I. Analytical uses of N-bromosuccinimide. II. Distribution studies of palladium in Poa pratensis L.

Mohammad Sarwar

University of Windsor

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I ANALYTICAL USES OF N-BROMOSUCINIMIDE
II DISTRIBUTION STUDIES OF PALLADIUM IN
POA PRATENSIS L.

BY
MOHAMMAD SARWAR

A Dissertation
Submitted to the Faculty of Graduate Studies through the
Department of Chemistry in Partial Fulfillment of the
Requirements for the Degree of Doctor of
Philosophy at the University of
Windsor

Windsor, Ontario, Canada
1969
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ABSTRACT

PART I

ANALYTICAL USES OF N-BROMOSUCCINIMIDE

N-Bromosuccinimide has been employed as a titrimetric reagent in the determination of various sulfur organic compounds. Thiourea and thioacetamide were determined by back-titration as well as by direct method using Bordeaux Red as an indicator. Sulfur amino acids, cysteine, cystine and methionine were determined by direct method. In another method the simultaneous determination of cysteine and cystine was carried out in pure solutions and also in an enzymatic system: cystine reductase. The error in each case was less than ±1%. The fact was also established that N-bromosuccinimide can be used as a primary standard.

PART II

EFFECT OF PALLADIUM CHLORIDE ON THE GROWTH OF POA PRATENSIS L.

A spectrophotometric method for the determination of palladium in biological systems was developed. The sensitivity of the method was 0.5 ppm using 1-cm path length.
The effect of palladium chloride on the growth of *Poa pratensis* has been investigated. The optimum value of pH has been determined for its growth in presence of PdCl₂. The minute quantities of palladium stimulate the growth while higher doses were toxic.
ACKNOWLEDGMENTS

Grateful acknowledgement is made to Professor R.J. Thibert, Ph.D., for his many constructive suggestions regarding the design and direction of these research projects.

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Gratitude is also due to my parents for their financial and moral support.

I must thank God Almighty, for His help visible and invisible.

Thanks are also due to the Canadian International Development Agency, and to the Government of Pakistan for awarding me a scholarship for higher studies.

Finally I must record my special thanks to my wife, Tahira, who assisted in the preparation of manuscripts, assignments and slides.
DEDICATED

To my wife, Tahira Sarwar, whose love gave me courage and strength, and who graciously tolerated long periods of separation.
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PART I

ANALYTICAL USES OF N-BROMOSUCCINIMIDE
CHAPTER I
GENERAL INTRODUCTION

Wohl (1) was the first person to report the uses of N-bromoacetamide for the bromination of allylic compounds. In the year 1942 a detailed investigation on the application of N-bromosuccinimide (NBS) as an agent for allylic bromination was published by Ziegler and his co-workers (2). This type of halogenation is presently known as the Wohl-Ziegler reaction. An excellent review on the subject has been published by Djerassi (3). Under various conditions, these N-halo compounds also react with olefins to add bromine to the double bond, or act as a source of hypohalous acid in aqueous solution. The earlier investigations stimulated tremendous interest in this group of compounds, which also includes N-chlorosuccinimide, N-iodosuccinimide, and N-bromophthalimide.

Aside from the advantages of these compounds as halogenating agents, several of them have been found to be useful for oxidations and dehydrogenations. The reactions and uses of N-bromosuccinimide have been reviewed in 1951 by Waugh (4) and an excellent survey of the chemistry of this compound has been published by Horner (5). N-Bromosuccinimide has both brominating and oxidative bond cleavage properties. Formic acid is attacked by N-bromosuccinimide in aqueous solution with the formation of HBr, succinimide and carbon dioxide (6).
Oxalic acid behaves similarly, and with higher homologs the same products are obtained, although heating is required. Maleic and fumaric acids are oxidatively cleaved to give acetaldehyde in addition to CO₂ and HBr (7). In aqueous medium NBS reacts on heating with aliphatic α-ω-hydroxy acids to give aldehydes and ketones containing one less carbon atom (8). It has also been used for the titrimetric determination of vitamin C (9), by taking advantage of the observation that ascorbic acid is oxidized selectively by NBS before other reducing substances which are present. Oxidative degradation of phenylacetic acid by NBS in carbon tetrachloride produces benzaldehyde. Polycarboxylic acids form aldehydes and ketones under these conditions. Thus citric acid was degraded to acetone (10). Carbon-nitrogen bond scission in tertiary amines by NBS occurs readily with the formation of aldehydes and secondary amines, frequently in good yields. The reaction was carried out in aqueous dioxane (11). The facile oxidative degradation of α-ω-amino acids to aldehydes by NBS and N-bromophthalimide in aqueous medium involves both carbon-carbon and carbon-nitrogen bond cleavage. Alanine was oxidized to acetaldehyde in 50% yield (12). In spite of its vast uses in organic chemistry, it did not draw the attention of analytical chemists until Barakat et al. (9) used it for the quantitative determination of vitamin C in 1955.

Qualitative and quantitative studies were made of the gases evolved in the bromodecarboxylation of amino acids.

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and their derivatives by NBS (13). The major contribution in the study of peptide cleavages by NBS has been conducted by Witkop and his colleagues (14-18). Their studies represent an important advance in peptide structure determination, for it affords a specific method for rupturing peptide chains at linkages involving the carboxyl group of tyrosine and tryptophan. During the course of their qualitative studies, Tagaki et al. (19) found that when divalent sulphides were treated with NBS in aqueous media they were oxidized to the corresponding sulfoxides in high yields. The oxidation of bromide and iodide ions to the free halogen by N-haloimides forms the basis of a convenient method for the qualitative and quantitative identification of these ions (4, 20, 21). It has been shown that the powerful reducing agent Cr(II) is quantitatively oxidized to Cr(III) in the presence of NBS in oxygen-free aqueous perchloric acid (22). It was found by Barakat et al. (23), that in the presence of dilute sulphuric acid, NBS readily and quantitatively oxidizes aqueous solutions of hydrazine salts and certain of their derivatives. They also found that NBS in presence of dilute HCl quantitatively oxidizes tervalent antimony (24).
REFERENCES


CHAPTER II

TITRIMETRIC DETERMINATION OF THIOUREA AND THIOACETAMIDE USING N-BROMOSUCCINIMIDE

KEY WORDS: thiourea, thioacetamide, N-bromosuccinimide, volumetric determination

M. Sarwar and R. J. Thibert
Department of Chemistry
University of Windsor,
Windsor, Ontario, Canada

ABSTRACT

A titrimetric procedure is described for the determination of thiourea and thioacetamide. N-Bromosuccinimide is used to quantitatively oxidize the sulfur in these molecules to sulfate, the extent of reaction being monitored iodometrically. From 4-15 mg. of thiourea and thioacetamide were analyzed with an average standard deviation of about 0.05 mg.

INTRODUCTION

N-Bromosuccinimide (NBS) is a common reagent which has been used frequently for the bromination of organic compounds and the titrimetric determination of inorganic compounds such as antimony(III). The oxidative bond cleavage properties of NBS have also been useful in many applications.

In this laboratory the bond cleavage property of NBS has been found useful for the determination of thiourea and thioacetamide, two compounds which are frequently used for analytical determinations. Previously, Chloramine-T has been used for the quantitative determination of thiourea and
thioacetamide, but quite stringent conditions are required. It has been found that N-bromosuccinimide quantitatively oxidizes the sulphur to sulphate in a basic medium, forming the basis of a method for the standardization of thiourea and thioacetamide.

EXPERIMENTAL

Reagents

N-Bromosuccinimide - 0.2 g. of NBS was dissolved in warm, deionized water and diluted to 500 ml. The solution was standardized by a method similar to that of Bishop and Jennings against sodium thiosulphate using starch as an indicator. The solution was prepared fresh daily.

Thioacetamide and Thiourea - solutions were prepared from analytical grade reagents and standardized iodometrically.

Standard solutions of sodium thiosulphate were prepared by the usual method. All other reagents were of analytical grade.

PROCEDURE

A 5-ml. aliquot of the test solution was transferred to an iodine flask. The solution was made alkaline with 5 ml. of 1 M sodium hydroxide and then an excess volume (75 ml.) of a standard N-bromosuccinimide solution was added. The resulting solution was allowed to stand for one minute with occasional shaking, acidified with 6 N acetic acid, and 25 ml. of 10% potassium iodide solution were added. The liberated iodine was titrated with the standard sodium thiosulphate solution using starch as an indicator. The amount of thiourea or thioacetamide was calculated from the amount of...
DETERMINATION OF THIOUREA AND THIOACETAMIDE

NBS used, which is the milliequivalents of total NBS added initially minus the meq. of excess NBS titrated iodometrically.

RESULTS AND DISCUSSION

The results of a series of determinations on synthetic samples are shown in Table 1.

