THE MICRODETERMINATION OF PHOSPHORUS IN BIOLOGICAL MATERIALS

A Thesis

Presented to

The School of Pharmacy

University of Manitoba

In Partial Fulfillment

of the Requirements for the Degree

Master of Science (Pharmacy)

by

KEITH JOHN SIMONS

April 1969

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to the following individuals:

To Dr. J. A. M. Shaw for his guidance and supervision of the entire project.

To Dr. N. N. De for his help in obtaining the samples of rabbit serum, and to Dr. Shaw and Miss R. Ewart for providing the biological fraction of Proteus OX-19.

To Dr. J. W. Steele, Dr. W. Alexander and Dr. J. Templeton for their useful suggestions with regard to the use of the instruments and the analytical techniques.

To the other staff members of the School of Pharmacy for their suggestions and encouragement during the project.

ABSTRACT

A method has been developed for the determination of total phosphorus in biological materials. The samples were ashed in test tubes with N/1 sulfuric acid, cleared with nitric acid containing 50 mg. calcium carbonate per litre, and the phosphorus complexed with molybdenum. The source of heat for the ashing procedure was a sand bath heated with a hot plate. The phosphomolybdic acid was then extracted into an organic solvent composed of xylene 65 per cent and isobutanol 35 per cent and read spectrophotometrically at 310 mu.

This method has been developed after an extensive literature survey and investigation. Other methods tested were the heteropoly or molybdenum blue method and the molybdovanadophosphoric acid method. These methods were rejected because of complications during the ashing procedure and the spectrophotometric determination.

This new extraction method has been applied to the determination of phosphorus in serum, urine, and a trichloroacetic acid extract of Proteus OX-19, and shows good reproducibility. The range of this method is 1 - 5 ug. if used as presented here, but could easily be expanded to handle larger samples with higher percentages of phosphorus.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
The Importance of Phosphorus Determination	1
Evaluation of Methods of Phosphorus Determination	3
Heteropoly Blue Methods	3
Modified Heteropoly Blue Methods	13
Molybdovanadophosphoric Acid Methods	16
Automatic Methods	19
Colourimetric and Titrimetric Methods	20
Flame Spectrophotometric Methods	22
Atomic Absorption Methods	23
Neutron Activation Methods of Phosphorus Analysis	24
Miscellaneous Qualitative Methods	24
EXPERIMENTAL	25
Outline of the Method of Vogler [99]	25
Outline of the Method of Lindner and Edmundsson [62]	28
Methods of Ashing	31
Extraction Methods (Modified Heteropoly Blue Methods)	41
Determination of Phosphorus in Serum and Urine	66
Determination of Phosphorus in Fraction C of *Proteus* OX-19.	73
Complete Details of Developed Method	76
Method of Baginski et al [9] Used to Confirm Results	82
DISCUSSION	84
The Ashing Procedure (Using the Heteropoly Blue Method)	86
The Modified Heteropoly Blue Method	93
Determination of Phosphorus in Serum and Urine	101
Determination of Phosphorus in Fraction C of *Proteus* OX-19.	104
Confirmation of Results using the Method of Baginski et al.[9]	106
CONCLUSIONS	108
BIBLIOGRAPHY	111

LIST OF TABLES

Page

I. Preparation of a Standard Phosphorus Curve..... 26
(Vogler [99])

II. Preparation of a Standard Phosphorus Curve..... 31
(Lindner and Edmundsson [62])

III. Results of Ashing Organic Material in a Variety of Vessels over Different Sources of Heat..... 32

IV. Effect of Varying Concentrations of Sulfuric Acid on the Absorbance of the Molybdenum Blue Colour..... 32

V. Comparison of Effectiveness of Various Ashing Techniques..... 34

VI. Comparison of Boiling Points of Acids used in Phosphorus Determination..... 34

VII. Comparison of Effects of Different Heating Temperatures Over Various Lengths of Time on Molybdenum Blue Absorbance..... 34

VIII. Checking for Loss of Phosphorus During the Ashing Technique, either by Volatilization of Phosphoric Acid or from Spurting..... 36

IX. Effect of Overheating during Ashing Procedures on the Molybdenum Blue Colourimetric Determination of Phosphorus..... 37

X. Reproducibility of Phosphorus Determination using the Method of Vogler (99)..... 37
(100 ug. Phosphorus)

XI. Reproducibility of Phosphorus Determination using the Method of Vogler (99)..... 38
(10 ug. Phosphorus)

XII. Time for Stability of the Molybdenum Blue Colour..... 38

XIII. Determination of Phosphorus by Colourimetric Analysis after Neutralization of the Ashed Samples with Ammonia 39

XIV. Effect of Neutralization with Ammonia on the Development of the Molybdenum Blue Colour..... 39

XV. Effect of pH on the Intensity of the Molybdenum Blue Colour in the Determination of Phosphorus..... 40

XVI. Plots of Standard Curve for Phosphorus Determination by the Modified Heteropoly Blue Method..... 43

LIST OF TABLES (Cont')

Page

XVII. Variation of Length of Time of Shaking for Extraction of the Molybdophosphoric Acid Complex..... 43

XVIII. Determination of Phosphorus by the Extraction Method after Neutralization with Ammonia..... 45

XIX. Determination of the Quantity of Dilute Ammonia Necessary to Neutralize the Samples, and Promote Optimum Extraction of the Heteropoly Acid..... 46

XX. Determination of Phosphorus in Samples that have been Ashed to Remove Organic Material then Neutralized with Ammonia before Extraction..... 46

XXI. Effect of Neutralization on the Extraction of Molybdophosphoric Acid in this Micro-Method..... 50

XXII. Effect of Small Amounts of Concentrated Sulfuric Acid on the Efficienty of Extraction of the Heteropoly Acid 50

XXIII. Determination of Phosphorus in the Presence of Organic Material without Neutralization before Extraction..... 52

XXIV. Determination of the Optimum Amount of N/1 Sulfuric Acid that Will Promote Maximum Extraction of the Heteropoly Acid..... 52

XXV. Determination of Phosphorus in the Presence of Organic Material that is Removed by Ashing with 2 ml. N/1 Sulfuric Acid..... 53

XXVI. Determination of Phosphorus in the Presence of Organic Material Removed by Ashing with N/1 Sulfuric Acid..... 61

XXVII. Determination of Phosphorus in the Presence of Different Organic Substances, Ashed with N/1 Sulfuric Acid..... 62

XXVIII. Problems of Phase Separation During Phosphorus Determination by Extraction of the Heteropoly Acid... 63

XXIX. Effect of Excessive Heating to Destroy Peroxide on the Determination of Phosphorus by the Heteropoly Acid Extraction Method..... 63

XXX. To Determine if a Larger Amount of N/1 Sulfuric Acid Can be Used to Ash the Organic Material without Affecting the Extraction or Absorbance..... 64

LIST OF TABLES (Cont')

Page

XXXI. Determination of Phosphorus in the Presence of Various Organic Substances that are Digested with 3 ml. of N/1 Sulfuric Acid..... 65

XXXII. To Determine Whether Starch Alone Increases the Absorbance of the Heteropoly Acid During Phosphorus Determination..... 65

XXXIII. Determination of Phosphorus in Glucose-1-Phosphate.... 66

XXXIV. Prelinimary Tests to Determine the Optimum Dilution and Volume of Rabbit Serum for Repeated Phosphorus Determinations..... 67

XXXV. Preliminary Tests to Determine the Optimum Dilution and Volume of Urine for Repeated Phosphorus Determinations..... 67

XXXVI. Determination of Phosphorus in Rabbit Serum..... 68

XXXVII. Determination of Phosphorus in Human Urine..... 69

XXXVIII. Determination of Phosphorus in Urine and Serum Using a New Reagent for Digestion..... 70

XXXIX. Determination of Phosphorus in Serum and Urine Using a Further Modification of the Ashing Technique..... 71

XL. Determination of Phosphorus in Serum Using the Modified Ashing Technique..... 71

XLI. Determination of Phosphorus in Fraction C of Proteus OX-19. Preliminary Tests..... 73

XLII. Determination of Phosphorus in 2 ml. Samples of a Solution of Fraction C, Containing 10.03 mg./ 100 ml.. 74

XLIII. Determination of Phosphorus in Samples of Fraction C Weighed Directly on a Micro-Balance..... 74

XLIV. Determination of Phosphorus in Intact Proteus OX-19 Bacterial Cells..... 75

XLV. Calculations and Figures for the Standard Curve used for ALL Calculations of this Particular Modified Heteropoly Acid Method..... 79

LIST OF TABLES (Cont')

Page

XLVI. Results to Plot a Standard Phosphorus Curve..... 83

XLVII. Determination of Phosphorus in Samples of Fraction C of Proteus OX-19..... 83

XLVIII. Determination of Phosphorus in Serum..... 83

LIST OF FIGURES

Page

1. Calibration Curve for Determination of Phosphorus (Vogler [99]) 27

2. Calibration Curve for Determination of Phosphorus (Lindner and Edmundsson [62]) 29

3. Graph to Determine the Wavelength of Optimum Absorbance of the Molybdovanadophosphoric Acid Complex..... 30

4(a) Apparatus for Determination of Temperature Reached by Samples During Ashing..... 35

4(b) Apparatus for Determination of Any Loss of Phosphorus by Volatilization or Spurting..... 35

5. Graph to Determine the Wavelength of Optimum Absorbance of the Extracted Heteropoly Blue Colour..... 42

6. Graph of Calibration Curve for the Determination of Phosphorus by the Modified Heteropoly Blue Method..... 44

7. Illustration to Show How Organic Material was Ashed in Test Tubes on Sand Baths..... 48

8. Graph to Determine the Wavelength of Optimum Absorbance of the Extracted Heteropoly Acid in the Organic Phase..... 49

9. Graph to Determine the Wavelength of Optimum Absorbance of the Unreduced Heteropoly Acid in the Organic Phase..... 51

10. Graph to Determine the Absorbance of Isobutanol Against Air. 54

11. Graph to Determine the Absorbance of Xylene Against Air..... 55

12. Graph to Determine the Absorbance of Xylene 65 % - Isobutanol 35 % Against Air..... 56

13. Graph to Determine the Absorbance of Phosphomolybdic Acid Extracted into Xylene-Isobutanol (65:35) in the Organic Phase, Unreduced..... 57

14. Graph to Determine the Absorbance of Phosphomolybdic Acid Extracted into Cyclohexane-Isobutanol (65:35) in the Organic Phase, Unreduced..... 58

15. Graph to Determine the Absorbance of Phosphomolybdic Acid Extracted into Hexane-Isobutanol (65:35) in the Organic Phase, Unreduced..... 59

LIST OF FIGURES (Cont')

Page

16. Calibration Curve for the Determination of Phosphorus by the Extraction of the Heteropoly Acid into Xylene-Isobutanol (65:35)..... 81

17. Calibration Curve for Determination of Phosphorus..... 84

INTRODUCTION

The Importance of Phosphorus Determination

The determination of traces of orthophosphate ions is one of the most widely performed analytical techniques, despite the fact that phosphorus occurs only to the extent of about 0.12 per cent in the lithosphere.

Phosphorus is an important component in many biological structures and reactions. It is essential for the formation of bone, resulting in the formation of a tricalcium phosphate complex. Also, compounds such as creatine phosphate are presumably involved in muscle contraction and in carbohydrate utilization. As phosphates play an important role in buffering the blood at the proper pH, the determination of micro amounts of phosphorus in body tissues, blood, serum, urine and other body fluids is often performed.

A large proportion of enzymic reactions either require inorganic phosphorus, or energy-donating, phosphorus-containing compounds such as adenosine triphosphate (A.T.P.) and nicotinamide adenine dinucleotide phosphate (N.A.D.P.) for the reaction to proceed or else release phosphorus or pyro-phosphate during the reaction. Some enzyme kinetics can therefore be calculated by quantitative determination of the uptake or release of phosphorus compounds during the reaction.

Many biological compounds such as the nucleotides, phospholipids (phosphatides) and some polysaccharides contain phosphorus, often present at the site of combination such as the polynucleotide bonds. One fraction investigated was isolated from Proteus OX - 19 as containing the endotoxic properties (labeled as fraction C). Quantitative determination of the phosphorus content may perhaps be linked to the reason for its presence and role in the endotoxin, perhaps as the binding site.

As phosphate compounds, particularly "superphosphate" $Ca(H_2PO_4)_2$ are used as fertilizers, the determination of the phosphorus content of soils and plants is important in studying plant growth and soil fertility. Also in the field of agriculture with the developement of many organo-phosphorus pesticides, the role of phosphorus determination is valuable in calculating pesticide residue contamination in plants, soils, animal products and water.

Phosphorus determination in water has several other important features. The extent of industrial and human waste contamination of streams and rivers can often be calculated by the amount of phosphorus present in the water. Secondly, phosphorus (phosphate) is often added to industrial boiler waters in concentrations seldom exceeding 30 ppm. of phosphorus or 100 ppm. phosphate in order to precipitate the calcium ions present in the water as hydroxyapatite $3Ca_2(PO_4)_2.Ca(OH)_2$, and thus prevent scale formation and the embrittlement of boilers. Also the addition of sodium hexametaphosphate $Na_6P_6O_{18}$ to "hard" industrial water will "soften" the water by sequestering the calcium by the formation of a chelate complex. Consequently the consumption of soap by calcium ions is prevented.

Information concerning the phosphate content in rocks is important to the geologist, especially in iron ore, and limestone used as flux in the production of steel. Phosphorus does not occur in the free state in nature so that its content in rocks, such as apatite $3Ca_3(PO_4)_2.CaF_2$, as well as its trace contamination in other rocks such as granite and basalt often need to be calculated as a source of phosphorus.

Determination of phosphorus in industry is important, especially in the production of iron and steel and the fractionation of petroleum.

Phosphorus is usually present in the form of iron phosphide Fe_3P which is soluble in the ferrite and has the disadvantage of causing a decreasing solubility of the carbon in iron and embrittlement of steel, the effect being more pronounced with increasing percentages of carbon in the steel. Phosphorus is also found to some extent in nonferrous metallurgical material, such as the phosphor bronzes used in bearings. Determination of phosphorus content in the range of one to twenty ppm. is often necessary. The petroleum industry makes use of metal salts of organophosphorus compounds added to serve as dispersant-detergents or corrosion inhibitors, generally in concentrations of 0.05 to 0.5 per cent.

The object of this investigation was to develop a method for the determination of phsophorus in a trichloroacetic acid extracted fraction (Fraction C) of Proteus OX-19. This fraction is an endotoxin having pyrogenic properties, and appears to be composed of a phosphorus-containing lipo-polysaccharide. It was also planned that this technique, once developed, would be versatile enough so that is could be adapted to the determination of phosphorus in other biological materials.

Evaluation of Methods of Phosphorus Determination

Heteropoly Blue Methods:

Due to the realization of the biological significance of phosphorylation processes and the role of phosphate determination in this field, the majority of the earlier analytical techniques have been developed by biochemists. However, with the advances in the field of instrument analysis and the development of techniques such as neutron activation and atomic absorption, the analytical chemist has invaded this area with more sensitive and delicate manipulations. The existance of a great variety of such methods can be ascribed not only to the large number of combinations in which phosphates occur in animate and inanimate material, but also to the manifold situations with which the analyst is faced when attempting to transform his substance into a form suitable for quantitative evaluation. The two main problems are the degradation of the organic or inorganic compound in such a way as to give rise to inorganic phosphate and a method of quantitatively determining the inorganic phosphate.

Most of the earlier methods to be discussed here took advantage of the property of orthophosphates to form a complex with molybdic acid according to the reaction:

$$H_3PO_4 + 12\ H_2MoO_4 \longrightarrow H_3Mo_{12}PO_{40} + 12\ H_2O$$

A property of interest of this phosphomolybdic acid complex, recognized by Taylor and Miller (1914)[96], is that it could be converted by reduction into a deep blue substance called molybdenum blue. The intensity of the blue colour was shown to be proportional to the phosphate content, which could then be determined by colourimetric analysis.

In the following years, application of this technique was adapted by workers for the determination of phosphorus in urine and blood.

Using trichloroacetic acid to precipitate protein and sulfuric acid to ash other organic material, Bell and Doisy (1920) [16] developed the molybdenum blue with hydroquinone plus sulfite as the reducing agent in and alkaline medium, but Briggs (1922) [22] suggested a modification of this technique by substituting an acidic medium which produced greater stability of the blue colour. This method was reviewed and rejected by Fiske and Subbarow (1925) [36] in their classical experiments which utilized 1-amino-2-naphthol-4-sulfonic acid as the reducing agent while the acidity from the sulfuric acid used for wet ashing the organic material was maintained. Modification of the amount of trichloroacetic acid from the Bell-Doisy [16] method was studied and the danger of the presence of alkali which leached silicon from the glassware was investigated. Silicon will react similiarly with molybdic acid and thus affect the colour intensity and accuracy. As the colour formation is affected by the acidity, King (1932) [53] and Allan (1940) [2] suggested the use of perchloric acid instead of sulfuric acid, whereby the procedure can be rendered less sensitive to variations in acidity.

A weakness of the methods proceeding under these conditions is that the high acidity present during the preliminary steps of the determination of inorganic phosphate may cause splittage of the acid labile organo-phosphates, thus raising the inorganic phosphate level. This splittage is intensified by the presence of molybdate. In order to modify these methods to a method for the determination of inorganic phosphate in the presence of acid labile phosphate esters, Lowery and Lopez (1946) [63] have adjusted the pH from 0.65 to 4.0, reduced the molybdate concentration from 0.25 to 0.1 percent, substituted ascorbic acid for the original mixture of bisulfite and aminonaphtholsulfonic acid and determined the colour

intensity spectrophotometrically at 700 mμ.

Boltz and Mellon (1947) [19] performed a spectrophotometric study of the heteropoly blue method for the determination of not only phosphorus but also germanium, silicon and arsenic. After standardizing the molybdate concentration, acid concentration, time and temperature of heating and introducing hydrazine sulfate as the reducing agent, a procedure was outlined for the determination of phosphorus in the presence of diverse ions. The transmittancy was measured at 830 mμ. The term "heteropoly blue" was introduced and defined as the reduction product having maximum absorbance at 830 mu to differentiate from the term "molybdenum blue" used to designate the blue reduction product exhibiting absorbance in the 650 - 700 mμ region. These same workers (1948) [20] also investigated the possibility of using the yellow colour of the unreduced molybdophosphoric acid as a means of colourimetric analysis reading the transmission at 380, 400 or 420 mμ.

An extensive study of the colourimetric determination of phosphorus was outlined by Fogg and Wilkinson (1958) [38] using ascorbic acid as the reductant instead of stannous chloride. The effect of the diverse silicate, sulfate, nitrate, perchlorate, ferric and arsenate ions was also investigated thoroughly. Sims (1961) [91] compared the activity of 1:2:4-triaminonaphtholsulfonic acid, p-methylaminophenol sulfate and 2:4-diaminophemol dihydrochloride to hydrazine sulfate as a reducing agent. However none of these were selected as the reductant of choice from this evaluation. The optimum concentrations of perchloric acid, ammonium molybdate and the reducing agent were also investigated and the nature of the molybdophosphate complex discussed.

A modification of the Lowery-Lopez [63] method was later presented

by Mokrasch (1961) [72] for the determination of phosphate in the presence of highly labile phosphorus compounds. This is achieved by substituting N,N-dimethylformide for most of the water in the assay which virtually arrests the hydrolysis. After reduction with ascorbic acid the colour is read at 335 mμ to yield a much more sensitive method. Following this technique, May (1959) [68] developed a procedure for the determination of quinquivalent, trivalent, and organic phosphorus in the atmosphere and in aqueous solutions. The amount of the three types of compounds in a sample can be calculated from the difference in the quinquivalent phosphorus found in three separate aliquots: 1) without oxidizing, and following 2) hydrochloric acid, and 3) alkaline persulfate oxidation. A further developement in this area was presented by Anton (1965) [3] in which the total acid normality is adjusted and the initial method modified so that hypophosphite can be determined in the presence of phosphates.

Wooster and Rakestraw (1951) [106] adapted the molybdenum blue method for the estimation of dissolved phosphate in sea water using tin (Sn^{++}-stannous chloride) as the reducing agent in a concentration of 2.1 mg. Sn^{++}/ ml. and reading the absorbance at 700 mμ, while Hansen and Robinson (1953) [43] used the same method for the determination of organic phosphorus in sea water by perchloric acid oxidation and read the absorbance at 625 mμ. Levine et al (1955) [61] investigated the molybdenum blue method for the determination of phosphorus in waters containing the diverse ions of arsenic, silicon and germanium, which will react similiarly with molybdic acid. After dry ashing the phosphorus was precipitated with aluminum hydroxide to eliminate the interference, complexed with molybdenum and reduced with stannous chloride or aminonaphtholsulfonic acid. A wavelength of 735 mμ was used to measure the absorbance. Recent work by

Menzel and Corwin (1965) [69] evaluates the use of persulfate oxidation to liberate the organically bound fractions before preceeding with the colourimetric analysis.

Vogler (1965) [98] discussed the problems of phosphate analysis in limnology and proposed a new technique for the determination of dissolved orthophosphate in the presence of condensed phosphates and organic esters of phosphoric acid. After filtration through sintered glass, the water was treated with a complex reagent containing sulfuric acid, sulfanilic acid, ammonium molybdate, antimony (Sb^{+++}) and tartaric acid. Reduction was achieved with ascorbic acid and the absorbance read at 665 mμ. Further work by the same investigator [100] was performed on the determination of condensed and organic phosphates. Based on these investigations, methods were presented for the determination of phosphorus compounds in water.[101] A new technique valuable for the determination of phosphorus in sea water, adapted by Armstrong et al (1965),[4] utilizes ultra-violet irradiation for photo oxidation of the organic matter. Exposure of sea water to ultra-violet irradiation for 1 - 2 hours liberates phosphorus rapidly from organic matter without the necessity of adding any reagents, except one or two drops of 30 per cent peroxide, introduced to ensure the presence of an excess of oxygen. Using the method of Murphy and Riley (1962) [76], and their single molybdate-antimony-ascorbic acid reagent, Armstrong and Tibbits (1968) [5] further investigated this technique.

Using Amberlite IRA 400 Resin, P^{32}, hydrous zirconium oxide and the molybdenum blue method, Rigler (1968) [84] proved by three different tests that the concentration of inorganic phosphate in water is lower than indicated by the molybdenum blue method alone. Hydrolysis of organic phosphorus compounds is suspected as the source of error. Hargrave and

Geen (1968) [44] used the method described by Strickland and Parsons (1965) [95] for the determination of phosphate uptake by zooplankton, both for total and dissolved inorganic phosphate.

Barrow (1967) [11] used the technique of Murphy and Riley [76] to determine the relationship between the uptake of phosphorus by plants and the phosphorus potential and buffering capacity of the soil, while Hislop and Cooke (1968) [48] used an anion resin, De Acidite FF 510 less than 0.5 mm. as a phosphate sink before determination by the Fogg-Wilkinson [38] method of the phosphorus status of soils.

For the determination of phosphorus in high purity iron, Gates (1954) [39] used electrolysis in a Malaeven Cell for the separation of the phosphorus from the iron, after treatment of the sample with perchloric and hyriodic acids. The molybdenum blue colour was produced by reduction with hydrazine sulfate. Despite the fact that the optimum absorbance wavelength is considered to be 850 mμ, readings were taken at 650 mμ. Using a wavelength of 825 mμ, Hoffman et al (1958) [49] applied essentially the same technique to the determination of phosphorus in gasolines containing the additive tritolyl phosphate.

As mentioned before, the importance of phosphorylation stimulated investigation into the determination of phosphorus in organic and biological compounds. Beveridge and Johnson (1949) [18] adapted the method of Boltz and Mellon [19], by using the molybdate-hydrazine sulfate reagent for the determination of phosphorus in phospholipids. The colour intensity was measured at 830 mμ. For the conversion of organic phosphorus to ortho-phosphate, Simmons and Robertson (1950) [90] refluxed the sample with hydriodic acid for a calculated period of time. Using aminonaphthol-sulfonic acid as the reducing agent Griswold et al (1951) [42] have

modified the Boltz and Mellon [19] heteropoly blue method for the determination of inorganic phosphate and phosphate esters in tissue extracts, while Chen et al (1956) [25] applied the Lowery-Lopez [49] modified technique of Fiske and Subbarow [36] using ascorbic acid as the reducing agent and reading the absorbance at 820 mμ for the determination of phosphorus in whole blood, serum and urine.

Dryer et al (1957) [33], investigating the determination of phosphorus and phosphatase, substituted N-phenyl-p-phenylamine (p-semidine) as a reducing agent in place of aminonaphtholsulfonic acid, stannous chloride and the mixed molybdate-hydrazine sulfate reagent. It was claimed to be fast, yielding a stable colour with a maximum absorbance reached at 345 or 770 mμ. Using the Schöniger Technique (1957) [88] of destroying the organic material by combustion in an oxygen-filled flask, Fleischer et al (1958) [37] compared its simplicity and superiority to the conventional Pregl Digestion for several complex organic phosphorus containing compounds including Ilidar* and Syntrophan* Phosphate (*Reg. - Hoffman la Roche). Phosphorus was quantitatively determined in a semi-micro titration method and a micro colourimetric method using ascorbic acid as the reducing agent. However Kolmerton and Epstein (1958) [55] determined the phosphorus content of the tetramethylphosphonium ion, oxidizing the sample by boiling it with sodium hydroxide, reducing to the molybdenum blue with stannous chloride and assaying by colourimetry.

In a slight modification of the Boltz-Mellon [19] method, Goodwin et al (1958) [41] emphasized the use of chloric acid as a most suitable digestant for serum and serum extracts in the determination of serum phosphorus and phospholipid, while Dellamonica et al (1958) [29] using the Fiske-Subbarow [36] method with the modified ferrous sulfate reducing

agent demonstrated the adverse effect that trichloroacetic acid, often used as a protein precipitant, has both on the silicate contamination by intensification of the blue colour as well as on the intensity of the reduced phosphomolybdic blue colour. Bartlett (1959) [12] also adapted and modified the phosphorus assay method of Fiske-Subbarow [36] to facilitate multiple total phosphorus analyses on elutes from chromatographic columns. Dry ashing was used with the addition of one or two drops of 30 per cent hydrogen peroxide while the molybdenum blue colour was developed with aminonaphtholsulfonic acid and read at 830 mμ.

Oliver and Funnell (1961) [78] have described a method for the determination of phosphorus in biological material based on the evolution of phosphorus by heat in a non-oxidizing (nitrogen) atmosphere. The phosphorus is collected on mercuric bromide, then eluted with iodine as phosphoric acid and determined colourimetrically as the heteropoly blue with molybdenum and hydrazine sulfate. For the same purpose, Morrison (1964) [73] has outlined a simple technique especially useful for lipid materials. After ashing with concentrated sulfuric acid and 30 per cent hydrogen peroxide, the heteropoly blue is developed with ammonium molybdate and ascorbic acid, and the absorbance read at 822 mμ. Another new digestion reagent was developed by Saliman (1964) [86] containing hydriodic acid, calcium iodide, water, phenol and acetic acid for the determination of phosphorus in organic compounds. Colourimetric analysis followed using molybdenum and hydrazine sulfate, absorbance being determined at a wavelength of 830 mμ.

Using the Schöniger [88] method of pyrolysis in an oxygen atmosphere to destroy the organic material, Lebedeva et al (1966) [58] reduced the molybdophosphoric acid with ferrous ammonium sulfate (Mohr's Salt) then

determining the phosphorus content spectrophotometrically. Using a modified Fiske-Subbarow [36] method for the determination of phosphorus, Vreman and Jöbsis (1966) [102] noted that mannitol interferes with the molybdenum blue formation by complexing the molybdenum. A general investigation of the problem is presented along with warnings and suggestions on how to overcome this interference.

Crouch and Malmstead (1967) [27] have described the major chemical steps in the molybdenum blue method for the determination of phosphorus using precision spectrophotometric measurements. A stoichiometric study of the reaction between phosphate and molybdate to form 12-molybdophosphoric acid is described and the kinetics of the reaction with the reducing agents 1-amino-2-naphthol-4-sulfonic acid and ascorbic acid for the heteropoly blue species are discussed. Further work by the same investigators [28] outlined a new method for the determination of phosphate in aqueous solution and in blood serum that utilizes an automatic digital readout of the initial rate of formation of molybdenum blue from phosphate, molybdate and ascorbic acid.

