AN INVESTIGATION OF METHODS FOR THE DETERMINATION OF OXALIC ACID IN URINE

by

Rodolfo Bongiovanni

Submitted in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

in the

Chemistry

Program

Elmer Poldoway 9/24/76

Adviser Date

Dean Land 9/24/76

Dean of Graduate School Date

YOUNGSTOWN STATE UNIVERSITY

March, 1977

ABSTRACT

The urinary oxalate excretion on a twenty-four hour basis has been investigated by three methods. The range of oxalate concentration in aqueous solutions studied was 0 to 100 mg/L. That range defines the expected levels of oxalic acid in urine. Each of those methods investigated involves direct precipitation of oxalic acid and a specific method of detection.

The first, a colorimetric method, involves the precipitation of oxalic acid from urine with calcium sulfate and absolute ethanol. The precipitated oxalate anion is reduced to glycolic acid by boiling with sulfuric acid in the presence of a zinc pellet. The glycolic acid from that reaction step is reacted with chromotropic acid, and the absorbance of the resulting chromophore is determined at 570 nm. Thirteen subjects were analyzed by this method, and the N.M.V. was 28.8 $\pm$ 3.2 mg/24 hr. That N.M.V. is comparable to other reported values.

The second, a spectrofluorimetric method, involves the precipitation of urine oxalate with calcium sulfate in the presence of absolute ethanol. The precipitated oxalate is extracted with tributyl phosphate to remove the interfering metabolites. The oxalate is reacted with Ce (IV), and the fluorescence of the Ce (III) produced is determined. Seven subjects were analyzed by this method; the normal range of oxalic acid per twenty-four hours was 15.6 to 30.1 mg with a N.M.V. of 21.7 $\pm$ 1.4 mg/24 hr.

The third, a colorimetric method, involves the direct precipitation of urine oxalate with calcium chloride. The calcium oxalate precipitate is reacted with Ce (IV), and the decrease in absorbance is determined. This method was applied to urines containing known amounts of oxalic acid. The percent recovery of oxalate from urine compared to that of the recovery from aqueous oxalic acid solutions was ninety percent. The relative merits of each method are discussed, and conclusions about the applicability to a clinical laboratory are presented.

ACKNOWLEDGEMENTS

I would like to extend my appreciation to Dr. Elmer Foldvary for the invaluable guidance and assistance in the completion of this research project.

I would also wish to extend my gratitude to Dr. George Homer for his numerous contributions, and Doctors John Van Norman and James Reeder for reading this manuscript and for making many necessary and worthwhile suggestions.

Finally, I would like to thank Mr. Dale Manos for his invaluable technical assistance.

TABLE OF CONTENTS

LIST OF SYMBOLS

SYMBOL	DEFINITION	UNITS
Å	Angstrom	10^{-10}
Ave.	Average	none
B_1	Vitamin (contains Thiamine Group)	none
B_6	Vitamin (contains Pyridoxal Group)	none
b	Intercept	See Eq. (5)
F	Female	none
I	Ionic strength	none
l	Liter	cm^3
Ma	Male	none
ml	Milliliter	10^{-3} l
M	Molar Solution	mole/l
m	Slope	See Eq. (8)
NAD	Nicotinamide Adenine Dinucleotide	none
N.A.	Not applicable	none
N. Av.	Not available	none
nm	Nanometer	10^{-9} meters
N	Normal Solution	Equivalent/l
[o]	Oxidation	none
pKa	Log of dissociation constant	none
RPM	Revolutions per minute	none
R	Correlation Coefficient	none
S	Standard Deviation	none

SYMBOL	DEFINITION	UNITS
STD	Standard deviation	none
μg	Microgram	10^{-6} gram
Y_f	Statistically best straight line	See Eq. (10)

LIST OF KEY WORDS

Anomalous	Irregular; marked by deviation from the natural order. Applied particularly to congenital and hereditary defects.
Anabolic	A constructive process by which simple substances are converted by living cells into more complex compounds, especially into living matter.
Catabolism	A destructive process by which complex substances are converted by living cells into more simple compounds.
Cirrhosis	Liver disease characterized pathologically by loss of the normal microscopic lobular architecture, with fibrosis and modular regeneration. The term is sometimes used to refer to chronic interstitial inflammation of an organ.
Diurnal Variation	A daily quantity or value subject to change.
Diabetes	A familial constitutional disorder of carbohydrate metabolism characterized by inadequate secretion or utilization of insulin by polyuria and excess amounts of sugar in the blood and urine, by thirst, hunger and loss of weight.
Electrolyte	A substance that dissociates into ions when fused or in solution and, thus, becomes capable of conducting electricity, an ionic solute.
Hyperoxaluria	A genetic disorder characterized by urinary excretion of large amounts of oxalate, with nephrolithiasis, nephrocalcinosis, early onset of renal failure, and often a generalized deposit of calcium oxalate (oxalosis), resulting from a defect in glyoxalate metabolism.
Diopathic	Self-originated; of unknown causation.
N.E.D.	Normal Equivalent Deviate (Appendix B).
Nephrolithiasis	A condition caused by the presence of renal calculi.

Nephrocalcinosis	A condition characterized by precipitation of calcium phosphate in the tubules of the kidney, with resultant renal insufficiency.
Oxalaemia (oxalemia)	The presence of an excess of oxalates in the blood.
Oxalosis	Primary hyperoxaluria
Oxaluria	Hyperoxaluria
Renal Stones	Stones formed in the kidneys.
Urolithiasis	The formation of Urinary Calculi--the diseased condition associated with the presence of urinary calculi.
Urinary	Pertaining to the Urine; containing or secreting urine.

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

Properties of Oxalic Acid

The presence of kidney stones and oxalate crystals have been identified in urine as early as 1839 by Donné.[1] Several attempts throughout the years have been made to correlate kidney stone formation and oxalic acid excretion with clinical symptoms.

The term oxaluria was introduced to describe the presence of excessive quantities of calcium oxalate crystals in urine and, by implication, an increased excretion of oxalic acid. The term oxalaemia was also used to describe a high concentration of oxalic acid in blood. Oxalaemia has been reported in a variety of conditions such as diabetes, cirrhosis of the liver and cardiac failure.[2]

Recently, oxalic acid has attracted interest in relation to the formation of calcium oxalate stones in the kidney and to the occurrence of calcium oxalate deposits in soft tissues, notably in patients with primary hyperoxaluria.[3] The purpose of this chapter is to bring into focus the current knowledge of biochemistry and physiology of oxalic acid in man. Oxalic acid is a dicarboxylic acid (HOOC • COOH) which crystallizes from aqueous solution as a white dihydrate. It is a relatively strong acid, the $pKa_1 = 1.23$ and $pKa_2 = 4.19$. Oxalic acid is moderately soluble in water (8.7 g per 100 g of water at 20°C).[4] It is oxidized by ferric compounds, potassium permanganate and ceric sulfate to carbon dioxide

and water. Oxalic acid forms soluble salts with alkali metal ions (Li^+, Na^+, K^+) and ferrous salts. With calcium ion, the oxalate ion forms a practically insoluble salt at neutral or alkaline pH; calcium oxalate is soluble to the extent of 0.67 mg per 100 g of water at pH 7.0 and 13°C.

In aqueous solution the solubility of calcium oxalate is increased by the presence of urea and various ions, particularly citrate, magnesium and to a lesser extent by lactate, sodium, potassium and sulfate.[2] The solubility is also increased by ethylene diamine tetra acetic acid (EDTA).

Clinical Features

Primary hyperoxaluria is a genetic disorder of oxalate metabolism characterized by the early onset of calcium oxalate nephrolithiasis and nephrocalcinosis and by progressive renal damage to death in uremia. At post-mortem examination, calcium oxalate deposits may be found in renal tissue, a pathological condition termed oxalosis. The basic defect is impairment of glyoxylate metabolism with secondarily increased synthesis and excretion of oxalic and glycolic acids. The pattern of inheritance of primary hyperoxaluria suggests its transmission as an autosomal recessive trait.[5] Patients with primary hyperoxaluria usually seek medical care because of some symptom of urinary calculi, although in a minority, renal failure is the main symptom. Eventually renal failure develops until uremia becomes established. The uremic phase is usually fairly short. As renal failure develops, the urinary excretion of oxalate diminishes and may return to a normal level. Diagnosis of primary hyperoxaluria in

life must be based partially on the demonstration of an elevated urinary oxalate excretion. The presence or absence of calcium oxalate crystals in the urinary sediment cannot be relied upon as evidence of kidney stone formation as those stones may be absent in hyperoxaluria or, conversely, present in normal urine.[5] The twenty-four hour urinary oxalate excretion is one of the most important laboratory tests in diagnosis of hyperoxaluria with ultimate formation of kidney stones.

Statement of the Problem

It has been stated that the determination of urinary oxalic acid concentration is one of the most important clinical tests in the diagnosis of kidney stones. The analytical procedures published until now are lengthy and require a multitude of steps to achieve satisfactory results. Two of the most reliable and best documented procedures are to be the object of study in the following work.

The first is a colorimetric procedure.[6] The determination involves 1.0 ml of urine. The oxalic acid is co-precipitated by calcium sulfate and aqueous ethanol, reduced to glycolic acid by boiling with dilute sulfuric acid and a zinc pellet, then estimated colorimetrically with chromotropic acid. Even though this procedure is specific in the determination of oxalate by chromotropic acid, one of the drawbacks is that the precipitation requires a twenty-four hour period for its maximum precipitation.

The second method is a direct precipitation of oxalic acid.[7] In this method an aliquot (50 ml) of urine is taken and adjusted to pH 5.2. To the urine is added 2.0 ml of five percent calcium chloride

and calcium oxalate is allowed to precipitate overnight at 4°C. The calcium oxalate is centrifuged, washed and titrated with potassium permanganate. The problem here centers around the titration in that it is extremely difficult to titrate small quantities.

It will be the object of this work to analyze the procedures discussed above, to improve them and finally to develop a new procedure with a new method of detection of oxalic acid.

The following chapter presents the metabolic pathway by which oxalate is produced. Although the oxalic acid levels in urine are important in identifying the condition of nephrolithiasis, a better correlation of that condition is recognized when the ratio of certain electrolytes is also considered. The significance of the ratios Mg/Oxalic and $\frac{Mg}{Ca}$ x Oxal are discussed.

CHAPTER II

HISTORICAL BACKGROUND

Metabolism of Oxalate

Several enzymes which will oxidize oxalic acid to carbon dioxide and water have been isolated and identified.[8] However, no catabolism of oxalic acid has been demonstrated in mammalian tissue. In man, injected isotopic oxalate is recovered almost quantitatively in the urine and none of the isotope can be detected as labeled carbon dioxide in expired air.[9]

The basic defect of primary hyperoxaluria is of a continuous excessive biosynthesis of oxalate.[5] The major pathways of oxalate synthesis in mammalian systems are summarized in Figure 1.[5] That figure clearly shows the glycine and ascorbate are the primary precursors in man.

Ascorbic Acid

In a study of human subjects, Hockaday et al[5] found that excreted oxalate is the major urinary metabolite of infused doses of L-ascorbate-^{14}C. After two days, about fifty percent of the labeled L-ascorbate has appeared as labeled oxalate in the urine.[5,10] It has been estimated that thirty-five to fifty percent of oxalate normally excreted is derived from ascorbic acid.[11]

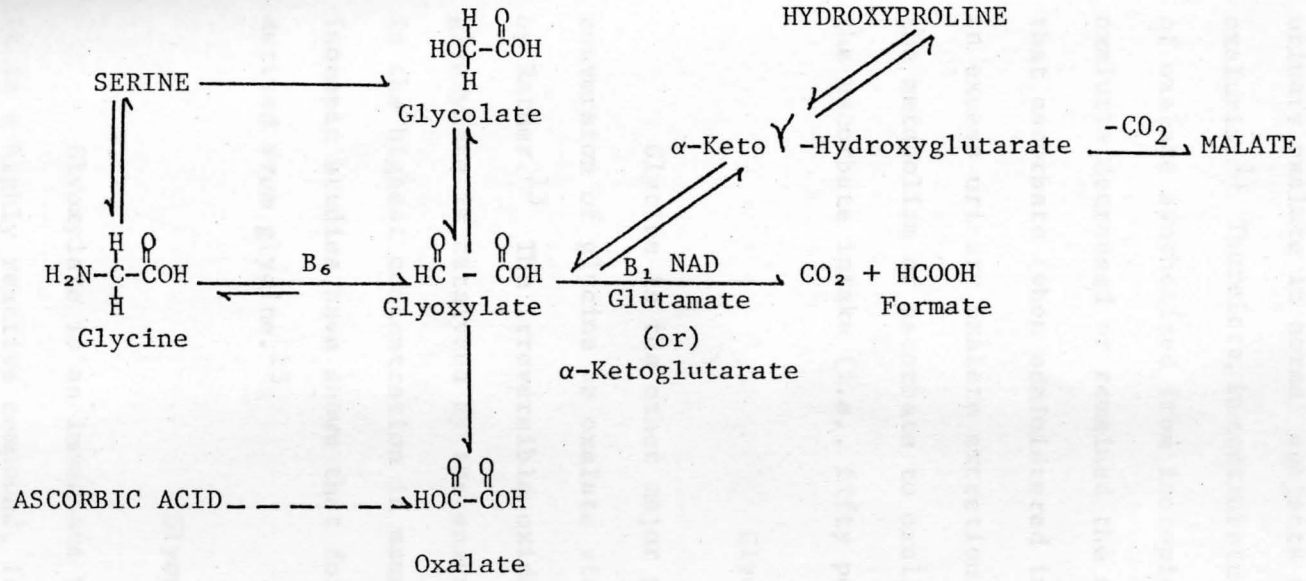


Fig. 1. Major pathways in glyoxylate metabolism and oxalate synthesis in mammalian systems.