TABLE 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amt. taken, mg.</th>
<th>Amt. found, mg. (Avg. of 9 Results)</th>
<th>Std. Deviation mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiourea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11.15</td>
<td>11.14</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>6.32</td>
<td>6.32</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>4.72</td>
<td>4.71</td>
<td>0.03</td>
</tr>
<tr>
<td>Thioacetamide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.28</td>
<td>15.23</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>9.36</td>
<td>9.34</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>8.43</td>
<td>8.41</td>
<td>0.09</td>
</tr>
</tbody>
</table>

The quantitative oxidation of sulphur to sulphate requires, in both cases, 8 equivalents of NBS per mole of thiourea or thioacetamide. The stoichiometry is illustrated by the following half-reactions:

\[
\begin{align*}
\text{NH}_2 - \text{C} - \text{NH}_2 + 10 \text{OH}^- & \rightarrow 2\text{NH}_3 + \text{SO}_4^{2-} + \text{CO}_2 + 4\text{H}_2\text{O} + 8e^- \\
\text{CH}_3 - \text{C} - \text{NH}_2 + 11 \text{OH}^- & \rightarrow \text{CH}_3 - \text{C} - \text{O}^- + \text{NH}_3 + \text{SO}_4^{2-} + 5\text{H}_2\text{O} + 8e^- 
\end{align*}
\]

The reaction proceeds rapidly at room temperature and the results indicate that the oxidation is quantitative in both cases when approximately a 20% excess of NBS is used. Stringent
control of the solution variables is not necessary for quantitative results.

It is believed that hypobromous acid is initially formed from NBS, and that this substance is the active oxidant that converts the sulfur in thiourea and thioacetamide to sulfate. Preliminary observations indicate that other sulfur containing organic compounds, such as methionine, cysteine and cystine, are also oxidized by NBS. Results of this study will be published in the near future.

ACKNOWLEDGMENT

The authors would like to thank the National Research Council of Canada for their financial support of this research, and Professors W. J. Holland and K. G. Rutherford of the Chemistry Department of the University of Windsor for their fruitful discussions regarding this work.

REFERENCES


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CHAPTER III

N-BROMOSUCCINIMIDE AS A DIRECT TITRANT FOR
THIOUREA AND THIOACETAMIDE USING
BORDEAUX RED AS AN INDICATOR

By

R. J. Thibert and M. Sarwar,
Department of Chemistry,
University of Windsor,
Windsor, Ontario, Canada.


Running Title: Microdetermination of Thiourea and Thioacetamide
I. INTRODUCTION

Methods for the determination of thiourea and thioacetamide utilizing hypobromite, hypoiodite and chloramine-T have been reported (1-4). The great disadvantage of using these reagents is that none can be used as a primary standard. The methods, therefore, are time consuming, since they require standardization of the titrant and the reagents are not very stable. In this laboratory N-bromosuccinimide was used for the determination of various sulfur amino acids (7) and also for the indirect determination of thiourea and thioacetamide (6). An excess of N-bromosuccinimide was added to the test solution and the residual amount was back-titrated with sodium thiosulfate. Eight equivalents of the oxidant were used for the complete oxidation, but the spread of error for an individual result was large when working with smaller quantities. A need was felt to devise a direct method for thiourea and thioacetamide which is more precise and more accurate. In this investigation Bordeaux Red has been found to be a suitable indicator in the direct titration of thiourea and thioacetamide with N-bromosuccinimide. The big advantage of employing N-bromosuccinimide is that it can serve as a primary standard (7). In the present method
N-bromosuccinimide is the only standard solution required. The end point with Bordeaux Red is very sharp (it changes from a rose red color to distinct yellow). N-Bromosuccinimide is stable for several days when protected from light and kept refrigerated.

II. EXPERIMENTAL

Reagents

Thiourea and thioacetamide solutions were prepared from analytical grade reagents and standardized to check the concentrations.

N-Bromosuccinimide was prepared by dissolving 0.124 g of recrystallized reagent in deionized water and diluting to 100 ml.

Sodium bicarbonate solution, 10% in water.

Bordeaux Red solution, 0.05% in water.

All other reagents used were of analytical grade.

Procedure

An accurately measured volume (1 ml) of the test solution was taken in a 50-ml Erlenmeyer flask. To this solution, 10 ml of 10% sodium bicarbonate solution and two drops of Bordeaux Red were added. The solution was then
titrated with a standard solution of N-bromosuccinimide, added dropwise from a microburette graduated at 0.02 ml intervals. The end point was reached when the rose red color of Bordeaux Red was changed to distinct yellow by the slightest excess of N-bromosuccinimide. In another flask, the same number of drops of Bordeaux Red were added in 10 ml of sodium bicarbonate solution and titrated with N-bromosuccinimide, and this reading was subtracted from the titer before calculation.

Calculations

\[
\text{Amount of thiourea or thioacetamide in mg} = N \times E \times V
\]

\[
N = \text{Normality of N-bromosuccinimide}
\]

\[
V = \text{Volume of N-bromosuccinimide used for titer}
\]

\[
E = \text{Equivalent weight of the test substance}
\]

III. RESULTS

Table I shows the oxidation of thiourea and thioacetamide by N-bromosuccinimide.
Table I. Determination of Thiourea and Thioacetamide

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sample, mg.</th>
<th>Found, mg., avg. of 5 results</th>
<th>Standard Deviation, mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiourea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.834</td>
<td>4.818</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>2.563</td>
<td>2.554</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>1.282</td>
<td>1.282</td>
<td>0.005</td>
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<tr>
<td></td>
<td>0.641</td>
<td>0.636</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>0.321</td>
<td>0.321</td>
<td>0.004</td>
</tr>
<tr>
<td>Thioacetamide</td>
<td>5.241</td>
<td>5.229</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>4.637</td>
<td>4.621</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>3.296</td>
<td>3.289</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>1.648</td>
<td>1.637</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>0.824</td>
<td>0.817</td>
<td>0.006</td>
</tr>
</tbody>
</table>
IV. DISCUSSION

In aqueous solutions, N-bromosuccinimide hydrolyzes to form succinimide and hypobromous acid (5) which is thought to be responsible for the oxidizing properties of this reagent. The reactions using N-bromosuccinimide in the determination of thiourea and thioacetamide as shown in Table I are quantitative. At a pH of 8 the following reactions take place:

\[
\text{CH}_2\text{C} = \text{C} - \text{NH}_2 + \text{NH}_2^- + 6\text{NaHCO}_3 \rightarrow
\]

\[
\text{CH}_2\text{C} = \text{C} - \text{NH}_2 + \text{Na}_2\text{SO}_4 + 7\text{CO}_2 + 4\text{NaBr} + 2\text{NH}_3 \quad (1)
\]

\[
\text{CH}_2\text{C} = \text{C} - \text{NH}_2 + \text{CH}_3\text{C} = \text{NH}_2 + 7\text{NaHCO}_3 \rightarrow
\]
These are in agreement with oxidation by chloramine-T (1,3).

In the equations (1) and (2) it can be seen that the sulfur in thiourea and thioacetamide is in the oxidation state of -2, which on reaction with N-bromosuccinimide increases to +6 oxidation state. There is a loss of a total of 8 electrons. In N-bromosuccinimide, the bromine changes from an oxidation state of +1 to -1 with a gain of 2 electrons. When calculating the equivalent weights of the respective substances these changes of oxidation state were taken under consideration. The reactions were not quantitative at neutral or acid pH levels, unlike the case at pH 8 as shown in Table I.

The error is less than ±1% and in most cases it is even less than ±0.5%. The method is quite rapid, precise, and accurate. Small quantities of thiourea and thioacetamide can be readily standardized. N-Bromosuccinimide has been shown to be stable for at least three weeks or more depending on the concentration, when refrigerated and protected from light (7).
SUMMARY

A rapid, precise and accurate method for the determination of small amounts of thiourea and thioacetamide is described. Bordeaux Red has been found to be a suitable indicator when N-bromosuccinimide was used as direct titrant. From 5-0.3 mg. of thiourea and thioacetamide were analysed with an average standard deviation of about 0.005 mg.