Baginski et al (1968) [9] described a simple procedure for the determination of nucleic acid phosphate using nitric acid-calcium nitrate reagent to digest the organic material, molybdenum to form the molybdophosphoric acid, citrate-arsenate reagent to complex and thus remove the excess molybdate, and an ascorbic acid-trichloroacetic acid reagent to reduce the complex to form the molybdenum blue colour which is then read at 700 or 840 mμ. A modified oxygen-flask procedure was developed by Scroggins (1968) [89] for the determination of phosphorus in organic compounds, difficult to do by conventional methods. The orthophosphate formed is determined spectrophotometrically as the molybdenum blue complex

formed by molybdate and hydroquinone, at a wavelength of 650 mμ. Using principally the method of Bartlett [12], Aalbers and Bieber (1968) [1] described a method for the quantitative determination of phosphonates (compounds posessing a carbon-phosphorus bond). Instead of separation by a lengthy chromatography procedure, two assays are employed. One assay is for total phosphorus and the other for total nonphosphonate produced by controlled digestion. The difference represents the amount of phosphorus present in a carbon-phosphorus linkage.

Modified Heteropoly Blue Methods:

Instead of reducing molybdophosphoric acid in aqueous solution Berenblum and Chain (1938) [17] extracted the molybdophosphoric acid with an immiscible solvent, isobutyl alcohol, and then reduced this to the heteropoly blue by shaking the extract with chlorostannous acid. This procedure for inorganic phosphate was modified by Martin and Doty (1949) [67] by the substitution of isobutyl alcohol-benzene (1:1) as the extractant, while retaining the stannous chloride reducing agent. The efficiency of the extraction was not impaired by the benzene, and the number of extractions was reduced from three to one with a shaking time of 15 seconds. The absorbance was measured between 625 and 725 mμ. Other organic extractants investigated were n-butyl alcohol, isoamyl alcohol and benzyl alcohol. Ernster et al (1952) [34] adapted this method for the determination of P^{31} and P^{32} in biological material.

A study of the catalytic effect of molybdate on the hydrolysis of organic phosphate bonds was performed by Weil-Malherbe and Green (1951) [104] using the procedure of Martin and Doty.[67] The ratios of hydrolysis constants in the presence and absence of molybdate for substances such as acetyl phosphate, creatine phosphate and the terminal phosphate of adenosine triphosphate were 20 to 100 times higher than for substances such as glucose-1-phosphate, glucose-6-phosphate, phosphoenol pyruvic acid etc. Further work by Weil-Malherbe [105] clarified the catalytic effect, especially in the determination of both inorganic and organic phosphate, where the inorganic phosphate was precipitated by magnesia in the presence of molybdate.

From an extensive study of the extraction of heteropoly acids including molybdophosphoric, molybdoarsenic, molybdosilicic and

molybdogermanic acids, Wadelin and Mellon (1953) [103] devised a method for the determination of phosphorus in steels. After destruction of the steel with nitric acid, the molybdophosphoric acid was extracted with 1-butanol 20 per cent $^{v}/_{v}$ in chloroform, and without reduction, the heteropoly acid extract was read at 310 mμ. This experiment was not seriously affected by the presence of diverse contaminating ions. Lueck and Boltz (1956) [64] however preferred to extract with pure isobutanol, reduce with chlorostannous acid and measure the absorbance at 725 mμ, while Theakston and Bandi (1966) [98] only modified this method by incorporation of techniques for the removal of contaminating ions such as titanium and chromium which interfered with the extraction of the molybdophosphoric acid.

Using perchloric acid to digest the organic material, Rhodes (1955) [83] developed the molybdenum blue colour by Allen's [2] method, extracted this blue colour into methyl-isobutyl ketone and then read the absorbance in the ketone layer after 30 minutes at 680 mμ. Henricksen (1964) [45] verified the usefulness of a modified reducing agent suggested by Sletten and Bach (1961) [94]. This reducing agent was comprised of stannous chloride dissolved in glycerol instead of a strong mineral acid, better suited for the reduction of molybdophosphoric acid extracted by the Martin-Doty [67] method. The omission of benzene in the extractant due to its toxicity was also stressed, so Dreisbach (1965) [67] modified the Martin-Doty [67] method by the substitution of the less toxic xylene. Using a 65:35 xylene-isobutanol ratio, the molybdophosphoric acid was extracted after the organic material had been precipitated with trichloroacetic acid. Then by omission of the reduction, the absorbance was measured at 310 mμ according to the technique

of Wadelin and Mellon [103]. Pennial (1966) [81] however used the unmodified Martin-Doty [67] method for the determination of inorganic phosphate after the protein had been precipitated with silicotungstic acid.

Djurkin et al (1966) [31] modified the extraction procedure of Wadelin and Mellon [103] by using 2-amino-4-chlorbenzenthiol to convert the 12 molybdate ions released from the extracted molybdophosphoric acid by alkali, to a green molybdenum VI complex that could be assayed spectrophotometrically at 710 mμ. Large excesses of silicon, germanium, arsenic or antimony do not interfere with this determination.

Recent investigation on the estimation of organic phosphate in the presence of adenosine triphosphate has been done by several workers using the method of Wadelin-Mellon [103] where reduction is omitted and the yellow colour assayed spectrophotometrically. Marsh (1959) [66] used citrate to remove molybdate from the solvent phase of pure butanol and to prevent molybdate catalysis of adenosine triphosphate hydrolysis in the aqueous phase. Absorbance was measured at 310 mμ. For the determination of orthophosphate as an assay for adenosine triphosphate activity, Mozerski et al (1966) [74] modified the method of Martin-Doty[67] by removal of the protein, precipitated with perchloric acid at pH 1.5-1.8 prior to the formation of the molybdophosphoric, acid and measured the molybdophosphoric acid after extraction into isobutanol-benzene (1:1) in the unreduced form, at 313 mμ. Further work by the same investigators [75] utilized a trichloroacetate buffer for deproteinization. After extraction by an isobutanol-benzene-ethanol reagent in special extraction tubes, described in the article, absorbance of the unreduced phosphomolybdic acid was read at 310 mμ.

Molybdovanadophosphoric Acid Methods:

Misson (1908) [71] proposed a method of phosphate analysis in steels using the technique of forming a mixed heteropoly acid by adding molybdate solution to an acidic solution containing orthophosphate and vanadate ions. This method was thoroughly studied by Kitson and Mellon (1944) [54] who made recommendations concerning the optimum concentration of reagents. Optimum acidity is about 0.5 N in nitric acid or equivalent acid, final vanadate concentration should be 0.002 M and molybdate concentration should be 0.01 M. The optimum concentration range is 5-40 ppm. of phosphorus, measuring the absorbance at 460 mμ. Barton (1948) [13] has adapted this technique for the photometric analysis of phosphate rock, reading the absorbance at 400 mμ, while Gee and Deitz (1953) [40] thoroughly investigated the method of differential spectrophotometry using the same technique, reading the absorbance at 390 mμ. Very few diverse substances have any effect on this method.

For the determination of phosphorus in steels, Baghurst and Norman (1955) [8] have applied the technique involving the formation of the molybdo-vanado-phosphoric acid complex after oxidizing the steel with nitric acid and permanganate. A thorough investigation of the effect of temperature on these results led to the conclusion that all methods were temperature dependent except for the method where the formation of sulfate by oxidation with ammonium persulfate occurred. Using a modified Schöniger [88] oxidizing scheme, Barney et al (1959) [10] have developed a method for the determination of phosphorus in motor oils and additives by the phosphomolybdovanado complex formation, reading the results spectrophotometrically at 391 mμ.

An intensive study of the molybdovanadophosphoric acid technique

of determining phosphorus has been performed by Quinlan and DeSesa (1955) [82]. Using perchloric acid and hydrofluoric acid the sample was destroyed in a platinum dish. Reagents must be added in the order of acid, vanadate and molybdate to prevent the formation of other complexes. The concentrations of acid, vanadate and molybdate were thoroughly investigated to determine the optimum concentration, while the study of interfering ions was limited to chromium, which can be removed as the red fumes of chromyl chloride after the addition of sodium chloride. Spectrophotometric measurements were made at 400 mμ. Michelson (1957) [70] continuing the study of the molybdovanado complex, suggested one reagent containing hydrochloric aicd, vanadate, and molybdate. This reagent was diluted 25 times thus allowing absorbance to be read at 315 mμ, the optimum wavelength, as the blank absorbance was reduced, over the 400 mμ usual wavelength, which was well down on the shoulder of the curve. The effect of the acid concentrations at 315 mμ was also investigated.

Christopher and Fennell (1965) [26] have applied the molybdovanado-phosphoric acid technique to the determination of phosphorus in organic material on the centimilligram scale. After ashing with sulfuric and perchloric acids, the colour is developed and the absorbance measured at 315 mμ. Using the combined reagent proposed by Michelson [70] except for the substitution of perchloric (or trichloroacetic) acid to precipitate protein, Lecocq and Inesi (1966) [59] have adopted this method for the determination of inorganic phosphate in the presence of adenosine triphosphate over the usual heteropoly blue methods. A Beckman DU Spectrophotometer with a photomultiplier attachment was used for determination as the absorbance of the blank was quite high at 350 mμ.

For the determination of inorganic bound phosphorus in the presence of alkylated phosphorus, Lindner and Edmundsson (1967) [62] used the molybdovanadophosphoric acid technique. Total phosphorus was measured by oxidation of the sample with sulfuric, nitric and perchloric acids using molybdate as a catalyst. Acid, vanadate and molybdate reagents were added separately and the absorbance measured at 445 mμ. Using milder oxidation with sulfuric acid and permanganate, high concentrations of inorganic bound phosphorus (0.1-0.5 mg) were determined by the same technique, reading the absorbance at 425 mμ, while low concentrations (10-100 ug.) were determined by the heteropoly blue method using the same digesting reagents and animonaphtholsulfonic acid and sulfite as reducing agent, and the absorbance was read at 660 mμ. Terman and Khasawuch (1968) [97] used the molybdovanadophosphoric acid technique for the calculation of crop uptake of fertilizer and soil phosphorus on dilute nitric acid extracts after ashing the samples in an oven at 400° C.

Automatic Methods:

Using the Technicon Auto Analyzer, Henricksen (1965) [46] has developed a method for the automatic determination of low levels of orthophosphate and acid-hydrolysable phosphates in fresh and saline water. The procedure is based on the Martin-Doty [67] method using isobutanol as extractant and dissolving the stannous chloride in isobutanol rather than glycerol as was previously suggested.[46] Sampling rate is 12 samples per hour as opposed to 2-4 per hour manually. The following year, the same investigator [47] modified the automatic technique due to the significant interference by silica. The acidity and molybdate concentrations were reduced and a correlation coefficient was calculated to correct the interference.

Hoppe-Seyler and Gundlach (1968) [50] have adapted the Auto Analyzer for the determination of inorganic phosphorus in serum, urine and other biological fluids using hydrazine sulfate as a reducing agent to develope the heteropoly blue complex, while Yee (1968) [107], also using the Auto Analyzer, depended on ferrous ammonium sulfate and thiourea as a reducing agent for phosphorus analysis of serum and urine.

Colourimetric and Titrimetric Methods:

Soyenkoff (1952) [87] developed a sensitive method of colour development in phosphorus determination by substituting 2-p-dimethylaminostyrylquinoline ethosulfate, a readily soluble dye, for quinoline red. The dye complexes with molybdophosphoric acid to yield a colour that can be measured at 510 mμ, after the organic samples have been ashed with sulfuric acid. A method for the determination of acyl phosphates was developed by Pechere and Capony (1968) [80] in which the reddish colour, produced by the addition of ferric chloride to the sample in acid medium after the conversion of the anhydride into the corresponding hydroxamate by succinhydroxamate, can be read spectrophotometrically at the optimum predetermined wavelength.

Canic et al (1965) [24] have developed a method for the chromatographic separation of condensed phasphates on a thin layer of starch, both in the ascending and circular techniques. Two solvent systems were developed, one for the separation of mono-, di-, tri- and tetraphosphates and the other for the separation of the cyclic and chain phosphates. Further work on chromatography was investigated by Koser and Oesper (1966) [56] who used bisulfite ion-exchange columns to separate glyceraldehyde-3-phosphate from inorganic phosphate.

Among the titrimetric methods, quinoline molybdate is widely used to precipitate the phosphorus present in a sample. Belcher and MacDonald (1958) [14] adapted this technique for organic material after destroying the organic matter in the Schöniger [88] oxygen flask technique. After precipitation, the assay is completed by a back titration with 0.05 N hydrochloric acid using phenolphthalein as the indicator. This method was also applied by Lench (1967) [60] for the determination of phosphorus

in highly alloyed steel, using gravimetric and volumetric determination techniques. MacDonald and Van der Voort (1968) [65] modified this technique for the successive determinations of silicon and phosphorus present in the same compound, If present in an organic compound, the organic material is first digested with peroxide. The phosphorus is removed by precipitation with molybdate, complexed with quinoline and assayed titrimetrically. Silicon,in the filtrate can then also be determined.

A unique titrimetric method was described by Yofe and Rappart (1968) [108] in which phosphate is complexed and precipitated with lanthanum and then the excess lanthanum is back-titrated with 0.1 M EDTA at pH 5 where chloride, sulfate, alkali and alkaline-earth metals do not interfere. A similiar technique was also described by Sinha et al (1968) [92] using an excess of zirconium as the precipitating agent and back-titrating the excess with 0.02 M EDTA. Iron, titanium, thorium and bismuth do not interfere, but fluorine must be eliminated when present in excess of 1.4 mg per 0.7 - 88 mg. of P_2O_5. The nature of the zirconium complex was also extensively investigated.

With the object of amplifying the reactions of trace amounts of phosphorus, Belcher and Uden (1968) [15] extracted the heteropoly molybdophosphoric acid into isobutyl acetate, back-extracted the molybdenum with an alkaline ammonia solution and complexed it with oxine. This precipitate was then dissolved and titrated with potassium bromate and sodium thiosulfate.

Flame Spectrophotometric Methods:

Pagliassotti and Porsche (1951) [79] developed a method for the determination of phosphorus in lubricating oil, by exposing the sample to a high-voltage spark discharge in a nitrogen atmosphere. The resulting discharge is focused and the spectrum photographically recorded on film. When developed, measurements were made on the phosphorus line at 2535.65 Å, and the content calculated.

Application of flame photometry in the determination of phosphorus was made by Dippel et al (1954) [30]. After the removal of cations on an ion-exchange column, the phosphorus content was measured by its effect on a standard calcium flame at 422.7 mμ. This could be applied to a standard curve formed by known amounts of phosphorus. Skogerboe et al (1967) [93] developed a flame spectrophotometric method for the determination of phosphorus. The analytical utilities and the interference effects were evaluated and the method applied to several biological materials.

Karmen (1964) [52] developed a hydrogen flame ionization detector sensitive to compounds containing the halogens or phosphorus, by heating a wire mesh treated with an alkaline hydroxide in a hydrogen flame. These elements increase the rate of volatilization of the metal vapour which can be detected in a second hydrogen flame. This technique can be calibrated so nanogram quantities of these elements can be detected. A microwave-powered helium plasma has been used by Bache and Lisk (1967) [7] to fragment and excite organic phosphorus compounds, among other, eluting from a gas chromatograph. The most sensitive and selective atomic line has been determined and it has been shown that elemental emission response is quantitative so the content of phosphorus can be calculated. Further work by Bowman and Beroza (1968) [21] has developed a dual flame photometric

detector that monitors gas chromatographic effluents responding to phosphorus and sulfur. The phosphorus response is linear, thus allowing for quantitative determination.

Atomic Absorption Spectrophotometric Methods:

Zaugg and Knox (1966) [109] developed an indirect method for the determination of phosphorus. After the formation of molybdophosphoric acid, it was extracted into 2-octanol. Citrate was added to complex the excess molybdenum and the organic phose was aspirated directly into the atomic absorption spectrophotometer where the molybdenum content was evaluated and from which the phosphorus content could be calculated. The same investigators [110] expanded this technique to the application of the determination of phosphorus in serum, blood, bone and other biological tissues and enzyme-reaction mixtures by previous ashing or protein precipitation by sulfuric and perchloric acids.

Using a molybdenum hollow cathode lamp, Kumamaru et al (1967) [57] were able to determine the phosphorus content by extracting it into n-butyl acetate which was then aspirated directly into the atomic absorption spectrophotometer and measured using molybdenum line 3133 Å as the light source. Hurford and Boltz (1968) [51] applied the technique of indirect ultra-violet spectrophotometry at 230 mμ or atomic absorption spectrometry using the 313.3 mμ line of molybdenum for phosphorus and silicon determination simultaneously. The phosphorus is reacted with the molybdate and extracted with diethyl ether. With a basic buffer the molybdenum is stripped and then determined by one of the two spectrophotometric methods.

Neutron Activation Methods of Phosphorus Analysis:

Rison et al (1967) [85] used the $^{31}P(n,\alpha)^{28}Al$ reaction method for the fast neutron activation analysis of phosphorus in a composite propellant consisting of ammonium perchlorate, aluminum and binder. A similiar method was used by Brunfelt and Steinnes (1968) [23] for the determination of phosphorus in rocks. After irradiation, the standard and sample were treated and the phosphorus precipitated with ammonium molybdate. The phosphorus content was evaluated by counting the β (beta) irradiation using a Geiger-Mueller counter. The same technique with modifications for the determination of phosphorus in alloy steel was used by Nadkarni and Halder (1968) [77].

Miscellaneous Qualitative Techniques:

Two techniques can be catagorized here for the determination of phosphorus poisons and pesticides. Fischl et al (1968) [35] used strips of filter paper impregnated with a buffered acetyl choline substrate solution containing phenol red as the indicator. This test is based on the inhibition of acetylcholinesterase by these poisons. With the addition of normal serum a yellow spot appears if the test is negative and a red-to-violet colour if positive.

Babad et al (1968) [6] investigated the nuclear magnetic resonance N.M.R. of phosphorus pesticides. By comparison of forty phosphorus-containing compounds they were able to correlate the structure and proton chemical shift data, as well as the structure, with the phosphorus coupling constants.

EXPERIMENTAL

Taking into account the object of the experiment, the various factors, and the wide range of available techniques, it was decided to develope a new, or modify an existing, method of colourimetric determination. The scope of the problem was divides into four areas:

1) the technique or apparatus for digesting the organic material,

2) the reagents to be used to achieve this digestion efficiently,

3) the method of developing a phophorus-molybdenum complex that could be assayed spectrophotometrically, and

4) the optimum wavelength for spectrophotometric determination.

In order to evaluate the efficiency of results from areas 1 and 2, the method of I Vogler [99] and II Lindner and Edmundsson [62] were chosen as simple techniques.

I Vogler's Method:

Reagents-

1. Molybdate-Sulfuric Acid Reagent.

144 ml. of concentrated sulfuric acid/ 300 ml. water.
10 g. sulfanilic acid suspended in 10 ml water.
12.5 g. ammonium molybdate tetrahydrate $(NH_4)_6Mo_7O_{24}.4\ H_2O$ / 100 ml. water.
0.4 g. antimony potassium tartrate)) 100 ml. water.
0.5 g. tartaric acid)

This reagent was made up to 1 litre. with water.

2. Ascorbic Acid Reagent (Reductant)

10 g. ascorbic acid / 100 ml. water

3. Standard Phosphorus Solution

0.0405 g. sodium dihydrogen phosphate $NaH_2PO_4.H_2O$ / 1 litre water.
This yields a solution containing: 0.01 mg. P./ ml. (10 µg./ ml.)

The volumes shown in Table I were measured by burette into 50 ml. volumetric flasks. To this, 4 ml. of molybdate-sulfuric acid reagent were added, followed by 1 ml. of ascorbic acid solution. The volumes were made up with water and the flasks shaken. Samples were taken into

1 cm. quartz cuvettes and the absorbance measured at 665 mμ. using a red filter, against blank reagents prepared similiarly with the omission of the phosphorus solution. See Table I for the results and Fig. 1 for the Standard Curve, plotted using the least squares formula.

Table I. Preparation of a Standard Phosphorus Curve. (Vogler (99)

	ml. P. Sol'n Added	μg. P. Added	Absorbance
Trial 1.	0.5	5	0.056
	1.0	10	0.104
	2.0	20	0.207
	3.0	30	0.319
	5.0	50	0.529
	10.0	100	0.938
Trial 2.	1.5	15	0.157
	6.0	60	0.619
	9.0	90	0.922

Calculations:

$$b = \frac{n(\Sigma xy) - (\Sigma x)(\Sigma y)}{n(\Sigma x^2) - (\Sigma x)^2}$$

$$= \frac{8(163.955) - (280)(2.913)}{8(15850) - (280)^2}$$

$$b = \underline{0.01}$$

$$a = \bar{y} - b(\bar{x})$$

$$= 0.364 - 0.01(35)$$

$$a = \underline{0.014}$$

$$y' = a + bx$$

$$= 0.014 + 0.01\ (10)$$

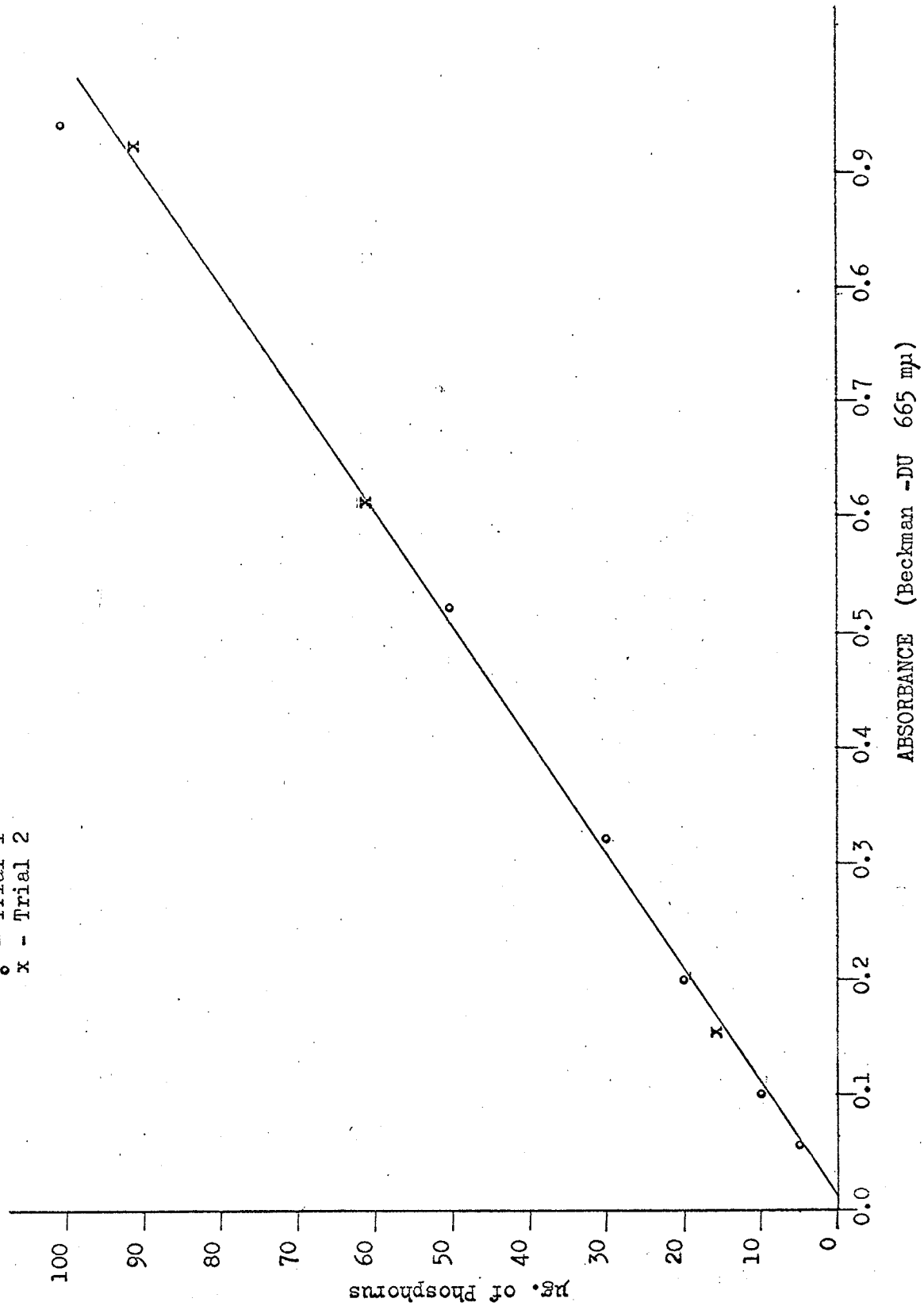
$$y' = 0.114$$

Similiarly when $x = 60$ $y' = 0.614$

N.B. The 100 ug. reading was omitted because the Beer-Lambert Law was no longer being obeyed.

Figure 1.

Calibration Curve for Determination of Phosphorus (Vogler (99))
o - Trial 1
x - Trial 2



II Lindner and Edmundsson's Method:

Reagents-

Standard Phosphorus Solution

0.4508 g. sodium dihydrogen phosphate were dissolved in 1 litre of water to yield a solution containing: 0.1 mg P./ ml. or (100 µg. P./ ml.)

I. Nitric Acid Reagent.

concentrated nitric acid diluted 1:2

II Ammonium Vanadate Reagent.

2.5 g. ammonium vanadate (NH_4VO_3) were dissolved in 500 ml. of warm water. After cooling, 20 ml of conc. nitric were added and the volume made up to 1 litre.

III Ammonium Molybdate Reagent.

50 g. ammonium molybdate were dissolved in 500 ml. water. It was made up to volume and filtered if necessary.

From the standard phosphate solution samples were pipetted into 50 ml. volumetric flasks. (Table II) To each flask was added 5 ml. each of reagents I, II, and III, in that order. Each flask was made up to volume and shaken. A blank was perpared in the same way with the omission of the phosphate standard. The standard was determined spectrophotometrically by assaying the samples against the blank at a wavelength of 445 mµ. using a blue filter and a tungsten lamp. The results are shown in Table II and the standard curve plotted in Figure 2.

However, this method was not used, for scanning to determine the wavelength of optimum absorbance revealed it to be 315 mµ. (Figure 3.), but when readings were attempted at this wavelength the blank had so much absorbance that readings were impossible.

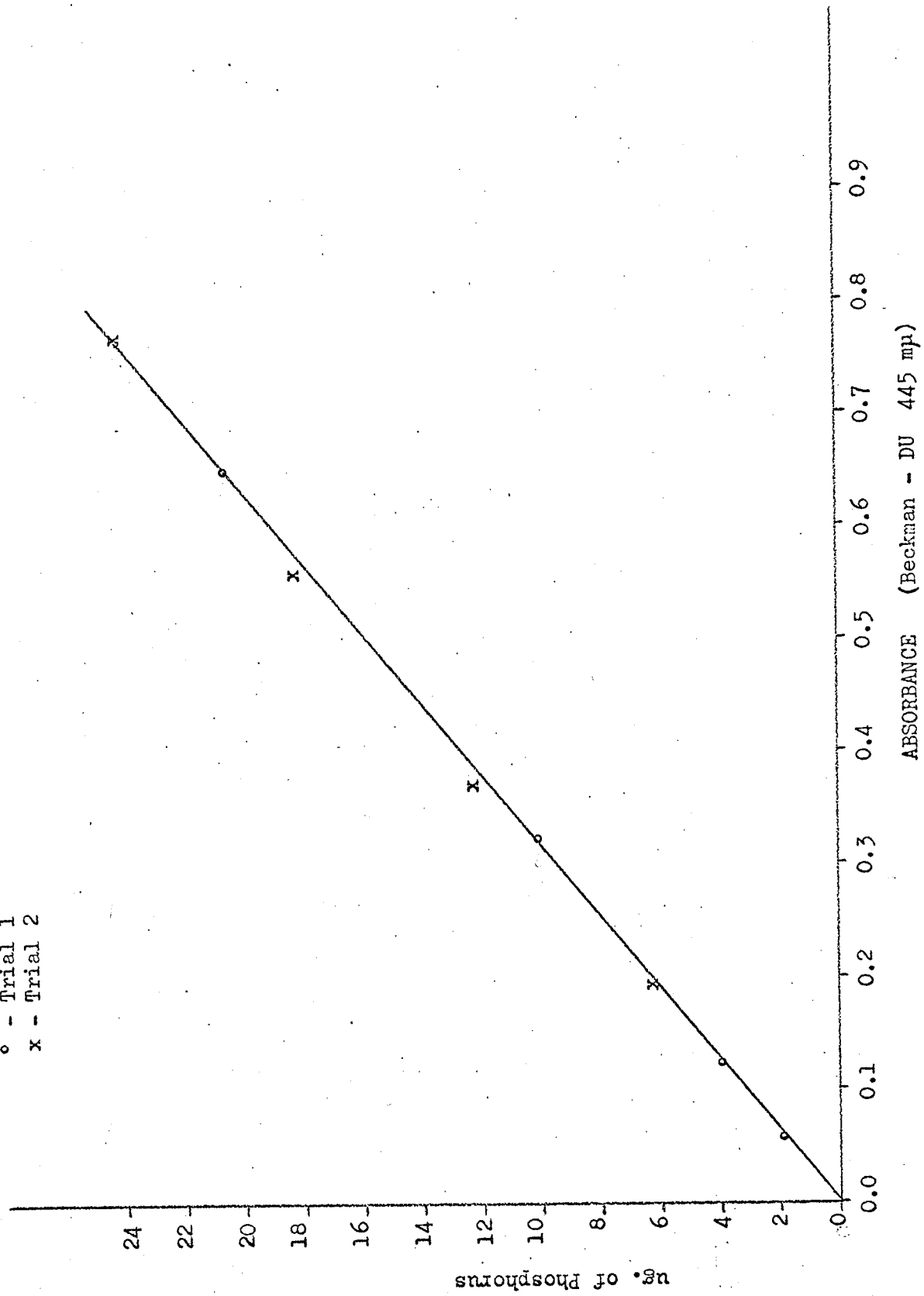


Figure 2. Calibration Curve for Determination of Phosphorus (Lindner & Edmundsson [62])
° - Trial 1
x - Trial 2

Figure 3. Graph to Determine the Wavelength of Optimum Absorbance of the Molybdovanadophosphoric Acid Complex.