The isotopic studies cited above indicate that L-ascorbic acid is a major precursor of oxalate. It is anomalous, therefore, that large ascorbate loads (i.e., four grams) do not materially increase urinary oxalate in normal subjects or in patients with primary hyperoxaluria.[11] Therefore, in control studies, it was shown that the amount of oxalate synthesized from isotopic ascorbate in patients with hyperoxaluria decreased or remained the same.[11] That study indicates that ascorbate (when administered in large quantities) is not a factor in excess urinary oxalate excretion. Further, in primary hyperoxaluria the metabolism of ascorbate to oxalate was unaltered with respect to the ascorbate intake (i.e., fifty percent conversion).[12]

Glycine

Glycine is the other major precursor of urinary oxalate. The conversion of glycine to oxalate via glyoxylate was first demonstrated by Ratner.[13] The irreversible oxidative deamination of glycine to glyoxylate is catalyzed by the enzyme glycine oxidase which is found in the highest concentration in mammalian kidney and liver tissue. Isotopic studies have shown that forty percent of urinary oxalate is derived from glycine.[13]

Glyoxylate

Glyoxylate is an immediate precursor of oxalate (Figure 1). It is a highly reactive compound, forming metabolites with urea, cysteine and certain alpha-keto acids, as well as undergoing non-enzymatic transamination to form glycine.[14] Glyoxylate has been reported present in human urine from the formation of a

dinitrophenylhydrazone with characteristics similar to those of the authentic compound.[14] Using isotope dilution techniques, it may be estimated from the excretion of oxalate and the proportion of infused glyoxylate converted to oxalate that 150 to 600 mg of glyoxylate is synthesized daily. Glyoxylate has many possible fates (Figure 2).

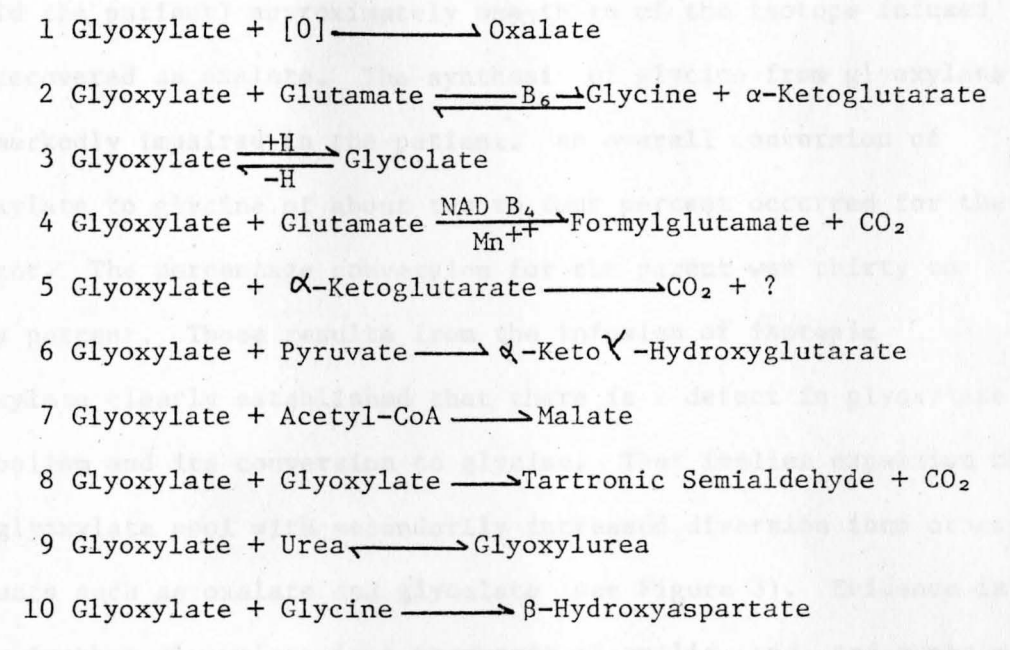


Fig. 2. Enzymatic reactions described in the metabolism of glyoxylic acid.

The reactions 7 through 10 have not been demonstrated in mammalian systems. Glyoxylate may undergo transamination to glycine with pyridoxal phosphate as a co-factor, and, therefore, enter the larger number of anabolic and degradative pathways followed by the formation of various amino acids.

Hockaday et al[5] have done a complete study of the metabolism of glyoxylate-^{14}C on control subjects (a parent of the patient) and patients with primary hyperoxaluria to establish whether a defect in

glyoxylate metabolism is responsible for the abnormal oxalate excretion. Those results are shown in Figure 3.[5] It was found that the patients excreted a much larger percent of the labeled glyoxylate (8.7 vs. 0.4 percent) and glycolate (16.4 vs. 3.4 percent) than did the parent. Unexpectedly, the parent formed as much glyoxylate-^{14}C as did the patient) approximately one-third of the isotope infused was recovered as oxalate. The synthesis of glycine from glyoxylate was markedly impaired in the patient. An overall conversion of glyoxylate to glycine of about two to four percent occurred for the patient. The percentage conversion for the parent was thirty to sixty percent. Those results from the infusion of isotopic glyoxylate clearly established that there is a defect in glyoxylate metabolism and its conversion to glycine. That implies expansion of the glyoxylate pool with secondarily increased diversion into other products such as oxalate and glycolate (see Figure 3). Evidence is shown in that glyoxylate is a precursor of oxalic acid, and experimental evidence has been presented in that an expanded glyoxylate pool exists in those patients which have hyperoxaluria. The formation of calcium oxalate (kidney stones) is a system which involves not only the oxalate ion but also other factors (e.g., electrolytes). In the next section, statistical data on twenty-four hour excretion of oxalic acid are presented, tables of normal and abnormal subjects are analyzed, the influence of electrolytes is discussed and an interpretation of the results is presented.

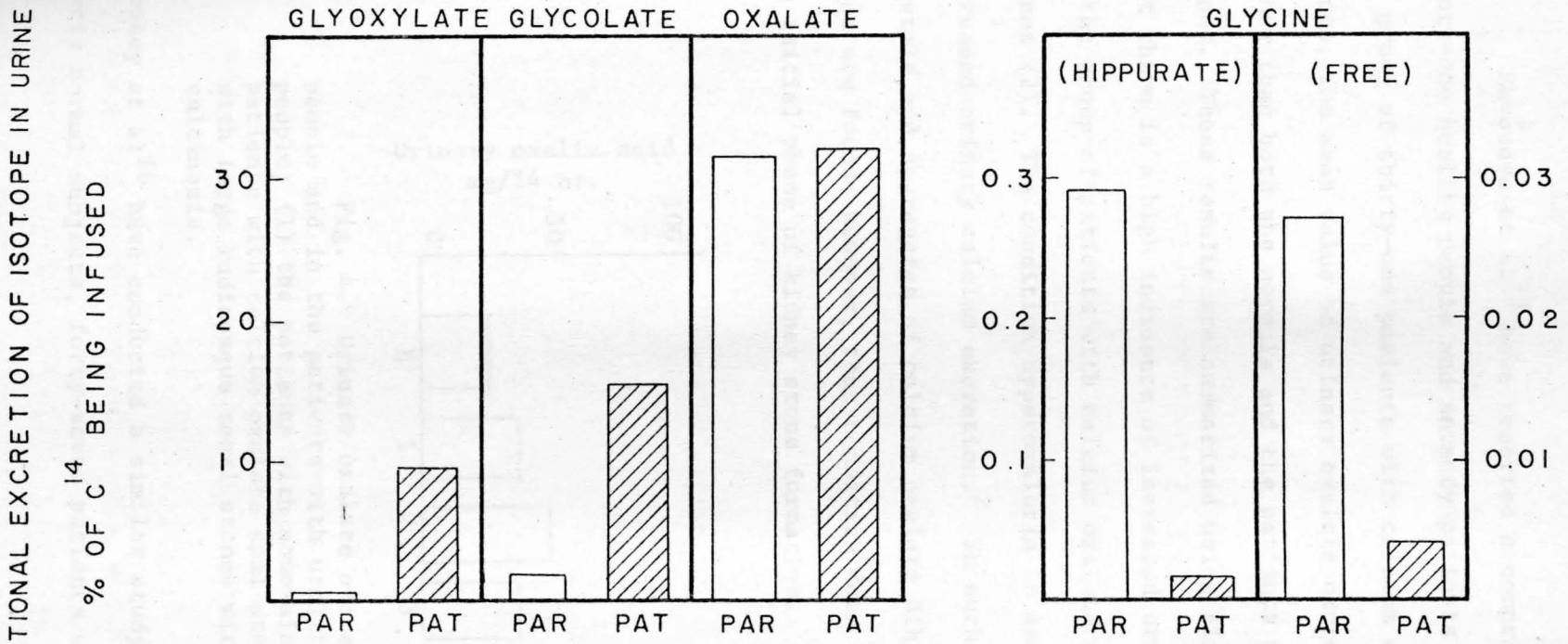


Fig. 3. Fates of Glyoxylate. Incorporation of intravenously administered carboxyl-labeled glyoxylate-^{14}C into urinary glycolate, oxalate and glycine and its excretion as unaltered glyoxylate (Par = Parent, Pat = Patient).

Urinary Oxalic Acid Excretion of Normal and Abnormal Subjects

Revúsová et al[15] have reported a comprehensive study of twenty-one healthy people and seventy patients with renal stones. In the group of thirty-one patients with calcium oxalate containing renal stones, the mean value of urinary oxalate excretion was significantly higher than both the normals and the patients without oxalic renal stones. Those results are summarized using Figure 4. Figure 4 shows that there is a high incidence of increased urinary oxalate excretion in the group of patients with calcium oxalate containing renal stones (2). The condition hyperoxaluria is associated with an increased urinary calcium excretion.[2] In such conditions large crystals and aggregates of calcium oxalate dihydrate in freshly voided urine are found; however, that condition does not necessarily indicate the initial phase of kidney stone formation.

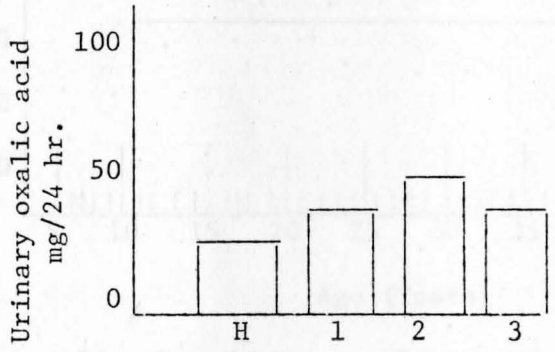


Fig. 4. Urinary oxalate excretion in healthy people and in the patients with urolithiasis. (H) healthy people; (1) the patients with nonoxalate renal stones; (2) the patients with calcium oxalate enal stones; (3) the patients with large radiopaque renal stones with or without nehrocalcinosis.

Dempsey et al[16] have conducted a similar study and found that in twenty normal subjects, forty-seven patients with renal stones and

forty-two patients with other disease states, the oxalate excretion was 15 to 50 mg per day (average 31 mg per 24 hour). Since oxalate excretion was within the normal range in all but three of the forty-seven patients with calcium oxalate stones, the correlation between the magnitude of oxalate excretion and the occurrence of calcium oxalate stones is poor.[16] However, the high concentration of oxalic acid excreted is indicative that kidney stones have been formed.

An important factor to be considered in the variation of urinary oxalate excretion is age. The urinary excretion of oxalate increases with age during childhood, and adult values are attained at the age of about fourteen years.[17] This is presented with a plot of urinary oxalate excretion versus age (Figure 5).

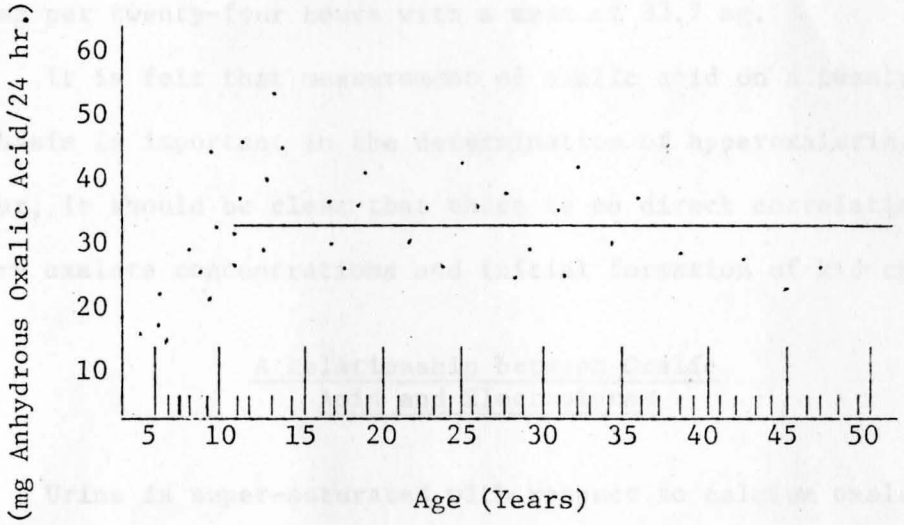


Fig. 5. Urinary oxalate excretion with age.

When the urinary oxalate excretion per unit of surface area is converted to the corresponding value for the adult standard body surface (1.73 m^2), the results for children lie in the same range as those obtained for adults, and there is no significant difference between the sexes (see Figure 6).

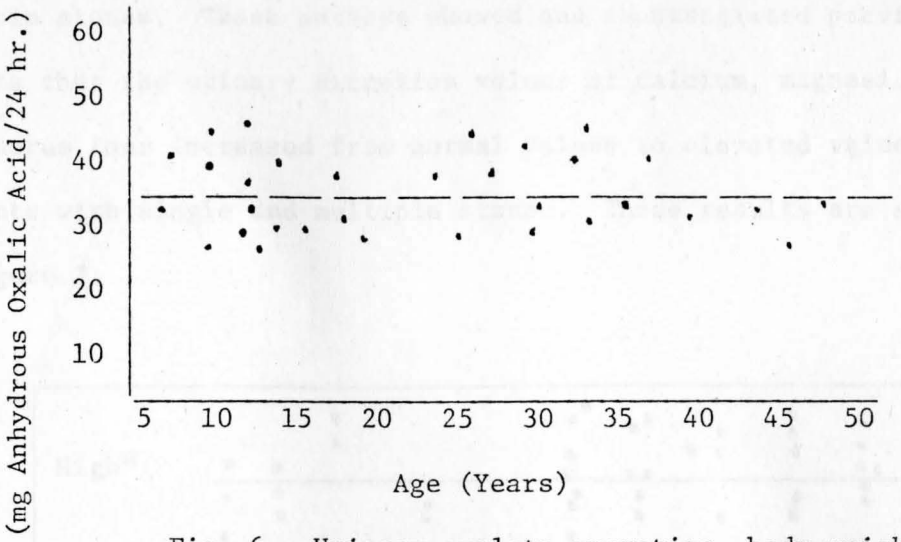


Fig. 6. Urinary oxalate excretion, body weight corrected.