ACKNOWLEDGEMENT

The financial support of the National Research Council of Canada is gratefully acknowledged.
REFERENCES


CHAPTER IV

Department of Chemistry, University of Windsor, Windsor, Ontario, Canada

Microdetermination of Sulfur Amino Acids with
N-Bromosuccinimide Using Bordeaux Red as an Indicator

By

R. J. Thibert and M. Sarwar

(Received May 29, 1968)

Introduction

Various titrimetric, colorimetric and electrometric methods are available for the quantitative determination of sulphydryl and sulfide groups. In the titrimetric procedures, numerous oxidizing agents have been used but these methods suffer from certain defects, e.g., either the oxidizing agents used are not stable or the method itself is complicated and time consuming. Sodium tetrathionate has been used by Anson to determine the —SH content of various native and denatured proteins, but it is unstable. Iodosobenzoate and porphyrindin have been used at a pH of 7 but the end points are not sharp. Iodoacetamide has also been used but the conditions must be stringently controlled. A similar procedure was used by Sampey, Slage, and Reid employing bromine water, but the main disadvantage of this method lies in the fact that bromine water is not a stable standard solution. Siggia and Edsberg have described a method for the determination of dialkyl sulfides which involves the oxidation of the sulfide to sulfoxide by means of standard bromide-bromate solution. This procedure suffers from the disadvantage of having an end point difficult to distinguish and is thus inapplicable to even slightly colored compounds. Ferricyanide, iodine, iodate, perbenzoic acid and hydrogen peroxide have also been used as oxidizing agents. In this laboratory N-bromosuccinimide has been used for the determination of sulfur amino acids. The use of this reagent for the determination of thiourea and thioacetamide was reported previously. N-Bromosuccinimide is an oxidizing agent which is sometimes very selective. It has been utilized for the preferential oxidation of tervalent antimony. In this laboratory, it was felt desirable to develop a method for the determination of sulfur amino acids which should be simple,
rapid and accurate. N-Bromosuccinimide was employed as a direct titrant using Bordeaux Red as an indicator. Comparison was also made by adding an excess of the oxidant and then back-titrating with sodium thiosulfate. Bordeaux Red was found to be superior to Crystal Violet as an indicator. N-Bromosuccinimide reacts preferentially with sulfur amino acids in the presence of Bordeaux Red. The red color of the indicator remains unchanged until all the sulfur amino acid has reacted. The slightest excess of N-bromosuccinimide added after all the amino acid has been oxidized changes the red color to a distinct yellow. A few experiments were also made to check the validity of N-bromosuccinimide as a primary standard. It has already been used as a primary standard, but no attempt has been made to establish its stability. In this work the stability of aqueous solutions of N-bromosuccinimide has also been investigated.

**Experimental**

**Reagents**

L-Cysteine hydrochloride: Standard solutions were prepared by dissolving an appropriate amount (National Research Council, Analytical Grade – Assay 637) in deionized water.

L-Methionine: Standard solutions were prepared by dissolving an appropriate amount (National Research Council, Analytical Grade – Assay 720) in deionized water.

L-Cystine: This was prepared by dissolving an appropriate amount of L-Cystine (National Research Council, Analytical Grade – Assay 681) in 0.1 N hydrochloric acid.

The above amino acids were purchased from General Biochemicals, Chagrin Falls, Ohio.

Standard solution of N-bromosuccinimide: 0.164 g recrystallized reagent was dissolved in deionized water and diluted to 100 ml.

Bordeaux Red: 0.05% solution in water.

Crystal Violet: 0.04% solution in water.

Sodium thiosulfate: 0.1 N solution.

All other reagents used were of analytical grade.

**Procedure**

An accurately measured volume (1 ml) of the test solution was taken in a 50-ml Erlenmeyer flask. Two drops of Bordeaux Red indicator were added. The solution was titrated with standard N-bromosuccinimide, added dropwise from a microburette graduated at 0.02 ml intervals. The end point was reached when the red color of the Bordeaux Red was changed from red to distinct yellow by the slightest excess of N-bromo-
succinimide. In another flask, the same number of drops of Bordeaux Red were added in acid medium and titrated with N-bromosuccinimide, and this reading was subtracted from the titer before calculation. In case of L-cystine, when the red color of the indicator faded, another drop of the indicator was added and the titration continued until the red color changed into distinct yellow by the addition of a single drop of N-bromosuccinimide.

**Calculations**

Amount of sulfur amino acid $\text{mg} = N \times V \times E$.

$N = \text{Normality of N-bromosuccinimide}$;

$V = \text{Volume of N-bromosuccinimide used for titration}$;

$E = \text{Equivalent weight of the amino acid}$.

In the case of Crystal Violet the procedure was exactly the same as described above. In the back titration a known excess (20%) of N-bromosuccinimide was added to the test solution and after 60 seconds the excess N-bromosuccinimide was back-titrated iodometrically. The amount of N-bromosuccinimide consumed for the reaction was then calculated.

**Results and Discussion**

The data given in all the tables for each experiment is the mean of three results. In Table I it is shown that N-bromosuccinimide can be used as a primary standard. Table II shows the stability of N-bromosuccinimide as a function of time and concentration. The results for the determination of three sulfur amino acids are given in Table III.

In aqueous solutions, N-bromosuccinimide decomposes to form succinimide and hypobromous acid which is thought to be responsible for the oxidizing properties of this reagent. The reactions using N-bromosuccinimide in the determination of sulfur amino acids are quantitative as shown in Table III. With bromine oxidation of such compounds the formation of sulfonic acids is proposed and the results reported here are in agreement with this. In the case of methionine the formation of sulfoxide is thought to occur. This is in agreement with Siggia and Edsberg who claimed the formation of sulfoxide when compounds of the type $R-S-R$ were oxidized with bromate–bromide mixture. The

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample mg</th>
<th>Found</th>
<th>Deviation</th>
</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>2</td>
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<td>2.399</td>
<td>+ 0.001</td>
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<tr>
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<td>1.964</td>
<td>0.000</td>
</tr>
<tr>
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<td>1.609</td>
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</tr>
<tr>
<td>6</td>
<td>1.391</td>
<td>1.391</td>
<td>0.000</td>
</tr>
<tr>
<td>7</td>
<td>1.226</td>
<td>1.226</td>
<td>0.000</td>
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</table>
Table II. Stability of N-bromosuccinimide

<table>
<thead>
<tr>
<th>No. of Days</th>
<th>Volume of 0.1 N Sodium Thiosulfate ml*</th>
<th>Volume of 0.1 N Sodium Thiosulfate ml**</th>
<th>Volume of 0.01 N Sodium Thiosulfate ml***</th>
</tr>
</thead>
<tbody>
<tr>
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<td>4.00</td>
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<tr>
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<td>0.92</td>
<td>4.00</td>
</tr>
<tr>
<td>3</td>
<td>3.24</td>
<td>0.92</td>
<td>4.00</td>
</tr>
<tr>
<td>4</td>
<td>2.22</td>
<td>0.92</td>
<td>4.00</td>
</tr>
<tr>
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<td>4.00</td>
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<td>4.00</td>
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<td>10</td>
<td>3.01</td>
<td>0.90</td>
<td>4.00</td>
</tr>
<tr>
<td>11</td>
<td>3.00</td>
<td>0.89</td>
<td>4.00</td>
</tr>
<tr>
<td>12</td>
<td>3.00</td>
<td>0.86</td>
<td>4.00</td>
</tr>
</tbody>
</table>

Constant up to 23 days

* 5 ml of 2.8836 g/liter solution titrated with 0.1 N sodium thiosulfate.
** 5 ml of 1.6376 g/liter solution titrated with 0.1 N sodium thiosulfate.
*** 5 ml of 0.712 g/liter solution titrated with 0.01 N sodium thiosulfate.

decarboxylation of amino acids has already been studied by Chapelle and Luck who determined amino acids by measuring the carbon dioxide liberated when they were treated with N-bromosuccinimide.

Table III. Microdetermination of sulfur amino acids

<table>
<thead>
<tr>
<th>Sulfur Amino Acid</th>
<th>No. of Experiments</th>
<th>Range mg</th>
<th>Deviation of % Back-titration</th>
<th>Deviation % Direct titration with N-bromosuccinimide using Crystal Violet as an Indicator</th>
<th>Deviation % Direct titration with N-bromosuccinimide using Bordeaux Red as an Indicator</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Cysteine Hydrochloride</td>
<td>10</td>
<td>0.54-12.31</td>
<td>— 3.70 to + 3.28</td>
<td>— 1.73 to + 1.85</td>
<td>— 0.80 to + 0.86</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>10</td>
<td>0.76-14.73</td>
<td>— 3.40 to + 1.87</td>
<td>— 1.42 to + 1.70</td>
<td>— 0.85 to + 0.10</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>10</td>
<td>1.83-15.79</td>
<td>— 2.30 to + 3.50</td>
<td>— 1.17 to + 1.18</td>
<td>— 0.79 to + 0.69</td>
</tr>
</tbody>
</table>
When N-bromosuccinimide reacts with L-cysteine, L-methionine and L-cystine in aqueous medium the following reactions possibly take place.

\[
\begin{align*}
6 \text{NBr} + 6 \text{H}_2\text{O} + 2 (\text{H} - \text{S} - \text{CH}_2 - \text{C} - \text{H}) & \rightarrow \\
& \text{COOH}
\end{align*}
\]

(1)

\[
\begin{align*}
6 \text{NH} + 2 (\text{H}_2\text{O} - \text{CH}_2 - \text{CH}_2 - \text{NH}_2) & \rightarrow \\
& + 2 \text{CO}_2 + 6 \text{HBr}
\end{align*}
\]

(2)

\[
\begin{align*}
5 \text{NBr} + 6 \text{H}_2\text{O} + [\text{S} - \text{CH}_3 - \text{C} - \text{COOH}]_2 & \rightarrow \\
& \text{NH}_2
\end{align*}
\]

