STUDIES ON THE DETERMINATION OF TOTAL 5-OXOPROLINE IN BLOOD SERUM

by

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ABSTRACT

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This study deals with the development of a solution technique for the quantitative determination of 5-oxoproline and is partly based upon existing qualitative methods. An attempt was made to render this procedure applicable to routine use in the clinical laboratory.

The assay involves a series of steps leading to the ultimate formation and measurement of triiodide ion in aqueous solutions. First, N-chlorination of 5-oxoproline is achieved by the use of sodium hypochlorite. After the excess sodium hypochlorite is reduced by ethanol, acetate buffer is added for pH control. Potassium iodide is added and is immediately oxidized to iodine. The iodine complexes with the excess iodide ion to form triiodide ion. The characteristic yellow solution can then be measured spectrophotometrically at 390-393 nm.

In order to obtain workable standard curves, it was necessary to optimize the conditions of the reactions--namely the concentrations of reagents and the reaction times. Experimentation revealed that a 10 minute chlorination reaction followed by a 45 minute period for the reduction of excess sodium hypochlorite produced the best results.

After obtaining a standard curve, an attempt was made to assay for 5-oxoproline in extracts of serum obtained from normal individuals

and patients suffering from renal failure. The assay values obtained were higher than the reported literature values for 5-oxoproline and no significant difference was evident between the assay values for the normal individuals and the patients.

The sensitivity of the assay procedure and the reliability of the extraction process were tested by assaying solutions of caffeine, urea, and glutamic acid, their "extracts," and their acid hydrolysates. The acid hydrolyzed serum of one normal individual and five anephric patients was also assayed.

Thin layer chromatography experiments supported the results and conclusions of the solution experiments. The presence of 5-oxoproline in serum was indicated by this procedure. In addition, the N-chloro derivative of 5-oxoproline was also detected.

This work demonstrated that the assay procedure could be used as a method for the determination of 5-oxoproline. However, in order to be of practical use, some other extraction technique must be used for the separation of 5-oxoproline from other interfering compounds in the serum.

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ABBREVIATIONS AND DEFINITIONS

ADP	Adenosine Diphosphate
Anephric	A condition in which one or both kidneys are completely dysfunctional due to disease or have been surgically removed.
ATP	Adenosine Triphosphate
5-Oxo-D-[2-^{14}C] proline	The D enantiomer of 5-oxoproline labeled at position 2 with carbon-14.
5-Oxo-DL-[U-^{14}C] proline	The racemic mixture of 5-oxoproline uniformly labeled at all carbon atoms with carbon-14.
5-Oxo-L-[U-^{14}C] proline	The L enantiomer of 5-oxoproline uniformly labeled at all carbon atoms with carbon-14.
Renal Failure	An acute or chronic condition in which kidney functioning is reduced below the level necessary for physiological homeostasis.
R_f	In thin layer chromatography, a numerical value obtained for a sample. The distance which the sample spot has traveled is divided by the distance the solvent front has moved to obtain the R_f.

CHAPTER I

INTRODUCTION

Structure of 5-Oxoproline

Pyroglutamic acid, 5-oxo-2-pyrrolidinecarboxylic acid, 2-pyrrolidone-5-carboxylic acid, glutimic acid, glutiminic acid, ∝-amino glutaric acid lactam, and glutamic acid lactam[1] are synonyms for the cyclic anhydride of glutamic acid which will be consistently referred to as 5-oxoproline in this thesis. Figure 1 shows the conventional structure used to represent 5-oxo-L-proline and gives the correct numbering of the atoms in the molecule as a derivative of the amino acid proline. The carbon atom at position 2, which has the carboxylic acid substituent, is asymmetric and is responsible for the fact that 5-oxoproline can exist in the form of D and L enantiomers. In Figure 1, the wedge representation indicates that the carboxyl group lies above the plane of the paper, while the dotted line indicates that the hydrogen atom lies below the plane of the paper. For the D form, the substituents on the carbon atom at position 2 would be reversed. One can also see from the diagram that 5-oxoproline is a five-membered heterocyclic ring which contains an internal amide bond between the nitrogen atom at position 1 and the carbonyl carbon at position 5. Detailed work on the crystal structure of 5-oxoproline has been reported by Pattabhi and Venkatesan.[2]

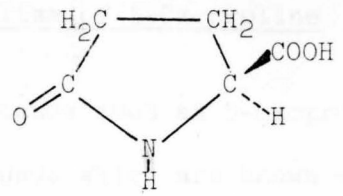


Fig. 1. Structure of 5-Oxo-L-proline

Chemistry of 5-Oxoproline

In the 1890's Menozzi and Appiani[3,4] reported the correct structure of 5-oxoproline and were able to show by the use of specific rotation data, that it existed in enantiomeric forms. Their work confirmed and expanded the experiments of Haitinger,[5] who had first obtained 5-oxoproline by heating glutamic acid at 180-190°C. Later experimenters, notably Wilson and Cannan,[6] have demonstrated that 5-oxoproline and glutamic acid are present in equilibrium when in aqueous solutions. The equilibrium system is dependent upon pH and temperature.

Interest in the study of 5-oxoproline caused other workers to study the possibilities of 5-oxoproline arising from other starting materials and by means of other reactions. It was found that glutamine, glutathione, and various substituted forms of glutamic acid, especially γ-glutamyl derivatives, could form 5-oxoproline--usually when heated in aqueous solutions. These chemical methods for the formation of 5-oxoproline have been reviewed by Orlowski and Meister.[7]

Water, alcohol, and acetone can be used as solvents for 5-oxoproline. The molecular weight of this compound is 129.11. The reported value for the specific rotation of 5-oxo-L-proline at 20°C and using the D line of sodium, $[\alpha]_D^{20}$, is -11.9° when the concentration is 2 g/100 ml water. The melting point of 5-oxo-L-proline is 162-163°C[1] while that of 5-oxo-DL-proline is approximately 182°C.[3]

Metabolism of 5-Oxoproline

The fact that a substance such as 5-oxoproline can be formed in aqueous solutions from compounds which are known metabolites in living systems usually leads to investigations of that particular substance in mammalian organisms. An interesting experiment reported by Sarah Ratner[8] in 1943 proved that D-glutamic acid was metabolized to 5-oxo-D-proline by rats. After feeding DL-glutamic acid containing ^{15}N to rats, Ratner analyzed their urine and found that 73% of the ingested D-glutamic acid had been excreted as 5-oxo-D-proline. Care had been taken to prevent non-enzymic formation of 5-oxoproline in the experiment, therefore some enzyme must have been responsible for the conversion. The L-glutamic acid did not form 5-oxo-L-proline; the only evidence of metabolized L-glutamic acid in the urine existed as ^{15}N labelled urea and ammonia. A group of Indian investigators expanded upon Ratner's work. When Ramakrishna, Krishnaswamy, and Rao[9] injected rats intraperitoneally with 5-oxo-D-[2-^{14}C]proline they found that it was excreted unchanged in the urine. However, when 5-oxo-L-[U-^{14}C]proline was injected into the rats in the same manner, it became apparent that the compound was being metabolized. Of the total dose of administered radioactivity, 64% was recoverable. Most of the recoverable radioactivity (87% of the 64%) was present in expired CO_2. The rats were sacrificed after the respiratory air had been analyzed and the remaining recoverable radioactivity was found in the urine and feces as well as incorporated into lipids and proteins of tissues. When fresh kidney and liver slices were incubated with 5-oxo-DL-[U-^{14}C]proline some of the radioactivity was found in CO_2, while 2-2.5% of the radioactivity was present in the form of L-glutamic acid. The D isomer was not oxidized.

The importance of the work done by the Indian investigators clearly lies in their demonstration of the apparent enzymic degradation of 5-oxo-L-proline. The work of Ratner[8] had demonstrated the formation of 5-oxo-D-proline from D-glutamic acid but indicated no subsequent degradation of the product. Orlowski and Meister[7] have hypothesized that the enzyme (now recognized as D-glutamic acid cyclotransferase) responsible for the conversion of D-glutamic acid to 5-oxo-D-proline has as its primary purpose the detoxification of D-glutamic acid. Their reasoning is that D-glutamic acid, which is present in mammals due to the activity of intestinal bacteria or from dietary sources, is a very poor substrate for D-amino acid oxidase. Thus the more specific enzyme is utilized in its detoxification, instead of the D-amino acid oxidase.

A significant breakthrough in the study of 5-oxoproline came in 1970 when Jellum, et al.,[10] found a 19-year-old male patient suffering from 5-oxoprolinuria. The 5-oxoprolinuria was attributed to an inborn error in metabolism and may have contributed to the fact that the patient was mentally retarded. When Van Der Werf, Orlowski, and Meister[11,12] announced their discovery of 5-oxoprolinase (pyroglutamic hydrolase) and its ability to convert 5-oxo-L-proline to L-glutamic acid, Eldjarn et al.,[13] again examined their patient. They concluded that their patient's error in metabolism was probably due to a lack of 5-oxoprolinase or its inactivity.

From the pooled data of these investigators, his own co-workers, and a number of other experimenters, Meister[14] proposed the γ-glutamyl cycle. The cycle is a sequence of enzyme-catalyzed reactions which functions in mammalian tissues, primarily in the kidney, as an amino acid transport system.

The functioning of the γ-glutamyl cycle, as illustrated in Figure 2, begins with the activity of γ-glutamyl transpeptidase (1) on an intracellular molecule of glutathione and an extracellular amino acid. Through the activity of this membrane-bound enzyme the γ-glutamyl moiety is cleaved from glutathione and binds to the amino acid, bringing it into the cell. This step has been named the "translocation step." The cysteinylglycine portion of glutathione remains in the cytoplasm where peptidase (6) action splits it into glycine and cysteine. The "release" of the amino acid to the cellular pool and subsequent cyclization of the γ-glutamyl moiety to 5-oxoproline is catalyzed by γ-glutamyl cyclotransferase (2). In the next three "recovery" steps, which are exergonic, energy is supplied by the cleavage of ATP to ADP and inorganic phosphate (P_i). The first recovery step is the catalyzed hydrolysis of 5-oxoproline to glutamic acid by 5-oxoprolinase (3). Next, glutamic acid and cysteine are bound through the action of γ-glutamylcysteine synthetase (4). The cycle is completed when glutathione is reformed by the action of glutathione synthetase (5) on glycine and γ-glutamylcysteine.[14]

Through the functioning of the γ-glutamyl cycle, the importance of the metabolic role of 5-oxoproline is realized. Eldjarn, et al.,[15] however, have speculated that alternate pathways of 5-oxoproline degradation may exist. Figure 3 shows three pathways which they have proposed. The first pathway supposes the acetylation (7) of 5-oxoproline to yield N-acetyl-5-oxoproline and its subsequent hydrolysis (8) to N-acetylglutamate. The second pathway would involve the conversion (9) of 5-oxoproline to 5-hydroxyproline followed by reduction (10) to Δ^1-pyrroline-5-carboxylate. Finally, 2-hyroxy-5-oxoproline formed by the 2-hydroxylation (11) of 5-oxoproline could be expected to undergo further

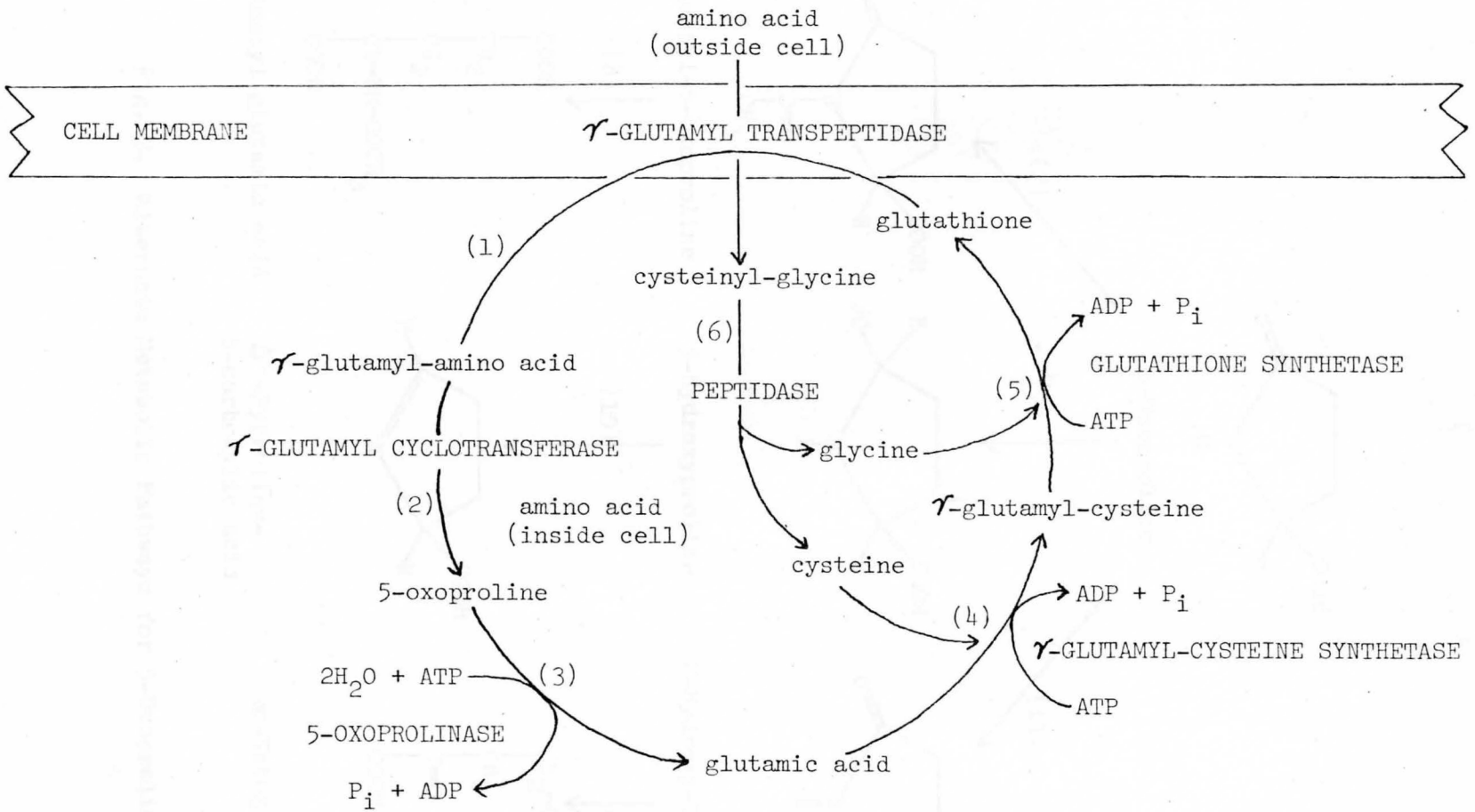


Fig. 2. The γ-Glutamyl Cycle[14]