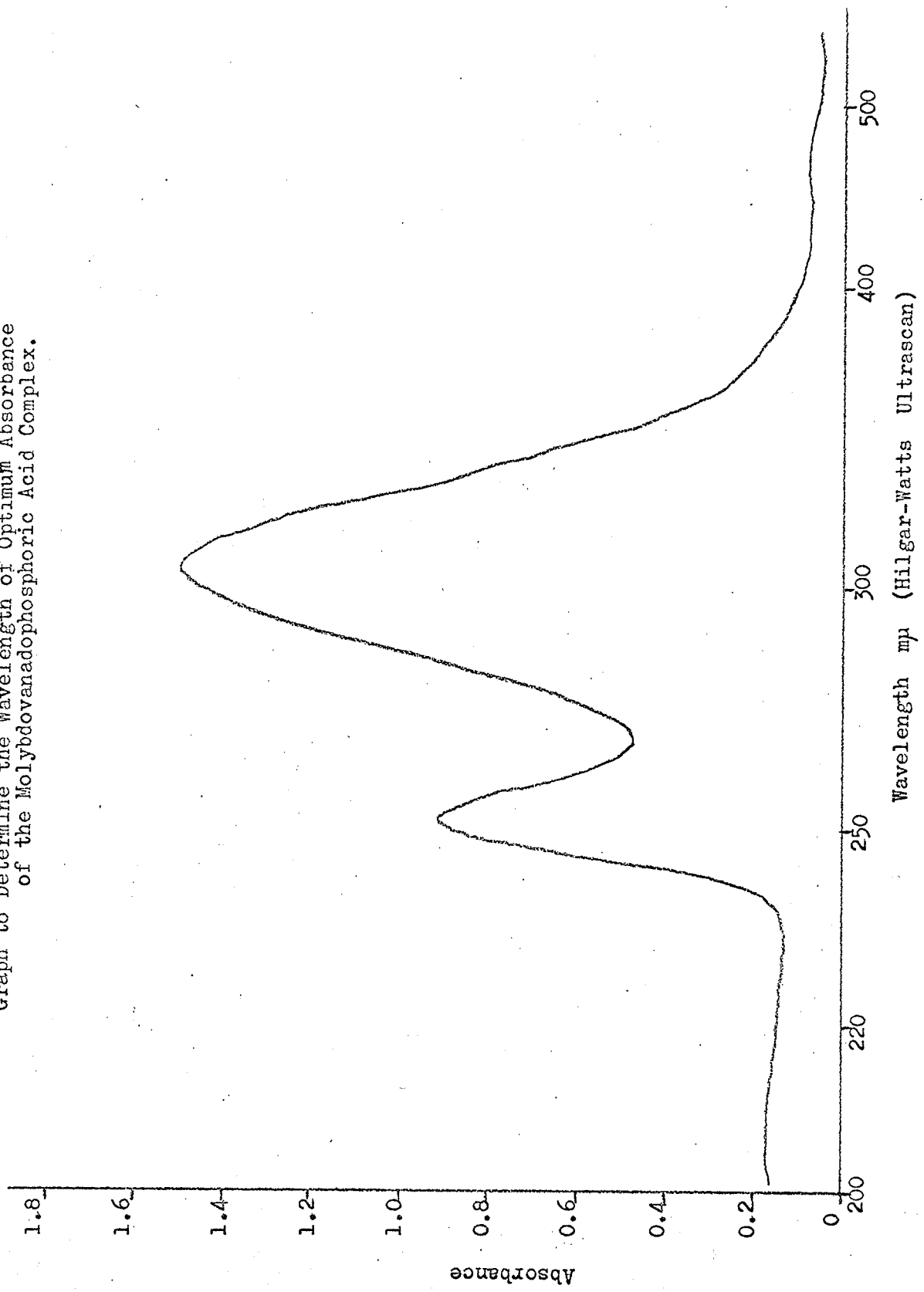


Table II. Preparation of a Standard Phosphorus Curve. Lindner and Edmundsson (62)

	ml. P. Sol'n Added	µg. P/ml. (50 ml. Dil'n)	Absorbance
Trial 1.	1	2	0.068
	2	4	0.130
	5	10	0.312
	10	20	0.615
Trial 2.	3	6	0.184
	6	12	0.368
	9	18	0.550
	12	24	0.738

Methods of Ashing:

Using 2 ml. of a 2.5 per cent aqueous solution of dextrose as organic material, and 2 ml. concentrated sulfuric acid as the digestant, methods of ashing were investigated using crucibles, evaporating dishes, beakers, Erlenmeyer flasks, round-bottom flasks, micro-Kjeldahl flasks, offset micro-Kjeldahl flasks and test tubes, heated by bunsen burners and hot plates. The results of this test are shown in Table III.

As the method of Vogler [99] was being adapted to total phosphorus determination in organic material, and the development of the molybdenum blue is dependent on pH, a test was performed to determine the amount of sulfuric acid that could be added without affecting the colour intensity.

To each 50 ml. volumetric flask was added 1 ml. of standard phosphorus solution (0.01 mg./ml.), 1 ml. of varying sulfuric acid concentrations, 4 ml. of molybdate-sulfuric acid reagent and 1 ml. of ascorbic acid reagent. The solutions were assayed spectrophotometrically at a wavelength of 665 mµ, using a red filter, against an

Table III. Results of Ashing Organic Material in a Variety of Vessels over Different Sources of Heat.

Type of Vessel	Sources of Heat	
	Bunsen Burner	Hot Plate
#00 Crucible	charred and boiled over	charred and boiled over
95 mm. Evaporating dish	popped and spurted before charring	charred and spurted
50 ml. Beaker	popped and spurted	popped and spurted
25 ml. Erlenmeyer flask	spurted before charring commenced	spurted before charring commenced
50 ml. Round-bottom flask	spurted and boiled over after charring	spurted and popped before charring
30 ml Kjeldahl flask	spurted, boiled over ashing not complete	spurted and popped
30 ml. offset Kjeldahl	spurted and boiled over	spurted and popped, ashing not complete
20 X 150 mm Test tube	spurted almost right away	spurted almost right away

Table IV. Effect of Varying Concentrations of Sulfuric Acid on the Absorbance of the Molybdenum Blue Colour.

Conc. of Sulfuric Acid	µg. P. Added	Absorbance	ug. P. Recovered
100%*	10	negative	-
50%	10	0.005	-
25%	10	0.103	9
10%	10	0.104	9
5%	10	0.104	9
1%	10	0.104	9
0 (Standard)	10	0.104	9

*Concentrated sulfuric acid taken as 100%

identically perpared blank with the omission of the phosphorus. Results are shown in Table IV.

Further work on the ashing techniques was carried out, restricting the vessels to Kjeldahl flasks and Erlenmeyer flasks and the sources of heat, to hot plates and sand baths.(sand baths heated with bunsen burners). To each flask was added 1 ml. of standard phosphorus solution (0.01 mg.P/ml.), 1 ml. 25 per cent sulfuric acid and 1 ml. of 1 per cent dextrose solution. The flasks were heated until charring was complete, then cleared with 1-2 drops of perchloric acid (72%). The contents were carefully transferred to 50 ml. volumetric flasks with sufficient washing. Four ml. of molybdate-sulfuric acid reagent and 1 ml. of ascorbic acid reagent were added after which the flasks were made up to volume, shaken, and the colour assayed spectrophotometrically, at 665 mμ using a red filter, against a phosphorus-deficient blank. Results are shown in Table V.

Other factors investigated were temperature and length of heating and the question of loss of phosphorus during heating due to the volatilization of phosphoric acid. (Table VI)

Because of the lower boiling point of phosphoric acid, trials were performed to determine heating temperature. To each 30 ml. Kjeldahl flask was added 1 ml. of standard phosphorus solution (0.1 mg. P/ ml.) 0.3 ml. of concentrated sulfuric acid, and to samples 3 an 4, 25 mg, of dextrose. The flasks were laid on their sides in sand baths with thermometers inserted as shown. (Figure 4(a) When ashing was completed the samples were cleared with perchloric acid and colourimetrically assayed as before. Results are shown in Table VII.

Table V. Comparison of Effectiveness of Various Ashing Techniques.

Sample.	Type of Flask	Source of Heat	µg. P Added	Absorbance	µg. P. Recovered	Comments
1.	50 ml. Erlenmeyer	Hot Plate	10	0.082	6.5	spurted before charring commenced.
2.	50 ml. Erlenmeyer	Sand Bath	10	0.107	9.6	10-15 min. before charring no spurting.
3.	30 ml. Kjeldahl	Hot Plate	10	0.091	7.5	evidence of spurting ashing not complete
4.	30 ml. Kjeldahl	Sand Bath	10	0.042	3.0	as above. particles in volumetric flask.
5.	30 ml. Kjeldahl	Hot Plate	10	0.104	9.0	satisfactory
6.	30 ml. Kjeldahl	Sand Bath	10	0.110	9.7	satisfactory
7.	Standard		10	0.104	9.0	

Table VI. Comparison of Boiling Points of Acids Used in Phosphorus Determination.

Acid	Boiling Point	Decomposition
Concentrated Sulfuric Acid	290°	340°
Concentrated Phosphoric Acid	261°	300°
Concentrated Perchloric Acid	19°	

Table VII. Comparison of Effects of Different Heating Temperatures Over Various Lengths of Time on Molybdenum Blue Absorpance.

Sample	Time of Heating (min.)	Temperature	µg. P Added	Absorbance	µg. P. Recovered	% Error
1.	150	240-250° C	100	0.748	73.4	26.6
2.	90	240-250° C	100	0.730	71.5	28.5
3.	120	240-275° C	100	0.760	74.6	25.4
4.	60	240° C	100	0.748	73.4	26.6
5.	standard	-	100	0.720	70.5	29.5

Figure 4(a) Apparatus for Determination of Temperature Reached by Samples During Ashing

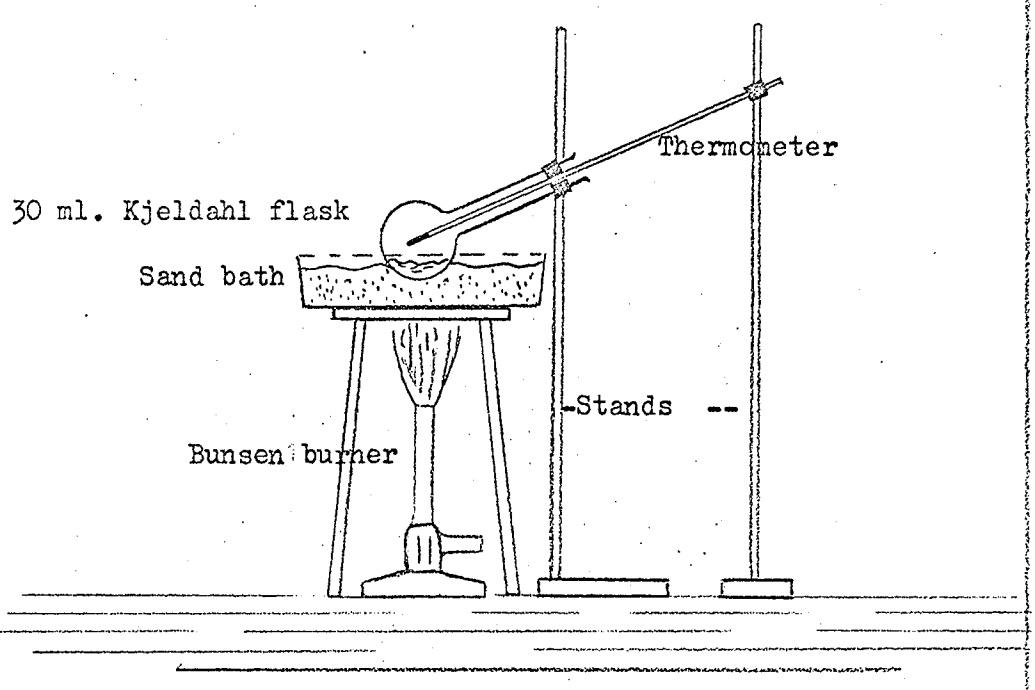
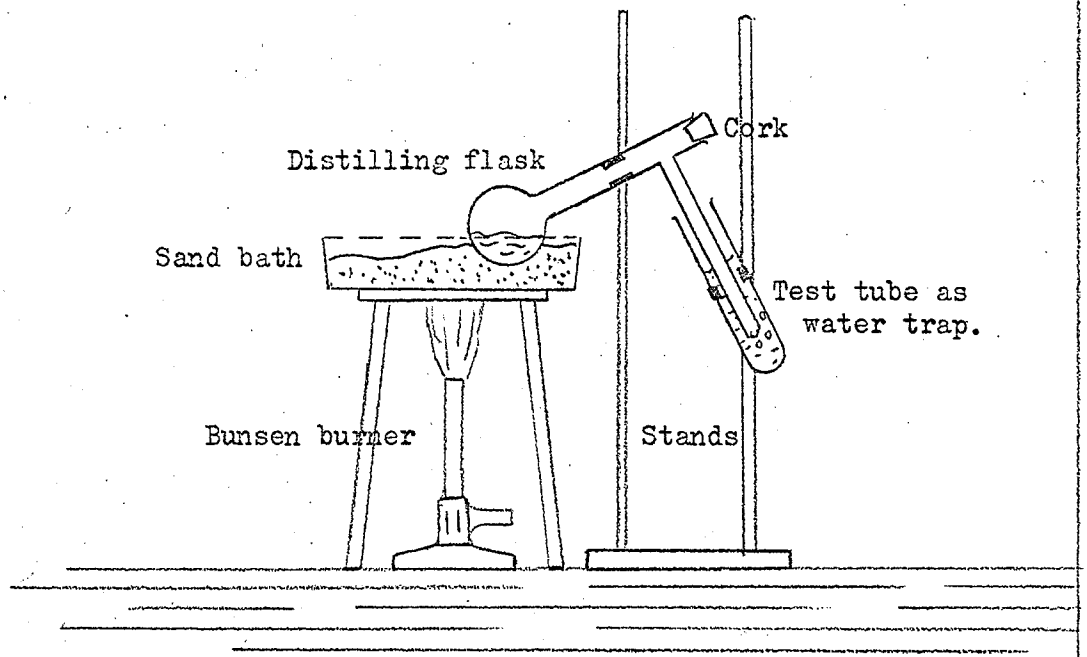


Figure 4(b) Apparatus for Determination of Any Loss of Phosphorus by Volatilization or Spurting.



In order to determine whether phosphorus could be lost during ashing by volatilization, the test was repeated, using distilling flasks with the side arms directed into water traps. (Figure4(b).The phosphorus content was then determined after the ashing, both in the sample flask and in the water trap as well. Results are shown in Table VIII.

Table VIII. Checking for Loss of Phosphorus During the Ashing Technique, either by Volatilization of Phosphoric Acid or from Spurting.

Sample	µg. P. Added	Absorbance	µg. P. Recovered	% Error
Standard	100	0.875	85.1	14.9
Flask 1	100	0.770	75.5	24.5
Trap 1	-	0.005	-	
Flask 2	100	0.792	77.8	22.2
Trap 2	-	0.001	-	
Flask 3	100	0.850	83.5	16.5
Trap 3	-	0.000	-	
Flask 4	100	0.725	71.0	29.0
Trap 4	-	0.005	-	

The test was then repeated and carried a step further by drastically extending the time of heating after ashing was complete. Using exactly the same procedure but a new phosphorus standard (10 µg. P./ ml.), the samples, containing dextrose, were ashed and cleared on the sand bath and then overheated for varying lengths of time. Colourimetric determinations were as before. Results are shown in Table IX. The pH of the final solution was also determined as a reference for application to further work.

An experiment was then performed to determine the reproducibility of Vogler's method, unmodified, with the addition of acid equivalent to 1 ml. of 25 per cent sulfuric acid and after an ashing procedure.

Table IX. Effect of Overheating during Ashing Procedures on the Molybdenum Blue Colourimetric Determination of Phosphorus.

µg. P. Added	mg. Dextrose Added.	Time (hr.) Overheating.	Absorbance	µg.P. Rec'd	% Error	pH
10	1	4	0.113	9.9	1	0.7
10	1	5	0.097	8.2	18	0.5
10	1	6	0.102	8.7	13	0.5
10	1	7	0.099	8.5	15	0.5

Four trial were performed.

Trial 1 and 2: to 50 ml. volumetric flasks were added 1 ml. of standard phosphorus solution (0.1 mg. P./ml.), 0.3 ml. concentrated sulfuric acid, 4 ml. of molybdenum reagent and 1 ml. of ascorbic acid.

Trial 3: was the same as Trials 1 and 2 with the omission of the sulfuric acid.

Trial 4: these samples were the same as Trials 1 and 2 except 25 mg. of dextrose was ashed as before, before the colourimetric determination was carried out.

Results are shown in Table X.

Table X. Reproducibility of Phosphorus Determination using Vogler's Method. (100 ug. Phosphorus)

	Trial 1		Trial 2		Trial 3		Trial 4	
µg. P. Added	Absorb.	µg. P. Found	Absorb.	µg. P. Found	Absorb.	µg. P. Found	Absorb.	µg. P. Found
100	0.840	82.6	0.795	78.2	0.895	88.2	0.850	83.6
100	0.850	83.6	0.800	78.7	0.900	88.6	0.830	81.6
100	0.870	85.5	0.815	80.6	0.910	89.8	0.825	81.0
100	0.815	80.6	0.823	80.8	0.900	88.6	0.830	81.6
100	0.865	85.1	0.800	78.7	0.908	89.5	0.825	81.0
Mean:		83.5		79.4		88.9		81.3

Trial 3 was repeated using a new phosphorus standard containing 10 µg. P./ ml., see Table XI.

Table XI. Reproducibility of Phosphorus Determination using Vogler's Method. (10 ug. Phosphorus)

µg. P. Added	Absorbance	µg. P. Recovered	% Error
10	0.095	8.0	20
10	0.095	8.0	20
10	0.095	8.0	20
10	0.095	8.0	20
10	0.095	8.0	20

The stability of the colour solution was also tested by reading the absorbance at varying intervals after the addition of the molybdenum blue forming reagents. See Table XII.

Table XII. Time for Stability of the Molybdenum Colour.

Sample	µg. P. Added	Time (min.) after Add'n Reagents	Absorb.	µg. P. Rec'd	% Error
1	10	5	0.97	8.2	18
2	10	15	0.97	8.2	18
3	10	30	0.97	8.2	18
4	10	60	0.97	8.2	18

Further tests were carried out to determine the optimum pH that will produce the maximum intensity and stability of the molybdenum blue colour.

Standard Reagent: 1 ml. of standard phosphorus solution (10 µg. P./ ml.) was diluted to 50 ml.
The pH of this solution was 6.2

Test Samples: 1 ml. standard phosphorus solution (10 µg. P./ml.)
1 ml. dextrose solution (1 mg. / ml.)
1 ml. concentrated sulfuric acid.

The ashing was performed in Kjeldahl flasks on sand baths, using 1-2 drops of perchloric acid to clear the solution. When

ashing was complete, the samples were washed into 50 ml. beakers. The pH was adjusted to pH 6.2 (same as standard solution) with dilute ammonia, and the samples were then transferred to 50 ml volumetric flasks and the colourimetric determination procedure was followed as outlined before. Results are shown in Table XIII.

Table XIII. Determination of Phosphorus by Colourimetric Analysis after Neutralization of the Ashed Samples with Ammonia.

Sample	µg. P. Added	Absorbance (30 min.)	µg. P. Rec'd	% Error	Absorbance (60 min.)	µg. P. Rec'd	% Error
Standard	10	0.110	9.7	3	0.125	11.1	11
1.	10	0.100	8.5	15	0.114	10.0	0
2.	10	0.112	9.9	1	0.119	10.2	2
3.	10	0.118	10.1	1	0.123	10.9	9
4.	10	0.125	11.1	11	0.127	11.2	12

Variations of these tests on the effect of neutralization with ammonia after ashing were performed. The samples were ashed with 1 ml. concentrated sulfuric acid, then neutralized to a pH of 7 before addition of molybdenum for colourimetric determination. The results are shown in Table XIV.

Table XIV Effect of Neutralization with Ammonia on the Development of the Molybdenum Blue Colour.

Sample	µg. P. Added	ml. Conc. Acid	ml. Dextrose sol'n (1 mg./ml.)	pH after ashing	pH after neutralization	Absorb.
Standard	10	1	-	0.9	7	?*
1.	10	1	1	0.4	7	0.110
2.	20	1	1	0.4	7	0.105
3.	30	1	1	0.5	7	cloudy*
4.	40	1	1	0.6	7	cloudy*

* samples have an unstable colour or else are cloudy and unreadable.

The cloudiness of samples 2 and 3 from Table XIV was thought to be caused by precipitation of the sulfanilic acid in the molybdenum reagent, so a new modified reagent was made in the same way, only with the omission of the sulfanilic acid. Then a test was designed and performed to demonstrate the effect of pH on the development and intensity of the molybdenum blue colour.

To each 50 ml. volumetric flask was added 2 ml. of standard phosphorus solution (10 µg.P/ml.) and 4 ml. of the modified molybdenum reagent. Then, using dilute ammonia, various pH levels were attained, 1 ml. of ascorbic acid reagent was added and the volumes were made up to 50 ml. Colourimetric analysis was performed as before and the results were shown in Table XV.

Table XV. Effect of pH on the Intensity of the Molybdenum Blue Colour in the Determination of Phosphorus.

µg. P. Added	Colour (before reduction)	Original pH	pH Wanted	Actual Modified pH	Absorbance.
20	faint greenish-blue	0.5	1	1.9	infinite
20	bluish tinge	0.6	2	2.6	infinite
20	faint blue	1.0	3	3.0	infinite
20	very faint blue	0.9	4	3.6	0.885
20	colourless	0.9	5	4.6	0.294
20	colourless	0.5	6	5.6	0.025
20	colourless	0.5	7	5.7	0.014
20	colourless	1.2	8	7.8	0.000

Extraction Methods (Modified Heteropoly Blue Methods)

Because of the problems that developed in the molybdenum blue method, variations of the modified heteropoly blue method were tested. The method to be used is outlined below, developed by many trial and error experiments. The reagents were the same as used before, including the modified molybdenum reagent.

To a separatory funnel was added 1 ml. of standard phosphorus solution (10 µg.P./ml.), 4 ml of modified molybdenum reagent and 20 ml of water. To this mixture 25 ml of a xylene-butanol (65:35) reagent were added and the funnel was shaken for 5 minutes. The funnel was then placed in a rack for at least 2 minutes to allow the phases to separate after which the lower aqueous phase was drawn off. One ml. of the ascorbic solution diluted to 25 ml was then added and the funnel shaken for 5 minutes. After separation, the aqueous phase was drawn off and the phosphorus content assayed spectrophotometrically on the blue colour in the aqueous phase, against a similiarly prepared blank. The optimum wavelength for maximum absorbance was determined on the Ultrascan was found to be 315 mµ. (Figure 5.) and the results of the standard curve determination are shown in Table XVI with the standard curve plotted in Figure 6 by means of the Least Squares Formula.

This experiment was then repeated to determine whether the length of time of shaking could be reduced without affecting the efficiency of the extraction. Results are shown in Table XVII.

Figure 5. Graph to Determine the Wavelength of Optimum Absorbance of the Extracted Heteropoly Blue Colour.

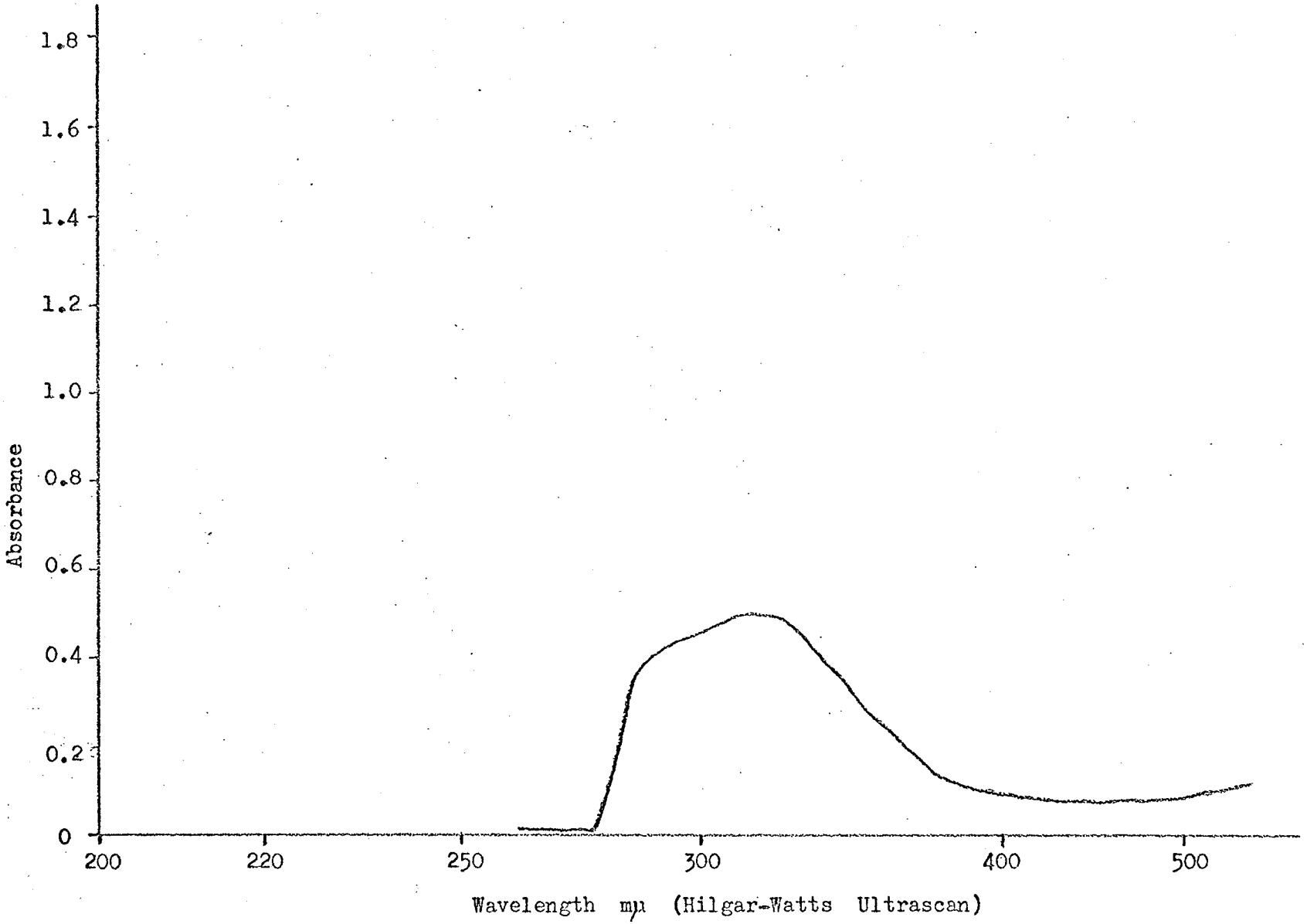


Table XVI. Plots of Standard Curve for Phosphorus Determination by the Modified Heteropoly Blue Method.