Gibbs et al[17] show data which is consistent with other workers.[2,3,15,16] Their corresponding values lie between 21.8 to 49.3 mg per twenty-four hours with a mean of 33.7 mg.

It is felt that measurement of oxalic acid on a twenty-four hour basis is important in the determination of hyperoxaluria. However, it should be clear that there is no direct correlation between urinary oxalate concentrations and initial formation of kidney stones.

A Relationship between Oxalic Acid and Electrolytes

Urine is super-saturated with respect to calcium oxalate and calcium phosphate. Calcium and magnesium ions are excreted in urine on effect against oxalate urolithiasis since it increases the solubility of calcium oxalate in urine.[2] Takasaki et al[18] have done a study of a group of normal adult patients with single stones and patients with

multiple stones. Those authors showed and substantiated previous reports that the urinary excretion values of calcium, magnesium and phosphorus ions increased from normal values to elevated values for patients with single and multiple stones. Those results are summarized in Figure 7.

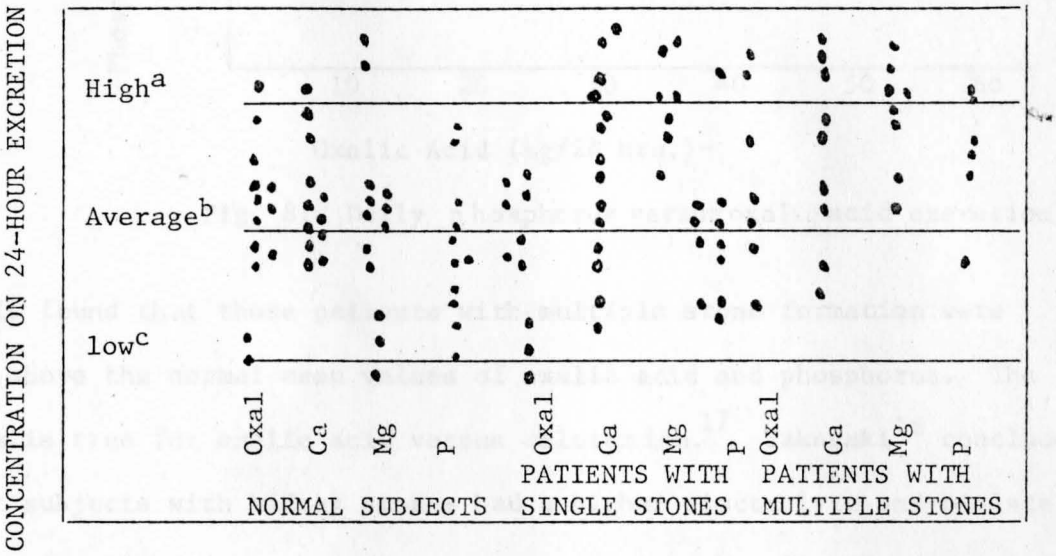


Fig. 7. Oxalic acid and electrolytes (24 hour excretion.)

	c	b	a
Oxalic Acid	<20 mg	20-40	>40
Calcium	<100 mg	100-300	300-400
Magnesium	<30 mg	30-60	60-90
Phosphorus	<500 mg	500-800	>800

In figure 8 the same values are plotted to show a relationship of phosphorus versus oxalic acid (Figure 8).

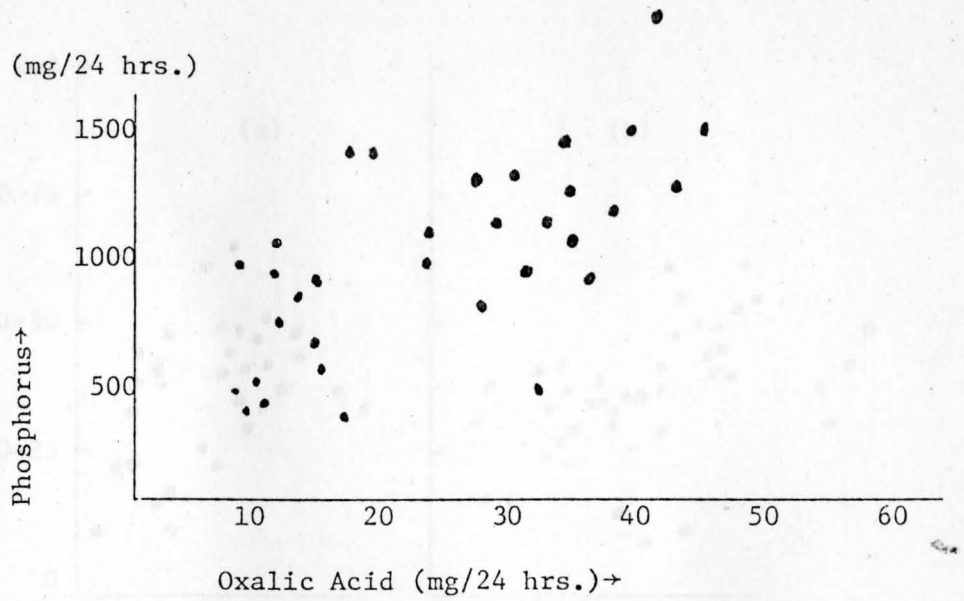


Fig. 8. Daily phosphorus versus oxalic acid excretion.

It is found that those patients with multiple stone formation were well above the normal mean values of oxalic acid and phosphorus. The same is true for oxalic acid versus calcium ion.[17] Takasaki[18] concluded that subjects with kidney stones had a higher electrolyte and oxalate excretion values than normal subjects. That high values lead to stone formation is speculative, since normal subjects also show the random spread of values; Hodgkinson[19] pointed out, with a thorough incidence study, that a group of normal subjects vs. a group of kidney stone formers give similar results. Those results are summarized in Figure 9 [note the likeness of (a) and (b)].

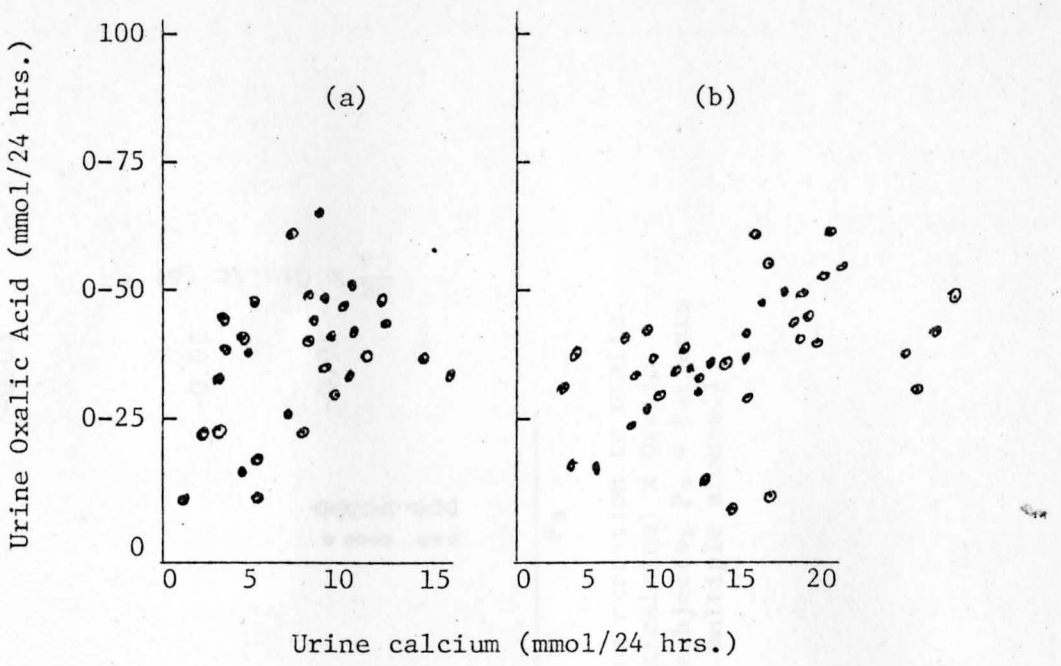


Fig. 9. Relation between daily excretion of calcium and oxalate. (a) Control subjects; (b) stoneformers. (Control subjects - normal daily activity.)

This can be interpreted to mean that high calcium and oxalate concentrations in urine do not necessarily correspond to calcium oxalate stone formation, the former is not necessarily the cause of the latter (stone formation).

What has been shown is that when magnesium is divided by calcium and multiplied by oxalic acid (Figure 10), it gives a ratio which is indicative of the condition of renal stone formation.[18,19] It must be kept in mind that there is no concrete data to show conclusively that the ratio $\frac{Mg}{Ca}$ x Oxal is directly related to whether a subject is going to form renal stones or not. Hodgkinson[19] summarized his findings with Figure 10. Those findings as they are represented show that those patients with the most severe cases of stone formation have the lowest values (i.e., Ratio = (Mg/Ca) x Oxalic Acid). There is strong evidence that points toward accepting that ratio [(Mg/Ca) x Oxalic

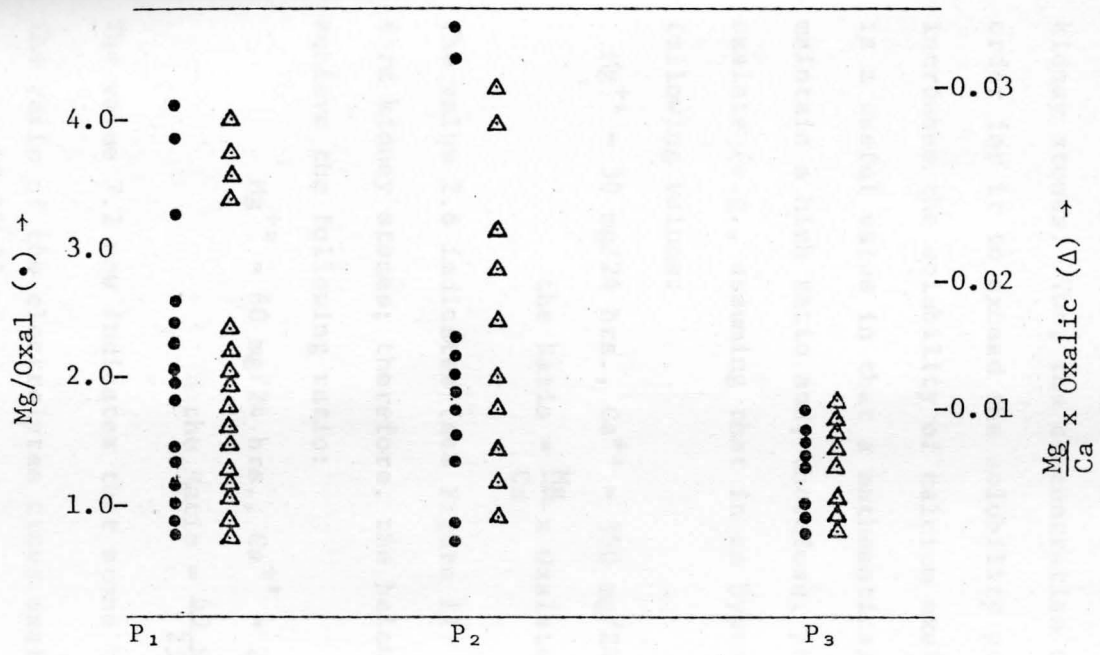


Fig. 10. The ratio of magnesium excretion to oxalic excretion (•). The ratio of (magnesium:calcium) x Oxalic Acid in the Urine (Δ). (P_1 = healthy subjects, P_2 = Patients with single stones, P_3 = Patients with multiple stones.)

Acid] as a meaningful clinical value. Three parameters which have been defined previously must be kept in mind in order to fully appreciate what the above discussed ratio means. One, higher calcium concentrations increase the chances of calcium oxalate to precipitate and form kidney stones. Two, the concentration of oxalic acid is critical in order for it to exceed the solubility product. Three, magnesium increases the solubility of calcium oxalate in urine. The ratio is a useful value in that a mathematical treatment can be set up to maintain a high ratio and, therefore, prevent formation of calcium oxalate (e.g., assuming that in an hypothetical case a subject had the following values:

$$Mg^{+2} = 30 \text{ mg/24 hrs.}, \quad Ca^{+2} = 350 \text{ mg/24 hrs.}, \quad C_2O_4^{=} = 30 \text{ mg/24 hrs.} \quad (1)$$

$$\text{the Ratio} = \frac{Mg}{Ca} \times \text{Oxalate} = \frac{30 \times 30}{350} = 2.6$$

The value 2.6 indicates (see Figure 11) that the subject is prone to form kidney stones; therefore, the balance of ions can be improved to achieve the following ratio:

$$Mg^{+2} = 60 \text{ mg/24 hrs.}, \quad Ca^{+2} = 250 \text{ mg}, \quad C_2O_4^{=} = 30 \text{ mg}; \quad (2)$$

$$\text{the Ratio} = \frac{60 \times 30}{250} = 7.2$$

The value 7.2 now indicates that stone formation is not possible. The ratio of the electrolytes times oxalic acid has been proposed[18,19,20] as a method of interpreting the patient's condition.

The determination of electrolytes in the laboratory is routine. Where the determination of oxalic acid is very difficult clinically, Takasaki[18] has suggested the possible use of urinary magnesium to phosphorus ratio (Mg÷P) as a substitute for the urinary magnesium to oxalic acid ratio (Mg÷Oxal) because urinary phosphorus output is

relatively well correlated with oxalic acid output. The possible use of the ratio (Mg÷P) has been confirmed by Hodgkinson,[19] and it is in total agreement with an earlier report by Evans et al.[20] In the present study two methods of analysis to detect oxalic acid are presented.