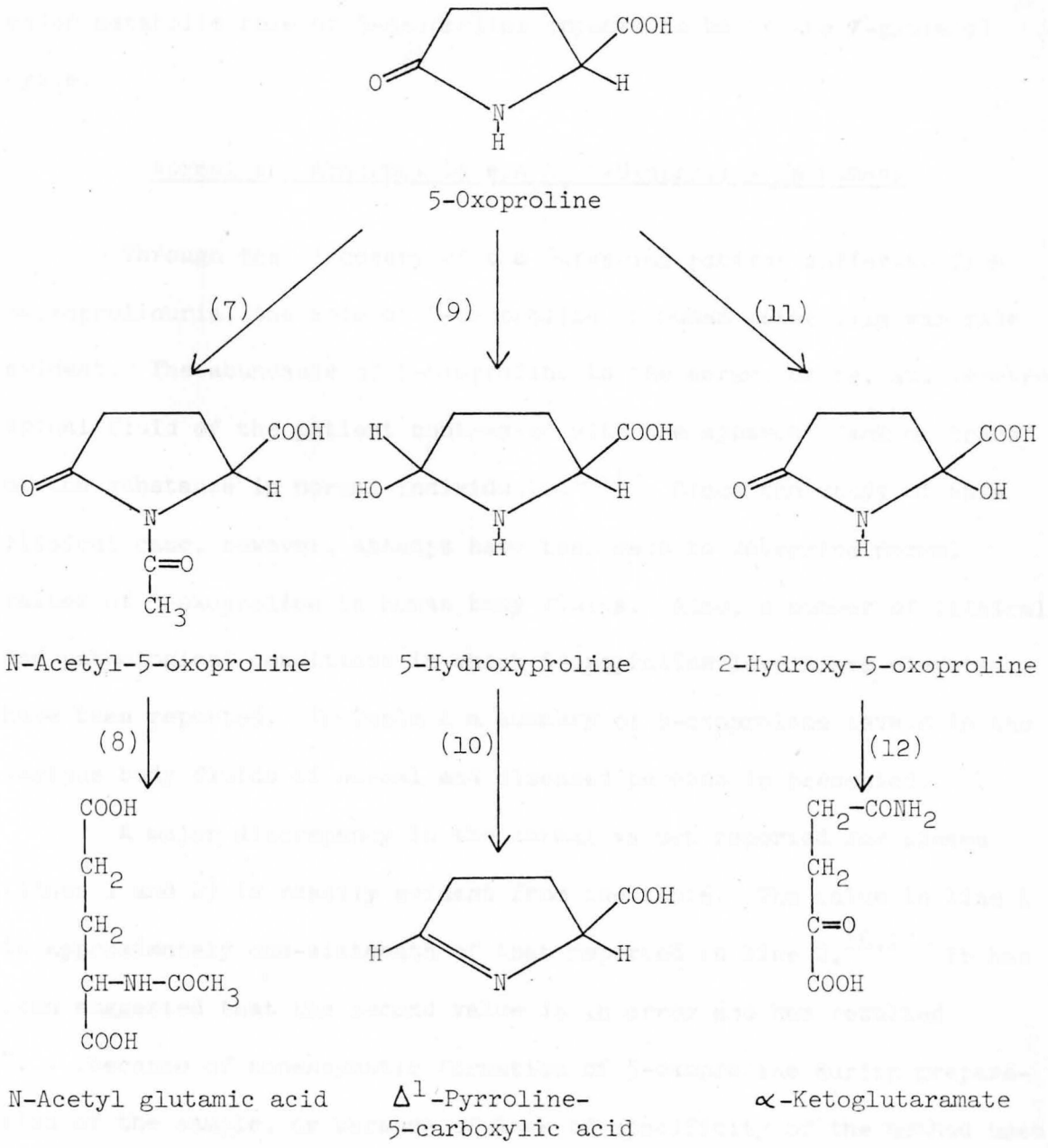


Fig. 3. Alternate Metabolic Pathways for 5-Oxoproline[15]

reaction (12) to yield α-ketoglutaramate. In vitro experiments in which attempts were made to isolate the intermediates and products of the hypothesized pathways were unsuccessful. Therefore, at present, the major metabolic role of 5-oxoproline appears to be in the γ-glutamyl cycle.

Normal and Abnormal Levels of 5-Oxoproline in Humans

Through the discovery of the Norwegian patient suffering from 5-oxoprolinuria, the role of 5-oxoproline in human metabolism was made evident. The abundance of 5-oxoproline in the serum, urine, and cerebrospinal fluid of the patient contrasted with the apparent lack or trace of the substance in normal individuals.[10,13] Since the study of this clinical case, however, attemps have been made to determine normal values of 5-oxoproline in human body fluids. Also, a number of clinical and pathological conditions in which 5-oxoproline levels are elevated have been reported. In Table 1 a summary of 5-oxoproline levels in the various body fluids of normal and diseased persons is presented.

A major discrepancy in the normal values reported for plasma (lines 1 and 2) is readily evident from the table. The value in line 1 is approximately one-sixteenth of that reported in line 2.[16,17] It has been suggested that the second value is in error and has resulted ". . .because of nonenzymatic formation of 5-oxoproline during preparation of the sample, or because of lack of specificity of the method used, or both."[18] The value reported in line 3, although derived from serum,[19] corresponds better with line 1 and supports its accuracy.

Comparison of the normal value in line 1, with the total 5-oxoproline levels of surgically anephric patients and those patients

TABLE 1

5-OXOPROLINE LEVELS FOUND IN HUMAN BODY FLUIDS

No.	Source of 5-Oxoproline	Concentration		
		D Form	L Form	DL Form (Total)
1	Normal Plasma[16]	1.3 ± 0.2 μM		13.9 ± 4.0 μM
2	Normal Plasma[17]			216 ± 44 μM
3	Normal Serum[19]			20 μM
4	Anephric Patients' Plasma[16]	31.0 ± 14.3 μM	41.5 ± 12.2 μM	72.5 ± 16.9 μM
5	Renal Failure Patients' Plasma[16]	32.7 ± 11.7 μM	52.9 ± 21.6 μM	85.6 ± 17.9 μM
6	Patient #1 Serum[13,18]			370-387 mM
7	Patient #2 Plasma[20]			4.5-5.0 mM
8	Normal Cerebrospinal Fluid[19]			60 μM
9	Patient #1 Cerebrospinal Fluid[13]			230 mM
10	Normal Urine (estimated)[21]	variable	500 μmol/day	3.9-39 μmol/day
11	Patient #1 Urine[10]			240 mmol/day
12	Patient #2 Urine[20]			50 mmol/day

suffering from renal failure shows that there is a 5- to 6-fold increase over the normal value occurring in the patients. The elevated level is not due to 5-oxo-L-proline alone as might be expected. While the 5-oxo-L-proline level is three or four times that of the normal value, the 5-oxo-D-proline level in both types of patients exhibits an enormous 25-fold increase over the normal value of 5-oxo-D-proline.[16] It can be concluded from this data, nonetheless, that elevation of 5-oxo-L-proline levels does occur in patients with renal failure or those patients who are anephric.

The 19-year-old patient, mentioned earlier, who suffered from an inborn error in 5-oxoproline metabolism, as well as mental retardation, has increased levels of 5-oxoproline in his serum, as well as in his cerebrospinal fluid[13] and urine.[10] The data for this patient is referred to as patient #1 in the table. Patient #2 refers to a female patient of approximately one year of age. This patient exhibits abnormal values of 5-oxoproline in both plasma and urine, however they are not as elevated as those of the 19-year-old. No signs of mental retardation are present in the younger patient;[20] her condition has been attributed to an inborn error in metabolism, probably in the synthesis of glutathione.[21]

Lin, Shieh, and Tung[23] have suggested that "Chinese restaurant syndrome" might be caused by the ingestion of 5-oxoproline. Nonenzymatic cyclization of monosodium glutamate, a common ingredient in Chinese cuisine, is thought to occur when Chinese food is cooked. The symptoms of the syndrome include nausea, rapid heartbeat, and sensations of tightness and burning. The syndrome apparently affects only certain individuals.[24,25,26]

The implications of the preceding paragraphs indicate that our understanding of human biochemistry would be greatly enhanced by the definition of normal, abnormal, and possibly toxic levels of 5-oxoproline in the body. Additional work in this area at present is limited only by the methods used in the determination of this compound.[18]

Current Methods for 5-Oxoproline Detection

A number of qualitative and quantitative methods for the detection of 5-oxoproline appear in the literature. Orlowski and Meister[7] and Ellfolk and Synge[27] have reviewed these methods quite thoroughly. A brief survey of some of these methods follows.

Palekar, et al.,[16] determined total 5-oxoproline, after acid hydrolysis, as glutamate using a Durrum amino acid analyzer. The action of L-glutamate decarboxylase on the L-glutamate derived from 5-oxo-L-proline allowed for the subsequent determination of the D isomer alone. The samples which were assayed for 5-oxoproline were extracts of plasma that had been obtained from heparinized blood. Picric acid deproteinization of the plasma left a filtrate that was subjected to column chromatography, using cation and anion exchange resins, for the separation of 5-oxoproline.

Wolfersberger and Tabachnik[17] have used similar separation techniques on plasma. However, they have assayed 5-oxoproline as glutamic acid by the use of ninhydrin.

Wilk and Orlowski[19] have assayed for total 5-oxoproline in serum and cerebrospinal fluid using gas chromatography. Extraction procedures were similar to those described by Palekar, et al.,[16] above. 5-Oxoproline was determined as an esterified derivative after reaction with

pentafluoropropanol in pentafluoropropionic anhydride.

By far the most refined method employed to date for the detection of 5-oxoproline has been reported by Jellum, Stokke, and Eldjarn.[28] Total 5-oxoproline in extracts of urine and serum were determined by using gas chromatography, mass spectrometry, and computer analysis.

Ramakrishna and Krishnaswamy[29] have quantitatively determined 5-oxoproline colorimetrically. Hydroxylamine hydrochloride was applied to paper chromatograms containing 5-oxoproline that had been separated from urine extracts. Through this process γ-glutamyl hydroxamate was formed and became detectable by the application of ferric chloride. The ferric chloride-hydroxamic acid complex was measured in a colorimeter at 535 nm after elution from the chromatogram.

Taking advantage of the internal amide bond in 5-oxoproline, Orlowski, et al.,[30] have determined total 5-oxoproline using absorbance measurements at 205 nm. Their kinetic studies of γ-glutamyl cyclotransferase employed this method of 5-oxoproline detection. The only drawback to this method, however, is that the absorbance measurements are not at the absorbance peak, which is at a lower wavelength.

In 1952, Rydon and Smith[31] reported the detection of peptides and amino acids on paper chromatograms by the ultimate formation of a starch-iodine complex. Peptides and amino acids were N-chlorinated in chlorine gas and sprayed with a solution of 1% starch-1% potassium iodide after sufficient aeration. In the reaction that occurred, the chloro-derivative oxidized the iodide to iodine which in turn complexed with the starch to produce a blue-black color. Ellfolk and Synge were the first ones to use the Rydon-Smith method in the detection of 5-oxoproline.[27] Later work on the method itself showed that chlorine

dioxide could also be used as a chlorinating reagent with detection facilitated by the use of o-toluidine or benzidine.[32,33] Mazur, et al.,[34] and Schwartz and Pallansch[35] have described the use of tert-butyl hypochlorite as a suitable chlorinating reagent, and Pan and Dutcher[36] have reported the use of sodium hypochlorite. These three groups of workers have continued to use the starch-potassium iodide solution as the detection reagent employed. The Rydon-Smith method of detection, along with its modifications, has only been used qualitatively however.

Lin, et al.,[23] have determined 5-oxoproline quantitatively by a reaction similar to the one employed by Rydon and Smith.[31] Figure 4 outlines their work. First, 5-oxoproline was brominated (13) using bromine in aqueous acetic acid. After the extraction of the unreacted bromine into chloroform, the N-bromo-5-oxoproline was mixed with a solution of potassium iodide. Hydroiodic acid (HI) formed in solution from the potassium iodide was oxidized to iodine (I_2) and N-bromo-5-oxoproline reduced (14) to 5-oxoproline. A yellow solution resulting from the iodine formed was measured spectrophotometrically at 400 nm. By this method a standard curve was obtained which exhibited linearity in the range of 1 to 8 mM which is not sensitive enough for biological fluid determinations. No determinations were done on biological fluids.

The great amount of variety in the techniques employed to determine 5-oxoproline covers the range from simple chromatography to the refinement of instrumentation and computer analysis. None of these methods, however, seem to have allowed for large scale clinical determinations of 5-oxoproline in biological fluids.

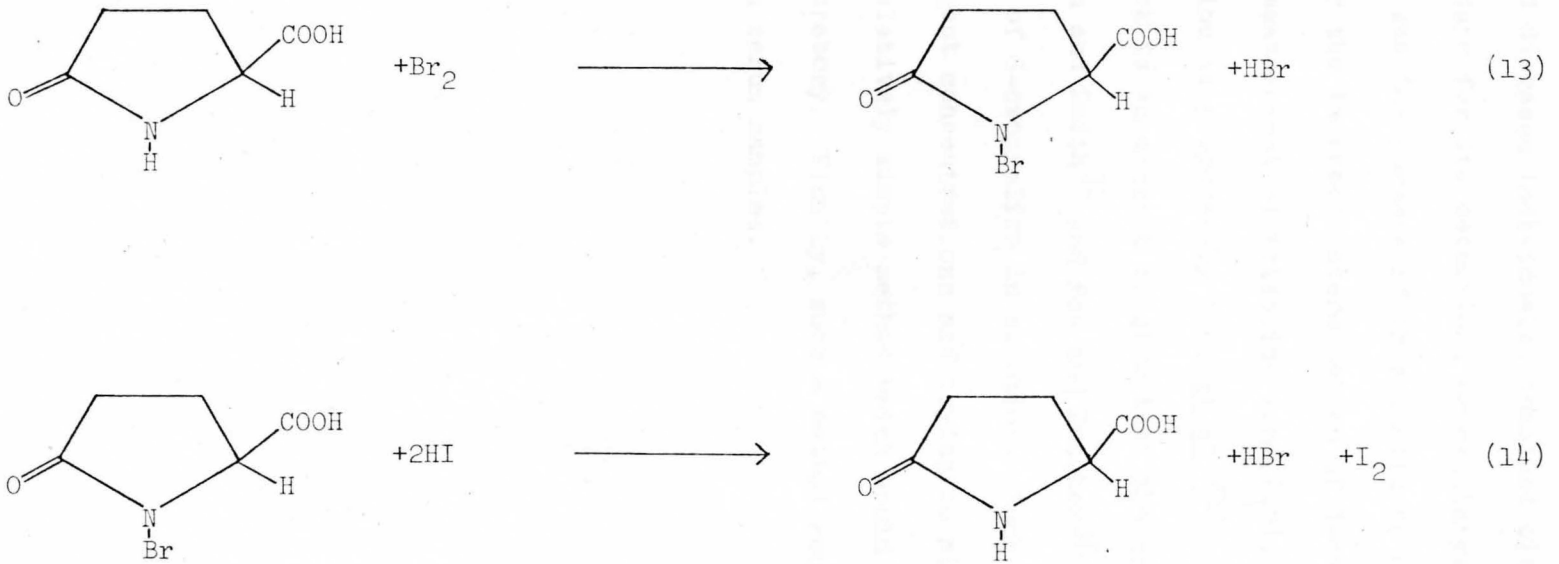


Fig. 4. Reactions in Lin's, et al., Experiment[23]

Statement of the Problem

The proven occurrence of 5-oxoproline in the biological fluids of both normal and diseased individuals, combined with the lack of a simple clinical procedure for its detection, necessitates further research in this area. It was the purpose of this thesis to develop a more sensitive method for the indirect determination of 5-oxoproline based on the formation and measurement of triiodide ion (I_3^-), instead of the measurement of iodine as proposed by Lin, et al.[23]

This work is an attempt to show that the chromatographic techniques of Rydon and Smith[31] and Pan and Dutcher[36] can be applied to determinations of 5-oxoproline in solution. Such an attempt involved maximizing reagent concentrations and trying to minimize time in order to develop a relatively simple method which would lend itself to use in a clinical laboratory. Finally, such a method required testing the method on human serum samples.