Sample	µg. P. Added	Absorbance
1	10	0.242
2	20	0.502
3	30	0.750
4	40	1.000
5	50	1.25

Calculations:

$$b = \frac{n(\Sigma xy) - (\Sigma x)(\Sigma y)}{n(\Sigma x^2) - (\Sigma x)^2} = \frac{5(137.46) - (150)(3.744)}{5(5500) - (150)^2}$$

$$b = 0.025$$

$$a = \bar{y} - b(\bar{x}) = 0.749 - (0.025)(30) = -0.001$$

$$y' = a + b(x) = -0.001 + 0.025(20)$$

$$y' = 0.499$$

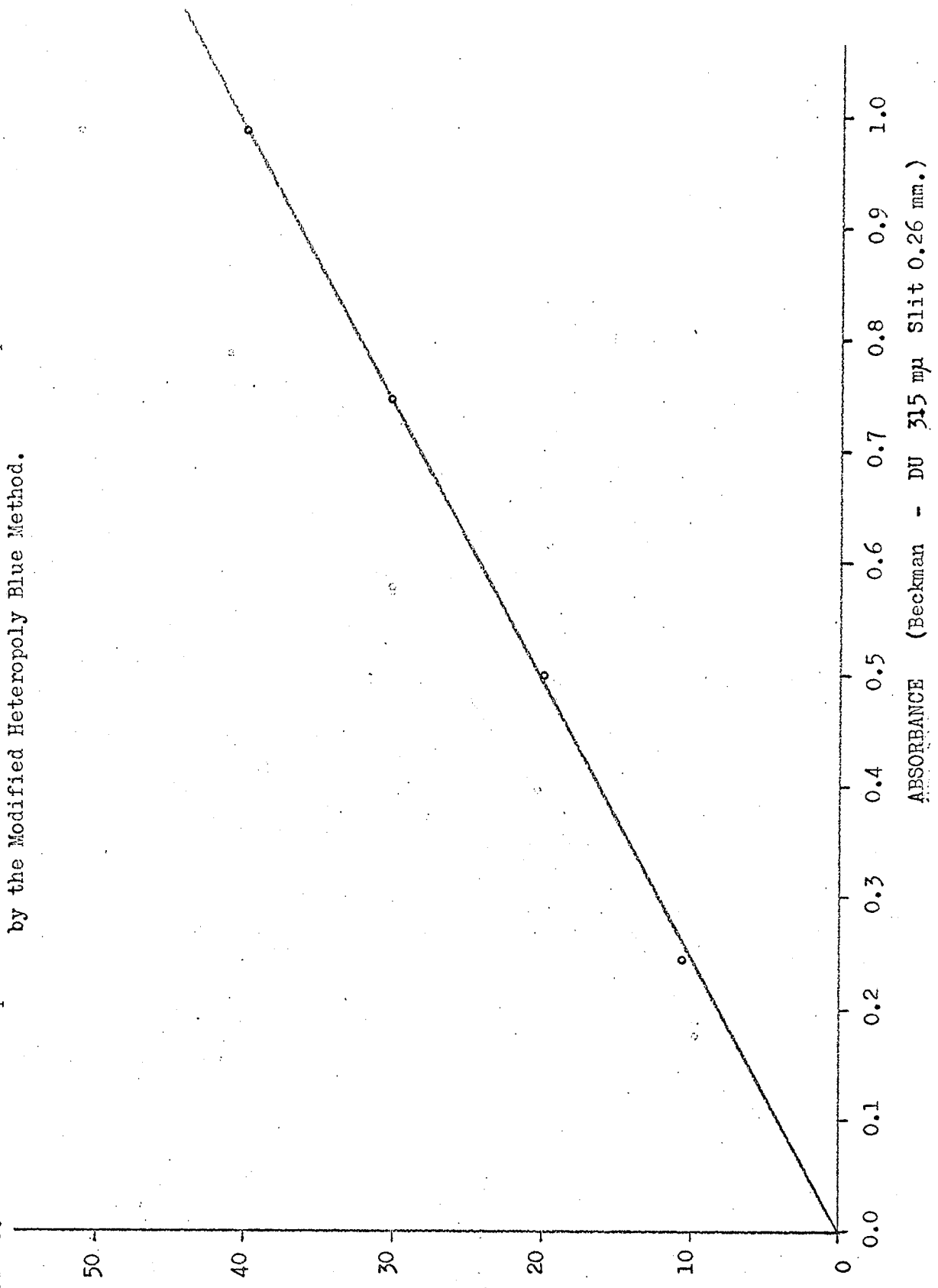
Similiarly when $x = 50$, $y' = 1.25$

Table XVII. Variation of Length of Time of Shaking for Extraction of the Molybdophosphoric Acid Complex.

µg. P. Added	1 st. Extraction Time (min.)	Absorb.	µg. P. Rec'd	2 nd. Extraction * Time (min.)	Absorb.	µg. P. Rec'd
10	1	0.225	9.0	1	0.246	9.8
10	2	0.245	9.8	2	0.246	9.8
10	3	0.227	9.1	3	0.241	9.6
10	4	0.241	9.6	4	0.243	9.7
10	5	0.236	9.5	5	0.241	9.6

* Second extraction means the back extraction of the reduced phosphomolybdic acid into the aqueous phase.

Figure 6. Graph of Calibration Curve for the Determination of Phosphorus by the Modified Heteropoly Blue Method.



An assay was carried out using this modified heteropoly blue method. Each sample contained 10 µg. phosphorus and 100 mg. dextrose. Ashing was carried out using 1 ml. of concentrated sulfuric acid in a 30 ml. micro-Kjeldahl flask on a sand bath heated with a hot plate. When ashing was complete, the samples were cleared with 1-2 drops of perchloric acid and transferred to separatory funnels with sufficient washing. then varying amounts of dilute ammonia were added and the extraction and spectrophotometric determination performed as before. The results are seen in Table XVIII.

Table XVIII. Determination of Phosphorus by the Extraction Method after Neutralization with Ammonia.

Sample	µg. P. Added	ml. dil. NH_3 Added	Absorbance
1	10	5	0.730
2	10	10	0.555*
3	10	6	0.609

* No visible colour evident in the aqueous phase on which the absorbance was measured.

Following this preliminary trial, an experiment was set up to determine the optimum quantity of dilute ammonia necessary to promote the maximum extraction of the heteropoly acid. The results are shown in Table XIX. There was no organic material in these samples so ashing was not necessary.

Assays were then carried out using 100 mg. of dextrose as the organic material and sulfuric acid as the digestant. The procedure was followed using the micro-Kjeldahl flasks on the sand baths. After ashing, the samples were neutralized with 5 ml. of dilute ammonia and extracted as before. The phosphorus content was then determined spectrophotometrically yielding results shown in Table XX.

Table XIX. Determination of the Quantity of Dilute Ammonia Necessary to Neutralize the Samples, and Promote Optimum Extraction of the Heteropoly Acid.

Sample	µg. P. Added	ml. conc. H_2SO_4	ml. dil. NH_3	Absorbance	µg. P. Rec'd	% Error
1	10	0	0	0.411	16.5	65
2	10	1	2	0.135	5.4	46
3	10	1	3	0.151	6.0	40
4	10	1	5	0.415	16.7	67
5	20	0	0	0.633	25.4	27
6	20	1	5	0.669	26.8	34
7	50	0	0	1.42	51.7	3.4
8	50	1	5	1.35	51.5	3.0
9*	Organic phase from Sample 7			0.000	-	
10*	Organic phase from Sample 8			0.000	-	

*To check completeness of back-extraction.

Table XX. Determination of Phosphorus in Samples that have been Ashed to Remove Organic Material then Neutralized with Ammonia before Extraction.

Sample	µg. P. Added	mg. Dextrose	ml. conc. H_2SO_4	ml. dil. NH_3	Absorb.	µg. P. Rec'd	% Error
1	10	100	1	5	0.238	9.5	5
2	10	100	1	5	0.248	10.0	0
3	10	100	1	5	0.223	9.0	10
4	10	100	1	5	0.242	9.7	3
5	20	100	1	5	0.464	18.5	7.5
6	20	100	1	5	0.489	19.5	2.5
7	20	100	1	5	0.475	19.0	5
8	20	100	1	5	0.453	18.2	9

Since the main object of this experiment was to developd a micro method for the determination of phosphorus in minute samples, modifications were applied to this extraction procedure to:

1) reduce the volume of the reagents,

2) check the possibility of performing all steps of the procedure in one vessel, thus omitting the error of transfer, and

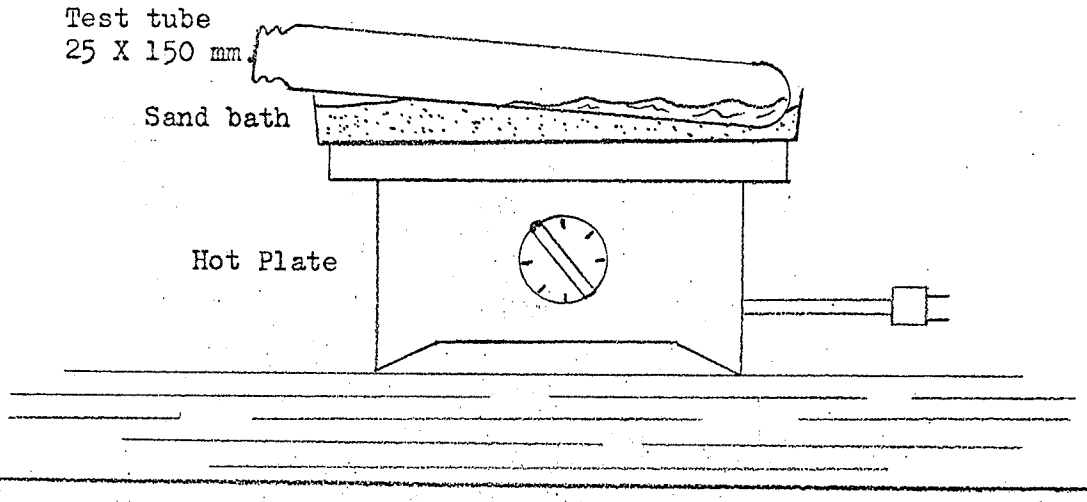
3) see if the absorbance can be measured successfully in the organic phase following this procedure.

The organic material was added to a 20 ml. quick-fit test tube along with measured amounts of phosphorus standard and 0.1 ml. of concentrated sulfuric acid. The samples were ashed on a sand bath heated by a hot plate (Figure 7) and cleared with 1-2 drops of perchloric acid (72%). The samples were then heated further in order to remove any excess perchloric acid that might remain. After the samples had cooled, some were neutralized with varying amounts of dilute ammonia, 1 ml. of modified molybdenum reagent was added, volumes were made up to 5 ml. with water and then 5 ml. of xylene-butanol (65:35) were added.

The tubes were stoppered and shaken for 1 minute, 1 ml. of ascorbic acid solution was added and the tubes were stoppered and shaken again for 1 minute. The organic phase was drawn off by Pasteur teat pipette into 1 cm. quartz cuvettes and the absorbance measured at the optimum wavelength of 335 mμ (Figure 8) against a similiarly prepared blank. The results are shown in Table XXI.

Further testing was carried out to determine whether smaller amounts of sulfuric acid would affect the colour intensity or efficiency of the extraction, so neutralization could be avoided. See Table XXII for the results.

Figure 7. Illustration to Show How Organic Material was Ashed in Test Tubes on Sand Baths.



Overhead View of Above to Illustrate How Several Samples were Ashed at the Same Time.

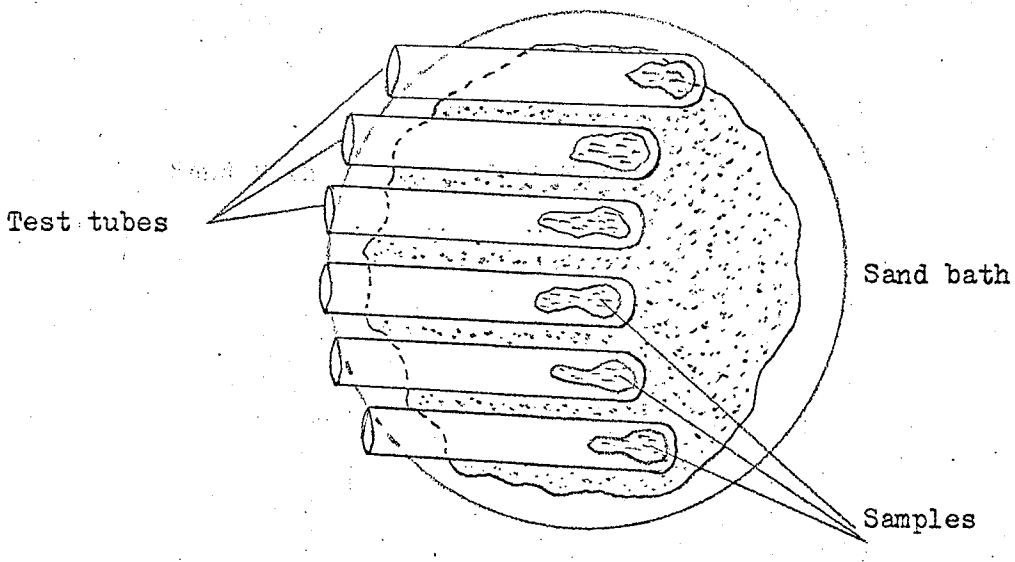


Figure 8. Graph to Determine the Wavelength of Optimum Absorbance of the Extracted Heteropoly Acid in the Organic Phase.

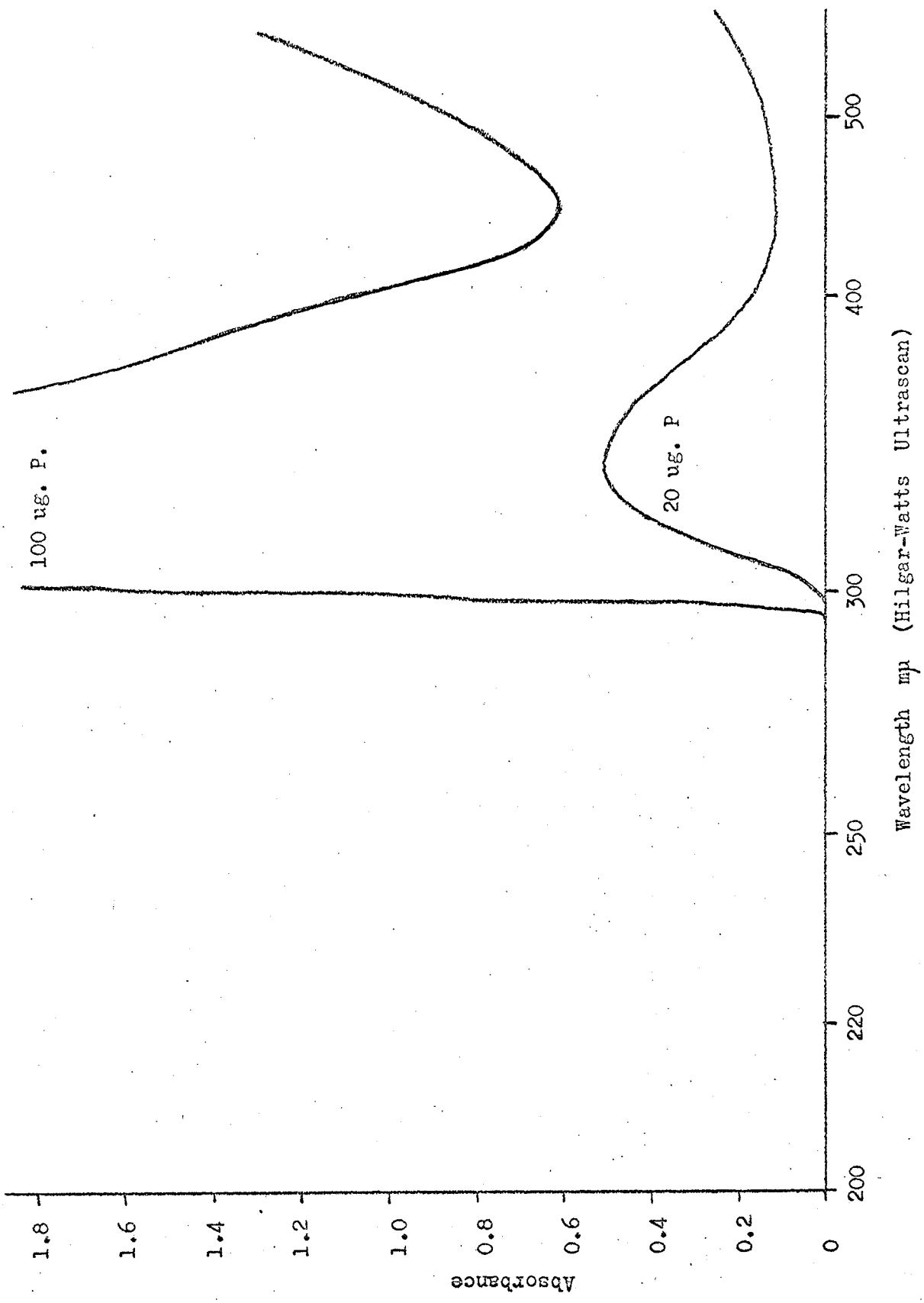


Table XXI. Effect of Neutralization on the Extraction of Molybdophosphoruc Acid in this Micro-Method.

Sample	µg P. Added	ml. Conc. H_2SO_4	ml. dil. NH_3	ml. Ascorbic Acid	Absorbance
1	10	-	-	-	1.80
2	10	0.1	1	-	1.85
3	10	-	-	1	0.653
4	10	0.1	1	1	0.400
5	10	0.1	1	1	0.195
6	20	0.1	1	-	infinite

Table XXII. Effect of Small Amounts of Concentrated Sulfuric Acid on the Efficiency of Extraction of the Heteropoly Acid.

Sample	µg. P. Added	Drops conc. sulfuric acid	Absorbance 350 mµ.
1	10	2	0.728
2	10	3	0.755
3	10	4	0.810
4	10	5	0.760
5	10	6	0.000

Testing was then carried out using 2 drops of concentrated sulfuric acid to ash organic material. Dextrose and pepsin were used, and after ashing the phosphorus content was determined by extracting the heteropoly acid as before. Results can be seen in Table XXIII.

From these results, further modification was deemed necessary. First of all N/1 sulfuric acid was substituted for concentrated sulfuric acid so larger, easier-to-measure volumes could be used. Reduction of the extracted phosphomolybdic acid was omitted as well and the absorbance measured in the organic phase at 315 mµ (Figure 9.)

Figure 9. Graph to determine the Wavelength of Optimum Absorbance of the Extracted Unreduced Heteropoly Acid in the Organic Phase.

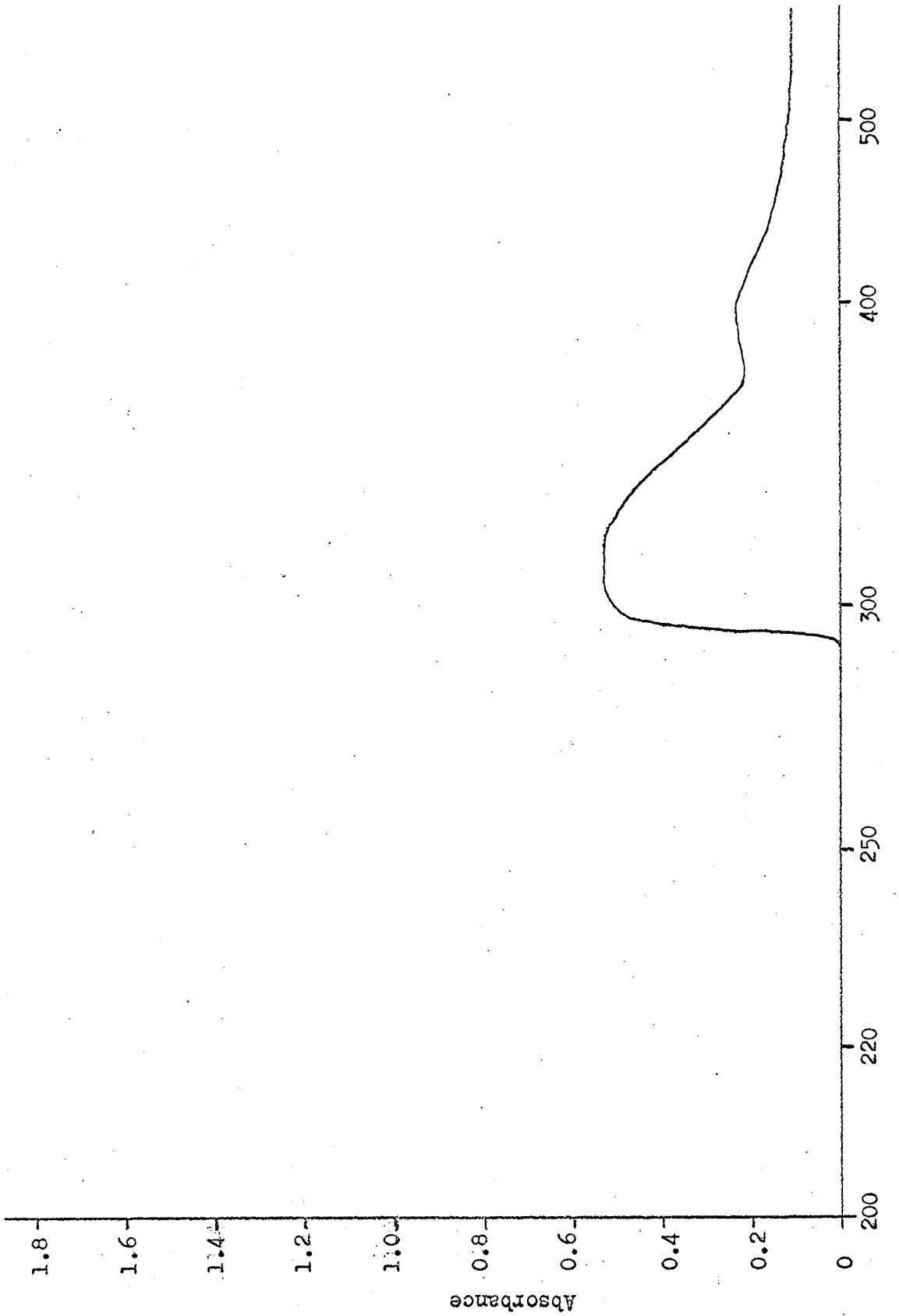


Table XXIII. Determination of Phosphorus in the Presence of Organic Material without Neutralization before Extraction.

Sample	µg. P. Added	Mg. Organic Material	Absorbance 350 mu.
		Dextrose	
1	10	30	0.292
2	10	30	0.190
3	10	30	1.48
4.	10	30	0.495
5	10	30	0.970
		Pepsin	
6	10	20	0.439
7	10	20	0.330
8	10	20	0.275
9	10	20	0.277
10	10	20	0.690

using a blue filter. The molybdenum reagent was modified also to contain only 1 per cent ammonium molybdate tetrahydrate in water, and only 1 ml. was used in the spectrophotometric determination. A preliminary test was carried out to determine the amount of N/1 sulfuric acid necessary to promote maximum extraction. (Table XXIV)

Table XXIV. Determination of the Optimum Amount of N/1 Sulfuric Acid that Will Promote Maximum Extraction of the Heteropoly Acid.

Sample	µg. P. Added	ml. N/1 H_2SO_4	Absorbance	µg. P. Rec'd	% Error
1	5	0.5	0.835	4.42	11.6
2	5	1.0	0.915	4.84	3.2
3	5	1.3	0.930	4.90	2.0
4	5	1.5	0.930	4.90	2.0
5	5	2.0	0.930	4.90	2.0

Following this experiment, tests were carried out to determine whether the quantity of N/1 sulfuric acid that seemed to promote maximum extraction of the heteropoly acid was sufficient to ash the organic material. Results are shown in Table XXV.

Table XXV. Determination of Phosphorus in the Presence of Organic Material that is Removed by Ashing with 2 ml. N/1 Sulfuric Acid.

Sample	ug. P Added	mg. Dextrose	Heat Time (min.)	Absorbance	ug. P. Rec'd	% Error
1	5	-	-	0.825	4.36	12.8
2	5	-	-	0.860	4.55	9.0
3	5	-	-	0.855	4.52	9.6
4	5	-	45	0.822	4.30	14.0
5	5	-	45	0.930	4.90	2.0
6	5	-	45	1.05	5.30	6.0
7	5	30	45	0.865	4.57	8.6
8	5	30	45	0.930	4.90	2.0
9	5	30	45	0.880	4.65	7.0

The graph plotted by the Ultrascan (Figure 9) has a curiously flattened peak that aroused suspicion that one of the organic solvents used as an extractant may be masking the true peak by its own absorbance. Consequently scan trials were performed on isobutanol and xylene alone as well as on the xylene-isobutanol (65:35) mixture and on a sample extracted into these solvents. Results are shown in Figures 10, 11, 12, and 13. Two other solvents were investigated as possibilities to replace the xylene fraction and results using cyclohexane and hexane are plotted in Figures 14 and 15.

Figure 10 Graph to Determine the Absorbance of Isobutanol Against Air

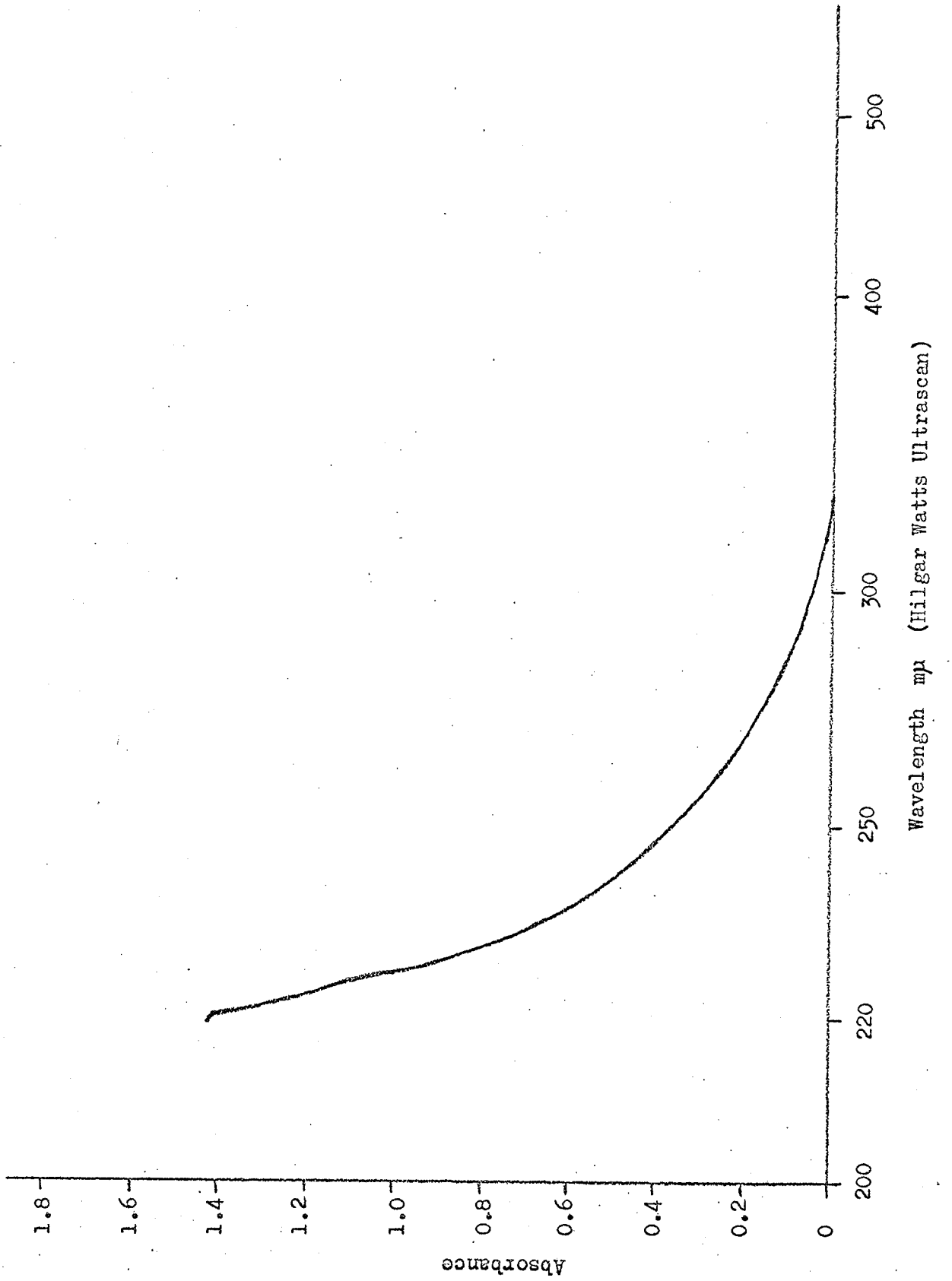


Figure 11. Graph to Determine the Absorbance of Xylene Against Air.