CHAPTER III

COMPOSITE LIST OF REAGENTS AND INSTRUMENTS

The aim of the present chapter is to present a detailed, composite list of reagents and instruments used throughout the laboratory work.

It is understood that all solutions are prepared and standardized by standard analytical techniques. Therefore, there is no need to spell out the techniques.

The instruments used are again listed, and pertinent information concerning each instrument is provided only if needed.

All reagents used in this research are analytical reagent grade as a minimum for quality standards.

Reagents

1. Calcium Carbonate, 0.025M; $CaCO_3$ mol. wt. 100.089. In a 100 ml volumetric flask add 0.249 g of Calcium Carbonate and 2 ml of distilled water with several drops of concentrated HCl; dissolve the solute and bring to mark (Ca^{+2}= 1,000 mg/l).

2. Calcium Oxalate, 0.011 M; $CaC_2O_4H_2O$ mol. wt. = 146.118. In a 100 ml volumetric flask add 0.146 g of $CaC_2O_4 \cdot H_2O$ dissolve in distilled water and bring to mark (Ca^{+2} = 455 mg/l, $C_2O_4^{=}$ = 1,000 mg/l).

3. Ceric Ammonium Sulfate, 0.01M; $Ce(SO_4)_2 \cdot 2(NH_4)_2 SO_4 \cdot 2H_2O$ Mol. wt. 632.59. In a (1) one liter volumetric flask add 50 ml of distilled water and 3 ml of concentrated sulfuric acid, with stirring

add 6.326 g of Ceric Ammonium Sulfate, bring to volume with distilled water.[21]

4. Iodine Monochloride Catalyst, in a volumetric flask, add 0.058 g of potassium iodate (KIO_3) and 0.089 g of potassium iodide (KI) to 125 ml of distilled water; mix thoroughly. All at once 125 ml of concentrated HCl is added, cool to room temperature and adjust the solution as follows (add 5 to 10 ml of chloroform and titrate very carefully with either dilute potassium iodate or dilute potassium iodide as the case may require until the chloroform layer shows only a very faint iodine color after vigorous shakings).

5. Sodium Hydroxide Solution, 2N; NaOH mol. wt. 39.99. In a (1) one liter volumetric flask partially filled with distilled water add 79.99 g of sodium hydroxide, mix and bring to volume.

6. Ceric Ammonium Sulfate, 0.1M; $Ce(SO_4)_2 \cdot 2(NH_4)_2 SO_4 \cdot 2H_2O$ mol. wt. 632.59. In a (1) one liter volumetric flask add 50 ml of distilled water and 3 ml of concentrated Sulfuric Acid; with stirring add 63.26 g of ceric ammonium sulfate, bring to volume with distilled water.

7. Arsenious Oxide, 0.011 M; As_2O_3 mol. wt. 197.84. In a 250 ml Erlenmeyer flask add 0.22 g of pure dry arsenious oxide and add 15 ml of 2N(NaOH) sodium hydroxide; warm gently to hasten the solution. When the sample has completely dissolved, cool to room temperature, and add 20 ml of concentrated (HCl) hydrochloric acid; transfer the solution to a 100 ml volumetric flask and bring to mark.

8. Potassium Biphthalate, 0.1M; $KHC_8H_4O_4$ mol. wt. 204.23. In a (1) liter volumetric flask add 20.42 g of potassium biphthalate and dilute to mark with distilled water (Potassium Biphthalate is dried in the oven

(120°C) for one hour prior to weighing).

9. Hydrochloric Acid, 12N HCl mol. wt. 36.457 concentrated thirty-seven percent. In a (1) liter volumetric flask place 400 to 500 ml of distilled water; then slowly add 166.67 ml of concentrated HCl, mix, bring to mark.

10. Ferroin Solution, 0.025 M; $C_{12}H_8N_2$ mol. wt. 198.21. In a 100 ml volumetric flask add 0.695 g of $FeSO_4 \cdot 4H_2O$; dissolve with the addition of 15 ml of distilled water. While mixing, add 0.495 g of 1,10-phenanthroline, mix well and bring to mark with distilled water.

11. Calcium Sulfate Saturated Solution; $CaSO_4$ mol. wt. 136.144. Enough calcium sulfate is added to one liter of distilled water in an Erlenmeyer flask to make a saturated solution. The solution is stirred (Magnetic Stirrer) for twenty-four hours. Finally, the solution is filtered and the pH adjusted to 7.0. A few grams of $CaSO_4$ is added to the filtered solution and stored in a glass bottle.

12. Oxalic Acid, 0.139N; $H_2C_2O_4 \cdot 2H_2O$ mol. wt. 126.07. In a 100 ml volumetric flask (1) add 6.30 g of Oxalic Acid and distilled water; mix and bring to mark.

13. Calcium Chloride, 0.025 M; $CaCl_2 \cdot 2H_2O$ mol. wt. 147.02. In a (1) liter volumetric flask add 3.668 g of calcium chloride and bring to mark with distilled water.

14. Ammonium Hydroxide, 0.5%; mol. wt. 35.0 (SP. Gr. 0.9016, 30% concentration). In a 100 ml volumetric flask add 1.85 ml of ammonium hydroxide to approximately 20 ml of distilled water; mix and

bring to volume with distilled water.

15. Calcium Chloride, 5 percent w/v; $CaCl_2 \cdot 2H_2O$ mol. wt. 147.02. In a 100 ml volumetric flask add 6.624 g of $CaCl_2 \cdot 2H_2O$ and bring to mark with distilled water.

16. Electrolytic zinc pellets weighing approximately 250 mg. Immediately before use the zinc is cleaned by immersing briefly in freshly prepared 10N HNO_3 (two volumes of concentrated HNO_3 to one volume of water). After washing thoroughly in distilled water the zinc is ready for use.

17. Oxalic Acid Standard, 5 mg/ml; dissolve 1.0231 g of potassium oxalate monohydrate in 100 ml of water. Store at 4° and prepare fresh once a week (this solution contains 5 mg of anhydrous oxalic acid per ml).

18. Chromotropic Acid Solution, one percent; dissolve one g of 4,5-dihydroxynaphthalene-2,7—disulphonic acid (disodium salt) in 100 ml of water. Store at 4° and prepare fresh once a week.

19. Bromo-thymol Blue, 0.04 percent (scientific name) Dibromo-thymolsulfonphthalein to a 100 ml volumetric flask add 0.04 g of Bromo-thymol Blue and dilute to mark with distilled water.

Apparatus

1. A Spectrophotometer (Beckman DU).

2. A Spectrophotometer (Beckman DB) with a 100 mV Recorder.

3. A Fluorescence Spectrophotometer (Turner Model 430) equipped with a recorder.

Instrument Setting

Recorder--chart speed medium, mV Span 100, Filter 2, variable span off.

Fluorimeter--blank low, sensitivity high, meter damp 2, Range varied, Excitation 260, Emission 350.

4. A Spectrophotometer with visible, Ultra Violet, Infra Red capabilities (Cary 14).

5. A Colorimeter, Spectrophotometer "Spectronic 20," Standard Model (B & L 33-31-21).

6. A centrifuge (GLC-1 Sorvall).

7. Analytical Balance (Mettler H33).

8. Tubes, 50 ml Round bottom, plain with pennyhead stopper (Pyrex brand 8424).

9. Tubes, conical, 15 ml plain, with stopper (Pyrex brand 8060A, No. 13).

10. Separatory funnels, squibb, with teflon® plug, Kimax (Kimble 45210F).

11. Flasks, Volumetric, Stopper, Class A, Kimax (Kimble 28014).

12. Test paper, Acid Alkali, pH indicating.

13. Burets, Precision Bore, Straight Glass Stopcock with teflon® plug, Dust cap, Kimax, Class A (Kimble 17029F).

14. Hot plate (Thermolyne HPA 1915B).

15. pH Meter (Analytical).

16. Pipets, Serological, Color Coded, tempered tip, Van-Lab® Pipets, Volumetric, Glass A, Corex®, Micro Pipetting System, Sampler, Oxford (53503-10).

CHAPTER IV

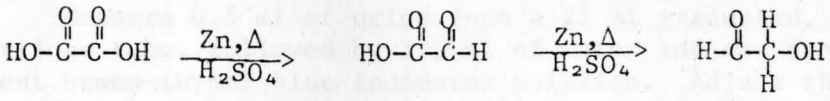
COLORIMETRIC ANALYSIS USING CHROMOTROPIC ACID

Principle

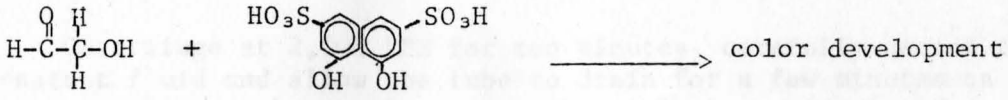
Hodgkinson and co-workers[6,22,23] have developed methods for determination of oxalic acid concentrations in urine. Hodkinson's[22] latest method of oxalate determination has been investigated in the present research.

Urine is supersaturated with respect to calcium and oxalate ions.[2] This condition of supersaturation is destroyed by addition of excess calcium ions in the form of calcium sulfate by the common ion effect. Furthermore, the solubility of calcium oxalate is reduced by addition of ethanol (salting out). The precipitated oxalate anion is reduced to glycolic acid by boiling with 2N H_2SO_4 in the presence of a zinc pellet. The resulting glycolic acid is reacted with chromotropic acid and the absorbance of the resulting chromophore is determined at 570 nm.

Reactions



OXALIC ACID GLYOXILIC ACID GLYCOLIC ACID



GLYCOLIC ACID

2, 7 - disulfonic 4,5 dihydroxy Naphtalene*
(chromotropic acid)

*Chromtropic Acid heated with dilute mineral acid and glycolic acid will give the corresponding dihydroxynaphtalene by loss of the sulfonic acid groups; the resulting dihydroxy-naphthalene reacts with glycolic acid to give a blue to violet color.[24]

Hodkinson's[22] method (Procedure A) follows. That procedure is presented in its original form and the findings presented.

Procedure A

Reagents and Apparatus

1. Reagents are listed in Chapter II.
2. Beckman DU Spectrophotometer.

Collection of Samples

Urine samples were collected in (1) one gallon brown glass bottles with 10 ml of concentrated hydrochloric acid. The urines tested were from normal subjects requesting routine clinical test profiles. The subject's sex and age were selected so that a cross section of the population could be adequately represented.

Method[6]

Measure 0.5 ml of urine into a 25 ml graduated, stoppered centrifuge tube, followed by 1.5 ml of water and one drop of 0.04 percent bromo-thymol blue indicator solution. Adjust the solution to pH 7.0 (green) by the addition of dilute NaOH or dilute acetic acid solutions. Add 2 ml of saturated aqueous solution of calcium sulfate, followed by 14 ml of ethanol, mix gently and allow the solution to stand at room temperature overnight.

Centrifuge at 2,000 RPM for ten minutes, carefully decant the supernatant fluid and allow the tube to drain for a few minutes on a filter paper. Wipe the mouth of the tube with clean tissue and dissolve the precipitate in 2 ml of 2N H_2SO_4 solution. Add a piece of freshly cleaned zinc and heat in a boiling water bath for thirty minutes. (The tube is left unstoppered to allow evaporation to occur, and the final volume should be less than 0.5 ml to ensure full color development.)

Remove the zinc with a bent glass rod. Wash the zinc with 0.5 ml of one percent chromotropic acid solution, adding the washings to the tube. This operation is most conveniently carried out by fixing the tube almost horizontally in a retort clamp and sliding the piece of zinc to the mouth of the tube where it can be washed with the chromotropic acid solution before final removal.

Add 5 ml of concentrated H_2SO_4 slowly with mixing, and heat in a boiling water bath for thirty minutes. (The tubes do not need to be stoppered.) Cool, dilute to 20 ml with 10N H_2SO_4 and determine the optical density absorbance at 570 nm. The color is stable for several hours.

Standard Curve

Dilute the stock standard oxalic acid solution (1:100 ratio) to give a solution containing 50 mg of oxalic acid per 1. Prepare six tubes containing 00,0.2, 0.4, 0.6, 0.8 and 1.0 ml of the dilute standard solution (0, 10, 20, 30, 40 and 50 mg of anhydrous oxalic acid). Add water to make the final volume 1 ml, followed by 1 ml of 4N H_2SO_4 and a piece of freshly cleaned zinc; then proceed as described above. The experimental data for procedure A are given in TABLE 1.

TABLE 1

STANDARD CURVE DATA FROM PROCEDURE A

Sample	Absorbance	Concentrations
Blank	0	0
STD_1	0.063	10 mg
STD_2	0.156	20 "
STD_3	0.225	30 "
STD_4	0.304	40 "
STD_5	0.375	50 "

Those data are also displayed in Figure 11. The absorbance versus oxalic acid concentration as determined obeys Beer's Law as shown by the correlation coefficient (R = 0.997) over the range of oxalic acid concentrations investigated (10 to 50 mg/l). Figure 12 summarizes procedure A.

Procedure A has been found to be in good agreement with other published values of twenty-four hour urinary oxalate excretion. However, it is desirable to shorten the time of analysis. That can best be done by reducing the time for quantitative precipitation of calcium oxalate. Absolute ethanol has been substituted for seventy-five percent ethanol to achieve that result, that method (procedure B) follows.