(3)

\[
\begin{align*}
5 \text{NH} + 2 (\text{H}_2\text{O} - \text{CH}_2 - \text{CH}_2 - \text{NH}_2) & \rightarrow \\
& + 2 \text{CO}_2 + 5 \text{HBr}
\end{align*}
\]
As can be seen from the equations (1), (2), and (3), the cysteine sulfur goes from $-2$ oxidation state to $+4$, with a loss of a total of 6 electrons. In the case of methionine the sulfur goes from $-2$ to zero with the loss of 2 electrons. In cystine there are two sulfur atoms whose oxidation state changes from $-1$ to $+4$, with the loss of 10 electrons. The equivalent weights of the respective substances were calculated taking these changes into consideration. The equivalent weight of N-bromosuccinimide was calculated considering the fact the bromine undergoes a change of oxidation state of $+1$ to $-1$. N-Bromosuccinimide has been used as a primary standard and for comparative purposes it was standardized\textsuperscript{15}, but there was no significant difference, as is clear from Table I, between the directly weighed sample and the standardized one. It has already been used as a primary standard\textsuperscript{18} but in the study reported here the comparative runs were made to check the validity of its use as a primary standard. In previous studies\textsuperscript{15,18} freshly prepared solutions of N-bromosuccinimide were used, but in the present investigation it is clear from Table II that solutions of N-bromosuccinimide protected from light and kept in refrigerator are stable over a period of several days. When an excess of N-bromosuccinimide was used and then the residual amount back-titrated iodometrically, it is found that in all the cases the error is more than $\pm 3\%$, and when directly titrated with N-bromosuccinimide using Crystal Violet as an indicator the error was more than $\pm 2\%$. When Bordeaux Red was used as an indicator the error was much less and the end point was very sharp. The error in this case is less than $\pm 1\%$ and in most cases it is even less than $\pm 0.5\%$. It has been found that aqueous solutions of N-bromosuccinimide of low concentrations are more stable than those of higher concentrations.

Acknowledgements

The authors gratefully acknowledge financial support from the National Research Council of Canada, and also wish to express their appreciation to Professor W. J. Holland of the Chemistry Department, University of Windsor, for his many helpful discussions regarding this investigation.

Summary

**Microdetermination of Sulfur Amino Acids with N-Bromosuccinimide Using Bordeaux Red as an Indicator**

A rapid and convenient method for the determination of sulfur amino acids is described. N-Bromosuccinimide is used as a direct titrant using Bordeaux Red as an indicator. The red color of the indicator changes to distinct yellow with a sharp end point. The error is less than $\pm 0.5\%$ in most cases, and in a few cases it is near $\pm 1\%$. Comparison of three
methods has been made: back-titration; direct method using either
Crystal Violet or Bordeaux Red as indicators. Bordeaux Red proved
to be the more suitable end point indicator. Aqueous solutions of N-bromo-
succinimide are stable over a period of several days when kept refrigerated
and protected from light.

Zusammenfassung

Eine zweckdienliche Methode zur Bestimmung schwefelhaltiger Amino-
säuren wurde beschrieben. N-Bromsuccinimid dient als Maßlösung, Bordeaux-
rot als Indikator, dessen rote Farbe scharf nach gelb umschlägt. Der Fehler
ist meist kleiner als $\pm 0.5\%$, in wenigen Fällen ungefähr $\pm 1\%$. Vergleichs-
weise wurden erprobt: 1. die Rücktitration; 2. direkte Titration mit Kristall-
violett oder Bordeauxrot. Letzteres hat sich besser bewährt. Wässrige
Lösungen von N-Bromsuccinimid sind in der Kälte vor Licht geschützt
einige Tage haltbar.

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CHAPTER V

THE SIMULTANEOUS DETERMINATION OF CYSTEINE
AND CYSTINE USING N-BROMOSUCCINIMIDE; APPLICATION
IN AN ENZYMATIC SYSTEM - CYSTINE REDUCTASE

By


Introduction

Various titrimetric, colorimetric, amperometric, and polarographic methods for the quantitative determination of sulfhydryl groups are available. Titrimetric procedures, using suitable oxidizing agents, are frequently employed, but they often suffer from numerous defects, e.g., either the oxidizing agents used are not stable or the oxidation goes beyond the disulfide stage. Sodium tetrathionate has been used by Anson for the determination of sulfhydryl content, but the method is time consuming and sodium tetrathionate is not a stable compound. When o-iodosobenzoate is used for the determination of sulfhydryl groups, it is added in excess, and the residual amount is determined iodometrically. It is essential that the excess be controlled carefully to get reliable results. The oxidation might occur to sulfinic or sulfonic acids leading to high results. Iodine and iodate have also been employed. Iodine solutions are not stable and consequently must be repeatedly standardized. These procedures suffer from the disadvantage of having an endpoint difficult to distinguish and thus are inapplicable to even slightly colored compounds.

Serious difficulties arise in the determination of cysteine and cystine when present together in certain systems.
No method is available for their simultaneous determination. N-Bromosuccinimide is an excellent oxidizing agent and has been used previously in this laboratory for various determinations. Sulphur amino acids were determined using Bordeaux Red as an indicator. It is also known that N-bromosuccinimide is often selective in its reactions.

In this investigation N-bromosuccinimide has been used for the selective oxidation of iodide to iodine which in turn oxidizes cysteine to cystine at pH 7. The same reagent is used to determine cystine using Bordeaux Red as an indicator. The simultaneous determination of cysteine and cystine was also performed. N-Bromosuccinimide is the only reagent used for these determinations, and it can be used as a primary standard.

The method was applied to the determination of cysteine produced from the reduction of cystine by the enzyme cystine reductase (EC 1.6.4.1). Previous methods used to detect cystine in this enzymatic system have centered mainly around the use of iodine or iodate. These methods only determine the amount of cysteine present at any specific time, but the remaining cystine was not determined by an independent method. In this investigation, cysteine and cystine were simultaneously determined at various time...
intervals. A comparative study was also made using both potassium iodate and N-bromosuccinimide to follow the course of the enzymatic reduction of cystine. Values determined by potassium iodate are high while N-bromosuccinimide gives comparatively true results.

The method permits the simultaneous determination of cysteine and cystine in pure solutions and also allows the monitoring of the cysteine levels during the catalytic reduction of cystine by the enzyme cystine reductase.

Experimental

Reagents

L-Cysteine hydrochloride: Standard solutions were prepared by dissolving an appropriate amount (National Research Council, Analytical Grade - Assay 637) in deionized water.

L-Cystine: Standard solutions were prepared by dissolving L-cystine (National Research Council, Analytical Grade - Assay 681) in 0.1N hydrochloric acid. The above amino acids were purchased from General Biochemicals, Chagrin Falls, Ohio.

Standard solution of N-bromosuccinimide: 0.175 g recrystallized reagent was dissolved in deionized water and diluted to 100 ml.
Bordeaux Red: 0.05 per cent (w/v) solution in deionized water.

Potassium iodide: 10 per cent (w/v) solution.

Buffers: phosphate buffers (0.05 and 0.025 M) of appropriate pH.

Starch solution: one per cent (w/v) starch solution.

Potassium iodate: a 0.001N solution was prepared from recrystallized and dried analytical grade reagent.

Phospho–24-tungstic acid: a 10 per cent (w/v) solution in 5 per cent HCl (v/v) was used to stop the enzymatic reaction.

Enzymatic suspension: prepared from bakers' yeast obtained from the Fleischmann Company in the form of pressed cakes. An acetone powder was made according to the procedure as given by Umbreit, Burris and Stauffer. A 20 per cent (w/v) suspension of the acetone powder in 0.025 M phosphate buffer (pH 6.2) was stirred for 30 minutes on a magnetic stirrer at 25°C and used as the enzyme suspension.

All other reagents used were of analytical grade.

Procedure

Determination of cysteine

An accurately measured volume (1 ml) of the test solution was placed in a 150-ml Erlenmeyer flask with 10 ml
of 10 per cent potassium iodide solution and 1 ml of starch solution. Sufficient buffer (30 ml) was added to maintain a pH of 7 during the titration. The solution was then titrated with standard N-bromosuccinimide added dropwise from a microburette graduated at 0.02-ml intervals. The end point was reached when the first blue color appeared and persisted for 30 seconds.

**Determination of cystine**

Another aliquot of the test solution was taken in a 150-ml Erlenmeyer flask and two drops of Bordeaux Red were added as an indicator. The solution was then titrated with standard N-bromosuccinimide. When the red color of the indicator faded, another drop of the indicator was added. The titration was continued until the red color of the indicator changed into a distinct yellow color by the addition of a single drop of N-bromosuccinimide. The same number of drops of Bordeaux Red, used in the titration, were titrated with N-bromosuccinimide and this volume was subtracted from the titer before calculations.