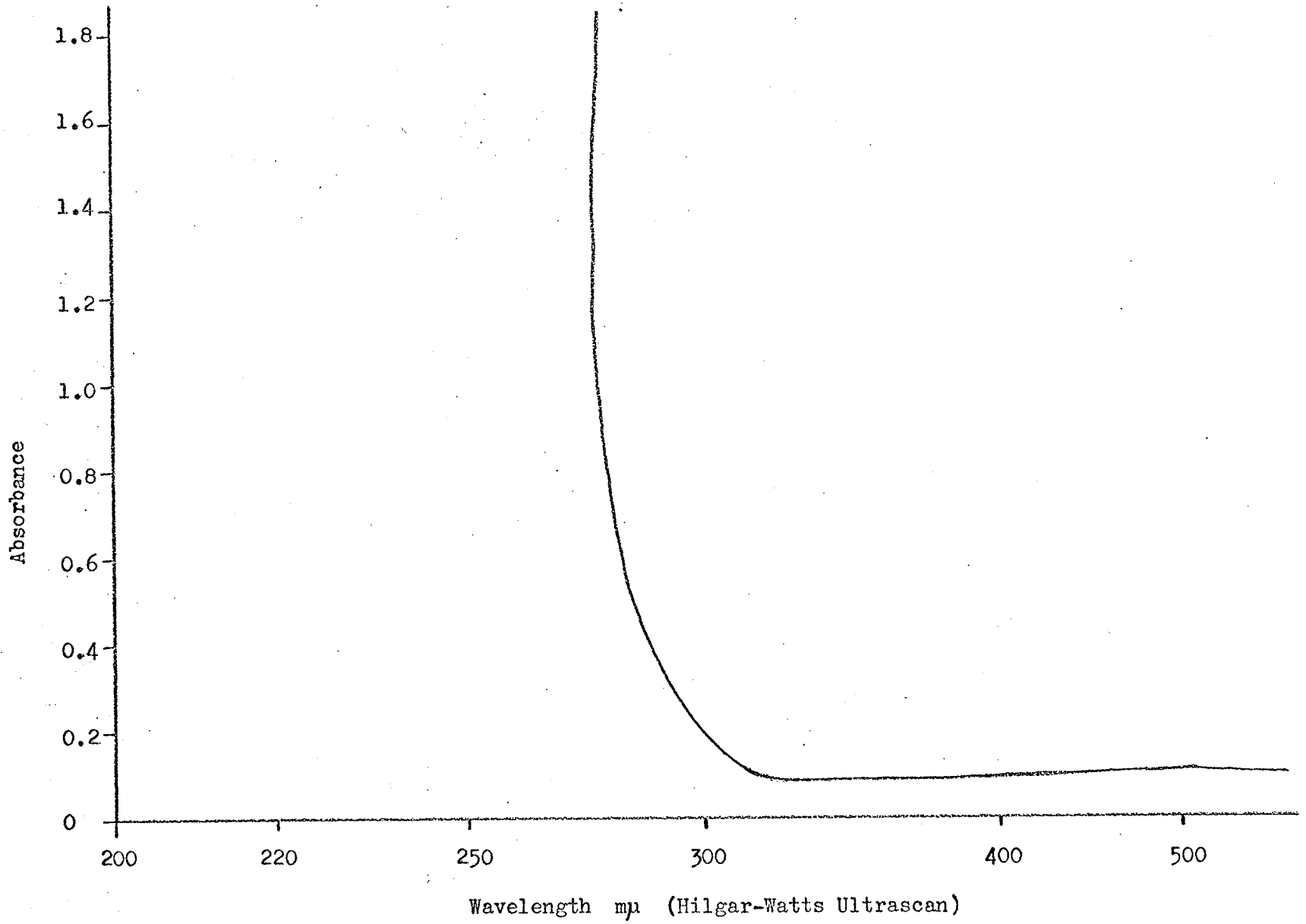


Figure 12. Graph to Determine the Absorbance of Xylene 65% - Isobutanol 35% Against Air.

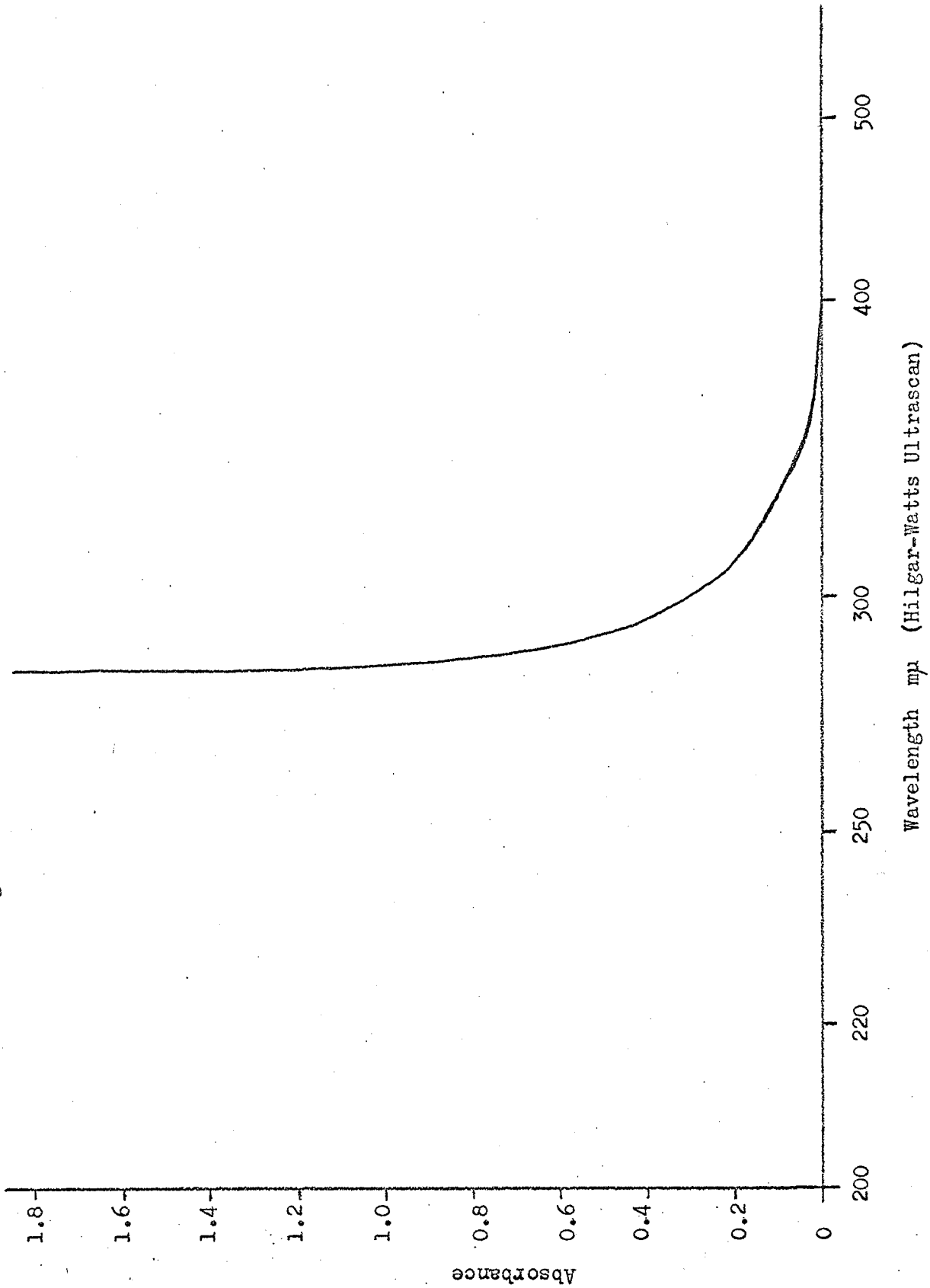


Figure 13. Graph to Determine the Absorbance of Phosphomolybdic Acid Extracted into Xylene-Isobutanol (65:35) in the Organic Phase, Unreduced.

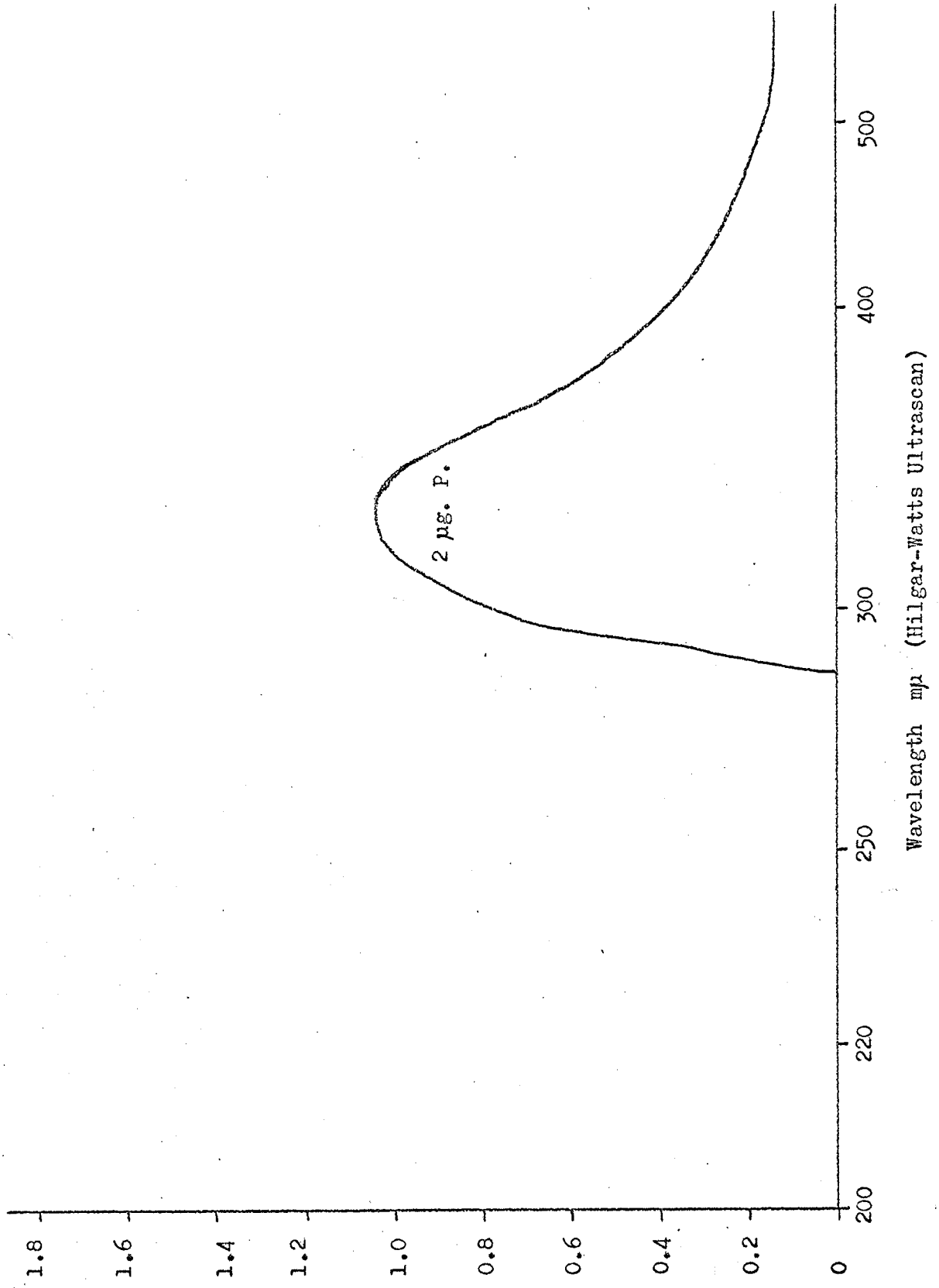


Figure 14. Graph to Determine the Absorbance of Phosphomolybdic Acid Extracted into Cyclohexane-Isobutanol (65:35) in the Organic Phase, Unreduced.

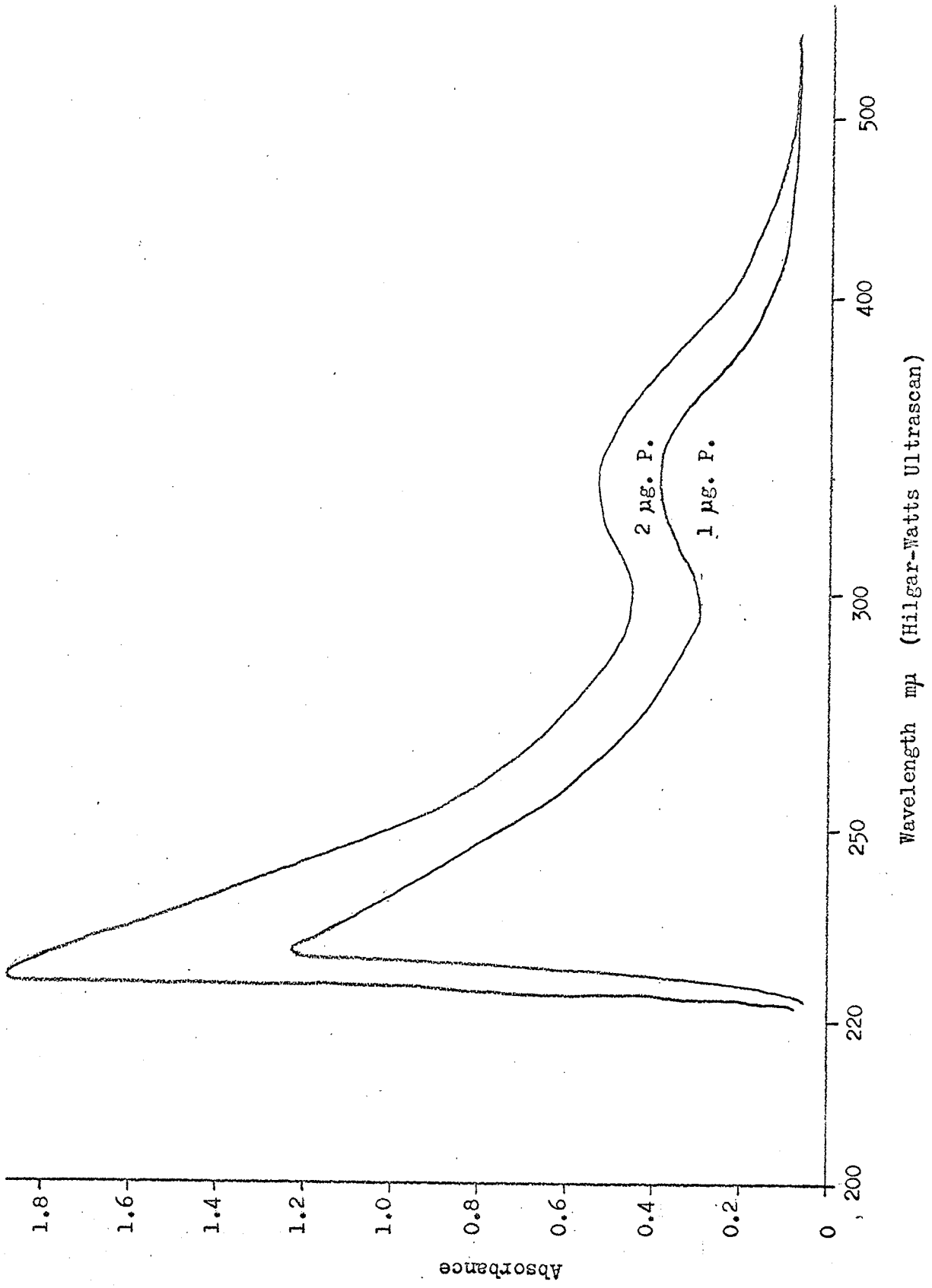
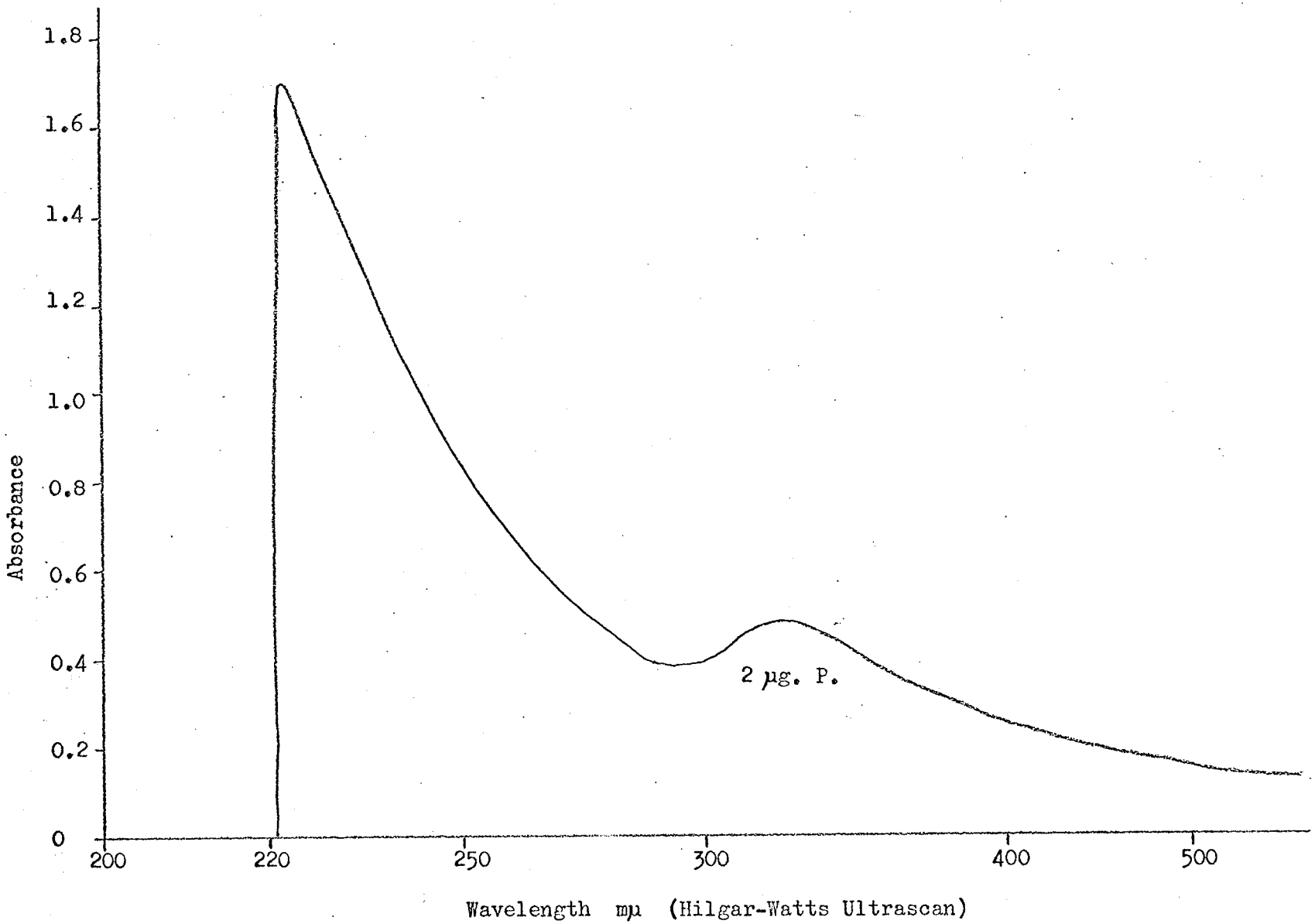


Figure 15. Graph to Determine the Absorbance of Phosphomolybdic Acid Extracted into Hexane-Isobutanol (65:35) in the Organic Phase Unreduced.



Following these preliminary experiments this method was developed and used.

Equipment:

Beckman D-U Spectrophotometer.
25 X 150 mm pyrex screw cap test tubes (rubberlined caps)
Sand bath and Hot Plate.

Reagents:

Organic Materials- Dextrose Solution 100 mg./ml.
Sucrose Solution 100 mg./ml.
Starch Solution 100 mg./ml.
Pepsin Solution 100 mg./ml.

Standard Phosphorus Solution- 0.0446 g. sodium dihydrogen phosphate monohydrate dissolved in 1 litre of water to yield a solution containing 10 µg. P./ml.

Molybdenum Reagent- 1 per cent ammonium molybdate tetrahydrate dissolved in water.

N/1 Sulfuric Acid- 2.64 ml. sulfuric acid to 1 litre of water.

72% Perchloric Acid and 30% Hydrogen Peroxide.

Organic Reagent- Xylene 65% -Butanol 35%.

Varying amounts of phosphorus and organic material were added to the test tubes and ashed with 2 ml. N/1 sulfuric acid on a sand bath heated by a hot plate. Clearing was accomplished by 1-2 drops of perchloric acid or hydrogen peroxide. On cooling, the volume was made up to 4 ml. with water which was also used to rinse down the sides of the test tubes. Then 1 ml. of molybdenum reagent was added, the tubes were shaken and 5 ml. of organic reagent added from a burette. The tube was then capped and shaken for 30 - 60 seconds. After 10 minutes the organic phase was transferred into 1 cm. quartz cuvettes by means of Pasteur teat pipettes and the absorbance was determined at a wavelength of 310 or 315 mµ. The results are shown in Table XXVI, measured from a

similiarly prepared standard curve plotted in Figure 16.

Table XXVI. Determination of Phosphorus in the Presence of Organic Material Removed by Ashing with N/1 Sulfuric Acid.

Sample	µg. P. Added	mg. Dextrose Added	Trial 1 Absorb. 315 mµ.	µg. P. Rec'd	% Error	Trial 2 Absorb. 315 mµ.	µg. P. Rec'd	% Error
		Clear with $HClO_4$						
1	1	10	0.168	0.93	7	0.175	0.97	3
2	2	20	0.325	1.75	12.5	0.364	1.95	2.5
3	3	30	0.478	2.55	15	0.558	2.97	1
4	4	40	not done			not done		
5	5	50	0.880	4.65	7	0.910	4.80	4
6	10	50	infinite			infinite		
		Clear with H_2O_2	310 mµ.	Trial 3		310 mµ.	Trial 4	
7	1	10	0.208	1.14	14	0.110	0.62	38
8	2	20	0.366	1.97	1.5	0.349	1.87	7.5
9	3	30	0.565	3.00	0	0.500	2.67	11
10	4	40	0.670	3.55	11	0.663	3.52	12
11	5	50	0.900	4.75	5	0.860	4.55	9

Following this preliminary trial shown above a more diversified test involving other organic material was carried out with results shown in Table XXVII.

A problem that arose during this procedure was the incomplete separation of the two phases after shaking. Suspended droplets of water in the organic phase clung to the vessel walls and vice versa. The organic phase was often more cloudy than at other times again suggesting incomplete separation of the phases. A test to try and rectify this problem was developed by application of different methods of shaking during extraction. The results are shown in Table XXVIII.

Table XXVII. Determination of Phosphorus in the Presence of Different Organic Substances, Ashed with N/1 Sulfuric Acid.

Sample	µg. P. Added	mg. Org. Added DEXTROSE	Heat (min,)	Trial 1 Absorb. 310 mµ.	Trial 1 µg. P. Rec'd	Trial 1 % Error	Trial 2 Absorb. 310 mµ.	Trial 2 µg. P. Rec'd	Trial 2 % Error
1	5	-	-	0.710	3.75	25	0.745	3.95	21
2	5	-	60	0.710	3.75	25	0.860	4.55	9
3	5	50	60	0.850	4.50	10	0.865	4.57	8.6
4	5	50	60	0.879	4.65	7	0.880	4.65	7
5	5	50	60	0.900	4.75	5	0.905	4.77	4.6
6	5	100	115	0.753	4.00	20	0.773	4.09	18.2
7	3	-	-	0.485	2.58	14	0.500	2.67	11
8	3	-	115	0.550	2.93	2.3	0.553	2.95	1.7
9	3	30	115	0.558	2.97	1	0.475	2.53	16
10	3	60	115	0.558	2.97	1			
11	3	90	115	0.550	2.93	2.3			
12	3	100	115	0.475	2.53	16	0.483	2.57	11
		SUCROSE							
13	1	20	115	0.400	?*				
14	3	30	115	0.480	2.55	15	0.488	2.60	13.
15	3	60	115	0.469	2.50	17	0.500	2.66	11
16	3	100	115	0.408	2.18	27	0.584	3.08	3
17	5	100	115	0.800	4.24	15			
		STARCH							
18	1	20	115	0.195	1.07	7			
19	2	40	115	0.400	2.15	7.5			
20	3	30	115	0.072*	-		0.509	2.71	10
21	3	60	115	0.530	2.82	6	0.595	3.16	5
22	3	100	115	0.665	3.52	17	0.710	3.75	25
23	5	100	115	1.03	5.30	6	1.08	5.32	6.5
		PEPSIN							
24	1	20	115	0.820	-?+				
25	3	60	115	infinite+			infinite+		

*Incomplete removal of H_2O_2 used to clear samples; when molybdenum reagent added a yellow colour appeared in the aqueous phase.

+ Samples hard to clear; some inorganic salts causing interference.

Table XXVIII. Problems of Phase Separation During Phosphorus Determination by Extraction of the Heteropoly Acid.

Sample	µg. P. Added	Shake	Absorb. 310 mµ.	µg. P. Rec'd	% Error	Comments
standard	3	30 sec. hard	0.565	3	0	taken later when problem solved.
1	3	30 sec. hard	0.443	2.37	21	reasonably clear
2	3	15 sec. hard	0.455	2.43	19	reasonably clear
3	3	20 X med.	0.600	3.18	6	quite cloudy, clears on stand.
4	3	20 X gent.	0.638	3.38	13	bubbles present, cloudy.
5	3	30 X gent.	0.532	2.83	6	quite cloudy with bubbles.
6	3	15 sec. hard	0.495	2.64	12	reasonably clear on standing
7	3	15 sec. hard	0.510	2.72	9	reasonably clear on standing
8	3	invert 20 X	0.501	2.67	11	slightly cloudy
9	3	invert 20 X	0.490	2.60	13	slightly cloudy with bubbles

As incomplete removal of the hydrogen peroxide used to clear the samples after ashing appears to lower the efficiency of the extraction, (Table XXVII*) a test was designed to determine the length of time necessary to "overheat" the samples in order to completely destroy the excess peroxide. It was also necessary to find out whether this excessive heating would affect the final results by driving off phosphorus as phosphoric acid or else removing some of the sulfuric acid by decomposition into sulfur dioxide, thus affecting the pH. Results are shown in Table XXIX.

Table XXIX. Effect of Excessive Heating to Destroy Excess Peroxide on the Determination of Phosphorus by the Heteropoly Acid Extraction Method.

Sample	µg.P. Added	ml N/1 H_2SO_4	Drops of H_2O_2 30%	Heat (min.) after clearing	Absorb.	µg.P. Rec'd	% Error
1	3	2	-	-	0.540	2.87	4
2	3	2	2	120	0.530	2.82	6
3	3	2	2	240	0.550	2.93	2
4	3	2	2	-	0.233*	-	

* Effect of H_2O_2 unremoved by heating.

On account of the problem that developed with the hydrogen peroxide used as a clearing agent, a test was carried out to ascertain if a larger quantity of N/1 sulfuric acid could be used to ash the organic material thus removing the necessity of using too much peroxide for clearing. Results are shown in Table XXX.

Table XXX. To Determine if a Larger Amount of N/1 Sulfuric Acid Can be Used to Ash the Organic Material without Affecting the Extraction or Absorbance.

Sample	µg. P. Added	mg. Dextrose Added.	ml. N/1 H_2SO_4	Trial 1 Absorb.	Trial 1 µg. P. Rec'd	Trial 1 % Error	Trial 2 Absorb.	Trial 2 µg. P. Rec'd	Trial 2 % Error
1	3	-	2	0.494	2.64	12	0.453	2.43	19
2	3	-	3	0.510	2.72	9	0.508	2.71	10
3	3	30	2	0.475	2.53	16	0.465	2.49	17
4	3	30	3	0.506	2.70	15	0.510	2.72	9
5	5	-	2	0.710	3.75	25	0.745	3.95	21
6	5	-	3	0.890	4.70	6	0.893	4.74	5

As the results using 3 ml. of N/1 sulfuric acid seem more reproducable the tests with the organic materials were repeated using 3 ml. of N/1 sulfuric acid to digest the organic material. (Table XXXI)

N.B. At this point the Isobutanol arrived and was substituted for the n-Butanol which was used up to this time.

The problem of higher absorbance in samples containing starch as the organic material was investigated. A test was performed in which the absorbance of ashed starch samples was determined in the absence of any phosphorus. Results are shown in Table XXXII

The effectiveness of this method was further verified by determining the amount of phosphorus in an organic material that contains an amount of phosphorus that can be calculated from its emperical formula. Glucose-1-phosphate disodium anhydrous ($C_6H_{11}O_5PO_4Na_2$) was

Table XXXI. Determination of Phosphorus in the Presence of Various Organic Substances that are Digested with 3 ml. of N/1 Sulfuric Acid.