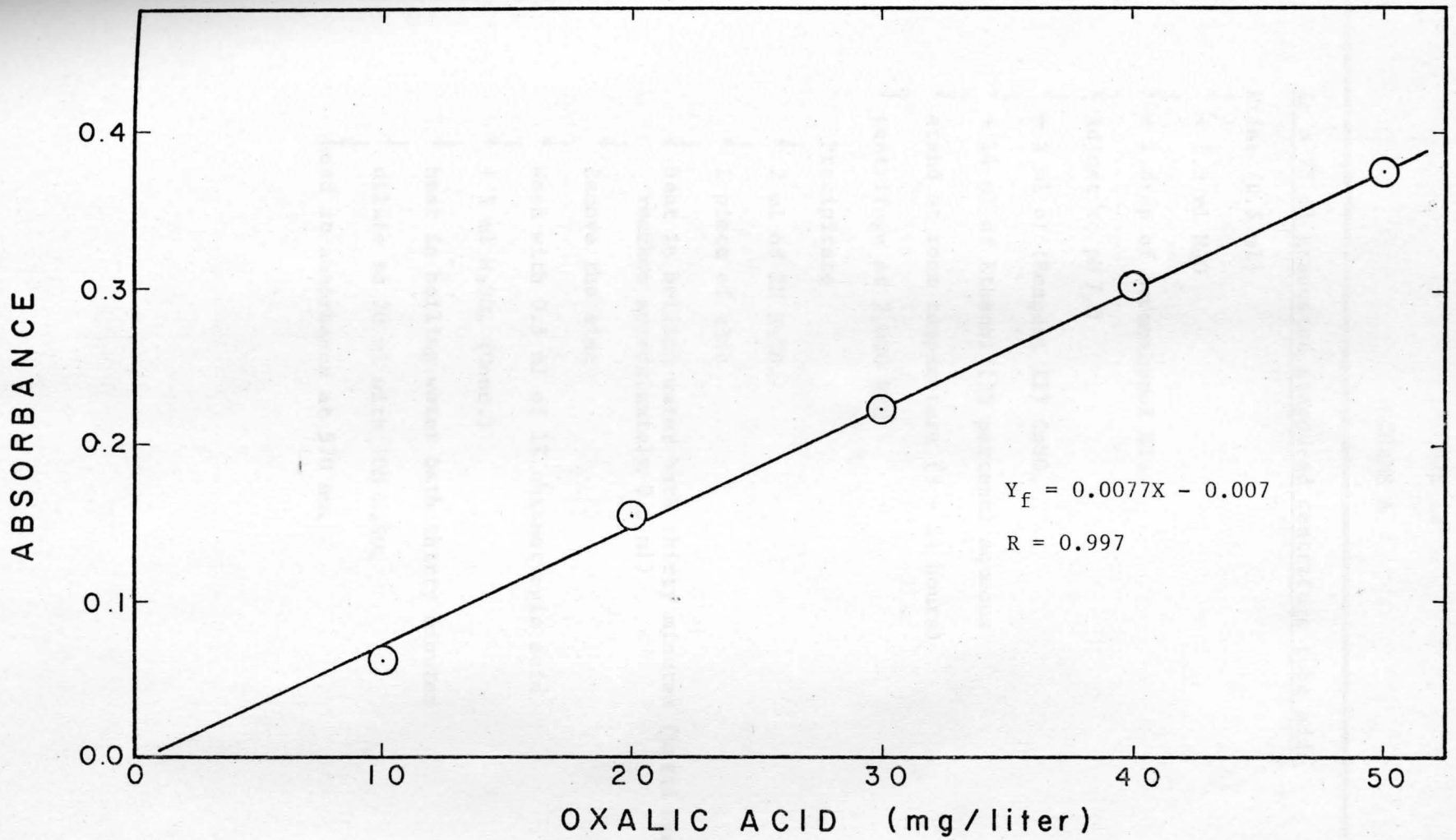


Fig. 11. Absorbance versus oxalic acid concentration.

FIGURE 12

SCHEME A

In a 25 ml graduated, stoppered centrifuge tube add:

Urine (0.5 ml)

↓ + 1.5 ml H_2O

↓ + 1 drop of Bromothymol Blue

↓ Adjust to pH 7.0

↓ + 2 ml of (Reagent 11) $CaSO_4$

↓ + 14 ml of Ethanol (75 percent) aqueous

↓ stand at room temperature (3 - 24 hours)

↓ centrifuge at 2,000 RPM

Precipitate

↓ 2 ml of 2N H_2SO_4

↓ 1 piece of zinc

↓ heat in boiling water bath thirty minutes (until the volume reaches approximately 0.5 ml)

↓ Remove the zinc

↓ Wash with 0.5 ml of 1% chromotropic acid

↓ + 5 ml H_2SO_4 (Conc.)

↓ heat in boiling water bath thirty minutes

↓ dilute to 20 ml with 10N H_2SO_4

↓ Read at absorbance at 570 nm.

Procedure B

Modified Method of Precipitation

Measure a 0.5 ml of each urine sample into a 15 ml graduated, stoppered centrifuge tube, followed by 1.5 ml of water and one drop of 0.04 percent bromothymol blue indicator solution. Adjust the solution to pH 7.0 (green) by the addition of dilute NaOH or dilute acetic acid solutions. Add 2 ml of a saturated aqueous solution of calcium sulfate followed by 12.5 ml of absolute ethanol (the test tube will fill to the base of the ground glass neck), invert slowly only twice and allow the solution to stand at room temperature for exactly 2.5 hours.

Centrifuge at 2,000 RPM for ten minutes, then carefully decant the supernatant fluid and allow the tube to drain for a few minutes on a filter paper. Wipe the mouth of the tube with clean tissue and dissolve the precipitate in 2 ml of 2N H_2SO_4 solution. Add a piece of freshly cleaned zinc and heat in a boiling water bath for thirty minutes. Leave the tube unstoppered to allow evaporation to occur, and the final volume should be less than 0.5 ml to ensure full color development.

Remove the zinc with a bent glass rod. Wash the zinc with 0.5 ml of chromotropic acid solution, adding the washings to the tube. This operation is most conveniently carried out by fixing the tube almost horizontally in a retort clamp and sliding the piece of zinc to the mouth of the tube where it can be washed with the chromotropic acid solution before final removal.

Add 5 ml of concentrated H_2SO_4 slowly with mixing, and heat in a boiling water bath for thirty minutes. (The tubes do not need to be

stoppered.) Cool, dilute to 20 ml with 10N H_2SO_4 and determine the optical density absorbance at 570 nm. The color is stable for several hours. Procedure B is outlined in Figure 13.

Figure 13

SCHEME B

In a 15 ml graduated, stoppered centrifuge tube add:

Urine (1.0 ml)

↓ + 1.0 ml H_2O

↓ 1 drop of Bromothymol Blue adjust to pH 7.0

↓ + 2 ml of $CaSO_4$

↓ + 12.5 of ethanol (absolute)

↓ Stand at room temperature (2.5 hours)

Centrifuge at 2,000 RPM

Precipitate

↓ +2 ml of 2N H_2SO_4

↓ +1 piece of zinc

↓ heat in boiling water bath thirty minutes (until the volume reaches 0.5 ml)

↓ Remove the zinc

↓ Wash with 0.5 ml of 1% chromotropic acid

↓ + 5 ml of concentrated H_2SO_4

↓ heat in boiling water bath thirty minutes, cool

↓ dilute to 20 ml with 10N H_2SO_4

↓

Read absorbance at 570 nm.

Comparison of Methods

The results for the two methods (A and B) were identical.

It was desired to show that similar quantitative results can be achieved with urine samples, a correlation study comparing method A and B was carried out. A range of twenty-four hour urinary oxalate excretion for a limited number of subjects was established using procedure A (precipitation time 24 hours). Those results are summarized in TABLE 2. The normal range as defined in Appendix B was 18 to 27.8 mg/24 hr., and the N.M.V. was 22.4 $\pm$ mg/24 hr. To show the effect of using absolute ethanol in the modified procedure (B), thirteen subjects were analyzed by both methods (A and B) where the precipitation time was fixed to 2.5 hours. TABLE 3 summarizes those results. It was noted that in almost every case the urinary oxalate concentration determined by procedure B was twice as large as those found by procedure A. The normal range is 12.5 to 66.5 mg/24 hr. and the N.M.V. was 28.8 $\pm$ 2.3 mg/24 hr. To show that maximum recovery was achieved in 2.5 hours by procedure B, a comparison of three selected urine samples were treated by procedure A with a precipitation time of 24 and 2.5 hours in both cases using 75(w/v)percent ethanol. Those results are summarized in TABLE 4. It is significant to note that urine oxalate concentrations determined by procedure B (2.5 hr. precipitation time using absolute ethanol) are equivalent to those determined by procedure A (24 hr. precipitation time using 75% ethanol). It is noted that procedure A with a 2.5 hour precipitation period gives incomplete urinaryoxalate recovery. It should be noted that previous work by Hodkinson[23] has shown that by procedure A a seventy-five to ninety-eight percent recovery with a mean value of 85.6 percent of urinary

oxalate was achieved. Within experimental error, procedure B gives the same values of urine oxalate concentrations, and the time required for that procedure has been significantly reduced.

TABLE 2

URINE OXALATE PER 24 HRS. BY PROCEDURE A
(PRECIPITATION PERIOD 24 HRS.)

Subjects	Absorbance	Urine Volume (ml)	Oxalic Acid mg/24 hr.
1. K.E.	0.020	1760	10.60
2. R.R.	0.365	1720	17.20
3. D.B.	0.098	820	21.00
4. B.L.	0.072	1110	21.09
5. L.B.	0.108	1120	32.85
6. B.E.	0.102	970	25.80
7. K.G.	0.083	1890	41.50

Sample Calculation

The concentration of oxalic acid in the original sample of urine is given by the following:

$$\text{mg of anhydrous oxalic} = \text{Reading from calibration curve (mg)} \times \frac{100}{0.5} \times \frac{1}{1000} \text{ acid per 100 ml.}$$

Procedure B gives the same values of urine oxalate concentrations, and the time required for that procedure has been significantly reduced.

TABLE 3

URINARY OXALIC ACID EXCRETION PER 24 HOURS
BASED ON PROCEDURE A AND B

Experimental Results			
Subject	Urine (ml)	(Procedure A)[a] Oxalic Acid mg/24 hrs.	(Procedure B)[b] Oxalic Acid mg/24 hrs.
1. K.V.	2260	6.1	15.8
2. N.W.	1590	14.3	28.6
3. S.C.	1540	9.8	20.0
4. P.T.	1330	15.4	30.8
5. S.R.	1440	11.4	22.7
6. H.G.	3100	32.2	55.8
7. H.K.	1200	14.4	16.8
8. D.A.	1000	26.0	29.6
9. P.S.	1400	33.6	36.4
10. R.L.	3170	7.6	25.4
11. R.L.	1760	N.AV.	24.3
12. M.H.	1680	21.8	45.4
13. F.D.	2350	23.5	51.7

[a]Procedure A using aqueous ethanol (75%), where the precipitation time is 2.5 hours.

[b]Procedure B using absolute ethanol, where the precipitation time is 2.5 hours.

TABLE 4

COMPARISON OF URINARY OXALIC ACID EXCRETION
WHERE THE TIME AND ALCOHOL CONCENTRATIONS
ARE VARIED

a. Time Allowed for Precipitation 2.5 hrs.

Subject	Ethanol %	Absorbance	Oxalic Acid mg/24 hrs.	Ethanol %	Absorbance	Oxalic Acid mg/24 hrs.
1. N.W.	75	0.035	14.3	100	0.067	28.6
2. C.S.	75	0.023	9.9	100	0.049	20.0
3. K.S.	75	0.014	6.2	100	0.026	15.8

b. Time Allowed for Precipitation 24 hrs.

1. N.W.	75	0.065	27.0
2. C.S.	75	0.043	17.3
3. K.S.	75	0.022	13.6

CHAPTER V

SPECTROFLUORIMETRIC ANALYSIS USING CERIC ION

A method of determining urine oxalate concentrations is discussed. One is fluorimetric, the other colorimetric; both methods are based on the Ce(IV)-Ce(III) couple. The reduction of Ce(IV) to Ce(III) is a one electron transfer reaction:

$$Ce^{+4} + e^{-} \longrightarrow Ce^{+3} \qquad (1)$$

The oxidation reduction reaction between oxalate and Ce(IV) can be expressed by the following reaction:

$$2Ce^{+4} + C_2O_4^{=} \longrightarrow 2Ce^{+3} + 2CO_2 \qquad (2)$$

Equation (2) represents the overall reaction. Cerium(IV) is a one electron acceptor and oxalate is a two electron donor.

Spectrofluorimetric

Kirckbright[25] and others have reported the spectrofluorimetric detection of oxalate in aqueous solutions by detecting the Ce(III) ion produced by reaction (2). In dilute sulfuric acid Ce(III) exhibits a characteristic fluorescence with an excitation maximum at 260 nm and a fluorescence emission of maximum intensity at 350 nm. In the present application reaction (2) is catalyzed using iodine-monochloride.[21,25]

Following the procedure of Kirckbright[25] a range (2 to 80 mg/l) of aqueous oxalic acid solutions were prepared. The solutions were

reacted with Cerium (IV) ammonium sulfate in the presence of 10^{-5}M H_2SO_4 with ICl ($3x10^{-4}$M) at 50°C. The reactions were carried out and the fluorescence read at thirty minutes. The details of that procedure follow.

Standard Curve

To a series of 50 ml test tubes add 47 ml of distilled water and 1 ml of each oxalic acid acid solution, stopper each test tube and wait until the solution has reached 50°C. When temperature equilibrium has been reached, add 2 ml of ceric solution with catalyst to each test tube (include an aqueous blank), stopper, invert several times and place in the water bath. At the end of thirty minutes the fluorescence is recorded.

The results are presented in Figure 14. The slope of those data is 1.267 1/mg and the correlation coefficient is +0.996. So, it is evident that there is a linear relationship between Ce(III) fluorescence and oxalic acid concentration.