**Cysteine from cystine in an enzymatic system**

In the enzymatic system, eight 125-ml Erlenmeyer flasks each contained 0.025 M phosphate buffer (pH 6.2), $2 \times 10^{-3}$ M cystine, and 20 ml of enzyme suspension. The cystine was
dissolved in 0.5 N HCl and an equal volume of 0.5 N NaOH was added to neutralize the acid. The final volume of the mixture was 30 ml. The enzyme was added last, the flasks tightly stoppered, and placed in a water bath at 37 ± 1°C for the appropriate time interval. Eight flasks containing no cystine served as controls. The reaction was stopped and the proteins precipitated by the addition of 4 ml of 10 per cent phospho-24-tungstic acid in 5 per cent HCl. The mixtures were centrifuged at 10,000 rev./min. for 10 minutes and the clear, yellow supernatant used for titration. Aliquots (5 ml) were taken from both the controls and the experimental flasks and titrated for cysteine using N-bromosuccinimide and also with potassium iodate. Values for the controls were subtracted from the experimental values before calculation of cysteine according to equation (1). The method for the determination with N-bromosuccinimide consisted of adding 30 ml of 0.05 M phosphate buffer (pH 7.0) to the 5-ml aliquot, 10 ml of a 10 per cent solution of potassium iodide, 1 ml of a 1 per cent starch solution, and titrating with 0.001N N-bromosuccinimide to a blue endpoint which persists for 30 seconds.

The method used for the determination of cysteine with potassium iodate was essentially the same as that of Woodward.
and Pry. No buffer was added in the case of potassium iodate since iodine is only liberated in an acid medium. One ml of 5 per cent HCl was added to lower the pH to 2. On a third aliquot, cystine was determined using N-bromosuccinimide with Bordeaux Red as the indicator. To a one-ml aliquot was added 20 ml of deionized water and two drops of Bordeaux Red. Buffer was not necessary as the method is independent of pH. The solution was then titrated with standard N-bromosuccinimide until the last drop just caused the red color to turn yellow. The controls were titrated in the same manner and the values subtracted from the experimental titers before calculations of cystine according to equation (2).

Calculations

Cysteine titration employing the N-bromosuccinimide displacement of iodine

\[
\text{mg cysteine} = \text{N} \times \text{V} \times \text{E}
\]

(1)

\(\text{N} = \text{Normality of N-bromosuccinimide.}\)

\(\text{V} = \text{Volume (ml) of N-bromosuccinimide used for titration.}\)

\(\text{E} = \text{Equivalent weight of cysteine (mol. wt./l).}\)

N-Bromosuccinimide titration of cystine and cysteine mixture to the Bordeaux Red end point

\[
\text{mg cystine} = \text{N}(\text{V}_2 - \text{V}_1)\text{E}_2
\]

(2)

\(\text{N} = \text{Normality of N-bromosuccinimide.}\)

\(\text{V}_2 = \text{Volume (ml) of N-bromosuccinimide required to titrate cysteine and cystine to the Bordeaux Red end point.}\)
\( V_1 = \text{Calculated volume (ml) of N-bromosuccinimide required to titrate only the cysteine to the Bordeaux Red end point.} \)

\[
\text{Milligrams of cysteine from iodine displacement titration} = \frac{N \times E_1}{N} \quad (3)
\]

\( N = \text{Normality of N-bromosuccinimide.} \)

\( E_1 = \text{Equivalent weight of cysteine (mol. wt./6).} \)

\( E_2 = \text{Equivalent weight of cystine (mol. wt./10).} \)

**Results and Discussion**

The simultaneous determination of cysteine and cystine was carried out in pure solutions. Table I shows the pH effect on the determination of cysteine by an iodine displacement titration. The results indicate that at pH 7 the determination of cysteine is quantitative. Table II shows the determination of various concentrations of cysteine alone at pH 7. The data in Table III illustrates the determination of cysteine in the presence of large amounts of cystine. The simultaneous determination of cysteine and cystine is shown in Table IV. Table V provides the data for the simultaneous determination of cysteine and cystine in an enzymatic system. Figure I represents the increase of cysteine concentration as a function of time due to the catalytic reduction of cystine by the enzyme cystine reductase.
Table I. The pH effect on the determination of cysteine

Details are given in the text. Every value is the average of five determinations.

<table>
<thead>
<tr>
<th>pH</th>
<th>Mg cysteine taken</th>
<th>Mg cysteine found</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
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<td>0.06</td>
</tr>
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<td>4</td>
<td>3.18</td>
<td>3.76</td>
<td>0.13</td>
</tr>
<tr>
<td>5</td>
<td>3.18</td>
<td>3.65</td>
<td>0.07</td>
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<td>4.79</td>
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<td>8.46</td>
<td>0.15</td>
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</table>

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Table II. The Determination of various concentrations of cysteine at pH 7

Details are given in the text.

<table>
<thead>
<tr>
<th>Sample mg</th>
<th>Found mg</th>
<th>Deviation per cent</th>
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</tr>
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<td>13.06</td>
<td>13.02</td>
<td>-0.36</td>
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</table>
Table III. The determination of cysteine in the presence of cystine
Details are given in the text.

<table>
<thead>
<tr>
<th>Mg cysteine</th>
<th>Cystine added</th>
<th>Cysteine found</th>
<th>Deviation per cent</th>
</tr>
</thead>
<tbody>
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<td>1.18</td>
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<td>4.71</td>
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<tr>
<td>11.83</td>
<td>63.24</td>
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<tr>
<td>11.83</td>
<td>1.32</td>
<td>11.84</td>
<td>+0.09</td>
</tr>
<tr>
<td>11.83</td>
<td>2.64</td>
<td>11.80</td>
<td>-0.26</td>
</tr>
</tbody>
</table>
Table IV. The simultaneous determination of cysteine and cystine. Details are given in the text. Cysteine and cystine were each determined on separate aliquots from the sulfur amino acid mixture.

<table>
<thead>
<tr>
<th>Sulfur amino acid mixture taken</th>
<th>Sulfur amino acid found</th>
<th>% Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine mg</td>
<td>Cystine mg</td>
<td>Cysteine mg</td>
</tr>
<tr>
<td>1.27</td>
<td>5.27</td>
<td>1.28</td>
</tr>
<tr>
<td>2.55</td>
<td>2.63</td>
<td>2.54</td>
</tr>
<tr>
<td>2.55</td>
<td>5.27</td>
<td>2.54</td>
</tr>
<tr>
<td>3.18</td>
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<td>15.81</td>
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<td>10.1</td>
</tr>
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<td>15.87</td>
<td>1.32</td>
<td>15.80</td>
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<tr>
<td>15.87</td>
<td>2.63</td>
<td>15.78</td>
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</table>
Table V. The simultaneous determination of cysteine and cystine in an enzymatic system

Details are given in the text. Each value is the average of five titrations.

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Cystine taken (µg/ml)</th>
<th>Cysteine found (µg/ml) NBS*</th>
<th>Cystine found (µg/ml) KIO₃</th>
<th>Cystine found (µg/ml) NBS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>457</td>
<td>5</td>
<td>12</td>
<td>436</td>
</tr>
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<td>10</td>
<td>457</td>
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<td>30</td>
<td>457</td>
<td>214</td>
<td>265</td>
<td>229</td>
</tr>
<tr>
<td>60</td>
<td>457</td>
<td>392</td>
<td>478</td>
<td>48</td>
</tr>
<tr>
<td>90</td>
<td>457</td>
<td>463</td>
<td>588</td>
<td>2</td>
</tr>
</tbody>
</table>

*NBS stands for N-Bromosuccinimide*
Legend to Figure I

The determination of cysteine from cystine as a function of time in an enzymatic system

The procedure is given in the text.

- Cysteine determined with N-bromosuccinimide as titrant

▲ Cysteine determined with potassium iodate as titrant.

--- Theoretical cysteine possible.
In aqueous solution, N-bromosuccinimide hydrolyzes to form succinimide and hypobromous acid \(^{32}\) which is thought to be responsible for the oxidizing properties of this compound. The reactions using N-bromosuccinimide in the determination of sulfur amino acids are quantitative \(^{22}\). N-Bromosuccinimide is often selective in its reactions \(^{22-25}\), and this property has been utilized in this investigation.

In the determination of cysteine, 10 per cent potassium iodide was added to the test solution and the pH was adjusted to 7. When titrated with N-bromosuccinimide, iodine is liberated preferentially from potassium iodide and then reacts with cysteine. The reaction is as shown below:

\[
2\text{NH}_2\text{H} - 2\text{COOH} + \text{I}_2 \rightarrow (\text{-S-CH}_2\text{-C-H})_2 + 2\text{HI} \quad (1)
\]

It has been found that at pH 7 the oxidation of cysteine (R-SH) does not go beyond cystine (R-S-S-R). N-Bromosuccinimide, used as a primary standard, can serve to liberate iodine from potassium iodide quantitatively at pH 7. Potassium iodate cannot be used at pH 7, and if used at low pH values, oxidizes the RSH beyond the R-S-S-R stage as shown in Table V and Fig. I.