Sample	µg.P. Added	mg. Organic	Trial 1 Absorb.	µg. P. Rec'd	% Error	Trial 2 Absorb.	µg. P. Rec'd	% Error
		DEXTROSE						
1	1	10	0.155	0.85	15	0.170	0.94	6
2	2	20	0.340	1.83	9			
3	3	30	0.528	2.82	6	0.541	2.87	4
		SUCROSE						
4	2	20	0.322	1.74	13	0.333	1.79	11
5	3	30	0.520	2.77	8	0.565	3.00	0
		STARCH						
6	2	20	0.409	2.19	10	0.405	2.17	9
7	3	30	0.660	3.50	17	0.695	3.68	23

Table XXXII. To Determine Whether Starch Alone Increases the Absorbance of the Heteropoly Acid During Phosphorus Determination.

Sample	mg. Starch	Absorb.	Modification of Table XXXI Results Absorb.	µg. P. Rec'd	% Error	Absorb.	µg. P. Rec'd	% Error
1	10	0.019						
2	20	0.041	0.368	1.97	1.5	0.364	1.95	2.5
3	30	0.076	0.584	3.10	3	0.619	3.29	10
4	40	0.116						
5	50	0.140						

was taken as the phosphorus containing substance, containing about 10.2 per cent total phosphorus on a calculated emperical formula basis. A solution was made containing 0.098 mg. glucose-1-phosphate per ml. which yields a solution concentration of 10 µg. phosphorus per ml. Using the procedure developed for the determination of phosphorus by the modified heteropoly method, the phosphorus content of the glucose-1-phosphate was determined experimentally (Table XXXIII).

Table XXXIII. Determination of Phosphorus in Glucose-1-Phosphate

Sample	µg. G-1-P. Added	µg. P. Calc.	ml. N/1 H_2SO_4	Absorb.	µg. P. Rec'd	% Error
1	30	3	3	0.462	2.51	17
2	30	3	3	0.450	2.47	18
3	30	3	3	0.460	2.49	17
4	40	4	3	0.770	4.15	4
5	50	5	3	0.920	4.95	1

Determination of Phosphorus in Serum and Urine

The Determination of phosphorus in serum and urine was one of the first applications of the method of phosphorus determination. However, from the literature survey, it can be seen that this particular extraction technique has not been applied to serum and urine. Therefore, it was decided to test this procedure on rabbit serum and human urine before assaying Fraction C of Proteus OX-19 of which very little was available.

The samples of serum or urine, suitably diluted, (Tables XXXIV and XXXV) were pipetted into the test tubes as used before. Ashing was accomplished by heating the test tubes on a sand bath heated by a hot plate in the presence of 3 ml. N/1 sulfuric acid. When charring was complete, clearing was accomplished by 1-2 drops of 30 per cent hydrogen peroxide followed by further heating to destroy the excess peroxide. When the samples were cool, the sides of the test tubes were rinsed down with 4 ml. of water, 1 ml. of 1 per cent ammonium molybdate reagent was added and the tubes were shaken on a Vortex mixer. Following this, 5 ml. of xylene-isobutanol (65:35) reagent were added and the tubes were capped and shaken for 20 seconds.

Then the organic phase was drawn off into 1 cm. quartz cuvettes by means of Pasteur teat pipettes and the absorbance was determined at 310 mμ. on a Beckman-DU Spectrophotometer with a slit width of 0.13 mm. and a blue filter, against suitably prepared blanks. Results can be seen in Tables XXXVI and XXXVII.

Table XXXIV Preliminary Tests to Determine the Optimum Dilution and Volume of Rabbit Serum for Repeated Phosphorus Determinations.

Sample	ml. undil. serum	Absorb.	ml. 1:10 serum	Absorb.	ml. 1:50* serum	Absorb.
1	0.1	1.88	1	1.59	1	0.295
2	0.2	infinite	1	1.59	2*	0.602*
3	0.3	infinite	1	1.59	3	0.915
4	-	-	-	-	4	1.24
5	-	-	-	-	5	1.58

Table XXXV. Preliminary Tests to Determine the Optimum Dilution and Volume of Urine for Repeated Phosphorus Determinations.

Sample	ml. 1:50 urine	Absorb.+	ml. 1:100 urine	Absorb.	ml. 1:200* urine	Absorb.
1	1	0.450	1	1.41	1*	0.712*
2	2	0.835	2	infinite	2	1.45
3	3	infinite	3	infinite	3	infinite
4	4	infinite	4	infinite	4	infinite
5	5	infinite	5	infinite	5	infinite

* Optimum dilutions, volumes and absorbances.
\+ These readings were taken at 400 mμ. as there was infinite absorbance at 310 mμ. in these dilutions.

Table XXXVI. Determination of Phosphorus in Rabbit Serum.

Sample	ml. 1:50 serum	Trial 1 Absorb.	Trial 1 µg. P. Rec'd	Trial 1 mg. P/ 100 ml.	Trial 2 Absorb.	Trial 2 µg. P. Rec'd	Trial 2 mg. P/ 100 ml.
1	2	0.600	3.25	8.13	0.640	3.42	8.55
2	2	0.621	3.36	8.40	0.645	3.49	8.73
3	2	0.611	3.31	8.28	0.636	3.45	8.63
4	2	0.615	3.34	8.35	0.605	3.27	8.18
5	2	0.685	3.70	9.25	0.638	3.45	8.63
	Means:	0.626	3.39	8.48	0.633	3.41	8.54

		Trial 3		
1	2	0.612	3.32	8.28
2	2	0.632	3.41	8.53
3	2	0.648	3.50	8.75
4	2	0.642	3.47	8.68
5	2	0.634	3.43	8.58
6	2	0.613	3.32	8.28
	Means:	0.630	3.41	8.51

Calculations:

Trial 1. Average Absorb. 0.626 ≡ 3.40 µg. P. ≡ 8.50 mg. P./ 100 ml. serum.

Average Equivalent of P. 3.39 µg. P., S.D. 0.18 ≡ 8.48 mg. P.

Average Amount of P./ 100 ml. is 8.48 mg., S.D. 0.44

Trial 2. Average Amount of P./ 100 ml. is 8.54 mg., S.D. 0.20

Trial 3. Average Amount of P./ 100 ml. is 8.51 mg., S.D. 0.21

Total Average Amount of P./ 100 ml. is 8.51 mg., S.D. 0.28

Relative Variation: $\frac{\text{Standard Deviation}}{\text{Mean}} \cdot 100 = \frac{0.28}{8.51} \cdot 100 = \underline{3.29\ \%}$

Table XXXVII. Determination of Phosphorus in Human Urine.

Sample	ml. 1:200 urine	Trial 1 Absorb.	µg. P. Rec'd	mg. P/ 100 ml.	Trial 2 Absorb.	µg. P. Rec'd	mg. P/ 100 ml.
1	1	0.740	3.93	78.6	0.670	3.55	71.0
2	1	0.705	3.74	74.8	0.688	3,65	73.0
3	1	0.685	3.64	72.8	0.688	3.65	73.0
4	1	0.685	3.64	72.8	0.668	3,54	70.8
5	1	0.705	3.74	74.8	0.663	3.52	70.4
	Means:		3.74	74.7		3.58	71.6
			Trial 3				
1	1	0.660	3.50	70.0			
2.	1	0.660	3.50	70.0			
3	1	0.645	3.42	68.4			
4	1	0.660	3.50	70.0			
5	1	0.652	3.46	69.2			
	Means:		3.47	69.5			

Calculations:

Trial 1. Average Amount of P./ 100 ml. is 74.7 mg., S.D. 2.37

Trial 2. Average Amount of P./ 100 ml. is 71.6 mg., S.D. 1.04

Trial 3. Average Amount of P./ 100 ml. is 69.5 mg., S.D. 0.72

Total. Average Amount of P./ 100 ml. is 71.9 mg., S.D. 2.68

Relative Variation: $\frac{\text{S. D.}}{\text{Mean}} \cdot 100 = \frac{2.68}{71.9} \cdot 100 = \underline{3.72\ \%}$

The method outlined before for the determination of phosphorus in serum and urine was modified slightly in the ashing procedure by substituting 2 ml. of concentrated nitric acid containing 50 mg. of calcium carbonate per litre [9] for the 3 ml. of N/1 sulfuric acid. No clearing was necessary and the samples were heated until brown fumes no longer evolved. When the ashing was completed, the 3 ml. of N/1 sulfuric acid were added, replacing 3 ml. of the water used to rinse down the sides of the test tube. Then the procedure was followed as outlined before. Results using this new reagent are seen in Table XXXVIII.

Table XXXVIII. Determination of Phosphorus in Urine+ and Serum* Using a New Reagent for Digestion.

Sample	ml. 1:50 serum	Absorb.	µg. P. Rec'd	mg. P/ 100 ml.	ml. 1:200 urine	Absorb.	µg. P. Rec'd	mg. P/ 100 ml.
1	1	0.448	2.39	11.95	1	0.444	2.37	47.4
2	1	0.478	2.55	12.75	1	0.449	2.40	48.0
3	1	0.473	2.53	12.65	1	0.495	2.64	52.8
4	1	0.441	2.36	11.80	1	0.530	2.82	56.4
5	1	0.335	1.81	9.05	1	0.530	2.82	56.4
			Mean:	11.64			Mean:	52.2

+ Urine sample 15 days old.
* New sample of Serum.

Calculations:

Serum- Average Amount of P./ 100 ml. is 11.64 mg., S.D. 1.66

Relative Variation: $\frac{S.D.}{Mean} \cdot 100 = \frac{1.66}{11.64} \cdot 100 = \underline{14.3\ \%}$

Urine- Average Amount of P./ 100 ml. is 52.2 mg., S.D. 4.37

Relative Variation: $\frac{S.D.}{Mean} \cdot 100 = \frac{4.37}{52.2} \cdot 100 = \underline{8.4\ \%}$

Following this experiment the ashing procedure was modified further. The 3 ml. of N/1 sulfuric acid used to char and destroy the organic material were restored. Then the samples were cleared with the 2 ml. of concentrated nitric acid containing the calcium carbonate. The samples were then heated as before until no more brown fumes evolved. Results of this experiment are shown in Table XXXIX.

Table XXXIX. Determination of Phosphorus in Serum and Urine Using A Further Modification of the Ashing Technique.

Sample	ml. 1:50 serum	Absorb.	µg. P. Rec'd	mg. P/ 100 ml.	ml. 1:200 urine *	Absorb.	µg. P. Rec'd	mg. P/ 100 ml.
1	1	0.445	2.38	11.9	2	1.38	5.47	54.7
2	1	0.440	2.35	11.75	2	1.43	5.45	54.5
3	1	0.440	2.35	11.75	2	1.38	5.47	54.7
4	1	0.442	2.36	11.8	2	1.38	5.47	54.7
5	1	0.445	2.38	11.9	2	1.40	5.48	54.8

*Urine sample 15 days old.

Using this modified technique, further samples of serum were tested with results shown in Table XL.

Table XL. Determination of Phosphorus in Serum Using the Modified Ashing Technique.

Sample	ml. 1:50 seum.	Absorb.	µg. P. Rec'd	mg. P/ 100 ml.	Absorb.	µg. P. Rec'd	mg. P/ 100 ml.
1	1	0.417	2.23	11.2	0.491	2.37	11.9
2	1	0.398	1.87	9.4	0.571	3.03	15.2
3	1	0.425	2.27	11.4	0.477	2.54	12.7
4	1	0.460	2.49	12.5	0.453	2.42	12.1
5	1	0.435	2.32	11.6	0.448	2.39	12.0

Calculations:

Table XXXIX

Serum- Average Amount of P./ 100 ml. is 11.82 mg., S.D. 0.08

$$\text{Relative Variation: } \frac{\text{S. D.}}{\text{Mean}} \cdot 100 = \frac{0.08}{11.82} \cdot 100 = \underline{0.7\ \%}$$

Urine- Average Amount of P./ 100 ml. is 54.7 mg., S.D. 0.12

$$\text{Relative Variation: } \frac{\text{S.D.}}{\text{Mean}} \cdot 100 = \frac{0.12}{54.7} \cdot 100 = \underline{0.2\ \%}$$

Table XL

Total- Average Amount of P./ 100 ml. is 12.00 mg., S.D. 1.45

$$\text{Relative Variation: } \frac{\text{S.D.}}{\text{Mean}} \cdot 100 = \frac{1.45}{12.0} \cdot 100 = \underline{12.0\ \%}$$

As the two samples # 2 appear to be well outside the range of the other samples, the calculations were repeated with the omission of the two samples.

Total- Average Amount of P./ 100 ml. is 11.92 mg., S.D. 0.52

$$\text{Relative Variation: } \frac{\text{S.D.}}{\text{Mean}} \cdot 100 = \frac{0.52}{11.92} \cdot 100 = \underline{4.4\ \%}$$

Determination of Phosphorus in Fraction C of Proteus OX-19

From the subjection of this method to the determination of phosphorus in serum and urine it was then applied to the determination of phosphorus in Fraction C of Proteus OX-19. For the preliminary testing 1.172 mg. of the lyophylized fraction was dissolved in 50 ml. of water. Samples were taken and subjected to the modified ashing technique before the phosphorus content was determined spectrophotometrically as before. Results are shown in Table XLI.

Table XLI. Determination of Phosphorus in Fraction C of Proteus OX-19. Preliminary Tests.

Sample	ml. of F.c solution	Absorb.	µg. P. Rec'd	% P. in Fract.
1	1	0.035	0.23	0.98
2	1	0.038	0.24	1.02
3	2	0.068	0.40	0.85
4	2	0.065	0.38	0.81

From this preliminary test, a suitable sample was calculated to be approximately 10 mg. of Fraction C (accurately weighed) dissolved in 100 ml of water. Two ml. of this sample were taken, ashed as before and the phosphorus content determined spectrophotometrically. Results of this experiment are shown in Table XLII of 2 ml. samples taken from a solution containing 10.03 mg. of Fraction C dissolved in 100 ml. of water.

Because 10 mg. of Fraction C did not completely dissolve in the 100 ml of water, rather formed a suspension, the experiment was repeated. Samples were taken containing 0.3 - 0.6 mg. of the lyophylized Fraction C, weighed on a micro-balance. These samples were treated as before in order to determine the phosphorus content. Results are shown in Table XLIII.

Table XLII. Determination of Phosphorus in 2 ml. Samples of a Solution of Fraction C, Containing 10.03 mg./ 100 ml.

Sample	ml. sol'n	Trial 1 Absorb.	Trial 1 µg. P. Rec'd	Trial 1 % P. in fraction	Trial 2 Absorb.	Trial 2 µg. P. Rec'd	Trial 2 % P. in fraction
1	2	0.329	1.77	0.88	0.348	1.87	0.93
2	2	0.348	1.87	0.93	0.359	1.93	0.96
3	2	0.327	1.76	0.88	0.359	1.93	0.96
4	2	0.326	1.75	0.87	0.359	1.93	0.96
5	2	0.367	1.97	0.98	0.374	2.00	1.00
6	2	0.362	1.95	0.97	0.348	1.87	0.93
7	2	0.365	1.96	0.98	0.360	1.93	0.96
8	2	0.353	1.90	0.95	0.408	2.18	1.09
9	2	0.331	1.78	0.89	0.320	1.73	0.86
10	2	0.367	1.97	0.98	0.304	1.64	0.82

Mean: 0.94% S.D. .02

Relative Variation: $\frac{0.02}{0.94} \cdot 100 = \underline{2\ \%}$

Table XLIII. Determination of Phosphorus in Samples of Fraction C Weighed Directly on a Micro-Balance.

Sample.	Weight mg.	Absorb.	ug. P. Rec'd.	% P. in fraction	Weight mg.	Absorb.	ug. P. Rec'd	% P. in fraction
1	0.3234	0.538	2.86	0.88	0.2962	0.485	2.59	0.87
2	0.2748	0.447	2.39	0.87	0.2542	0.452	2.42	0.95
3	0.3266	0.547	2.91	0.89	0.4760	0.890	4.70	0.99
4	0.2322	0.386	2.08	0.89	0.3460	0.621	3.30	0.95
5	0.3136	0.520	2.77	0.88	0.4268	0.690	3.65	0.86

Mean: 0.90% S.D. .04

Relative Variation: $\frac{0.04}{0.90} \cdot 100 = \underline{4.4\ \%}$

Phosphorus content of the intact Proteus OX-19 bacteria cells was also determined by a modified technique. Approximately 100 mg. of whole cells (lyophylized), accurately weighed in a test tube were ashed with 2 ml concentrated sulfuric acid on a sand bath heated by

a hot plate. When charred, 2 ml of concentrated nitric acid containing 50 mg. of calcium carbonate per litre were added and the samples were heated until no more reddish-brown fumes evolved. When cool, the contents were transferred with sufficient washing to a 250 ml volumetric flask, followed by 4.6 ml of concentrated sulfuric acid and made up to volume with water. This procedure yields the phosphorus in a solution of N/1 sulfuric acid.

One ml of this sample was pipetted into a 25 X 150 mm. screw cap test tube. To this was added 2 ml. of N/1 sulfuric acid, 1 ml. of water and 1 ml. of 1 per cent ammonium molybdate reagent. The tube was shaken on a Vortex mixer. Then 5 ml. of xylene-isobutanol (65:35) reagent were added and the tube was capped and shaken for 20 seconds. After waiting 10 minutes the samples were assayed spectrophotometrically as before. Results are shown in Table XLIV.

Table XLIV. Determination of Phosphorus in Intact Proteus OX-19 Bacterial Cells. (0.117 g. / 250 ml.)

Sample	Absorb.	µg. P. Rec'd	% P. in bacteria cells.
1	0.780	4.13	0.88
2	0.782	4.14	0.88
3	0.782	4.14	0.88

Mean: 0.88% S.D. 0

Complete Details of Developed Method.

Apparatus:

Screw-topped byres test tubes - 20 X 150 mm. with rubber-lined plastic screw caps.

Hot plates
Sand Baths
Teflon-stopcock burettes
Matches 1 cm. quartz spectrophotometric cuvettes

Hilgar-Watts Ultrascan
Beckman - DU Spectrophotometer
Gram-Actic Analytical Balance
Cahn Gram Electrobalance.
Magnetic Stirrer with Teflon-coated stirring bars.
Vortex Jr. Mixer

Reagents:

Concentrated Sulfuric Acid H_2SO_4

N/1 Sulfuric Acid - 2.64 ml. conc. sulfuric acid diluted to 1 litre.

Concentrated Nitric Acid HNO_3 to which has been added 50 mg. / 1. calcium carbonate $CaCO_3$

Molybdenum Reagent - 1% ammonium molybdate tetrahydrate in water.

$(NH_4)_6Mo_7O_{24}.4H_2O$

Xylene

Isobutanol

Deionized water was used for all dilutions, solutions and final washings of apparatus.

N.B. All reagents were Reagent Grade.

Standard Phosphorus Solution - 0.04864 g. sodium dihydrogen phosphate monohydrate dissolved in 1 litre of water to yield a solution containing 10 µg. P./ ml. $NaH_2PO_4.H_2O$

Procedure:

The range of this method has been limited to 1 - 5 µg. of phosphorus in order to preserve the sensitivity and its use for very small samples, although it can easily be expanded if necessary.

Samples can be prepared by digestion of the organic material in three ways.

Ashing Techniques:

Sample Type I

Micro samples low in phosphorus content can be weighed on a micro-balance (less than 1 mg.) and digested in a screw top test tube with 3 ml. of N/1 sulfuric acid added by burette. The test tube was laid on its side in a sand bath as shown in Figure 7. When all of the water had evaporated and the charred sulfuric acid remained (approximately 30 min.), 2 ml of the nitric acid reagent were added and the digestion continued until the disappearence of the brown fumes.

Sample Type II

Larger samples soluble in water, e.g. glucose-1-phosphate, or readily miscible with water, e.g. serum and urine, were diluted before digestion. The solutions were prepared so that 1 - 2 ml of the dilution contained a calculated or predetermined experimentally 1 - 5 µg. of phosphorus.

Glucose-1-phosphate - 0.0985 g. dissolved in 1 litre of water.

Serum - diluted 1:50 and Urine - diluted 1:200

The serum and urine dilutions were constantly stirred with a magnetic stirrer while the aliquots were withdrawn by pipette. One ml. of the sample was added to the test tube with 3 ml. of N/1 sulfuric acid and the ashing technique followed as before.

Sample Type III

Larger samples insoluble in or immiscible with water, e.g. intact

lyophylized Proteus OX-19 bacterial cells, were treated in this manner. One hundred mg. (predetermined experimentally) of the bacterial cells were weighed in a screw top test tube. (to avoid the problems of quantitative transfer). To this 2 ml. of concentrated sulfuric acid were added and the sample was ashed on the sand bath as before, using 2 ml. of the nitric acid reagent to clear the solution. When digestion was complete the sample was transferred to a 250 ml. volumetric flask with sufficient washing. To this 4.6 ml. of concentrated sulfuric acid were added and the volume made up with water. This procedure yields the sample in N/1 sulfuric acid.

Preparation for Spectrophotometric Determination:

Samples types I and II were treated similiarly. The sides of the test tubes containing the ashed sample were washed with 4 ml. of water. To this 1 ml. of the molybdenum reagent was added by burette. The contents were shaken manually without the tops or mixed by using a Vortex mixer. Then 5 ml of the organic extractant xylene- isobutanol (65:35) were added by burette and the tops were screwed on tightly.

Sample type II was treated in a little different way. One ml. of the 250 ml sample was pipetted into a screw top test tube. Two ml. of N/1 sulfuric acid were added and 1 ml. of water. Then 1 ml. of the molybdenum reagent was added and the samples were then treated as before.

Spectrophotometric Determination:

Blanks were prepared similiarly with the omission of any sample or phosphorus. The samples were shaken for 20 seconds and allowed to stand for 10 minutes to ensure complete separation of the two phases. Using Pasteur teat pipettes, sufficient quantities of the upper organic

phase were drawn off to fill the 1 cm. cuvettes. Using a cuvette filled with pure xylene-isobutanol (65:35) as the blank, measurements were taken on the Beckman - D-U Spectrophotometer using a wavelength of 310 mμ (predetermined as the optimum wavelength - Figure 13.) from a hydrogen lamp with a slit width of 0.13 mm. and using a blue filter.

A standard curve was also prepared (Figure 16) by taking 1, 2, 3, 4, 5, ml. samples of the standard phosphorus solution and diluting them to 10 ml. Then 1 m. of these 10 ml. dilutions were treated as the samples above with the omission of the organic material and ashing to yield results shown in Table XLV.

Table XLV. Calculations and Figures for the Standard Curve used for ALL Calculations of this Particular Modified Heteropoly Acid Method.

Sample.	1 μg. P. Absorb.	2 μg. P. Absorb.	3 μg. P. Absorb.	4 μg. P. Absorb.	5 μg. P. Absorb.
1	0.184	0.385	0.551	0.752	0.945
2	0.184	0.375	0.558	0.752	0.935
3	0.180	0.385	0.552	0.760	0.945
4	0.186	0.380	0.558	0.770	0.945
5	0.182	0.373	0.560	0.752	0.950
6	0.183	0.384	0.550	0.750	0.955
7	0.184	0.384	0.558	0.748	0.960
8	0.184	0.385	0.558	0.750	0.960
9	0.184	0.388	0.565	0.730	0.945
10	0.185	0.381	0.550	0.730	0.975
Mean:	0.183	0.382	0.556	0.749	0.952

Calculations:

These are the calculations for the Least Squares Line to yield the Standard Curve (Figure 16).

x	y	x^2	xy
1	0.183	1	0.183
2	0.382	4	0.764
3	0.556	9	1.668
4	0.749	16	2.996
5	0.952	25	4.760
15	2.822	55	10.371

$\bar{x} = 3$

$\bar{y} = 0.564$

$$b = \frac{n(\Sigma xy) - (\Sigma x)(\Sigma y)}{n(\Sigma x^2) - (\Sigma x)^2}$$

$$= \frac{5(10.371) - (15)(2.822)}{5(55) - (15)^2}$$

$$b = \underline{0.19}$$

$$a = \bar{y} - b\bar{x}$$

$$= 0.564 - 0.19(3)$$

$$a = \underline{-0.009}$$

$$y' = a + bx$$

$$= -0.009 + (0.19)(1)$$

$$y' = 0.182$$

Similiarly when $x = 2$ $y' = 0.373$

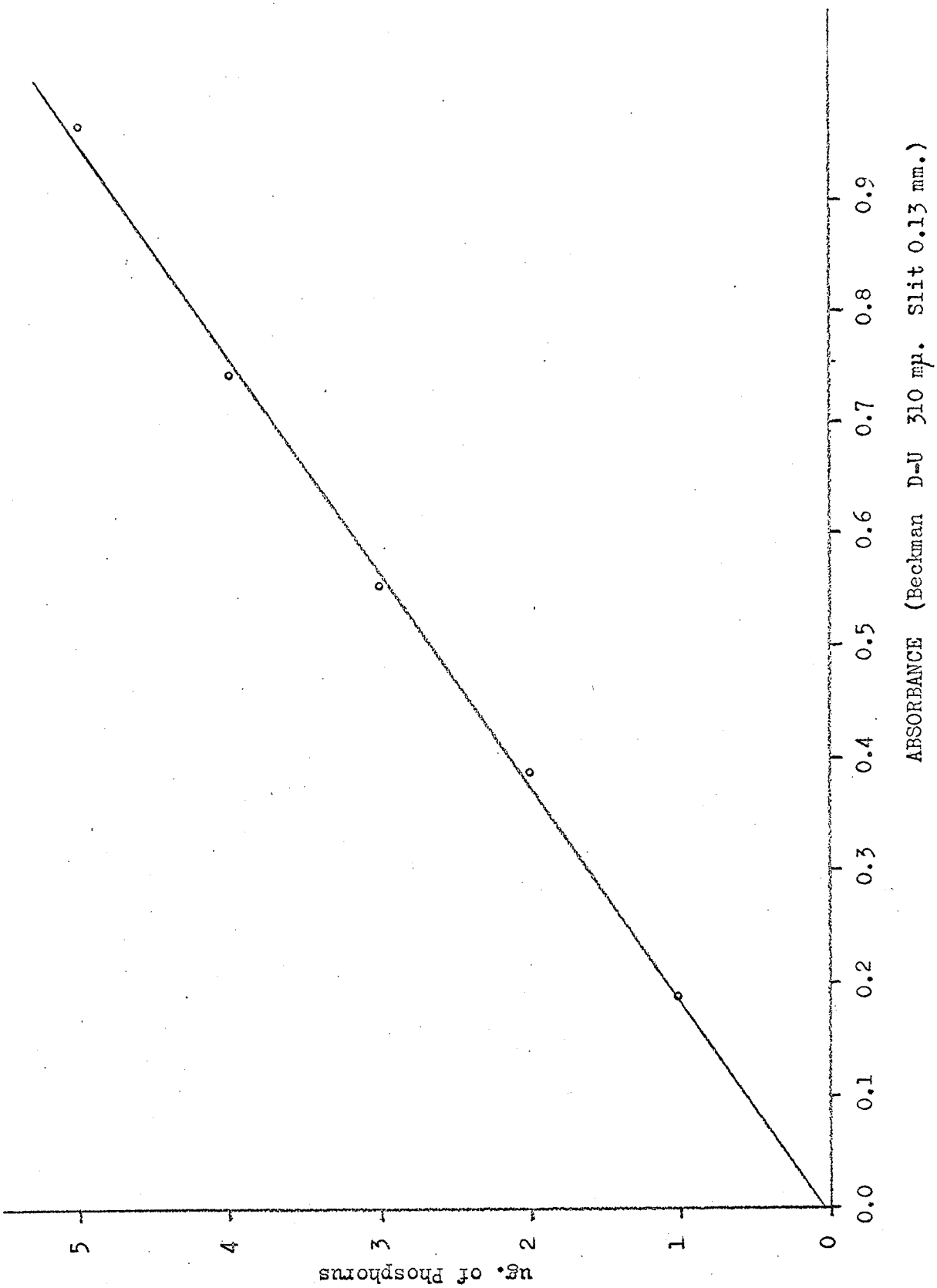
3 0.564

4 0.755

5 0.946

These points were plotted on Figure 16 to yield the optimum line that would satisfy the absorbances shown in Table XLV.

Figure 16. Calibration Curve for the Determination of Phosphorus by the Extraction of the Heteropoly Acid into Xylene-Isobutanol (65:35)



Method of Baginski et al [9] Used to Confirm Results

This method was presented late in 1968 and a slightly modified version of it was used to confirm some of the results presented before.

Reagents:

Standard Phosphorus Solution - 0.04477 g. of sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$) was dissolved in 1 litre of water; equivalent to 10 µg. P./ ml.

Nitric acid-calcium nitrate solution (NAC) - 50 mg. of calcium carbonate were dissolved in 1 litre of concentrated nitric acid.

Arsenite-citrate solution (AC) - 2.0 g. of sodium citrate dihydrate and 2.0 g. sodium arsenite were dissolved in 100 ml. of 2 per cent acetic acid.