It was then desired to adapt this fluorescence technique to the determination of urinary oxalic acid concentrations. It is necessary to establish that the reagents and procedure used in the extraction of calcium oxalate do not interfere with the fluorescence measurements. The separation procedure was first carried out with aqueous calcium oxalate solutions. The direct precipitation of procedure B was used to separate oxalic acid as calcium oxalate from aqueous solutions. The calcium oxalate obtained was dissolved in 2N H_2SO_4. The solutions were then treated as above. The fluorescence was recorded at 30, 60, 90, 180, 300 minutes to determine when reaction (2) was complete. The summary of

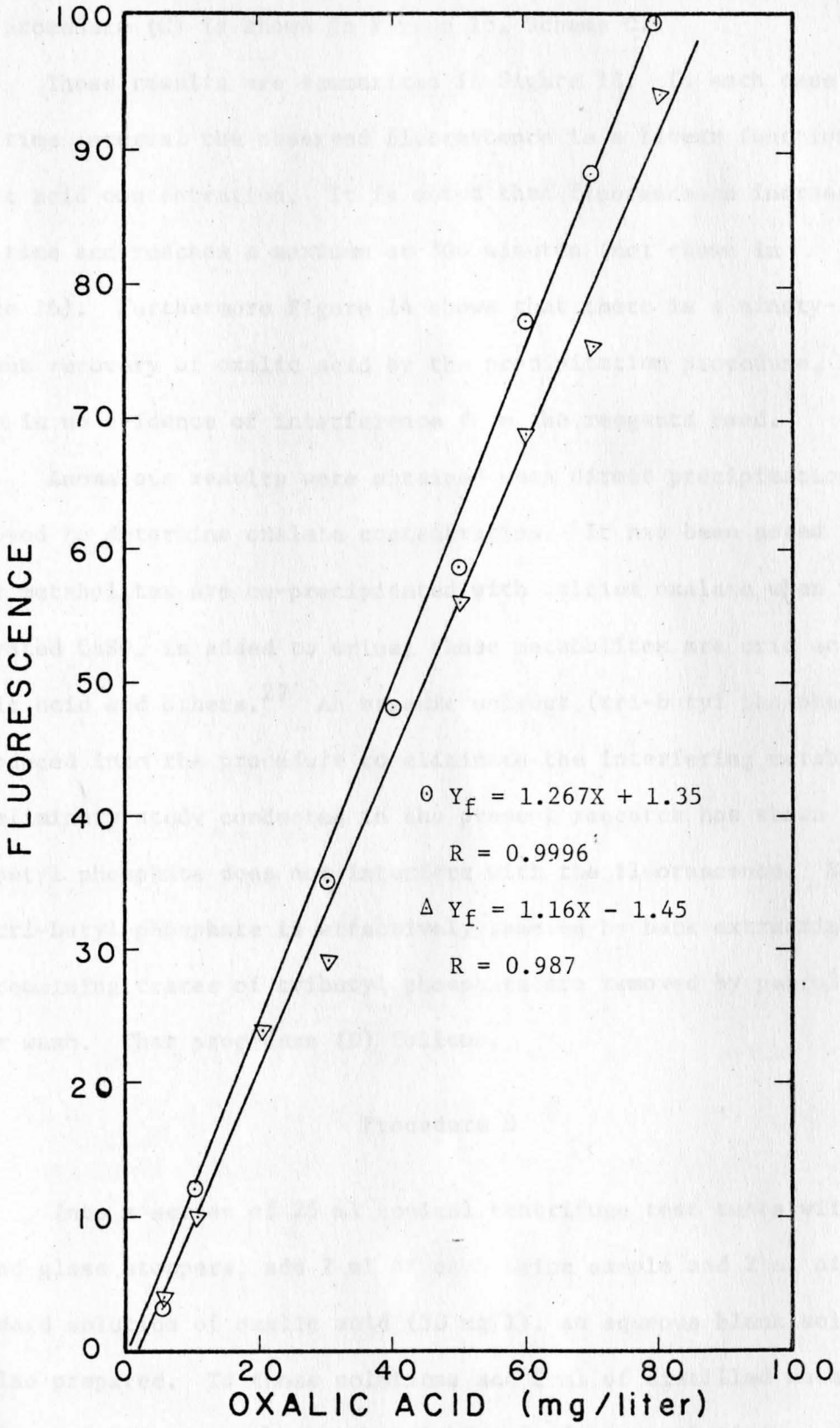


Fig. 14. Fluoresence versus oxalic acid concentration; (△) aqueous oxalic acid solutions precipitated and (⊙) aqueous oxalic acid solutions.

that procedure (C) is shown in Figure 15, Scheme C.

Those results are summarized in Figure 16. In each case at each time interval the observed fluorescence is a linear function of oxalic acid concentration. It is noted that fluorescence increases with time and reaches a maximum at 300 minutes (not shown in Figure 16). Furthermore Figure 14 shows that there is a ninety-five percent recovery of oxalic acid by the precipitation procedure, and there is no evidence of interference from the reagents used.

Anomalous results were obtained when direct precipitation was employed to determine oxalate concentration. It has been noted that other metabolites are co-precipitated with calcium oxalate when saturated $CaSO_4$ is added to urine; those metabolites are uric acid, citric acid and others.[27] An organic solvent (tri-butyl phosphate) was introduced into the procedure to eliminate the interfering metabolites. A preliminary study conducted in the present research has shown that tri-butyl phosphate does not interfere with the fluorescence. Since the tri-butyl phosphate is effectively removed by back extraction, the remaining traces of tributyl phosphate are removed by petroleum ether wash. That procedure (D) follows.

Procedure D

Into a series of 25 ml conical centrifuge test tubes with ground glass stoppers, add 2 ml of each urine sample and 2 ml of a standard solution of oxalic acid (50 mg/l), an aqueous blank solution is also prepared. To those solutions add 2 ml of distilled water and 1 drop of 0.04 percent bromo-thymol blue indicator solution.

Figure 15

SCHEME C

1 ml of Sample (Standard and Blank)

↓ + 1 ml of $CaCl_2$ Solution

↓ + 2 ml of $CaSO_4$ Solution

↓ + 12.5 ml of absolute ethanol

↓ Let it stand for 2.5 hrs.

↓ Centrifuge at 2000 RPM, 10 minutes

↓ Decant supernatant (discard)

Precipitate

↓

+ 2 ml of H_2SO_4

↓

+ 46 ml of H_2O

↓

Equilibriate temperature (50±2)°C

↓

+ 2 ml of Cerium solution

↓

Read fluorescence at time intervals (30, 60, 90, 180, 300 minutes).

Adjust the solution to pH 7.0 (green) by the addition of dilute sodium hydroxide or dilute acetic acid solution. Add 4 ml of a saturated aqueous solution of calcium sulfate followed by 17 ml of absolute ethanol. The test tubes are slowly inverted twice and the solutions are allowed to stand for 2.5 hours at room temperature. Centrifuge at 2000 RPM for ten minutes; carefully decant the supernatant and allow the tube to drain for a few minutes. To each test tube add 2 ml of H_2O, 0.1 ml of 98 percent formic acid, 0.22 ml of 10N HCl and 10 ml of tributyl phosphate, stopper each tube immediately. Vortex each test tube for two minutes at high speed, then centrifuge at 3000 RPM for fifteen minutes and discard

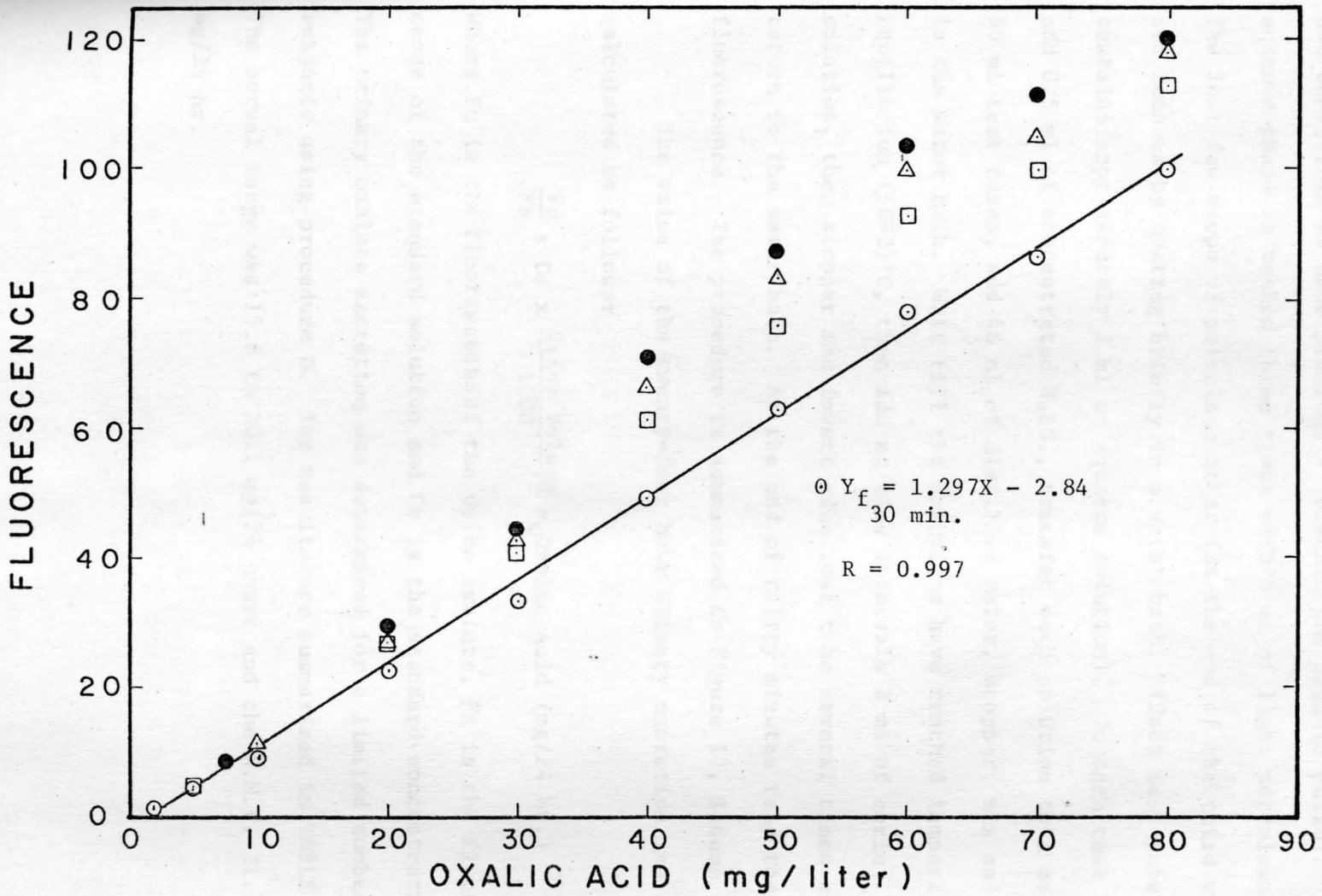


Fig.16. Fluorescence versus oxalic acid concentration at various time intervals. The circles, squares, triangles and solid circles correspond to 30, 60, 90, 180 min., respectively.

the aqueous phase. To the organic phase add 1.9 ml of 2N NaOH, vortex and centrifuge as done previously (discard the organic phase). The aqueous phase is washed three times with 5 ml of light petroleum ether. The last few drops of petroleum ether (at the end of the third wash) are removed by heating briefly in a water bath. (Each test tube contains approximately 2 ml of aqueous solution). To each test tube add 0.5 ml of concentrated H_2SO_4, transfer each solution to a series of 50 ml test tubes, add 46 ml of distilled water, stopper, mix and place in the water bath. Wait till the solutions have reached temperature equilibrium $(50 \pm 2)°C$, then add at time intervals 2 ml of ceric solution, then stopper and invert each test tube several times and return to the water bath. At the end of thirty minutes read the fluorescence. The procedure is summarized in Figure 17, Scheme D.

The value of the twenty-four hour urinary excretion are calculated as follows:

$$\frac{Fu}{Fs} \times Cs \times \frac{\text{Urine Volume}}{1000} = \text{Oxalic Acid (mg/24 hr.)}$$

Where Fu is the fluorescenseof the urine oxalate, Fs is the fluorescence of the standard solution and Cs is the standard concentration. The urinary oxalate excretion was determined for a limited number of subjects using procedure D. The results are summarized in TABLE 5. The normal range was 15.6 to 30.1 mg/24 hours and the N.M.V. 21.7 ± 1.4 mg/24 hr.

Figure 17

SCHEME D

2 ml of Sample (Urine, Standard and Blank)

↓ + 2 ml of distilled water

↓ Adjust to pH 7.0 bromo-thymol blue

↓ + 4 ml $CaSO_4$ solution

↓ + 17 ml of absolute ethanol

↓ Let it stand for 2.5 hours

↓ Centrifuge at 2000 RPM 10 minutes

Precipitate [decant supernatant (discard)]

↓ + 2 ml distilled water

↓ + 0.1 ml of 98 percent Formic Acid

↓ 0.22 of 10N HCl

↓ 10 ml of tributyl phosphate

↓ Stopper, vortex (high speed for 2 minutes), centrifuge (3000 RPM 15 minutes)

Aqueous phase (discard) ← Ester Phase

Ester Phase →

↓ + 1.9 ml of 2N NaOH, vortex, centrifuge

↓ Aqueous <u>washed</u> 3 times with petroleum ether

Ester phase (discard) ← ↓ + 0.5 ml of concentrated H_2SO_4

↓ + 46 ml of distilled water

↓ Equilibriate at $(50 \pm 2)°C$

↓ + 2 ml of cerium solution

↓ Read fluorescence at time specified (30 minutes)

TABLE 5

NORMAL SUBJECT'S URINARY OXALIC ACID EXCRETION

Sample	Urine Volume (ml)	Fluorescence	Oxalic Acid (mg/24 hr.)
1.	1550	16.5	12.8
2.	1720	21.0	18.0
3.	2420	26.0	31.4
4.	515	37.0	9.5
5.	1940	33.0	37.0
6.	4570	19.0	43.0
7.	810	49.0	19.9
Standard Solution	1000	100.0	50.0

CHAPTER VI

COLORIMETRIC ANALYSIS USING CERIC ION

Principle

During the titration of oxalate ions with Cerium(IV) the yellow color of the solution fades as the colorless Cerium(III) ions are produced.[21] That reaction is best carried out at 50°C in the presence of iodine monochloride catalyst and sulfuric acid. Ohlweiler[28] has reported a photometric standardization of Ce(IV) solutions with standard sodium oxalate with a 0.01 percent relative error. Cerium (IV) has been used to determine urinary oxalate concentrations by titration of precipitated calcium oxalate with Cerium (IV) by means of an indicator.

In the present work a spectrophotometric technique is developed to determine twenty-four hour urinary excretion levels. Ohlweiler[28] has shown that excellent results are obtained in a spectrophotometric determination of Ce (IV) ions by aqueous oxalate standards. In order to apply that method of detection to urine oxalate concentrations, it was first necessary to determine if that method is applicable to a range of concentrations which is expected in urine samples. Various concentrations (0 to 80 mg/l) of aqueous oxalic acid were prepared. The method that was employed for recovery of urine oxalates was applied to those solutions. That precipitation method follows.

