acid: (A) 0.3 mg., (B) 3.0 mg., and (C) 5.0 mg. of pyruvic acid. Within 30 minutes of mixing, these solutions were analyzed for cystine by the Sullivan (6), the Okuda (7), and the Folin-Marenzi (8) procedures. The cystine was estimated practically quantitatively by all three methods, even in Solution C, with a molar ratio of pyruvic acid to cystine of approximately 14:1. In fact, with 24 hours contact, 100 per cent of the cystine was recovered colorimetrically in Solution C. Likewise, with relatively concentrated solutions of cystine (1 mg. in 1 cc. of 0.1 N HCl and proportions of pyruvic acid as given above) there was little if any effect on the determination of cystine even in 24 hours standing of the mixture. Thus with 14 moles of pyruvic acid to 1 of cystine, the cystine findings were 97 per cent of the theoretical.

Experiments were also carried out at pH 6.8, the pH of the distilled water, and in phosphate buffer, pH 7.4, the solutions

being so made that each 5 cc. contained 1 mg. of cystine and 0.73 mg. of pyruvic acid (2 moles). After 18 hours standing of the reactants, with the controls respectively in distilled water and buffer at pH 7.4, colorimetric estimation on 5 cc. samples indicated 92 per cent of the theoretical cystine at pH 6.8 and 89 per cent at pH 7.4.

At pH 6.8 and 7.4 the solubility of cystine in the presence of pyruvic acid is questionable, so the results were interpreted to indicate little if any effect of pyruvic acid on the estimation of cystine; in short, no combination such as occurs between cysteine and pyruvic acid, presently detailed.

Pyruvic Acid and Cysteine, Dilute Solution—Mixtures were made of cysteine hydrochloride and pyruvic acid so that each 5 cc. contained 1.0 mg. of cysteine and 0.73 mg. of pyruvic acid, that is mole for mole, with the solutions adjusted to pH 1, 2, and 3 respectively. Analyzed by the Sullivan cysteine (9) procedure, at intervals up to 24 hours, the cysteine was recovered quantitatively within the limits of error, at all three pH ranges. The minimum finding of cysteine even after 24 hours standing of the pyruvic acid and cysteine at pH 1, 2, and 3 respectively was 95 per cent of the theoretical. The Folin-Marenzi method run as for cystine showed marked falling off at the end of 6 hours at pH 2.0 (84 per cent return) and at pH 3.0 at the end of 6 hours (80 per cent return).

Pyruvic Acid and Cysteine, Concentrated Solutions—Mixtures were made of cysteine hydrochloride and pyruvic acid so that 2.5 cc. of solution contained 20 mg. of cysteine weighed as the hydrochloride and 15 mg. of pyruvic acid, adjusted respectively to pH 1, 2, and 3. Analyzed by the Sullivan method at the end of 6 hours, 70 per cent, 63 per cent, and 59 per cent of the cysteine were recovered at pH 1, 2, and 3 respectively and at 24 hours the results were practically the same. Less reactive cysteine was found by the Folin-Marenzi procedure without sulfite than by the Sullivan procedure. At the end of 2 hours standing the respective percentage recoveries of cysteine at pH 1, 2, and 3 were, Sullivan, 84, 80, and 76; Folin-Marenzi, 75, 60, and 53. The Okuda method without reduction at the end of 24 hours showed 60, 50, and 50 per cent recovery at pH 1, 2, and 3 respectively. However, when the Okuda cystine procedure, reduction with zinc and hydro-

chloric acid, was employed, 100 per cent return of the cysteine was obtained even at the end of 24 hours contact of the reactants at each pH. The Okuda values indicated several possibilities: either the phenomenon was due to greater oxidation of cysteine in the presence of pyruvic acid or the pyruvic acid-cysteine complex was split by the action of zinc and hydrochloric acid or by the acid alone. In order to determine just which of these possibilities was correct the cysteine-pyruvic acid complex was prepared according to the method of Schubert (2).