CHAPTER II

MATERIALS AND APPARATUS

Materials

A number of special chemicals were obtained from various chemical supply companies. 5-Oxo-L-proline was obtained under the name L-2-pyrrolidone-5-carboxylic acid from Aldrich Chemical Co., Inc, Milwaukee, WI, in 97% pure form. Bio-Rad Laboratories, Richmond, CA, was the source of Dowex 50W-X4 cation resin (H^+ form, 50-100 mesh). Potassium iodide was obtained in "analyzed reagent" grade from the J.T. Baker Chemical Co., Phillipsburg, NJ. Sodium Hypochlorite (4-6%) was obtained from the B&A division of Allied Chemicals, Morristown, NJ. Commercial Solvents Corp., Terre Haute, IN was the source of 95% ethanol. All other chemicals were reagent grade.

Serum from anephric patients was provided by Dr. Robert A. Bacani, of the Youngstown Hospital Association. Other serum was obtained from Mrs. George Bennett, of St. Elizabeth Hospital Medical Center or from student and faculty volunteers of Youngstown State University. All human sera was collected in Vacutainer tubes purchased from Becton, Dickinson, and Co., Rutherford, NJ. Bovine serum containing a bactericidal agent was provided by Mr. William Gennaro of the Youngstown Hospital Association. The water used in all experiments was distilled and deionized.

Apparatus

Thin layer chromatograms were produced on chromatogram sheets coated with silica gel, without fluorescent indicator, from the Eastman Kodak Co., Rochester, NY. The silica gel was coated on poly-(ethylene terephthalate) sheets using polyvinyl alcohol as a binder. The chromatograms were developed using Eastman chromatogram chamber plate sets. The spots were made on the chromatogram sheets using Ziptrol microliter delivery systems from Drummond Scientific Co., Broomall, PA. Microdispenser tubes used with the Ziptrol, were purchased from Helena Laboratories, Beaumont, TX.

The pH measurements were made on a Sargent-Welch model 1P pH meter using a Sargent/Jena combination elecrode (model S-30070-10) from Sargent-Welch Scientific Co., Skokie, IL. Centrifugations were carried out with GLC-1 centrifuge, Sorvall., Newtown, CT.

A Multi-Temp-Block (#2093) was obtained from Lab-Line Instruments, Inc. Melrose Park, IL. Evaporation of acetone extracts under air was done with a Silli-Vap distributed by the Pierce Co., Rockford, IL. The Vortex apparatus was a product of Vortec Corp., Cincinnati, OH.

Absorbance measurements were made on a Cary-14, Varian Assoc., Palo Alto, CA, or a Beckman 26 recording spectrophotometer, Beckman Instruments, Fullerton, CA. The cuvettes used were matched Suprasil 10 mm cells obtained from the Sargent Welch Co.

CHAPTER III

EXPERIMENTAL

Preparation of Solutions

All of the solutions were prepared from reagents described in the previous chapter. The materials were used without further purification or preparation except in the case of the Dowex 50.

Acetate buffer was prepared from equal molar aqueous solutions of potassium acetate and acetic acid. Acid was added to the salt solution with stirring until a pH of 5.0 was obtained. The concentrations of acetate buffer solutions were 200 mM, 800 mM, 1.0 M, and 3.0 M.

The 5-oxoproline solutions used in the experiments were prepared in the following ways. Water or 200 mM acetate buffer was added to the mark of 100 ml volumetric flasks containing 646 mg of 5-oxoproline to obtain 50 mM solutions. These solutions were diluted 1:100 with water or buffer, respectively, to obtain 500 μM solutions.

Two aqueous solutions of 5-oxoproline needed for standard curves were prepared by diluting 1.292 g of 5-oxoproline to 100 ml and further diluting these solutions 1:100. The concentrations of the working standard were 1.0 mM.

Potassium iodide (KI) solutions that were 1.0 M were prepared from 16.6 g of KI in 100 ml volumetric flasks filled to the mark with water or 200 mM acetate buffer. KI solutions that were 100 mM and 10 mM were prepared by diluting the 1.0 M solutions 1:10 and 1:100 with water or buffer, respectively. Similar techniques were used to prepare 50 mM

and 500 mM solutions of KI in buffer. Aqueous solutions of KI that were 125 mM and 250 mM were prepared in liter quantities from 20.8 g and 41.5 g KI, respectively.

Iodine (I_2) solutions were used in preliminary investigations of I_3^- formation and were prepared as follows. To 140.9 mg I_2 in a volumetric flask was added enough water to bring the volume to 100 ml. Next, 1.0 ml of 100 mM KI in buffer and 1.0 ml of 1.0 M KI in water were added to the solution. The KI solutions were added in this manner because the iodine did not dissolve in water alone. The final concentrations of the species in solution were: 5.5 mM I_2, 11 mM KI, and 2.0 mM acetate buffer. Another solution that was 5.1 mM I_2, 11 mM KI, and 200 mM acetate buffer was prepared in a similar way using 129.9 mg I_2, 1.0 ml each of 1.0 M and 100 mM KI in buffer and substituting 200 mM acetate buffer for water. A 1:20 dilution of this solution with buffer was also used; its final I_2 concentration was 260 μM.

The chlorinating reagent in all of the experiments was sodium hypochlorite. It was used either full strength (4-6%) as obtained from the manufacturer or used in dilute solution. The dilutions were prepared in 100 ml volumetric flasks using 2.0 ml of the reagent grade NaOCl and enough water or 200 mM acetate buffer to bring the volume to the mark. A liter of this solution was prepared using 20 ml NaOCl reagent diluted to 1000 ml. The final concentration of NaOCl in these solutions was approximately 15 mM.

Glutamic acid solution with a concentration of 10 mM was prepared from 150 mg of the compound and water in a 100 ml volumetric flask. The use of 1.94 g of caffeine diluted to 100 ml gave a 100 mM solution of caffeine. Both the glutamic acid and caffeine solutions were warmed in a

37°C water bath for approximately one-half hour to facilitate dissolving the substances.

Two solutions of different concentrations of urea were prepared. The first solution was 100 mM and was prepared in a 200 ml volumetric flask using 1.21 g of urea and water as the solvent. The second solution was 10 mM and was the result of a 1:10 dilution of the first solution.

When ethanol (EtOH) was used in the experiments it was 95% pure and was used undiluted. Dowex 50 was equilibrated with 1.0 M HCl and washed with water prior to use. The washings were repeated until the pH of the water was neutral.

Preparation of Samples

The samples used to test the assay method developed in this thesis were extracts of human sera from normal, healthy individuals and patients suffering from renal failure, as well as pooled bovine serum. Blood from volunteers and patients was collected in red top Vacutainer tubes and allowed to clot for approximately one hour followed by centrifugation. The blood of the normal individuals was centrifuged at 3000 rpm for 10 minutes and the clear yellow sera collected in 1.0 ml aliquots. The blood of the patients required centrifugation at 6000 rpm for 10 minutes before clear sera could be obtained. The increased speed was used because of the formation of large fibrin clots. Since all of the patients were undergoing hemodialysis, some therapeutic drug may have been the cause of the fibrin clots.

To the 1.0 ml aliquots of human or bovine sera was added 150 µl of 3.0 M HCl and 6.0 ml of acetone. After centrifugation at 3000 rpm for 10 minutes, protein-free extracts of sera were obtained. These extracts

were evaporated to dryness in a heating block at 60°C under a stream of filtered air. All samples were then reconstituted with 1.0 ml water and mixed to homogeneity using a Vortex apparatus. Lipids were extracted into an equal volume of methylene chloride and the aqueous (upper) layer containing 5-oxoproline was applied to 1.0 ml of Dowex 50. The solution and Dowex 50 were mixed gently and equilibrated for 15 minutes, using the "batch method" for cation exchange. The colorless solutions were then drawn into disposable pipets and transferred to graduated centrifuge tubes. After the volume of the solution was recorded, water was added to bring the final volume of all samples to 1.0 ml, making them ready for assay.

In addition to the serum samples, stock solutions of 5-oxoproproline, urea, caffeine, and glutamic acid were also treated according to the extraction procedure in some cases. When this was done the procedure began with the addition of 150 µl of 3.0 M HCl and proceeded as described.

The addition of the HCl prior to the addition of acetone was the suggestion of Dr. Sylvan Sax.[37] The acid prevented the formation of a milky aqueous layer during the extraction with methylene chloride. Instead a narrow white interface was obtained between the methylene chloride and aqueous layers. The white material may have been small molecular weight polypeptides which had not been precipitated by the treatment.

Preparation of Hydrolysis Samples

Samples of human sera, 5-oxoproline, urea, caffeine, and glutamic acid were hydrolyzed in one set of experiments prior to assay.

These samples were prepared by the extraction methods described in the previous section with a couple of modifications.

After evaporation of the acidified acetone extracts, the samples were reconstituted with 1.0 ml of 3.0 M HCl instead of water and placed in glass-stoppered test tubes in a boiling water bath. After one hour the samples were removed from the bath. Hydrolysis was stopped by the addition of 1.0 ml of 3.0 M sodium hydroxide (NaOH). After cooling approximately 10 minutes the solutions were mixed with 2.0 ml of methylene chloride for extraction of lipids. From the aqueous layers, 1.0 ml aliquots were taken and applied to 1.0 ml of Dowex 50. The rest of the procedure was carried out as described in the previous section.

Thin-Layer Chromatographic Techniques

Thin layer chromatograms were developed to support solution techniques of the triiodide assay procedure. Eastman sheets were spotted with 1 µl samples of solutions at positions 2.0 cm from the edge of the sheet, sandwiched between Eastman glass plates, and developed by ascending chromatography in a solution of 1-butanol: acetic acid: water (100:25:25). When the solvent had migrated 10 cm from the origin of the spots, the chromatograms were removed and dried in air for 20 minutes. Visualization of the compounds was obtained by one of two methods. One method involved spraying the dried chromatogram with a solution of 0.2% ninhydrin in 1-butanol and heating in an 80°C oven for 10 minutes. The other method was the Rydon-Smith[30] method. Chromatograms were placed in a glass chamber and chlorinated in vapor for 10 minutes. The chlorine vapor was obtained by adding concentrated hydrochloric acid to potassium permanganate. After chlorination, the chromatograms were removed from

the chamber, air-dried for 5 minutes, and then sprayed with a solution of 1% starch-1% KI.

After the sample spots became visible their positions were marked and their migration distances recorded. The R_f values were then calculated from this data.

CHAPTER IV

RESULTS AND DISCUSSION

UV Spectra of 5-Oxoproline and N-Chloro-5-oxoproline

Aqueous solutions of 5-oxoproline and N-chloro-5-oxoproline were scanned in the UV spectrum from 250 to 180 nm against water blanks. Scanning speed was 100 nm/min and chart speed was 5 in/min. The concentration of the 5-oxoproline solution was 500 μM. The solution of N-chloro-5-oxoproline was prepared by adding 2.5 ml of 15 mM NaOCl to 2.5 ml of 10 mM 5-oxoproline. After chlorination for 10 minutes, the solution was diluted 1:20 to give a final N-chloro-5-oxoproline concentration of 250 μM.

The spectra of the two solutions are presented in Figures 5 and 6. The 5-oxoproline solution peaked at approximately 196 nm with an absorbance of 1.9. The N-chloro-5-oxoproline peaked at 192-194 nm with an absorbance of 1.65. These absorbance maxima are typical of the peptide bond which is known to absorb below 210 nm.[28] The molar absorptivities calculated as 3800 and 6600 for 5-oxoproline and N-chloro-5-oxoproline, respectively.

The results show that both compounds absorb with a maximum very close to 200 nm, which is usually the lower limit of UV scans without purging with nitrogen gas. The wavelengths and molar absorptivities, therefore, can only be considered as rough approximations. Since many other compounds absorb UV light below 200 nm, only pure solutions of 5-oxoproline or N-chloro-5-oxoproline could be measured by this method.