Ammonium molybdate solution (AM) - 1.0 g. of ammonium molybdate tetrahydrate was dissolved in 100 ml. of water.

Ascorbic acid - trichloroacetic acid reagent - (A-TCA) - 1.0 g. of ascorbic acid was dissolved in 10 per cent trichloroacetic acid.

Procedure:

The sample was added to a 25 X 150 mm. pyrex test tube along with 2 ml of NAC. Digestion was carried out on a sand bath heated by means of a hot plate, until all the yellow fumes disappeared. When the tube had cooled, 1 ml. of water, 1.0 ml. of A-TCA, 0.5 ml. of AM and 1.0 ml. of AC solution were added respectively. After 15 minutes the colour intensity was measured at 700 mu. against a blank prepared similiarly with the omission of sample or phosphorus.

To prepare a standard curve, 1, 2, 3, 4, 5, ml. portions of the standard phosphorus solution were pipetted into 10 ml. volumetric flasks, yielding solutions containing 1 - 5 µg. P./ ml. One ml. from each flask was pipetted into the test tubes and the procedure followed as above with the omission of the sample. Results of this

experiment are shown in Table XLVI and the standard curve is plotted in Figure 17.

Table XLVI Results to Plot a Standard Phosphorus Curve.

Sample	µg. P. Added	Trial 1+ Absorbance	Trial 2x Absorbance
1	1	0.144	0.150
2	2	0.297	0.314
3	3	0.460	0.460
4	4	0.642	0.630
5	5	0.760	0.778

+Trial 1. Using the Unicam S.P. 600 Spectrophotometer.
xTrial 2. Using the Beckman D-U Spectrophotometer.

Phosphorus determination was carried out on samples of Proteus OX-19 (Table XLVII) and Serum (Table XLVIII).

Table XLVII. Determination of Phosphorus in Samples of Fraction C of Proteus OX-19.

Sample	Trial 1+ Weight mg.	Absorb.	ug. P. Rec'd	% P. in fraction	Trial 2x Weight mg.	Absorb.	ug. P. Rec'd	% P. in fraction
1	0.3960	0.721	4.64	1.17	0.2596	0.385	2.47	0.95
2	0.3282	0.579	3.72	1.13	0.5908	0.865	5.57	0.94
3	0.1956	0.355	2.29	1.17	0.3331	0.461	2.97	0.89
4	0.5068	0.891	5.74	1.13	0.3951	0.618	3.98	1.00

Trial 1. Mean: 1.15% S.D. 0.02 Rel. Var. 1.7 %
Trial 2. Mean: 0.93% S.D. 0.04 Rel. Var. 4.3 %

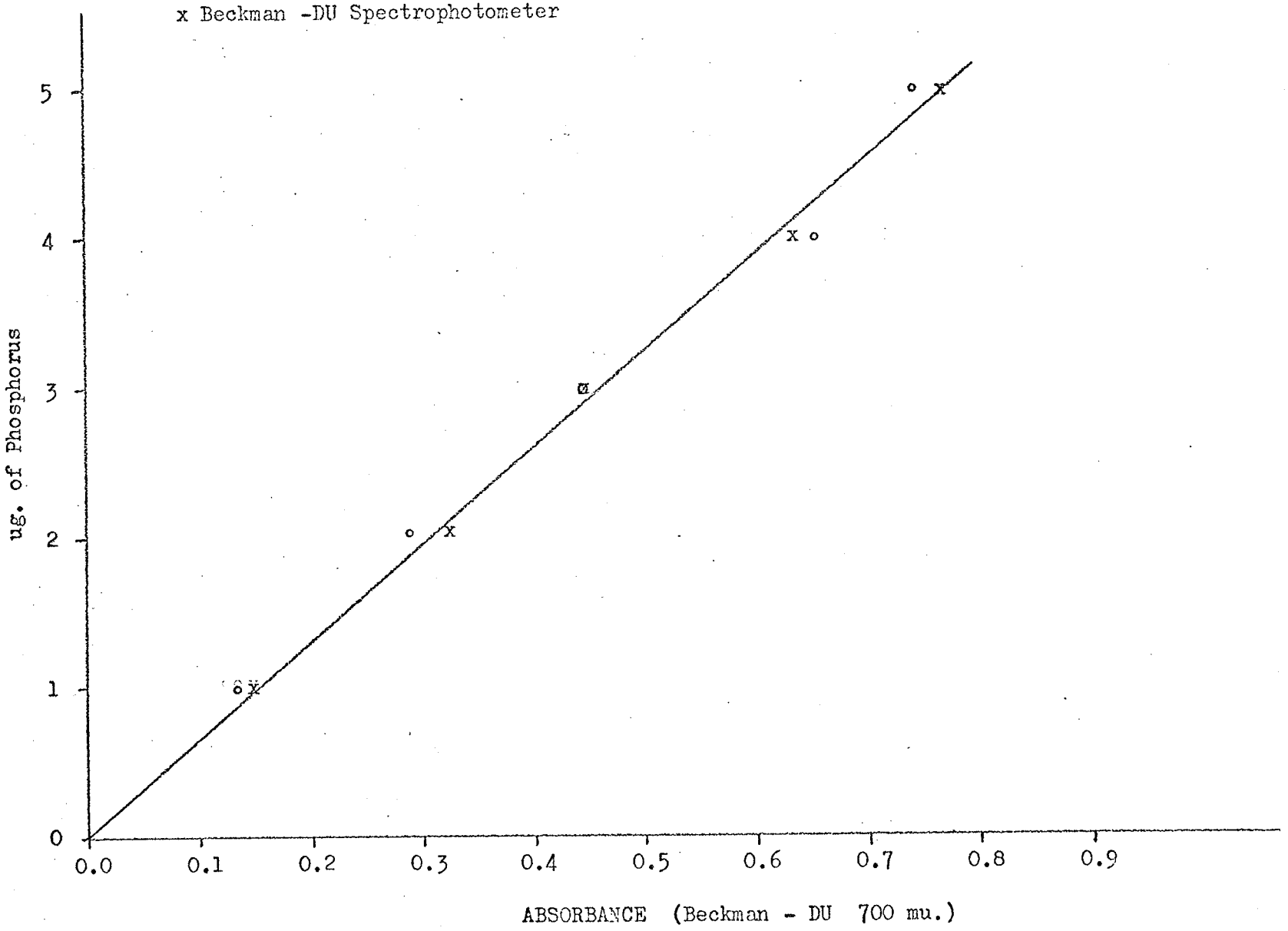
Table XLVIII. Determination of Phosphorus in Serum.*

Sample	ml. 1:100 serum	Absorb.	ug. P. Rec'd	mg. P/ 100 ml.	
1	2	0.276	1.77	8.85	Mean: 8.95
2	2	0.256	1.65	8.25	S.D. 0.76
3	2	0.303	1.95	9.75	Rel. Var. 8.5 %

* Serum sample the same as Table XXXVIII now 5 mos. old.

Figure 17. Calibration Curve for Determination of Phosphorus (Baginski et. al. [18])

° Unicam S.P. 600 Spectrophotometer
x Beckman -DU Spectrophotometer



DISCUSSION

The object of this investigation was to develop a method for the determination of phosphorus in a trichloroacetic acid extracted fraction (Fraction C) of Proteus OX-19. This fraction is an endotoxin having pyrogenic properties, and appears to be composed of a phosphorus-containing lipo-polysaccharide. It was also planned that this technique, once developed, would be versatile enough so that it could be adapted to the determination of phosphorus in other biological materials.

There are several factors to be considered. As the fraction composes approximately about 2.5 per cent of the total dried bacteria weight, only micro amounts are available, so the method should be sensitive enough to determine micro-amounts of phosphorus in minute quantities of organic material. As well as being sensitive, it was hoped that a technique could be developed which would be easy to use, involve a minimum of manipulations and would not require expensive, highly-specialized instruments, so that any well-equipped laboratory would be able to perform this assay.

Another factor to be considered is that often many samples require assaying. If each sample has to be handled individually, the method could become very time-consuming as well as expensive. In this case it was necessary that the method, once developed would require only the minimum of attention during certain procedures so that several samples could be assayed simultaneously without loss of accuracy.

Considering all of the factors and the range of available techniques, it was decided to develop a colourimetric technique, where, after digestion of the organic material, the phosphorus could

be complexed with molybdenum and assayed spectrophotometrically.

The scope of the problem was divided into four areas:

1) the technique and apparatus to be used in order to digest the organic material,

2) the digesting reagents to be used in this ashing technique,

3) the method of complexing the phosphorus with the molybdenum so efficiently that it could be assayed spectrophotometrically, and

4) the method of spectrophotometric determination.

Although it is very easy to divide a problem into specific areas, often in practice, the procedure followed in one area may have a direct bearing on, or affect the procedure in another area. In this experiment it is often the case, so some overlap of the four areas may occur.

The Ashing Procedure (Using the Heteropoly Blue Method.)

Many investigators (19, 20, 31, 38, 40, 64, 70, 91) have restricted their experimentation to the determination of inorganic phosphate, by using simple phosphate salts as sources of phosphorus. In these cases, destruction of the organic material was not a problem. Other workers have limited their investigation to the determination of inorganic phosphate in rock (13), or iron (39) and steel (8, 98, 103) requiring drastic digestion of the extraneous material, or in water (61, 106), in which case little or no organic material is present to interfere.

Other investigators have developed techniques for the determination of inorganic phosphate in the presence of labile phosphate esters (63, 72), the determination of hypophosphate in the presence of phosphates (3) the determination of quinquevalent, trivalent and organic

phosphorus (68), the determination of phosphonate phsophorus in the presence of organic or inorganic phosphates (1) or the determination of inorganic bound phosphorus in the presence of alkylated phosphorus.

The purpose of this investigation, however, was simply to determine total phosphorus in biological material, especially in the Fraction C of Proteus OX-19. In order to accomplish this, it is necessary to destroy all of the organic material in such a way as to leave the phosphorus as orthophosphate which can be quantitatively determined by spectrophotometry. The type of vessel to use for this digestion, the source of heat and the digestion reagent have been thoroughly investigated.

Workers with large samples suggest the micro-Kjeldahl Digestion Appartus (73), while others recommend the Schöniger Oxygen Flask Technique (10, 37, 88) or its modified version (89). A very simple method involved digestion of the sample in a test tube or ignition tube over a bunsen flame or micro-flame (73). However, this suggests individual ashings, and one of the factors to be considered was to develope a method in which several samples could be analysed simultaneously.

The digesting reagent usually suggested was a strong oxidizing agent, and many investigators invariably chose sulfuric (16, 18, 33, 36, 49, 62, 73), while others recommended nitric (9) or combinations of the two acids (25). Some more complex procedures involved refluxing the organic material with hydriodic acid for periods up to 11 days (90), destruction of the organic material with ammonium persulfate and sodium hydroxide (55), using chloric acid (41) and a new reagent containing

hydriodic acid, calcium iodide, phenol and acetic acid (86). In another procedure the sample is ashed in a platinum dish using hydrofluoric acid and perchloric acid as digestants (82) while other workers suggest removing the phosphorus from the biological material in a non-oxidizing atmosphere of nitrogen and collecting it on mercuric bromide from which it is later eluted as phosphoric acid with iodine and determined colourimetrically as molybdenum blue (78). The use of 60 per cent perchloric acid alone has been recommended by some workers (2, 53) and in combination with nitric and sulfuric acids by others (62).

In the determination of inorganic phosphate only in the presence of organic material, many investigators suggested the use of trichloro-acetic acid as a protein precipitant (32, 42, 59, 75) although others have demonstrated that this acid affects the production of the molybdenum blue colour (29, 32) so chloric acid (74) or silicotungstic acid (81) have been used in its place.

From this wide selection of digesting reagents, sulfuric acid was chosen for this experiment, not only because of its popularity but also because of its seeming equal effectiveness to other more complicated methods. Using dextrose as an organic substance and sulfuric acid as the digestant, several different types of vessels were tested for their effectiveness using bunsen burners and hot plates as sources of heat. (Table III) From these results it can be seen that all of them must be discarded as none of these reactions could be left to ash without removal of the heat from time to time or constant adjustment of the heat.

Even though these methods were rejected ashing was carried through to completion. However, there were still charred particles in most of the vessels so a clearing agent was required. Perchloric acid 72 per cent (25), hydrogen peroxide 30 per cent (18, 33, 73) and potassium permanganate (62) have been suggested by several workers. In these experiments, 1-2 drops of perchloric acid were used to clear the samples and seemed satisfactory. Evaluation of the effectiveness of these ashing procedures required a spectrophotometric method for the determination of phosphorus and the methods of Vogler [99] and Lindner and Edmundsson [62] were chosen as simple methods.

However the second method (62) was not used beyond the determination of a standard phosphorus curve because when the optimum wavelength was determined (Figure 3.) to be 305 mμ., measurements were unreadable because of the high absorbance of the blank. Although the readings taken at 445 mu. were recommended by the investigators (62) as being effective it was felt that this wavelength was too far down on the shoulder of the peak, thus perhaps adding to the source of error. Modifications by other workers included diluting the reagents (70) in order to be able to take measurements at 315 mu., but even with this adjustment the method was rejected.

On the other hand, the other method (99) required a single complex molybdenum reagent with ascorbic acid as the reducing agent. Among the many recommended reducing agents available such as; hydroquinone and sulfite (16, 22), aminonaphtholsulfonic acid (36, 42, 61, 63), hydrazine sulfate (18, 19, 39, 41, 49, 78, 86), stannous chloride (43, 55, 61, 91, 106) and N-phenyl-p-phenylenediamine (33),

ascorbic acid appeared to be the one of choice (9, 25, 37, 38, 73, 99).

Knowing that the 2 ml. of concentrated sulfuric used in the ashing technique (Table III) would undoubtedly affect the intensity of the molybdenum blue colour (19, 36, 99) a test was performed to demonstrate these effects. (Table IV). From these results it seems that 1 ml. of 25 per cent sulfuric acid will not affect the colour. Therefore the test of ashing techniques was repeated with a more limited range of vessels and the addition of a sand bath (25) as a source of heat. The results were then calculated (Table V) to yield a more promising situation and some possibilities. Even though the results were lower than the actual quantity of phosphorus added, samples 2 and 6 had only a 4 per cent error.

As these results had errors all on the low side, it seemed to indicate either a low reading of the molybdenum blue colour or else a loss of phosphorus due either to overheating (Table VI) or loss through spurting and bumping during the ashing procedure. Even though the results shown in Table VII after the over-heating test show a rather high percentage error, these results are fairly consistant having only about a 4 per cent error between the tests. Confirmation of these results shown in Table VIII also seems to indicate that no phosphorus is lost during the ashing precedure for a similiar range of errors is achieved while the water traps did not indicate the presence of any phosphorus either from volatilization or spurting or bumping. On the other hand, further overheating tests (Table IX) yield one result that has only a 1 per cent error. The 0.3 ml. of concentrated sulfuric acid in these last three tests

replaced the 1 ml. of 25 per cent sulfuric acid, as it contains an equivalent amount of acid and it was hoped that reduction of the water content would lessen the frequency of samples spurting.

These latter tests seemed to indicate that the ashing technique was quite efficient so investigation into the colourimetric determination method was then carried out. This experiment was performed to determine the reproducibility of the molybdenum blue colour and from the results in Table X it can be seen that this was the source of the problem. The results from Trial 3 should yield almost perfect reproducibility, as it is a simple assay for the determination of inorganic phosphate, even though Trials 1 and 2 were modified and Trial 4 determination followed an ashing technique. However, the results from Trial 3 show a distinct lower error indicating that even the 0.3 ml. of concentrated sulfuric acid present in Trial 1, 2 and 4 affects the molybdenum blue colour production in some way. As confirmation the results from the experiments listed in Table XI yield the consistant 20 per cent lower results, while the stability of the colour over 1 hour does not seem to be a source of discrepancy or error. (Table XII)

That pH is a very important factor in the intensity of the molybdenum blue colour is clearly demonstrated in the results from the neutralization tests shown in Table XIII. Not only has one sample a 0 per cent error, but for the first time some of the results are on the high side instead of being consistantly low. The effect of the variation of the pH is clearly demonstrated in the results of Tables XIV and XV and although the results of

Table VIII are quite promising, so many transfers are involved that the simple technique has completely disappeared. The sample has to be transferred from the ashing flask to a beaker for neutralization, and from there to the volumetric flask for colourimetric determination. Furthermore the pH readings taken with the Beckman pH Meter are very time consuming causing each sample to be handled individually.

The modification of the molybdenum reagent, after the cloudy samples appeared in the results of Table XIV, by the omission of the sulfanilic acid (never very soluble), seemed effective. The results in Table XV, although subjected to great extremes of pH, did not yield any cloudy samples. From here on this modified reagent was used as the molybdenum reagent.

The Modified Heteropoly Blue Method

As more and more problems were arising from the investigations of the heteropoly blue method, and as the molybdovanadophosphoric acid technique had been rejected, it was decided to investigate the possibility of a modified heteropoly blue or extraction method as suggested by several workers.(32, 64, 66, 67, 81, 103). The ashing procedure from before seemed quite adequate so it was temporarily retained.

Isobutanol appears to be the extractant of choice (17, 64, 67) with the addition of various proportions of benzene, (67, 81) chloroform (103) or xylene (32) to hasten the phase separation. Benzene was declared too toxic by several workers (45, 32) so xylene was used in these experiments in the optimum ratio of xylene 65 per cent: isobutanol 35 per cent.(32). Isobutanol was not available at first so n-butanol was used in the same percentage and seemed fairly effective for the preliminary testing. The disadvantage of n-butanol is that it is slightly more soluble in water than the isobutanol.

Although 2 extractions were found necessary when isobutanol was used alone (64) with the addition of benzene or xylene this was reduced to only one extraction (64).

The preliminary tests made use of the modified molybdenum reagent and ascorbic acid solution from the heteropoly blue method. Although the standard curve was determined using 5 minutes shaking time for both the extraction and back-extraction, (Table XVI) further testing revealed that at least 1 minute's shaking was effective for both extractions (Table XVII) while many investigators only used 15 - 30 seconds. (32, 67, 81, 103).

Most investigators reduced the extracted phosphomolybdic acid in the organic phase with stannous chloride, dissolved either in strong mineral acids (67, 81) or glycerol. (45, 94). However, the effect noted here of the phosphomolybdic acid, reduced with ascorbic acid, being back extracted into the aqueous phase was not mentioned by any investigators. The closest method is perhaps that of Djurkin et al [31] where the extracted molybdenum was stripped from the phosphomolybdic acid complex with ammonia and then complexed with 2-amino-4-chlorobenzenethiol hydrochloride and determined spectrophotometrically in the aqueous ammonia phase as the molybdenum VI green complex.

From experiments carried out using this technique it was determined that if 1 ml. of concentrated sulfuric acid is used as a digestant, then 5 ml. of dilute ammonia are necessary to promote the optimum extraction of the phosphomolybdic acid. (Table XIX). These results are partly confirmed by the results shown in Table XX as samples 2, 4, and 6 show only a 0 - 3 per cent error. Although the higher 5-10 per cent errors of the other samples must not be overlooked, samples 9 and 10 in Table XIX did not indicate that any of the extracted phosphomolybdic acid remains in the organic phase on the back extraction.

Although these results seemed promising, as with the final experiments on the heteropoly blue method, too many manipulations and transfers were involved. Determination to develope a method that was as simple as possible, yet still effective, coupled with the fact that this method, when developed, should be a micro-method, led to further investigation with a view to modification of this so-called modified heteropoly blue method.

Although the test tube had been eliminated as a vessel for ashing almost immediately when bunsen burners and hot plates were used as sources of heat, the possibility of heating a test tube on a sand bath had not been investigated here before (25). This in combination with the fact that some workers (32, 81) used capped test tubes to do the shaking and extracting while determining the absorbance in the organic phase led to investigation of these suggestions.

Preliminary tests were carried out simply by reducing the volumes of reagents of the extracted heteropoly blue method. The results are shown in Table XXI. With a lack of reproducibility between like samples 4 and 5, even though samples 1 and 2 show possibilities, it was evident that further modification was necessary. The effect of very small amounts of concentrated sulfuric acid was examined to determine whether neutralization with ammonia was really necessary. As the results in Table XXII show little variation e.g. between samples 2 and 4, even including sample 1, tests were carried out to see if 2 drops of concentrated sulfuric acid were sufficient to ash a quantity of organic material. The results shown in Table XXIII were so divergant e.g. a ranges of absorbances from 0.292 to 1.48 on identical samples, that further modification was apparently necessary.

The first modification applied was the substitution of N/1 sulfuric acid for the concentrated sulfuric acid. It was hoped that larger, easier-to-measure volumes of a very dilute acid would yield better reproducibility of the actual volume of undiluted acid present.

One ml. of N/1 sulfuric acid contains 0.026 ml of concentrated sulfuric and can be measured with far more accuracy than 1-2 drops of concentrated sulfuric acid. Spurting does not appear to be a problem now, using the sand bath as a source of heat, so the addition of 1-2 ml. of water should not have an adverse effect.

Omission of the reduction of the extracted phosphomolybdic acid has been suggested by several investigators (32, 103) who noted that the extracted heteropoly acid has an absorbance of its own in the organic phase which can be measured using ultra-violet light and a low wavelength.

Modification of the molybdenum reagent was instigated to remove as many substances as possible, so that no interference can be attributed to the molybdenum reagent. Several investigators used simple solutions of sodium (64) or ammonium (32, 75, 81) molybdate. One per cent ammonium molybdate tetrahydrate (81) was suggested and used here.

Preliminary testing to determine the optimum quantity of N/1 sulfuric acid revealed that at least 2.0 ml. could be used to produce a stable colour, comparable to Dreisbach [32] who had, however, no ashing step in his procedure. One ml. of the 1 per cent molybdate reagent was used although other workers using similiar techniques (32, 81) used only 0.5 ml. Although the quantity of molybdate is quite critical (103) excess must be present, and as this would remain in the aqueous phase after extraction, it should not interfere with the procedure.

After the determination of the optimum quantity of N/1 sulfuric acid (Table XXIV) further testing was necessary to see if this quantity

was sufficient to ash an amount of organic material. Results from this experiment are encouraging. (Table XXV) as two samples, numbers 5 and 8 yield similiar 2 per cent errors as shown by samples 3, 4, and 5 in Table XXIV where the optimum quantity of acid was determined.

Before further testing was carried out using this method, one factor remained to be clarified. The strangely flattened peak produced by the Ultrascan (Figure 9) used to determine the wavelength of maximum absorbance, seemed to suggest some interference. Consequently, the extractant solvents were tested to determine their own absorbance. From the results of Figures 10, 11, 12, and 13, it can be seen that the xylene definitely could mask the absorbance, but not enough to seriously affect the results which are being read at 310 - 315 mu. Nevertheless, two other solvents were tested, cyclohexane and hexane, to determine whether they could be substituted for the xylene, but did not yield such marked absorbances (Figures 14, 15) as the xylene-isobutanol extract (Figure 13). These tests were performed using the isobutanol, which had arrived by this time, whereas several of the following experiments still had to be performed with butanol which has been used before by other workers. (66)

Due to the flatness of the peak from the scan to determine the wavelength of maximum absorbance, tests were carried out using the wavelengths of 310 and 315 mu. In the same test, the clearing agents were also checked by using 72 per cent perchloric acid and 30 per cent hydrogen peroxide. From the results shown in Table XXVI, it can be seen that the readings taken at 315 mu., appear to be the best, using perchloric acid as the clearing agent.

However, as many workers using a similiar system used 310 mu., (32, 66, 75), further experiments were carried out using this wavelength. Hydrogen peroxide was also substituted for the perchloric acid, as it was felt that fewer diverse ions were being added. Using several organic substances with a standard phosphorus solution, tests were performed, yielding the results shown in Table XXVII. It is surprising to note, that dextrose, the simplest organic substance used, yielded the best results, (Trial 1- samples 9, 10) and close to the poorest results (Sample 6, Trials 1 and 2.) Another noteworthy fact is that the results from the samples containing starch, were nearly all on the high side. It can also be seen that peroxide was not as good a clearing agent as was hoped from the results marked (*). It appeared that the peroxide must be completely removed or destroyed or it will affect the extraction. The samples using pepsin as a source of organic material (protein) were very hard to ash and also were affected by what appeared to be an inorganic salt, which could not be removed. It was not used again as its purity was also questionable.

The results shown in Table XXVIII demonstrate one of the problems experienced in reading the results of Table XXVII. The incomplete separation of the phases and the presence of bubbles made some samples very hard to read. However it was noticed that only samples not ashed, seemed to be especially bad. The tubes in which samples were ashed were exposed to what amounted to fuming sulfuric acid which probably cleaned the sides of the tubes very thoroughly indeed. In future the tubes were cleaned with chromic acid or fuming sulfuric acid and the problem was solved.

From the different methods of shaking tested (Table XXVIII) it can be seen that a great variety of methods of shaking seem available and results differ from method to method. Other investigators have also used different method of shaking and extraction. Shaking times varied from 5 - 10 seconds (32, 66) to 20 - 30 seconds, shaking about 20 - 30 times (75) to 60 seconds (98). Another worker suggested inversion of the tubes 20 times or mixing on a Vortex Mixer. (81). It was decided, from the review of the literature and results conducted here, (Table XXVIII) to shake each sample hard for 20 seconds.

Also, on standing the samples appeared to become clearer, (Table XXVIII) so a time for allowing the samples to stand after shaking was determined. As the spectrophotometric determination of each samples takes about 5 minutes, samples were shaken and allowed to stand while two other samples were read. The first 2 samples were also allowed to stand for 10 minutes. This time appears to give the maximum separation of the two phases and the removal of bubbles.

The problem of the interference of the hydrogen peroxide used as a clearing agent was also investigated and overcome. By heating the samples for at least two hours after clearing had been accomplished, the danger of interference from excess peroxide was removed. (Table XXIX). The serious effect of unremoved peroxide was well demonstrated in the results from Sample 4. Excessive heating does not seem to affect the results, in fact, the sample heated for the longest time, yielded the best results. (Sample 3).

The possibility of the addition of larger amounts of sulfuric acid was investigated in order to determine whether better destruction

of the organic material with the acid would reduce the necessity of having to add too much peroxide. Results shown in Table XXX reveal that at least 3 ml. of N/1 sulfuric acid can be added to the samples without adversely affecting the results. Also, the samples with the excess acid appear to be stabilized better, and have lower percentages of error.

With the substitution of Isobutanol for the n-Butanol, which had now arrived, and the increased volume of acid, some of the trials with results shown in Table XXVII were repeated to yield the results shown in Table XXXI. Although some percentage errors are still high Trial 2. Sample 7, at least one result shows 0 per cent error. The samples containing starch still appeared to have high absorbances, so an experiment was performed using starch as the organic substance, but without the addition of a phosphorus sample. Results shown in Table XXXII, demonstrate that starch has its own absorbance. Recalculation of the starch samples in Table XXXI yield lower percentages of error, thus approving this method for futher testing.

The results from the determination of phosphorus in glucose-1-phosphate were not promising in the lower amounts. However a 1 per cent and 4 per cent error in the larger samples are equivalent to the previous results obtained with the organic material and phosphorus solution. As these results were calculated assuming 100 per cent purity of the sample, another source of error may have been that the sample of glucose-1-phosphate was not 100 per cent pure. Also biological variation of the sample may account for some of the error. Nevertheless, further testing of this method was applied by using it to determine the phosphorus content of urine and serum.

Determination of Phosphorus in Serum and Urine

Although many investigators have been interested in the determination of phosphorus in serum and urine, (16, 36, 41, 107) the application of such an extraction method has only been used for inorganic phosphate after the precipitation of protein with perchloric acid. (32). Aslo, as only a very small quantity of the fraction from Proteus OX-19 is available, it was decided to use serum and urine as examples of complex organic material, containing phosphorus, on which this method could be further tested, before its application to the determination of the phosphorus content of the Proteus OX-19 fraction.

The preliminary testing (Tables XXXIV and XXXV) was carried out to determine the best dilutions of serum and urine to use. As measurements on most spectrophotometers are more accurate between absorbances of 0.300 - 0.700, dilutions were made and volumes taken of the serum and urine in order to maintain absorbances in this range. From the results in Tables XXIV and XXXV, it can be seen that 2 ml. of serum 1:50 and 1 ml. of urine 1:100 seem to be the best volumes and dilutions. Large enough volumes were also taken so that the problem of measuring very small volumes of viscous liquids was eliminated.