Procedure E

Determination of Oxalate in Aqueous Solutions

To a series of 80 ml test tubes (conical) add 50 ml of each standard solution and using a glass electrode adjust the pH to within the range 5.0 to 5.2 with dilute NH_4OH or dilute HCL. $CaCl_2$ (2 ml of 5 percent w/v solution) was added and mixed thoroughly; the specimen was left at room temperature for about sixteen hours. The precipitated calcium oxalate was separated by centrifuging at 3,000 RPM for twenty minutes. To a series of 50 ml test tubes add 50 ml of 0.005 M Ce(IV) with 0.0004 M ICl catalyst; let the solution temperature come to $(50\pm2)°C$. Transfer the calcium oxalate quantitatively to a 15 ml conical centrifuge tube and washed with two 4 ml portions of approximately 0.35N NH_4OH. The washed precipitate is allowed to drain. To each washed test tube transfer the ceric solution, stopper, shake vigorously and again transfer the ceric solution to its original 50 ml test tube; place the test tubes back to their original position in the water bath. Wait two to five minutes, then read the absorbance at 3800 Å. It has been previously reported that recovery of oxalate from aqueous solutions by the above procedure is ninety to ninety-two percent.[29]

The results of Procedure (E) applied to the photometric determination of oxalate in aqueous solutions are displayed in Figure 18. From the correlation coefficient (R = 0.996), there is a linear response to the oxalic acid concentration employed in this study.

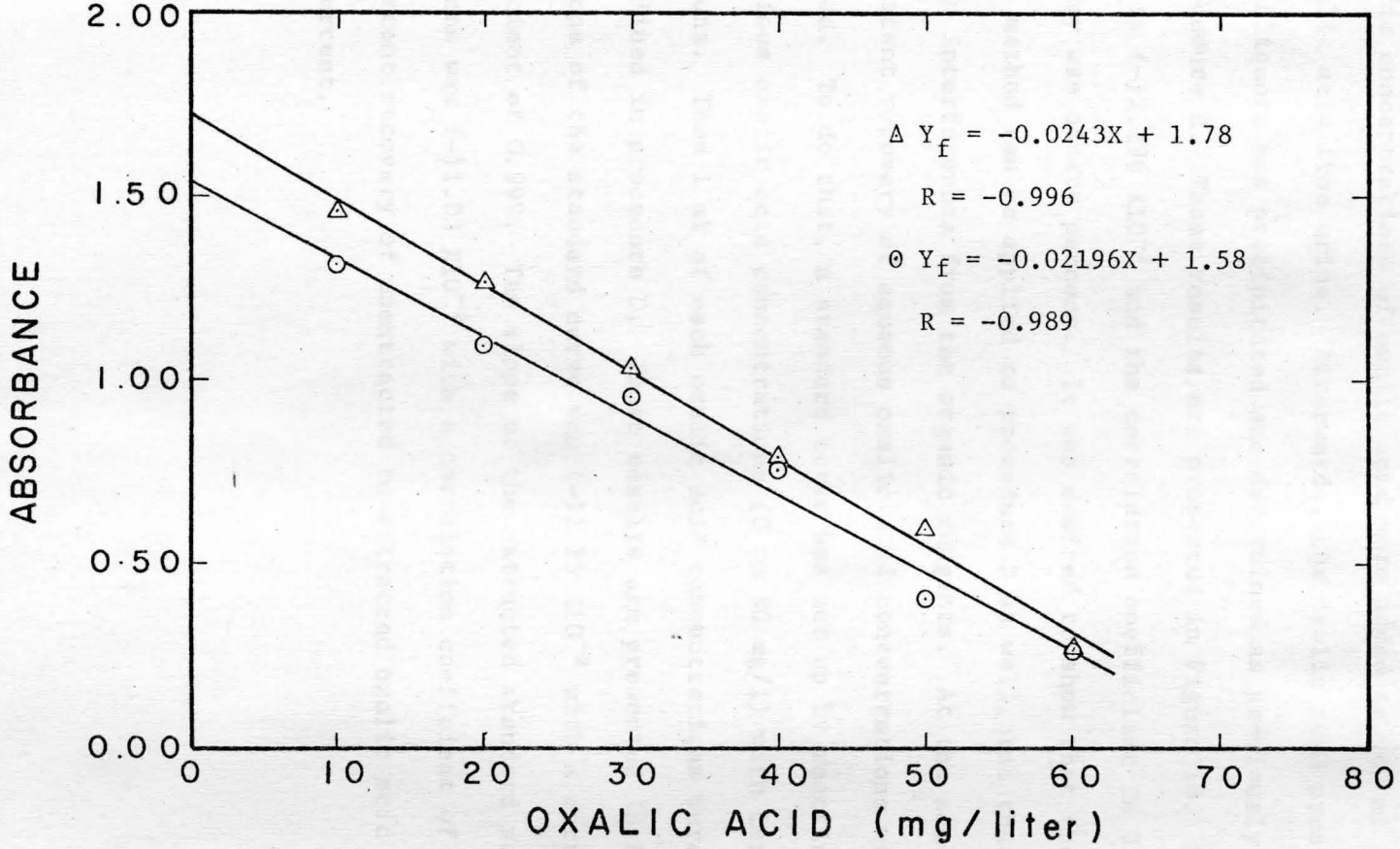


Fig. 18. Absorbance of Cerium(IV) versus oxalic acid concentration; (Δ) aqueous oxalic acid solutions precipitated and (0) urine oxalic acid precipitated.

It was next desired to do an oxalate recovery study from urine. To insure that urine contained from 0 to 100 mg of oxalate, calcium oxalate (endogenous) was removed as above (procedure E). Specific concentrations of oxalic acid were added to 100 ml aliquots of oxalic acid free urine. Afterwards, the oxalic acid present in urine aliquots was precipitated and determined as previously described in procedure E. Those results are presented in Figure 18. The slope is (-)2.196 $X10^{-2}$ and the correlation coefficient is 0.989. The recovery was ninety percent. It was desired to show that the photometric method can be applied to procedure D as well, and that there were no interferences from the organic reagents. At the same time, the percent recovery of aqueous oxalic acid concentrations were obtained. To do that, a standard curve was set up by reacting a 1 ml of various oxalic acid concentrations (0 to 80 mg/l) with Cerium(IV) solutions. Then 1 ml of each oxalic acid concentrations were analyzed as outlined in procedure D. Those results are presented in Figure 19. The slope of the standard curve was (-)1.25 $X10^{-2}$ with a correlation coefficient of 0.999. The slope of the extracted standard aqueous solutions was (-)1.03 $X10^{-2}$ with a correlation coeficient of 0.995. The percent recovery of unextracted to extracted oxalic acid was 82.4 percent.

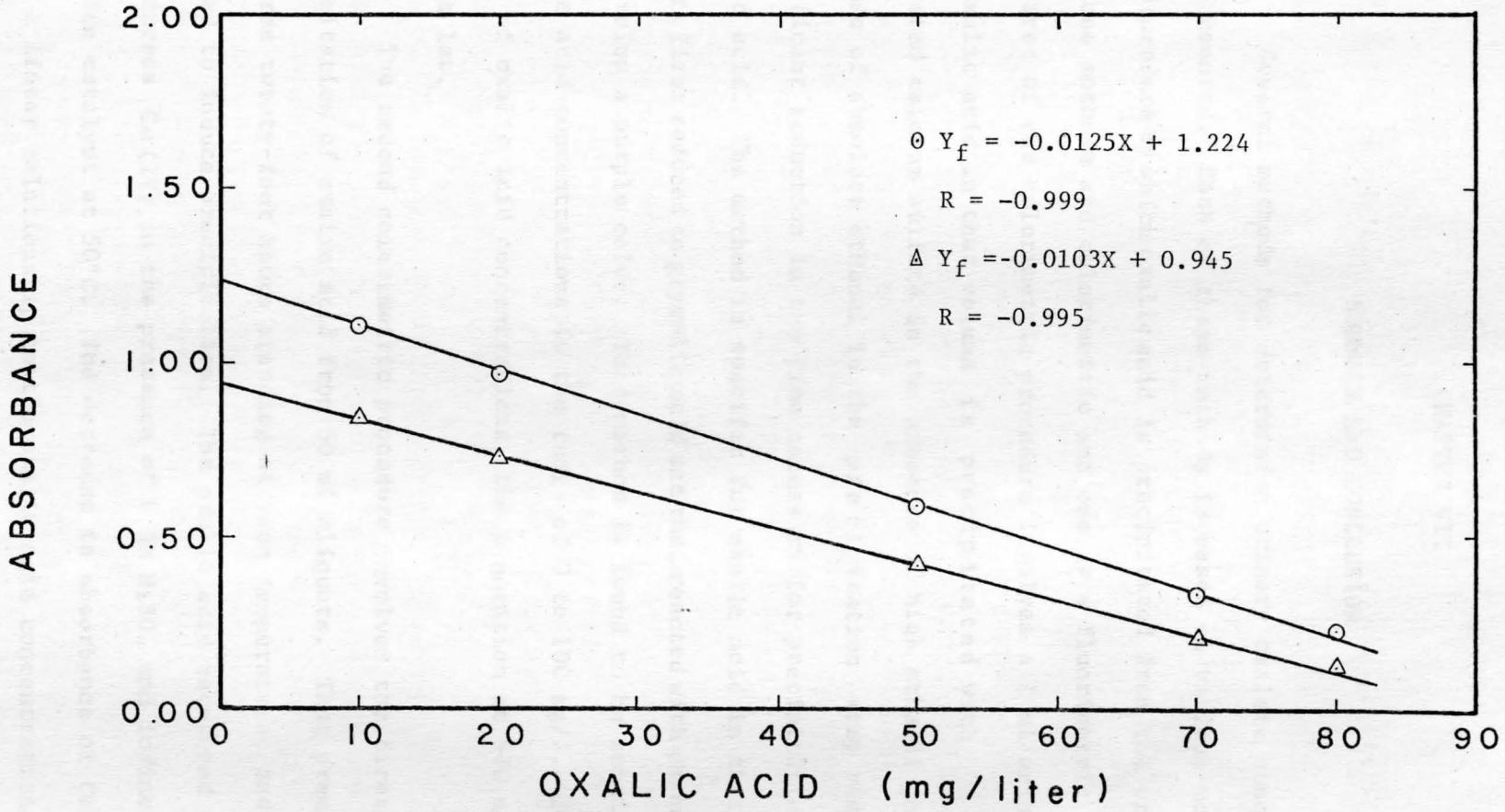


Fig. 19. Absorbance of Cerium(IV) versus oxalic acid concentration; (Δ) standard aqueous oxalic acid solutions precipitated (procedure D), (0) aqueous standard oxalic acid solutions.

CHAPTER VII

SUMMARY AND CONCLUSION

Several methods for determining urinary oxalate concentrations are presented. Each of those methods is based on various modifications of a procedure by which oxalic acid is precipitated from the urine. Two of those methods are colorimetric and one is a fluorimetric technique. The first of the colorimetric procedure involves a 1 ml urine sample. The oxalic acid in that volume is precipitated with saturated calcium sulfate in the presence of high ethanol concentration. The use of absolute ethanol in the precipitation step results in a significant reduction in the time necessary for precipitation of oxalic acid. The method is specific for oxalic acid in that oxalic acid is first reduced to glycolic acid and then reacted with chromotropic acid to develop a purple color. That method is found to be sensitive to oxalic acid concentrations in the range of 0 to 100 mg/l. In that range of oxalic acid concentrations, the absorption at 570 nm obeys Beer's law.

The second colorimetric procedure involves the direct precipitation of oxalic acid from 50 ml aliquots. That precipitation requires twenty-four hours standing at room temperature, and no alcohol is used to induce precipitation. The oxalic acid recovered is reacted with excess Ce (IV) in the presence of 0.5M H_2SO_4 and iodine monochloride catalyst at 50°C. The decrease in absorbance of Ce(IV) ions shows a linear relationship to the oxalic acid concentration range (0 to 80 mg/l) employed in the present study. The fluorimetric

technique is also based on the redox reaction between Ce(IV) and recovered urine oxalate. In this case, the fluorescence of the Ce-(III) ion produced by reacting the recovered oxalic acid with Ce(IV) in dilute sulfuric acid at 50°C is measured. However by this technique it is necessary to extract the oxalic acid precipitate with tributyl phosphate since co-precipitated metabolites which were found to interfere with measured fluorescence of Ce(III) must be removed.

Of the techniques investigated in the present research, the fluorimetric method is the most susceptible to erroneous results. It appears that all the metabolites which are co-precipitated with calcium oxalate may be removed by the tributyl phosphate extraction step. Good results were obtained with careful laboratory techniques. The normal mean values were found to be comparable to the other two methods employed herein. However, it is not certain that all interfering ions have been eliminated.

The time necessary for the determination of urine oxalate by chromotropic acid, as previously reported, has been reduced from 24 hours to 2.5 hours for the precipitation by substituting absolute ethanol for seventy-five percent ethanol with no sacrifice of oxalate recovery. It has been shown that with aqueous ethanol, complete precipitation is achieved only after twenty-four hours. Furthermore, both methods show a normal mean value comparable to previously reported urinary oxalate values. Finally, the improved method (procedure B) is favored when analysis is conducted by this technique.

When the time element is of secondary importance to the clinical test results, then the second colorimetric method involving the direct precipitation is preferred. First of all, both colorimetric

methods are equally specific for oxalic acid. However, a greater number of steps are required for the first method, and it is also a more hazardous procedure since it involves boiling with sulfuric acid. The second method [using Cerium (IV)] involves very few steps, and the results are quite satisfactory. The latter method of analysis is felt to be the preferred method since a large number of analyses can be done routinely without the involvement of a tedious analysis.

APPENDIX A

Linear Regression and Simple Linear Correlation[30]

The following least squares linear regression is designed to minimize the sum of the squares of the deviations of the actual data points from the straight line of best fit.[30] Essentially a plot is constructed of the variables (called a scatter diagram) and drawing the best straight line which uniformly divides the data points as shown below (Figure 20). Since the data may not be best represented by a straight-line curve, it is desirable to measure how well the linear curve actually does not fit the data. This measure is called the correlation coefficient and may be calculated from the independent variables and the linear equation parameters.