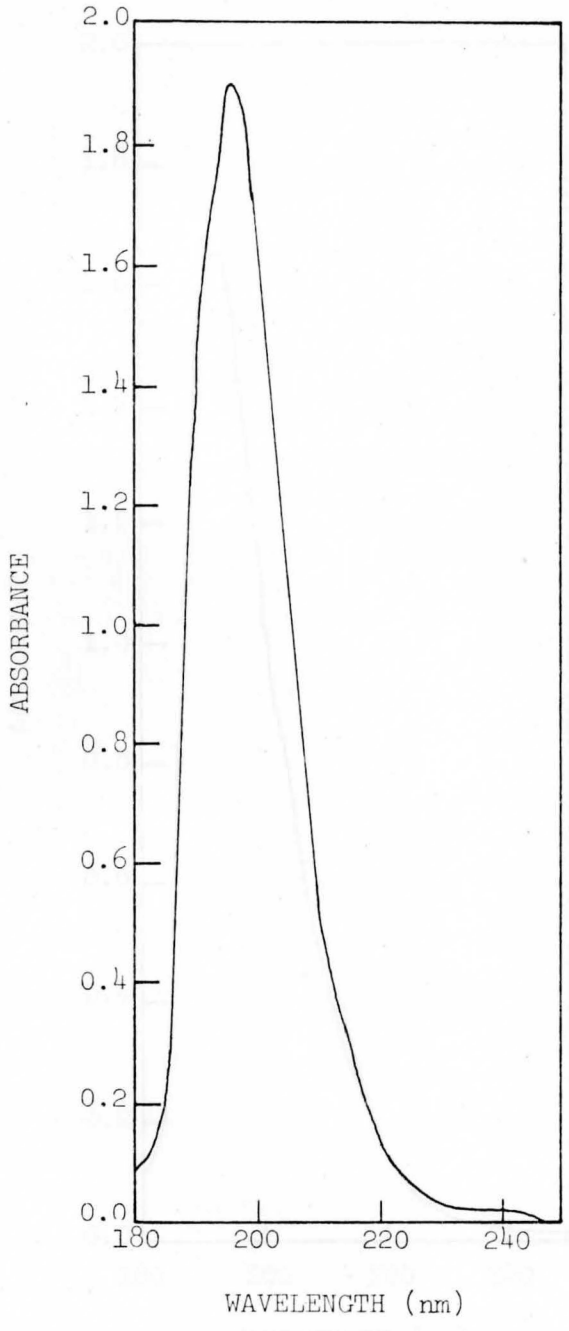


Fig. 5. UV Spectrum of 500 μM 5-Oxoproline

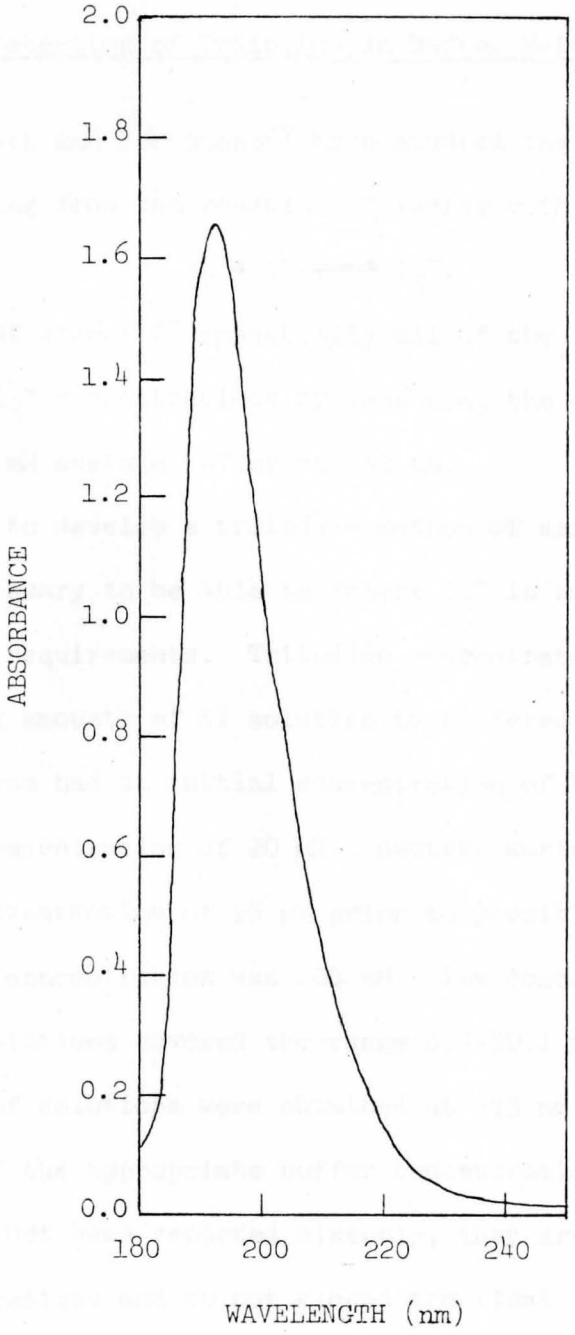


Fig. 6. UV Spectrum of 250 μM N-Chloro-5-oxoproline

This fact alone indicates the necessity of an assay method for 5-oxoproline by some other procedure.

Detection of Triiodide in Buffer Solution

Schildcrout and Fortunato[39] have studied the formation of triiodide as resulting from the reaction of iodine with iodide ion (I^-):

$$I_2 + I^- \rightleftharpoons I_3^- . \qquad (15)$$

In the presence of excess I^- essentially all of the I_2 reacts to form I_3^-. They determined I_3^- concentrations by measuring the absorbance of I_3^- solutions in 200 mM acetate buffer at 353 nm.

In order to develop a triiodide method of assay for 5-oxoproline it was first necessary to be able to detect I_3^- in solution and to ascertain buffer requirements. Triiodide concentrations were varied by adding increasing amounts of KI solution to buffered I_2 solutions. One series of solutions had an initial concentration of 55 μM and final acetate buffer concentration of 20 μM. Another series of solutions had an initial I_2 concentration of 55 μM prior to possible reaction with I^-, while the buffer concentration was 200 mM. The concentration of KI in each series of solutions covered the range 0.2-90.1 mM. Absorbance values for each series of solutions were obtained at 353 nm on the Cary 14 against blanks of the appropriate buffer concentration. While I_3^- concentrations have not been reported directly, they are proportional to final KI concentrations and do not exceed the final iodine concentration in each tube. The pH value of each solution was also measured.

From Table 2 it can be seen that, in general, the absorbance values increased with increasing KI concentration for the solutions of lower buffer concentration. This indicated that the equilibrium of

equation (15) favored I_3^- formation at higher I^- concentrations. However, the absorbance values of solutions with KI concentrations of 3.1 mM and greater, do not steadily increase, but rather exhibit quite a degree of variability. The pH values of these solutions can also be seen to fluctuate as the buffer capacity is exceeded by the increasing KI concentration.

TABLE 2

ABSORBANCE AND pH MEASUREMENTS OF TRIIODIDE SOLUTIONS IN 20 μM ACETATE BUFFER

No.	Final KI Concentration (mM)	A at 353 nm	pH
1	0.2	0.14	6.6
2	0.4	0.23	6.6
3	0.6	0.36	6.2
4	0.8	0.42	6.0
5	1.0	0.44	6.0
6	1.1	0.57	6.0
7	3.1	1.04	5.7
8	5.1	1.00	5.7
9	7.1	1.27	5.9
10	9.1	1.27	6.0
11	10	1.09	5.8
12	30	1.75	5.8
13	50	1.91	---
14	70	1.10	6.1
15	90	1.17	6.1

On the other hand the pH values of the solutions with 200 mM acetate buffer concentration were 5.0 in all cases. The absorbance values exhibited a steady increase and approached linearity, when plotted against KI concentration, as can be seen in Figure 7.

It was evident from the data that the acetate buffer was necessary for obtaining good absorbance values. It was decided to use at least 200 mM acetate buffer concentration in all subsequent experiments.

Dependence of Triiodide Formation on KI Concentration

While the data from the previous experiments indicated increases of I_3^- concentrations in buffer solutions with increasing KI concentration, the data from this experiment indicates that a limit is reached where additional KI only decreases the absorbance values for I_3^-. Solutions having a final I_2 concentration of 26 μM were obtained by adding aliquots of 50 and 500 μM KI solution in 200 mM acetate buffer to 1.0 ml of 260 μM I_2 and bringing the final volume to 10 ml with 200 mM acetate buffer. The final concentrations of KI ranged from 5 mM to 450 mM. Absorbance readings of the solutions were measured against a blank of 200 mM acetate buffer at 353 nm on the Beckman 26.

The data obtained appears in Table 3 along with the final molar ratio $[KI]/[I_2]$. It can be seen that the absorbance values for I_3^- have reached their limit in solutions where the ratio $[KI]/[I_2]$ is between 580 and 1300. Solutions in which the ratio $[KI]/[I_2]$ was higher, due to increased amounts of KI, showed a steady decrease to zero absorbance. The decrease to zero absorbance could be attributed to the formation of a tetraiodide ion (I_4^{2-}). Genser and Connick[40] have expressed the formation of tetraiodide by the following equation:

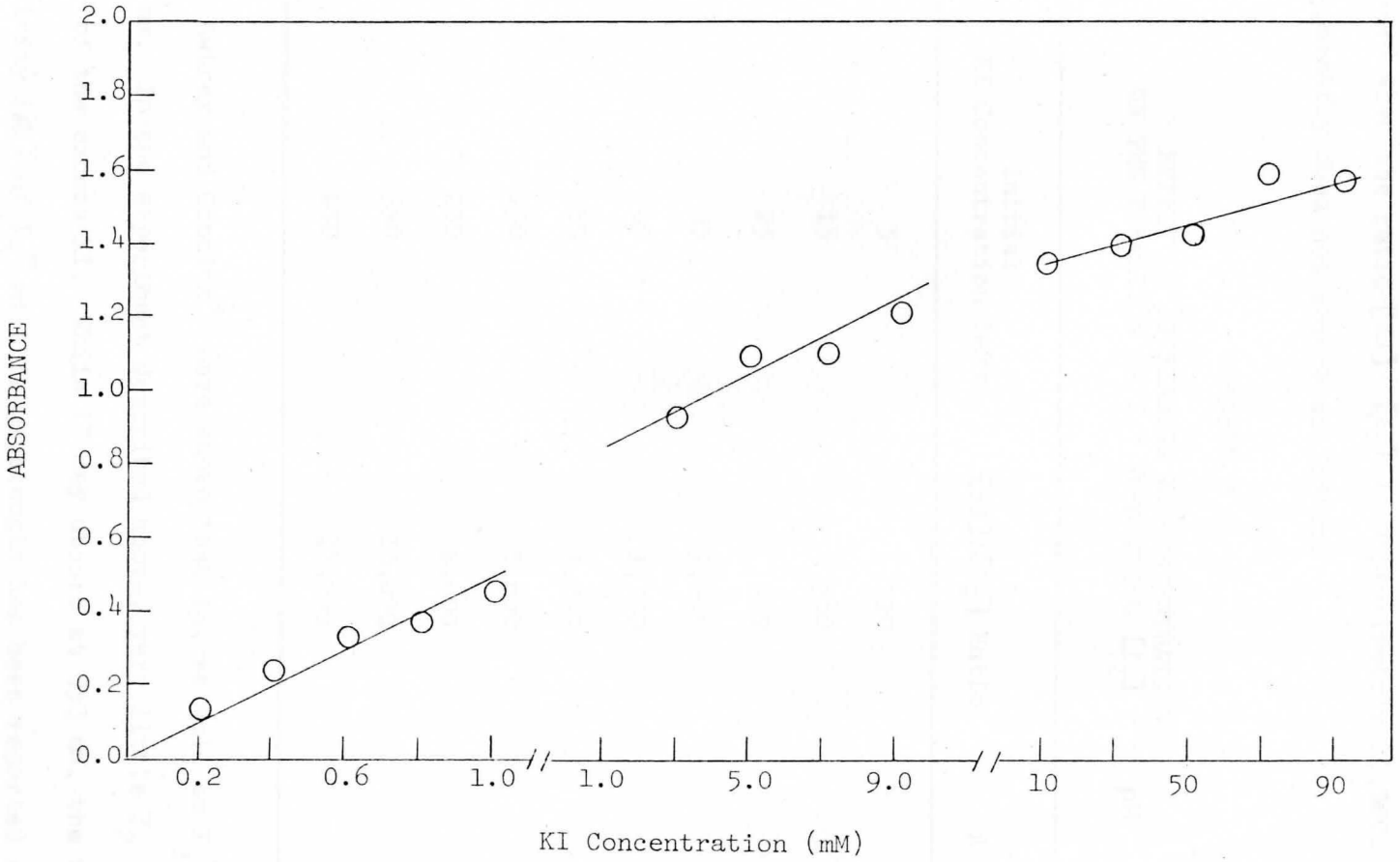


Fig. 7. Plots for Absorbance Values of I_3^- at Different KI Concentrations in 200 mM Acetate Buffer.

$$I_3^- + I^- \rightleftharpoons I_4^{2-}. \qquad (16)$$

When the ratio $[KI]/[I_2]$ is apparently 580 then I_4^{2-} begins to be formed while I_3^- begins to disappear. The absorbance values eventually reach zero when the ratio $[KI]/[I_2]$ is approximately 10,000, because I_4^{2-} apparently does not absorb at 353 nm.

TABLE 3

EFFECT OF INCREASING KI CONCENTRATIONS ON THE FORMATION OF I_3^- WHEN TOTAL $[I_2]$ = 55 μM

No.	Initial KI Concentration (mM)	$[KI]/[I_2]$ Ratio	A at 353 nm
1	5	190	0.390
2	15	580	0.436
3	25	960	0.434
4	35	1,300	0.430
5	45	1,700	0.415
6	50	1,900	0.410
7	150	5,800	0.268
8	250	9,600	0.099
9	350	13,000	0.000
10	450	17,000	0.000

Awtrey and Connick[41] have shown that I_2, as well as I_3^-, absorbs at 353 nm. In the experiment described above, very little I_2 is present because of the excess KI. While I^- may absorb at 353 nm, the molar absorptivity (ϵ) of I_3^- at this wavelength has been reported as 2.6×10^4 by Genser and Connick.[40] The corrected molar absorptivity for

the absorbance due to I^- is given as

$$\epsilon = 2.64 \times 10^4 (1 + 0.164 [I^-])$$

and may be considered to be negligible here.

Because of the data in Table 3, it was decided that in subsequent experiments an initial KI concentration of 25 or 12.5 mM would be used.

Effect of Acetate Buffer on 5-Oxoproline and Sodium Hypochlorite

The effect of acetate buffer, pH 5.0, on 5-oxoproline and NaOCl was determined by comparing UV spectral scans of each compound in buffered and unbuffered solutions. Unbuffered solutions of 50 mM 5-oxoproline and 15 mM NaOCl were scanned against a water blank from 360 to 200 nm on the Beckman 26. Similar solutions made with 200 mM acetate buffer were scanned against a buffer blank in the same region of the spectrum. For all scans the chart speed was 5 in/min and the scanning speed was 100 nm/min. The 200 mM acetate buffer was also scanned in a similar manner against a water blank.

Buffer scanned against a water blank gave absorbance values which sharply climbed to 2.0 at approximately 250 nm and continued to give "off-scale" absorbance values down to 200 nm. The fact that acetate buffer absorbs in the UV region of the spectrum presents no problem as long as it is compensated for in the blank, as was done in later experiments as well as this one.