When cysteine and cystine are directly titrated using
N-bromosuccinimide with Bordeaux Red as the indicator, the titrant reacts preferentially with the sulfur amino acids. The color of the Bordeaux Red, therefore, remains unchanged until all the sulfur amino acid has reacted. The slightest excess of N-bromosuccinimide then changes the color of the Bordeaux Red to a distinct yellow. The reactions of N-Bromosuccinimide with cysteine and cystine are as follows:

\[
\begin{align*}
6 \text{N-Br} + 6\text{H}_2\text{O} &+ 2(\text{H-S-CH}_2\text{-C-H}) \rightarrow \text{COOH} \\
6 \text{NH} &+ 2(\text{HO}_3\text{S-CH}_2\text{-CH}_2\text{-NH}_2) + 2\text{CO}_2 + 6\text{HBr} \\
5 \text{NBr} + 6\text{H}_2\text{O} &+ [\text{-S-CH}_2\text{-C-COOH}]_2 \rightarrow \\
5 \text{NH} &+ 2(\text{HO}_3\text{S-CH}_2\text{-CH}_2\text{-NH}_2) + 2\text{CO}_2 + 5\text{HBr}
\end{align*}
\]
From equations (4) and (5) it can be seen that cysteine sulfur goes from an oxidation state of -2 to -1 with the loss of one electron. In equation (5), the sulfur changes its oxidation state from -2 to +4 with a loss of 6 electrons. In cystine there are two sulfur atoms which change their oxidation state from -1 to +4 with a loss of 10 electrons. This is shown in equation (6). In N-bromosuccinimide, the oxidation state of bromine goes from +1 to -1 with the gain of 2 electrons. The equivalent weights of the respective substances were calculated taking these changes into consideration.

N-Bromosuccinimide has been used as a primary standard\textsuperscript{22-24}. It has been shown that N-bromosuccinimide is stable for several days when protected from the light and refrigerated\textsuperscript{22}. This is the only standard solution required in the simultaneous determination of cysteine and cystine. As can be seen from Tables III, IV, and V, cysteine and cystine can be determined in the presence of each other with reasonable accuracy. The error in pure solutions is less than \( \pm 1 \) per cent.

Table V points out the fact that when N-bromosuccinimide is used as a titrant for the enzymatic determination of cysteine as a function of time, the values of cysteine and cystine add up to the amount of cystine originally put in within reasonable accuracy. The comparative study with
potassium iodate as titrant indicates relatively high results. As is shown in Fig. I, after 57 minutes of catalysis the amount of cysteine, as titrated with potassium iodate, exceeds that theoretically possible. Potassium iodate and other methods can measure the appearance of cysteine but these values may not be the true values since none of these methods determine residual cystine. Lucas and King point out that iodine titrations for the determination of cysteine are very pH dependent and it is not known at which pH the values are correct, if at any. This study has shown that at pH 2 the results with potassium iodate are indeed incorrect while those with N-bromosuccinimide appear to be quantitative.

Summary

1. A new, rapid, convenient, and accurate method for the simultaneous determination of cysteine and cystine has been developed for use in pure solutions as well as in the enzymatic system, cystine reductase. N-Bromosuccinimide is the only standard solution employed in these determinations. Cysteine was determined at pH 7 by displacement of iodine from potassium iodide with N-bromosuccinimide. The total cysteine plus cystine was determined by N-bromosuccinimide using Bordeaux Red as an indicator. A comparative study to determine cysteine using N-bromosuccinimide and potassium iodate was performed.
2. It has been found that the values obtained with potassium iodate in the enzymatic system are relatively high. N-Bromo-succinimide gives more accurate results as shown by determining the residual amount of cystine after a particular time of incubation. When N-bromosuccinimide is used as a titrant for cysteine and/or cystine in pure solutions the error is less than ± 1 per cent. When N-bromosuccinimide is used as a titrant in the enzymatic system for the determination of cystine, the method is more precise and more accurate when compared to the use of potassium iodate.

Acknowledgments

The authors gratefully acknowledge financial support from the National Research Council of Canada, and also wish to express their appreciation to Mr. R.J. Walton of the Chemistry Department, University of Windsor, for his helpful discussions regarding this investigation.
References


DISTRIBUTION STUDIES OF PALLADIUM IN

POA PRATENSIS L.
CHAPTER I

EFFECT OF PALLADIUM CHLORIDE ON
THE GROWTH OF *POA PRATENSIS* L.

M. Sarwar and R.J. Thibert
Department of Chemistry,
and
W.G. Benedict
Department of Biology,
University of Windsor,
Windsor, Ontario, Canada.
Abstract

The effect of palladium chloride on the growth of Kentucky bluegrass (*Poa pratensis* L.) was investigated. A pH of 5.5 was optimum for its growth in various concentrations of PdCl$_2$. Minute quantities of palladium (50 µg-11,500 µg/tray) stimulated growth of Kentucky bluegrass, whereas, high doses (115,000 µg-575,000 µg/tray) were toxic.
Introduction

Major reasons for investigating trace elements in plants are to improve yields and to increase the kinds of plants that can be grown in a given deficient environment. Other reasons involve the transfer of trace elements for nutrient purposes from the soil to animals and man via edible plants. Diets frequently reflect the importance of the trace mineral content of staple food plants (1-4).

Various studies on the importance of macro- and micro-nutrients for plants have been reported, and if one examines these, one finds that few elements of the periodic chart have been neglected. However, one of the elements studied for which there are very few references is palladium. These few studies have been made on the physiological, pharmacological, and toxicological effects of palladium in animals and humans (5-10). For example, Meek et al. (5) performed tests with palladium on human skin to study its toxic effects, and also injected palladium chloride subcutaneously and intravenously to determine toxic levels, but their method of palladium determination in tissues is tedious and has many interfering factors. Ascoli and Orestano (6-8) have also studied the toxic effects of palladium in humans and rabbits but the
descriptions of their methods are rather incomplete. Howarth and Cooper studied the toxic effects of colloidal palladium in cats (9). Colloidal palladium was injected intravenously into the cats and the distribution and level of palladium was followed in the animal's blood by use of radioactive tracers.

Even fewer studies have been made on the effects of palladium in plants. Somers (10) observed the toxic effects of palladium on fungi and correlated the toxicity with the electronegative response of the metal. Brenchley (11) also used palladium on plants in a very limited way to study its toxicity.

We felt that an investigation of the physiological, pathological, and toxic effects of palladium on plants was possible. A method that determined the amount of palladium present at a certain time in biological systems already has been reported from this laboratory (12). The present report is a study of the uptake of palladium by Poa pratensis L. and its effects on the plant grown under controlled environmental conditions. Factors governing the uptake are discussed.
Materials and Methods

**Palladium**

This element was purchased from Fisher Scientific Company, Ltd. and was used as palladium chloride ($\text{PdCl}_2$). An appropriate amount of the compound was dissolved in concentrated hydrochloric acid and then diluted to the desired volume for use in the experiment. Before use in plants the pH of the various solutions of palladium chloride was adjusted with sodium carbonate.

**Kentucky bluegrass (Poa pratensis L.)**

This bluegrass was selected as the experimental plant because the seed is readily available and seedlings can be easily grown. A complete description of the plant is given in Gray's Manual (13).

**Growth of plants**

Locally constructed plant growth chambers (14) provided day and night temperatures of 26.7°C and 22.2°C, respectively. A relative humidity of 80% and a 14-hour day of 32,000 lumens per m$^2$ at plant height was maintained. The seedlings were grown in 20-cm$^2$ plastic trays and filled 5-cm deep with crumb perlite that was sterilized, pressed down, and watered. Three grams of seed were then sown on the perlite in each tray.
The trays were covered with wet paper towels until the seed germinated. Germination was 17% which gave a good stand of seedlings.

**Nutrient solution**

After the seed had germinated, which was about one week after sowing, the first 150 ml of nutrient solution was added to each tray. The nutrient solution required the preparation of stock solutions A and B, according to Ranson (15).

Solution A:

<table>
<thead>
<tr>
<th>Substance</th>
<th>ml/liter of nutrient solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>M KH$_2$ PO$_4$</td>
<td>1</td>
</tr>
<tr>
<td>M KNO$_3$</td>
<td>5</td>
</tr>
<tr>
<td>M Ca(NO$_3$)$_2$</td>
<td>5</td>
</tr>
<tr>
<td>M Mg SO$_4$</td>
<td>2</td>
</tr>
</tbody>
</table>
Solution B:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Grams dissolved/liter of water</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{H}_3\text{BO}_3$</td>
<td>2.86</td>
</tr>
<tr>
<td>$\text{MnCl}_2\cdot\text{H}_2\text{O}$</td>
<td>1.81</td>
</tr>
<tr>
<td>$\text{Zn SO}_4\cdot7\text{H}_2\text{O}$</td>
<td>0.22</td>
</tr>
<tr>
<td>$\text{Cu SO}_4\cdot5\text{H}_2\text{O}$</td>
<td>0.08</td>
</tr>
<tr>
<td>$\text{H}_2\text{Mo O}_4\cdot\text{H}_2\text{O}$</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Final Solution:

To each liter of solution A, 1 ml of solution B was added. Then 1 ml of iron tartrate, 0.5% (w/v), was added to each liter of final solution. The pH of this solution was adjusted to 6 by adding 0.1M $\text{H}_2\text{SO}_4$. All chemicals used were of analytical grade.