Cysteine-Pyruvic Acid Complex—The crystalline compound melted at 149–151° uncorrected and gave a negative nitroprusside reaction with dilute ammonia, while the addition of aqueous sodium cyanide slowly produced a faint color, as did the addition of dilute sodium hydroxide. For quantitative work 17.2 mg.[1] of the compound were dissolved in 50 cc. of 0.1 N HCl, making a solution equivalent to 200 parts per million of cysteine. The Sullivan cysteine reaction on this solution was very faintly positive. If the cystine reaction was used (2.0 cc. of 5 per cent NaCN in N NaOH, 10 minutes contact) and the solution compared with a 200 parts per million cysteine solution similarly treated, a color was obtained indicating a liberation of 44 per cent of the cysteine. This experiment was repeated but with an increase of the contact time of the sodium cyanide from 10 to 30 minutes. Complete splitting of the complex resulted and a colorimetric estimation of 100 per cent of the cysteine. Substituting 2.0 cc. of N NaOH for the sodium cyanide and giving 30 minutes contact gave a return of 77 per cent of the cysteine. Heating 5 cc. of the pyruvic acid-cysteine complex (equivalent to 1 mg. of cysteine) at 60° for 10 minutes with 2 cc. of 5 per cent NaCN in N NaOH completely liberated the cysteine and gave 100 per cent matching of 5 cc of cysteine (1 mg.) similarly heated, both being cooled to room temperature before the colorimetric determination. Heating with 2 cc. of 4 N NaOH similarly gave 100 per cent return of the cysteine by the Sullivan cysteine procedure when matched against a cysteine standard similarly treated with both standard and pyruvic acid complex run without any sodium cyanide.

[1] Later work showed the presence of 8.15 per cent potassium chloride, so the actual amount of ash-free material weighed out was 15.8 mg. See "Addendum."

The Okuda method without reduction gave a negative reaction with the cysteine-pyruvic acid product. Application of the Okuda method after reduction with zinc and HCl or simply boiling for 15 minutes with 2 per cent HCl gave 100 per cent return of the cysteine.

The complex can thus be split either by relatively long contact with NaCN and NaOH or by heating with weak acid or relatively strong alkali. By use of 5 per cent NaCN in alkali, cystine, if present, would be estimated and such estimation for the present purpose was undesirable. Accordingly, a more satisfactory procedure for splitting the complex into cysteine and pyruvic acid was used; namely, boiling the material with dilute hydrochloric acid, 2 per cent, and estimating the cysteine by the Sullivan cysteine procedure without cyanide. The procedure used is as follows: 25.9 mg. of cysteine-pyruvic acid complex were dissolved in 75 cc. of distilled water. To 10 cc. was added 0.5 cc. of concentrated HCl and the mixture boiled 10 minutes on the hot-plate, cooled, and neutralized by adding 5 N NaOH dropwise with stirring to pH 3.5 and made to 10 cc. with water. 5 cc. were used for colorimetric work as follows: To 5 cc. add 1 cc. of a 1 per cent aqueous solution of 1,2-naphthoquinone-4-sodium sulfonate, shake for 10 seconds, and add 5 cc. of 10 per cent Na_2SO_3 in 0.5 N NaOH, mix, and wait 30 minutes. Then add 1 cc. of 2 per cent $Na_2S_2O_4$ in 0.5 N NaOH. Match against a cysteine standard (1 mg. in 5 cc. of 0.1 N HCl) similarly treated. By this procedure cysteine only is estimated. A cystine solution containing 1 mg. in 5 cc. of 0.1 N HCl was negative in this procedure. The recovery of cysteine was 95.4 per cent of the theoretical.

Complete Combining of Pyruvic Acid and Cysteine—As previously mentioned, cysteine and pyruvic acid, mixed mole for mole and kept for 24 hours at pH 1, 2, and 3, did not become negative in the Sullivan reaction for cysteine. Accordingly the effect of higher pH and higher amounts of pyruvic acid was tried. Thus 20 mg. of cysteine and 15 mg. of pyruvic acid were dissolved in 2.5 cc. of phosphate buffer at pH 6. After 3 hours contact at room temperature, 28–30°, and suitable dilution with 0.1 N HCl for colorimetric work, 59 per cent of the cysteine was found by the Sullivan cysteine method and the same finding obtained after 24 hours contact of the reactants, Solution A. When the pyruvic

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