By comparing the scans of the unbuffered and buffered solutions of 5-oxoproline, Figures 8 and 9, it can be seen that different species are present in each of the solutions. The scan of unbuffered 5-oxoproline gave "off-scale" absorbance values from approximately 232 to 200 nm.

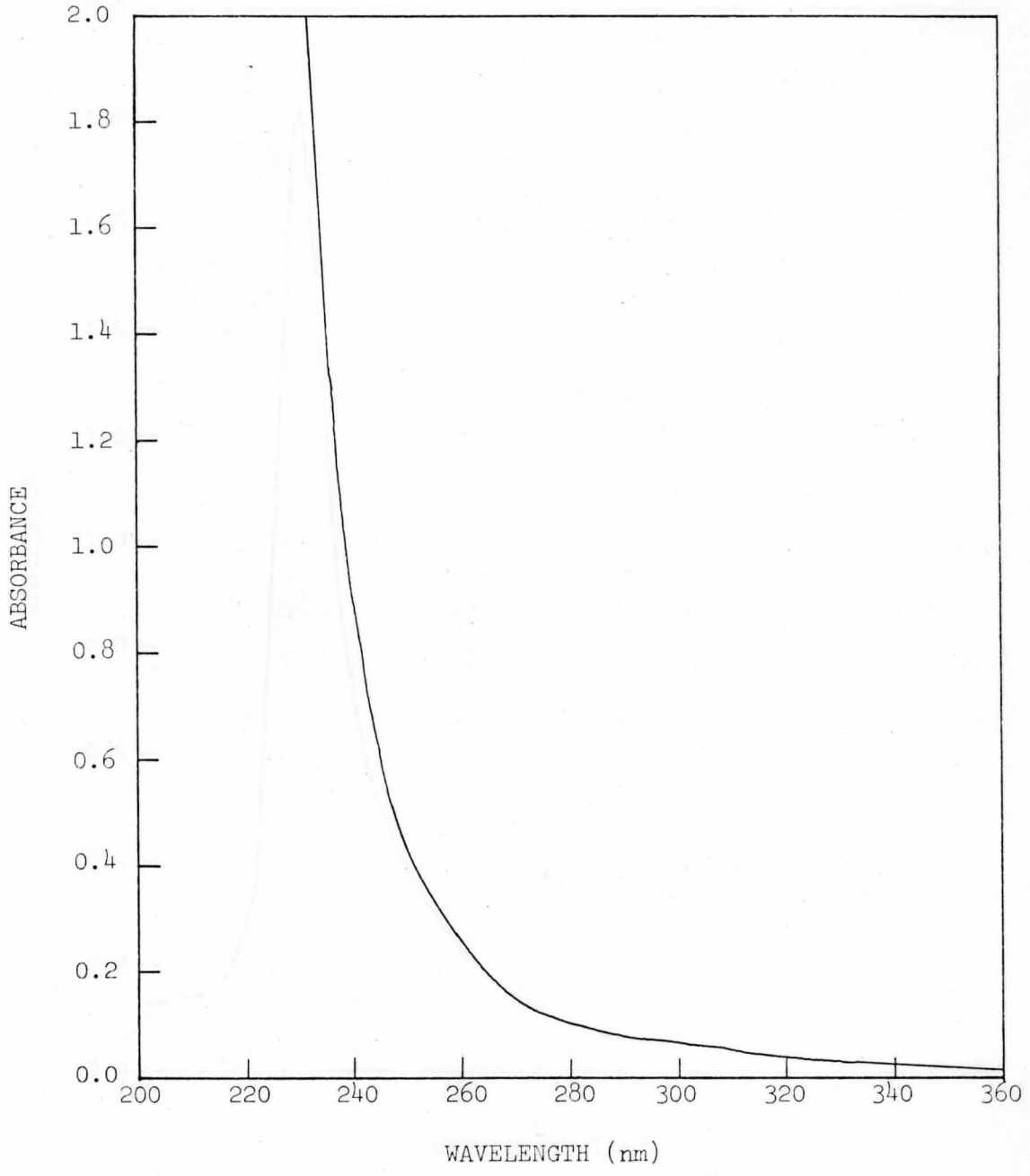


Fig. 8. UV Spectrum of 50 mM 5-Oxoproline in Unbuffered Solution at pH of 3.2.

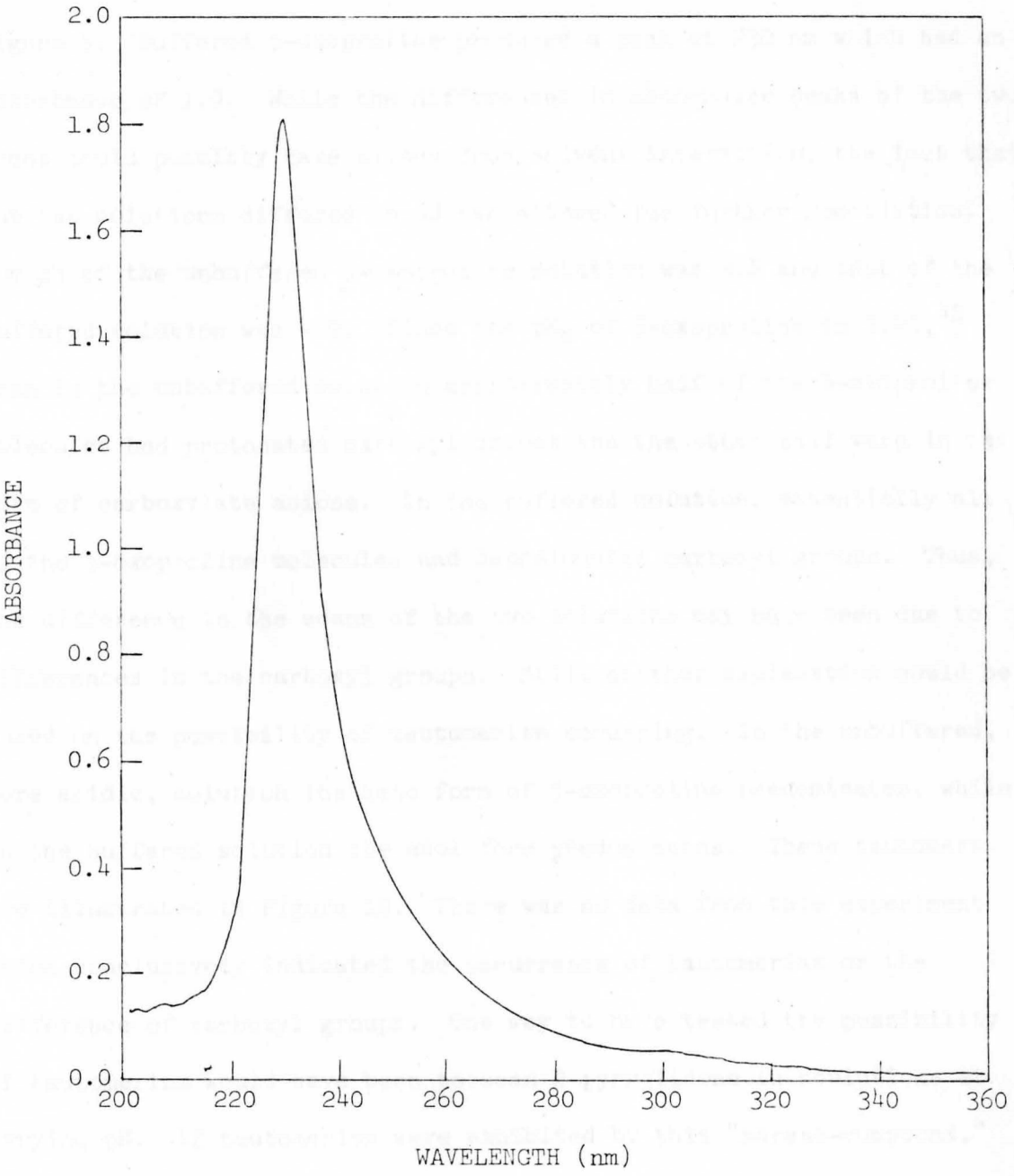


Fig. 9. UV Spectrum of 50 mM 5-Oxoproline in 200 mM Acetate Buffer at pH of 4.9.

This indicated the possibility of the true peak of 5-oxoproline lying near 200 nm and is supported by the scan of 5-oxoproline shown in Figure 5. Buffered 5-oxoproline produced a peak at 230 nm which had an absorbance of 1.9. While the differences in absorbance peaks of the two scans could possibly have arisen from solvent interaction, the fact that the two solutions differed in pH has allowed for further speculation. The pH of the unbuffered 5-oxoproline solution was 3.2 and that of the buffered solution was 4.9. Since the pK_a of 5-oxoproline is 3.25,[42] then in the unbuffered solution approximately half of the 5-oxoproline molecules had protonated carboxyl groups and the other half were in the form of carboxylate anions. In the buffered solution, essentially all of the 5-oxoproline molecules had deprotonated carboxyl groups. Thus, the difference in the scans of the two solutions may have been due to differences in the carboxyl groups. Still another explanation could be based on the possibility of tautomerism occurring. In the unbuffered, more acidic, solution the keto form of 5-oxoproline predominates, while in the buffered solution the enol form predominates. These tautomers are illustrated in Figure 10. There was no data from this experiment which conclusively indicated the occurrence of tautomerism or the difference of carboxyl groups. One way to have tested the possibility of tautomerism would have been to scan 2-pyrrolidone in soulutions at varying pH. If tautomerism were exhibited by this "parent-compound," it would strongly support the occurrence of tautomerism in 5-oxoproline.

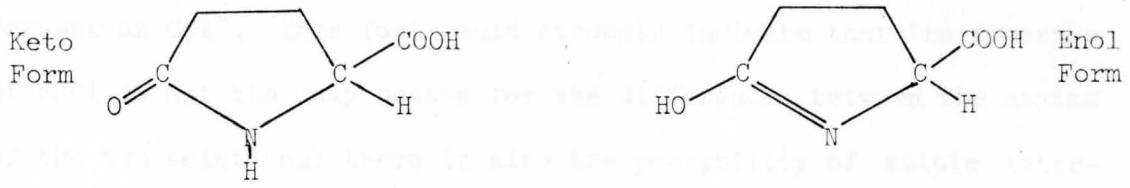


Fig. 10. Tautomerism in 5-Oxoproline

Regardless of the reason for the differences in the two scans, the possibility exists that the N-chlorination of 5-oxoproline occurs to the greatest extent in only one of the two environments: unbuffered or buffered. This can be supported by comparing the scans of unbuffered and buffered solutions of NaOCl, Figures 11 and 12.

Unbuffered NaOCl produced a peak at 290 nm which had an absorbance of 1.84 and a pH of 11.8. Buffered NaOCl, with a pH of 8.3, gave a peak at 235 nm which had an absorbance of 0.50. Again, two different species are represented by the scans. An explanation of which species exist in each of the solutions is more readily arrived at here than in the case of 5-oxoproline. The pK value for the dissociation of hypochlorous acid according to the equation.

$$HOCl \rightleftharpoons H^+ + OCl^- \qquad (17)$$

is 7.53.[43] Therefore, in the unbuffered solution, NaOCl exists almost exclusively in the form of Na^+ and OCl^-; OCl^- causes the maximum at 290 nm. In the buffered NaOCl solution the pH of 8.3 is nearer the pK value of hypochlorous acid, indicating that less than 50% of the NaOCl exists in the form of HOCl. The result is a shift in the maximum wavelength. The presence of some OCl^- is indicated from the shoulder appearing from 300-270 nm in the scan of NaOCl in buffer. However, the shoulder has an absorbance of only 0.14, less than half the absorbance of unbuffered NaOCl. A larger absorbance should have occurred at 290 nm if greater than 50% of the species in the buffered solution were present as OCl^-. This fact would strongly indicate that the presence of HOCl is not the only reason for the differences between the maxima of the two solutions: there is also the possibility of solute interactions between the acetate buffer and either HOCl or NaOCl.

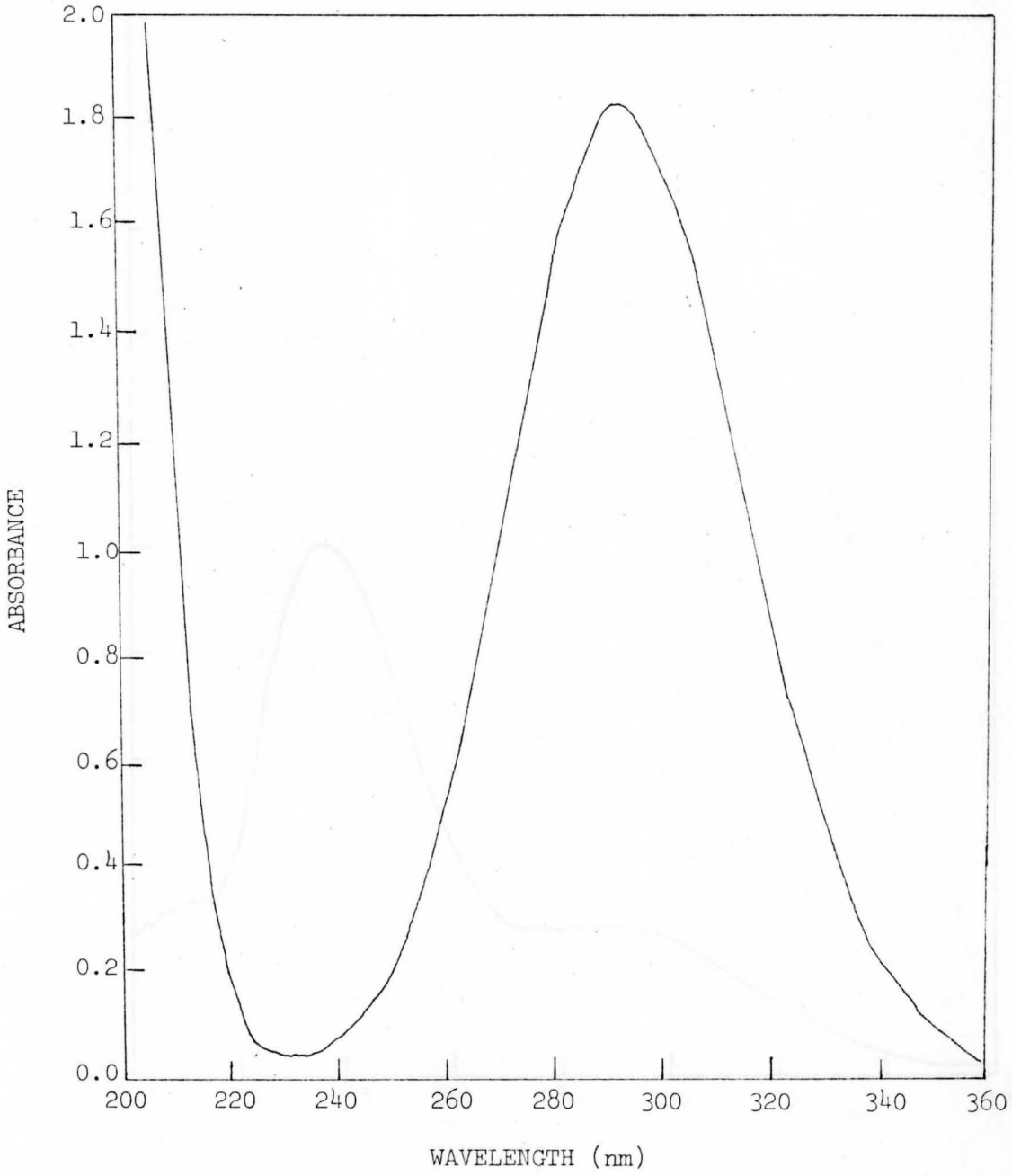


Fig. 11. UV Spectrum of 15 mM NaOCl in Unbuffered Solution at pH of 11.8.