Treatment of month-old seedlings with palladium chloride

Trays which showed uniform growth were selected and arranged in a random design in the growth chamber. According to the requirements of the experiment, the appropriate amount of palladium chloride, in micrograms, was mixed with 150 ml of nutrient solution, adjusted to the desired pH, and added to the tray. Additional solution was added to the trays every three days.
After two months of growth, the height of the grass in each tray was measured, harvested, and weighed immediately. Then it was wrapped in wax paper, labelled according to treatment, and stored in a cold room at -18°C prior to analysis. The roots were carefully removed from the perlite in each tray by washing in tap water and in 0.01 N HCl. The roots were then dried in an oven at 80°C for five hours. After the dried roots from each tray were weighed, they were stored at room temperature to await analysis.

**Uptake of palladium**

In order to determine the uptake in palladium-treated grass, certain trays of variously treated and control grass were cut each week during the four weeks of the second month of growth. The grass was handled in the manner described above. All analyses for palladium followed the method reported by Thibert and Sarwar (12) using a Beckman Model DU Spectrophotometer with Gilford Model 220 absorbance indicator, Gilford Model 210 automatic cuvet positioner and a temperature control unit. The pH measurements were made with a Fisher Accument pH meter Model 19 by emersing the microelectrodes into the trays.
The three parameters investigated in the analyses for palladium uptake in Kentucky bluegrass grown in nutrient solution with palladium chloride added were: different ambient pH and \( \text{PdCl}_2 \) in fixed amount; different constant pH and \( \text{PdCl}_2 \) in fixed amount; maintained optimum pH and \( \text{PdCl}_2 \) in various amounts.

Results and Discussion

**Experiment 1**

The effect that germinating Kentucky bluegrass seeds had on an initial pH of 4, 5 and 6 of the nutrient medium with 2920 \( \mu \text{g} \) palladium chloride added per tray is shown in Table I. During the first five days the average change in pH was 0.4 units.

**Experiment 2**

The effect that a constant pH of 4 to 9 of the nutrient medium with added palladium chloride had on the growth of Kentucky bluegrass is shown in Table II. In this experiment the pH was adjusted daily as required with either 0.1 N HCl or M \( \text{Na}_2\text{CO}_3 \) solution. Analyses for palladium showed that about 16 \( \mu \text{g/g} \) of roots could be found in plants grown at pH 5 and 6. No trace of the element could be detected in...
TABLE I

Effect of germinating Kentucky bluegrass seed on the pH of the nutrient medium with added palladium chloride

<table>
<thead>
<tr>
<th>Days after seeding on perlite</th>
<th>(nutrient medium and PdCl$_2$ added*)</th>
<th>pH of nutrient medium**</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
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<td>6.2</td>
</tr>
<tr>
<td>4</td>
<td>5.7</td>
<td>6.5</td>
</tr>
<tr>
<td>5</td>
<td>6.0</td>
<td>6.9</td>
</tr>
</tbody>
</table>

* 2920 μg PdCl$_2$/tray.

** pH ambient.
TABLE II

Effect of pH on the growth of Kentucky bluegrass in nutrient medium with added palladium chloride

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH of nutrient medium</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
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<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>did not grow</td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td></td>
<td>5</td>
<td>27.5</td>
<td>25.0</td>
<td>7.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
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<td>nil</td>
<td>45</td>
<td>43</td>
<td>nil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PdCl&lt;sub&gt;2&lt;/sub&gt; (2920 μg)</td>
<td></td>
<td>2.5</td>
<td>35.0</td>
<td>32.5</td>
<td>7.2</td>
<td>did not grow</td>
<td></td>
</tr>
<tr>
<td>Height (cm)</td>
<td>nil</td>
<td>nil</td>
<td>59</td>
<td>52</td>
<td>nil</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>nil</td>
<td>nil</td>
<td>16.8</td>
<td>15.9</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> 2920 μg PdCl<sub>2</sub>/tray.

<sup>b</sup> pH kept constant.

All weight and height measurements are based on the average values for two trays.

Palladium analyses were performed in duplicate on each tray used in the experiments.
the leaves even though the grass grew 35 cm high and weighed 59 gm in a tray treated with palladium chloride at pH 5. This experiment showed that Kentucky bluegrass could be successfully grown only at pH 5 and 6. Addition of 2920 µg PdCl₂ per tray of grass did not affect its growth adversely.

**Experiment 3**

The effect that an ambient pH of 5 of the nutrient medium with added palladium chloride had on the growth of Kentucky bluegrass is shown in Tables III and IV. The effect of lower concentrations of PdCl₂ in the range 50 to 2,000 µg/tray are shown in Table III, and of higher concentrations in the range 3,000 to 575,000 in Table IV. In this experiment the grass was grown in nutrient solution of pH 5 that was replenished every three days. After one month of growth without palladium, PdCl₂ was added with the nutrient solution, adjusted to pH 5, every 2 days. After one month of growth in nutrient solution with added PdCl₂ the palladium uptake by the plants was determined from analyses of the roots of the grass in each tray.

Table III shows that palladium stimulated the growth of Kentucky bluegrass in concentrations of PdCl₂ up to 2,000 µg
TABLE III

Effect of lower concentrations of palladium chloride on the growth of Kentucky bluegrass in nutrient solution of pH 5

<table>
<thead>
<tr>
<th>Conc. PdCl$_2$/tray (µg)</th>
<th>No. of weeks in PdCl$_2$</th>
<th>Height (cm)</th>
<th>Fresh Weight (g)</th>
<th>Palladium (µg/g roots)</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
<tr>
<td></td>
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<td>-</td>
</tr>
<tr>
<td></td>
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<td>17.5</td>
<td>-</td>
<td>-</td>
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<td></td>
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<td></td>
<td>2</td>
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<td>4</td>
<td>35.0</td>
<td>62</td>
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... continued
Table III continued

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<tr>
<td></td>
<td>1</td>
<td>20.0</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>2000</td>
<td>2</td>
<td>22.5</td>
<td>55</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>25.0</td>
<td>58</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>30.0</td>
<td>62</td>
<td>10.3</td>
</tr>
</tbody>
</table>

All weight and height measurements are based on the average values for two trays.

Palladium analyses were performed in duplicate on each tray used in the experiments.
TABLE IV

Effect of higher levels of palladium chloride on the growth of Kentucky bluegrass in nutrient solution of pH 5

<table>
<thead>
<tr>
<th>Conc. PdCl₂ /tray (µg)</th>
<th>Height (cm)</th>
<th>Weight (g)</th>
<th>Palladium (µg/g roots)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>27.0</td>
<td>47</td>
<td>-</td>
</tr>
<tr>
<td>3,000</td>
<td>41.2</td>
<td>61.5</td>
<td>14.1</td>
</tr>
<tr>
<td>4,500</td>
<td>40.0</td>
<td>72</td>
<td>15.8</td>
</tr>
<tr>
<td>7,000</td>
<td>41.2</td>
<td>64</td>
<td>19.0</td>
</tr>
<tr>
<td>9,000</td>
<td>40.0</td>
<td>72</td>
<td>18.7</td>
</tr>
<tr>
<td>11,500</td>
<td>45.0</td>
<td>73</td>
<td>18.5</td>
</tr>
<tr>
<td>12,000</td>
<td>42.5</td>
<td>74</td>
<td>18.5</td>
</tr>
<tr>
<td>115,000</td>
<td>Died after a week</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>575,000</td>
<td>Died after 2nd day</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

All weight and height measurements are based on the average values for two trays.

Palladium analyses were performed in duplicate on each tray used in the experiments.
per tray. The concentration of palladium was then raised to determine whether a toxic level existed. The plants were grown as before but higher concentrations of PdCl\textsubscript{2} were added. The results are shown in Table IV. The concentration of palladium was toxic at 115,000 μg per tray causing the plants to die one week after PdCl\textsubscript{2} was added. At the highest concentration used, 575,000 μg PdCl\textsubscript{2} per tray, the grass died two days after the solution with palladium was added. No analyses could be made of the roots of the dead grass. Otherwise palladium analyses were made in duplicate on each tray of the two trays used for each concentration. Table IV again shows that palladium stimulated the growth of Kentucky bluegrass until toxic concentrations were reached.

A summary of the data is illustrated in Figs. 1-3. Figure 1 A,B shows the increased growth of Kentucky bluegrass in nutrient solution with PdCl\textsubscript{2} added in concentrations of 50 to 2,000 μg/tray. Apparently, trace amounts of PdCl\textsubscript{2} permitted growth of the largest plants. Figure 2 shows that palladium uptake could be detected during the second and following weeks. Only a slight increase in the amount was found during the third and fourth week. Figure 3 shows that PdCl\textsubscript{2} uptake varies directly with concentration of palladium in the roots until about 18.5 μg/g is reached.
Fig. 1 A, B.