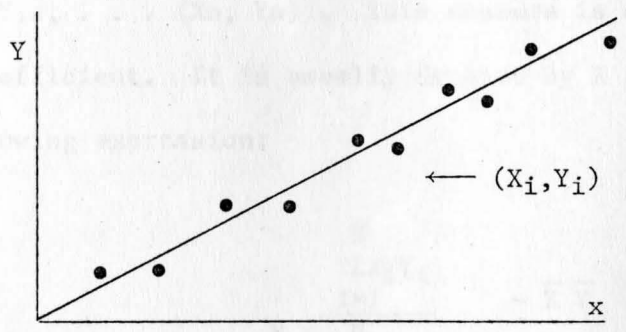


Fig. 20. Scatter diagram

A linear equation of the form (3) is used to represent the diagram

$$Y = mx + b \tag{3}$$

It can be shown that the slope and Y - intercept are determined as follows:

$$m = \frac{\frac{\sum_{i=1}^{N} X_i Y_i}{N} - \bar{X}\,\bar{Y}}{x^2} \tag{4}$$

$$b = \bar{Y} - m\bar{X} \tag{5}$$

$\bar{X}$ = Average X value = $\frac{\sum_{i=1}^{N} X_i}{N}$

$\bar{Y}$ = average Y value = $\frac{\sum_{i=1}^{N} Y_i}{N}$

x^2 = variance of the X values = $\frac{\sum_{i=1}^{N} X_i^2}{N} - \bar{X}^2$

After the linear regression curve is determined, there is a method to measure the degree of association between the random variable (X_1, Y_{1o}, . . . (Xn, Yn)). This measure is called the correlation coefficient. It is usually denoted by R and is calculated using the following expression:

$$R = \frac{\frac{\sum_{i=1}^{N} X_i Y_i}{N} - \bar{X}\,\bar{Y}}{x\ y} \tag{6}$$

Where

y = variance of the y values

$$y = \frac{\sum_{i=1}^{N} Y_i^2}{N} - \bar{y}^2$$

by rewriting equation six in terms of equation 4, one may show that

$$R = \frac{m\,x}{y} \tag{7}$$

or

$$m = \frac{R\,y}{x} \tag{8}$$

Using this expression, the linear regression curve, equation 1, may be expressed as

$$Y = \frac{R\,y}{x}\,X + b \tag{9}$$

Equations seven and nine define an efficient method for both determining the best linear curve fit and assessing the degree of association between the variable and the linear equation.

When the values of the constants m and b are known, a best fitted Y variable can be calculated as

$$Y_f = mX + b \tag{10}$$

Y_f are the fitted values for the statistically best straight line (10). The estimated absolute standard deviation of Y_f and b will be taken equal to S,

$$S^2 = \frac{\Sigma(Y-Y_f)^2}{N-2} \tag{11}$$

So that the standard deviation of $Y_f - b$ will be $\sqrt{2}\cdot S$. The estimated variance of the slope m of the standard curve is given by

$$Sa^2 = \frac{NS^2}{N\Sigma X_i^2 - (\Sigma X_i)^2} \tag{12}$$

The estimated standard deviation of X in percent is accordingly given by equation 13.

$$\frac{100\,Sx}{X} = \frac{100}{a}\left[\frac{2S^2}{X^2} + Sa^2\right]^{1/2} \tag{13}$$

The number of degrees of freedom is two less than the number of Y values because two parameters, a and b, have been calculated.

Sx, Sa are the estimated standard deviations of X and the Slope m.

APPENDIX B

NORMAL VALUES

Theoretical and Practical Aspects

The first step in determining normal values is the data collection or sampling. Ideally a normal value or range would be derived from the entire population to which it is applied.[31] Since this is impractical, only a few samples are used. The criteria used to select the samples of normal subjects are as follows: (1) that the real life process of sampling determines the theoretical meaning of normal values and (2) that "normal" is used only as a statistical term and does not imply a deterministic concept, such as the state of health. Therefore, random samples have been selected from clinical laboratory routine specimens and students in this laboratory.

To derive a normal range from a small set of data, a statistical treatment suggested by Tietz[31] is used. This method requires that the values obtained be arranged in order of increasing magnitude. Each number is then plotted on the horizontal axis of a linear graph paper against the normal equivalent deviate (N.E.D.)[31] on the vertical axis. After plotting all values, a straight line is drawn representing all points. If the data obtained represents a curve rather than a straight line, a logrithmic transformation of all data is made and these values are plotted against the N.E.D. If there is a random distribution of these values from the straight line, the distribution of values is considered to be normal.

If the deviation from the straight line is systematic, the data probably did not come from a homogenous population. To show the N.E.D. method a sample calculation in set up (from TABLE 3, procedure B). Once it has been ascertained that the distribution follows a log normal distribution, a mathematical approach (Appendix A) will be used to determine the mean and standard deviation (see Figure 21).

TABLE 6

THE N.E.D. NORMAL DISTRIBUTION

N	X	Log X	Log $(\overline{X} - X)$	Log $(X - X)^2$	N.E.D.
1	15.82	1.199	0.26	0.0676	-0.935
2	16.80	1.225	0.23	0.0529	-0.755
3	20.02	1.302	0.157	0.025	-0.598
4	27.75	1.357	0.102	0.010	-0.454
5	24.30	1.386	0.073	0.005	-0.319
6	25.40	1.073	0.054	0.003	-0.189
7	28.62	1.457	0.002	0.000	-0.063
8	29.60	1.471	0.012	0.000	+0.063
9	30.86	1.489	0.029	0.001	+0.189
10	36.40	1.561	0.102	0.010	+0.319
11	45.40	1.657	0.198	0.039	+0.454
12	51.70	1.714	0.255	0.065	+0.598
13	55.80	1.750	0.291	0.085	+0.755

Sample Calculations

X = Oxalic Acid Concentration (Mg/24 hr.)

$$\log X = \frac{\Sigma \log X}{N} = \frac{18.97}{13} = 1.459 \qquad S = \frac{\Sigma \log (\overline{X} - X)^2}{-N - 2} = \frac{0.364}{11} = 0.182$$

$$\log \overline{X} \pm 2S = 1.459 \pm 0.364$$

Normal Range 1.459 + 0.364 = 1.823 and 1.459 - 0.364 = 1.095

Normal Mean = 1.459 Standard deviation = 0.364

taken the antilog of the above

Range = 12.45 to 66.5 Mean = 28.77 ± 2.3

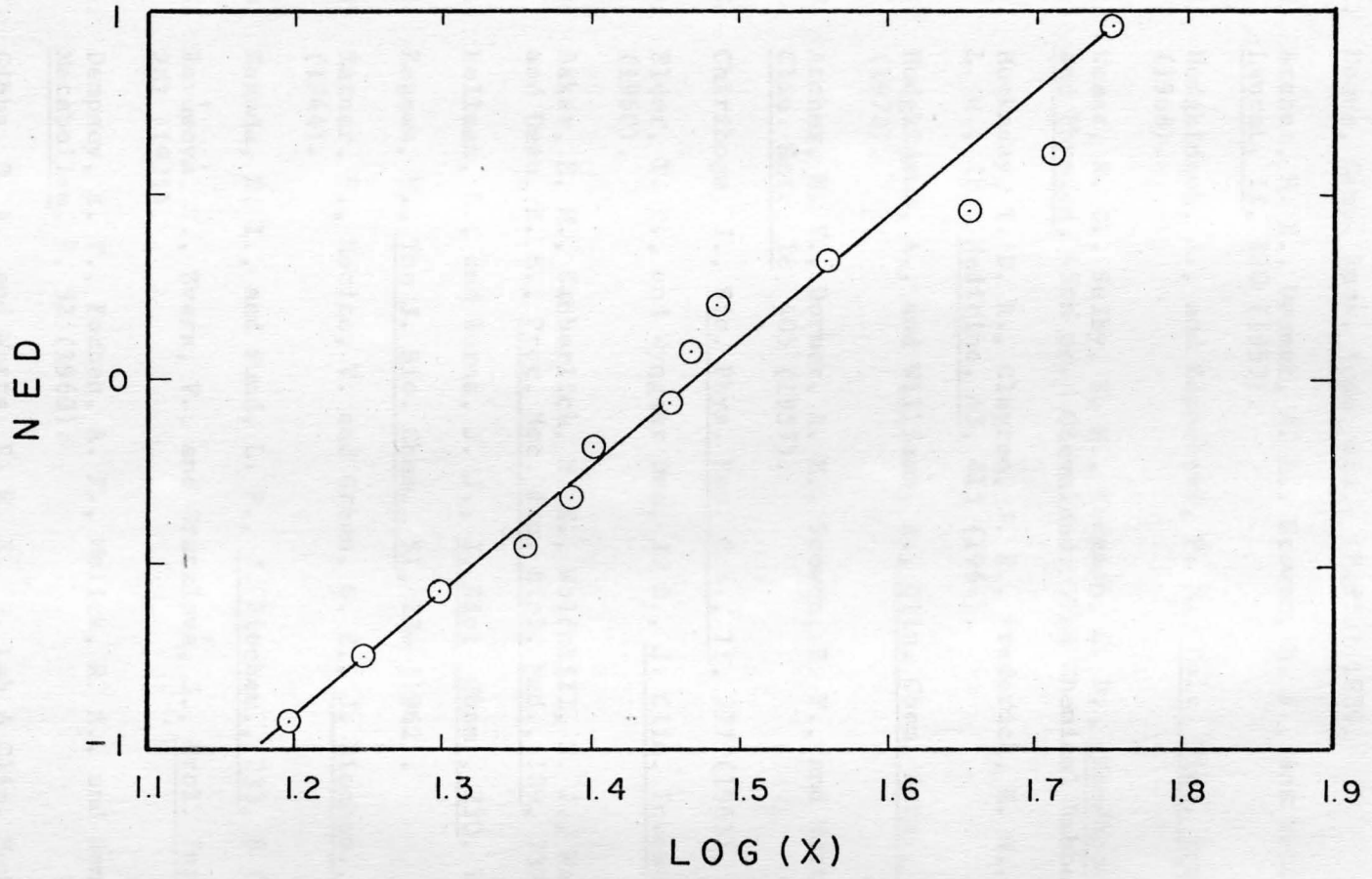


Fig. 21. Normal equivalent deviate (NED) versus log (X).

REFERENCES

1. Donné, Comp. Reus. Acad. Sci., (Paris) 1839.

2. Archer, H. E., Dormer, A. E., Scowen, E. F., and Watts, R. W. E., Lancet, ii, 320 (1957).

3. Hodgkinson, A., and Zarembski, P. M., Calc. Tiss. Res., 2, 115-132 (1968).

4. Weast, R. C., Selby, S. M., Hodgman, C. D., Handbook of Chemistry and Physics, 45th ed. Cleveland: The Chemical Rubber Co., 1964.

5. Hockaday, T. D. R., Clayton, J. E., Frederick, E. W., and Smith, L. H., Jr., Medicine, 43, 415 (1964).

6. Hodgkinson, A., and Williams, A., Clin. Chem. Acta., 36, 127-132 (1972).

7. Archer, H. E., Dormer, A. E., Scowen, E. F., and Watts, R. W. E., Clin. Sci., 16, 405 (1957).

8. Chiriboga, J., Bio. Phys. Res. Com., 11, 277 (1963).

9. Elder, T. D., and Wyngaar Deu, J. B., J. Clin. Invest., 39, 1337 (1960).

10. Baker, E. M., Sanberlick, H. E., Wolfskill, S. J., Wallace, W. T., and Deau, E. E., Proc. Soc. Exp. Biol. Med., 109, 737 (1962).

11. Hellman, L., and Burns, J. J., J. Biol. Chem., 230, 923 (1958).

12. Kagawa, Y., The J. Bio. Chem., 51, 134 (1962).

13. Ratner, S., Nocito, V. and Green, D. E., J. Biochem., 152, 119 (1944).

14. Nakada, H. I., and Sund, L. P., J. Biochem., 233, 8 (1958).

15. Revúsová, V., Zvara, V., and Gratzlová, J., Urol. Int., 26, 277-282 (1971).

16. Dempsey, E. F., Forbes, A. P., Melick, R. A., and Henneman, P. H., Metabolism, 9, 52 (1960).

17. Gibbs, D. A., and Watts, R. W. E., J. Lab & Clin. Med., June, (1969).

18. Takasaki, E., Shimand, E., Investigative Urology, 5, 3, 303-312 (1967).

19. Hodgkinson, A., Clin. and Mol. Med., 46, 357-367 (1974).

20. Evans, R. A., Forbes, M. A., Sutton, R. A. L., and Watson, L., Lancet, iv, 958-961 (1967).

21. Kolthoff, I. M., Sandell, E. B., Meehan, E. J. and Bruckenstein, S., Quantitative Chemical Analysis, 4th ed., p. 838.

22. Hodgkinson, A. and Zarembski, P. M., Analyst, 86, 16 (1961).

23. Zarembski, P. M. and Hodgkinson, A., Biochem. J., 96, 17 (1965).

24. Goldsmith, J. N., Thorpe's Dict. Appl. Chem., 8, 367 (1949).

25. Kirckbright, G. F., West, T. S., and Woodward, C., Anal. Chim. Acta, 36, 298-303 (1969).

26. Leach, Carolyn, S., Rarnbault, Paul, C., and Fischer, C. L., Clin. Biochem., 8, 2, 108-117 (1965).

27. Dutt, Eswara, V. V. S., and Mottola, H. A., Biochemical Medicine, 9, 148-157 (1974).

28. Ohlweiler, L. A., Revista Clinica Espanola, 125, 19-26 (1972).

29. Archer, H. E., Clin. Sci., 16, 405 (1957).

30. Werner, Mario, Marsh, L., CRC Critical Reviews in Clinical Laboratory Sciences, 81-100 (1975).

31. Tietz, W. Norbert., Fundamentals of Clinical Chemistry. Philadelphia, Pa.: W. B. Saunders Co., p. 69, 1970.