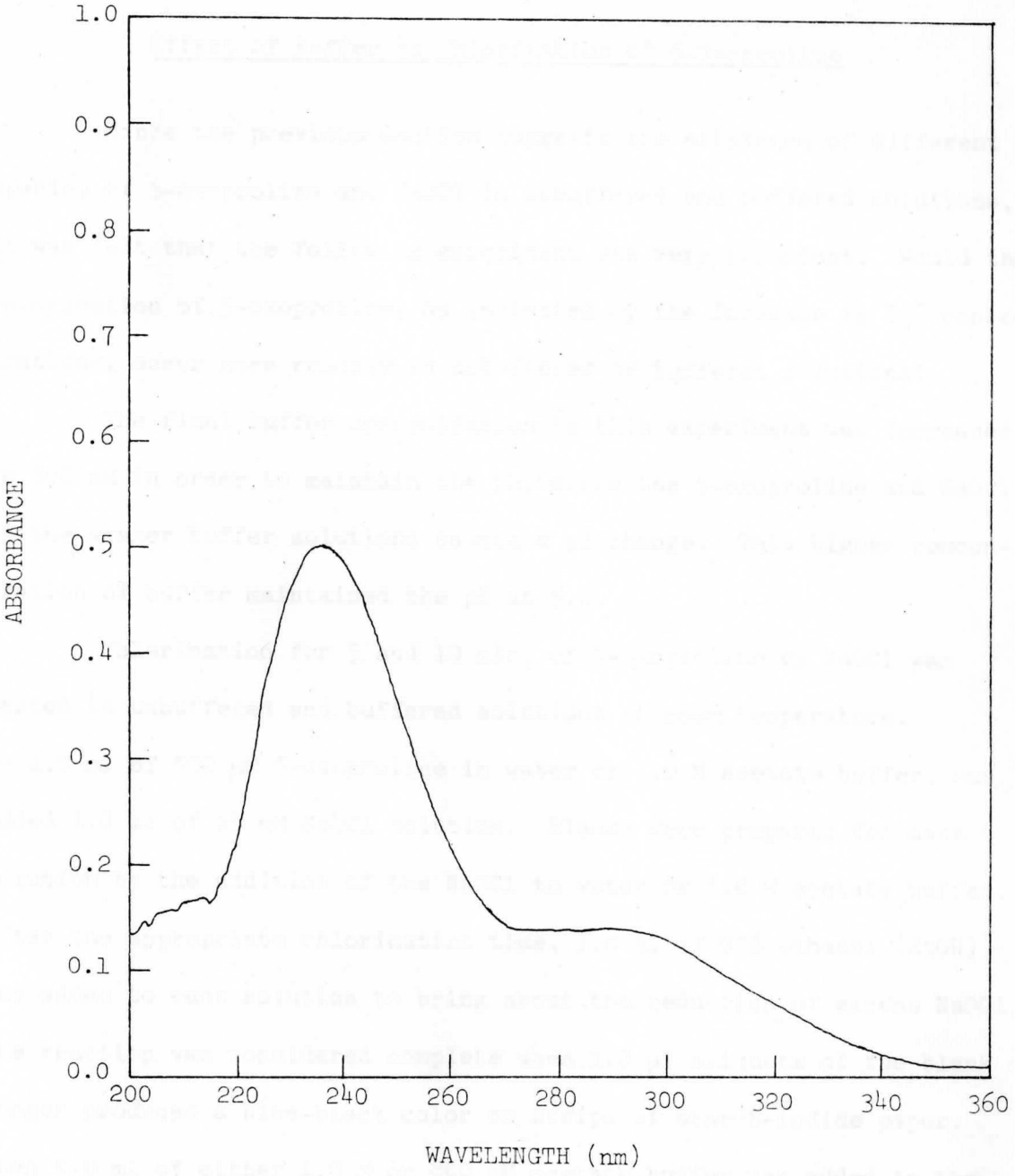


Fig. 12. UV Spectrum of 15 mM NaOCl in 200 mM Acetate Buffer at pH of 8.3.

Nevertheless, the possibility remains that NaOCl will be a better chlorinating reagent in only one of the two environments.

Effect of Buffer on Chlorination of 5-Oxoproline

Since the previous section suggests the existence of different species of 5-oxoproline and NaOCl in unbuffered and buffered solutions, it was felt that the following experiment was very important. Would the chlorination of 5-oxoproline, as indicated by the increase in I_3^- concentrations, occur more readily in unbuffered or buffered solutions?

The final buffer concentration in this experiment was increased to 500 mM in order to maintain the pH, since the 5-oxoproline and NaOCl in the weaker buffer solutions caused a pH change. This higher concentration of buffer maintained the pH at 5.0.

Chlorination for 5 and 10 min, of 5-oxoproline by NaOCl was tested in unbuffered and buffered solutions at room temperature. To 1.0 ml of 500 μM 5-oxoproline in water or 1.0 M acetate buffer, was added 1.0 ml of 15 mM NaOCl solution. Blanks were prepared for each solution by the addition of the NaOCl to water or 1.0 M acetate buffer. After the appropriate chlorination time, 1.0 ml of 95% ethanol (EtOH) was added to each solution to bring about the reduction of excess NaOCl. The reaction was considered complete when 1.0 μl aliquots of the blank no longer produced a blue-black color on strips of starch-iodide paper. Then 5.0 ml of either 1.0 M or 800 mM acetate buffer was added to the solutions. Upon the addition of 2.0 ml of 125 mM KI solution the final acetate buffer concentration became 500 mM. Since the formation of I_3^- is dependent on buffer concentration, the buffer was added just prior to the addition of the KI solution instead of at the same time. The

formation of triiodide was immediate upon the addition of the KI solution as evidenced by the instantaneous change from colorless to yellow solutions. Only the blanks of the solutions which had been chlorinated in an aqueous environment remained colorless. The results of the experiment appear in Table 4.

TABLE 4

CHLORINATION OF 500 mM 5-OXOPROLINE IN UNBUFFERED vs BUFFERED SOLUTION AT ROOM TEMPERATURE

No.	Chlorination Environment	Chlorination Time (min)	A at 353 nm
1	Unbuffered	5	1.45
2	Buffered	5	>2.00
3	Unbuffered	10	1.55
4	Buffered	10	>2.00

The solutions which were chlorinated for 5 minutes required 165 minutes for the reduction of excess NaOCl by EtOH, while those chlorinated for 10 minutes required 152 minutes. The differences in times required for reduction of excess NaOCl indicated that there was a greater excess of NaOCl in solutions 1 and 2, but more N-chlorinated 5-oxoproline in solutions 3 and 4. The absorbance values of the unbuffered solutions confirms this. It was decided that, since the unbuffered solutions gave "on-scale" absorbance values, the chlorination step to be used in the development of the triiodide assay would be limited to unbuffered solutions.

It is possible that the chlorination of 5-oxoproline in buffer may have been a better choice, but it evidently would have required more time to obtain colorless blanks. It appears that EtOH does not reduce the NaOCl species in buffered solution as readily as in unbuffered solution. Therefore, in order to minimize the time required for the assay procedure, chlorination in unbuffered solution was used.

Since there was a small difference between the absorbance values for the solutions of 5-oxoproline chlorinated for different lengths of time, it was decided to study the time of chlorination more thoroughly.

Chlorination of 5-Oxoproline at 37°C

In Table 5, the results of three different chlorination times on the production of I_3^- are given. The solutions that were measured were prepared as described in the previous section and chlorination of 5-oxoproline was carried out for 10, 20, or 30 minutes in unbuffered solution at 37°C. The times required for reduction of excess NaOCl were 58 minutes, 54 minutes, and 54 minutes, respectively, at 37°C. Each solution was measured against its own blank, at 353 nm, on the Beckman 26. The blanks had been prepared in the same manner as the solutions from 1.0 ml water instead of the 1.0 ml of 5-oxoproline solution. Upon the addition of KI solution the samples turned yellow, while the blanks remained colorless.

The absorbance values of the three solutions show close agreement, although the solution which had been chlorinated 20 minutes gave

the highest value. Since there was no large difference (less than 3%) between the absorbance values of the solutions chlorinated for 10 and 20 minutes, it was concluded that the 10 minute chlorination time was sufficient.

TABLE 5

EFFECT OF CHLORINATION TIME ON I_3^- ABSORBANCE IN UNBUFFERED SOLUTION OF 500 mM 5-OXOPROLINE AT 37°C

No.	Chlorination Time (min)	A at 353 nm
1	10	0.824
2	20	0.843
3	30	0.840

When the absorbance values for the solutions listed in Table 4 are compared to those of solutions 1 and 3 in Table 5, it can be seen that the values in Table 5 are significantly lower. A plausible explanation for this is that both N-chloro-5-oxoproline as well as excess NaOCl are reduced by EtOH, and in effect, compete for the reducing reagent. NaOCl, however, is apparently reduced more readily than N-chloro-5-oxoproline. In the blanks, there is no competition for the reducing reagent and the excess NaOCl is reduced at a faster rate than in the solutions containing N-chloro-5-oxoproline. At 37°C, all of the reactions occur at a faster rate than at room temperature; the increased rate of reduction of N-chloro-5-oxoproline by EtOH resulted in lower absorbance values. Since less time was involved by allowing the reactions to proceed at the higher temperature, and since fairly high absorbance

values were still obtained, 37°C was chosen as the temperature for the chlorination and NaOCl reduction steps. The choice of this temperature required further experimentation with the NaOCl reduction step and is described in the next section.

Reduction of Excess Hypochlorite by Ethanol

In order to determine the extent of reduction of NaOCl by EtOH, the time allowed for the reduction reaction was varied from 16 minutes to 125 minutes, unbuffered solutions containing either 1.0 ml of 1 mM 5-oxoproline or 0.5 ml of 1 mM 5-oxoproline with 0.5 ml of water were chlorinated for 20 minutes using 1.0 ml of 15 mM NaOCl at 37°C. This was followed by the addition of 1.0 ml of 95% EtOH. After these solutions were incubated for various time intervals at 37°C, 5.0 ml of 1.0 M acetate buffer pH 5.0 was added followed by 2.0 ml of 250 mM KI solution. Blanks containing 1.0 ml of water were treated in the same way as each pair of 5-oxoproline solutions. Absorbance values were measured at 353 nm on the Beckman 26 spectrophotometer. All of the blanks were colorless except the one in which the NaOCl reduction proceeded for 16 minutes. The results of the experiment appear in Table 6. The absorbance values of the two different 5-oxoproline concentrations for each reduction time interval were plotted on the graph shown in Figure 13.

It can be seen from the diagram, that the curves formed by any two sets of points intersect the same graph at different positions. Curve A intersects the "Absorbance" axis at approximately 0.7 units, while curves E and F intersect the "Concentration" axis near the origin. The origin of the graph, while not actually included in any of the

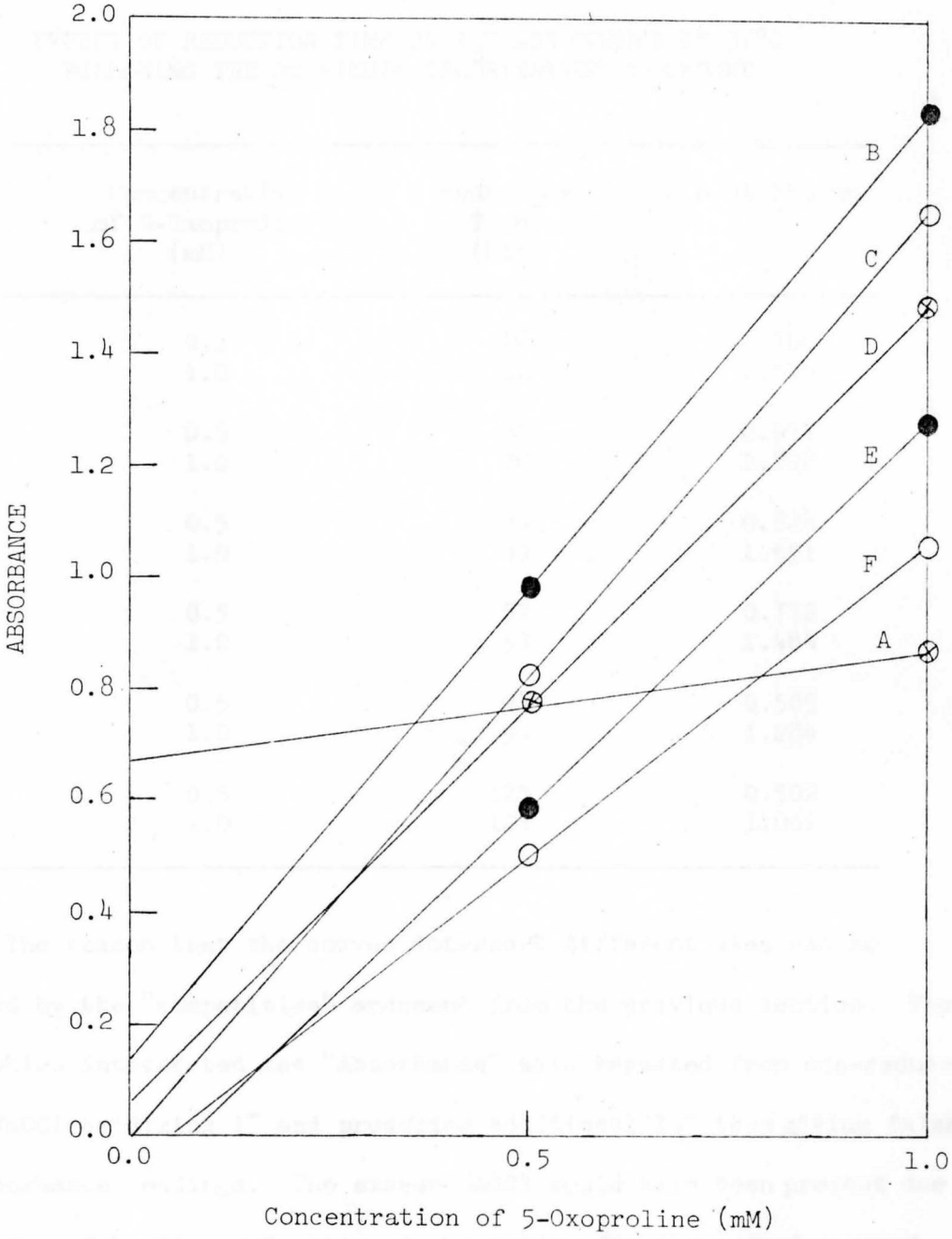


Fig. 13. Effect of Reduction Time on Standard Curves of 5-Oxoproline.

curves, is more nearly included in curve C.