As the phosphorus content of serum and urine varies from sample to sample, the exact content can only be determined by repeated testing. Therefore, these results were tested for reproducibility, rather than accuracy to a standard phosphorus content. From the results shown in Table XXXVI and the calculations, it can be seen that it does not seem to matter whether the samples are averaged first, and then one total phosphorus content determined, or if each sample is calculated

through to the final content, and then averages. In these experiments, however, each sample was totally calculated, and then the results averaged. The reproducibility was very good, showing only a difference of 0.06 mg./ 100 ml. between the highest and lowest average of the three trials. The results from the urine samples were also very reproducible (Table XXXVII) such that the relative variations between the two experiments only differed by 0.43 per cent. The three trials on the urine samples were performed on consecutive days and it should be noted that while the results became slightly lower from day to day, the reproducibility improved. Nevertheless, even when these results were averaged together, the total deviation was only slightly higher than that of the more stable urine samples. (Table XXXVI).

The calcium ion-containing nitric acid digestant of Baginski et al [9] did not appear to be as effective as the sulfuric acid digestant used here in the previous trials. (Table XXXVIII). The standard deviations and relative variations were noticably higher. It was determined that when nitric acid is used as a digestant, the samples must be heated almost to dryness before ashing is complete. It can be seen that the results obtained from experiments using this new extraction method were more reproducible when the phosphorus was contained in a very small quantity of concentrated sulfuric acid. Unfortunately as a new sample of serum was used, and the results from the urine samples were even lower, direct comparison with the results in Tables XXXVI and XXXVII was not possible.

The use of calcium to bind the phosphorus as orthophosphate during ashing procedures and prevent evaporation has been mentioned by several investigators. (9, 86). The N/1 sulfuric acid reagent

with calcium ions added yielded erratic results, but when the regular N/1 sulfuric acid was used for ashing, and the concentrated nitric acid reagent of Baginski et al [9] for clearing, very good results were obtained as shown in Table XXXIX. Although these results were really exceptional, further testing of this new digestant on samples of serum yielded the results as shown in Table XL. These conformed to the results obtained before (Tables XXXVI, XXXVII) showing that extreme care was necessary in order to achieve the results of Table XXXIX.

From these experiments performed on samples of serum and urine, it was felt that application of this extraction method could be made to the determination of phosphorus in the fraction of Proteus OX-19. This was a trichloroacetic acid extract obtained from the lyophylized bacteria cells of Proteus-OX-19. It was lyophylized and stored in a dessicator under a vacuum in order to keep it in the dry state. This extract is thought to be a phosphorus- containing lipo-polysaccharide and is fairly water soluble. Only a limited amount of this material was available, so few pertinant trials were performed. This fraction will be referred to from here on as Fraction C.

Determination of Phosphorus in Fraction C of Proteus OX-19

As with the serum and urine samples, an amount of Fraction C was needed that would yield readable results by this extraction method of phosphorus determination. The preliminary trials with the results shown in Table XLI indicated this optimum amount.

It is surprising to note that the results of Table XLII yielded a lower relative variation than the results of Table XLIII, when the reverse was expected. However both series of experiments yielded a high degree of accuracy with a difference of only 0.04 per cent between the averages of the two different methods. The relative variations also compare favourable with the results of the determinations of phosphorus in urine and serum. It can be seen that the total phosphorus content of Fraction C of Proteus OX-19 is approximately 0.9 per cent. As most biological contents are only given to one decimal place, because of the variation of the composition and content from sample to sample, the results from this method are quite satisfactory.

Determination of the phosphorus content of the intact, lyophylized bacterial cells and the results shown in Table XLIV demonstrated a modification of this extraction method which seems to retain the accuracy. From the results in Table XLIV it can be seen that the intact bacteria cells have approximately the same percentage of of phosphorus, as 0.88 per cent would be rounded off to 0.9 per cent. As the fraction comprises about 2.5 per cent of the intact cells, the percentage of the phosphorus contained in this fraction as a percentage of the total phosphorus contained in the bacterial cells can be determined. One hundred mg. of intact bacteria cells would contain 0.9 mg. of

which 0.02 mg. would be contained in fraction C.

Confirmation of these results was necessary and it was felt that the method of Baginski et al [9] would be suitable for this task. Not only was it a fairly simple method but made use of the heteropoly blue method, so that the results obtained by the method developed here would be subjected to a more intense comparison than if another extraction method was used.

Two spectrophotometers were used for this experiment, the Unicam S-P 600 and the Beckman D-U. Results from the two instruments compare favourably in the determination of a standard curve Table XLVI. This curve, as with the others was plotted used the least squares line principle, and both sets of results formed exactly the same line when calculated from this formula.

However, surprising results were noted on the determination of phosphorus using these two instruments. The results using the Unicam (Table XLVII. Trial 1) were about 0.15 per cent higher than the results using the Beckman D-U. Also the results from the Unicam show less deviation and a lower relative variation. This is probably due to the fact that the Unicam S-P 600 is a less sensitive instrument than the Beckman D-U, so it would not record the slight variations that would be noted by the Beckman D-U spectrophotometer.

On the other hand, the results from Table XLVII. Trial 2, correspond very favourably with the results obtained using the extraction method, developed here, as shown in Table XLIII. The mean is only 0.03 per cent higher, the relative variation is is only 0.1 per cent lower, and the standard deviation is the same.

The determination of phosphorus in serum by the method of Baginski et al [9] was carried out as another check on the efficiency of this modified extraction method. However, as found before with the urine samples, the serum also appears to have a lower phosphorus content on standing. The results can be compared to the modified results of Table XL where the standard deviation is 0.52 as can be compared to the standard deviation of 0.76 from Table XLVIII. The relative variations however,cannot be compared as the difference between the two means, (Table XL. 11.92 and Table XLVIII 8.95) is too great.

It was felt that one of the disadvantages of the method of Baginski et al [9] was the ashing procedure. When this procedure was applied to the extraction technique,(Table XXXVIII) the results became very erratic. The ashing procedure must be watched closely for the termination of the evolution of the brown fumes, otherwise it appears that if heated to dryness, some phosphorus is lost or cannot be detected by this determination procedure. However, when the N/1 sulfuric acid is used as a digestant, the phosphorus is contained in a very small amount of concentrated sulfuric acid, which can be heated excessively with no apparent los of phosphorus or accuracy of determination.(Table XXXIX) In this way it is felt that the ashing technique using sulfuric acid is superior, although use is made of the calcium ions in the nitric acid to increase accuracy. (Table XXXIX)

The use of nitric acid as a clearing agent in place of peroxide appeared to be very beneficial, as the elimination of the nitric acid completely could be detected by the evolution of the brown fumes, while the excess peroxide could only be removed by prolonged arbitrary

periods of heating with the hope that it was destroyed. Detection was only possible during the spectrophotometric determination, at which time it was too late to destroy the excess peroxide by heating.

It should also be noted that all the results of the extraction heteropoly method of phosphorus determination were calculated from one standard curve (Figure 16). As can be seen from the results in Table XLV, there is variation from time to time in plotting the standard curve. It was felt that the variation from one slightly modified technique to another would be represented better if the results were all calculated from one standard curve. Consequently, several trials were performed to develope a standard curve and the average of these results used, by means of the least squares line formula, to plot the standard curve in Figure 16. Therefore all results from Table XXVI to Table XLIV were calculated using this standard curve (Figure 16).

CONCLUSIONS

The object of this experiment was to develop a new, or modify an existing method for the determination of phosphorus in biological materials, especially the trichloroacetic acid extract of Proteus OX-19 designated as Fraction C. From the four areas of investigation, a method was developed that is easy to use, relatively fast (an average of 5 - 6 samples in 2 hours) and reproducible.

The ashing procedure reuqires only screw-cap test tubes, a sand bath heated by a hot plate and sulfuric and nitric acids. In order to achieve rapid destruction of the organic material, as much of the sample as possible should be exposed to a high, even source of heat. This is accomplished by laying the test tubes on their sides in the sand bath. (Figure 7). Up to 5 ml of solution cna be treated in this manner without danger of spurting. Micro-Kjeldahl flasks can also be used, but should be reserved for larger samples. The completeness of the ashing technique can be determined as when brown fumes are no longer evolved from the samples, while excessive heating during ashing does not affect the results.

Another advantage of this technique, is that all the procedures are performed without the transfer of samples from one vessel to another, eliminating the problem of quantitative transfers. All reagents can be added by burette, thus eliminating the constant use of graduated cylinders or pipettes. The samples must, however, be added by pipette if liquid, or be accurately weighed on a micro-balance if solid.

The complex molybdenum reagent of the heteropoly blue method

was replaced by a 1 per cent solution of ammonium molybdate in water. This reagent was made daily to ensure maximum effectiveness while the other reagents were stable for as long as required.

The use of screw-cap test tubes was superior to quick-fit test tubes, as grease was not required for complete sealing. The quick-fit test tubes had a tendency to leak around the stoppers if not sealed with silicone grease. Both the leakage and the grease unfortunately had an adverse affect on the phosphorus determination results. The caps were rubber-lined plastic that were resistant to autoclaving, so sufficiently strong enough to be used in these experiments. In order to ensure complete separation of the two phases after extraction, the tubes were always thoroughly cleaned between each experiment with chromic acid or fuming sulfuric acid.

There was no interference from the organic extractants in the spectrophotometric determination. This was clearly demonstrated not only by the ultrascan readings (Figures 10 - 13) but also by the very narrow slit width (0.13 mm.) used during these readings. The quartz cuvettes were completely dried before each spectrophotometric determination as there was insufficient volume of sample with which they could be rinsed.

In these experiments, de-ionized water was used for all solutions, dilutions and final rinsings of all containers, even though many other investigators used just single distilled water. (32, 66, 81). This ensured that no contamination could be attributed to reagents.

The range of phosphorus determination has been limited to 1 - 5 ug. in these experiments, but the method can easily be expanded

by dilution of the organic extractant or the samples before extraction, provided volumes and acidity are maintained in proportion.

Comparison of the accuracy of this method to other methods was difficult because of the various means of determining accuracy used by different investigators. However, this extraction method can be compared to the method of Baginski et al [9] in the determination of phosphorus of Fraction C of Proteus OX-19.

These comparisons were made using Tables XLIII and XLVIII. The percentage means differed only by .0.03 per cent, the standard deviations were exactly the same, and the relative variations had only a 0.1 per cent difference. The difference between the range of the samples was only 0.01 per cent. Although the phosphorus determination of the samples of urine and serum could not be compared directly to methods used by other investigators, the reproducibility of the results in this extraction method indicated that it was an effective method for these analyses.

From these comclusions it can be seen that this extraction method is effective for the determination of phosphorus in biological material with a high degree of accuracy.

BIBLIOGRAPHY

1. Aalbers, J. and Bieber, L., A Method for Quantitative Determination of Phosphonate Phosphorus in the Presence of Organic and Inorganic Phosphates. Anal. Biochem. 24: 443-447 (1968).

2. Allan, R., The Estimation of Phosphorus. Biochem. J. 34: 858 (1940).

3. Anton, A., Spectrophotometric Determination of Hypophosphites in the Presence of Phosphates. Anal. Chem. 37: 1422-1423 (1965).

4. Armstrong, R., Williams, P. and Strickland, J., Photo-Oxidation of Organic Matter in Sea Water by Ultra-Violet Radiation. Analytical and Other Applications. Nature. 211: 481-483 (1966)

5. Armstrong, F. and Tibbits, S., Photochemical Combustion of Organic Matter in Sea Water, for Nitrogen, Phosphorus and Carbon Determination. J. Mar. Biol. Ass. 48: 143-152 (1968)

6. Babad, H., Herbert, W. and Goldberg, M., Nuclear Magnetic Resonance Studies of Phosphorus (V) Pesticides. Anal. Chim. Acta 41: 259-268 (1968)

7. Bache, C. and Lisk, D., Selective Emission of Spectrophotometric Determination of Nanogram quantities of Organic Bromide, Chlorine, Iodine, Phosphorus and Sulfur Compounds in a Helium Plasma. Anal. Chem. 39: 786-789 (1967)

8. Baghurst, H. and Norman, V., Effect of Temperature on Photometric Determination of Phosphorus in Steel. Anal. Chem. 27: 1070-1073 (1955).

9. Baginski, E., Foa, P., Weiner, L. and Zak, B., Microdetermination of Nucleic Acid Phosphate. Microchem. J. 13: 115-119 (1968)

10. Barney, J., Bergman, J. and Tuskan, W., Rapid Determination of Phosphorus in Motor Oils and Additives. Anal. Chem. 31: 1394-1396 (1959)

11. Barrow, N., Relationship between Uptake of Phosphorus by Plants and Phosphorus Potential and Buffering Capacity of the Soil. Soil Sci. 104: 99 (1967)

12. Bartlett, G., Phosphorus Assay in Column Chromatography. J. Biol. Chem. 234: 466-468 (1959)

13. Barton, C., Photometric Analysis of Phosphate Rock. Anal. Chem. 20: 1068-1073 (1948)

14. Belcher, R. and MacDonald, A., A Rapid Micro Method for the Determination of Phosphorus in Organic Compounds. Talanta 1: 185-188 (1958)

15. Belcher, R. and Uden, P., The Determination of Trace Amounts of Phosphorus by a Double Amplification Method. Anal. Chim. Acta 42: 180-182 (1968)

16. Bell, R. and Doisy, E., Rapid Colourimetric Methods for the Determination of Phosphorus in Urine and Blood. J. Biol. Chem. 44: 55-67 (1920)

17. Berenblum, I. and Chain, E., Biochem. J. 32: 295 (1938)

18. Beveridge, J. and Johnson, S., The Determination of Phospholipid Phosphorus. Can. J. Res. 27: 159-163 (1949)

19. Boltz, D. and Mellon, M., Determination of Phosphorus, Germanium, Silicon and Arsenic by the Heteropoly Blue Method. Anal. Chem. 19: 873-877 (1946)

20. Ibid. Spectrophotometric Determination of Phosphorus as Molybdodiphosphoric Acid. Anal. Chem. 20: 749-751 (1948)

21. Bowman, M. and Beroza, M., Gas Chromatographic Detector for Simultaneous Sensing of Phosphorus and Sulfur-Containing Compounds by Flame Photometry. Anal. Chem. 40: 1448-1452 (1968)

22. Briggs, A., A Modification of the Bell-Doisy Phosphate Method. J. Biol. Chem. 53: 13-16 (1922)

23. Brunfelt, A. and Steinnes, E., The Determination of Phosphorus In Rocks by Neutron Activation. Anal. Chim. Acta 41: 155-158 (1968)

24. Canic, V., Turčic, M., Petrovic, S.M. and Petrovic, S.E., Separation of Condensed Phosphates on Thin Layer of Starch. Anal. Chem. 37: 1576-1577 (1965)

25. Chen, P., Jr., Torihara, T. and Warner, H., Microdetermination of Phosphorus. Anal. Chem. 28: 1756-1758 (1956)

26. Christopher, A. and Fennell, T., Determination of Phosphorus in Organic Compounds on the Centimilligram Scale. U.S. Govt. Res. Develpp. Rept. 41: 5-19 (1966)

27. Crouch, S. and Malmsteadt,. H., A Mechanistic Investigation of Molybdenum Blue Method for Determination of Phosphate. Anal. Chem. 39: 1084-1089 (1967)

28. Ibid. An Automatic Reaction Rate Method for Determination of Phosphate. 39: 1090-1094 (1967)

29. Dellamonica, E., Bingham, E. And Zittle, C., Effect of Trichloroacetic Acid on Determination of Silicate and Phosphate with Molybdate Reagent. Anal. Chem. 30: 1986-1988 (1958)

30. Dippel, W., Bricker, C. and Furman, N., Falme Photometric Determination of Phosphate. Anal. Chem. 26: 553-556 (1954)

31. Djurkin, V., Kirkbright, G. and West, R., A Sensitive and Selective Spectrophotometric Procedure for the Determination of Phosphorus. Analyst 91: 89-93 (1966)

32. Dreisbach, R., Submicrogram Determination of Inorganic Phosphate in Biological Material. Anal. Biochem. 10: 169-171 (1965)

33. Dryer, R., Tammes, A. and Routh, J., The Determination of Phosphorus and Phosphatase with N-Phenyl-p-Phenylenediamine. J. Biol. Chem. 225: 177-183 (1957)

34. Ernster, L., Zetterstrom, R. and Lindberg, O., On the Determination of P^{31} and P^{32} in Biological Material. Acta Chem. Scand. 6: 804-805 (1952)

35. Fischl, J., Pinto, N. and Gordon, C., Rapid Detection of Organic Phosphorus Poisons. Clin. Chem. 14 371-373 (1968)

36. Fiske, C. and Subbarow, Y., The Colourimetric Determination of Phosphorus. J. Biol. Chem. 66: 375-400 (1925)

37. Fleischer, K., Southworth, B., Hodecker, J. and Tuckerman, M., Determination of Phosphorus in Organic Compounds. Anal. Chem. 30: 152-154 (1958)

38. Fogg, D. and Wilkinson, N., The Colourimetric Determination of Phosphorus. Analyst 83: 406-414 (1958)

39. Gates, O., Determination of Micro Amounts of Phosphorus in High-Purity Iron. Anal. Chem. 26: 730-732 (1954)

40. Gee, A. and Deitz, V., Determination of Phosphate by Differential Spectrophotometry. Anal. Chem. 25: 1320-1324 (1953)

41. Goodwin, J., Thibert, R., McCann, D. and Boyle, A., Estimation of Serum Phospholipid and Total Phosphorus using Chloric Acid. Anal. Chem. 30: 1097-1099 (1958)

42. Griswold, B., Humoller, F. and McIntyre, A., Inorganic Phosphates and Phosphate Esters in Tissue Extracts. Anal. Chem. 25: 192-194 (1951)

43. Hansen, A. and Robinson, R., The Determination of Organic Phosphorus in Sea Water with Perchloric Acid Oxidation. J. Marine Res. 12: 31-41 (1953)

44. Hargrave, B. and Geen, G., Phosphorus Excretion by Zooplankton. Limnology and Oceanography 13: 232 (1968)

45. Henriksen, A., Application of a Modified Stannous Chloride Reagent for Determining Orthophosphate. Analyst 88: 898 (1964)

46. Henriksen, A., An Automatic Method for Determining Low-level Concentrations of Phosphates in Fresh and Saline Waters. Analyst 90: 29-34 (1965)

47. Henriksen, A., Interference from Silica in Phosphate Analysis. Analyst 91: 290-291 (1966)

48. Hislop, J. and Cooke, I., Anion Exchange Resin as a Means of Assessing Soil Phosphate Status. A Laboratory Technique. Soil Sci. 105: 8 (1968)

49. Hoffman, F., Jones, L., Jr., Robbins, O., Jr. and Alsberg, F., Colorimetric Determination of Phosphorus in Gasolines Containing Tritolyl Phosphate. Anal. Chem. 30: 1334-1336 (1958)

50. Hoppe-Seyler, Von G. and Gundlach, G., Eine automatisierte Bestimmung von anorganischem Phosphat. Z. klin. Chem. u. klin. Biochem. 6: 256-258 (1968)

51. Hurford, T. and Boltz, D., Indirect Ultraviolet Spectrophotometric and Atomic Absorption Spectrometric Methods for Determination of Phosphorus and Silicon by Heteropoly Chemistry of Molybdate. Anal. Chem. 40: 379-382 (1968)

52. Karmen, A., Specific Detection of Halogens and Phosphorus by Flame Ionization. Anal. Chem. 36: 1416-1421 (1964)

53. King, E., Biochem. J. 26: 292 (1932)

54. Kitson, R. and Mellon, M., Ind. Eng. Chem., Anal. Ed. 14: 155 (1944)

55. Kolmerton, J. and Epstein, J., Colourimetric Determination of Tetramethyl Phosphonium Ion. Anal. Chem. 30: 1536-1537 (1958)

56. Koser, B. and Oesper, P., Separation of Glyceraldehyde-3-Phosphate from Inorganic Phosphate on Bisulfite Ion-Exchange Columns. Anal. Biochem. 17: 119-124 (1966)

57. Kumamaru, T., Otani, Y. and Yamamoto, Y., A Method for the Determination of Phosphorus by Atomic Absorption Spectrophotometry Using Molybdenum as a Light Source. Bull. Chem. Soc. Japan 40: 429 (1967)

58. Lebedeva, A., Novozhilova, I. and Aksenova, N., Colourimetric Method of Phosphorus Determination in Organophosphorus Compounds. Arm. Khim. Zh. 19: 743-746 (1966)

59. Lecocq, J. and Inesi, G., Determination of Inorganic Phosphate in the Presence of Adenosine Triphosphate by the Molybdo-Vanadate Method. Anal. Biochem. 15: 160-163 (1966)

60. Lench, A., Determination of Phosphorus in Highly Alloyed Steels with Quinoline Molybdate. Anal. Chem. 39: 1456-1458 (1967)

61. Levine, H., Rowe, J. and Grimaldi, F., Molybdenum Blue Reaction and Determination of Phosphorus in Waters containing Arsenic, Silicon and Germanium. Anal. Chem. 27: 258-262 (1955)

62. Lindner, J. and Edmundsson, I., Analytical Determination of Inorganic Bound Phosphorus in the Presence of Alkylated Phosphorus. Acta Chem. Scand. 21: 136-142 (1967)

63. Lowery, O. and Lopez, J., The Determination of Inorganic Phosphate in the Presence of Labile Phosphate Esters. J. Biol. Chem. 161: 421-428 (1946)

64. Lueck, C. and Boltz, E., Spectrophotometric Study of Modified Heteropoly Blue Method for Phosphorus. Anal. Chem. 28: 1168-1171 (1956)

65. MacDonald, A. and Van der Voort, F., Separation of Silicon and Phosphorus with Ammonium Molybdate and their Successive Determination. Analyst 93: 65-71 (1968)

66. Marsh, B., The Estimation of Inorganic Phosphate in the Presence of Adenosine Triphosphate. Biochim. Biophys. Acta 32: 357-361 (1959)

67. Martin, J. and Doty, D., Determination of Inorganic Phosphate. Anal. Chem. 21: 965-967 (1949)

68. May, R., Determination of Quinquevalent, Trivalent and Organic Phosphorus in the Atmosphere and in Aqueous Solutions. Anal. Chem. 31: 308-310 (1959)

69. Menzel, D. and Corwin, N., The Measurement of Total Phosphorus in Seawater, Based on the Liberation of Organically Bound Fractions by Persulfate Oxidation. Limnology and Oceanography 10: 280-282 (1965)

70. Michelson, O., Photometric Determination of Phosphorus as Molybdo-vanadophosphoruc Acid. Anal. Chem. 29: 60-62 (1957)

71. Misson, G., Chem. Ztg. 32: 633 (1908)

72. Mokrasch, L., Spectrophotometric Determination of Phosphate in the Presence of Highly Labile Phosphorus Compounds. Anal. Chem. 33: 432-434 (1961

73. Morrison, W., A Fast, Simple, and Reliable Method for the Micro-determination of Phosphorus in Biological Materials. Anal. Biochem. 7: 218-224 (1964)

74. Mozersky, S., Pettinati, J. and Krolman, S., An Improved Method for the Determination of Orthophosphate, Suitable for Assay of Adenosine Triphosphate Activity. Anal. Chem. 38: 1182-1187 (1966)

75. Ibid. Micromethod for Determination of Orthophosphate in Presence of Adenosine Triphosphate after Deproteinization with Trichloro-acetate Buffer. Anal. Chem. 40: 788-791 (1968)

76. Murphy, J. and Riley, J., A Modified Single Solution Method for the Determination of Phosphate in Natural Waters. Anal. Chim. Acta 21: 31-36 (1962)

77. Nadkarni, R. and Haldar, B., Determination of Silicon, Phosphorus and Sulfur in Alloy Steel by Neutron Activation Analysis. Anal. Chim. Acta. 42: 279-284 (1968)

78. Oliver, W. and Funnell, H., Determination of Phosphorus in Biological Material. Anal. Chem. 33: 434-435 (1961)

79. Pagliassotti, J. and Porsche, F., Spectrographic Determination of Phosphorus in Lubricating Oil by Solution Excitation. Anal. Chem. 23: 198-200 (1951)

80. Pechere, J. and Capony, J., On the Colourimetric Determination of Acyl Phosphates. Anal. Biochem. 22: 536-539 (1968)

81. Pennial, R., An Improved Method for the Determination of Inorganic Phosphate in Biological Material. Anal. Biochem. 14: 87-90 (1966)

82. Quinlan, K. and DeSesa, M., Spectrophotometric Determination of Phosphorus as Molybdovanadophosphoric Acid. Anal. Chem. 27: 1626-1629 (1955)

83. Rhodes, D., Microdetermination of Phosphorus. Nature 176: 215-216 (1955)

84. Rigler, F., Further Observations Inconsistant with the Hypothesis that the Molybdenum Blue Method Measures Orthophosphate in Lake Waters. Limnology and Oceanography 13: 7 (1968)

85. Rison, M., Barber, W. and Wilkness, P., Determination of Trace Amounts of Phosphorus in a Composite Propellant by Fast Neutron Activation Analysis. Anal. Chem. 39: 1028-1030 (1967)

86. Saliman, P., A New Digestion Reagent for the Determination of Microgram Quantities of Phosphorus in Organic Compounds. Anal. Chem. 36: 112-114 (1964)

87. Sayenkoff, B., An Improved Micromethod of Phosphate Determination. J. Biol. Chem. 198: 221-227 (1952)

88. Schöniger, W., Midatlantic Analytical Symposium, Philidelphia, Pa., Feb. 8, 1957.

89. Scroggins, L., Spectrophotometric Microchemical Phosphorus Determination. Microchem. J. 13: 385-301 (1968)

90. Simmons, W. and Robertson, J., Determination of Total Phosphorus in Organic Phosphates. Anal. Chem. 22: 294-296 (1950)

91. Sims, R., Formation of Heteropoly Blue by Some Reduction Procedures used in the Microdetermination of Phosphorus. Analyst 86: 584-590 (1961)

92. Sinha, B., Das Gupta, S. and Kumar, S., A Titrimetric Method for the Determination of Phosphorus. Analyst 93: 409-412 (1968)

93. Skogerboe, R., Gravatt, A. and Morrison, G., Flame Spectrophotometric Determination of Trace Amounts of Phosphorus. Anal. Chem. 39: 1602 (1967)

94. Sletten, O. and Bach, C., J. Am. Wat. Wks. Ass. 53: 1031 (1961)

95. Strickland, J. and Parsons, T., A Manual of Sea Water Analysis 2 nd. Ed. (Bull. of the Fisheries Research Board of Canada, Ottawa, Canada 1965) p. 203

96. Taylor, A. and Miller, C., J. Biol. Chem. 18: 215 (1914)

97. Terman, G. and Khasawuch, F., Crop Uptake of Fertilizer and Soil Phosphorus in Relation to Calculated A Values. Soil. Sci. 105: 346 (1968)

98. Theakston, H. and Bandi, W., Spectrophotometric Determination of Trace Amounts of Phosphorus in High-Alloy Steel. Anal. Chem. 38: 1764-1767 (1966)

99. Vogler, P., Problems of Phosphate Analysis in Limnology and a New Method for the Determination of Dissolved Orthophosphate in the Presence of Condensed Phosphates and Organic Esters of Phosphoric Acid. Intern. Rev. Ges. Hydrobiol. 50: 33-48 (1965)

100. Vogler, P., Analysis of Condensed and Organic Phosphates in Limnological Investigations. Int. Rev. Ges. Hydrobiol. 51: 775-785 (1966)

101. Vogler, P., Analysis of Phosphorus Compounds in Water. Limnologica 4: 437-444 (1966)

102. Vreman, H. and Jöbsis, F., Interferences by Mannitol and Other Compounds with Phosphate Determination. Anal. Biochem. 17: 108-118 (1966)

103. Wadelin, C. and Mellon, G., Extraction of Heteropoly Acids: Application to the Determination of Phosphorus. Anal. Chem. 25: 1668-1673 (1953)

104. Weil-Malherbe, H. and Green, R., The Catalytic Effect of Molybdate on the Hydrolysis of Organic Phosphate Bonds. J. Biol Chem. 49: 286-292 (1951)

105. Weil-Malherbe, H., Ibid. 55: 741-745 (1953)

106. Wooster, W. and Rakestraw, N., The Estimation of Dissolved Phosphate in Sea Water. J. Marine Res. 10: 91-100 (1951)

107. Yee, H., A Simplified Method for Automated Phosphorus Analysis of Serum and Urine. Clin. Chem. 14: 898-903 (1968)

108. Yofe, J. and Rappart, B., Complexiметric Titration of Phosphate with Lanthanum as Precipitant. Anal. Chim. Acta 43: 346-347 (1968)

109. Zaugg, W. and Knox, R., Indirect Determination of Inorganic Phosphate by Atomic Absorption Spectrophotometric Determination of Molybdenum. Anal. Chem. 38: 1759-1760 (1966)

110. Zaugg, W. and Knox, R., Determination of Phosphate in Biological Materials and Reaction Mixtures by Atomic Absorption Spectrophotometry. Anal. Biochem. 20: 282-293 (1967)