Growth of Kentucky bluegrass in various concentrations of \( \text{PdCl}_2 \) added to nutrient solution at pH 5.

Legend to 1-A

- \( \circ \) -- Control
- \( \bullet \) -- 200 \( \mu \text{g} \) \( \text{PdCl}_2 \)
- \( \triangle \) -- 1,000 \( \mu \text{g} \) \( \text{PdCl}_2 \)
- \( \square \) -- 2,000 \( \mu \text{g} \) \( \text{PdCl}_2 \)

1-B

- \( \circ \) -- Control
- \( \bullet \) -- 50 \( \mu \text{g} \) \( \text{PdCl}_2 \)
- \( \triangle \) -- 100 \( \mu \text{g} \) \( \text{PdCl}_2 \)
- \( \square \) -- 500 \( \mu \text{g} \) \( \text{PdCl}_2 \)
Fig. 2.

Uptake of palladium by roots of Kentucky bluegrass grown in nutrient solution at pH 5 with various concentrations of PdCl$_2$ added.

Legend

- $\bullet$ -- 500 µg PdCl$_2$
- $\square$ -- 1,000 µg PdCl$_2$
- $\triangle$ -- 2,000 µg PdCl$_2$
FIGURE 2

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Fig. 3.

Variation of palladium uptake by roots of Kentucky bluegrass with concentration of PdCl$_2$ in the nutrient solution.
The results clearly show that higher concentrations of palladium chloride are phytotoxic to Kentucky bluegrass. Whether this is due to the toxicity of the palladium ion or a non-specific ionic effect would require further investigating.

Acknowledgements

The authors gratefully acknowledge the financial assistance of the National Research Council of Canada and the technical assistance of Mr. E. Treverton.
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CHAPTER II

Recovery of Added Palladium from Plant and Animal Tissues

R. J. THIBERT AND M. SARWAR

Department of Chemistry, University of Windsor, Windsor, Ontario, Canada

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There are many spectrophotometric methods reported in the literature (1) for the determination of palladium in pure solutions, but none is available for biological systems. From the several procedures generally recommended for the determination of palladium at the microgram level, Beamish (2) indicates that the most sensitive reagent is p-nitrosodiphenylamine (3), but exact pH control is required and interference occurs from high salt concentrations, gold, silver, and platinum (II).

Reeds and Banks (4) used a-furildioxime for the gravimetric determination of palladium and nickel, and they also indicated that the reagent could be used for the qualitative identification of these elements.

Menis and Rains (5) used this reagent for the spectrophotometric determination of palladium. Rigid pH control is not required in this method, and many interferences have been eliminated by extraction of the palladium complex with chloroform.

The purpose of this investigation was to determine microgram quantities of palladium in biological systems applying the method of Menis and Rains (5), in which destruction of the organic matrix by sulfuric acid digestion and subsequent evaporation is involved.

EXPERIMENTAL

Apparatus

Spectrophotometer: Beckman model DU monochromator with Gilford model 220 absorbance indicator and Gilford model 210 automatic cuvet positioner. The temperature of the cuvet chamber was maintained constant at 25 ± 0.02°C using thermostapers and Colora model K-3248 constant-temperature circulator. All absorbance measurements were done with cuvets of 1-cm path length.

Reagents

Standard palladium solution: PdCl₂ from Fisher Scientific Company can be used as a primary standard (6) after drying it over sulfuric acid
for 4 hr. Palladium solution was made by dissolving 0.14602 gm PdCl₂ in 10 ml concentrated hydrochloric acid and diluting to 100 ml with deionized water. The quantities of palladium used in this work were appropriate dilutions of aliquots of this stock solution.

\( \alpha \)-Furildioxime: A 1\% solution of \( \alpha \)-furildioxime was prepared by dissolving 1 gm analytical-grade reagent (Eastman Organic Chemicals) in 30 ml absolute ethyl alcohol and then diluting to 100 ml with deionized water.

All other reagents were of analytical grade.

**Procedure**

Two to three grams of plant or animal tissue were placed in a 250-ml Kjeldahl flask and a known amount of palladium added to this from the stock solution. Concentrated sulfuric acid (25 ml) was then added

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Palladium added, µg</th>
<th>Palladium found, µg</th>
<th>Average % difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tobacco leaves</td>
<td>35.0</td>
<td>34.8</td>
<td>-0.4</td>
</tr>
<tr>
<td>Tobacco stem</td>
<td>51.6</td>
<td>51.3</td>
<td>-0.6</td>
</tr>
<tr>
<td>Tobacco roots</td>
<td>51.6</td>
<td>51.1, 51.3, 51.0</td>
<td>-0.9, -0.6, 0.0</td>
</tr>
<tr>
<td>Beef kidney</td>
<td>35.0</td>
<td>35.2, 35.4, 34.2</td>
<td>-1.2, -0.7, -0.9</td>
</tr>
<tr>
<td>Rat kidney</td>
<td>51.6</td>
<td>51.9, 50.6</td>
<td>-0.7</td>
</tr>
<tr>
<td>Rat brain</td>
<td>51.6</td>
<td>52.0, 51.2</td>
<td>0.0</td>
</tr>
<tr>
<td>Rat blood</td>
<td>51.6</td>
<td>52.5, 51.9, 51.5</td>
<td>0.7, 0.0, 0.0</td>
</tr>
<tr>
<td>Rat heart</td>
<td>51.6</td>
<td>51.1</td>
<td>-1.0</td>
</tr>
<tr>
<td>Rat liver</td>
<td>51.6</td>
<td>51.6, 52.1, 51.1</td>
<td>0.0, 0.7, -1.0</td>
</tr>
</tbody>
</table>
slowly followed by 25 ml of deionized water. Two or three Pyrex glass beads were also put into the flask along with 2 to 3 drops of capryl alcohol (7) as antifoaming agent. The flask was at first slowly heated on a microburner and then, after boiling had commenced, was vigorously heated. Digestion was complete within 2 hr. To this brown solution small quantities of 30% hydrogen peroxide solution were added to decolorize it (8). The contents of the flask were then strongly heated to dryness. The residue was dissolved in 15 ml concentrated hydrochloric acid by heating for 5 min and shaking occasionally. The solution was quantitatively transferred to a 250-ml separatory funnel by washings with deionized water. \( \alpha \)-Furildioxide solution (10-15 ml) was added and the whole solution mixed well. After 2 min a yellow color formed and was extracted by vigorous shaking with chloroform using five 4-ml portions; the extract was transferred into a 25-ml volumetric flask. The volume of the extract was adjusted to 25 ml with chloroform and the extract was dried by adding 1 gm of anhydrous sodium sulfate. The absorbance was measured at 380 \( \mu m \) against a chloroform blank. The absorbance of the samples was measured at 25 ± 0.02°C.

RESULTS

Leaves, stems, and roots of tobacco plant, as well as beef and rat kidney, and rat brain, blood, liver, and heart were used. Known amounts of palladium were added to these tissues. Determinations were performed in triplicate and the values are given in Table 1.

DISCUSSION

The absorbance of the unknown solution was measured and knowing the absorptivity (223) the concentration was calculated according to the method of Menis and Rains (5). The method is quite feasible for the determination of palladium in biological systems. The error is within the limit of ±1% and the interference due to cyanide is eliminated because of the acid digestion. The digestion procedure does not affect the recovery of palladium. The procedure is simple, applicable to a large number of samples, and very sensitive. It permits the determination of palladium in biological systems during the course of distribution studies.

Attempts to determine endogenous palladium in beef liver and kidney using up to 200-gm samples were unsuccessful. Similar experiments with up to 25 gm of tobacco plant were carried out and palladium could not be determined.

The level of palladium has been reported in mammalian muscle at 0.002 ppm using activation analysis (9, 10). Menis and Rains state that the sensitivity of the \( \alpha \)-furildioxide method for palladium is 0.1 ppm.
The spectrophotometric method for palladium is thus not as sensitive as the activation analysis procedure. Therefore, using 1-cm path length cells, the sensitivity of the method reported here is 0.5 ppm.

**SUMMARY**

Palladium can be determined in biological systems with α-furildioxime. Plant and animal tissues are digested with concentrated sulfuric acid and decolorized with 30% hydrogen peroxide. The palladium complex is extracted with chloroform and the absorbance measured at 380 mμ against a chloroform blank.

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**REFERENCES**

VITA AUCTORIS

Born: June 10, 1936, Lyallpur, West Pakistan, Son of Mr. and Mrs. Wali Muhammad.

High School: D.C. High School Maurusipur, Lyallpur, West Pakistan.


Academic roll of honour in 1962.

Merit certificate from the University of the Punjab in 1961.


Professional Societies: The Chemical Institute of Canada.


Future Plans: After receiving a Ph.D. in chemistry, the author will return to Pakistan as Senior Research Officer in Council of Scientific and Industrial Research, West Regional Laboratories, Lahore, West Pakistan.