TABLE 6

EFFECT OF REDUCTION TIME ON I_3^- ABSORBANCE AT 37°C FOLLOWING THE 20 MINUTE CHLORINATION PROCEDURE

No.	Concentration of 5-Oxoproline (mM)	Reduction Time (Min)	A at 353 nm
A1	0.5	16	0.766
A2	1.0	16	0.870
B1	0.5	30	0.977
B2	1.0	30	1.828
C1	0.5	39	0.824
C2	1.0	39	1.651
D1	0.5	57	0.772
D2	1.0	57	1.484
E1	0.5	94	0.585
E2	1.0	94	1.284
F1	0.5	125	0.502
F2	1.0	125	1.062

The reason that the curves intersect different axes can be explained by the "competition" argument from the previous section. Those curves which intersected the "Absorbance" axis resulted from non-reduced, excess NaOCl oxidizing I^- and producing additional I_3^- thus giving falsely high absorbance readings. The excess NaOCl would have been present due to competition of NaOCl and N-chloro-5-oxoproline for the reducing agent. Since there was no such competition in the blanks, curves A and B falsely indicate that the blanks had a measureable absorbance. In the case of curves E and F intersecting the "Concentration" axis, it is

apparent that, after all of the excess NaOCl has been reduced, the N-chloro-5-oxoproline is also susceptible to reduction by EtOH. As more N-chloro-5-oxoproline becomes reduced, the curve intersects the "Concentration" axis farther from the origin.

One of the requirements for a standard concentration curve is the inclusion of the origin in the curve. From Figure 13, it can be concluded that the reduction of excess NaOCl is an important factor in obtaining such a curve. Therefore, having taken into consideration the possibility of experimental error, the approximate average of reduction times for curves C and D, 45 minutes, was chosen as the amount of time allotted for the reduction of excess NaOCl in future experiments.

Standard Triiodide Assay

The standard triiodide assay for the determination of 5-oxoproline, as presented in this section, was used in all of the subsequent determinations presented in this thesis. The method takes into consideration the results of all the previously described experiments. Concentrations of reagents were adjusted so that when they were added to 1.0 ml samples containing 5-oxoproline the final volume was 6.0 ml. Absorbance measurements were taken at 350 nm, instead of the previous 353 nm, as the difference observed between the two values was always negligible. There appeared to be no interference from other compounds in spectral scans of the resulting triiodide solutions.

In its final form the triiodide assay entails six basic steps.

1. Use 1.0 ml of sample containing 5-oxoproline.
2. Add 1.0 ml of 15 mM NaOCl solution and incubate at 37°C for 10 minutes. This is the chlorination step.

3. Add 2.0 ml of 95% EtOH and incubate at 37°C for 45 minutes. This step reduces the excess NaOCl.

4. Add 1.0 ml of 3.0 M acetate buffer that has been adjusted to pH of 5.0.

5. Immediately following step 4, add 1.0 ml of 75 mM KI solution.

6. Read absorbance values for the solutions at 350 nm on the Beckman 26, 20 to 45 minutes after the addition of the KI solution. Dilute samples, when necessary, with 0.50 M acetate buffer to obtain "on-scale" absorbance values.

Upon the addition of each reagent, solutions are thoroughly mixed on a vortex apparatus for at least 5 seconds. While the solutions are incubating in the 37°C bath they are covered to prevent evaporation. The reagents used in steps 2 through 4 were made or obtained in liter quantities and dispensed from automatic pipetting bottles.

The chemical reactions which proceed according to this method can be hypothesized on the basis of the work done by Rydon and Smith[31] and Lin, et al.[23] They are presented in Figure 14. Chlorination of the amino nitrogen of 5-oxoproline is brought about by reaction with NaOCl to produce hydroxide ion (OH^-) and N-chloro-5-oxoproline. Excess NaOCl is reduced by EtOH to chloride ion. Buffer is added to maintain pH. Upon the addition of KI, I^- is oxidized to I_2 with the simultaneous reduction of N-chloro-5-oxoproline to 5-oxoproline. Since KI is present in excess, essentially all of the I_2 formed complexes with I^- to form I_3^-.

Reproducibility of the Triiodide Method

In order to test the reproducibility of the triiodide method, measurements of I_3^- formed in solutions derived from two separate 1 mM solutions of 5-oxoproline were determined. The samples tested were prepared by diluting aliquots of the solution to a final volume of 1.0 ml.

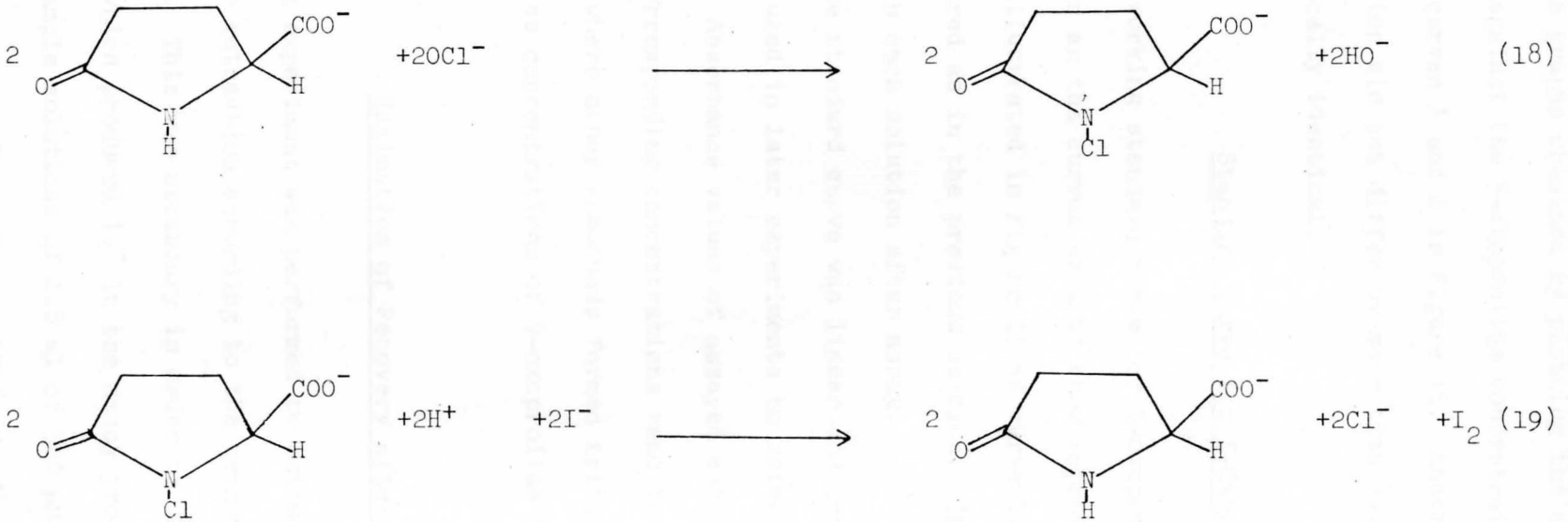


Fig. 14. Reactions in the Triiodide Assay Procedure.

The graphs obtained by plotting the absorbance of the final solutions against the 5-oxoproline concentration of the 1.0 ml samples appear as curves A and B in Figure 15. Absorbance values for any concentration did not differ by more than 4%, proving that the curves are practically identical.

Standard Curve for 5-Oxoproline

A working standard curve for 5-oxoproline was prepared in the same manner as the curves used to show reproducibility. This standard curve is illustrated in Figure 15 as curve C. The 5-oxoproline samples were prepared as in the previous section. The pH value of 5.0 was measured in each solution after assay.

The standard curve was linear and included the origin. This curve was used in later experiments to determine 5-oxoproline concentrations. Absorbance values of assayed solutions were found on the curve and the corresponding concentrations read from the graph. In the assayed solutions where other compounds formed triiodide the results were expressed as concentrations of 5-oxoproline for the purpose of comparison.

Estimation of Recovery after Extraction

An experiment was performed to estimate the recovery of 5-oxoproline after extraction according to the method described in Chapter III, pp. 20-21. This was necessary in order to correct for loss of any compound which produced I_3^- in the assay procedure.

Sample solutions of 1.0 ml of 500 μM 5-oxoproline were assayed after extraction. The volumes obtained after extraction were 0.75 and

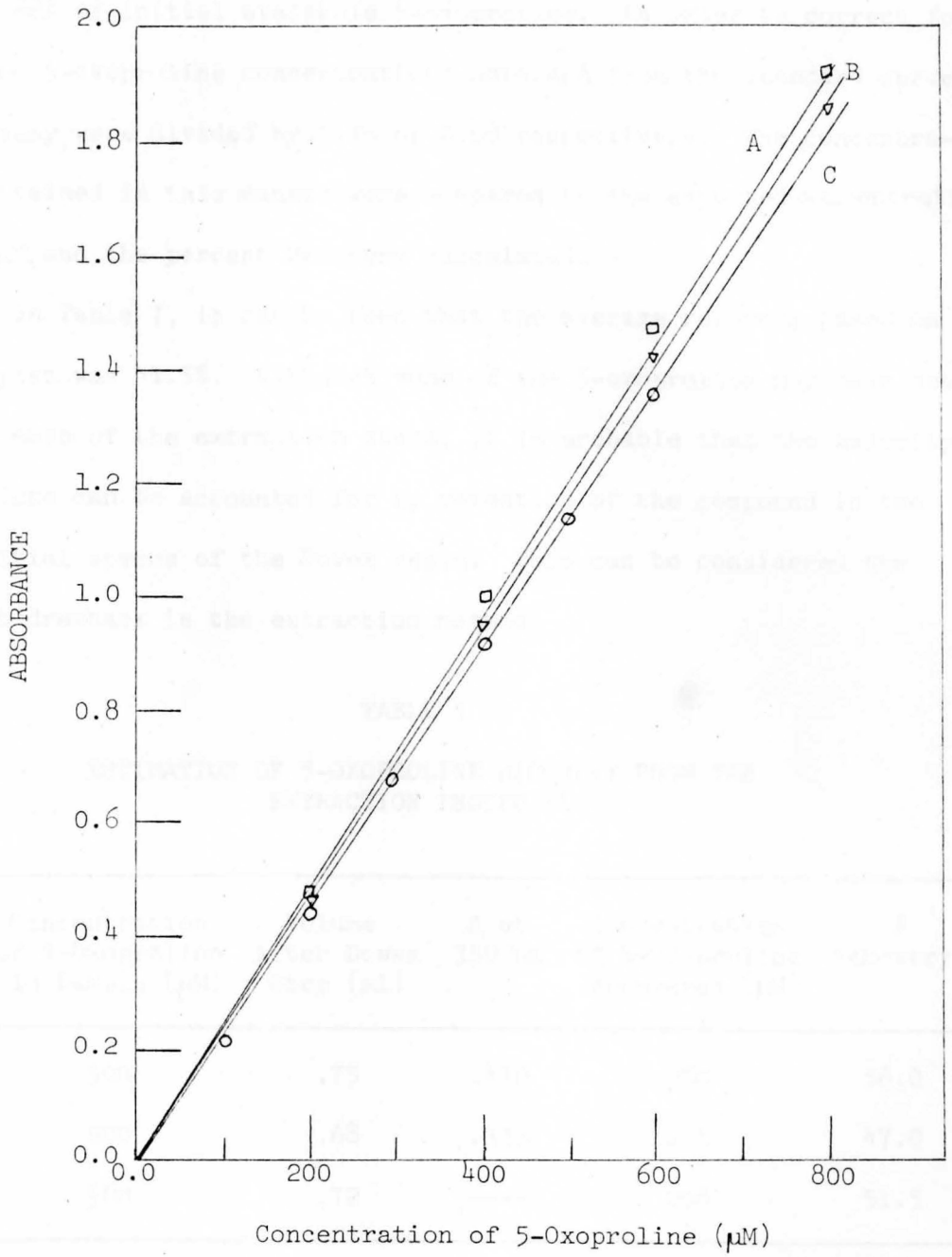


Fig. 15. Reproducibility of Standard Curves of 5-Oxoproline

0.68 ml and were brought to a volume of 1.0 ml for assay. Therefore, 5-oxoproline concentrations obtained after triiodide assay represented 75% and 68% of initial available 5-oxoproline. In order to correct for this, the 5-oxoproline concentrations obtained from the standard curve after assay were divided by 0.75 or 0.68 respectively. The concentrations obtained in this manner were compared to the expected concentration of 500 μM and the percent recovery calculated.

In Table 7, it can be seen that the average recovery based on two samples was 51.5%. Although some of the 5-oxoproline may have been lost in each of the extraction steps, it is probable that the majority of the loss can be accounted for by retention of the compound in the interstitial spaces of the Dowex resin. This can be considered the greatest drawback in the extraction method.

TABLE 7

ESTIMATION OF 5-OXOPROLINE RECOVERY FROM THE EXTRACTION PROCEDURE

No.	Concentration of 5-Oxoproline in Sample (μM)	Volume After Dowex Step (ml)	A at 350 nm	Concentration of 5-Oxoproline Recovered (μM)	% Recovery
1	500	.75	.470	280	56.0
2	500	.68	.373	235	47.0
Avg.	500	.72	----	258	51.5

In calculating the equivalent 5-oxoproline concentrations in later experiments the following equation was used:

$$\text{Final Concentration of 5-Oxoproline} = \frac{\text{Concentration from standard curve x dilution factor}}{\text{Volume after extraction x recovery factor}}$$

This equation accounts for all the correction factors needed to obtain the final 5-oxoproline concentration. The dilution factor was necessary when dilution with acetate buffer was required for obtaining "on-scale" absorbance measurements. Thus a 1:5 dilution had a dilution factor of 5. Where no dilution was necessary the dilution factor was 1.

Assay of Normal Sera

In this experiment an attempt was made to determine 5-oxoproline concentrations in human sera. The sera of six volunteers were extracted according to the method described in Chapter III and the extracts were assayed by the triiodide method. Five of the six samples were done in duplicate. In addition to the normal sera pooled bovine serum and water were also extracted and assayed on three successive days as a control. The results of this experiment appear in Table 8. The final 5-oxoproline concentration was calculated according to the equation in the last section.

The water that was "extracted" and assayed for 5-oxoproline gave negligible absorbance measurements. These values can be attributed to experimental error and it is not necessary to correct for them. The bovine serum assayed, showed some fluctuation in "5-oxoproline concentrations" on different days, but the fluctuations were not any greater than those obtained from duplicates of normal sera assayed on the same day.

The values obtained as "final 5-oxoproline concentrations" for normal sera are enormously high compared to the "normal" values given in the Introduction (cf., Table 1). While the non-enzymic cyclization of other serum metabolites to 5-oxoproline is possible, it is just as likely

TABLE 8

"5-OXOPROLINE" CONCENTRATIONS IN NORMAL SERA

No.	Sample	Volume after Dowex Step (ml)	Dilution Factor	A at 350 nm	Final Concentration of "5-Oxoproline" (mM)
1	Water	0.80	1	0.004	---
2	Water	0.80	1	0.002	---
3	Water	0.83	1	0.003	---
4	Bovine Serum	0.70	5	1.018	6.1
5	Bovine Serum	0.70	5	1.110	6.7
6	Bovine Serum	0.85	5	1.179	5.6
7	Normal #1	0.65	5	0.856	5.6
8	Normal #1	0.52	5	0.897	7.4
9	Normal #2	0.60	5	0.953	6.7
10	Normal #2	0.60	5	0.898	6.4
11	Normal #3	0.60	5	0.982	7.0
12	Normal #3	0.80	5	1.137	6.1
13	Normal #4	0.60	5	0.856	6.1
14	Normal #4	0.95	5	1.008	4.5
15	Normal #5	0.80	5	1.135	6.0
16	Normal #5	0.75	5	1.012	5.7
17	Normal #6	0.80	5	1.132	6.0

that some other compounds which are sensitive to the assay method have contributed to the high values obtained.

Other compounds which could be sensitive to the triiodide assay include amino acids, urea, and nitrogenous compounds such as purines and pyrimidines. However, Dowex 50 was used in the extraction process to adsorb positively charged substances, especially glutamic acid. Since many other amino acids will also be positively charged it is likely that they would be adsorbed by the Dowex resin too. The concentration of urea in serum is 2.5-6.7 mM[44] and would present a formidable source of error. The concentration of non-protein nitrogen in serum is 20-35 mg/100 ml[45] and compounds represented by this classification may also be sensitive to the triiodide assay. Investigation of the sensitivity of the triiodide assay procedure was clearly warranted.

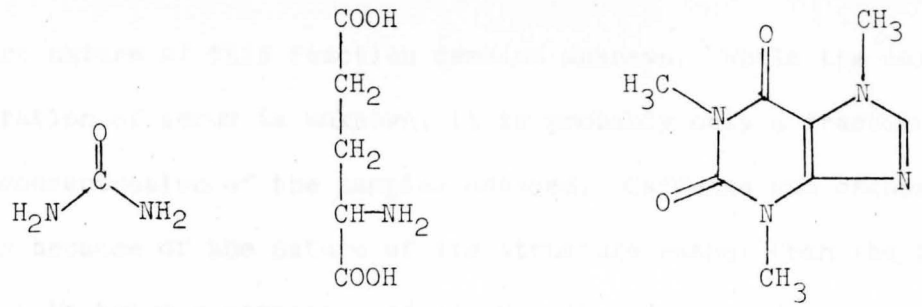
Possible Interfering Compounds in Triiodide Assay of 5-Oxoproline in Serum

On the basis of the Rydon-Smith method and its modifications,[30-35] the number of compounds that could be sensitive to the triiodide assay appears to be quite large. In order to gain better insight into the sensitivity of the assay method, 1.0 ml samples of 10 and 100 mM urea, 10 mM glutamic acid, and 100 mM caffeine were assayed with and without the extraction procedure. In addition, some samples, including 500 μM 5-oxoproline, were also assayed after acid hydrolysis by the method described in Chapter III, pp. 21-22. The structures of glutamic acid, urea, and caffeine appear in Figure 16, and the results of the experiment are listed in Table 9.

TABLE 9

ASSAY OF VARIOUS COMPOUNDS BY THE TRIIODIDE METHOD

No.	Sample and Concentration	Volume after Dowex Step (ml)	Dilution Factor	A at 350 nm	Final Concentration "5-Oxoproline" (mM)
1	500 µM 5-Oxoproline, acid hydrolysate	1.0	3	0.000	0.0
2	10 mM Glutamic acid, without extraction or hydrolysis	---	1	0.000	0.0
3	100 mM Caffeine, without extraction or hydrolysis	---	1	0.466	0.21
4	100 mM Caffeine, with extraction	0.9	5	0.887	4.2
5	100 mM Caffeine, acid hydrolysate	1.0	10	0.470	4.1
6	10 mM Urea, with extraction	0.9	5	1.252	5.9
7	10 mM Urea, acid hydrolysate	1.0	10	0.897	7.7
8	100 mM Urea, without extraction or hydrolysis	---	1	0.769	0.33
9	100 mM Urea, with extraction	0.9	9	1.040	8.9
10	100 mM Urea, acid hydrolysate	1.0	18	1.024	15.6



Urea Glutamic acid Caffeine

Fig. 16. Structures of Urea, Glutamic Acid, and Caffeine

The results indicate that 10 mM glutamic acid did not promote the formation of I_3^- by the assay method. The concentration of glutamic acid was well in excess of the concentration found in serum. (Total α-amino nitrogen concentration in serum is 3.7-5.5 mg/100 ml.[46])

The 100 mM urea solution assayed without extraction or hydrolysis has a concentration greater than that found in normal serum.[44] It gave an assay concentration equivalent to 330 μM 5-oxoproline. This value is only a fraction of the concentrations of "5-oxoproline" reported for normal sera in Table 8. The samples of urea that were assayed after extraction and hydrolysis, however, could have accounted for the high values reported for normal sera in Table 8. In fact these values indicate that hydrolysis occurs during the extraction process. It can be concluded that urea is sensitive to the triiodide assay method. Also, it is an important source of error in assaying serum by this method, especially in combination with the extraction procedure used.

Caffeine samples, assayed as "5-oxoproline," gave a low concentration value without extraction or hydrolysis, and high values when assayed after hydrolysis or extraction. Again, it can be hypothesized that some hydrolysis reaction occurs during the extraction process.

The exact nature of this reaction remains unknown. While the caffeine concentration of serum is unknown, it is probably only a fraction of the 100 mM concentration of the samples assayed. Caffeine was chosen for analysis because of the nature of its structure rather than the possibility of it being a component of serum. It was assumed that the methylated nitrogen atoms of caffeine would not be chlorinated. Therefore the only nitrogen atom which could be chlorinated would be the one participating in the double bond. Since this nitrogen atom apparently was sensitive to the triiodide assay, there is strong indication that many other nitrogen compounds would also be sensitive.

Since the acid hydrolysate of 5-oxoproline gave no absorbance, it was assumed to be completely hydrolyzed to glutamic acid. There was apparently no re-formation of 5-oxoproline after its hydrolysis.

Assay of Normal and Abnormal Sera

The blood of five anephric individuals with renal disease was collected prior to the patients' hemodialysis treatments. The assay of the sera of these blood samples was performed along with one normal serum. Acid hydrolysis of these sera, followed by assay, was also performed.

From the data of this experiment, presented in Table 10, it is apparent that the values, reported as "5-oxoproline" concentrations, for the patients were similar to the normal values obtained by this method. Also, the sera which had undergone acid hydrolysis prior to assay did not differ significantly in "5-oxoproline" concentration from the normal and abnormal sera assayed without hydrolysis. On the basis of these results, combined with the fact that hydrolyzed caffeine and urea gave

TABLE 10

ASSAY VALUES FOR NORMAL AND ABNORMAL SERA

No.	Sample	Volume after Dowex Step (ml)	Dilution Factor	A at 350 nm	Final "5-Oxoproline" Concentration mM
1	Normal #5	0.8	5	1.024	5.8
2	Normal #5, acid hydrolysate	0.8	10	0.462	4.9
3	Abnormal #1	0.7	9	0.530	5.7
4	Abnormal #1, acid hydrolysate	1.0	18	0.440	6.6
5	Abnormal #2	0.8	9	0.616	5.9
6	Abnormal #2, acid hydrolysate	1.0	18	0.500	7.7
7	Abnormal #3	0.8	9	0.460	4.4
8	Abnormal #3, acid hydrolysate	0.9	18	0.265	4.5
9	Abnormal #4	0.7	9	0.443	4.7
10	Abnormal #4, acid hydrolysate	1.0	18	0.345	5.3
11	Abnormal #5	0.9	9	0.738	6.3
12	Abnormal #5, acid hydrolysate	1.0	18	0.465	7.2

higher "5-oxoproline" concentrations than non-hydrolyzed samples, it seems likely that the concentration of the NaOCl reagent was a limiting factor.

If the concentration of compounds susceptible to chlorination exceeded the concentration of NaOCl, then all of the concentration values for sera would have been similar. It is unlikely that any of the other reagents would have produced such an effect. The problem with assaying sera by this method, then, lies in the separation techniques employed.

Support of Experimental Method by Chromatography

Samples of 5-oxoproline and glutamic acid were spotted on thin layer chromatogram sheets. The concentration of each solution was 1.0 mg/ml. A sample from a 1:2 dilution of 5-oxoproline with 4-6% NaOCl reagent was also spotted. In addition, normal serum which had been extracted and reconstituted with 0.1 ml of water was applied to the sheets. The chromatograms were developed by the method described in Chapter III, pp. 22-23.

Glutamic acid gave a purple spot when the ninhydrin visualization method was used. No spot was obtained by the Rydon-Smith method; this supported the negative results obtained by the triiodide assay procedure. The R_f value for glutamic acid was 0.47.

A blue-black spot was obtained from 5-oxoproline by the Rydon-Smith method and gave an R_f value of 0.68. A light spot representing an unknown compound and having an R_f of approximately 0.79 was also detected.

This spot was probably the source of the 3% impurity of 5-oxoproline since this compound is probably some product of a reaction

involving 5-oxoproline, it most likely has a similar molecular weight and does not represent a substantial source of error. Neither of these spots were visible when sprayed only with ninhydrin.

When the sheet containing N-chloro-5-oxoproline was sprayed with starch KI solution a blue-black spot became visible. It's Rf was approximately 0.54. The fact that this N-chlorinated form of 5-oxoproline withstood the development of the chromatogram seems to indicate that 1-butanol does not bring about the reduction of this compound, or else that it does so at an extremely slow rate. It may be of interest in future experiments to try to use 1-butanol instead of ethanol as a reducing agent for excess NaOCl.

The ninhydrin visualization method on a normal serum sample produced four spots with R_f values of 0, 0.14, 0.47, and 0.62. The ninhydrin-positive spot at the origin was probably due to some short-chain polypeptides, while the spot with R_f of 0.47 represents glutamic acid. It is not certain what compounds the other spots represent. A blue-black spot with an R_f of 0.67 became visible by the Rydon-Smith method and strongly indicated the presence of 5-oxoproline. The presence of a number of ninhydrin-positive spots in the serum sample did not exclude their possible interference in the triiodide assay. This is because the chromatogram was sprayed with ninhydrin and then subjected to the Rydon-Smith procedure. The spots first developed by ninhydrin remained while the spot representing 5-oxoproline became visible. It was not attempted to subject the serum sample to the Rydon-Smith method alone. Furthermore, it is possible that another compound might migrate with 5-oxoproline and also be sensitive to triiodide assay.

While the results of these chromatograms do not conclusively prove the existence of 5-oxoproline in serum, they do lend support to the results obtained when 5-oxoproline solutions were assayed by the triiodide method. They also suggest the use of 1-butanol as a reducing agent.

CHAPTER V

CONCLUSIONS

This thesis has presented the stepwise development of a solution method for the assay of 5-oxoproline by triiodide ion detection. The assay of serum obtained from normal individuals and patients suffering from renal failure was also discussed. While the assay method could be used to produce a standard curve for aqueous solutions of 5-oxoproline, its use on the serum samples gave values higher than those reported in the literature. The normal serum values reported as 5-oxoproline were similar to the values obtained for the patients with renal failure and no justification was found for use of the triiodide method in diagnosing kidney disease. The reason that the high values were obtained is probably due to the fact that other compounds present in serum interfere in the assay of 5-oxoproline. The extraction procedure that was used did not separate all of the interfering compounds.

The triiodide method can be used for the detection of micromolar quantities of 5-oxoproline in 1.0 ml samples of aqueous solutions. This method, therefore, is more sensitive than Lin's, et al.,[23] method in which millimolar concentrations were determined. Sensitivity of the triiodide assay could be increased further, but unless interfering substances are removed, they could then be even more of a problem.

The amount of time required for assay by the triiodide method is 65 minutes. The method requires 10 minutes for chlorination and 45 minutes for the reduction of excess chlorinating reagent by ethanol.

In order to shorten the time required for assay, the use of another reducing agent might be warranted. Such a reducing agent should be able to reduce sodium hypochlorite rapidly and still be mild enough so as not to react with 5-oxoproline or its N-chloro derivative. While 1-butanol might meet these requirements its insolubility in water is a major disadvantange.

Some revisions of the triiodide assay method which would make it more applicable to the determination of 5-oxoproline are possible. Eluting serum 5-oxoproline from Dowex 50 columns, instead of using the batch method, should certainly reduce the number of interfering compounds and increase the percent recovery. However, this method of separation would require larger sample volumes. It may also be possible to introduce the chlorinating reagent between one of the extraction steps. The subsequent use of anion exchange resins might then be appropriate. Urea could be removed in one of the steps by the use of the enzyme urease.

Should too many variables make the triiodide assay impractical for the assay of 5-oxoproline, then perhaps this method could be used for the determination of other nitrogenous compounds, urea, nitrogen-containing drugs, or total serum nitrogen. However, the proper extraction techniques would need to be employed.

Another type of procedure for the determination of 5-oxoproline that would be of interest is the use of high pressure liquid chromatography. In this case sample preparation would require deproteinization and lipid extraction.

As a whole, this thesis has contributed to the knowledge of the analytical chemistry of 5-oxoproline and has indicated that further study of this important physiological metabolite is necessary.

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